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THE ADEQUACY OF THE UNI–EXPONENTIAL MODEL IN CHARACTERIZING SPIN–LATTICE RELAXATION CURVES OF BIOLOGICAL TISSUES

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Summary. – *The aim of this study was to examine whether spin–lattice relaxation in biological tissues is adequately described by a single time constant T_1 and to define under what circumstances a multi–exponential approach is indicated. Relaxation curves were measured at 60 MHz for a range of tissues from tumour–bearing mice. Uni– and bi–exponential curves were fitted to the empirical data using chi–square as a criterion for the goodness of the fit. An F–test was applied to test the validity of each exponential term as it was added to the fitting function.*

Riassunto. – *Questo studio si propone di esaminare se il rilassamento spin–reticolo in tessuti biologici è descritto in modo adeguato da una singola costante di tempo T_1 e di definire in quali condizioni sia più indicato l'uso di un approccio multiesponenziale. Le curve di rilassamento sono state misurate a 60 MHz per diversi tessuti isolati da topi portatori di tumori. Sono state usate curve mono– e bi–esponenziali per interpolare i dati sperimentali, usando il test del chi quadro, come criterio di bontà del* fit.

Introduction

Spin–lattice relaxation times are usually measured by the inversion recovery method. In this method [1] T_1 is obtained from a least squares fit of the theoretical recovery curve:

$$M_z(\tau)=M_0(1-2\exp(-\tau/T_1)) \qquad (1)$$

to the empirical data with M_0 and T_1 as parameters. This approach is appropriate only when the relaxation is uni–exponential. Most biological tissues seem to satisfy this requirement and can be characterized by a single T_1. Substantial deviations from the uni–exponential relaxation model have been reported, however. Marriolhet and Moran [2], for instance, found a clear multi–component behaviour of T_1 in murine liver tissue, while Bovée *et al.* [3], Goldsmith *et al.* [4], Rasz *et al.* [5], and Edzes and Samulski [6] observed a distinct two–componency to T_1 in mammary tissue, lens tissue and muscle tissue, respectively.

The present study was undertaken to state precisely to what extent biological tissues satisfy the uni–exponential model and to define under what circumstances a multi–exponential approach is indicated.

Materials

Inversion recovery curves were measured *in vitro* for a range of tissues from tumour–bearing mice. The mice were of the C57Bl strain and had a subcutaneous, transplantable, fast–growing mammary adenocarcinoma –M8013– on one of the hindlegs. All animals were sacrificed by ether anaesthesia and subsequent cervical dislocation. Excision took place when the tumour had reached a volume of approximately 1 cm^3. Measurements were performed on the viable and necrotic parts of the tumour and on a wide range of normal tissues, including thigh muscle, liver, kidney, spleen, salivary gland, eyelens, and subcutaneous fat. Measurements took place within 5 hours from excision.

Methods

Measurement of spin–lattice relaxation time T_1

The T_1 measurements reported here were performed with a spin–echo spectrometer built at the Laboratory of Technical Physics of the University of Technology in Delft, operating at a frequency of 60 MHz. The sample cavity was maintained at 27° C. In the applied pulse sequence the width of the 180° and 90° pulses was of the order of 150 and 75 μs respectively. The height of the free induction decay signal was measured on oscilloscope immediately after the 90° pulse.

The spin–lattice relaxation curves used in this study were determined from 15 to 25 pulse experiments, the pulse separation τ varying from 10 ms to 10 s. The sequence repetition time was chosen long enough to allow the spin system to reach equilibrium between sequences.

The sensitivity, stability and accuracy of the spectrometer were checked regularly by monitoring the T_1 value of a reference solution of Mn^{2+} ions in water.

Multi–exponential analysis of relaxation curves

In a multi–exponential analysis of the inversion recovery experiment the parameters M_{0i} and T_{1i}, i=1,....., n are obtained from a weighted least squares fit of the theoretical curve

$$M_z(\tau) = \sum_{i=1}^{n} M_{0i}(1-2\exp(-\tau/T_{1i})) \qquad (2)$$

to the empirical data with M_{0i} and T_{1i} as parameters. In this formula n represents the number of exponentials required for an adequate description of the data, taking into account the experimental accuracy σ_i of the data points. Optimum values of the parameters M_{0i} and T_{1i} are obtained by minimizing the weighted sum of squares of deviations of the data from the fitting function with respect to each of the parameters simultaneously. The goodness of the fit is evaluated through the calculation of the reduced variance of the fit χ^2_ν

$$\chi^2_\nu = \frac{1}{N-2n} \sum_{i=1}^{N} \frac{1}{\sigma_i}(M_z^i - M_z(\tau_i))^2 \qquad (3)$$

with N the number of data points, 2n the number of parameters, ν=N–2n the number of degrees of freedom left after fitting the N data points to the 2n parameters M_{0i}, T_{1i}, σ_i the uncertainty in the data point M_z^i and M_z (τ_i) the theoretical value of M_z at pulse interval τ_i.

As discussed by Bevington [7] the value of the reduced chi–square should be approximately unity or less if the fitting function is an adequate approximation of the data.

Results

Proton spin–lattice relaxation curves were measured for a range of tissues from tumour–bearing mice. The curves were analysed according to the fitting procedure outlined in the preceeding section. Table 1 shows the mean values of the parameters M_{0i} and T_{1i} obtained by uni– and bi–exponential analysis of relaxation curves for a series of mice.

Adopting $\chi^2_\nu \lesssim 1$ as a criterion for the goodness of the fit, the uni–exponential model appears to be an adequate description of the data for necrotic tissue. Eyelens and fat show a distinct bi-exponentiality while liver, spleen, active tumour, muscle, kidney, and salivary gland present intermediate cases.

According to Table 1, the bi–exponential analysis yields a fastly relaxing minor component with $T_{11} \lesssim$ 20ms and a slowly relaxing major component with $T_{12} > 300$ ms. Fat presents a different case yielding a relatively slow minor component with $T_{12} \sim 200$ms. In neither case a third exponential could be resolved in our experiments. This does not necessarily imply their non–existence, however. The above analysis simply and solely shows the bi–exponential model to be a convenient parameterization of the experimental data, taking into account experimental accuracy, number of data points, etc. Inclusion of higher order terms is not justified by our analysis, as this would not yield a significantly better description of the data.

Table 1. – *Mean values and standard errors of the mean of the parameters M_0 and T_1 obtained by uni–and bi-exponential analysis of proton spin–lattice relaxation curves for a range of tissues from tumour–bearing mice. M represents the number of mice (taken from reference 8).*

		model 1		model 2		
Tissue	M	T_1 (ms)	M_{01} (%)	T_{11} (ms)	M_{02} (%)	T_{12} (ms)
Eyelens	10	351 ± 20	14.7 ± 0.8	15 ± 2	85.3 ± 0.8	466 ± 20
Fat	2	251 ± 2	81.1 ± 4.5	197 ± 6	18.9 ± 4.5	910 ± 202
Kidney	9	573 ± 15	5.7 ± 0.9	3 ± 1	94.3 ± 0.9	637 ± 16
Liver	8	526 ± 9	1.5 ± 0.7	10 ± 5	98.5 ± 0.7	540 ± 4
Muscle	6	816 ± 11	7.1 ± 0.4	17 ± 2	92.9 ± 0.4	933 ± 15
Necrosis	5	1029 ± 41	–	–	–	–
Salivary gland	8	416 ± 13	5.3 ± 1.0	12 ± 5	94.7 ± 1.0	454 ± 14
Spleen	8	811 ± 25	3.6 ± 0.9	9 ± 2	96.4 ± 0.9	870 ± 35
Tumour	6	1096 ± 37	3.4 ± 1.7	11 ± 6	96.6 ± 1.7	1163 ± 29

Discussion

The results presented in Table 1 indicate the adequacy of the bi–exponential model in describing spin–lattice relaxation data in various excised mouse tissues. A good fit to a particular model, however, does not guarantee the validity of that model. It will therefore be necessary to consider the results within the framework of current theories on spin–lattice relaxation in biological tissues. As described elsewhere [8] the results appear to be consistent with a model of tissue hydration described by Berendsen [9], assuming three types of tissue water, *viz.* specific and non–specific hydration water associated with the macromolecules in the tissue and general hydration water. In the case of fat, the bi–exponentiality is presumably due to the different relaxation behaviour of water and fat protons.

Evidently, more precise measurements of relaxation curves and additional information concerning the molecular composition and dynamics of tissues will be necessary to further elucidate spin–lattice relaxation processes in biological tissues.

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MEASUREMENT AND MEANING OF RELAXATION TIMES: SPECIFIC AND NON-SPECIFIC VARIATIONS IN CANCER

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Summary. — *A review is presented on the principal results obtained in proton NMR on tumor and non-tumor tissues in malignant tumor-carrying animals (systemic effect). An attempt will also be made to establish a link between biological variations and the different physical parameters that occur within the relaxation times.*

Riassunto. — *Viene presentata una rassegna sui principali risultati ottenuti, mediante ^1H NMR, su tessuti normali e tumorali di animali portatori di tumori maligni (effetto sistemico). Si tenta anche di stabilire correlazioni tra variazioni biologiche e i diversi parametri fisici che influenzano le proprietà di rilassamento magnetico nucleare di questi tessuti.*

Introduction

The hope of finding a new method for the diagnosis of malignant tumors was instrumental in the early development of biomedical applications of nuclear magnetic resonance (NMR). However, as often happens where a change is involved, the passing years and detailed research somewhat dampened the initial enthusiasm.

Research in this domain over the last ten years can be divided into several periods. The first began in 1971 when R. Damadian published an article describing differences in relaxation times between normal tissues and corresponding tumor tissues. Research had already been carried out on the structure of water in muscle [1] and, some 15 years previously, A. Szent-Gyorgyi [2] had hypothesized on the role of cell water in the malignant transformation process. Damadian tested this theory and his first results concerning tumor tissue raised a wave of enthusiasm for what then appeared to be a sure and rapid means of discriminating between normal and tumor tissue.

In the mid-seventies, this line of research went into almost total decline for two reasons. Firstly, NMR imaging systems made their appearance and completely eclipsed *in vitro* results. Secondly, during the same period, studies which queried NMR efficiency in diagnosing cancer were abundant. As of 1972, criticisms mainly concerned the specificity of the method. If an increase in relaxation time is only the result of an increase in water content (D.P. Hollis group's hypothesis [3], then NMR cannot be expected to be specific and it is therefore of no interest to oncologists.

Towards the end of the seventies, a third period began during which various studies attempted to explain variations in relaxation times. These resulted in an intermediate position between Damadian's enthusiasm and Hollis' disillusion. Simultaneously NMR tomography units became more accurate; separate relaxation times T_1 and T_2 could be obtained as could the density of spin using appropriate pulse sequences (inversion-recovery, saturation-recovery, spin-echo, etc.). Interest in understanding the causes of fluctuations in proton relaxation times in biological tissue revived.

We shall review briefly the principal results obtained in proton NMR on tumor and non-tumor tissues in malignant tumor-carrying animals (systemic effect). We shall then try to establish a link between biological variations and the different physical parameters that occur within the relaxation times.

I. Review of principal results

I.1. Tumor tissue

In 1971, Damadian measured the T_1 and T_2 of various tumors in animals and he compared these results with those obtained in normal tissue [4]. The following year, he published the first results obtained *in vitro* on human tumors [5]. Numerous *in vitro* studies on human tumors were conducted over the succeeding years: breast [6-8], lung [9], thyroid [10, 11] and intracranial tumors [12-14].

Damadian even proposed a "malignancy index" associating T_1 and T_2. According to him, easy discrimination between malignancy and benignity would thereby be possible [7-9]. Such an idea is a good illustration of

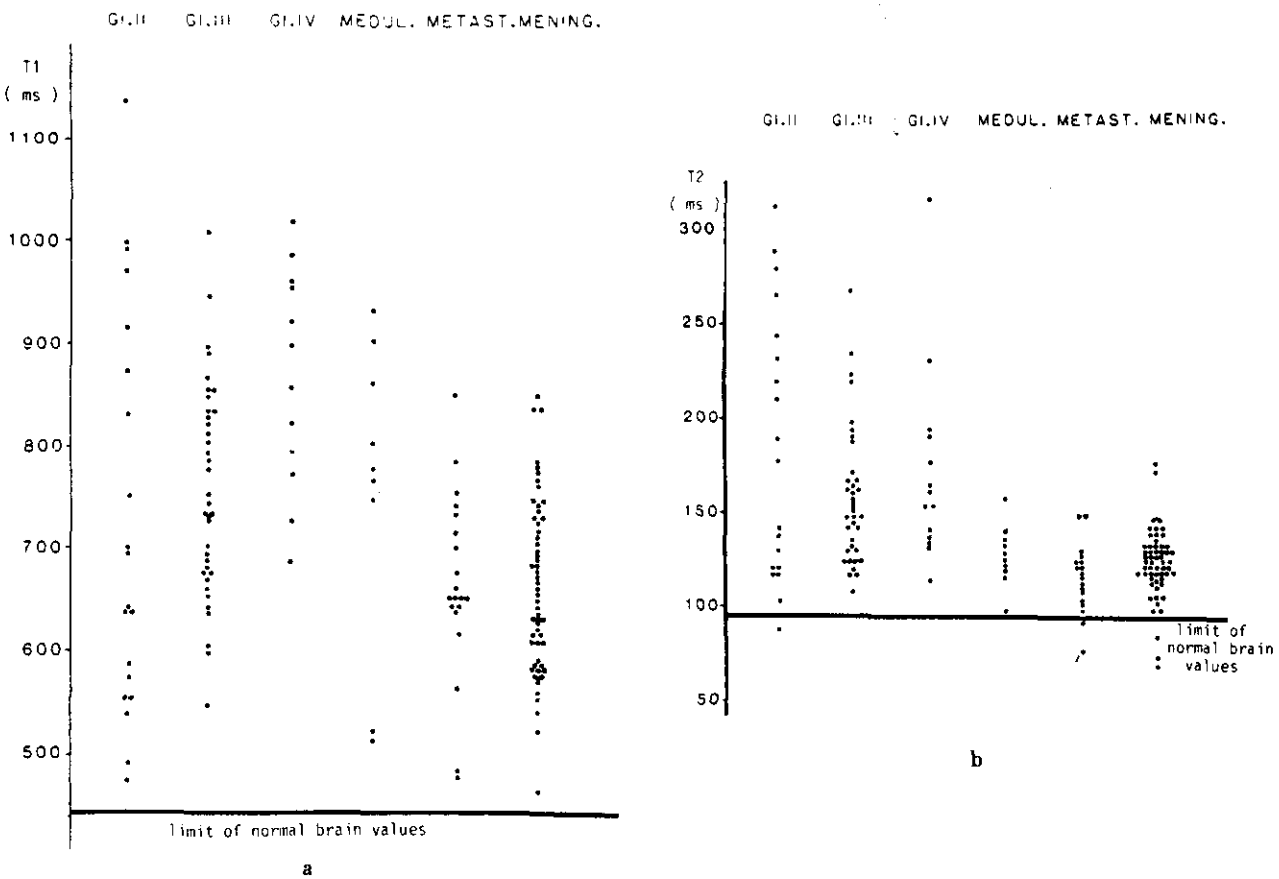

Fig. 1. — a) T_1 variations in human intracranial tumors (de Certaines *et al.*, 1982 unpublished): the results show there is no simple discrimination between malignant and benign tumors. b) T_2 variations in human intracranial tumors.

the initial enthusiasm for NMR that prevailed among oncologists. The association of the two relaxation times T_1 and T_2 is however, open to debate. T_1 and T_2 express fluctuations in the various physical parameters of tissue water differently [10-15]. Their association may well hide data specific to T_1 or T_2. Furthermore, many authors have shown that proton relaxation times do not allow a distinction to be made between malignant and normal tissue but between tumor, or, even more generally, altered tissue and healthy tissue [6, 8, 10, 12, 14] (Fig. 1).

Proton NMR way seems a sensitive method for detecting physiological fluctuations but it can no longer be considered a specific method for characterizing cancer.

I.2. Systemic effect

H.E. Frey and M.M. Pintar [16] were the first, in 1972, to show that the T_1 of different non-tumor organs (liver, kidney, spleen) increased by 10 to 20% in a cancer-carrying mouse. These results were confirmed by other researchers using different animals. We ourselves reached the same conclusion when we studied the liver, spleen, kidneys, heart and striated muscles in mice with Lewis lung tumor [17]. This systemic effect is more sensitive where T_1 is concerned. T_2 would seem a better *indicator of the tumor itself*. We also showed a sensitive T_1 and T_2 reaction in the peritumoral area (Fig. 2). C. R. Ling found the same alteration in T_1 in the peritumoral area and showed that allografts of normal tissue *were also accompanied by an increase in* T_1 in adjacent tissue. He attributed this effect to a local immune reaction. The systemic effect on tissues is not therefore necessarily specific to cancer. To give an example, we have shown that as the Lewis tumor grows, the splenic T_1 and T_2 increased; there was a good correlation with the splenic index (Fig. 3). Relaxation times in spleen also change, however, after exposure to radiation, and after immunomodulation due to various strains of *Brucella abortus*, etc.

It was then discovered that this systemic effect, which had previously been described in relation to various solid tumors, also existed in serum [18]. Hollis [19] and Hazlewood [20] later confirmed this result on animals models. Hazlewood further showed that the systemic effect on serum could also occur during the growth period of benign tumors.

To extend these results to human patients we measured the T_1 and T_2 of serum from 224 controls and from 310 carriers of various solid tumors at different stages of evolution. Our results [21] showed that:

i) the systemic effect described in animals also existed in man;

ii) as in animals, the effect resulted in an increase in T_1 (Fig. 4). The effect on T_2, however, was a change in its distribution but not in its mean average;

iii) it only appeared in advanced stages and ceased to exist in the residual stage;

iv) it could neither be correlated with a variation in

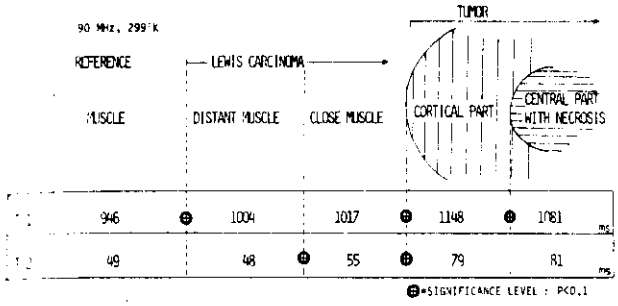

Fig. 2. – Intra and peritumoral T_1 and T_2 differentiation in Lewis lung carcinoma in mice [17]. Probability estimate of the significance of the difference in the means: $P < 0.1$.

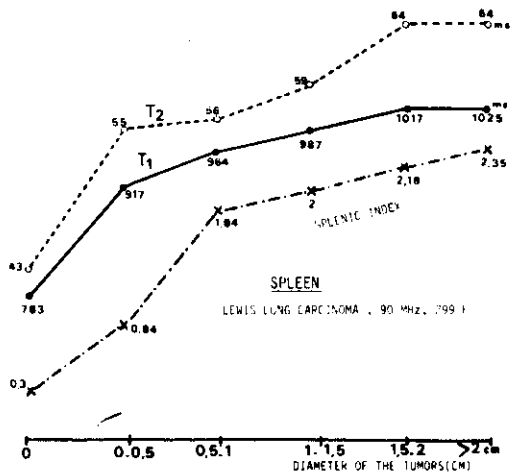

Fig. 3. – Systemic effect: T_1 and T_2 spleen variations and splenic index of Lewis tumor carrying mice as a function of tumor growth [17].

