JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Post-mortem Changes in Porcine M. Longissimus Studied by Solid-State ^{13}C Cross-Polarization Magic-Angle Spinning Nuclear Magnetic Resonance Spectroscopy

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Solid-state ^{13}C cross-polarization (CP) magic-angle spinning (MAS) nuclear magnetic resonance (NMR) experiments are carried out for the first time on rapidly frozen muscle biopsies taken in M. longissimus in vivo and at 1 min, 45 min, and 24 h post-mortem from three pigs. Two of the pigs were CO_2-stunned (control animals), and one was pre-slaughter-stressed (treadmill exercise) followed by electrical stunning to induce difference in metabolism post-mortem. ^{13}C resonance signals from saturated and unsaturated carbons in fatty acids, carboxylic carbons, and carbons in lactate and glycogen are identified in the solid-state NMR spectra. The ^{13}C CP MAS spectra obtained for post-mortem samples of the stressed, electrically stunned pig differ significantly from the post-mortem control samples, as the intensity of a resonance line appearing at 30 ppm, assigned to carbons of the methylene chains, is reduced for the stressed pig. This spectral difference is probably due to changes in lipid mobility and indicates altered membrane properties in the muscle of the stressed/electrically stunned animal when compared with the control animals already 1 min post-mortem. In addition, the post-mortem period changes in glycogen carbons can be estimated from the ^{13}C CP MAS spectra, yielding a correlation of $r = 0.74$ to subsequent biochemical determination of the glycogen content.

KEYWORDS: Glycogen; muscle biopsies; ^{13}C MAS NMR spectroscopy; membranes; lactate

INTRODUCTION

During the post-mortem period, muscles are converted to meat as a result of several biochemical and physical processes. It has long been recognized that the processes taking place during the post-mortem period are of great importance in meat animals, as they have decisive influence on the final quality of the meat (*1*−*3*). Pre-slaughter handling of animals affects the final meat quality, especially when the animals are exposed to high stress situations (*4*, *5*), and in particular poor water-holding capacity (WHC) is a well-documented problem in pre-slaughter-stressed pigs (*6*, *7*). Several studies have demonstrated that pre-slaughter stress causes increased metabolic rates (*8*−*10*). However, recent studies indicate that destabilization of important cell components, especially membrane structures, might also be a central factor (*11*, *12*). Measurement of electrical impedance, which can be considered an indirect measurement of membrane integrity (*13*), has revealed differences in membrane properties early post-mortem between muscles from pre-slaughter-stressed and normal pigs (*12*). Likewise, heat stress in live chickens is reported to give rise to the presence of creatine kinase in plasma, which indicates alterations in muscle membrane integrity upon stress (*14*). For these reasons, loss of membrane integrity upon pre-slaughter stress could also be hypothesized to be a significant contributor to inferior WHC in meat. In fact, this has been the reason to recommend supranutritional vitamin E supplementation to pigs due to a possible membrane stabilizing effect of this vitamin (*15*). Nevertheless, at present, alterations in membrane properties post-mortem and their relationship to pre-slaughter stress are greatly underexplored topics, mainly due to lack of appropriate noninvasive methods. Accordingly, detailed studies of both the dominating biochemical processes

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10.1021/jf020919x CCC: $25.00
Published on Web 02/21/2003

in terms of post-mortem energy metabolism and changes in muscle membrane characteristics are much required.

Muscle glycogen is of great interest because of glycogen's role in in vivo stress, exercise-related energy metabolism. In meat animals, muscle glycogen is furthermore of importance due to its crucial role in the anaerobic glycolysis and lactate formation post-mortem, which is known to be very decisive the final meat quality. In the investigation of muscle energy metabolism the use of biopsy sampling is the most established technique. The application includes a biochemical determination of the metabolites of interest performed after homogenization and extraction steps. However, it is well-known that the extraction procedures are associated with several disadvantages, including incomplete extraction, undesired hydrolysis, selectivity problems, slow analysis, and last, not but least, they are destructive to the sample.

