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pH Dependence of the Progression in NMR T_2 Relaxation Times in Post-mortem Muscle

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Continuous NMR T_2 relaxation measurements were carried out on seven rabbit longissimus muscle samples in the period from 25 min to 28 h post-mortem at 200 MHz for ^{1}H. To display differences in post-mortem pH progress and extent of changes in water characteristics during conversion of muscle to meat, three of the seven animals were pre-slaughter injected with adrenaline (0.5 mg/kg live weight 4 h before sacrifice) to differentiate muscle glycogen stores at the time of slaughter. Distributed analysis of T_2 data displayed clear differences in the characteristics of the various transverse relaxation components dependent on progress in pH, as did the water-holding capacity of samples 24 h post-mortem. This reveals a pronounced effect of the progressive change in pH on the subsequent development in physical/chemical states of water during the conversion of muscle to meat. Finally, the relaxation characteristics are discussed in relation to supposed post-mortem processes of protein denaturation.

KEYWORDS: Transverse relaxation; protein denaturation; water-holding capacity; rabbit

INTRODUCTION

Several studies have reported changes in the ^{1}H transverse relaxation times of water in muscle post-mortem (*1*−*3*). This can probably be ascribed to the physical and chemical changes taking place during the conversion of muscles to meat. However, the precise relationship between the processes taking place in muscles post-mortem and the changes in ^{1}H transverse relaxation times is still largely unexplored. Recently, it was shown that exercise-induced changes in ^{1}H transverse relaxation time can be explained by the simultaneous decrease in pH and resultant increase in intracellular volume (*4*). Likewise, it seems reasonable to suggest that the decrease in pH taking place post-mortem due to formation of lactate has a major effect on the simultaneous changes in ^{1}H transverse relaxation times, as has also been demonstrated in liver (*5*).

The ability of meat to retain water, also known as the water-holding capacity (WHC), is a major quality attribute of fresh meat as it determines potential drip loss, technological quality, and appearance of fresh meat. Moreover, it may affect the sensory properties of the cooked meat, as high cooking loss results in the meat being perceived as being less juicy (*6*). It has been suggested that post-mortem pH changes in muscle strongly influence the degree of protein denaturation, which in turn is found to determine the WHC of the meat (*7*−*9*). However, at present knowledge about how the progress in protein denaturation post-mortem affects the physical−chemical state of water is far from well understood.

The aim of the present study is to investigate the relationship between pH development post-mortem and the physical−chemical state of water in muscle during its conversion to meat using high-field NMR T_2 relaxation measurements. The results are discussed in terms of models of protein denaturation within the same period.

MATERIALS AND METHODS

Animals and Sampling. Seven rabbits weighing 3.5−4.0 kg were included in the present experiment. Three of the animals were subcutaneously injected with adrenaline (0.5 mg/kg of body weight) 4 h before sacrifice. All animals were sacrificed by intravenous injection with an overdose of Nembutal (Abbott Laboratories, Chicago, IL). A muscle sample 7 mm in diameter and 3 cm long in the fiber direction was taken from the M. longissimus dorsi as quickly as possible and placed in the probes used for the subsequent NMR relaxation measurements (see below). In addition, a muscle sample was taken simultaneously from the same muscle and used for continuous ^{1}H NMR MAS spectroscopy from which pH was calculated from the chemical shift

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of the histidine C4 proton of the dipeptide carnosine (β-alanylhistidine) using eq 1

$$\mathrm{pH} = \mathrm{p}K_a + \log\frac{\sigma H - \sigma}{\sigma - \sigma A} \quad (1)$$

where pK_a is the acid dissociation constant, σH and σA are the chemical shifts of the acid and alkaline, respectively, and σ is the observed chemical shift. pK_a was set to 7.10, σH was set to 7.25, and σA was set to 6.92 ppm, as reported by Pan et al. (*10*). For further details of the ^{1}H spectroscopic NMR measurements, see Bertram et al. (*11*).

The experiment was approved by the University of Queensland Animal Ethics Committee, and the animals were treated in accordance with the guidelines outlined by the same authority.

