AN NMR INVESTIGATION OF RIGOR IN PORCINE MUSCLE

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SUMMARY

Sections of porcine L. dorsi muscle were frozen to arrest rigor at various times post mortem. ^1H nmr spectra were recorded from -80 to +10°C. Thaw rigor was then allowed to proceed to completion at 0°C, the samples refrozen and the spectra re-recorded. Observable, 'liquid-like' nmr lines exhibited Arrhenius linewidth temperature dependence indicative of a single water phase of activation energy 6 to 8 kcals/mole, apparently independent of the state of rigor. No abrupt change in signal characteristics occurred at 0°C except on samples where thaw rigor had been induced where signal strength changes indicated a greater fraction of free, bulk water.

INTRODUCTION

It is known that over a period of hours after death a muscle system undergoes certain fundamental changes. The actin and myosin filaments of the contractile system cross link, imparting a more rigid structure, there is a loss of water, and the pH value of the system is reduced as a result of the conversion of glycogen to lactic acid. The ultimate pH value is determined by the condition of the muscle prior to death. The rigor process may be arrested by freezing the sample, but on restoration to a higher temperature the rigor process is resumed. The features of thaw rigor are that the rigor process is more rapid, the loss of water more drastic, and the physical size of the sample may be reduced considerably (1).

The width of an nmr spectral line is sensitive to molecular motion, the narrower the line the greater the rate of motion. Proton resonance therefore offers a method of investigating the motion of water molecules in the muscle system, and of the type of bonding in which the molecules participate. Szent-Gyorgi (2) and Bratton et al. (3) postulated that water in these

systems exists in more than one phase, and hence nmr spectra are expected to be complex. This has been confirmed by the deuteron nmr of frozen deuterated muscle (4), and more recently by relaxation measurements at room temperature (5). By using a Gill-Meiboom modification (6) of the Carr-Purcell pulse sequence (7) we have confirmed the result at room temperature on a sample from the batch used in the following experiment (figure 1). This particular sample was in a state of complete rigor. Three clearly distinguishable phases are observable with three values of T_2, the spin spin relaxation time. T_2 is a measure of the time taken for the nuclear spins constituting a phase to reach a common thermal equilibrium. Unless instrumentally broadened, cw linewidths are usually inversely proportional to T_2, which is in turn related to a correlation frequency describing molecular motion (8). However, unless linewidths are clearly different, cw measurements are less effective in resolving multi component spectra. Field and sample inhomogeneity effects broaden narrow lines. Wide lines particularly if they are weak are often hidden underneath the wings of more intense narrow lines.

In muscle systems concentrations of salts are such that freezing is expected to commence just below $0^{\circ}C$. As bulk ice is precipitated the amount of liquid phase giving a narrow nmr spectrum is reduced and salt concentration increases. At the lowest eutectic temperature of any salt present any remaining liquid component will freeze unless supercooling or vitrification occurs. In the deuterated muscle system (4) narrow liquid like lines were observed down to $-100^{\circ}C$ below any plausible eutectic temperature (9). This observation is consistent with calculations of Riedel that 8% water remains unfrozen at $-70^{\circ}C$ (10). In the deuterated muscle it was assumed that the liquid phase was attributable to water molecules within the myofibrils associated with the myofilaments.

In a proton cw measurement it might be expected that one of the phases (presumably the liquid phase) would be observed preferentially, and that

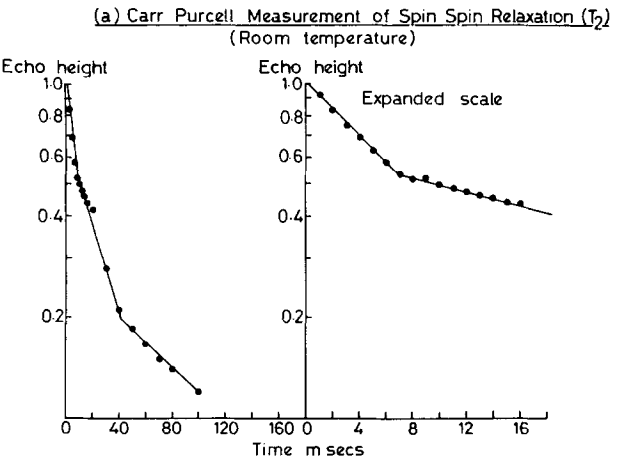

Figure 1 'H Spin Spin Relaxation of Post Rigor Muscle at 25°C.

proton nmr would be consistent with a single phase. The predictions are therefore that below 0°C the nmr signal should decrease in size over some 20 or 30°C and that below these temperatures only this "surface" water will remain. It might be expected that changes in protein conformation as a result of rigor will alter the amount of water interacting with the proteins and conceivably the rate of motion. On cooling there will be a gradual slowing down and therefore a broadening of the nmr line. The following measurements were performed to test the validity of these predictions.

