# " *in vivo* " and " *in vitro* " TWO DIMENSIONAL $^1H$ NMR SPECTROSCOPY.

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## I Introduction.

The nucleus most frequently used for Nuclear Magnetic Resonance (NMR) spectroscopy studies in living tissues has, until recently, undoubtedly been $^{31}P$. $^{31}P$ NMR is very sensitive, the spectra are simple because of the wide available spectral range, the relatively few accessible molecules and the small number of $^{31}P$ nuclei present in each molecule. The molecules displayed take part in the energy metabolism of the cell (ATP, ADP, phosphocreatine, inorganic phosphate and sugar phosphates). But $^1H$ proton spectra also have several potential advantages: sensitivity is high and, most important, they provide access to many important molecules (1). *In vivo* proton NMR has developed more slowly, both because of the major problem of selective suppression of the water signal, and also because of the great complexity of the $^1H$ spectra. This nucleus has a narrow spectral width (10ppm) and there are many protonated molecules accessible to measurement. Each molecule contains many $^1H$ , and the signals have a fine structure, because of scalar $^1H$-$^1H$ coupling. All these characteristics make the spectrum particularly crowded and complex.

## II Materials and Methods.

We have used two models to study cerebral metabolism by NMR.

### II.1 *In vitro* model : superfused slices of new born rat brain.

Detailed descriptions of the superfusion for brain slices have been published elsewhere (2,3,4). Our initial results were obtained using a commercial NMR probe which could accept tubes with a maximum diameter of 5mm (2). We have since constructed a special probe accepting 10mm tubes which is better adapted to tissue perfusion. The $^1H$ proton signal detecting coil is closest to the sample in this "reversed" probe, and a larger diameter coil allows verification of sample viability using a $^{31}P$ spectrum. Samples can be kept for up to 12 h under these conditions without detection of any inorganic phosphate in the $^{31}P$ spectrum

### II.2 *In vivo* rat model

The animal was positioned vertically in a custom built probe. Emission and detection were via a surface coil assembly placed on the rat's skull.

### II.3 NMR parameters.

All spectra were recorded using a BRUKER AM400 wide-bore 400 MHz spectrometer. The conditions for obtaining $^1H$ 1D spectra varied according to the model. The $^1H$ spectra of superfused brain slices were recorded using the sequence:

Select. sat. - 90 - D - 180 - D - acquisition with a selective saturation period of 0.6 sec and D of 68 ms.

For the *in vivo* rat studies, the 180° pulse was flanked by two composite selective pulses (- 90°) (5,6) and D of 136 ms. The acquisition parameters were in both cases: 2048 data points, spectral width of 4000 Hz.

The COSY 2D spectra were recorded using the SUPERCOSY sequence (7), preceded by a selective $H_2O$ signal saturation period of 0.6 sec :

Select. sat. - 90 - t1 - D - 180 - D - 90 - D - 180 - D - acquisition

with D = 0.3/J and J = 7 Hz.

A total of 128 FID (2K, 32 scans, total time of 1.25 h) were acquired. The spectral width in the F1 and F2 dimensions was 4000 Hz. The data were multiplied by an unshifted sine bell before Fourier transformation. All 2D spectra presented are absolute intensity spectra.

## III. Suppression of the Water signal.

The techniques for obtaining $^1H$ spectra in living tissues are now well established. We selected a two-stage water signal suppression. The water signal was first selectively saturated by a long low power decoupler pulse, it was then greatly reduced by introducing a spin-echo between the excitation pulse and the start of reception. The molecules which interest us are relatively small and have a long T2, while intracellular water has a short T2 and a long T1 (8,9), the spin-echo thus allows not only selective reduction of the intracellular water signal, but also those of large molecules such as phospholipids and proteins which interfere with the $^1H$ spectra.