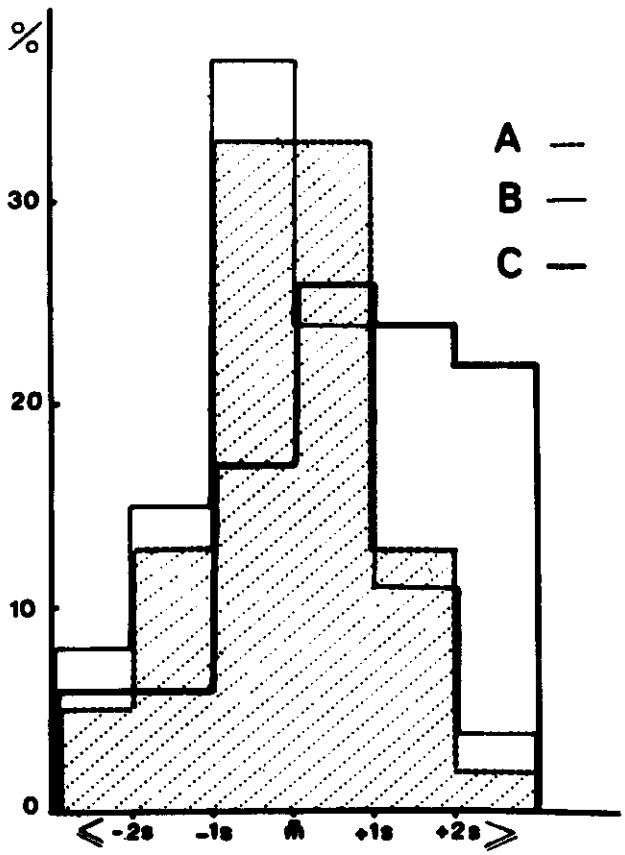

Fig. 4. – Systemic effect: serum T_1 variations in patients with solid tumors [21].
A - control population (n = 224) m = 1386 (SD = 68 ms)
B - imperceptible disease (n = 155) m = 1354 (SD = 77 ms)
C - evolutive disease (n = 156) m = 1428 (SD = 108 ms)
(significant difference between A and C for a threshold of $p < 0.001$).

seric ion nor with a drop in total protein. On the other hand, the different changes in T_1 and T_2 mean that we can consider that a correlation exists with the proteinogram insofar as each protein, depending on its size and structure, exerts a different influence on serum relaxation times.

II. Biological parameters likely to influence relaxation time

Cancer is a combination of complex and as yet ill-known biological phenomena which do not occur only in tumor cells.

II.1. Malignant transformation

A tumor cell is a little-differentiated cell whose growth is excessive and unaffected by regulation processes. Its different growth rate studied by NMR is the subject of the next paragraph. The main other cyto-chemical variations that can alter relaxation times may be: water content, ionic fluctuations, changes in molecules or in organized polymolecular systems (e.g. membranes or cytoskeleton).

a) Water content

Tumor tissue often has a higher water content than corresponding healthy tissue. But is this sufficient to explain the lengthening of relaxation times generally observed in tumor tissue? A rather lively discussion took place over this point between Hollis' group [22, 23] and Damadian's. For the latter, who had been inspired by Szent-Gyorgyi's theory [2], the T_1 and T_2 values observed in tumor tissue are linked to a structural modification of intracellular water. For Hollis, they are linked to a non-specific fluctuation in water content. The issue in this debate is whether NMR can detect highly specific alterations due to cancer and therefore whether it is of new interest in the diagnosis of malignant tumors. The Szent-Gyorgyi-Damadian hypothesis does not seem to have been confirmed experimentally, whereas relaxation times for tumors seem often, but not always, to correlate well with the water content [22, 25].

b) Ionic fluctuations

A qualitative and no longer a quantitative variation in intracellular water caused by the hydratation shells of the ions and macromolecule may be considered. Dama-

dian's point of departure [4, 5, 26] is that Na^+ ions are "structure-making" as regards water and K^+ ions are "structure-breaking". This is based on the circulation of the transition energy required by one molecule of water in the hydratation shell of an alkaline ion. The Na^+ and K^+ ratio fluctuates in biological fluids. The structure of water molecules and therefore their relaxation times would depend on the variations of these ions. To test this hypothesis, Damadian [5] put *Escherichia coli* into a culture medium enriched with either potassium or sodium. He noted an increase in the relaxation times of the potassium-enriched cultures. A study of the potassium content of different tumors led him to confirm L. Dunham's observation: "with few exceptions, the potassium content of malignant tumors increased".

However, Damadian himself in 1974 [27], and then B.M. Fung in 1975 [28], found that the concentration of potassium sometimes decreased in tumors and this explanation was not therefore acceptable. These results were also in contradiction with the importance accorded to sodium ions in Hazlewood's study of the maturation of muscular tissue published in 1971 [29].

B.M. Fung, in 1973, in his works on the hydratation shells of collagen in solution [30], and G.P. Raaphorst, in 1975 [31], indicate that ionic variations bring an indirect influence to bear. They cause transconformation of the macromolecules and thus variations in the importance of their hydratation shells. Within the limits set by the physiological concentrations, the contribution of paramagnetic impurities and free radicals to T_1 and T_2 variations has also been evoked, but has not been proved; at higher doses, modulating contrast in NMR imaging is possible.

c) Histo-physiological parameters

R.E. Block [32] compared cytosol extracts taken from hepatoma and from normal rat liver. He considered that the stronger concentrations of proteins and glycogen in the control explained a shorter T_1 compared with the hepatoma. For Block, variations in hydratation shells played a decisive role in the T_1 of cell extracts and could be applied to the measurement of solid tissue.

With regard to breast cancer, W.M. Bovée [33] thinks that the T_1 observed is the sum of a short T_1 (fat contained in the tissue) and of a long T_1 (cell water). He also remarked that the long T_1 does not seem to be statistically different in normal and tumor cells. He concluded that the fat content of the sample was the decisive element as this is lower in benign and malignant tumors than in normal tissue.

S.S. Ranade [34] measured the T_1 of cell nuclei of fibrosarcoma in mice, normal liver and liver taken from a fibrosarcoma-carrying mouse. He noted that as liver of the mouse with fibrosarcoma and was greater still in the tumor itself. The increase in T_1 could then reflect the altered nucleocytoplasmic ratio, the polyploidy and the rate of mitosis which characterizes cancer tissue. This resembles the role played by chromatin as described by Hazlewood and to which we shall return in the following paragraph.

In addition to these alterations which affect indirectly the bound water and the water content of the tissue, C.J. Lewa's work on normal or tumor dehydrated tissue should also be mentioned: this encourages research into the reasons for fluctuations in relaxation times in the properties or structure of a non-aqueous cell substances [35].

II.2. Growth rate of tumor tissue

If proton relaxation tissue T_1 and/or T_2 could be considered as "cell cycle markers", an important tool would be available for chemotherapy monitoring, for the use of G_0 cell recruiting agents and for the optimization of circadian rhythm dependant treatment.

Can cell division and differentiation loss (the two phenomena seem connected) lead to variations in water content or cause alterations in T_1 and T_2 when the water content is constant? Several studies to this effect have been published. Ranade [34] investigated the T_1 of isolated nuclei of tumor cells. Schara [11] the difference in T_1 between well-differentiated and little-differentiated thyroid cancers. Inch [36] compared embryon tissue with neoplastic formations and Hollis [3] studied hepatoma in rats.

Morgan and Cameron [37] showed that cell proliferation increased in the liver, kidneys and spleen of hepatoma-carrying mice. This result is not isolated [38, 39] and could explain the systemic effect described above.

a) NMR and the growth rate of organs or tumors

In 1974, Pintar's group showed that the average T_1 measured on rapid-growth transplanted tumor tissue was significantly higher than the average T_1 for slow-growth spontaneous tumors. He also confirmed that rapid-growth non-tumor tissue such as embryon or regenerating liver tissue after partial hepatectomy could have an increased T_1. Pintar's group is the first, if we except the suggestion made by Carver in 1973 [40], to ascribe T_1 fluctuations to a rapid growth process. Carver's demonstration was not definitive, however. His study of regenerating liver seems especially open to question: it only concerns T_1 and no use was made of relative laparotomized controls. Furthermore, a heterogenous sample of different embryon tissue cannot normally be compared to mature tissue (Fig. 5). We measured embryon tissue during the first weeks of life and confirmed this first result without going so far as to prove that cell division on its own is sufficient to explain the increased T_1. The same reasoning applies to regeneration following partial hepatectomy [15] where postoperative stress must be taken into consideration (Fig. 6).

We used another model of *in vivo* cell synchronization, planarian regeneration, and obtained a good correlation between variations in T_2 and the number of mitoses. T_1 fluctuated little (Fig. 7).

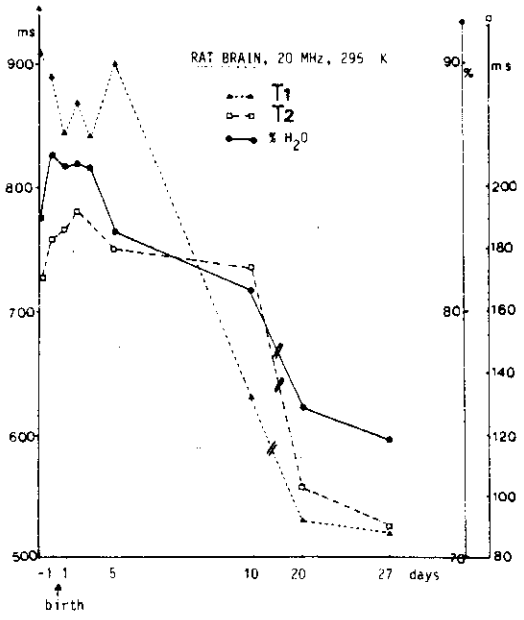

Fig. 5. – An example of relaxation time evolution: rat brain during neonatal period (de Certaines, 1981, unpublished).

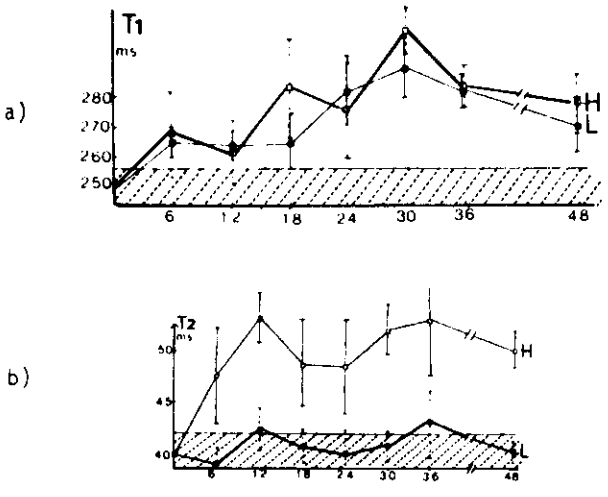

Fig. 6. – Study of *in vivo* cell cycle synchronization [15]:
a) T_1 of hepatectomized rats (H) and laparatomized control rats (relative controls L) depending on post-surgery hour. The striped area represents absolute controls (m ± DS).
b) T_2 variations.

Hollis [3] compared T_1, water content and the growth rates of different transplantable hepatoma cell lines. He found a good positive correlation but he emphasized that there is a difference between the growth of the tumor *in-situ* and the doubling time of its cell fraction outside of the G_0 phase. For Kiricuta [42], the high T_1 in mitosis could explain the increase in T_1 in rapidly growing tissue, both tumor and immature. In tumors, this effect would be particularly marked on the periphery (where there are more dividing cells) than at the center (where there are more cells in the G_0 or G_1 phase).

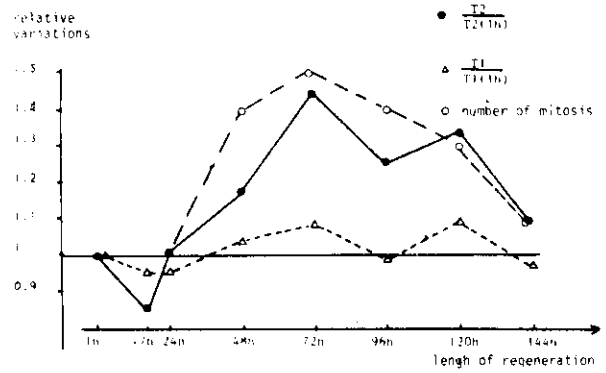

Fig. 7. – Study of *in vivo* cell cycle synchronization: planarian regeneration *(Polycelis tenuis-nigra)* after serial sectioning of animals [41].

b) Studies on in vitro cell cultures

P.T. Beall [42] showed that T_1 in synchronized Hela cells went from a minimum at the S phase to a maximum at mitosis. He went on the prove that these variations cannot be attributed to simple fluctuations of the water content. S and G_2 phase water content is very similar whereas T_1 is significantly different [44]. The same team [45, 46] put different human breast cancer cell lines into culture: they found a connection between the growth rate in culture and T_1. This may be of interest in prognosis.

Relaxation times vary during the cell cycle and especially during the tumor cell cycle. They also vary in normal cells. Beall demonstrated this in his work on Hamster ovary cells [47]. Mitotic cell population T_1 and T_2 are longer than those of cell populations at the interphase.

Other models have not always given the same results. R.N. Muller [48] found no significant T_1 change in rat hepatoma cells treated by Nocodazole. Can macrocellular transconformation during the cell cycle explain, at last in part, the T_1 variations described above? Beall let spermin act on S-phase synchronized HeLa cells: he condensed the chromatin and obtained a 30% increase in T_1 without altering the water content [49]. Furthermore, the difference between S-phase and G_2-phase T_1 in isolated nuclei is the same as in whole cells. Cytoskeleton molecules may also play a part as Beall correlated T_1 with the microtubule complexes in human breast cancer cell lines [50].

Are variations in water content during cell division the decisive element? Hollis found a good correlation between hepatoma growth rates, their T_1 and water content [3]. Beall, however, cannot explain the difference in T_1 at the S and G_2 phase and, to lesser degree, at the M and G_1 phase by water content variations. These only explain G_1-S and G_2-M differences.

The medical stakes involved in the NMR study of the cell cycle are considerable. The study of non-mitotic cell division can help to understand the mechanisms

concerned. B.S. Wong [51] used cultures of monocellular algae and found a drop in relaxation times T_1 and T_2 during the log-phase as compared with the stationary phase. At first sight, these results may seem in contradiction with those expressed above. They showed that T_1 increased with the growth rate. Monocellular algae cannot be compared with mammalian cells. They have no nucleus but a clear central area called mucleoplasma which is not surrounded by a membrane and which contains DNA. There is no nucleolus. Reproduction occurs by simple division. There are no chromosomes and no mitotic spindle.

These results should also be compared with those obtained by other physical technique e.g. the study of osmotically active water carried out by Dupree (50) on synchronize Ehrlich cells or Cerek's work on fluorescent polarizzation [53, 54].

II.3. Other histological alterations

Local histological characteristics such as vascularization, adipose tissue, inflammation, oedema, necrosis, fibrosis, can greatly alter relaxation times.

Ling and Foster have studied inflammation in particular [55, 56]. They implanted malignant or normal tissue and observed a brief increase in relaxation times around the graft. They explained this as being due to an inflammatory reaction of the adjacent tissue. They carried out the same experiment by injecting turpentine which provoked a localized inflammatory reaction. The increase in T_1 in the peritumoral zone in a Lewis tumor-carrying mouse (Fig. 2) can be ascribed to the same phenomenon [17].

Necrosis has been the subject of several *in vitro* studies [17, 57] which have been confirmed by imaging.

C.J. Lewa [58], K.G. Go and S. Naruse [57] have studied oedema. We ourselves have followed T_1 and T_2 fluctuations in rat brains during chemically (triethyletin salts) or osmotically induced oedema. Our results demonstrated that proton NMR is a sensitive indicator of oedema and can be used for pharmacological studies (Fig. 8) [60]. Oedema is important for the NMR detection of intracranial tumors, but the relaxation time variations observed in this tumor localization cannot be explained by the sole presence of oedema.

Vascularization [57], the presence of melanin [59], adipose tissue particularly in breast cancer [33, 57, 61], cytoskeleton changes (50), etc., are all factors which must be taken into consideration when interpreting relaxation times.

In human intracranial tumors (meningiomas), we used Masson Trichrome to colour the collagen content and showed that the latter could explain significant T_1 variations [13].

Thus, the biological parameters likely to alter malignant tumor biological parameters are numerous and generally non-specific. It is therefore necessary to try to clarify the different causes of variations, so that guidelines for the interpretation of clinical results can be defined.

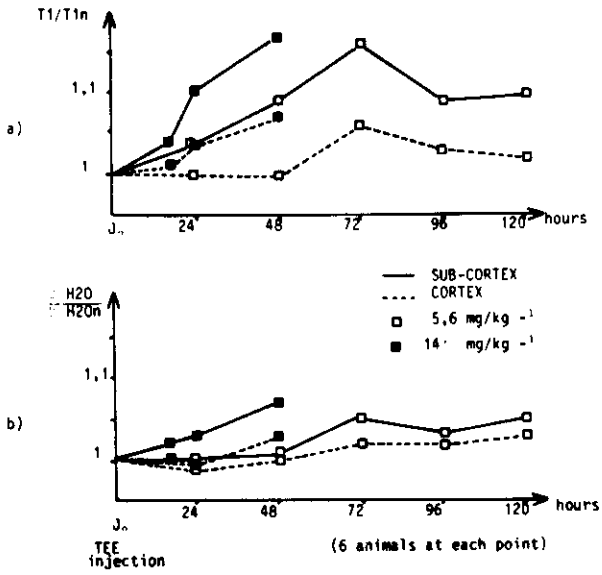

Fig. 8. – Oedema: evolution of proton NMR relaxation time T_1 measured 24, 48, 72, 96 and 120 hours after induction of cerebral oedema in a rat. Intoxication by triethyletin salts (TEE: 5.6 and 14 mg/ kg^{-1} by a single intravenous injection) [60].

III. Interpretation models of variations in relaxation times

III.1. Water in biological systems

Even if water protons alone are not responsible for variations in relaxation times [35], they play an essential part. The water content of living organisms varies between 50% in bacteria spores and 96 to 97% in certain marine invertebrates. The adult human body has a water content of 65 to 70%, but the water is divided unequally between the tissues.

Table I. *Water content of human tissues*

TISSUE	WATER CONTENT
Nervous tissue	84%
Muscle	77%
Liver	73%
Skin	71%
Conjonctive tissue	60%
Adipose tissue	30%

Water has long been considered a simple liquid filling intermolecular gaps. Its role as a regulator of molecular interaction was described by A. Szent-Gyorgyi, whose hypotheses served as a basis for Damadian's first studies.

a) The structure of water

Water has been widely studied and the number of publications on the subject is impressive. Nevertheless, certain major points still remain to be elucidated. The strong attraction that water molecules exert on each other can be explained in part by their dipolar nature. Thus, one water molecule form hydrogen bonds with four adjacent molecules. The structure of water in its liquid form differs little form that of ice crystals. Only a small proportion of hydrogen bonds is destroyed during transition from the solid to the liquid state. This leaves the intermolecular organization at short distance intact, but modifies it at a greater distance. For each water molecule, the distribution of its close neighbours is almost identical to that of an ice crystal. Beyond a radius of 10 Å, however, (that of the molecule is 1.5 Å), the movements of the other molecules are most coordinated in comparison with the molecule under consideration. The difference between water and ice, of which the structures are very similar, lies in the rate at which the hydrogen bonds are made and destroyed. If, at a given moment, most of the water molecules are linked by hydrogen bonds, the latter have a half-life of 10^{-10} to 10^{-11} s and the term "fluctuating aggregates" has been introduced to designate those structures in water whose life span is short and similar to those in ice.

b) Water in solutions

Numerous models have also been proposed in an effort to understand the structure of water in solution. As of 1945, Franks introduced the idea of "icebergs" to explain the water organized itself around solute molecules. Szent-Gyorgyi took this idea up and talked of an "ice-like" water surrounding proteins.