EXPERIMENTAL

The first sample was placed in a deep freeze unit at -30°C within 40 minutes of the slaughter of a sow that was in a rested condition. Other samples were stored at 0°C and at varying times these were lightly dried with filter paper to remove surplus water, inserted in an nmr sample tube and transferred to the deep freeze unit. pH values were measured in control samples. With the exception of the first sample they were subsequently not disturbed (the first sample was repacked somewhat more tightly after thaw rigor). Spectra were recorded from -80 to +10°C. Observation of nmr signals indicated that samples required 15 to 20 minutes to reach thermal

equilibrium, but to ensure that equilibrium was attained samples were left for one hour at each temperature before spectra were recorded. To minimise effects due to changes in the rigor status of the muscle during the measurement period only a limited number of measurements were made near or above 0°C. Spectrometer settings were chosen to avoid rf saturation and field modulation broadening; the modulation frequency was smaller than the linewidth, and thus the derivative of the absorption line was recorded. Spectra consisted of single lines, Lorentzian in shape. The area of an absorption line is proportional to the number of nuclei contributing to the nmr spectrum, and a double integration is required to determine this quantity from a derivative lineshape. This tedious and potentially inaccurate process has been circumvented by estimating relative areas as the peak to peak height multiplied by the square of the width. This assumption is valid if the lineshape remains unchanged.

RESULTS AND CONCLUSIONS

Linewidths and estimated signal areas are plotted for four samples in figure 2, thaw rigor being induced in the first three samples. At all temperatures linewidths are smaller than those of bulk ice (11, 12). Above 0°C linewidth is determined by field inhomogeneities (80 Hz) but may also be attributable to the dispersed nature of the system, magnetic susceptibility is anisotropic and the order of 1 or 2 ppm corresponding to 60 to 120 Hz linewidths. At sufficiently low temperatures linewidth is expected to reach a limiting value of the static linewidth; there is evidence of such a levelling off process. At intermediate temperatures linewidths are describable by Arrhenius plots. The activation energies 6 to 8 kcals/mole are similar to the value of 6.5 kcals/mole reported for the diffusion of water in frozen cod muscle (13). There is some evidence of thermal hysteresis at -30°C.

There are no observable breaks in the freezing region in the linewidth plots and only negligible changes in estimated signal area where thaw rigor