Due to the noninvasive and nondestructive character of the NMR technique, the use of NMR for studies on energy metabolism has been suggested as a potentially useful technique (*16*, *17*). However, because of the low natural abundance (1%) of the ^{13}C isotope, NMR measurements of the carbon energy metabolism are presently very sparse (*18*). However, using magic-angle spinning (MAS) combined with cross-polarization (CP) techniques, a considerable improvement in signal intensity can be obtained with samples frozen to very low temperatures. This has the advantage that samples can be stored and studied without degradation of metabolites and compounds of interest, and accordingly subsequent measurement is possible at any time.

The use of solid-state NMR techniques has proven to be successful in many areas of biochemistry and chemistry (e.g., peptides, metalloenzymes, and carbohydrates), materials science (e.g., polymers, timber, minerals, zeolites, and catalysts), and to some extent also in food science (for a review see ref *19*). The line broadening occurring in traditional solution NMR techniques for compounds with restricted motion may be reduced considerably by modern solid-state techniques. As a result, the resolution of larger molecule complexes in biological systems may be strongly enhanced, and ^{13}C solid-state methodologies have, for example, proven to be successful in providing information about cell wall constituents in yeast cells (*20*) and in measurements of alterations in membrane constituents in mammary tumor tissue and cells (*21*). However, to our knowledge, ^{13}C solid-state methodologies have so far not been applied to research related to muscle and meat, despite the fact that a number of possible and obvious applications can be foreseen, as for example demonstrated for liver tissue (*22*). The present work reports the first study of carbon constituents and metabolism in post-mortem muscles using a low-temperature solid-state ^{13}C CP MAS NMR technique of frozen muscle samples.

MATERIALS AND METHODS

Animals and Sampling. The experiments included three slaughter pigs, which were offspring of Duroc/Landrace boars cross-bred with Landrace/Yorkshire sows. Three weeks before slaughter, a muscle biopsy sample was taken in M. longissimus dorsi. Immediately after sampling, the biopsies were frozen in 7 mm o.d. zirconia rotors (225 μL sample volume) in liquid nitrogen and stored at −80 °C until further analysis.

The pigs were slaughtered in the experimental abattoir at Research Centre Foulum. At the time of slaughter, the pigs had a live weight of ∼100 kg. Two of the pigs served as control with minimal pre-slaughter stress and subsequent CO_2-stunning (80% CO_2 for 3 min). To induce pre-slaughter stress, the third pig was exercised on a treadmill at a speed of 3.8 km/h for 20 min (*23*) and subsequently electrically stunned, which was done by placing stunning tongs on the pig's head (220 V, 15 s, equipment from K. Schermer and Co., Ettlingen, Germany).

After stunning, the pigs were exsanguinated, and 1 min, 45 min, and 24 h post-mortem muscle biopsy samples were taken in M. longissimus dorsi and instantly frozen in 7 mm o.d. zirconia rotors (used for the CP MAS NMR spectroscopy) in liquid nitrogen before storage at −80 °C until further analysis. For the control pigs, one sample was taken at the points in time given above, whereas for the stressed pig, two samples were taken.

NMR Measurements. The ^{13}C CP MAS spectra were recorded on a Varian Unity INOVA-300 (7.05 T) spectrometer at 75.43 MHz. All experiments were performed with a 7 mm home-built high-speed spinning variable-temperature ^{1}H−X double-resonance probe tuned to 299.1 and 75.4 MHz. The measurements on the stored (−80 °C) sample rotors were performed at a temperature of −50 °C at the inlet to the probe. This corresponds to an actual sample temperature of −38 °C, determined in separate experiments as described elsewhere using ^{207}Pb MAS NMR of $Pb(NO_3)_2$ as an NMR thermometer (*24*). The rotors were rapidly (in <15 s) transferred to the precooled stator in order to avoid deterioration of the samples. A spinning speed of 4.5 kHz was used along with radio frequency (rf) field strengths of ∼40 kHz for the Hartmann−Hahn match and ∼65 kHz for ^{1}H decoupling. A contact time of 1 ms and a repetition delay of 4 s were employed. The contact time was initially optimized by varying it in the range from 0.2 to 5 ms on different types of samples (samples taken in vivo and samples taken 24 h post-mortem). These experiments showed that (i) independent of sample type, the same development in spectral intensities as a function of contact time was observed for the different carbon resonances and (*ii*) independent of sample type, maximum spectral intensity was observed for the same contact time. Therefore, possible differences in the spectral intensities must be ascribed to differences in concentrations or differences in CP efficiency caused by molecular or segmental motion. A contact time of 1 ms was chosen because it results in optimized spectral intensities of carbons with weak dipolar couplings to protons (e.g., carbonyls), as well as carbons with strong dipolar couplings to protons. Between 1200 and 1800 scans were accumulated for each sample. The spectral intensities of all resonances for each spectrum are normalized to the intensity of the carbonyl carbon resonance at 175 ppm.