What is certain is that biological molecules bring about change in the structure of water by surrounding themselves with a hydratation shell. The water molecules belonging to this shell are dynamically orientated and have a lesser degree of mobility. The arrangement and diffusion of the water are different from those of pure water. This becomes a fluctuation of correlation time τ_c or perhaps a continuous distribution of τ_c if it is accepted that movement within the hydratation shell is not uniformly restricted.

In macromolecular solutions, at temperatures greatly below the freezing point of water (as low --70° C), part of the water does not freeze. Kuntz and Kauzman [62] have identified this unfreezable water with the water of the hydratation shell or "bound water". The geometrical arrangement of water molecules in a protein surface is different from that imposed on water molecules in ice crystals. This would seem to prevent freezing. From the NMR viewpoint, the decrease in mobility of the molecules and therefore the lengthening of their correlation time τ_c gives shorter T_1 and T_2 relaxation times than in pure water.

c) Water in biological systems

Results similar to those of protein solutions are found in intracellular water and in biological tissue where T_1 and T_2 are lower than in pure water. Cooke and Wien [63] showed that the relaxation times measured on muscle cells were the same as those in a 20% protein solution. Therefore, in conformity with the data on macromolecular solutions, it is reasonable to suppose that the decrease in intracellular water relaxation times is due to the existence of one or several phases of water having a short relaxation time [64]. Experimental results go against the idea of one-phase intracellular water which has therefore been completely rejected. Consequently, the debate on the exact number of phases remains open (Table 2). In 1969, Hazlewood [1] carried out a high resolution NMR study and made it obvious that there were at least two phases of ordered water in skeleton muscle. One was a major phase in which the molecules lost a lot of freedom in comparison with the pure water molecules. In the second and minor phase, mobility was less than in the major phase but greater than in a solid. Cooke and Wien [63] showed that the measured values of T_1 and T_2 were compatible with a two-phase model free water-bound water, the latter representing 4 to 5% of the total water. They further suggest that there are rapid exchanges between these two phases. Fung [65] also accepted this two-phase rapid exchange bemodel and found that bound water represented about 10% of the total water. In 1974, Hazlewood [66] opted for a three-phase model based on the study of muscle T_2, where one of the phases had a T_2 too short to be detected by present measurement techniques.

The definitions of water as more or less "free" or more or less "bound" ("ice-like", "crystalline", "adsorbed", "ordered") are ambiguous. In an attempt to clarify matters, Damadian [26] proposed the term "QUERP water" (Quick Endocytic Relaxing Pulse) to define the phases of intracellular water based on NMR measurement of T_1 and T_2. This proposition did not catch on. It had the advantage of defining the concept by the experimental technique used. The inaccuracy which still dominates most of the work an water in biological systems would thus have been avoided.

An important question remains unanswered. Are the relaxation times observed in the intracellular water the result of a rapid exchange between "bound water" with proteins (short relaxation time) and "free water" molecules with longer relaxation times? In the event of a positive response, the descending magnetization curve (FID, free induction decay) is a simple exponential after the equilibrium is altered. On the other hand, if slow exchanges are thought to have occurred, a descending curve which is the sum of several exponentials should be seen.

III.2. Application of a 2-phase rapid exchange model

In an attempt to classify the different variations described above, we decided to use the two-phase rapid exchange model (Fig. 9) proposed by Fung [65]. The model only corresponds to what is voluntarily a very summary and often inaccurate representation of complex phenomena. Its advantage, however, is that it allows us

Table 2. — *Main models of water in biological tissue*

Number of phases in water	Type of exchange	
	Fast exchange	Slow exchange or no exchange
1	LING (1962) Contradicted by experimental results as this model led to a value of $\omega_0 \tau_C < 1$ for $T_1 \gg T_2$	
2	FUNG, PINTAR, COOKE, WIEN (1974): T_1 and T_2 exponential 2 phases: – free water (pure) – bound water with macromolecules having a longer T_2	COPE and HAZLEWOOD (1969) on striated muscle: T_2 not exponential
3	KOENIG, FRANKS (1972) 3 phases: – free water external hydratation shell – irrotationally bound water	HAZLEWOOD (1974) on striated muscle
n	PACKER (1977) – free water – n phases of bound water each with very different characteristics	

to reason on the basis of the different parameters likely to be involved in the physiological of physiopathological events we are trying to understand.

T_{1obs} = T_1 observed
T_{1f} = T_1 of free water
T_{1b} = T_1 of bound water
b = relative bound water content
p_i = parameter of cover of one molecule
c_i = concentration of molecule

Fung's model is expressed as follows:

$$T_{1obs}^{-1} = T_{1f}^{-1} + b\,(T_{1b}^{-1} - T_{1f}^{-1})$$

If the T_{1f}^{-1} which follows T_{1b}^{-1} is removed, we get:

$$T_{1obs}^{-1} = T_{1f}^{-1} + b\,T_{1b}^{-1}$$

now, $b = pc$.
Therefore, for i molecular types, we get:

$$T_{1obs}^{-1} = T_{1f}^{-1} + \sum_i p_i c_i T_{1bi}^{-1}$$

III.3. In vitro measurement of the 2-phase rapid exchange model

T_{1b} and b are two-phase rapid exchange parameters which can be obtained experimentally by the progressive lowering of temperature. T_{1obs} is the starting data obtainable by direct measurement. T_{1f}, representing free water, must be constant from one sample to another for the given temperature and frequency. In tissue, free water usually freezes at about - 10° C. The free water has become ice and its signal has disappeared. The relative bound water content (b) can be deduced from the difference in the signal emitted before and after freezing (Fig. 10 a). By measuring T_1 before and after freezing, T_{1obs} can be obtained for high temperatures and T_{1b} for low temperatures. If the temperature for measurement are chosen sufficiently close together, there is no need to correct the T_1 values according to their different temperature measurements. If the temperature measurements are greatly different, then the T_1 values for a same temperature have to be calculated (Fig. 10 b).

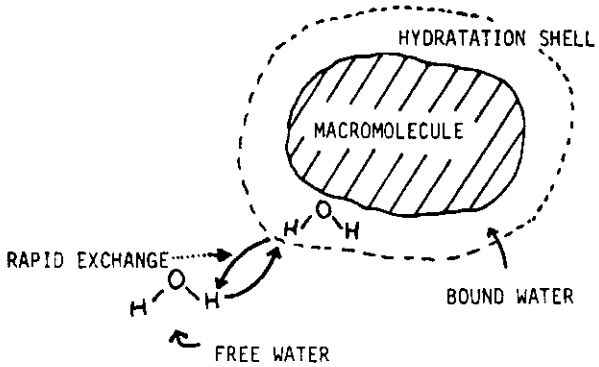

Fig. 9. — Two phases rapid exchange model of cellular water

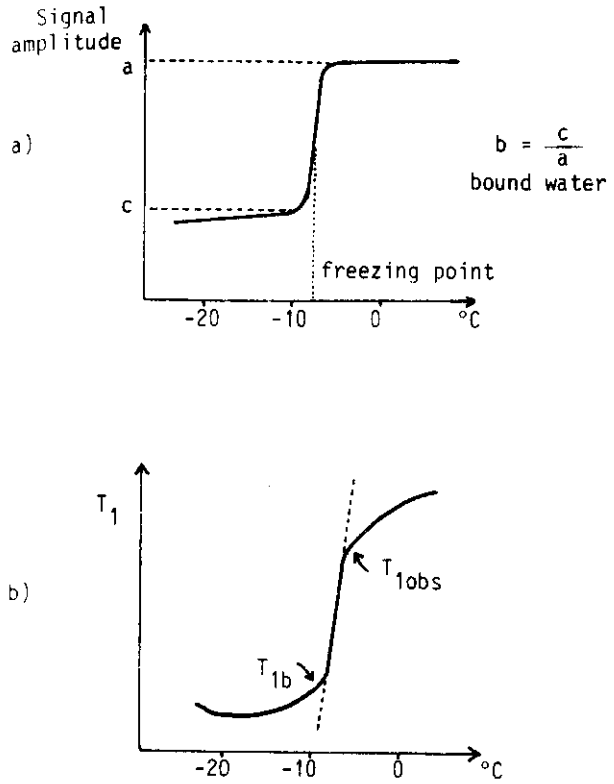

Fig. 10. – Experimental measurements of parameters b, T_{1obs} and T_{1b} in two phases rapid exchange model

a) b is measured by change in signal amplitude around frew water fusion point.

b) measurements of T_{1obs} towards - 5°C and of T_{1b} towards - 10°C

. Once the free water has been frozen, the signal emitted by certain macromolecular protons with very short relaxation times can also be observed.

T_{1f} can be deduced from parameters T_{obs}, T_{1b}, b and should not vary much from one tissue to another.

An experimental estimation of T_{1f} can be obtained for a protein solution. The concentration (c) dependant T_1 curve can be plotted and extrapolated for when c = 0.

Ratkovic and Sinadinovic [67] carried out a similar experiment on thyroid tissue according to tissue concentration in soluble proteins. When concentration was zero, a relaxation time superior to that of free water was found by extrapolation. The authors attributed this mainly to the membrane hydratation shells. This is a good illustration of the fact that, in the two-phase model, parameter b can be separated into two units i and j:

$$b = \sum_c p_i c_i + \sum_j p_j c_j$$

Unit i is likely to vary depending on cellular metabolism. Unit j corresponds to the minimum of constant molecular structure whilst the cell is alive.

Furthermore, the total water content can be measured by dehydrating the sample.

As an example, the following results were obtained from measurements made on a bovine serum albumin solution (BSA) and rat liver tissue (see Table 3).

This effort to discriminate between the different parameters seems indispensable if one wants to connect the variations to their biological causes and use proton NMR images as much as possible.

Table 3

	BSA solution		Rat liver tissue	
	10 MHz	90 MHz	20 MHz	90 MHz
T_1 measured	640 ms	800 ms	180 ms (– 9°C)	272 ms
T_{1b} measured	82 ms	160 ms	40 ms (– 12°C)	138 ms
b measured	6%	6%	14%	9%
T_{1f} estimated at transition	1130 ms (– 5°C)	1080 ms	400 ms (– 10°C)	310 ms
c	15%	15%	–	
p	0.4	0.4		
H_2O % total water content			67%	

III.4. Different biological causes of variations in the 2-phases model

We can now connect the biological variations described above to the different parameters they are likely to affect. One then discovers that most have multiple causes and that they do not specifically indicate the presence of a malignant tumor. Table 4 gives several examples (see Table 4).

The question then is whether the overall variation in mean relaxation time observed is useful in diagnosis. In most cases, several parameters will change simultaneously but they will not all have the same importance for T_1 and/or T_2 changes.

Conclusion

In conclusion, all physicians who interpret NMR tomography images must have a sound knowledge of the causes for fluctuations in whatever parameter is under consideration. The anatomical image may well seem satisfactory, but, unless each image is closely studied as to the exact nature of the contrasts which appear on it, the importance of NMR as a source of physiological and biological information will be lost to view.

When a major hydric variation (e.g. oedema) is the dominant indication of the physiological event to be detected, interpretation will be easier. When less perceptible alterations are sought (e.g. variations in b likely to express cell kinetics), the difficulty will be to derive information from fluctuations in water content which may well hide such alterations. This is where NMR enters into its own as it can modify the parameters measured by varying the pulse sequences. In order to do this, however, NMR must be accepted as an imaging technique different from the others. It is a biochemical technique capable of transforming minute physiological alterations into an image. To do this, it must be possible to obtain the NMR of nuclei other than proton, and to link *in vivo* NMR machines to more "anatomical" techniques by means of a system allowing data processing and images from different sources to be superimposed. Such a technique would then be highly original and would complete but not compete with tomodensitometry.

Table 4

		Variations specific to cancer or having great diagnostic or prognostic importance	Non-specific variations
T_1	b	Physico-chemical structure and environment of the molecule [4, 5, 26, 30, 31, 68]	
T_1	b		Oxygenation (death, ischemia, necrosis) Paramagnetic contrast media [23, 32, 69-72]
b	p	Macromolecular transconformations (e.g. chromatin condensation) [34, 44, 49, 50]	
	c		Overall variation of water content [3, 22, 23, 60] Proteinic synthesis [23, 32, 50] Macromolecular degradation or release [67]

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THE NMR PROTON RELAXATION IN BIOLOGICAL FLUIDS: A GOOD WAY TO IDENTIFY PRECISELY HEALTHY OR PATHOLOGICAL STATES

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Summary. — *A new approach of medical diagnosis by NMR is proposed and applied to a physiological fluid. It is well known that such fluids are relatively easy to study by NMR techniques, because they are mainly aqueous solutions of mineral ions and of macromolecules in the presence of suspended cells. If the extraction is correctly done, we have similar properties* in vivo *and* in vitro. *A given fluid generally has a well defined composition for a large healthy population, and the small qualitative or quantitative deviations show a weak influence on the relaxation properties. We should also mention that a given pathology can show qualitative changes of composition in direct connection with the evolution or with the seriousness of the pathology.*

We used an experimental procedure capable of precisely describing a given healthy physiological fluid, a pathological state of this fluid and the level of the latter by NMR relaxation times T_1 *and* T_2. *An appropriate strategy is developed in several steps precisely described in this paper. The particular physiological fluid explored is the amniotic fluid and its pollution by meconium (mainly polymucosaccharides) in foetal distress, in the last weeks of pregnancy.*

Some final remarks are made about the application of this procedure to other physiological fluids or soft tissues.

Riassunto. — *Viene qui proposto un nuovo approccio diagnostico, che utilizza la Risonanza Magnetica Nucleare per l'analisi di fluidi fisiologici. E' ben noto che questi fluidi possono essere studiati facilmente con tecniche NMR, perché consistono essenzialmente di soluzioni acquose di ioni minerali, di macromolecole e di cellule in sospensione. Se l'estrazione è eseguita correttamente, le proprietà* in vitro *ed* in vivo *sono molto simili. Un dato fluido ha in generale una composizione ben definita per un grande numero di individui sani, e le piccole deviazioni qualitative o quantitative mostrano solo lievi effetti sulle proprietà di rilassamento Si deve anche menzionare che una data patologia può essere associata a cambiamenti qualitativi e quantitativi di composizione, in diretta relazione con l'evoluzione della patologia stessa.*

E' stato qui usato un procedimento sperimentale capace di descrivere con precisione le proprietà di rilassamento magnetico nucleare di un dato fluido fisiologico in individui sani e in individui affetti da alcune patologie, a vari livelli di gravità. In particolare, è stato preso in considerazione il liquido amniotico e ne sono stati studiati gli stati di inquinamento da meconio, che si possono associare a casi di anomalie fetali nelle ultime settimane di gravidanza.

Vengono infine considerate le possibilità di applicare questo procedimento ad altri fluidi fisiologici o a tessuti molli.

Introduction

Since Damadian's discovery in 1971 [1], proton relaxation of physiological water has been largely investigated for medical diagnostics: its importance is crucial in spin imaging, and its possibilities seem very promising in the identification of pathological tissues.

In the present state of the development of research in this field, we think that more information can be extracted from relaxation measurements. In spin imaging, the intensity at each point is often a complicated function of M_0, the magnetization of protons, and of T_1 and T_2, their relaxation times. The significance of the images obtained is thus not clear and their interpretation is difficult. Measurements of T_1 and T_2 in soft tissues, which normally are inhomogeneous media, give "mean" values, generally deprived of any biochemical meaning. Moreover, the amplitude of the applied field H_0 is generally arbitrary or chosen without reference to the often sensitive dispersion of the relaxation times.

We propose here a more rigorous treatment of the relaxation of water protons in physiological aqueous solutions. After a brief survey of the physical principles of the involved parameters, this treatment is applied to a physiological fluid, namely the amniotic fluid (A.F.) of pregnant women. We are focusing on the

following problems and purposes: identifying pathologies of this fluid; applying the same procedure to other fluids and to soft tissues; obtaining a better interpretation in NMR imaging.

Our investigations on physiological fluids show that proton relaxation measurements in an appropriate H_0 field range can enable one: to distinguish different healthy tissues; to follow the evolution of a given tissue; to identify a particular pathology in a given tissue; to evaluate the degree of injury in this last case.

The relevant parameters in this approach are the magnetization M_0 of the nuclei in the applied field H_0 and the relaxation times T_1 and T_2, measured, if possible, in a large range of H_0 values: M_0 is directly connected to the abundance of water in the explored volume; the dispersion of the relaxation times gives the correlation times τ_c of the medium and the rate of energy exchange for each of the dynamical processes, precisely characterized by one correlation time.

The experimental procedure used will be given with reference to a specific case: the pollution of the amniotic fluid of pregnant women by meconium (faeces of children not yet born) in the last weeks of pregnancy, in the case of foetal distress. The main steps of that procedure will be developed for this example.

I. Experimental methods

I.1 Physical principles

Measurements are done on physiological fluids which are normally aqueous solutions of mineral ions, organic molecules, proteins and other macromolecules with some suspended material (e.g. cells). By proton NMR experiment, we detect a signal which is a function of M_0, the magnetization in a given field H_0, T_1 and T_2, the relaxation times in connection with energy exchanges between a given proton and its surrounding. T_1 is characteristic of the energy exchange between spins and lattice, while T_2 is connected with the life time of a given proton in a given energy state. These two relaxation times are, then, very sensitive to the microscopical motion of the molecules whose protons are studied (specifically, the water molecules of the solvent) or possibly to chemical exchanges in the system. All these dynamical transformations are characterized by correlation times τ_c, generally very small compared to the relaxation times. Consequently, in a liquid sample we normally observe only one relaxation time T_1 and one relaxation time T_2, which are the weighted means of local relaxation times in different possible states (different environment, mobility) of water molecules [2]. For example, in a solution of proteins, free water and water bound to protein molecules.

In the more frequent cases explored by our Group, the dominant relaxation mechanism is the dipolar magnetic interaction between the two protons of the same water molecule. In this kind of situation, T_1 is mainly sensitive to exchanges characterized by small correlation times ($\tau_c^{-1} > \nu_0$, the Larmor frequency) and T_2 is sensitive to all dynamical exchanges, the largest contribution being that of longer correlation times. The changes occurring in T_1 and T_2 when H_0 is changed, are known as relaxation dispersions, normally important for T_1 but smaller for T_2. Note that for water solutions at pH = 7, a dispersion near $\nu_0 \cong 1$ kHz is observed [3] (Fig. 1).

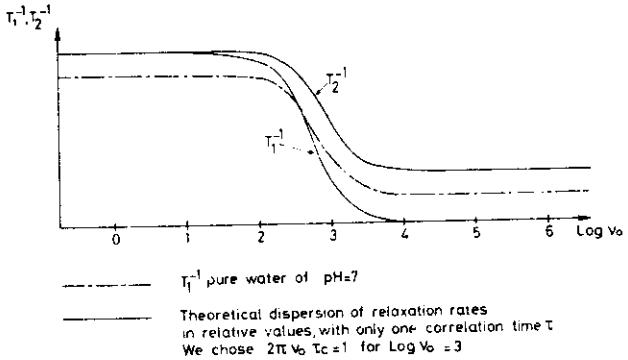

Fig. 1. – Dispersion of relaxation times.

The effect of the dissolved or suspended material on relaxation times and their dispersion with H_0, gives the opportunity to identify the dissolved material both qualitatively and quantitatively.

These phenomena are the foundation of the diagnostics of physiological fluids by NMR.

I.2 Experimental procedure

Measurements of equilibrium magnetization M_0 and of relaxation times T_1 and T_2, are made by three main techniques:

a) in the conventional H_0 range ($H_0 > 1$ KOe), T_1 is measured by inversion-recovery method and T_2 by Carr-Purcell-Gill-Meiboom sequence [4, 5]. M_0 is directly related to the area of the resonance signal, in the absence of saturation effects;

b) in the low frequency range, the analysis of the decay envelope of the free induction signal, obtained after prepolarization [6] in a large, perpendicular magnetic field, gives T_2 (see ref. [7] for the conditions required to have a correct T_2 value). The evolution of the maximum amplitude of the free induction signal with the duration of the magnetization gives an exponential curve from which T_1 may be evaluated. This procedure gives T_2 in the precessing field (here, the earth field) and T_1 in the polarizing field (~ 100 Oe).

