3:5 Post mortem energy metabolism in muscles studied by non-invasive phosphorus-31 nuclear magnetic resonance

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Introduction

Energy metabolites of post mortem muscle are conventionally analyzed by enzymatic spectrophotometric methods (Bergmeyer, 1974). This is a destructive and rather elaborate technique. Due to variations in the concentration of metabolites along the muscle, erroneous results can be introduced when sampling is repeated. Moreover, incomplete extraction or hydrolysis of labile bonds during extraction cannot always be avoided. Phosphorus-31 nuclear magnetic resonance spectroscopy (^{31}P-NMR) can be used to study, in a non-invasive manner, the metabolism of the major phosphorylated metabolites (>0.5 mM) such as ATP, creatine phosphate (CP), sugar-phosphates and related compounds (mainly glucose-6-phosphate, G6P) and inorganic phosphate (P_i). The method is quantitative and since it is non-destructive it permits repetitive measurements on the same sample thus circumventing some of the problems which occur when analysing extracts. Basing his ideas on earlier works with laboratory animals Gadian (1980) suggested that ^{31}P-NMR should be useful for studying post mortem events in muscles from slaughtered animals, but to our knowledge this technique has yet to be used for this purpose.

The aim of the present study was 1) to assess the usefulness of ^{31}P-NMR in the study of post mortem metabolism in slaughter carcasses, 2) to follow changes in muscle metabolism induced by electrical stimulation in intact samples by ^{31}P-NMR, and 3) to assess the possibility of following the metabolic events connected with thaw shortening.

Materials and Methods

Bovine M. longissimus dorsi was removed on one side of the carcass immediately after debleeding. Within 10 min of stunning low voltage electrical stimulation (85 volts peak for 5 ms at a frequency of 14 pulses/s) was applied for 32 s by using a clip in the interseptum of the nose and with the overhead rail acting as the negative electrode. M. longissimus dorsi on the other side of the carcass was then removed and approximately 200 g was cut from the non-stimulated (NS) and electrically stimulated (ES) muscles and transported to the laboratory for ^{31}P-NMR measurements. In the laboratory a core of about 9 mm in diameter was removed and placed in the 10 mm NMR glass tubes. These were placed in the magnetic field at room temperature and at an average of 1.0 h post mortem the first NMR spectrum was recorded. The rest of the muscles were kept at 22°C for 3 h and subsequently at 10°C for 9 h and sampled regularly for biochemical analysis (Fabiansson & Laser Reuterswärd, 1984, Vogel et al., 1984).

^{31}P-NMR spectra were obtained at 103.2 MHz on a home-built NMR spectrometer equipped with a 6T magnet (Oxford Instruments, bore 89 mm) operating in the Fourier transform mode. A 10 mm solenoidal probe as described earlier (Vogel et al., 1983) was used. Acquisition parameters were a repetition rate of 8 sec (>3 x T_1) combined with 90° (50 µsec) pulses and a spectral width of 20,000 Hz. Normally 160 scans were collected. All spectra were acquired at 23°C using the CP resonance (above pH 6.0) as an internal standard at 2.35 ppm.

^{31}P-NMR spectra of cell extracts were run at 23°C on a Nicolet-360 WB spectrometer operating at 145.7 MHz. The samples were placed in standard 12 mm NMR tubes (Wilmad). The acquisition parameters used were: pulse length 25 usec (60°), repetition rate 10.5 sec, spectral width 10,000 Hz using 8 K memory. Extracts, as prepared for biochemical analysis, were diluted with D_2O (Ciba-Geigy), containing 2 mM EDTA, the pH (meter reading) was adjusted to pH 8.5. These spectra were proton decoupled and the samples were locked on the D_2O resonance.

M. adductor and M. semimembranosus from pig and lamb were sampled as early as possible on the slaugterline. Post mortem metabolism was followed by ^{31}P-NMR, as above, at 25°C and 16°C. One sample of M. adductor from lamb was homogenized in a Moulinex food processor for 10 seconds before it was placed in the spectrometer.

For the thaw rigor experiments 2-6 g samples from hot boned bovine M. longissimus dorsi were frozen in liquid nitrogen. They were kept at -80°C until placed in the spectrometer and allowed to thaw. Spectra were collected as above.

Results and Discussion

Presence of phosphorylated compounds

Figure 1 depicts some representative ^{31}P-NMR spectra, recorded at different time intervals after slaughter, from a non-stimulated muscle. Six resonances can be observed in Figure 1A. These resonances have been assigned, from low field to high field, to the following metabolites: sugar phosphates (4.25 ppm), inorganic phosphate (2.15 ppm), creatine phosphate (-2.35 ppm), ATPγ (-4.95 ppm), ATPα + NAD(H) (-9.90 ppm), ATPβ (-18.70 ppm).

The assignment of the most downfield resonance to sugar phosphates was made based on ^{31}P-NMR and enzymatic studies of extracts. Figure 2 shows part of a ^{31}P-NMR spectrum of a cell extract. Note that the resolution is better here than in the intact muscle.

Comparing this spectrum with ^{31}P-NMR spectra of the appropriate standards and with enzymatic analysis of the extracts we have assigned the resonances to the following metabolites: (1) G6P, (2) Glyc3P, (3) FDP + 3PG, (4) AMP + IMP, (5) F6P. Thus G6P, Glyc3P and F6P are the most abundant phosphomonoesters present post mortem, thus agreeing with previously reported studies (Fabiansson & Laser Reuterswärd, 1984).