Relaxation Measurements. Relaxation measurements were performed on a Bruker MSL 200 spectrometer operating at 200 MHz for ^{1}H. Samples were placed in a sealed glass probe with a diameter of 7 mm. ^{1}H T_2 relaxation times were measured using the CPMG sequence. The T_2 measurements were performed with a τ value (time between subsequent 180° pulses) of 300 μs, and the pulse times were 7.4 and 14.8 μs for the 90° and 180° pulses, respectively. The amplitude of every second echo in a train of 8192 echoes was acquired as 32 scan repetitions. The repetition time between two succeeding scans was 5 s, which allowed the longitudinal relaxation to return to equilibrium.

The first spectra were recorded 25 min post-mortem, and thereafter spectra were recorded continuously until 28 h post-mortem, the first 4 h with a delay of 10 min between succeeding measurements and thereafter with a delay of 1 h between succeeding measurements until 28 h post-mortem.

Distributed exponential fitting analysis was performed on the T_2 relaxation data using the RI Win-DXP program (software release version 1.2.3) released from Resonance Instruments Ltd. A continuous distribution of exponentials for a CPMG experiment may be defined by eq 2

$$g_i = \int_0^\infty A(T) \times \mathrm{e}^{-t_i/T}\,\mathrm{d}T \quad (2)$$

where g_i is the intensity of the decay at time t_i and $A(T)$ is the amplitude of the component with transverse relaxation time T. The RI Win-DXP program solves this equation by minimizing the function

$$(g_i - \int_{x=1}^{m} f_x\,\mathrm{e}^{-\tau_i/T_x})^2 + \lambda \sum_{x=1}^{m} f_x^2 \quad (3)$$

where $f_x = {}^{T_x+1}\!\int_{T_x} A(T)\,\mathrm{d}T$. $\lambda\sum_{x=1}^{m} f_x^2$ is a linear combination of functions added to the equation to perform a zero-order regularization as described by Press et al. (*12*). The data were pruned from 4096 to 256 points using linear pruning, which on synthetic data was found to give robust solutions. This analysis resulted in a plot of relaxation amplitude for individual relaxation processes versus relaxation time. From such analyses, time constants for each process were calculated from the peak position, and the area under each peak (corresponding to the proportion of water molecules exhibiting that relaxation time) was determined by cumulative integration. Additionally, the width of the relaxation population was calculated as the standard deviation of the observed relaxation times for the given peak. All calculations were carried out using an in-house program written in Matlab (The Mathworks Inc., Natick, MA).

Determination of Water-Holding Capacity. At 45 min post-mortem, a sample was taken from M. longissimus dorsi and kept at 20 °C for the first 6 h and thereafter at 4 °C until 24 h post-mortem, when the sample was used for determination of the WHC, which was determined by centrifugation. From each sample, five subsamples, ~1 cm long and having a cross-sectional area of approximately 3 × 3 mm (weight ~0.3−0.5 g), were cut out parallel to the fiber direction. The subsamples were weighed and placed in tubes (Mobicols from MoBiTec, Göttingen, Germany) with a filter (pore size = 90 μm) in the bottom of the tubes to separate the meat from the expelled liquid. The samples were then centrifuged at 500*g* for 1 h at a temperature of 20 °C. After centrifugation, the samples were weighed again, and the centrifugation loss was calculated as the percentage difference in weight before and after centrifugation.

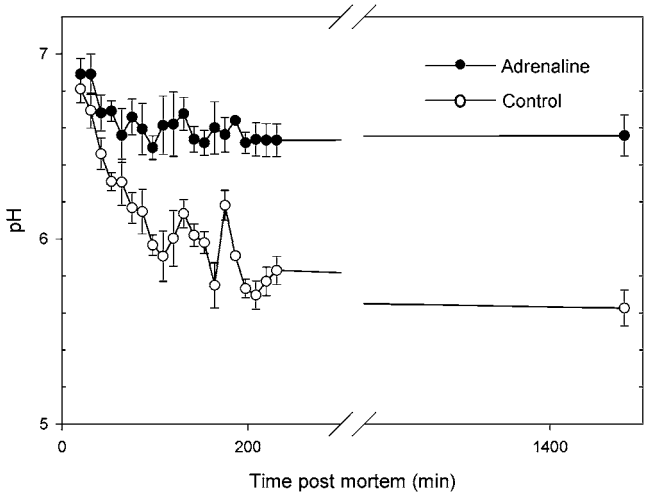

Figure 1. Development in pH as determined by ^{1}H magic angle spinning. LSMeans for muscle samples from adrenaline ($n = 3$) and control animals ($n = 4$), respectively, are given. Bars show standard errors.