The block diagram of this apparatus is given in Fig. 2;

c) the variation of T_1 with the amplitude of the H_0 field (relaxation dispersion) was determined in the following conditions:

- in a relatively small range of H_0 values (50 - 300 Oe), by changing the amplitude of the polarizing field in the free precession experiment;
- between 1 to 100 MHz by NMR in a conventional electromagnetic;

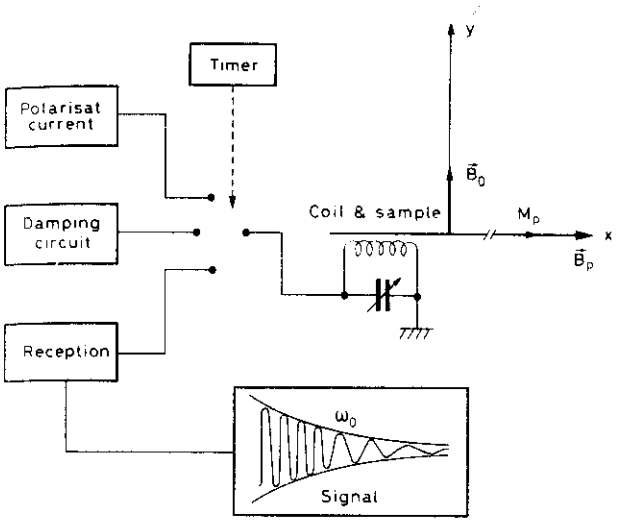

Fig. 2. – Block diagram of free precession apparatus

– in the largest ν_0 range, by using a field-cycling spectrometer, able to give T_1 between 20 Hz and 50 MHz.

In the following, measurements made by V. Graf and F. Noack with their home-made dispersion spectrometer, are described [8].

Note that this dispersion curve is able to give the values of correlation times τ_c encountered in the fluid, and the corresponding energy exchanges.

Measurements of T_1 and T_2 in the lowest field range are able to give an estimation of the largest τ_c encountered ($\tau_c > 10^{-2}$ s).

II. The steps of the procedure

II.1 Is it necessary to use NMR diagnostics?

The degree of pollution of the amniotic fluid by meconium is important to know in the case of foetal distress. Two methods are normally used [9]:

– an optical, purely qualitative method, the amnioscopy (estimation of the opacity of the A.F.) which is not always possible to use and generally unpleasant for pregnant women;
– an analytical method. After extraction by amniocentesis and preliminary filtration and/or centrifugation, the A.F. is spectrophotometrically explored [10]. A "meconial index" can be determined which is a guide mark and not a quantitative measurement.

The use of NMR gives three possibilities:

– a distinction of the polluted A.F. from the healthy one (different chemical composition) by change of relaxation times and correlation rates;
– an estimation of the meconium present, because relaxation times decrease drastically with the content of polluting material (all healthy A.F. have similar composition qualitatively and quantitatively);
– *in situ* measurements. There might be in fact not yet known injuries induced by using low steady or variable magnetic fields.

II.2 Biochemical analysis

During the last weeks of pregnancy the A.F. is mainly a dilute solution of serum proteins in the isotonic fluid. The concentration is normally in the range 3 to 0.5 g/l. A typical value is ~ 1.5 g/l. At the body temperature, these concentrations give for T_1 and T_2 measured in a given field, values concentrated in a very small range, which allow one to define the "healthy" A.F. If a pollution by meconium is present, intrauterine movements of the foetus are able to roughly dissolve the meconium in the A.F. The observed concentrations of meconium in the A.F. on a large sampling, typically range from 1 to 40 g/l. It should be mentioned that meconium is mainly (80 % or more) constituted by mucopolysaccharides (highly polyionic macromolecules), which have a large effect on the relaxation times of the solvent water protons. It is important to identify low concentrations of meconium, generally not seen by obstetricians.

II.3 Preliminary experiments on healthy AF and meconium solutions in the pathological range

Measurements of T_2 on healthy A.F. show that the dispersion of values is very small and related to the low content of serum proteins which constitute the main dissolved components. Typical values are 2.4 ± 0.45 in the MHz range (1-20 MHz) and 2 ± 0.59 in the earth magnetic field (2 KHz). Measurements on solutions of meconium in isotonic aqueous solution (8 g/l NaCl) show an important change of T_2 values, both in the MHz range as well as in the earth field with a monotonic decrease of T_2 with the increase of the concentration. Typical values for meconium concentration up to 40 g/l are (in all ranges of H_0 values) 1.8 s to 0.2 s. These preliminary experiments lend themselves to a) define an easy diagnostic test of the meconium pollution; and b) identify the possibility of estimating the meconium content by the variation of T_2 with concentration (Fig.3).

A precise study of the dispersion of T_1 in a very large range of Larmor frequencies (typically 20 Hz - 100 MHz) [11] gave a good confirmation of the preliminary experiments, with the additional possibility of determining correlation times and each corresponding energy exchange.

We can summarize these results on T_2 as follows (Fig. 4): in all the investigated fluids, a dispersion step is observed near $\nu_0 \approx 1$ KHz, typical of pure water at pH = 7; the A.F. as well as meconium solutions show several dispersion steps in the 10 KHz – 10 MHz range. The correlation times have very similar values, but the energy exchanges associated with each τ_c are very different.

The main step in the A.F. is in the range of 1 KHz and for meconium solution in the range of 100 KHz.

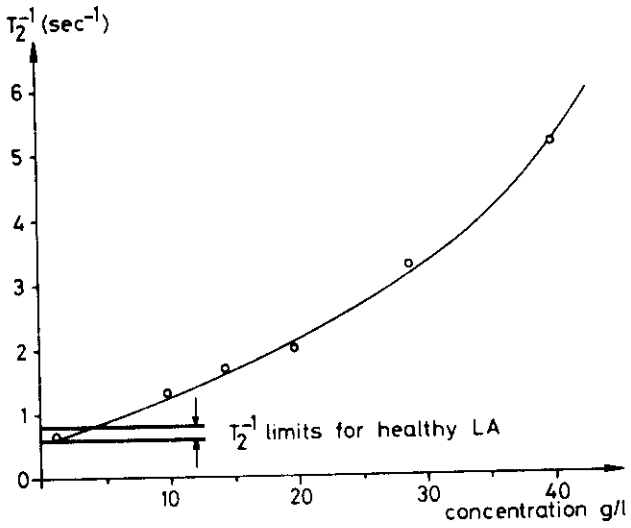

Fig. 3. – Evolution of T_2^{-1} of A.F. with increasing meconium concentration for $\nu_0 \approx 2$ KHz (at pH = 7).

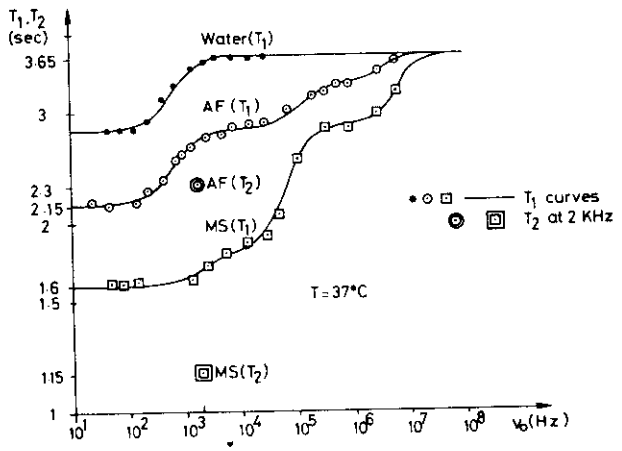

Fig. 4. – Dispersion in a very large Larmor frequency range of relaxation times in pure water(at pH = 7), in healthy A.F. and in a meconium dilute solution (MS).

In the very low field range, $\nu_0 \leq 1$ kHz, we note a similar value for T_1 and T_2 (~ 2.2 s) for the healthy A.F. but a large difference between T_1 and T_2 in the meconium solutions. This difference seems to be connected with a large correlation time ($> 10^{-3}$ s), as was also observed in other polysaccharides [12]. The interest of this very low field step of dispersion is the possibility of discriminating the meconium pollution (for which the step is present) from the other possible pollutions (blood or its degradation products) for which no dispersion step was observed in this range. It should in fact pointed out that a) when the pollution is certainly due to the meconium, T_2 measurements are able to detect such a pollution and evaluate the content of meconium in the A.F.; b) when other pollutions seem possible, a measurement of T_1 in the low Larmor frequency range ($\nu_0 < 500$ Hz) is able to discriminate blood from meconium pollutions: in fact, if $T_1 \approx T_2$ we can conclude that we have a blood pollution; if instead $T_1 > T_2$, we have a meconium pollution. In the latter case the mean ratio T_1/T_2 is ca. 1.4. We are able to study pollution of A.F. by meconium in real pathological cases.

II.4 Measurements on a very large sampling of healthy or polluted A.F. in order to evaluate the viability of this approach

Sampling was made for measurements: a) of T_2 in the low field range (typically $H_0 \cong 0.5$ Oe) in view of topical NMR by a free precession NMR spectrometer [5]; b) of T_1 and T_2 in the conventional field range ($\nu_0 \approx$ 10-20 MHz), in view of the identification and an estimation of meconium content in a spin-imaging scanner able to measure T_1 and T_2 in a steady magnetic field $H_0 \geq 10^3$ Oe.

The results of such measurements were the following:

i) the good ability of T_2 measurements in the low as well as in the high frequency range to identify a pollution of the A.F. by meconium in the last weeks of pregnancy;

ii) the ability of T_1 and T_2 measured together in the very low frequency range, to discriminate between blood and meconium pollution.

Works are in progress to identify blood pollution of the A.F. in the frequency range of scanners as well as other possible pollutions of the A.F.

II.5 In situ *measurements*

Two situations were explored:

- topical NMR in the low frequency range by free precession experiments (work done *in situ* by our Group) [12];
- relaxation time measurements with a spin imaging apparatus (preliminary *in vitro* experiments).

In the first case, the recorded signal not only reflects the modifications undergone by the proton magnetization of the A.F. but also contains information from other tissues in the explored area and noise. The recorded signal is *not* an exponential curve (Fig. 5). In order to determine the initial amplitude of the signal and the relaxation time T_2 of the A.F., the analysis of the A.F. exponential curve is made by an appropriate program of calculation [14]. Works are now in progress in order to refine such algorithms in view of their reliability, quantitative precision, speed and simplicity of use. The presently used program normally shows two exponential components of which only the A.F. exponential shows a large amplitude. We made experiments on about fifty pregnant women in the last stage of pregnancy; the results obtained show that:

a) a monotonic increase of the relaxation time T_2, as measured in the earth field with the normal evolution of the pregnancy. It is in fact well known that the healthy A.F. is mainly a dilute solution of serum proteins. The dilution increases with time: 5 to 7 g/l

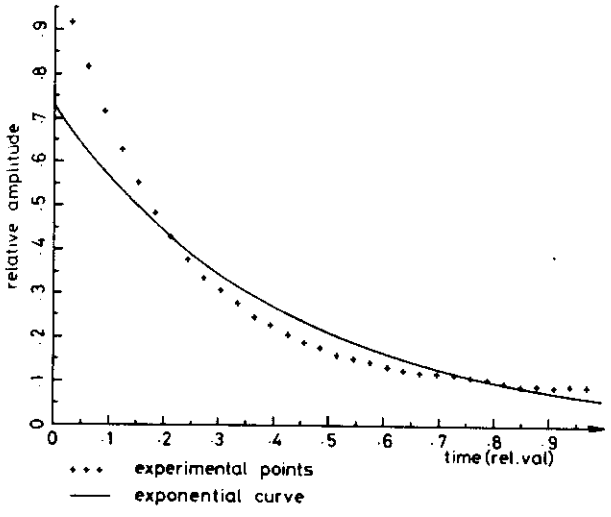

Fig. 5. – Non exponential record in free precession "*in situ*" experiments.

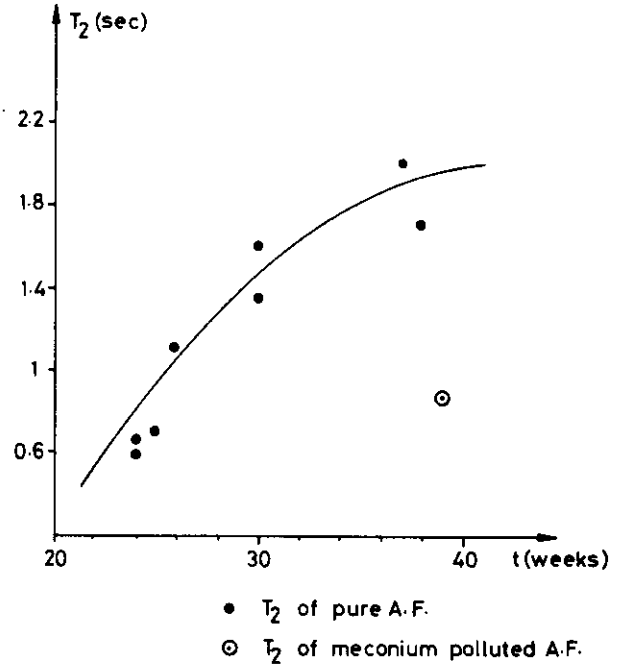

Fig. 6. – Evolution of T_2 of A.F. in last weeks of pregnancy. Effect of meconium pollution.

at 24 weeks to 1.5 g/l at 39 weeks (end of pregnancy) and consequently we observe an increase of T_2 [15];

b) if near the end of pregnancy we have a pollution of the A.F. by the meconium emitted by the not-yet-born child, T_2 shows a decrease which can be very important. In the *in situ* analysed case, this relaxation time dropped from 1.8 s to 0.8 s. An analysis made after the delivery, showed a concentration of meconium of 8 ± 2 g/l, in good agreement with the observed relaxation time value (Fig. 6).

In the particular case of spin imaging, working in the MHz range, but able to determine T_1 and T_2, we observed that T_2 is also a good parameter to clearly identify a pathological A.F. The precise identification of the polluting agent requires other measurements.

Conclusions

We present with some details the procedure used to obtain a clear determination of the kind of A.F. pollution and the contents of the polluted agent. The results can be used for a correct and quantitative diagnostic test of the pollution of the A.F. by the meconium. In addition the results show that a similar procedure may be used to study: other pollutions of the A.F.; other physiological fluids and their pollutions; possibly, some soft tissues.

Our laboratory is now active in the exploration of other physiological fluids (such as blood, saliva, urine, pleural and peritoneal fluids, etc.) and their important pathological states. The extension of this procedure to soft tissues requires more sensitivity and another set of relaxation time measurements which cannot always be obtained by free precession experiments, but rather by other NMR techniques in a higher field range.

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RELAXATION MEASUREMENTS IN EXCISED AND PERFUSED ORGANS

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Summary. — *The proton relaxation of freshly excised tissues and of perfused organs (kidney and heart) under different conditions of flow rate and osmotic pressure was studied, in order to assess functional significance of proton NMR signals emitted by the tissue, in the imaging experimental conditions. Preliminary results are reported in this work. The decays or recoveries of the magnetization have been obtained with a device allowing the automatical recording and processing of the free induction decay (FID) produced by the suitable pulse sequences. The longitudinal magnetization recovery curves are generally mono-exponential, but the transversal decay curves appear bi-exponential. The components of decay T_1 profiles have been assigned to water cellular compartments in which phases are in rapid exchange. The fast T_2 component would be due to intracellularly bound water and the slow one to the extracellularly free water. The changes of T_2 governed by the osmoticity and flow rate of perfusate seem to prove that the transversal relaxation is a more suitable probe in order to provide functional information on tissue through the structural states of the cellular water.*

Riassunto. — *E' stato studiato il rilassamento protonico di tessuti escissi di fresco e di organi perfusi (rene e cuore) in varie condizioni di flusso e di pressione osmotica, al fine di valutare il significato funzionale dei segnali NMR del protone nel tessuto in situazioni sperimentali simili a quelle realizzate nella tomografia. Vengono presentati i risultati preliminari di questo studio. Il decadimento o il ritorno all'equilibrio della magnetizzazione sono stati ottenuti con un apparato sperimentale che consente la registrazione e l'elaborazione automatiche del segnale di decadimento libero (FID) prodotto da opportune sequenze di impulso.*

Le curve di ritorno all'equilibrio del componente longitudinale della magnetizzazione sono in generale monoesponenziali, mentre le curve di decadimento trasversale appaiono bi-esponenziali. I componenti dei profili di decadimento T_1 sono stati identificati con compartimenti acquosi cellulari in cui le fasi sono in rapido scambio. Il componente veloce del decadimento T_1 è identificato con compartimenti acquosi cellulari in cui le fasi sono in rapido scambio. Il componente veloce del decadimento T_2 è identificato con l'acqua intracellulare legata e il componente lento con l'acqua libera extra-cellulare. Il fatto che i cambiamenti di T_2 siano regolati dai valori della pressione osmotica e della velocità di flusso del perfusato sembra indicare che il rilassamento trasverso sia più idoneo del rilassamento longitudinale a fornire informazioni sugli stati strutturali dell'acqua cellulare e quindi sullo stato funzionale di tessuti ed organi.

Introduction

The contrast in the proton NMR imaging of the living tissue arises from the different parameters which govern the signal amplitude: the proton density, ρ, the relaxation times T_1 and T_2 which characterise the dynamical structural environment, the self-diffusion coefficient D, and the flow rate v, which characterise the proton mobility. It is well known that the contribution of each of these parameters can be selectively enhanced by manipulation of the r.f. pulse sequences in order to produce images contrasted in proton densities, or in T_1 or T_2 and to obtain rheological images [1, 2].

Since water contains 70% and fat 16% of the protons in the tissue, proton NMR imaging provides a well suited probe for soft tissues.

A high resolution imaging method requires a molecular probe which is abundant in most of the organs and which possesses noticeable differences which can be responsible for the contrast. Such an abundant probe, however, cannot *a priori* play a specific biological role at the molecular level. In this sense X-ray absorption by electrons in matter has a high resolution imaging capability but a poor physiological investigative power, unless assisted by the invasive introduction of radio-opaque contrast media.

Water is the principal component of living tissue and is the medium in which most of the body chemistry and metabolism takes place. The body fluids are the carriers

of biomolecules to their active sites in living cells. In this sense they are often considered to have only a secondary role [3]. By its abundance and its role, mobile water reveals itself as a powerful probe for high resolution soft tissue imaging by NMR.

Early *in vitro* studies indicated that T_1 and T_2 of the tissue depend on structure and physiopathological states. Similarly, the image contrast monitored by T_1 or T_2 permits better visualization of different tissues in organs and assessment of pathological state, e.g. tumours, edema, infarct, sclerosis, etc.

At the present state of the NMR imaging art, attempts are being made to try to assess the clinical significance of the changes of the contrast factors T_1, T_2, ρ and v in the images of patients. Until the event of NMR imaging there was no way to study the fine structure of water in relation to the biological state of tissue *in vivo*.

To help to understand the physiological basis of this new semeiology, experimental studies were planned on normal and pathological tissues *in vitro* and on freshly excised organs perfused under physiological conditions. Two organs required for the transport of body fluids were studied: the kidney for water filtration and resorption, and the heart for muscle and pump functions.