The suppression of the water signal for *in vivo* and *in vitro* experiments may be considered separately. Two types of water can be readily distinguished in the superfused tissues: intracellular water and superfusion water. Intracellular water behaves as described above, superfusion water has a long T2 an suppression of its signal is mainly due to selective saturation (Fig. 1a). We took advantage of the intrinsic inhomogeneity of the *in vivo* rat model, by adding a sandwich of selective "-90°" pulses (5,6) to either side of the 180° pulse in order to prevent water signal refocussing (Fig. 1b).

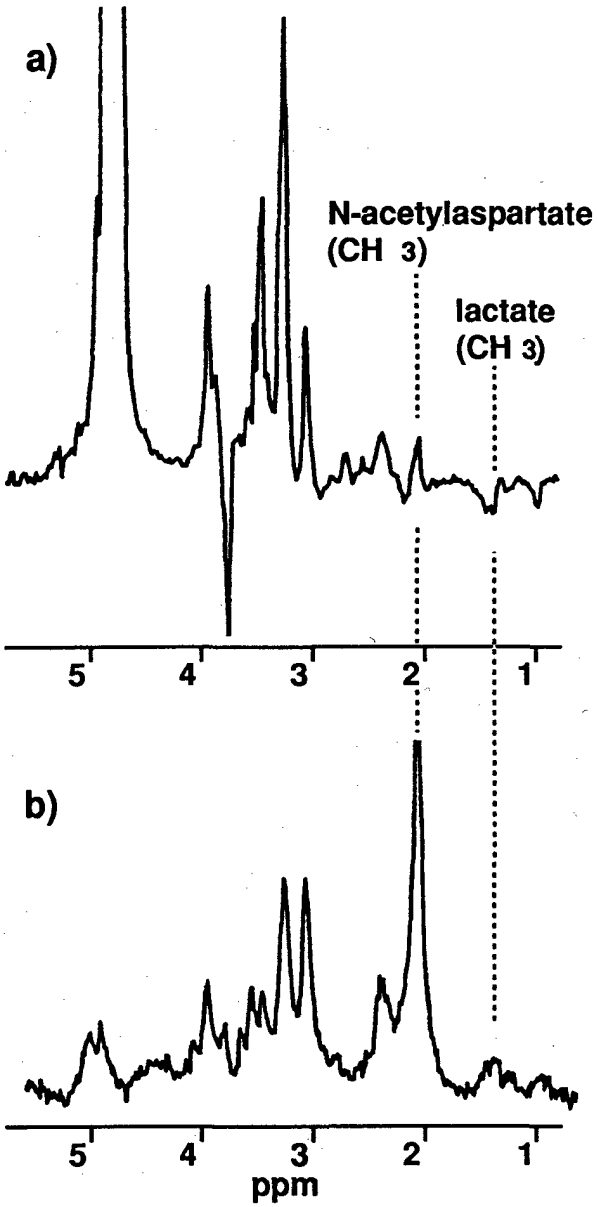


**Fig. 1 .** 1H NMR spectra of :
a) superfused brain slices (inverted signals because of spin echo delay of 68 ms )
b) live rat brain (spin echo delay : 136 ms)

## IV. Two-dimensional *in vitro* and *in vivo* NMR.

### IV.1 Spectrum Editing.

The most important factor limiting the development of *in vivo* $^1H$ NMR is the difficulty of extracting the required information from such complex spectra. The characteristic signal of a specific molecule must be isolated in order to follow its changes. This problem has, until now, mainly been overcome using editing techniques:

_ difference spectroscopy which involves alternating suppression of the J modulation of the spin-echo (10-17).

_ homonuclear polarisation transfer, without difference spectroscopy (18).

These techniques share a major inconvenience: they require the use of at least one frequency selective pulse. They can therefore only be correctly applied to molecules having relatively isolated signal in the spectrum. Such molecules do not really require the use of editing techniques as they are readily identified by their isolated signal. This is why the majority of studies carried out in this area have been on lactate, a molecule which perfectly satisfies with the criteria listed above because of the isolated signal of its methyl group . Lactate has also been studied using zero quantum, 1D (19) and 2D experiments (20). Finally, all these techniques can be used to follow only one molecule at a time.