^{13}C chemical shifts are relative to TMS, and the scale was referenced using hexamethylbenzene as a secondary substitution standard. To assign the resonances from lactate, a ^{13}C spectrum was recorded for a meat sample taken 24 h post-mortem followed by addition of exogenous lactic acid and recording of a second low-temperature ^{13}C CP MAS spectrum. Pronounced increases in signal intensity of the resonances at approximately 20.5, 67.4, and 175 ppm were observed following addition of lactate, and the resonances were assigned to C3, C2, and C1 in lactate, respectively. Following resolution and assignment of all the ^{13}C resonances to the various components, quantitative assessments of these constituents were accomplished using mathematical deconvolution. Fitting of the experimental spectra was performed on a SUN Sparc-10/51 workstation using the deconvolution curve-fitting procedure of the Varian VNMR software employing Gauss−Lorentzian distributions.

Biochemical Determination of Glycogen and Lactate. The concentration of muscle glycogen was determined in duplicate for 50 mg muscle samples. Samples were heated in a test tube with 5 mL of 1 M HCl at 100 °C for 2 h to hydrolyze the glycogen to glucose units and then centrifuged at 1500*g* for 10 min at 4 °C. The concentration of muscle lactate was determined in duplicate in 10 mg muscle samples, incubated for 30 min on ice in vials containing 600 μL of 3 M perchloric acid. The extraction procedure was stopped by adding 1000 μL of 2 M $KHCO_3$ to the vials, and the samples were centrifuged using the same procedure as described for glycogen. Both glucose and lactate were measured spectrophotometrically as outlined elsewhere (*25*) using a Cobas ABX Mira Plus autoanalyzer (ABX Diagnostics). The results are expressed in micromoles of glucose residues per gram of muscle (wet weight). The repeatability of the method using two 10 mg muscle samples was 0.86.

Statistics. Linear regression was performed using PROC REG, and Pearson correlation coefficients were calculated and significantly tested using the Statistical Analysis System (*26*).