Materials

An experimental imaging device for small objects has been home-built from components of Bruker Company [4]. The magnet is ironcored with a 18 cm gap. The magnetic field is 5 KG which corresponds to a frequency of 20 MHz for the ^1H resonance; homogeneity is 10^{-5}. The gradient system is composed of three coils providing linear gradients of 0.5 – 1.0 G. cm^{-1} in the three rectangular directions Ox, Oy, Oz.

The spectrometer is a Bruker CXP. 100b. A microprocessor assembled in our laboratory controls all the functions from gradient monitoring, to pulse sequence generation, to data collection transfer or visualization. The microprocessor is linked to a central Hewlett-Packard HP 1000 computer (192 K words - 16 bits - 50 M octets - 800 BPI taperecord). Visualization is displayed with a Barco CDTC 31 M. Biological samples are imaged by the zeugmatographic method [5].

Method

Measurement of the relaxation times: determination of T_1

T_1 is determined by the inversion-recovery pulse sequence $\pi/2 - \tau - \pi$, which is generated by a Rafi microprocessor, with τ varying from 25 msec to 1 sec by steps of 25 msec.

The free induction decay (FID) curves were automatically recorded on a Biomation 1010 wave form recorder (sampling time 0.4 msec, ADC 12 bits), linked to a HP 1000 computer in order to obtain T_1 from $M_\tau = \rho\,(1 - 2\exp(-\tau/T_1))$. The proton density, ρ, was obtained by extrapolation to the origin (total recording time, five minutes).

Determination of T_2

T_2 was measured by the classical Gill-Meiboom sequence, $\frac{\pi}{2} - \tau - (\pi - 2\tau - \text{echo})_N$, with a separation of 8 ms between successive π pulses and a number of echoes, N, equal to 40. The echoes were recorded on a Biomation waveform recorder. The experimental curves were fitted by least squares to the equation $M_N = \rho\exp(-8N\tau/T_2)$ (total recording time, two minutes).

Organ preparation

Rats were anaesthetised with nembutal. The organs were quickly excised and stored on ice until measurements, which were performed at room temperature.

Kidney preparation: Wistar rats were anaesthetised with nembutal (0.1 ml/100g) on a heated surgical table and a cannula was inserted into the renal artery *via* the descending aortic vessel. The kidney was excised and gently placed on a small cylindrical cell. The organ was oxygenated under a pressure of 1 atm and perfused with physiological solution using a peristaltic pump. The cell is then placed in the imaging emitter-receiver solenoidal coil of 76 mm diameter and 2 cm thickness (3 turns).

Heart preparation: Albino Sprague Dawley rats (200 g) were anaesthetised with nembutal (0.1 ml/100 g). The hearts were excised and a cannula was inserted into the aorta. The cannula was connected to a peristaltic pump and perfusion pressure was monitored by a mercury manometer. For NMR measurements the organ was inserted into a probe tube (diameter 2 cm) surrounded with the emitter-receiver solenoidal r.f. coil. The physiological medium was a Krebs Henseleit solution (120 mM NaCl, 2.4 mM KCl, 1.2 mM KH_2PO_4, 0.5 mM K_2HPO_4, 1.3 mM $CaCl_2$, 28 mM $NaHCO_3$, 1.2 mM Mg SO_4, 11 mM glucose) to which 60 g/l of Dextran was added, A gas mixture 95%O_2 – 5%CO_2 was bubbled continuously into the perfusate maintained at 37°C. The flow rate was 5.5 ml/min the cardiac rythm was monitored by electrocardiography.

Results

T_1 and T_2 measurements in vitro

The organs were freshly excised and gently placed in a sealed tube to be measured at room temperature. The decay curves were fitted as single or bi-exponentials.

Experimental relaxation curves of some organs were better fitted with a bi-exponential decay. For some of the others, the initial part of the decay curve showed a fast component which could not be determined with sufficient precision and these curves were treated as

mono-exponentials. The relaxation times of the different organs are presented in Table 1.

Usually, only one relaxation time is described for T_1 in the literature, which corresponds to the slow components presented here.

In the case where only one relaxation time was taken either for T_1 or T_2, we have performed a non-parametric rank test of Spearman [6] in order to compare the following data:

1. our T_1 values with those obtained by Ling (at a frequency very close to ours [7];
2. our T_1 and T_2 values when the latter were fitted to a mono-exponential decay curve;
3. our T_1 values and the content in water of the tissues as, taken from Biology Data book, 1964 [8].

The rank test confirms a good correlation in the three cases at a 5% significance level.

The decrease of the proton relaxation times in tissues in comparison with pure water (by a factor of 2.5 to 23 for T_1 and of 5.8 to 24 for T_2) is the consequence of the restricted motion of water molecules, both due to their interactions with biopolymers and to the limitation of diffusion by membranes barriers or ordered water structure. The last two effects reduce the diffusion coefficient by a factor of approximatively 2 when compared to the value obtained for pure water or dilute electrolyte solutions.

The bi-exponential longitudinal magnetization recovery profile of the tissues implies that existence of more than one compartment, which differ in the overall proton relaxation times, with no or very low proton exchanges between them. The compartment associated with the shorter relaxation time could be the lipids; the other compartment should probably correspond to two water phases in rapid exchange, according to the fast exchange limit model of Zimmerman and Brittin cited by c.f. Hazlewood [9]. Hydration and solvation of extra-and intra-cellular water could correspond to these two phases, because the liquid-like phases tend to dominate the longitudinal relaxation rate, i.e. the more external hydration shells of bound water.

Concerning the transversal magnetization decay curve large deviations from the mono-exponentiality are generally observed; these can also be attributed, as for T_1, to compartmentalization. Generally four or five components have been described in the literature [10]. Our results do not contain the faster components in the microsecond and millisecond range, which correspond to mobile protons in lipid and macromolecules. They are not detected due to limitation in shortening the interpulse delay. Thus, the components observed by us concern only water protons. The fast component is due to intracellular bound water in rapid exchange with a free water phase. Since the solid-like structure phase contributes mainly to the transversal relaxation governed by local field inhomogeneity, this fast component mainly characterises the bound water phase. The longer component corresponds to the extra- and trans-cellular water, in which a liquid-like structure predominates. The percent of proteins with long T_2 found by us for some organs are larger than those found by other authors: more investigations are needed in order to explain these differences.

Kidney perfusion experiments

In order to understand the variations and heterogeneity of relaxation times in the tissues and to eventually evaluate their structural and functional significance, the NMR parameters, measured from excised organs were correlated with physicochemical factors known to affect the cells at the molecular level: pH, ionic strength, temperature, O_2 pressure, osmoticity and the flow-rate of the perfusion liquid. These experiments have been mainly performed on the rat heart: in addition, studies of renal clearance have been attempted with the use of contrast agents. Preliminary experiments from kidney are reported in Fig. 1. The kidney was perfused at the normal flow rate of 11 ml min^{-1} gr^{-1} and at a pressure of 84 mm Hg with the physiological medium containing manganous ions at the concentration of 5.10^{-2} M. The amplitude of the signal decreases immediately after the outset of the perfusion. After 8 min it reaches a constant level approximatively equal to 15% of its initial value. After 40 min, the kidney was washed with a

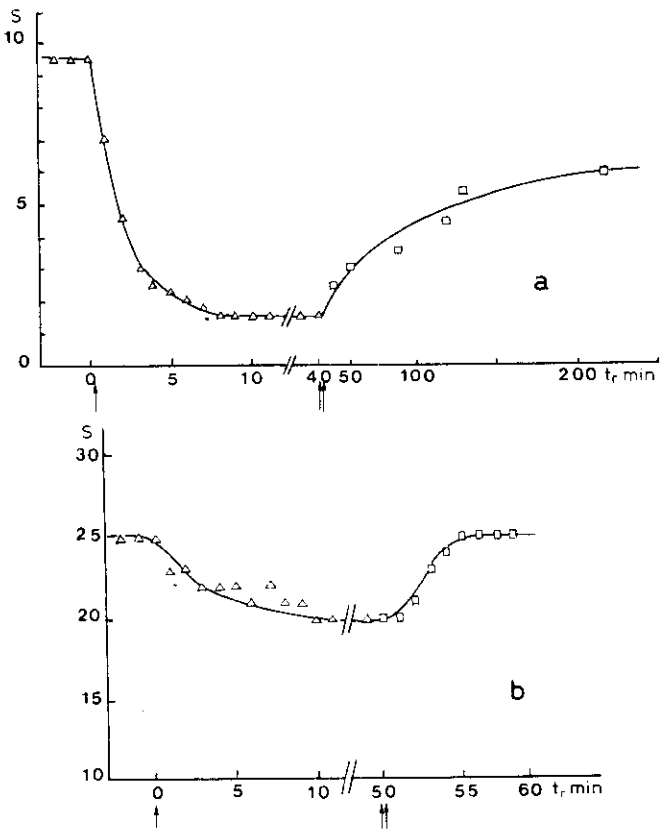


Fig. 1. — Kidney perfusion experiment: (1a) manganous ions effect. S amplitude of the signal (mixture ρ, T_2) in arbitrary units, t_r time of the perfusion (min). Perfusion with manganous ions solution 10^{-3} M (△) washing out with physiological solution (□). (1b) Iodine effect: perfusion with biodo 2-4-6-acetamido 5 N. Hydroxyethyl isophtalamic acid (30%) (△) washing out with the physiological solution (□).

Table 1. *Relaxation times of freshly excised organs*

ORGANS	T_1	T_2	Short T_1	% Short	Long T_1	% Long	Short T_2	% Short	Long T_2	% Long
STRIATED MUSCLE										
Skeletal muscle	570 ± 16	58 ± 6								
Intercostal muscle	483 ± 23	66 ± 10								
Diaphragm	619 ± 38	87 ± 15								
SMOOTH MUSCLE										
Ureter	634 ± 50	110 ± 13	131 ± 25	26 ± 7	746 ± 29	74 ± 7	32 ± 15	53 ± 4	173 ± 30	47 ± 14
Uterus	840 ± 40	125 ± 17	128 ± 36	7 ± 4	879 ± 76	93 ± 4	70 ± 20	57 ± 13	161 ± 40	43 ± 13
Trachea	694 ± 80	144 ± 15								
Oesophagus	645 ± 27	95 ± 9								
Stomach wall	478 ± 20	99 ± 13								
CARDIAC MUSCLE										
Ventricule	702 ± 45	114 ± 31					68 ± 22	68 ± 5	166 ± 34	32 ± 5
Auricle	779 ± 50	159 ± 23					46 ± 22	61 ± 20	286 ± 70	39 ± 20
NERVOUS SYSTEM										
Grey matter	636 ± 29	116 ± 17								
White matter	552 ± 54	104 ± 8								
Cerebellum	594 ± 23	105 ± 5								
Medulla oblongata	533 ± 29	96 ± 10	101 ± 26	7 ± 1	531 ± 21	93 ± 1				
KIDNEY										
Medulla	565 ± 68	98 ± 10	110 ± 10	21 ± 8	639 ± 57	79 ± 8	34 ± 11	59 ± 12	160 ± 13	41 ± 12
Cortex	434 ± 26	78 ± 12	167 ± 17	10 ± 2	510 ± 51	80 ± 2	67 ± 5	91 ± 2	299 ± 60	9 ± 2
Liver	339 ± 26	62 ± 4	159 ± 16	10 ± 4	329 ± 12	90 ± 4	53 ± 6	93 ± 4	155 ± 50	7 ± 4
Lung	636 ± 20	85 ± 11								
Spleen	569 ± 21	85 ± 12								
Heparinized blood	970 ± 56	245 ± 26								
Blood clot	590 ± 8	126 ± 17								
Serum	1608 ± 161	537 ± 46								

Table 2. *Spin-lattice, spin-spin relaxation time, and proton densities values of the perfused heart rat for high and low flow rate of the perfusion in function of the perfusion time. (Profiles of the T_1 and T_2 variations are shown in fig. 1)*

Flow rate ml/min	Perfusion time	T_1 s	Long T_2 s	Short T_2 ms	ρ_2^l (1)	ρ_2^S (1)	$\rho_2^l + \rho_2^S$ (1)
5.5	15	1.12	0.72	66	8.0	14.4	22.4
	30	0.97	–	–	–		
	45	1.23	0.82	73	12.0	16.7	28.7
	60	1.31	0.81	36	11.6	12.1	23.7
1.3	90	1.41	0.62	38	8.8	8.8	17.6
	105	1.40	0.60	39	9.0	11.8	20.8
	120	1.52	0.60	40	8.0	12.8	20.8
1.1	135	1.65	–		–	–	–
	150	1.37	0.60	38	8.8	7.2	16
	165	1.31		–		–	–
5.5	180	1.40			–	–	–
	195	1.54	0.80	32	8.8	11.6	20.4

(1) ρ_2^l and ρ_2^S are the proportions of proton density corresponding to long and short T_2 components, respectively.

physiological solution free of Mn^{++} ions. The amplitude of the signal increased due to the release of the paramagnetic ions, but did not reach its initial value, indicating a residual quenching, which is probably dependent on the manganese which is firmly bound to proteins (Fig. 1a). The quenching effect of manganese can also be seen on the corresponding NMR images (Fig. 2a). Because of this residual quenching and its toxicity, manganese does not seem to be appropriate for clearance studies. Alternatively, an iodine product (38% I_2), used as X-ray contrast agent, was tested as a quenching agent because of its quadrupolar moment. Effectively, a quenching effect was observed (Fig. 1b), but it was less pronounced than for the manganese ion; washing with the physiological solution fully restored the initial signal amplitude in a short time. The corresponding NMR image of the kidney shows this effect (Fig. 2b).

Heart perfusion experiments

The excised heart from Albino Sprague Dawley rat was perfused with the Krebs Henseleit solution as described in Materials and Methods. The perfusion flow rate was controlled by a peristaltic pump at values 5.5 ml/min and 1.1 ml/min during more than 3 hours. The perfusion experiment was divided into four periods, during which the flow rate was respectively adjusted to 5.5, 1.3, 1.1 and then again to 5.5 ml/min.

The relaxation times T_1 and T_2 as well as the proton density are reported in Table 2. The decay curves of the longitudinal relaxation remained mono-exponential. The T_1 values at high flow rate were lower than those at low flow rate, at the beginning of the perfusion; the increase of proton density observed at high flow rate cannot explain the T_1 shortening; this result could be due to a rise of the amount of oxygen delivered to tissue at higher flow rate. However the slight increase of T_1 observed after a long perfusion time could be due to edema formation (Fig. 3). On the other hand the transversal magnetization decay curves were bi-exponential. The two components corresponding to long and short T_2 decreased with flow rate. After a long time of perfusion at 1.1 ml/min., when the flow rate was restored to its initial value of 5.5 ml/min, the fast T_2 was not recovered, while the slow T_2 increased; this behaviour agreed with that of the slow T_1 value and was probably also a consequence of the edema formation. The proton density ρ_2 "short" remained constant. The total proton density (ρ_2 "short" + ρ_2 "long"), obtained from the T_2 measurement decreased with the flow rate, likewise the corresponding values obtained, within experimental errors, from T_1 measurements.

These observations confirm the T_1 insensitivity and the T_2 sensitivity to changes in the state of a muscle. Considering a multiphasic model with fast exchange inside each compartment, the increase of the T_2 can be explained by the release of bound water when the muscle is stretched at high flow rates. The fact that no variation of ρ_2 "long" was observed can be due to the small proportion of bound water in the compartment corresponding to the slow relaxation time T_2.

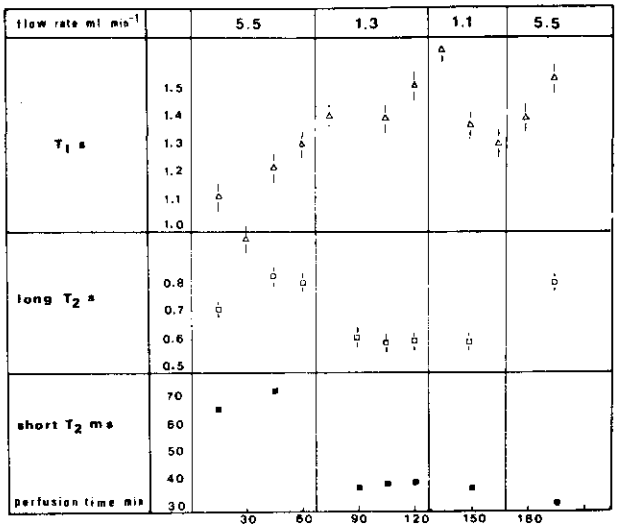


Fig. 3. Heart perfusion experiment. Influence of the flow rate. Variations of the relaxation times values for high and low perfusion flow rate in function of perfusion time.

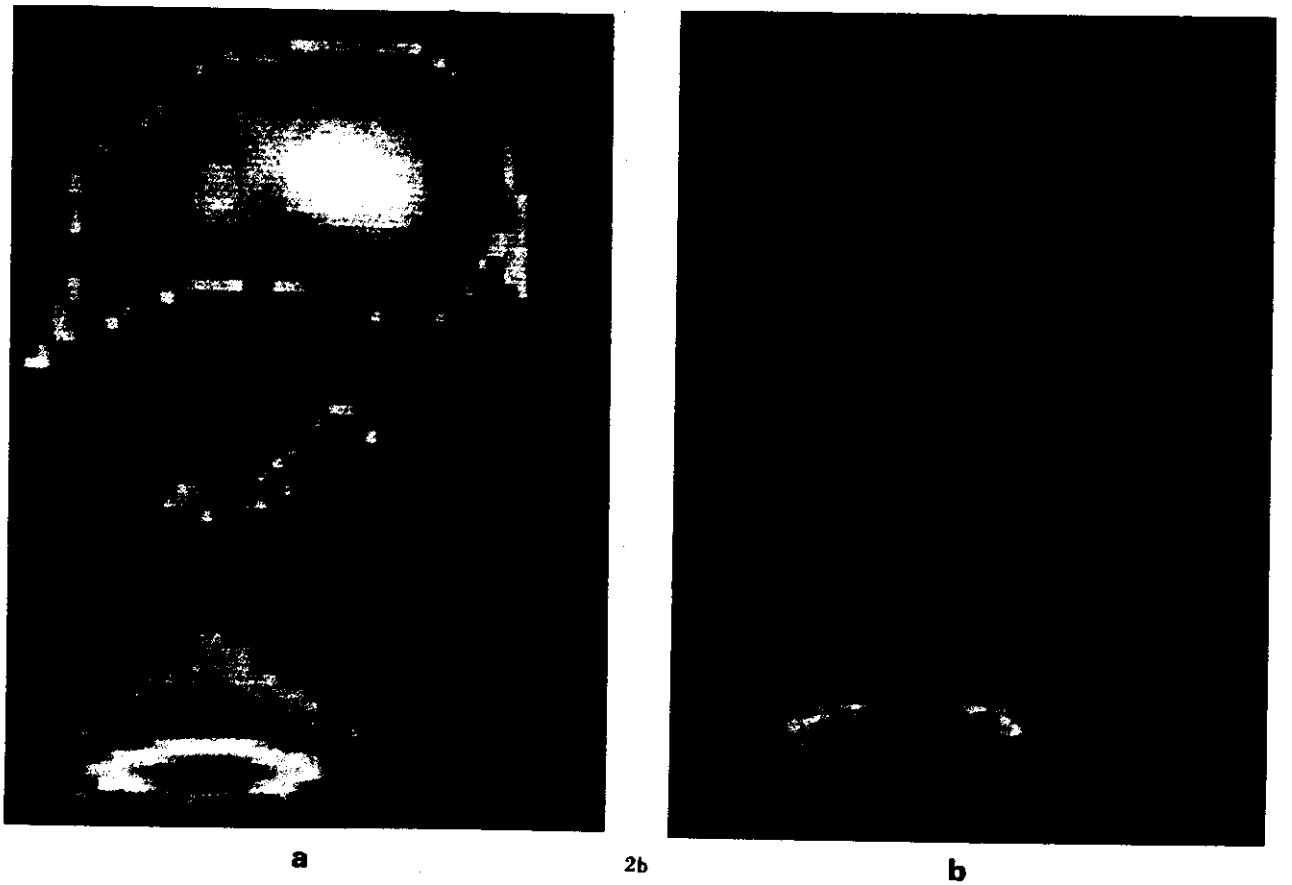
These results are in agreement with the increase of T_2 and the unchanged T_1 values reported by C.B. Bratton *et al.* [11] on the stretched and exhausted living muscle, as compared to the muscle at rest. These authors assume that the change in tension is associated with the release of about 20% of the bound water. This could result in a change in T_2 of the magnitude observed but would not affect, within the experimental error, the T_1 observed for a cellular compartment in which bound and free water phase are in fast exchange.