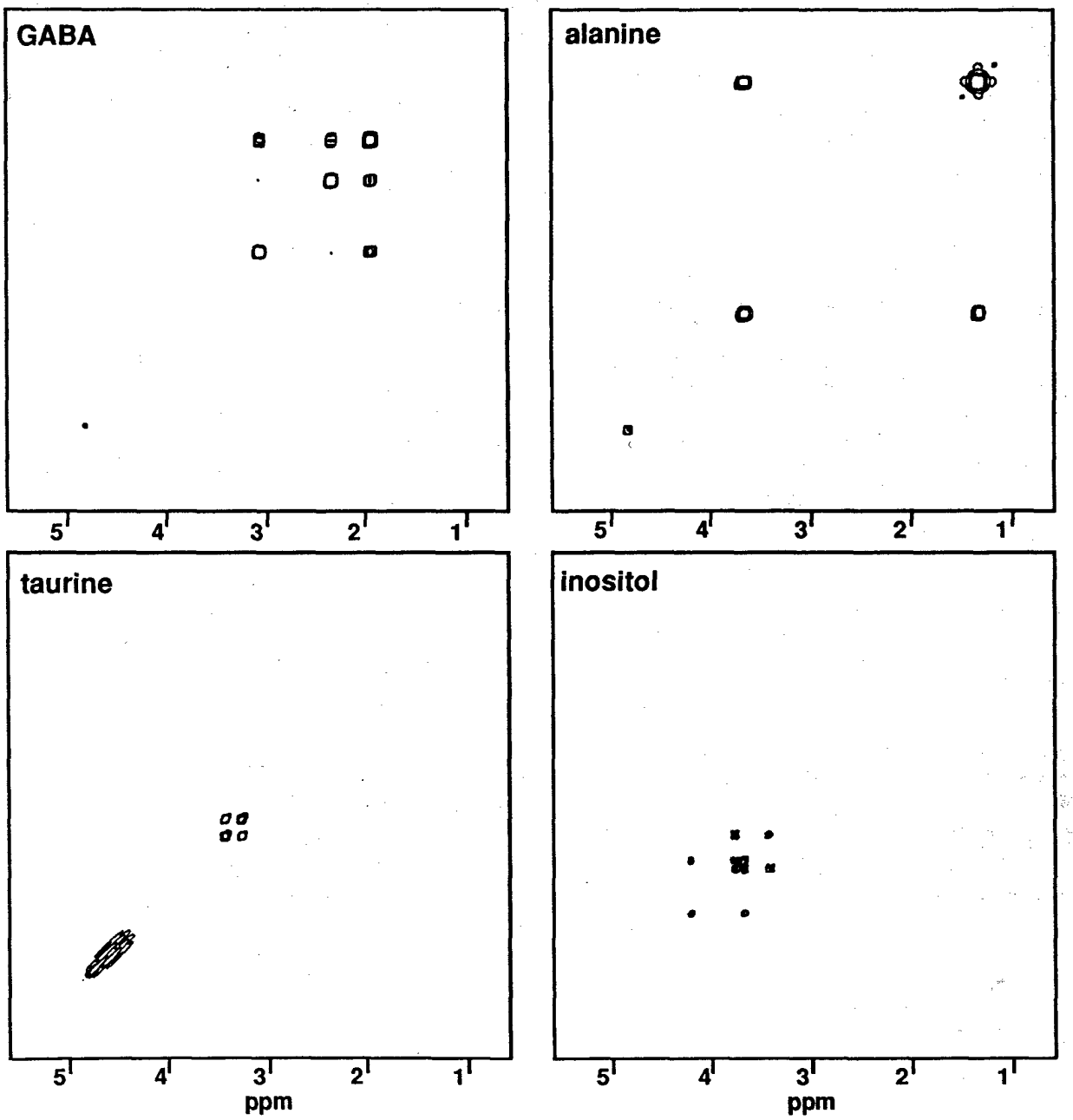


**Fig. 2 .** Cosy spectra of GABA, alanine, taurine, inositol.

## IV.2 COSY spectra in living tissues.(2,23,24)

Two dimensional NMR solves the problem of the complexity of the *in vivo* $^1H$ spectra by the increased resolution obtained by displaying the spectrum on a surface. Nevertheless, this technique has certain inconveniences : the length of the experiment (several hours) and the adaptability of the pulse sequences when using surface coils. There are two $^1H$ NMR experimental approaches suitable for *in vivo* studies: J-resolved experiments (21) and chemical shift correlations (COSY) (21). The former is less effective as it is limited to separating scalar couplings in the second dimension. COSY experiments, on the other hand, really expands the spectrum (scalar coupled signals) on a surface and are thus particularly suitable for studies of living tissues and for complex mixtures in general. The system of spins produced by the protons of a molecule is a simplified version of the graph of back bone bonds in the molecule because of the presence of quaternary carbons and heteroatoms. There is a non-direct relationship between the graph of a molecule and its COSY spectrum, but in fact this spectrum is sufficiently characteristic to permit unequivocable identification of molecules of interest (Fig. 2). Identification of a molecule's characteristic spectrum is thus made by simply superimposing the COSY spectrum of the molecule over that of the mixture under investigation. Superimposition contains both the classic information on signal position, which becomes much more readily accessible for 2D spectra , and also information on the carbon chain proximities, or $^1H$ - $^1H$ scalar coupling, which becomes directly readable. The same amount of information can only be obtained on 1D NMR by first identifying the $^1H$ signals of the molecule and then performing selective decoupling on each of these signals. Clearly, the advantages listed above are lost for a molecule having a system of uncoupled spins.