Statistical Analysis. Statistical analysis was carried out with the Statistical Analysis System (SAS Institute Inc., Cary, NC), using correlation analysis (Proc CORR) and analysis of variance (Proc GLM). The statistical model included the fixed effects of measurement time and treatment (adrenaline vs control) and the random effect of animal within treatment.

RESULTS

Figure 1 shows the intramuscular pH development in both the control and adrenaline-administered animals as determined by ^{1}H MAS NMR spectroscopy. The effect of ante-mortem adrenaline injection is highly significant, with the decrease in pH being less pronounced compared with control samples. In addition, the rate of decrease in pH is faster in muscle samples from the control animals compared with samples from adrenaline-treated animals. Centrifugation loss was significantly influenced by preslaughter treatment ($p = 0.02$), with centrifugation loss being 2.6% in samples from adrenaline-administered animals compared with 6.0% in the control group.

The ^{1}H T_2 relaxation time data were fitted using a continuously distributed exponential curve fitting method as described under Materials and Methods. Panels a and b of **Figure 2** show typical changes in these T_2 relaxation characteristics during the post-mortem period of muscle samples from control and adrenaline-administered animals, respectively. In all muscle samples, three major components were detected, namely, a component with a very short relaxation time between 1 and 10 ms, in the following called T_{2B}; a major component having a time constant between 30 and 60 ms, in the following called T_{21}; and finally a component with a time constant from 100 to 200 ms, in the following called T_{22}. In addition, a minor very slowly relaxing component with a relaxation time between 400 and 600 ms, in the following called T_{23}, was occasionally observed. Changes in both the T_{21} and T_{22} populations with time post-mortem were clearly affected by adrenaline treatment. In the muscle samples from control animals, the T_{21} and T_{22} components broadened and merged together during the first hours post-mortem, whereas in muscle samples from adrenaline-administered animals the two components remained clearly well-separated during the 24 h post-mortem period.

Figures 3, **4**, and **5** show the detailed changes in the T_{2B}, T_{21}, and T_{22} time constants, populations, and width of the

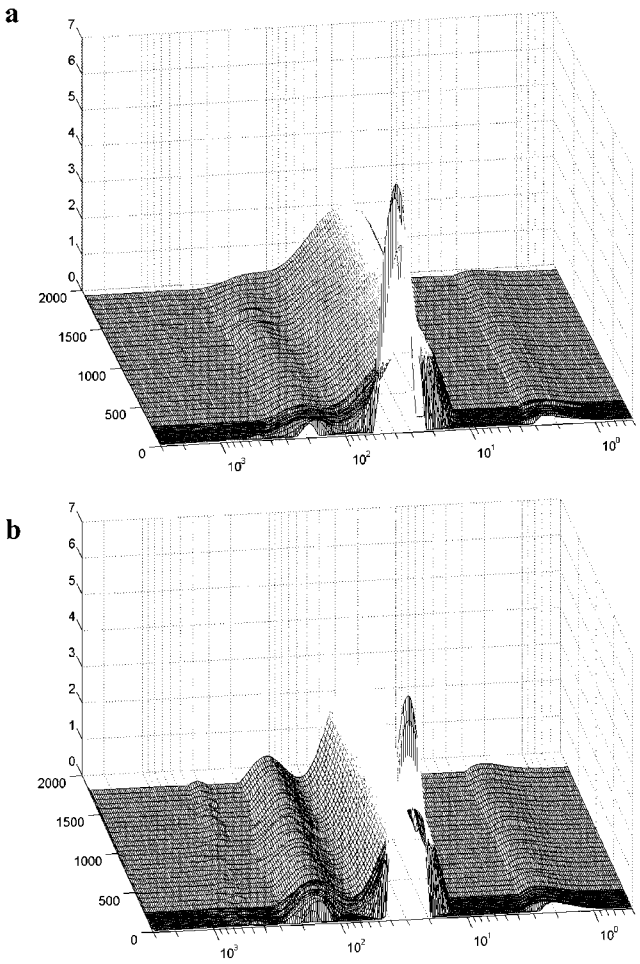

Figure 2. Typical example of three-dimensional plot showing the post-mortem progress of T_2 relaxation times after distributed exponential fitting analysis of data for the muscle samples with (a) rapid and pronounced decrease in pH and (b) moderate decrease in pH post-mortem, respectively. Time post-mortem (p.m.) is shown in minutes on the *X*-axis, relaxation time in milliseconds on the *Y*-axis, and signal intensity (*I*) on the *Z*-axis.