Influence of osmotic pressure

In order to study the free water phase, the heart was perfused with solution of different osmoticities adjusted by NaCl. Dextran was added to the perfusate to prevent edema formation.

The observation (Table 3 and Fig. 4a, b, c) that only ρ short follows the variation of intracellular water would imply that the short relaxation time observed in the T_2 measurement of the heart is related to intracellular water, which is reduced when the osmoticity of the perfusate is increased.

The T_1 values are independent of the osmoticity even when the intracellular water content decreases. The changes expected are probably within the limits of experimental errors. The short T_2 remains also independent of the osmoticity, but the long T_2 values are not in agreement with the tonicity effect on water content. However concerning this experiment a water flow governed by a dialysis effect on the echo amplitudes cannot be excluded and the T_2 determination could be biased [12].



Figs. 2a, 2b. – Images taken with a product contrast: 2a) manganese containing product; 2b) iodine containing product. In each set of figures:

a) Before the injection of the product

b) After the injection of the product.

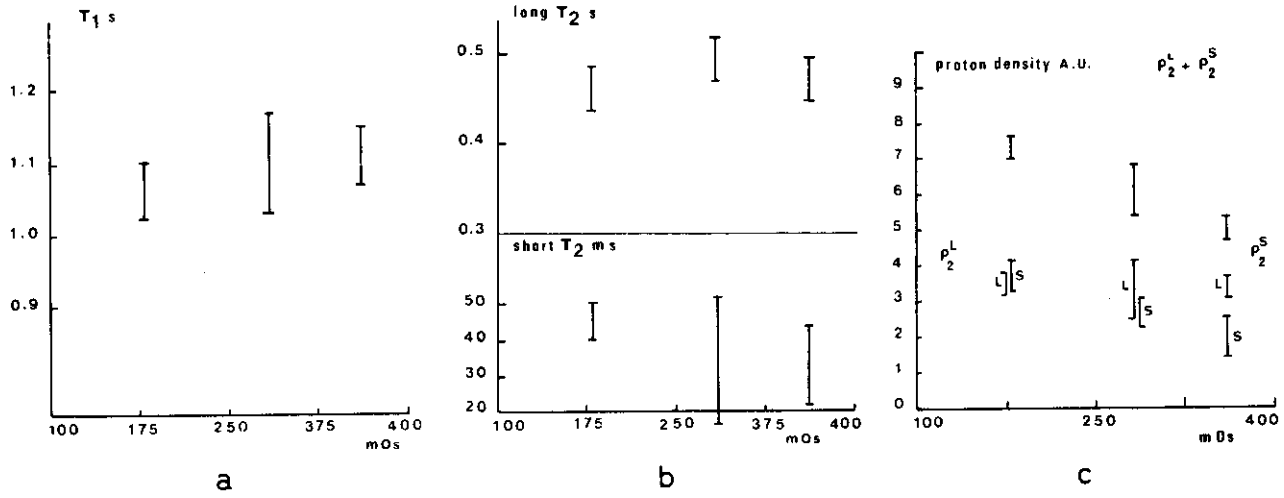


Fig. 4. Heart perfusion experiment: influence of the osmotic pressure. Mean values of spin-lattice (a), spin spin (b) relaxation times and proton densities (c) of rat heart in function of the perfusate osmoticity (calculated from Table 3 for each osmoticity).

Table 3. *Spin lattice, spin-spin relaxation time and proton densities values of the perfused rat heart for different perfusate osmolarities measured during the perfusion. The heart was perfused at a flow rate of 5.5 ml min^{-1} during about four hours.*

Perfusion Time min	Osmolarity (milliosmole)	T_1 s	Long T_2 s	Short T_2 ms	ρ_2^l %	ρ_2^s %
25	284	1.06	0.38	35	29	28
50	284	1.15	0.35	11	30	23
60	284	1.09	0.33	27	45	27
75	284	1.04	—	..	..	..
90	359	1.00	0.36	26	31	23
105	359	1.15	0.46	59	37	14
120	359	1.10	0.43	27	34	21
135	359	1.14	0.41	30	34	16
165	284	1.10	0.37	32	46	36
180	284	1.15	0.34	43	29	34
205	179	1.10	0.47	33	41	40
220	179	1.10	0.43	38	48	33
245	179	..	0.45	34	49	38
270	284	1.10	0.37	24	52	28

Discussion

The large number of publications [12-16] about relaxation behaviour of the proton in living tissues demonstrate that these phenomena are rather complex, and it appears that their biological significance is not yet properly understood. Due to the spatial and temporal heterogeneities of the tissue samples, analyzed magnetization decay profiles appear non exponential. In addition they are truly dependent, particularly for T_2, on the sensitivity of the device. With our system the T_1 decay curves cannot be resolved into more than two components which correspond, respectively, to the lipids and water tissular proton relaxation times (lipids and water represent two compartments with no or very low proton exchange between them). However, in the water compartment, characterized by the longer T_1, bound and free water phases coexist and are partially exchanged. However, the intra-cellular and extra- or trans-cellular water compartments are indistinguishable, because the corre-

lation times of the longitudinal relaxation times are distributed over a wide range, which can be characterized by a mean value only.

Concerning the transversal relaxation, the two components observed correspond only to the water protons due to the fact that the T_2 of the lipid protons in the millisecond time range cannot be measured with our device. The decay curves are resolved into two components which characterize two water compartments in slow proton exchange. The larger T_2 component is assignable to the extra-cellular water and the shorter to the intra-cellular water.

The different proportions of these two components of T_1 and T_2 are explained by the fact that the free water with the more mobile protons tends to dominate the longitudinal relaxation rates, while the bound water of the macromolecular hydration shell contributes mainly to the transversal relaxation rates [11]. In addition it is known that the long T_2 components are influenced by the longitudinal relaxation while the T_2 short components are mainly dependent on the local field inhomogeneity.

This simple interpretation helps us to interpret organ perfusion and particularly the cardiac muscle studies. Here T_1 reflects mobile free water and the short T_2 component reflects the more rigidly bound water. T_1 is a parameter which characterizes the water content of tissues and T_2 short a parameter which characterizes the structural bound water reflecting the physiological state of the muscle. The T_1 variation with the flow rate could be dependent on the oxygen supply, but after a long perfusion time this effect would be occulted by an edema formation. T_1 measurements are quite insentitive to osmoticity of the perfusate, in agreement with the fact that T_1 characterizes only the liquid, intra- and extracellular phases which are in rapid equilibrium. The liquid phase of the two extra- and intra-cellular compartments, in respect to the longitudinal magnetization phase, seems quite homogeneous.

T_2 short has been assigned to intracellular water and mainly to bound water, and long T_2 to free extracellular water. In our experiment the reduction of T_2 short is associated with a decrease of the flow rate; this fact could be due either to the stretching of the muscle with release of bound water or to oxygen concentration which decreases with flow rate. These experiments confirm that the cellular water repartition and its structural properties depend in a complex manner on many physicochemical factors like PO_2, osmotic pressure, and physiological factors, like stretching, exhaustion, etc.

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Discussion on experimental measurements in real tissues
Moderators: J. M. Lhoste and W. M. M. J. Bovée

DE CERTAINES – I have one remark to make on Dr. Bakker's paper. I think there is some problem with the mono–or multi–exponential curve in tissue samples. We are often confusing two different things: firstly, protons of water in tissue, and secondly, protons of macromolecules, especially fats and lipids. For instance, in breast tissues, we have seen Dr. Bovée's results and we have the same result from Canet and Escanye in Nancy at 20 MHZ. We have fat, and we have water of tissue. If we don't distinguish between macromolecule and water protons, it will not be possible to understand properly the variations of water in tissue, and therefore bound or free water. It is better to have a smaller sample to see if we have fat or if we have cells and try to see what is the role of protons of macromolecules and what is the role of protons of water. In my opinion, this is important for *in vitro* studies.

REISSE – I have a question for Dr. Foster. You have shown some values of T_1 obtained during *in vivo* and *in vitro* measurements, but do you know the precision of these data? You have not indicated the errors but you have given values with three figures.

FOSTER – The error on imaging results is something in the region of about 4% on most tissues, or perhaps I should say about 4% when we are looking at some of our phantom images. The variation on our *in vitro* measurements (those values were taken out of a paper we published some time ago) is in the region of 5 %. I'm afraid I must be less specific about the variation in our imaging measurements because it is tissue dependent and the tissue in general tends to vary.

REISSE – Is it possible to conclude from your results that NMR is not able to discriminate between rabbit and man?

FOSTER – Yes! It's just as simple as that.

ZIMMERMAN – Maybe I could also ask Dr. Bakker a question. I didn't catch the idea about what is filtered out by the band width of the receiver. Is that not the answer to the question of Dr. de Certaines?

BAKKER – Due to the incomplete inversion of the macromolecular magnetization by the relatively long 180° pulse and the slow reaction of the receiver, we did not observe the rapidly decaying FID of the macromolecular protons. The observed signal exclusively originated from the protons in the tissue water.

SEGRE – I would like to ask Dr. Bakker if he used the limit of confidence in the χ square test and the F test on 95 %, which is usually done, but is rather dangerous since the precision of T_1 measurements cannot usually be considered better than a 10 % confidence.

BAKKER – The χ^2 test was applied to evaluate the goodness of the fit of the uni and bi–exponential model at the 5 % significance level. To decide on the validity of adding exponential terms to the fitting function, an F test was applied. The 1 % level of significance relates to this test.

CHAMBRON – Dr. Foster, you have presented T_1 images of four abdomen slices, one at 1.7 MHz and the other at a higher frequency of 3.5 MHz, on which the blood vessels and their contents are clearly visible in front of the spine. It seems that the wall of the vessel is not resolved at low frequency in comparison with images produced at higher frequency. Are these differences a consequence of the dependence of the tissue T_1 values on the frequency or of a decreased signal–to–noise ratio?

FOSTER – You say that we can see the wall of the vessel at low frequency. It may look like the wall of a vessel, but I think that what you are getting are partial volume effects. As far as I know on those vessels the wall is so small that we wouldn't expect to be able to discriminate them anyway. The blood shows up very well because of simple flow effects.

DERBYSHIRE – A question for Dr. Bakker again. Did you see any evidence that you could not represent your spin–lattice relaxation by a sum of exponentials? The reason I'm asking this question is that, if one thinks of the work of Edzes and Samulsky on water in collagen, they saw that the spin–lattice relaxations of the water and collagen components started off separate, but after a spin exchange time they became the same. So one spin lattice relaxation curve was convex and the other was concave. Have you ever encountered anything like this?

BAKKER – Edzes and Samulsky did two types of experiments: one where both molecular magnetization and water magnetization were observed, and one in which only the water signal was observed. I think my type of experiment is the last one. According to Edzes and Samulsky cross–relaxation between the bulk of water protons and the bulk of macromolecular protons significantly perturbs water proton spin relaxation in tissues. Solution of the Bloch equations reveals that spin–lattice relaxation in either plase in described by a sum of two exponential decays, characterized by two apparent relaxation times T_1^+ and T_1^-. The results of our measurements are consistent with the cross–relaxation model when T_{11} and T_{12} are interpreted as T_1^+, and T_1^-.

ZIMMERMAN – I have a question for Dr. de Certaines. On which argument do you base your affirmation that systemic effects must have different causes?

DE CERTAINES – We essentially have some physiological arguments; we have in fact studied some physiological variations during the systemic effect in liver, spleen and in muscle, and there are different biological phenomena. For instance, in liver or in spleen there are cell divisions. In muscle you have hydro–electrolytic imbalance, and around the tumour you may have an inflammation due to immunological response. Sometimes the total water content varies greatly, sometimes it varies

little. I think there are many systemic effects and not just one. This is biologically normal.

ZIMMERMAN – My second question is addressed to Prof. Chambron. I suppose that your hypotheses about the difference in the supply of oxygen to tissues could be more easily verified by trying to keep the flow constant and to vary instead the oxygen flow derived from the tank.

CHAMBRON – Yes, Dr. Zimmerman, I agree with you, but if you keep the flow rate at a constant level during a longer experimental time in order to assess an effect of the oxygen concentration, you can observe changes in the results due to the fact that T_1 increases because of myocardial suffering.

PODO – Dr. de Certaines mentioned the existence of a French protocol. We are interested in knowing the criteria by which you defined this protocol, its history and if you can provide us with some documentation. Here in this meeting we are trying to do something like a European protocol, and if you already have a French protocol, then it is very interesting for us.

DE CERTAINES – Yes, we have only had it for two years in Nancy and Rennes, one year in Brest, and it is beginning at Angers. It's only on small surgical pieces of human tumours. At Nancy it concerns breast tumours, at Brest breast and brain tumours and at Rennes brain, liver, thyroid and lung tumours. The purpose of this protocol is to collect as much data as possible and to interpret histological variations using T_1 and T_2 measurements. It is only a beginning but we've seen that with only one center, it is not possible to obtain enough results for precise histological correlations. In my opinion, more centers should be included. They may be more than the four that have already adopted the protocol. We work at 20 MHz and 4° C and we measure the tissue sample (preserved at 4° C) less than 2 or 3 hours after sampling.

RADDA – I have a question for Dr. Chambron on this oxygen effect that he reports. First of all, after 180 min of perfusion at 1 ml per min of flow rate, you went back to 5 ml and your T_1 value remained elevated, which to me suggests that the heart by that stage is really beaten up with that low flow. The other aspect is that if one looks at the ^{31}P spectrum under these sorts of conditions, it tells you that the oxygen extraction remains reasonably constant, irrespectively of the flow rate. When you have high flow you extract a smaller percentage of the oxygen than if you have low flow. So I don't think that the tissue oxygen level is likely to change. Therefore I'm not sure if your explanation really stands up.

CHAMBRON – Yes, Dr. Radda, I thank you for your remarks about our hypothesis.

DE VRE – I have a question for Dr. Chambron. If I understood you correctly, I think you mentioned that T_1 is given mainly by the liquid phase. It would seem to me that actually the overall T_1 values or the T_1 variations would usually be much more complex, and depend on the bound water and the dynamic structure.

CHAMBRON – In the muscle you have 80 % of water in the free phase. It is well known that the contractile actomyosin complex is a polyelectrolyte dependant effect. When you increase the ionic strength, you can observe a contraction and perhaps a release of the bound water. T_1 should be increased, but if T_1 remains constant, I assume that we have a variation within error limits. Perhaps T_2 is a better parameter to probe the solid phase in this case, because transverse relaxation decay is dominating the solid-like phase.

ERMANS – I would like to ask Dr. Béné what is the clinical significance of the contamination of amniotic fluid with meconium.

BENE – Meconium is normally emitted after birth. But when there is foetal distress, especially respiration, we can have emission of meconium before birth. If the time is long before birth, namely more than one week, the meconium is able to give infection both to the child and to the mother. Another effect is that delivery takes place later. The situation is really difficult. Until now, when the delivery is not made at the predicted time, we make amnioscopy with an optical system, which is not always possible and not precise. Another solution is to make amniocentesis i.e. extraction of the amniotic fluid from the body of the pregnant woman, but this is not without risk. For this reason we were asked, when we began to work in this field, to try to observe the pollution of the amniotic fluid by meconium *in vivo* without any injury. In fact we observed practically 50 pregnant women and this experiment seems very well tolerated.

LHOSTE – Now we could start the more general discussion. I'd just like to propose one question on the difference between *in vivo* and *in vitro*. I was told that when you kill an animal, the image is quite different within a few minutes. Could someone comment on this point?

FOSTER – Well, I'm terribly sorry, but I just don't know what the T_1 of a soul is. But we have in fact done a fair amount of work on this. We've been looking at fat distribution in carcases as a method of assessment for the Meat Marketing Board. To do this, we've been taking pigs, doing a set of images *in vivo*, killing them *in situ* in the imager, and then taking a set of images after death at various times. As far as we are concerned, the deep body tissues seem to vary very little - you get temperature effects, and that's about all. You get a very big immediate effect in the subcutaneous fat. Again this is probably a temperature effect - it presumably cools down the quickest, but that's really the only difference that we see in any great detail. The T_1 values, once you allow for the fact that you are imaging at a different temperature, are virtually the same.

DERBYSHIRE – Can I take a point up that Dr. Foster has just mentioned. It could be that your technique at Aberdeen is not particularly sensitive to blood flow, is it? These reported measurements of differences between live and dead may be picking up differences due to blood flow, where yours is not particularly sensitive to that.

FOSTER – Yes, when I showed that set of values measured *in vivo* and *in vitro*, I commented that with a tissue like the kidney you are getting high pressure very

rapid blood flow; that is probably the reason why we see the greatest differences there. But I do agree, we are not particularly sensitive to blood flow.

DERBYSHIRE – Whereas people like Paul Lauterbur claimed to see big differences.

REISSE – I would like to come back to a comment I made this morning. Apparently, at least during biological studies and especially *in vitro* measurements, T_1 is obtained by the inversion - recovery method, using a semi-logarithmic linear least - square analysis. This method is extremely dangerous. Of course, if all the points fit a perfect exponential, and if you transform the exponential into a straight line by using a semi log representation, you obtain the same results by calculating the best exponential or the best straight line. But if your points are not exactly on an exponential, the situation changes dramatically. Moreover, from the statistical point of view this kind of variable transformation is completely forbidden. It is well–known in NMR literature that the T_1 you calculate from the best straight line is not equal to the T_1 you calculate from the best exponential. It is a fact which is easy to prove by computer simulation. The only significant T_1 value is the one you obtain by calculating the best exponential through the experimental points.

RADDA – Can I come back to this question of *in vitro* versus *in vivo*. Of course we do have an enormous amount of experience of how tissues behave in the isolated form in a biochemical sense, compared to *in vivo* in relation to organ transplantation, preservation of tissues, and this is known for a whole range of animal and human tissues. So it seems to me that if you are going to compare *in vitro* and *in vivo* there is no doubt at all that you can have biochemical conditions where the two systems are comparable. One can lay down a set of general rules that you should certainly never freeze your tissue, and once you have frozen it we can forget it being a good model for *in vivo*. Obviously you should try and cool your tissue as soon as you have let it run out of oxygen. We know that buffering the tissue is enormously helpful because that can preserve the materials, something that is routinely used in transplantation surgery. I think that the information is there, and it just needs to be used to develop a system for studying and systemically comparing the same tissue inside the animal and in the isolated form. I believe that it should be perfectly feasible to devise a protocol for studying small samples *in vitro* and still under physiologically meaningful conditions.

LUITEN – I am not expert in this field but I remember having seen data obtained by Dr. Foster in Aberdeen a couple of years ago. Are these still representative for the present day and present state of knowledge, or have they been replaced by other results? At that time, I remember, you should keep them on ice and they were okay for three hours. Is that right?