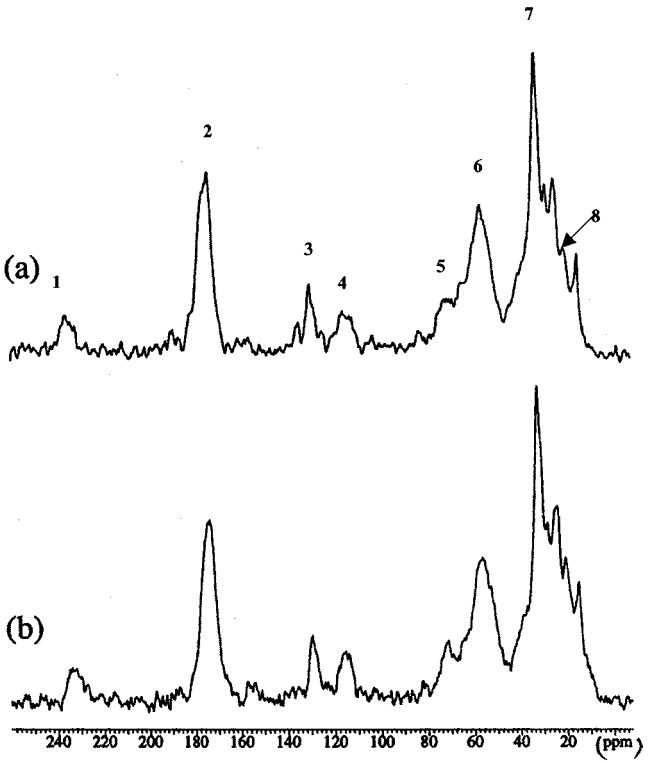

Figure 1. ^{13}C CP MAS NMR spectra of prefrozen M. longissimus biopsies taken in vivo from (a) control, CO_2-stunned pig, and (b) pre-slaughter-stressed, electrically stunned pig. Each spectrum required ~1500 scans and was collected at −38 °C with a spinning frequency of 4.5 kHz. Peaks 1 and 4 are assigned to spinning sidebands, whereas peak 2 is assigned to carbonyl carbons. Peak 3 is assigned to unsaturated carbons of fatty acids, peak 5 to C3, C4, C5, and C6 in glycogen, and peak 6 to methyl groups of choline and phosphatidylcholine; peak 7 is assigned to various saturated carbons of methylene chains of the fatty acids, and peak 8 is assigned to the CH_3 group in lactate.

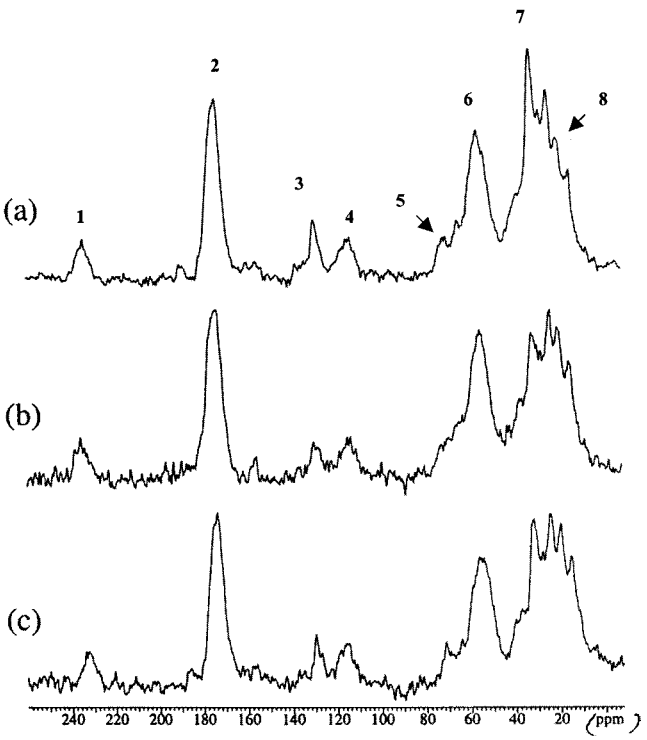

Figure 2. ^{13}C CP MAS NMR spectra of prefrozen M. longissimus biopsies taken 1 min post-mortem from (a) control, CO_2-stunned pig, (b) pre-slaughter-stressed, electrically stunned pig, and (c) replicate of (b) (see text). Each spectrum required ~1500 scans and was collected at −50 °C with a spinning frequency of 4.5 kHz. Peaks 1 and 4 are assigned to spinning sidebands, whereas peak 2 is assigned to carbonyl carbons. Peak 3 is assigned to unsaturated carbons of fatty acids, peak 5 to C3, C4, C5, and C6 in glycogen, and peak 6 to methyl groups of choline and phosphatidylcholine; peak 7 is assigned to various saturated carbons of methylene chains of the fatty acids, and peak 8 is assigned to the CH_3 group in lactate.

RESULTS

Figure 1 illustrates the ^{13}C CP MAS spectra obtained for the muscle biopsies taken in vivo from one of the control pigs and from the pig that was subsequently exposed to pre-slaughter stress. As expected, the spectra are quite identical. The resonance that appears at 175 ppm is identified as originating from the carbonyl carbons of peptides and from esterified and free fatty acids (*27*, *28*), and the associated first-order spinning sidebands from this resonance are seen at 235 and 115 ppm. Furthermore, the resonance that appears at 130 ppm is identified as originating from the unsaturated carbons of fatty acids (*27*, *28*). The resonance at 56 ppm most likely represents the methyl groups of choline and phosphatidylcholine, whereas the peak positioned at 72 ppm represents the unresolved signals from C3, C4, C5, and C6 in glycogen (*27*, *28*). A low-intensity peak from the anomeric carbon C1 in glycogen is observed at 100 ppm. The most intense resonance in the spectra is found at 30 ppm, with several shoulders on its high-field side. This resonance can be assigned to the various saturated carbons of fatty acids, in particular from the methylene chains (*27*, *28*). Lactate also contributes to the signal and has in a separate experiment exhibited a chemical shift at ~20.5 ppm (see section on NMR measurements).