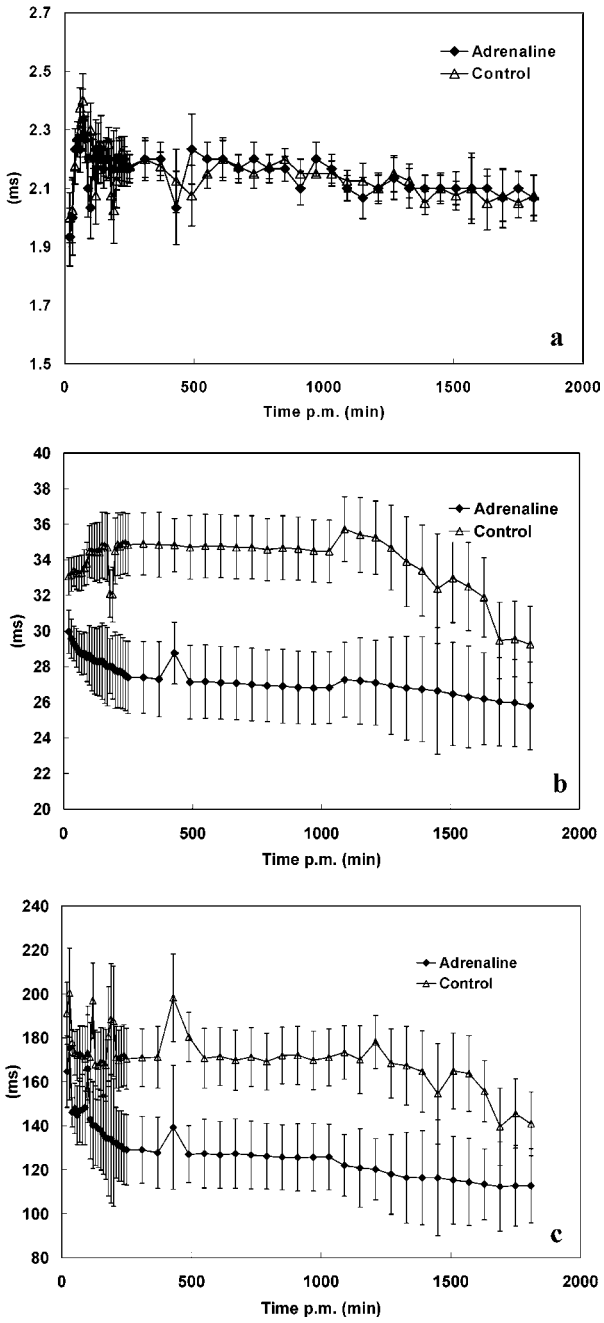

Figure 3. Post-mortem course of the characteristics of mean time constants (a) T_{2B}, (b) T_{21}, and (c) T_{22} for muscle samples with rapid and pronounced decrease in pH ($n = 4$) and moderate decrease in pH post-mortem ($n = 3$), respectively. LSMeans are given. Bars show standard errors.

populations, respectively, during the post-mortem period for the two treatments. The T_{2B} time constant initially increased and thereafter remained constant at ~2 ms for both treatments, that is, independent of change in pH (**Figure 3a**). **Figure 3b** shows that at all times post-mortem the T_{21} time constant was significantly higher in muscle samples from the control group compared with the adrenaline group. In addition, the progressive change in T_{21} was also significantly affected by pH development, as an initial increase was observed for muscle samples from the control group, whereas an initial decrease corresponding to faster relaxation was observed for the samples from the adrenaline group (**Figure 3b**). The T_{22} time constant was initially found to decrease with time post-mortem. This decrease in T_{22} was much more pronounced in muscle samples showing a smaller decrease in pH (adrenaline group), and from ~4 h post-mortem and afterward, T_{22} was significantly lower in these muscle samples compared with the control samples (**Figure 3c**).