### IV.2.1 *In vitro* COSY (superfused rat brain slices) (2).

Adaptation of the COSY pulse sequence (22) :

relaxation delay - 90 - t1 - 90 - acquisition

under conditions specific to *in vivo* studies requires extensive modification. We have chosen the technique for selective suppression of the water signal that was used for 1D spectra. Selective saturation of the water signal was first introduced during the relaxation period, plus a spin-echo between the two 90° pulses. This schedule effectively reduces the water signal intensity, but several expected correlations are not visible. In contrast to Arus et al. (23), we chose a non-phasable sequence requiring an absolute mode calculation, partly because of the poor intrinsic sample homogeneity and of the lower sensitivity of the double quantum filtered COSY sequence. The off-diagonal correlation signals of the COSY experiment have antiphase character, ie, under conditions of very poor homogeneity found in *in vivo* NMR these signals cancel each other because of their increased width. Mutual cancelling was also accentuated by the need for acquisition times compatible with sample survival, which made it necessary to reduce the number of FID in dimension 1 (128 FID), ie to reduce digitalization in this dimension (7). Calculating the final surface in absolute mode (24) does not detract from this phenomenon, the cancelling takes place during signal acquisition between the sinewaves of opposite phases (25). The only solution is that proposed by Kumar (7), to get the signals in phase before acquisition by adding two spin-echos symmetrical to the second 90° pulse. In phase signals are obtained for a given value of the spin-echo duration for a given spin system (AX, $AX_2$ or $AX_3$). These two echos also act to reduce the intensity of the water signal and to remove the broad signals. . In fact, the majority of molecules of interest contain a freely rotating carbon chain, corresponding to a coupling constant of 7 Hz. A mean value of 0.3/J with J = 7 Hz is suitable for the majority of the molecules studied (2).