FOSTER – It was just a set of things that we did when we first started the study to see just how badly we can mishandle our tissues before we started upsetting our results. We tortured them, as it were, to the extreme, and we found that most tissues are extremely hardy. If they were kept at 5° C we could keep them for anything up to twenty–four hours without any major change in the T_1. Possibly small changes, but nothing that would take them out of the clinically usable levels, which is, after all, what we are interested in. Our studies are not geared towards fine detail measurements. We do normally keep our tissues, either in the sample tubes on ice with a cover to prevent dehydration, or keep them in the fridge - we can keep them overnight quite happily. If we are doing an experiment in which we are comparing different things, we make sure that our conditions are very, very standardised. But if I take an animal to pieces it can take anything up to four hours to process all of the different tissues from it. I will repeat that experiment as I did with the rabbit ones that were published from our department a few years ago. From a series of rabbits I took the tissues in different orders, they were measured in different orders, and it made very little differences. It was within the normal standard deviation of the measurements. So we just can't detect them. Most tissues are very hardy - some show greater variations.

MARAVIGLIA – I would never dare to say that something is strictly dead or alive because I have the feeling it is a very complicated definition. You can establish some criteria for a piece of tissue which has been excised. One can say that it is alive if it can be used to be grafted back into the original organism. This can last a long time, as far as I've heard. There are also other situations which unfortunately are not good for most animals. In plants, you can take a piece of the plant and after a long time start a new organism from this single piece.

RADDA – I think I can tell the difference between dead and live tissue, and if you want to use NMR you can tell it by that. But in fact obviously functional studies and recovery studies are the main way. I don't think one can generalise. We know that animal kidneys cannot be preserved under any condition for more than perhaps 5 or 6 hours unless you oxygenate them and perfuse them, unlike human kidneys which can be maintained up to 24 hours, and they will still function after they are put into a recipient. If you do the same thing with heart, you can't keep it for more than 3 hours or it will be irreversibly damaged. So irreversible damage can be measured by functional recovery under defined conditions. I think we have pretty good ideas now of what kind of parameters are responsible, or at least are indicators, of reversible versus irreversible damage.

ERMANS – I would like to know if anyone has any experience of intact tissue and the same tissue after homogenisation? Are all the parameters lost or modified, or are some differences between the different tissues persisting?

LHOSTE – My question would be, is there any need for homogenisation?

DERBYSHIRE – I can answer that question in part. We have homogenised muscle tissue and looked at the proton water relaxation before homogenisation and afterwards, and we just see the weighted mean relaxation rate after homogenisation. We see single component expo-

nential relaxation after homogenisation, and is the weighted mean of the relaxation rates before homogenisation.

DE VRE – Why should the sample be homogenised? The only problem is to get the intact tissue into the NMR tubes. Those who have worked with 5 mm NMR tubes know the difficulty of introducing tissue samples to the bottom of the tube. Some of the samples get very sticky as soon as you excise the tissues. I believe this is one of the reasons why people have probably tried to homogenise them, plus the fact that you improve the precision on the relaxation time measurements. These are practical problems but very important when they come to accurate values for relative comparison.

FOSTER – Can I just say that we've not actually tried going as far as homogenising - we've tried quite a variety of different chopping techniques with our tissues, and providing that you've got quite a reasonable block of tissue and you've not smeared it all over your tube, it really does not make a great deal of difference. I suspect that a lot of the differences that at times I've managed to create by smearing things around have been by dehydration of the thing rather than by loss of its structural integrity. If you are trying to put something like grey brain tissue down to the bottom of a 5 mm tube, it takes you so long that you've probably lost half the water. If it's any use to anybody, I've just started using a new technique. I've cut off the bottoms of all of my 5 mm tubes, just a straight cut not flamed smooth. I now put my tissue in by taking it like a cork–borer into the piece of tissue and then ram it down hard into a piece of paraffin wax. The paraffin wax protons relax very fast and of course I don't see those with my method and it effectively seals the end. So it is overcoming some of this problem of getting the tissue into the tube in the first place without too much disturbance on it.

LHOSTE – That's a good trick. Now we could move on the second part and compare normal and pathological tissues.

PODO – Prof. Bovée, you have done studies showing that fat content is very important in making comparison between breast normal and tumor tissues. Could you please comment on this, because people are indeed generally talking about water, but fat is also a very important constituent of tissue.

BOVEE – We did experiments on rats and on human tissue. The rat experiments gave nice results - they reproduced very well and they gave quite a good distinction between normal and malignant tissue. But as soon as we started doing experiments on human tissue, this distinction disappeared, the reproducibility was bad, and it was impossible to draw any conclusion from the experimental data. Afterwards the reason for this appeared to be that the human breast tissue (contrary to that of rats) contained large, varying, amounts of fat. The fat and the remaining breast tissue had relaxation rates differing a factor 3 or more. We then analysed the samples also on fat content, under the microscope, and by NMR the latter was done by analysing the relaxation curves on double exponential behaviour, and abstracting from them the relative amounts of fat and remaining breast tissue and the time constant concerned. These time constants were very different and could therefore be separated very well. The samples measured by NMR were analysed by pathologists. They determined among other things fat content. From that time on we saw clear results and we could understand them. There was no significant difference between the relaxation behaviour of malignant and normal tissue, if you looked at the part of the signal stemming from the fatless tissue. The fat contents determined by the pathologist compared well with the NMR values.

PODO – What about the water content in that case? Moreover, do you think that this fact could be generalised through other cases as well?

BOVEE – No, we also determined the water content from this tissue. Taking into account the amount of fat we determined the water content of the non–fatty tissue. The water content of the tumorous tissue and the normal or benign tissue were the same within the experimental accuracy. There was some evidence that some benign tumours had long relaxation rates. I've forgotten the names, but there were some benign tumours which had considerably long relaxation rates, at high water contents.

PODO – By themselves?

BOVEE – Yes. We also corrected for the fat content.

DE CERTAINES – Experimental animal tumours give better results than spontaneous human tumours. This is the first reason, I think, for important differences between results obtained in man. The second reason is the histolological variations in samples. If you have a blood vessel, or some fat, or necrosis, or haemorrhage in the sample, then you will have large variations in relaxation times. It is easier to have histologically pure samples in animal experimentation than in human tumours. This is why it is so difficult to apply results obtained from experiments on animals to human samples. I don't think it is possible to discriminate between one tumour and another, or between benign and malignant tumors, but I do think it may be possible, if you have one kind of tumour, either to follow it after treatment, or to have some histological indications if you know exactly the tumour classification. For instance, in meningioma, there will be variations due to collagen content; in breast tumours you have variations with fat, etc. A lot of variations exist which do not have any direct relation with the cancer itself.

PODO – Has anyone followed in time the relaxation behaviour of tumours in regression under the action of drugs or physical agents?

LHOSTE – I am not sure that the relaxation times have been measured under therapy.

BOVEE – I think there are some examples in the literature of regression studies of tumours, and as far as I remember when they treated the tumour by radiotherapy or by chemicals, when the tumour started getting smaller, the relaxation time T_1 decreased.

BAKKER – We have done some experiments, again on mice with radiation treatment. A very large dose was given in one session and only one type of tumour was studied. In general the tumour exhibited a small decrease in relaxation time. We also looked at various normal tissues. A spectacular decrease in relaxation time after radiation was observed for the spleen. The spleen is a radio– sensitive organ - the lymphocytes in it die immediately or within a few hours after irradiation. Most other tissues did not show radiation effects.

LHOSTE – What do you call a large dose?

BAKKER – The dose was 15 Gy in one session.

FOSTER – Could I ask you - did you look at the red cell count at the same time? We've done quite a lot of work on cases in which we administer to animals drugs which can cause haemolytic anemia. In those cases you get a massive reduction in spleen T_1 and this is due to the deposition of red cell debris. Of course, giving a large radiation dose, you almost certainly get anemia as a short– term effect from it, and that could be at least one of the causes of the reduction in your spleen T_1.

BAKKER – We measured the radiation effect at one point in time, and it was 48 hours post–irradiation. Generally the mice were in good condition then and showed no observable radiation sickness from that dose.

FOSTER – That in fact wouldn't counter the argument, because just a very small anemia causes the effect. When I give dimethylaminoazobenzene it works within 48 hours to start the decrease in spleen T_1 and the animals at that stage appear normal in other respects.

DE VRE – Did you observe the effect on T_1 and T_2 or both after radiation?

BAKKER – Only T_1 measurements were performed.

DE VRE – Just the T_1? Because normally you would expect T_2 to be more sensitive than T_1 as a discriminant of the effect of radiation.

BAKKER – Results published by Kiviniitty in Strahlentherapie in 1975 indicate that both T_1 and T_2 are affected by irradiation. Their samples were taken from gynecological patients who underwent radiotherapy.

DE CERTAINES – I have also measured T_1 and T_2 in spleen after radiations and I have found a decrease in T_1 and T_2 in the spleen of mice after radiation. I think that for the NMR follow–up of tumour during therapy it is possible to have an effect on the therapy itself. During chemo–therapy, if we have variations of T_1 and T_2 of the cells during the cell cycle (according to P. Beall and some of our own results) it might be possible to synchronize or to try to synchronize *in vivo* cells or only to follow the effect of chemo– therapy, but perhaps this is a dream! Perhaps the most interesting for therapy monitoring will be phosphorus NMR.

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THE PROBLEMS OF CHARACTERIZATION OF TISSUES AND BIOLOGICAL MATERIALS

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Summary. – *It is becoming increasingly apparent that* in vitro *tissue characterization by high resolution NMR is an essential step for fully understanding the significance of* imaging *results. However, many difficulties are encountered in tissue characterization due to: (i) the multiparameter dependence of relaxation times including inherent biological factors and extrinsic physical parameters, (ii) the need for standardizing the conditions for measurements such as the resonance frequency and temperature, and (iii) the rapid degeneration of tissues after biopsy or dissection. It is clear that a careful study is needed to tackle these problems in order to obtain reproducible and comparable results of the relaxation times of tissue water from comparative studies in different laboratories.*

Riassunto. *Si va rendendo sempre più evidente come la caratterizzazione* in vitro *dei tessuti mediante NMR in alta risoluzione sia un passo fondamentale per comprendere a pieno il significato dei risultati dell'* imaging. *Tuttavia, nella caratterizzazione dei tessuti, si incontrano numerose difficoltà dovute a: (i) la dipendenza dei tempi di rilassamento da diversi parametri, compresi quelli inerenti a fattori biologici e i parametri fisici estrinseci, (ii) la necessità di standardizzare le condizioni di misura, quali la frequenza di risonanza e la temperatura, e (iii) la degenerazione rapida dei tessuti dopo la biopsia o dissezione. E' chiaro che sono indispensabili studi accurati per affrontare tali problemi, al fine di ottenere risultati riproducibili e sovrapponibili dei tempi di rilassamento dell'acqua nei tessuti, per studi comparativi in diversi laboratori.*

Tissue characterization implies correlating NMR relaxation times with the biological state of tissues. Such studies can lead to an explicit and precise identification of the underlying processes responsible for many pathological and physiological states of tissues, and provide a biophysical basis for imaging results on non empirical grounds. In a previous paper [1] the biophysical aspect of the relaxation behaviour of water in biological system were outlined. There is increasing apprehension now that in the interest of clinical evaluation of NMR for diagnosis and research, it is indispensable to complement imaging results with tissue characterization by high resolution spectroscopic techniques.

Tissue characterization is required to serve the following purposes:

1) comprehensive compilation of T_1 and T_2 data for a series of normal and pathological tissues, for *in vivo* and *in vitro* system, i.e. to build up a system of data base under known and well-defined experimental conditions;

2) to standardize the experimental protocol and methods of data analysis required for discrimination of tissues by T_1 and T_2 measurements;

3) to seek optimum conditions for maximum discrimination of tissue types;

4) to delineate the biophysical basis of discriminating parameters for detecting malignant tissues and other types of lesions;

5) to study the relevance of relaxation data from *in vitro* studies with *in vivo* results obtained from imaging.

Several problems are encountered in tissue characterization by measurements of T_1 and T_2 *in vitro* because of the multiparameter character of relaxation times. In fact T_1 and T_2 are sensitive to: i) a number of inherent biological factors that perturb in different ways the water balance; ii) extrinsic physical parameters, such as temperature, resonance frequency and sample treatment (e.g. preparation and storage).

Thus, for an explicit correlation of T_1 and T_2 variations with specific pathological conditions, it is important to recognize and/or to minimize the effects of irrelevant factors. Therefore, studies must be performed under controlled and normalized conditions in order to obtain comparable, reproducible and meaningful results. Many details related to experimental protocol will be discussed in subsequent papers of these Proceedings.

The following problems need to be tackled for the characterization of tissues *in vitro:*

a) the rapid degeneration of biological samples after biopsy or dissection of laboratory animals perturbs the state of water, and thereby affects the measured values of relaxation times. This introduces uncertainties in the studies dealing with relative comparison of relaxation times unless the methods of preparation and preservation are standardized and controlled. The effects of sample degradation is a deep rooted problem for *in vitro* studies of biological tissues;

b) due to the ease of sample degradation at high temperatures, the choice of temperature for measurements of T_1 and T_2 is an important problem. *In vitro* measurements at a temperature corresponding to the body temperature (37° C) are likely to give irreproducible results, hence measurements should be performed preferably at lower temperature (for example at +5° to +15° C), and the values extrapolated for comparison with *in vivo* results. Relaxation times are sensitive to temperature changes [1]. It is likely that the error introduced by extrapolating the values of the relaxation times observed at lower temperatures to higher temperatures (using accurately determined activation energies) is less important than the irreproducibility and uncertainties arising from measurements at high temperatures;

c) the frequency dependence of relaxation times of tissues water [1] requires that the values be compared at identical frequencies. Not only the true values of relaxation time T_1 and T_2, but the relative differences between normal and cancerous cells [2] are also dependent on the resonance frequency. The problem of comparing the results from different laboratories using spectrometers operating at different frequencies may be resolved by obtaining some calibration curves for the frequency dependence of T_1 and T_2 for a model system with the state of water akin to cell water and reproducible preparation;

d) the measurements of T_1 and T_2 by high resolution NMR are very sensitive to the inhomogeneity of the sample. Tissue samples packed in NMR tubes present a fairly inhomogeneous mass for precise measurements of relaxation times (particularly T_2), and may give rise to low accuracy of measurements in certain cases. Should the excised samples be homogenized before measurements? It should be noted that homogenization can drastically alter the integrity of the intra and extra cellular water. In this case the state of water persisting after homogenization does not represent the real state of the biological tissues under consideration, and the correlation of the relaxation times with the properties of water will not reflect the true differences in pathological and physiological conditions. It is expected that the discrimination of relaxation times will be attenuated in the homogeneized samples. Here one faces the dilemma of increasing the precision of measurements at the risk of losing the essential biological information;

e) the medical applications of proton NMR require devices to detect with high accuracy small variations in T_1 and T_2 values irrespective of the precision of absolute values;

f) the relaxation data from *in vitro* studies are often compared with *in vivo* results. This entails several problems such as: i) the state of "water balance" in living cells and tissues is likely to be significantly different from that in non-living tissues by the very difference in their biological states, and the degradation effects *in vitro;* ii) high resolution studies give very accurate but average values of T_1 and T_2, whereas imaging represents the spatial distribution of intrinsic T_1 and T_2 values in the heterogeneous biological space; iii) *in vitro* studies are often performed at higher frequencies than those normally employed for imaging setup for patients.

g) the problem of the appropriate choice of index parameters lies in the fact that they should be sensitive enough to detect small differences in the state of water and quick in manipulation to allow a series of measurements within a reasonable time. The complementary nature of T_1 and T_2 relaxation mechanisms necessitates that studies be performed using both T_1 and T_2. The commonly employed parameters are T_1 and T_2 at a fixed frequency and temperature. Certain functions of relaxation times are likely to be more informative discriminating parameters for identification of the biological state of tissues.

Conclusions

Several problems related to tissue characterization originate from the inherent biophysical background of the relaxation behaviour of water in biological systems, and from extrinsic physical parameters. It is clear that these problems need to be tackled genuinely in order to obtain reproducible and comparable results of the relaxation times of tissue water required for tissue characterization. It may be noted that while NMR imaging is useful for localizing in the heterogeneous biological space those soft tissues that exhibit an abnormal behaviour, tissue characterization helps in identifying explicitly the underlying processes responsible for such abnormalities.

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INFLUENCE OF AGE AND THERMAL TREATMENT ON THE PROTON NMR RESPONSE OF MUSCLE

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Summary. – *Major differences between the* in vivo *and* in vitro *NMR response of tissues may exist which are related to biochemical changes induced by death, thermal treatment and even by a careful handling of the sample. On the other hand, experimental evidence is presented indicating that the proton NMR response of muscular tissues is scarcely affected by the procedures of sample preparation followed in most laboratories. This fact suggests that* in vitro *studies may provide reliable estimates of how sensitive NMR tomography will be in discriminating between normal and diseased muscles. The spin–lattice and spin–spin relaxation processes of protons are, usually, "more exponential" in normal muscles than in diseased or damaged muscles. The significance of this observation is discussed and it is suggested that a properly defined "non–exponentiality factor" will show a great sensitivity to the pathological condition of the muscular tissue.*

Riassunto. – *Tra le risposte NMR* in vivo *ed* in vitro *possono esistere in linea di principio differenze rilevanti, dovute alle modifiche biochimiche indotte dalla morte, dal trattamento termico e persino da un accurato trattamento del campione. D'altra parte, vengono qui presentate evidenze sperimentali che indicano che la risposta ^1H NMR di tessuti muscolari è scarsamente influenzata dalle procedure generalmente usate per preparare i campioni. Questo fatto suggerisce che gli studi* in vitro *possono fornire stime affidabili della sensibilità della tomografia NMR nel discriminare tra muscoli normali e patologici. I processi di rilassamento spin–reticolo e spin–spin sono generalmente "più esponenziali" nei muscoli normali che in quelli patologici o danneggiati. Viene discusso il significato di questa osservazione e si suggerisce che un opportuno fattore di "non esponenzialità" possa presentare una grande sensibilità alle condizioni patologiche di un tessuto muscolare.*

Introduction

Broadband proton magnetic resonance (PMR) studies of tissues have a low level of technical difficulties and highly reliable data exist. Since most of the experiments have been performed *in vitro*, the question arises of whether handling of the sample has significantly altered the *in vivo* PMR response of the tissue. A further question concerns the PMR sensitivity to a pathological condition. It would be most helpful to have a definite feeling of the extent to which *in vitro* studies yield the response of living tissues and provide useful information for NMR tomography. Due to the great complexity of the samples, an answer to these questions should be derived from a critical analysis of existing experimental data. For this reason, a brief overview of the literature will be given on the effects of the sample handling procedures upon the NMR response, without commenting upon the interpretations offered for the experimental observations.

Overview of the literature

The spin–spin (or T_2) relaxation process appears to be highly sensitive to details of the sample preparation and different shape of decay for the transverse magnetization have been observed in different laboratories [1,2]. Pearson *et al.* [3] found a nearly exponential spin–spin relaxation process in freshly excised muscles: non–exponentiality developed after onset of rigor and/or freeze–thaw cycles. Qualitatively similar observations on the effect of rigor have been made by Fung and Puon [4]. Belton and Packer [5] noted variations of spin–spin relaxation process in the frog gastrocnemius as a function of the time spent by the frog in captivity. In a careful study of the spin–lattice (or T_1) process in skeletal muscles Chang *et al.* [6] found that this process is slightly non–exponential; both the effective T_1 and the amount of non–exponentiality undergo characteristic changes in the first few hours following extraction. While we were unable to observe any non–exponentiality in whole frog gastrocnemius [7], we could see such an effect on T_1 as a function of time after excision: while there were no significant differences between T_1 immediately after and 12 hours after excision, the spin–lattice relaxation time increased by about 10% in the first

2–3 hours and then decreased. The phenomenon was reproducible and fully unexplained.