Figure 2 displays the ^{13}C CP MAS spectra obtained for the samples taken 1 min post-mortem from the control pigs (**Figure 2a**) and the two samples taken from the pig exposed to pre-slaughter stress (**Figure 2b**,**c**). The spectra of the two samples from the pre-slaughter-stressed pig are quite similar, indicating fine repeatability of the NMR measurements within the muscle. The spectrum obtained for the sample from a control animal (**Figure 2a**) is nearly identical to the spectra obtained for the in vivo samples; only a minor decrease in the intensity of the resonance at ~34 ppm is observed (**Figure 1**). In contrast, the spectra obtained for the samples from the pre-slaughter-stressed pig (**Figure 2b**,**c**) differ, as is most noticeable from the considerable decrease in the intensity of the resonance at ~34 ppm.

The ^{13}C CP MAS spectra obtained for the muscle biopsies taken 45 min post-mortem show tendencies similar to those of the 1 min post-mortem spectra, that is, a marked difference is evident in the spectra from the pre-slaughter-stressed pigs, because the resonance at ~34 ppm is considerably lower than observed in in vivo samples. In contrast, for the control samples the intensity of the resonance at ~34 ppm is reduced only slightly in intensity compared with samples taken in vivo (spectra not shown). In contrast to the spectra obtained early post-mortem, the ^{13}C CP MAS spectra obtained for the muscle biopsies taken 24 h post-mortem are almost identical, independent of pre-slaughter treatment (spectra not shown).

Figure 3 represents an example of an expansion of the region between 0 and 80 ppm in a typical spectrum. Deconvolution of the expanded spectrum was carried out, and the peak at 72.4 ppm was used for quantification of the relative amount of glycogen in the muscle tissue. In addition, the peak at 20.5 ppm was used for quantification of the relative amount of lactate in