The COSY spectrum of the sample of superfused rat brain slices (Fig. 3a), whose viability has been checked by recording a $^{31}P$ spectrum (absence of inorganic phosphate), can be used to directly identify the following components in living tissue by superimposing their respective COSY spectra: lactate, GABA, N-acetylaspartate, aspartate, glutamine/glutamate, creatine/phosphocreatine, taurine, inositol, R-choline and R-ethanolamine (reference 2 contains an error: the correlation signal of choline and ethanolamine are not superimposed). Taurine, inositol, R-choline and R-ethanolamine could be easily identified despite the presence of 10 mM glucose in the superfusion solution, which masks this region of the spectrum (Fig. 3a). The lactate and GABA signals are relatively increased and alanine appears in the COSY spectrum of dead slices (Fig. 3b) (no glucose in the superfusion medium). The experiment time has been reduced to 40 min (16 scans and 128 FID) by

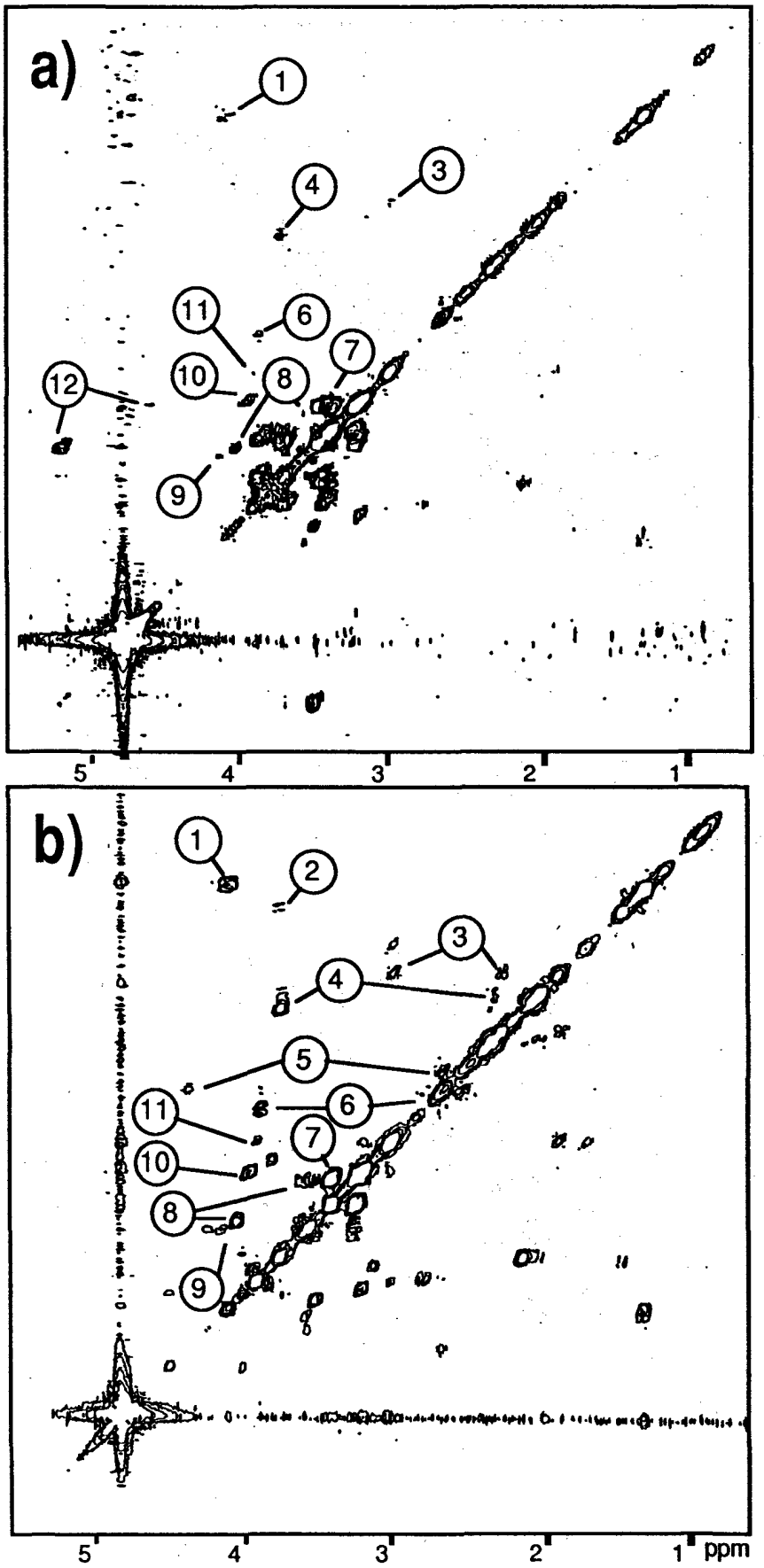


**FIG. 3.** COSY spectra of brain slices (SUPERCOSY). **a)** Superfused brain slices, **b)** Dead brain slices (without glucose in the superfusion medium). **1**/ lactate, **2**/alanine, **3** /GABA, **4**/glutamate/glutamine, **5** /N-Acetylaspartate, **6** /aspartate, **7**/taurine, **8**/inositol, **9**/R-choline, **10**/ R-ethanolamine. **11**/Creatine/Phosphocreatine. **12** /Glucose (superfusion medium.)