A decrease in the population of protons decaying with T_{2B} from an initial value of ~6% to approximately 3 and 4% for the control and the adrenaline group, respectively, was observed (**Figure 4a**). **Figure 4b** shows that for both treatments an initial decrease in the population of protons decaying with T_{21} was observed. Even though not significant, a tendency for a more pronounced decrease in the T_{21} population in the muscles from adrenaline-administered animals was observed. Independent of treatment, an increase in size of the T_{22} population from ~3% to ~12% was observed from 30 min post-mortem to 3.5 h post-mortem, which was followed by a slight decrease (**Figure 4c**).

The width of the T_{2B} populations increased initially followed by a slight narrowing (**Figure 5a**). The width of the T_{21} population immediately postslaughter was found to be identical in muscle samples from the two treatments; however, an increase in the width, revealing a more ill-defined T_{21} population, progressed rapidly in the muscle samples exhibiting the pronounced decrease in pH (**Figure 5b**).

As was seen for the T_{21} population, the width of the T_{22} population was initially found to be almost identical for the two

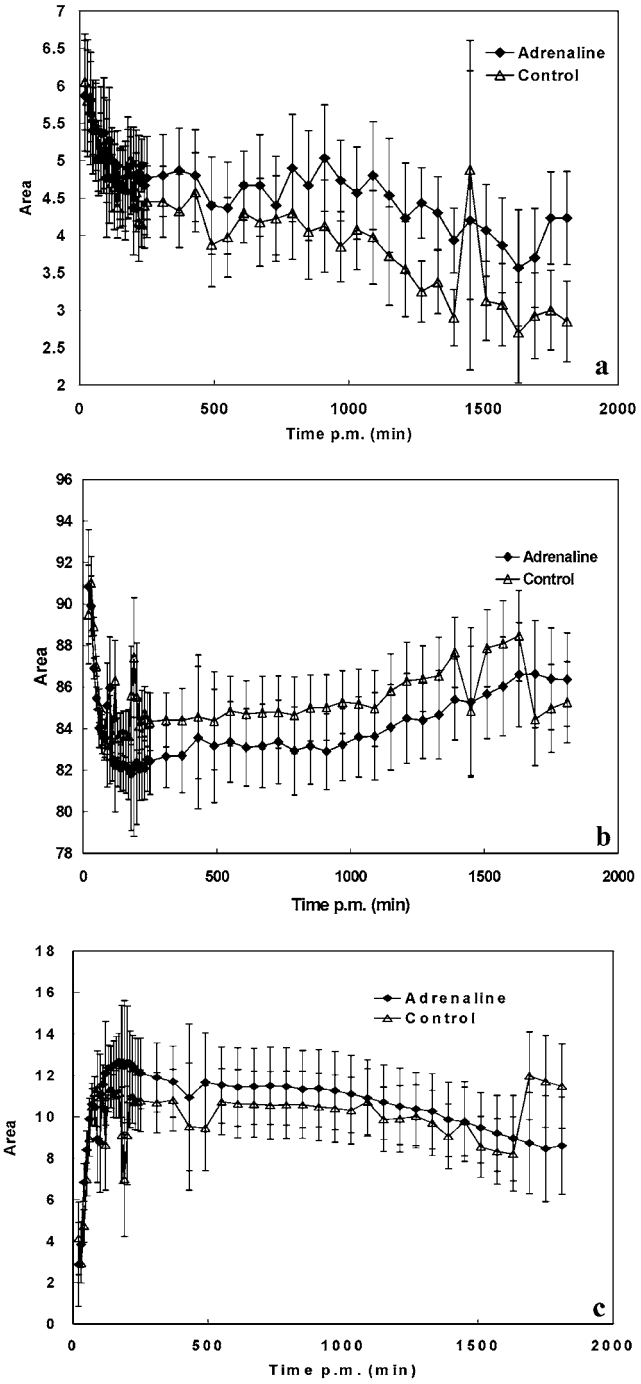

Figure 4. Post-mortem course of the T_2 populations (a) T_{2B}, (b) T_{21}, and (c) T_{22} for muscle samples with rapid and pronounced decrease in pH ($n = 4$) and moderate decrease in pH post-mortem ($n = 3$), respectively. LSMeans are given. Bars show standard errors.