In the literature quoted above there are often statements about the effects of storing the samples below room temperature. A favorite procedure [8] for keeping the samples over times of the order of one day is to store them over ice, which appears not to change T_1. Some Authors [8] prefer a storage at –30°C which is likely to keep the muscle over extended period of time. We were unable to detect differences larger than the variability among individuals in the T_1 behaviour of normal human muscles stored at –30° C in scaled containers for times between one week and six months. On the other hand, a batch of muscle biopsies left for a long time (one to four years) in the freezer showed major changes: brown–to–black colours, reduced water content and T_1 values up to five time shorter than those of well preserved muscles. Comparison between T_1 and T_2 in freshly excised tissues and in tissues kept one day at 4° C confirmed that the muscular tissue is rather insensitive to short term storage procedures [9]. On the other hand, other tissues, taken in particular from colon and lung, showed a PMR response which was modified by the storage. No unique pattern in these modifications was however found [9].

Pathological conditions are known to affect the PMR response of tissues and to cause changes of the relaxation times which can be rather substantial [10, 11]. Cancer is known to cause slightly increased relaxation times in most tissues. This change appear to be related to the higher water content of the tumoral tissues [12]. While longitudinal and transverse relaxation times taken separately do not allow a good discrimination between normal and tumoral tissues, a more reliable diagnosis can be made by using the sum of the relative changes with respect to normal of both T_1 and T_2. The longitudinal spin–lattice relaxation time of heart slightly increases in acute ischemia as well [13]. A recent *in vitro* analyses of the spin lattice relaxation behaviour in muscles of patients affected by a variety of neuropathies [14] mostly, buth not uniquely, showed a decrease of T_1 with respect to the control group. On the other hand, the T_1 process was substantially less exponential in diseased muscles than in the normal ones [14].

Discussion

The literature shows that handling procedures and physiological conditions have an effect upon the "shape" of decay of the transverse magnetization, while the spin–lattice relaxation process is much less affected by these factors. An explanation for this observation may be the following. NMR relaxation time in biological systems always represent an average of T_1 or T_2 over relatively large regions of the sample. A non–exponential relaxation process, or an increase of the non–exponential character means that the sample is not homogeneous over these "large regions". The size of these regions is of the order of the distance over which water molecule diffuses during the experimental time (i.e. times of the order of T_1 or T_2). Thus, a non–exponentiality in the proton T_2 relaxation in a tissue indicates that an inadequate average is obtained over a region 0.01 mm in diameter. The inhomogeneity should be 3–4 times larger in order to cause a non–exponentiality of the spin–lattice relaxation process. A number of phenomena can cause this inhomogeneity: preferential condensation of water in certain compartments, biochemical changes of macromolecules affecting different cells in different ways, etc. A discussion of this problem, with some rather preliminary conclusions, has been reported [4]. It is stressed here that the message contained in the non–exponentiality of the relaxation processes is eminently "macroscopic" and it is erroneous to extract from it populations of water fractions with different physical properties.

While the non–exponentiality of the relaxation processes does not give useful information about the state of water in a complex biological system, it provides a way of classifying the state of a tissue. Let be m (t) a normalized quantity that decays from of value of 1 (at time t=0) to 0. With $m_0(t)=\exp(-t/T_{\alpha 0})$ is indicated an exponentially decaying function that at t=0 has the same time derivative as m(t). The non–exponentiality factor can be defined as

$$\eta = (t_a - t_0) / t_a$$

where the times t_a, t_0 are defined by the equation

$$0.1 = m_0(t_0) = m(t_a)$$

Since information about pathological conditions are contained in the non–exponentiality factor, it is important to select a sample handling procedure which does not change η or which modifies it in a predictable way. It was found that a storage of muscles at –20°C in sealed containers for periods of 5–6 months did not affect the non–exponentiality factor of the spin–lattice relaxation process.

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Discussion on protocols for the preparation of biological samples for characterizing tissues
Moderator: J. S. Orr

ORR – We have to look at the possibilities of having some general agreement on procedures for preparation of samples for *in vitro* studies of proton properties. The two speakers appear to me to be saying almost the same thing – perhaps I could ask them if they could both agree to work to the same procedures.

VILLA – Yes, we basically agree, except that I have some difficulties now in accepting the lowest temperature possible. The liquid nitrogen passage is the only point where I don't agree, because all food storage experts say that the lowest is not always the best. It seems to me that one should avoid both storage at extremely low temperatures and too large a type of exclusion temperature which is provoked by the fast drying in liquid nitrogen. This is only because I found in the literature that this can adversely affect the conservation of the products. Maybe it is worthwhile to stick with simply the dry ice type of procedure, just for the transfer and immediate fast cooling, and avoid any passage in liquid nitrogen. For the rest I completely agree with M.me De Vré.

DE VRE – My point of proposing liquid nitrogen is to ensure fast freezing. In the beginning we used dry ice for freezing, but I think from the point of view of the rate of freezing, it is preferable to use liquid nitrogen. We stored the samples in a deep freezer at about –60° C.

ORR – Would workers be willing to make some small change in their prejudices in order to be working in exactly the same way?

DE VRE – I'd like to ask a question to Dr. Villa. In your experiments you wrapped all the samples in an aluminium foil and then transferred small pieces of them into NMR tubes when they were cold at –20 or –40° C. Wouldn't you expect some effect of moisture condensation during the transfer? This can be avoided by transferring samples in NMR tubes before freezing at –20° C and tightly closing them.

Dr. VILLA – The rationale of the aluminium foil is the following: first of all, it helps to define a small space in which the sample is kept, secondly it helps in fast cooling. The decision to manipulate the sample in the freezer, instead of inserting it immediately in the test tube depends on the choice taken by the medical school in which you are working. That means, if there are people that I can trust, able to directly insert in the surgical room the sample inside a 5 mm NMR test tube, I would go along with your suggestion. Or if I can have the type of test tube that you have, it would probably be possible to have the personnel in the surgical room to produce directly the NMR test tube. But I have found it much more flexible to have a slightly larger sample that I can store directly in the freezer. Then I can take a piece to determine the water content, a piece for electron microscopy, a piece for NMR, and so on. It is just a problem of choice, and as long as you have a procedure that does not change the temperature of the sample during manipulation – this is rather important – I am rather confident in the normal wrapping in freezer and manipulation and so on.

DERBYSHIRE – I think a great deal is going to depend on what you define as the object of the exercise. If you are talking about tissue viability, that is one thing. Alternatively, if you are working for the food industry (most of my sponsorship comes from the food industry), you are talking about the nutritional value of the food and the textural properties of the food after it has been frozen, and maintaining those. But to say one thing to Dr. Villa, if, for example, you take fish muscle – fish muscle will still degrade at –20° C, and the food industry is trying a compromise since it's a lot more expensive to store food at –30° than it is at –20°, and –25° may be an acceptable compromise. –30° would be better from the point of view of keeping the tissue intact. What we do find is that, whatever you define as the object of the exercise, cell viability or the mechanical properties afterwards, the rate of cooling and the rate of heating are critical. If you look at cell viability and the rate of cooling, you get a curve that goes to a peak, then down and then increases again. In order to explain that, you've got to think of the mechanisms of damage. At lower rates of cooling, you are exposing the cell to osmotic shock because, as the ice forms, the odds are, because of the sheer volume space, the first nucleation occurs in the extra cellular medium, and as a result of that, ice crystals start to grow in the extra cellular medium, water transfers from inside the cell to outside, the salt and the sugar contents of the cells increase, and this may induce some damage. That's one cause of damage, so what you are doing at the lower rates of cooling with an increased rate of cooling, you are decreasing the amount of cellular damage, because you are cooling more quickly. As you then go on to cool down even more rapidly, you've a better chance of forming an ice crystal inside the cell. There's a straight competition between intra and extra cellular ice formation. If you get an ice crystal formed inside the cell, I don't think there have been any reported cases where the cell has survived that formation of an ice crystal internally. So here we are seeing a sort of salt damage – osmotic shock, then we are seeing damage due to the ice crystal formation inside, and on this side we are really cooling so rapidly that we are forming a glass inside the cell, so we are not exposing it to ice crystal formation. The cell should survive this if we could cool sufficiently rapidly. But what happens is that this maximum and this minimum depend upon the particular cell you are talking about, and upon cell volume. If you are going to talk about a tissue, an object with this sort of shape, the rate of cooling that you can produce inside is very limited by thermal conductivity of the medium itself. So I'm slightly concerned about

your specification of a safe level, because some biodegradation occurs at even −20° ; but low temperatures would be better. I think we are going to have some problems if you try and specify a rate of cooling, an arbitrary rate of cooling for all sizes and all tissues.

DE CERTAINES – I don't entirely agree with the protocol of freezing. I find that in some cases you have, after freezing, the same results for T_1 but you may have more variations for T_2. Perhaps when a better protocol is not possible, freezing could be used. There have been some attempts in Grenoble, on brain tumors, but only for T_1. I know that for T_2, we have found some variations after freezing. As I said, it is perhaps all right in some cases when a better protocol is not feasible, but as a general rule, I don't think it is good to freeze and thaw.

DE VRE – The values do depend on the rate of freezing. We used different positions of a tissue sample, in half a dozen different NMR tubes and subjected them to different freezing conditions and degradation. The T_2 results were found to be irreproducible by freezing samples at a very slow rate (about −20° C) in an ordinary freezer. On the contrary, the T_2 values were more consistent for samples frozen in liquid nitrogen. I do agree that probably certain changes are induced, but we found the results more reproducible than for slow freezing.

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Discussion on a possible categorization of measured values
Moderator: F. Podo

PODO – The discussions held in the previous sections indicated the importance of reaching a common agreement in the definition of an appropriate standardization of measurements and of coordinating a collection of NMR relaxation data obtained on the basis of such criteria. Results reported in the previous literature could also be reviewed on these bases. Such a working plan should lead to a rapid and systematic assessment of the feasibility and applicability of the NMR approach to the problem of tissue characterization by NMR and hopefully provide the clinician with useful diagnostic tools. The question is now whether it would be useful to establish a data bank on NMR relaxation data or some other form of fast exchange of information among laboratories. M.me De Vré has given us a copy of a "Biological NMR Data Bank Form" distributed by the Society for Magnetic Resonance Imaging, Bethesda. The information required to be provided by this form is the following: 1) date of submission, investigators, address, city, state, area code and telephone. 2) General information: title of project; machine information: model, frequency, nucleus, sample temperature at measurement (°C), pulse sequence, parameters measured; method of data analysis: initial slope (ms), least square fit, multicomponent fit: *mode of imaging; sample information:* type of sample, volume (cc); time delay before measurement (hours); method of storage: fresh or refrigerated or frozen, etc.; time of storage; reproducibility of this type of tissue (± ms, % of error); patient information: designate in data table patient ID numbers, sex, age, weight, ethnic origin (optional), hormone status, medication and pathological diagnosis to facilitate comparison with other studies. 3)NMR data:sample; parameter; value (mean ± SD, ± SE); 4) Conclusions. 5) Reference to publication. One possibility would be that everyone of us get in touch with this data bank. Another possibility would be that anyone using his own librarian facilities would try to have a data bank in his own institute. A third possibility might be that of trying to establish a European data bank on the basis of common protocols, or to activate some other mechanism of fast exchange of information. I would like now to open the discussion on these points.

LHOSTE – Could I just add a comment. It would also be very useful to have standard samples which might be exchanged among laboratories in order to compare measurements from one centre to another. I remember this was done for EPR about fifteen or twenty years ago. We prepared samples which were sent over the world and two years later we came back with our results, and the spread was by a factor of 10, concerning the number of spins in the sample.

PODO – An exchange of standard samples would also be very useful and the protocols for their preparation and handling are very important.

ORR – As Dr. Podo said, protocols for sample preparation are vital. For these to be useful, we must also have protocols regarding the data that will be collected. The best way to set this up and to impose the discipline necessary is to have a standard data collection form. The best way to get people to fill these up is to have somewhere to send them. Therefore we need four things: we need the protocols for sample preparation, we need the protocols on the data to be collected, we need a mechanism for receiving and for analysing the data, and finally the fourth thing that Prof. Lhoste mentioned, a system for standardized calibration to be used in all the laboratories participating.

DE CERTAINES – There are two different problem here. The first concerns the protocols and the second the data bank itself. For protocols, it is clear that the same criteria cannot be applied to proton *in vitro* studies and to phosphorus studies, for instance. I shall only talk about proton T_1 and T_2 *in vitro* protocols. If you try to understand what causes T_1 and T_2 variations in tumours, it is fundamental to have a lot of studies on histological variations in each kind of tumour. It is not possible to obtain good results unless you have 300 or 400 patients. Only one center can never have so many patients over a short period of time. Therefore the protocols now being used by the three French centers is interesting for that reason. That is only a small part of the problem but it is pertinent. The data bank is another problem again. I am not sure that the information like that in the paper you have read is enough to understand the results. You ought to have the age of the patients, the conditions under which the tissue was extracted for *in vitro* studies, and a lot more information besides. It just isn't possible to have all that information on one small piece of paper. I think it is more interesting to have a specific bibliographic data bank to have rapid access to studies, on breast tumors *in vitro* at 20 MHz and 4°C, for instance. It is for this reason that we decided to try to constitute a data bank of bibliographical references, and not of direct data information. Our data bank (MEDAGRA-RMN) is now being elaborated, and it should be completely operative at the end of the year. It will also be accessible to international information processing.

CORTELLESSA – I am the Director of the Data Management Service here in our Institute, and I just want to make a quick observation. First of all, I think (and we are experimenting already in some fields from physics to medicine), I think that the present state of word processing allows us to skip the step of a data form. I would like to say that if the information is given, even if it is not fully standardized (I hope that you understand that I am trying to make an extreme statement when the truth is in between), word processing is so powerful today that we may not ask to fill in stan-

dardized forms. That has nothing to do with protocols of course - we are only talking about collecting information to be stored in the data bank. What is interesting is that there is the minimal set of information that you want to compare. This seems to be only a matter of informatics, but it tells us that it is easy to take the first step, just comparing the information as it is, as it has already been collected by everyone. We would like to volunteer to be one of I don't know how many who want to participate in this European data bank exercise. We would like to collect the information and try to order the information and try to distribute it - I mean, the first step before the data bank, that is simply a step that I call the clearing-house, where you collect the information even if it is not yet fully standardized or half standardized. Then from the first step, try to treat the information, distribute it and have back the observations from the audience or from the subjects who are interested in participating in the exercise, and then, as a next step, go to a real data bank. It is particularly important to process the existing information so that a minimal set may be extracted for further standardization. It seems to me that this is relevant for the specific subjects and topics you are talking about, because there is still some work to be done from the point of view of the standardization. So I don't know how far my proposal may go, but the proposal is to try to collect the information after, hopefully, a minimal effort of standardization, go on treating the information to see what happens, and then for the next step to try to have the information collected on a more standardized data collection form. We are trying this way especially with physicians in Italy, and you know that physicians are really difficult to treat because every physician has his own view, but it seems that we are succeeding in doing this, with a two-step approach. First, to see how valuable the treatment of information is and then convincing the subject that it is possible to standardize. We are ready, and of course if there is a step to be made we may divide the tasks e.g. in the field of bibliography (which I'm not going to enter into), with the Istituto Superiore di Sanità being the central focal point for many automatic bibliography systems and we know that bibliographic information is very valuable especially in order to keep people together on a specific subject.

FOSTER — As far as standardization is concerned, if I could address that question, we have seen all the various things that make us non-standard, and a great many of the things that we have heard about have been suggestions like, "I think this could make one set of results different, I think this could . . . ", etc.. I can't help feeling that there are two things that we really ought to try: one of them is perhaps to set up a sort of small research programme or at least something along those lines at a very small level, where one or two laboratories from our group who are approaching their studies in rather different ways attempt to get them to do the same type of study on the same material. I'm afraid I am thinking of biological material rather than just a bottle that could be handed round from one to another, because of the varations inherent in the biological system as well, but to set up, say, a standard breed of rat and take their samples as near standard as they can, and see what results they get. That would at least tell us how non-standard we actually are, because I don't think we appreciate that at the moment. The second thing is that I think it would be perhaps very useful if we could establish one or two laboratories in each of our countries and get them to have a lot of communication between each other so they can act as a national centre to which anyone who wants to refer their own particular method can go, so that someone in Britain sets up a standard system which is comparable to that in every other country, and if I want to cross-check on something I've got, I can go there and say "look, will you do a measurement on this for me?", and then I can know how far my own system is varying away from what it used to be and from what other people are doing. I think this would bring us towards a better understanding of, on the one hand, just how non-standard we are, and on the other hand help us to standardize our actual results. I'm thinking here entirely of course of *in vitro* measurements.

SCHMIDT — I see some difficulties in performing experiments in two countries with rats of the same breed, or something like that. I have a suggestion: we could use commercially available cell lines which can be bought by all the centres in the different countries, and we could cultivate the cells under standardized conditions and then measure *in vitro* T_1 or T_2 and compare those results. Potentially this is a way for reaching a standardization between different centres.

DE CERTAINES — Cells in culture are more difficult to measure by NMR than tissue samples, and I'm not sure that we can obtain the best results with them. For protocols in France, for instance, we have taken as reference the liver of Wistar rats of 200 grams, and we have not found important variations. With cells in culture, however, with centrifugation and other problems of separation, it is more difficult to define a reference system.

SCHMIDT — We could do both.

DE CERTAINES — Perhaps.

REINHARDT — I would like to suggest that it would also be interesting to have the image data available, not only the data in the protocol. It is very difficult to handle such a large amount of data, but if you try to apply statistical analysis procedures to have multiexponential analysis on these data, I think it's necessary to have a high number of images, and therefore it would be avantageous to use images which have been obtained at different institutions and places. But it's evident that there are other problems if you are using different measurements and so on. If you are planning some future activities, I think this point should be considered, namely to establish image data banks as well.

VILLA – I have a practical proposal. What it seems to me comes out from this proposal of establishing a data bank is that this should be started somehow, and probably it is convenient to start it in rather a preliminary form. To do this, I would say that the Istituto Superiore di Sanità should act as a centre for collecting this data base and I would suggest to do this in rather a "hybrid" type of form. That means to reach an agreement on some kind of protocol and some suitable standardization, to send data and/or references to Dr. Podo. These data can then be entered in the computer and the information distributed in a type of bulletin, maybe categorized in an appropriate way. After several months of this work, enough feeling about the utility of this data base can probably be gathered to make suggestions on how to organize it. In a second moment, one could probably set up a network in which every laboratory can feed directly the data to a central computer and can retrieve the data directly from the central computer.

LHOSTE – I think the best thing would be that people who seem more involved in this problem could discuss together tonight and propose something tomorrow, remembering that the spreading of data is very useful, but the spreading of preliminary or incomplete data is dangerous.

PODO – I don't think that only one centre should be in charge of this collection and exchange of information. It would be good to have at least one centre for each country, or maybe for two countries.

DE CERTAINES – If you have a multi-focus system, surely you must have the same codification for published data at the beginning of the bank. For example, we've had a codification system for six months and if we have to change, we will need to know the new codification rapidly because it will take us six months to do so. For a multi-focus system I think it is necessary to have exactly the same codifications right from the beginning.

CORTELLESSA – I'm sorry but there is no need whatsoever to have the same codification. If you give me the information and your code, the so-called transcodification is normal procedure. We're not asking anybody to use unique codes. We use word processing, and we use information processing. The only problem is that if you send us a set of numbers without telling us what the numbers are, it is difficult because it is cryptography - it is difficult but not impossible. But if each centre has its own codes and these codes are exchanged the problem is solved. Let me put it in another way, we have enough computing power to use all the other codes and to translate from one code to another. But this is another point, because it strongly depends on how large a computer you have in the focal point in each country. With a large enough computer - as we have - there is no problem whatsoever. To me, to try to ask to use the same codes means five years of discussion about the codes before we even begin. So I think that it is possible to have a multi-centre, multi-focus system if everybody is not going to ask the others to do this exercise on changing the codes, but just use the programmes to read other people's codes. I'm not saying that it is possible, I know that it is possible to do that.