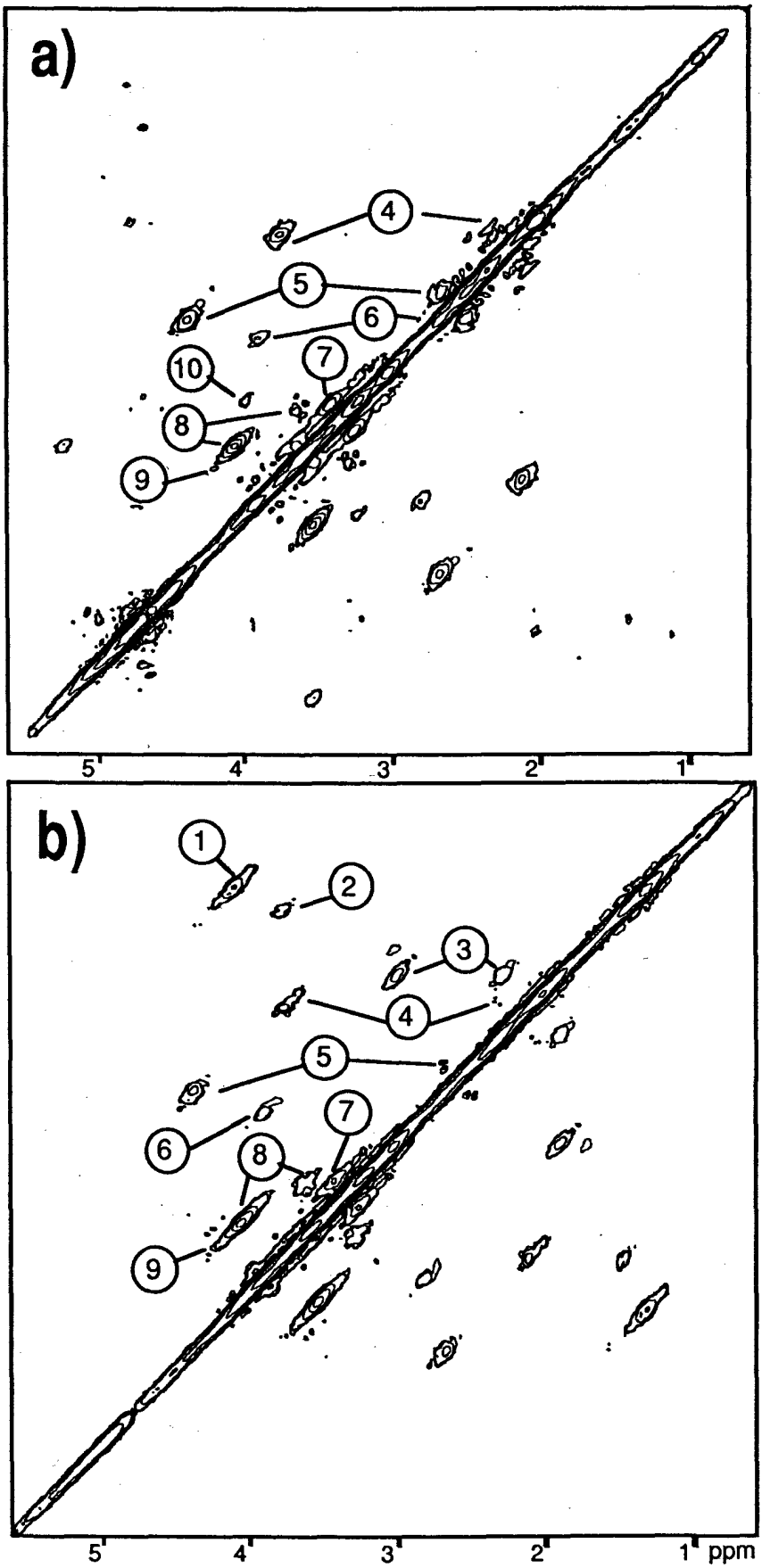


**FIG. 4.** COSY spectra of rat brain (SUPERCOSY, surface coil). **a)** live rat brain, **b)** Dead rat brain. **1/** lactate, **2**/alanine, **3** /GABA, **4**/glutamate/glutamine, **5** /N-Acetylaspartate, **6** /aspartate, **7**/taurine, **8**/inositol, **9**/R-choline, **10/** R-ethanolamine.

using a special probe which accepts wider tubes (10 mm).

IV.2.2 *In vivo* COSY (live rat, surface coils)

Studies on living animals present further difficulties, the $B_1$ excitation field produced by a surface coil is no longer homogeneous, but decreases rapidly with distance from the center of the coil. The magnetization tilt angle thus varies at each point in the sample. The SUPERCOSY sequence thus becomes:

$\Theta - t_1 - D - 2\Theta - D - \Theta - D - 2\Theta - D$ acq.

In fact a simplified calculation, using the formalism of product operators (26) shows that the signal is modulated by $(\sin \Theta)^3$, if the $2\Theta$ pulses are ignored. Thus, the COSY sequence behaves as a volume selective sequence when surface coils are used. The two $2\Theta$ pulses provide an even greater volume selectivity. The vast majority of the signals come from the region of the sample where $\Theta = 90°$. As the problem of sensitivity is less critical with $^1H$ protons than with other nuclei, this phenomenon is an advantage provided that an imaging system is used. It is possible to locate the area of measurement in the sample and then to move it by varying the emission power, thus avoiding spectral contamination with signals coming from the surface. Because of the high sensitivity of the model, the experimental time is limited by the phase program required to eliminate artefacts ( minimum: 16 scans). Elimination of the water signal is as effective as in the *in vitro* conditions, with no modifications of the sequence. The same components could be identified (Fig. 4) and there are similar increases in the signals for lactate, alanine and GABA after cell death.

## V. Conclusion

Despite considerable progress during the last few years, 1D NMR of proton $^1H$ in living tissues has not allowed exploitation of all the potential of this nucleus, especially in terms of the number of components accessible. The use of 2D NMR has made many molecules accessible to a single measurement, and their identification has become simpler and surer. The major disadvantage of 2D NMR compared to 1D NMR is its prohibitively long experimental time. At present, 1D NMR remains irreplaceable in all cases when temporal resolution of less than one minute is necessary, 2D NMR may be used to study stable states (durations over 30 min), or the passage from one stable state to another. Precise quantification of measurements (variations in component concentration) by 2D NMR is presently under investigation; it can be resolved, as demonstrated by Arus (23). The main problem is not quantification itself (peak volume measurement, for example) but the use of a stable internal reference. We are presently working along three lines : reduction in experimental time, adaptation of new sequences, and precise quantification of measurements.

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