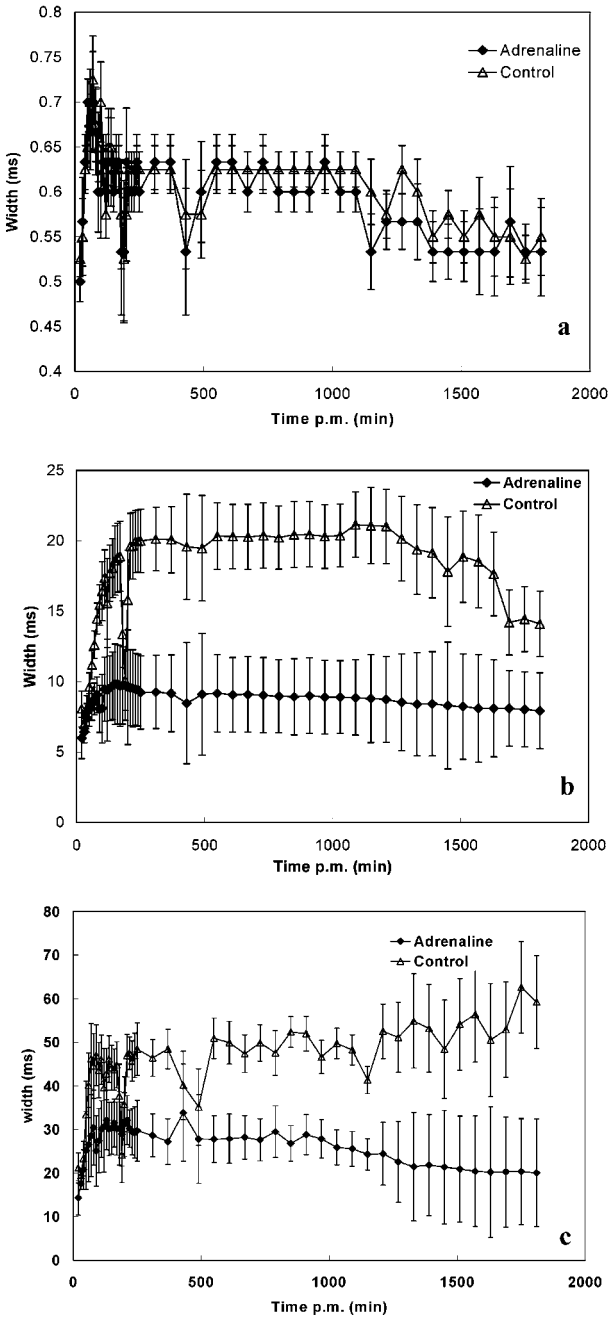

Figure 5. Post-mortem course of the characteristics of the width of T_2 populations (a) T_{2B}, (b) T_{21}, and (c) T_{22} for muscle samples from control ($n = 4$) and adrenaline-treated ($n = 3$) animals, respectively. LSMeans are given. Bars show standard errors.

treatments; however, the T_{22} population of the control group rapidly became more ill-defined (broader) compared with the T_{22} population in muscle samples from the adrenaline-administered animals (**Figure 5c**).

DISCUSSION

Using different preslaughter treatments, different glycogen levels and rates of glycolysis can be obtained in post-mortem muscle, also resulting in different pH progress post-mortem (*13*−*15*). By administration of adrenaline preslaughter, directly affecting glycogenolysis (*16*), two different post-mortem profiles of pH developments were obtained in the present study. Determination of the WHC by centrifugation of samples at 24 h post-mortem verified a strong, significant effect of the post-mortem pH changes on WHC.

NMR T_2 relaxation times have long been recognized as being very sensitive to the physical and chemical states of water in muscle and meat (*1*, *17*−*19*). In addition, NMR T_2 relaxation measurements have been shown to be able to predict the WHC of the meat (*20*−*23*), revealing that WHC is determined by the physical−chemical states of water in meat. In the present study distributed exponential fitting analysis of T_2 relaxation data revealed four different water fractions: a rapidly relaxing component ($T_{2B} \sim 1-10$ ms), a major component ($T_{21} \sim 30-60$ ms), and finally a slower component ($T_{22} \sim 100-200$ ms).

In addition, a fourth, minor component having a long relaxation time ($T_{23} \sim 500-1000$ ms) was occasionally observed.