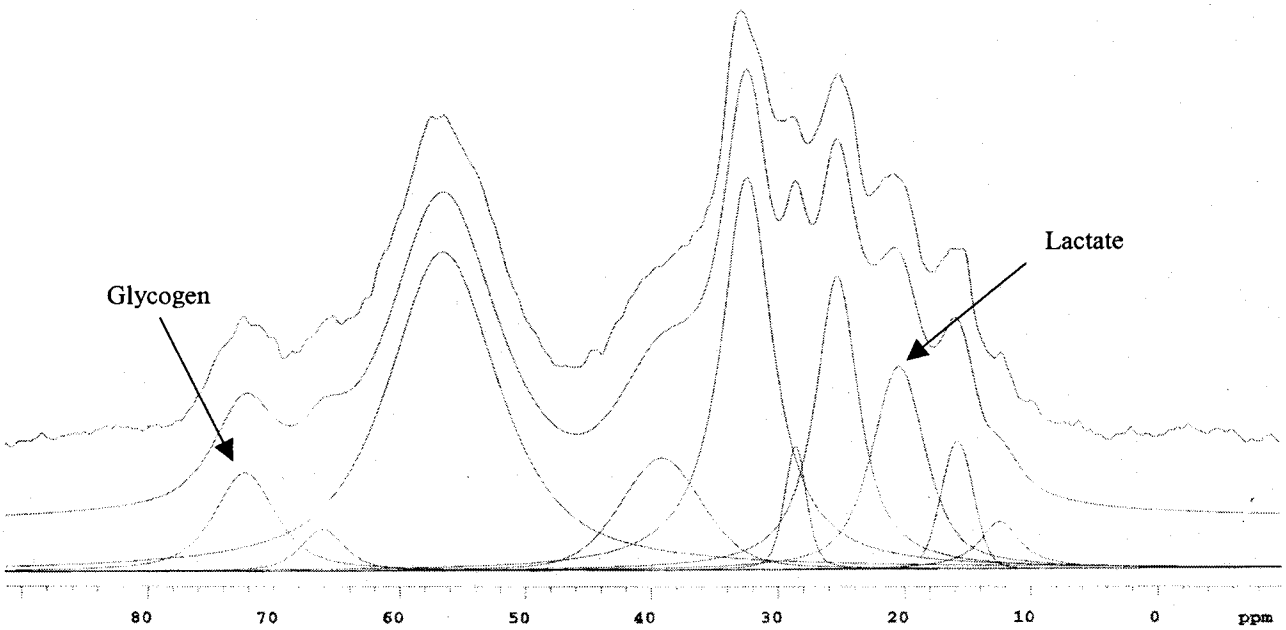

Figure 3. Expansion of the region between 0 and 80 ppm for a representative experimental ^{13}C CP MAS NMR spectrum. A multicomponent fit (deconvolution) is shown below the experimental spectrum.

the muscle tissue. In **Figure 4**, the relationship between the relative amount of glycogen (a) and lactate (b), respectively, determined from the NMR spectra and the absolute amount determined biochemically is displayed. A correlation analysis revealed a significant correlation between glycogen estimated from the MAS NMR spectra and biochemical determinations with a Pearson correlation coefficient of $r = 0.74$ (all samples included). In contrast, a poor correlation was observed between lactate estimated from the MAS NMR spectra and biochemical determinations. However, a weak correlation between lactate estimated from the MAS NMR spectra and biochemical determinations was observed for in vivo and pre-rigor samples ($r = 0.34$); that is, in particular samples taken 24 h post-mortem differed.

DISCUSSION

At present the effect of stress on muscle membrane properties post-mortem is an almost unexplored topic because of the lack of suitable analytical techniques, which allow studies on an intact sample. Using ^{13}C CP MAS NMR spectroscopy, the present study has demonstrated clear differences in the intensity of the resonance at ~34 ppm between muscle samples from minimally stressed, CO_2-stunned and pre-slaughter-stressed, electrically stunned pigs, respectively. The intensity of the resonance at 30 ppm, which can be ascribed to methylene carbons of the chains in fatty acids, is markedly reduced in samples taken both 1 and 45 min post-mortem from the pre-slaughter-stressed pig compared with identical muscle samples from the control pigs and with in vivo samples. Changes in spectral intensities for the ^{13}C CP MAS NMR experiments can be ascribed to minor differences in (i) sample concentrations or (ii) CP efficiency (see section on NMR experiments). As the concentration of methylene carbons can be assumed to be constant, the decrease in intensity of the 34 ppm resonance is probably a result of increased mobility of the methylene chains, which decreases CP efficiency for the common contact time (1 ms) used in all CP MAS experiments. This could reveal alterations in muscle membrane lipids caused by pre-slaughter stress combined with electrical stunning already 1 min post-mortem. It is noticeable that Wind et al. (*21*) observed a similar phenomenon when comparing normal and tumor mammary cells, as the tumor cells were also associated with decreased intensity of the resonance from methylene chains. Wind et al. (*21*) suggested that the increased mobility in tumor cells could be due to either (i) increased amounts of low molecular weight lipids or (ii) changes in the structural surroundings of the lipids, the latter eventually causing altered fluidity of the cell membranes. In the present study both explanations are equally possible. Considering post-mortem events in muscles, it is worth noting that this situation is comparable with anoxia/ischemia, which from other cell systems is known to be associated with increases in Ca^{2+} (*29*), which in turn increases the activity of Ca^{2+}-activated lipases, known to catalyze the hydrolysis of membrane lipids (*30*, *31*). Accordingly, an increased Ca^{2+} release in muscles from the stressed, electrically stunned pig could lead to increased amounts of low molecular weight lipids in the membranes. However, a more general change in the structure of membrane constituents cannot be excluded on the basis of the present data. The present data show for the first time the direct implications of pre-slaughter stress on membrane integrity and call for further ^{13}C CP MAS NMR spectroscopic studies on membrane function as a function of pre-slaughter stress in order to unravel the basic mechanistic understanding in relation to meat quality.