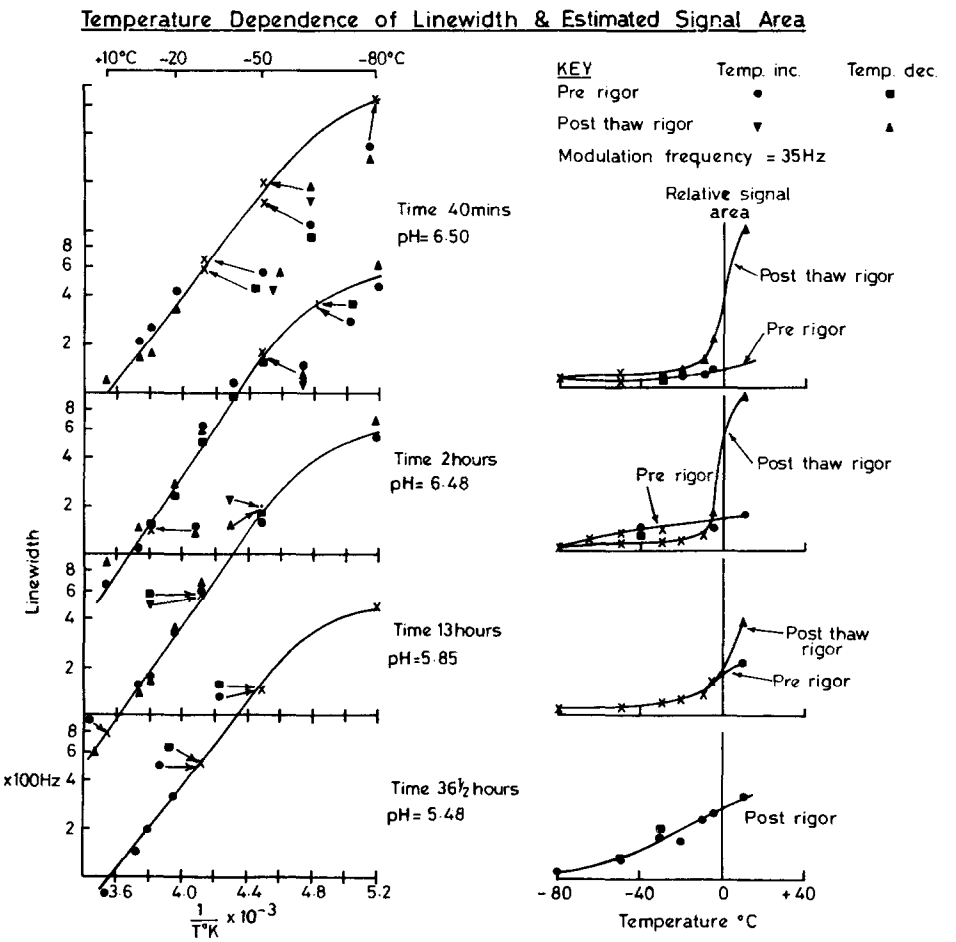

Figure 2 Temperature Dependence of Linewidth and Signal Area.

f_m = 33 Hz

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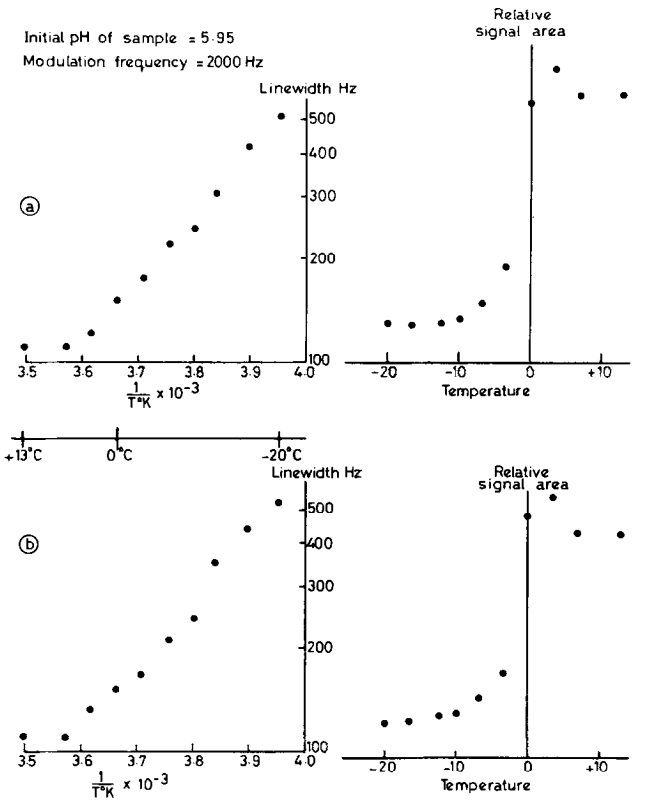
was not induced. In samples where thaw rigor has been induced bulk water is expected to be present and indeed an abrupt change of signal area is observed that is greater for the samples where rigor was arrested at an early stage by transfer to the deep freeze unit. Failure to observe a marked freezing phenomenon in samples frozen pre rigor was unexpected. Even if lineshape changes had occurred on thawing, invalidating the assumption used in calculating signal area, certainly a very significant fraction of the water molecules must remain unfrozen. To check any lineshape effect, signals were



(a) Spectra obtained on removal from deep freeze unit.
(b) Spectra obtained after a further 24 hours at 0°C.

Figure 3 Temperature Dependence of Linewidth and Signal Area.

f_m = 2000 Hz

recorded using a different technique where the modulation frequency was large and the sidebands gave the absorption lineshape. The results are plotted in figure 3. Signal area is now proportional to the product of height and linewidth, and any change in scaling factor as a result of lineshape changes on passing through the freezing region will be different. However, the intensity and linewidth plots are consistent with those of the sample frozen at pH 5.85 in the post rigor state suggesting that the effects observed are real. The sample used in this experiment was placed in a sample tube at the same time as the others, and had been stored in the deep freeze unit for

$2\frac{1}{2}$ weeks. The similarity of the intensity plot obtained on removal from the unit to that obtained after 24 hours at $0^{\circ}C$, suggests that the rigor process had proceeded either at $-30^{\circ}C$ over the storage period or during the time of the experiment and that water had been exuded.

Conclusions from the observations reported in this note are that a considerable proportion of water molecules maintain mobility below $0^{\circ}C$, and that the rate of motion is apparently unaffected by the rigor process.

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