The origin of multiexponential relaxation in muscle tissue and meat has been discussed extensively (*19*, *24*−*26*). The most widely accepted interpretation is due to the multicompartment model, in which the different T_2 components are suggested to originate from different anatomic compartments between which the water is in slow exchange (*17*, *20*, *25*−*26*). In muscle T_{21} is thought to arise from intracellular water, whereas the T_{22} time constant is due to extracellular water, as the cell membrane is believed to act as a physical barrier for rapid exchange (*17*, *26*, *27*). Recently, it was questioned whether this interpretation can be applied after the muscle has turned into meat (*19*), as cell membrane structures are known to disintegrate post-mortem, as revealed by microscopic studies (*28*). Accordingly, in meat T_{21} might more specifically represent water located within highly organized protein structures, for example, water in tertiary and quaternary protein structures and spaces with high myofibrillar protein densities including actin and myosin filament structures, whereas T_{22} more likely represents extramyofibrillar water (*20*). A recent study in which the structure of meat was manipulated by prerigor stretching or cold contraction showed that this interpretation is consistent with theoretical calculations (*29*).

Although the distributed exponential fitting analysis of the data in the present study indicates the presence of a number of distinct relaxation populations, it also clearly demonstrates that each population is not simply characterized by a single, discrete relaxation time, but rather by a continuous spectrum of relaxation times. A widely accepted theory of NMR relaxation in heterogeneous media such as muscle is that proposed by Brownstein and Tarr (*30*). They assumed that the relaxation rate was determined by exchange processes between water and macromolecules; the latter can be considered surfaces and are described as relaxation sinks, as described in more detail in ref *29*. These authors also suggested that the relaxation of water protons depends on the relaxation strength and the probability of the water protons meeting the surface, the latter expressed as the surface-to-volume ratio of the structure, which confines the water protons. Consequently, the T_2 relaxation time can be expressed as eq 4 (*30*)

$$\frac{1}{T_2} = \mu\frac{S}{V} \tag{4}$$

where μ is the relaxation sink strength, S is the surface area, and V is the volume. This approach has later been used by Hills et al. (*31*, *32*) and others (*29*, *33*, *34*) to analyze T_2 relaxation data quantitatively in a range of diverse systems. Moreover, in accordance with these ideas, Lillford et al. (*24*) explained the existence of a distribution of relaxation times as a result of heterogeneity of the system investigated, as such heterogeneity will result in a continuous distribution of distances between water molecules and macromolecules or surfaces. Likewise, in the present study the existence of a distribution of relaxation times within each population suggests a distribution of sizes of the different domains, which will result in a distribution of surface-to-volume ratios (S/V). However, the existence of a distribution of relaxation times within each population may also be the result of a distribution of relaxation sink strengths, as a whole range of different macromolecules or constituents will be present in the different domains; that is, in the case of the myofibrillar space the relaxation sink strength may vary for actin and myosin filaments and other proteins present.

It is noticeable in the present study that the progress and characteristics of especially the T_{21} and T_{22} populations are significantly influenced by the changes in pH post-mortem (**Figures 5b** and **4c**). In the muscle samples from control animals, the T_{21} and T_{22} components broadened and merged within the first few hours post-mortem, whereas in the muscle samples with the reduced decrease in pH (adrenaline group) the two components remained separated during the entire measuring period. It is worth noting that the samples were not chilled but were rather exposed to a relatively high temperature during measuring (the temperature was measured to be 32 °C in the probe). The combination of the high temperature and the rapid decrease in pH in the muscle samples from control animals resembles conditions known to develop in exudative pork, that is, PSE meat (pale soft exudative), characterized by a high drip loss. This was supported by the results of the WHC measurements, which showed inferior WHC in muscle samples undergoing a rapid decrease in pH compared with muscle samples with moderate change in pH post-mortem.