Muscle glycogen is of great interest because of glycogen's role in in vivo stress, exercise-related energy metabolism. In meat animals, muscle glycogen is furthermore of importance due to its crucial role in the anaerobic glycolysis and lactate formation post-mortem, which is known to be very decisive in the final meat quality. Consequently, development of suitable methods for assessment of muscle glycogen is essential with regard to different clinical and research areas. Although spectral intensities in ^{13}C CP MAS NMR experiments may be slightly affected by CP efficiency, a strong correlation ($r = 0.74$) between biochemical determinations of muscle glycogen and estimations from solid-state ^{13}C CP MAS spectra has been demonstrated in the present study. The spectral simulations used for the estimations from NMR spectra induce an error of ~5−10%, and the repeatability of the biochemical determinations is 0.86. Consequently, a correlation between the estimates obtained from the solid-state spectra and the biochemical

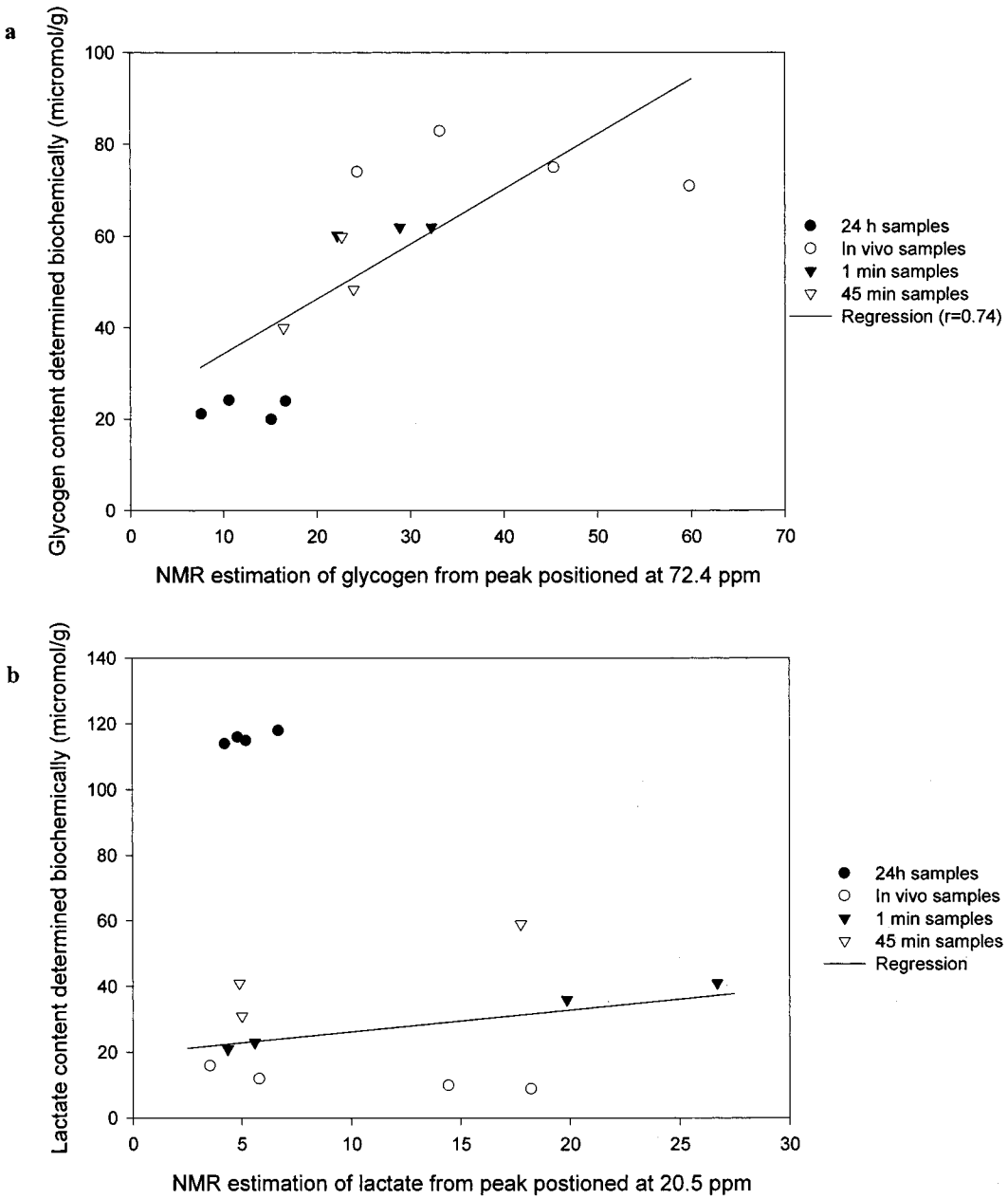

Figure 4. Relationship between the metabolites estimated from ^{13}C CP MAS NMR spectra and the absolute quantities determined biochemically (*25*) for (a) glycogen (the best linear fit corresponds to the equation $y = 1.2x + 22.3$ with a correlation coefficient of $r = 0.74$) and (b) lactate.

determinations higher than $r = 0.86$ can never be obtained, and therefore the correlation of $r = 0.74$ must be considered to be an adequate result. Concerning muscle tissue, no correlation between these two methods has been reported in the literature previously. In liver, Quistorff et al. (*22*) observed a correlation of $r = 0.98$ between solid-state ^{13}C CP MAS estimations and biochemically estimated glycogen content. The reason for the higher correlation is most probably related to the considerably higher quantities of glycogen in liver compared to skeletal muscle (*32*, *33*).

Formation of lactate upon anaerobic degradation of glycogen is the main cause for the decrease in pH post-mortem, which is a major factor determining the final meat quality. Unfortunately, the solid-state ^{13}C CP MAS spectra of muscle tissue gave rise to rather broad lines with several overlapping peaks, including the resonance from lactate at ~21 ppm, which overlaps with various carbons in fatty acids. Thus, the relationship between the area of a deconvoluted peak at 20.5 ppm and lactate determined