This study also shows that muscles undergoing a rapid decrease in pH at relatively high temperatures show broad distributions of the longer relaxation times (**Figure 5b**,**c**). This may be due to a larger distribution of surface-to-volume ratios (S/V) compared with normal meat (eq 3). The combination of the rapid decrease in pH and relatively high temperature will lead to more pronounced protein denaturation, giving rise to a more heterogeneous distribution of spaces in the myofibrillar structures. Another possibility is an increase in the range of surface relaxation sink strengths in such muscles compared with normal muscles. Knowledge of what determines the relaxation sink strength is very limited. However, as denaturation of proteins causes conformational changes, that is, change in hydrophobicity, it seems reasonable to expect changes in the rate of exchange with water protons and the rate of relaxation at their surfaces. Moreover, as the meat will possess both native and denatured proteins, changes in the relaxation sink strength on denaturation will result in a wider range of relaxation sink strengths and therefore a larger range of relaxation rates. In addition, the possibility that the protein denaturation also leads to a reduction in the chemical exchange of water cannot be ruled out. In fact, many NH groups of amino acids become accessible to water upon denaturation of proteins, and the proton exchange becomes possible and faster, which could be expected to change T_2.

The results presented in **Figures 3** and **5b**,**c** show that both the T_{21} and the T_{22} populations broaden in the muscles exposed to the combination of rapid decrease in pH and relatively high temperature. Consequently, proteins in both populations should be denatured if either of the above explanations is valid. Reduced extractability of actomyosin from so-called PSE meat was early discovered and was first believed to be due to a deposition of denatured sarcoplasmic proteins on the myofilaments (*35*). The sarcoplasmic protein creatine kinase was found to be especially susceptible to denaturation (*8*). However, Penny (*36*) also demonstrated that myosin becomes insoluble during exposure to the combination of high-temperature and low-pH conditions that will occur in the post-mortem PSE situation. Later it was shown that the solubility of both sarcoplasmic and myofibrillar proteins is reduced in PSE meat (*9*). Denaturation of the myofibrillar proteins would be expected to affect mainly the T_{21} component, whereas denaturation of sarcoplasmic proteins may be expected to affect both the T_{21} and the T_{22} components, as high amounts of sarcoplasmic proteins are found in the extrafibrillar fluid (*37*).

The reduced WHC associated with a high degree of protein denaturation is believed to be due to a reduced ability of the

proteins to bind the water (*7*, *35*); however, the exact mechanism of denaturation is far from well understood. Therefore, knowledge about whether protein denaturation gives rise to a larger distribution in sizes of domains and, furthermore, what eventually determines the relaxation sink strength of the constituents in the meat, is very limited. Myosin denaturation is often assessed as loss of ATP′ase activity (*9*, *38*). The destruction of ATP′ase activity indicates an altered structure of the heavy chains of myosin, which contains the enzyme activity. Penny (*36*) studied myosin exposed to various pH and temperature conditions and found that loss of ATP′ase activity is initiated at temperatures around 30 °C. In addition, differential scanning calorimetry has been used to show that chicken muscle myosin rods start to aggregate at 30 °C (*39*). Thus, it seems reasonable to suggest that the structures of several parts of the myosin molecule are changed, with simultaneous changes in relaxation sink characteristics, during exposure to high temperature and low pH early post-mortem.

Recently Saab et al. (*40*, *41*) reported the presence of three different T_2 components in in vivo skeletal muscle both at rest and during exercise in addition to a rapidly relaxing population corresponding to the population named T_{2B} in this work. The longer T_2 relaxation time constants were approximately 21, 40, and 110−140 ms, whereas the very fast relaxing component had a T_2 time constant below 5 ms. Saab et al. (*41*) suggested that the component with a time constant of 21 ms represents water associated with heavily hydrated glycogen, whereas the component with a time constant of 40 ms should represent cytoplasmic fluid. Depletion of muscle glycogen stores using adrenaline treatment in the present study made it possible to examine this hypothesis. Assuming that water associated with glycogen has a relaxation time of ∼20 ms, it should contribute to the T_{21} population detected in the present study. If this is the case, the T_{21} population should be shifted toward shorter relaxation times in the muscles from control animals having normal glycogen levels immediately post-mortem compared with muscle samples with glycogen depletion (adrenaline-administered animals). On the contrary, in muscle samples from the control animals the T_{21} population had longer relaxation times. This indicates that factors other than glycogen content control the value of the T_{21} in the present study, most likely spatial factors.

CONCLUSIONS

In conclusion, the present study demonstrated that the physical−chemical state of water in muscles during their conversion to meat is strongly affected by the progressive change in pH post-mortem. A pronounced decrease in pH post-mortem in muscle results in broader relaxation populations, which probably should be ascribed either to a more heterogeneous distribution of myofibrillar spaces or to an increase in the range of surface relaxation sink strengths upon severe protein denaturation.

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