biochemically gives rise to a rather poor correlation. However, eliminating the data from the 24 h samples improved the correlation ($r = 0.34$). The reason for the poor correlation between lactate estimated from MAS NMR and biochemical determinations can most likely be ascribed to interference from carbons in fatty acids with the resonance used for the estimation. Moreover, the improvement in correlation upon when data from the 24 h samples were eliminated indicates that the contribution from fatty acids to the resonance is different in peri-mortal muscle samples and meat samples. Most probably this may be ascribed to alterations in fatty acids within membranes progressing throughout the post-mortem period, thereby causing altered mobility characteristics detectable by ^{13}C CP MAS NMR spectroscopy.

CONCLUSION

In conclusion, the present study has demonstrated that ^{13}C CP MAS NMR spectra obtained for post-mortem samples can distinguish between different pre-slaughter treatments (CO_2-stunned vs pre-slaugther-stressed, electrically stunned pigs) on

the basis of intensity differences of the resonance at ~34 ppm. This may be ascribed to changed mobility of methylene chains in the membrane lipids. The decreased intensity of the resonance at ~34 ppm observed for samples from the stressed pig indicates altered membrane properties in the muscles already 1 min postmortem. Finally, solid-state ^{13}C CP MAS NMR of frozen muscle biopsies is a suitable method for determination of muscle glycogen, as comparison with biochemical determinations of glycogen contents reveals a strong correlation ($r = 0.74$) between the two methods.

ACKNOWLEDGMENT

The use of the facilities at the Instrument Centre for Solid-State NMR Spectroscopy, University of Aarhus, sponsored by the Danish Natural Research Council, the Danish Technical Science Research Council, Tegnologistyrelsen, Carlsbergfondet, and Direktør Ib Henriksens Fond, is acknowledged. Technicians Camilla Bjerg Pedersen and Marianne Rasmussen are gratefully acknowledged for performing biochemical determination of glycogen.

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Received for review August 29, 2002. Revised manuscript received December 19, 2002. Accepted January 6, 2003. We are grateful to the Danish Veterinary and Agricultural Research Council (SJVF 9702805) and to the Danish Directorates for Development (Product Quality Project to H.J.A.) for financial support of this project.

JF020919X