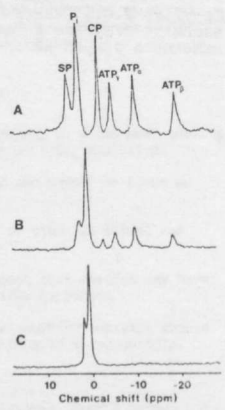

Fig. 1. Examples of ^{31}P-NMR spectra recorded at 3 (A), 6 (B) and 24h (C) after slaughter of a non-stimulated muscle.

Intracellular pH

The chemical shift of the inorganic phosphate resonance is sensitive to changes in pH near neutrality and can be used to measure the intracellular pH (Moon & Richards, 1973, Gillies et al., 1982). In addition, it is also possible to use the changes of the chemical shift of the sugar phosphate resonances, such as G6P, down to a lower pH than for P_i.

Using test solutions for calibration we followed the changes in pH in samples from M. longissimus dorsi during the first 10 hours post mortem by ^{31}P-NMR. The pH was also measured electrochemically in muscle homogenates (Fig. 3).

There was a difference between the determination of pH according to the chemical shift of P_i or G6P. It should be recalled however that the sugar phosphate resonance comprises, in fact, contributions from various compounds (See Figures 2B, 2C), hence the obtained pH values using the G6P standard curve are only an approximation. There is only a very small difference between the pH measured electrochemically and that determined by ^{31}P-NMR using the P_i resonance. Some discrepancies occur at the lower pH values. However, the changes in chemical shift with pH for the P_i standard are small in this region and hence pH measurements are less reliable in this pH region than around neutrality. It should also be realized that ^{31}P-NMR registers only the intracellular pH, whereas the other method is an average for the intra- and extracellular volume.

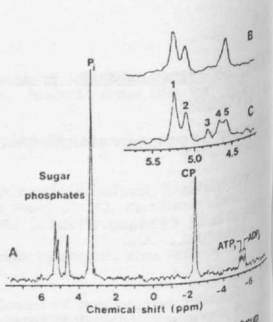

Fig. 2. Part of a ^{31}P-NMR spectrum of a muscle extract (A), expansion of the sugar phosphate area of the same sample (B) and an expansion of an extract of a non-stimulated muscle at 0.25h (C) showing several resonances in the sugar phosphate area.

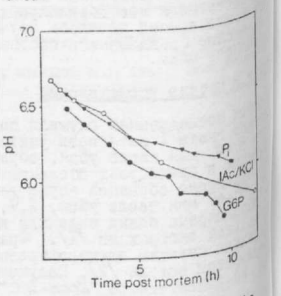

Fig. 3. Changes in pH during the first 10h post mortem as followed by ^{31}P-NMR (P_i and G6P) or electrochemically in muscle homogenates (IAc/KCl). Mean values from six experiments.

Results from the present investigation indicate that the true intracellular pH, as measured by the chemical shift of inorganic phosphate, is approximately the same during post mortem metabolism as the combined intracellular and extracellular pH measured electrochemically after homogenization in iodoacetate/KCl.

Quantitative analysis of the amounts of phosphorylated metabolites

Since all the ATP present is usually in the cytoplasmic compartment (Veech et al., 1979) we have used this metabolite to calculate a conversion factor based on the ATP levels measured enzymatically and by ^{31}P-NMR at 1.3 hours. With the possible pitfalls of such a calculation in mind we compared the values obtained by conventional enzymatic methods with those obtained by ^{31}P-NMR (Fig. 4).

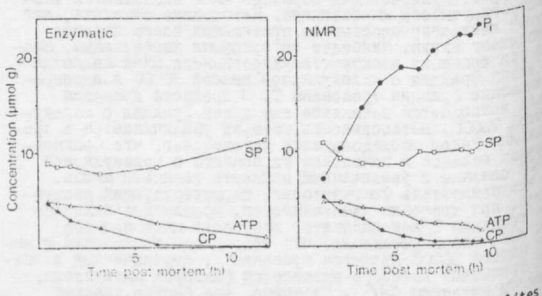

Fig. 4. Comparison of the amounts of some phosphorylated metabolites detected by enzymatic methods and by ^{31}P-NMR at different times post mortem (n=6). The arbitrary µnits of the NMR resonances have been converted to µmol/g using the amount of ATP at 1.3h as determined enzymatically.

There was a close resemblance between the course of the changes in the level of CP. Note also that the ATP levels as determined by both methods remain relatively constant until the CP level decreases to a very low level, suggesting that phosphocreatine kinase remains active. The NMR resonance for the sugar phosphates compared favourably with the sum of the amounts of G6P, F6P and Glyc-3P determined biochemically.

We have also found in this study evidence that P_i, not detected in spectra of intact muscle, is present. For example, a direct comparison of NMR spectra, taken directly after slaughter (15 m) of a cell extract, shows a higher P_i/CP ratio (Fig. 2A) than a spectrum taken 60 min after slaughter from an intact sample (see Fig. 5A). It is unlikely that the higher level of P_i in the extract was the result of hydrolysis during extraction, because we found comparable ATP and CP levels in intact samples and cell extracts.

Effects of electrical stimulation

Figure 5 compares the ^{31}P-NMR spectra obtained for two samples from one carcass, one non-stimulated (Fig. 5A) and one electrically stimulated (Fig. 5B).

Both spectra were recorded 1 hour after slaughter. The different ratios of the metabolites present are immediately apparent. Moreover, the more upfield chemical shift for the sugar phosphate and P_i resonance indicates a lower intracellular pH in the electrically stimulated muscle.

In Figure 6 a comparison of the changes in the phosphorus compounds as analyzed using ^{31}P-NMR in electrically stimulated muscles in relation to the non-stimulated counterparts is shown.

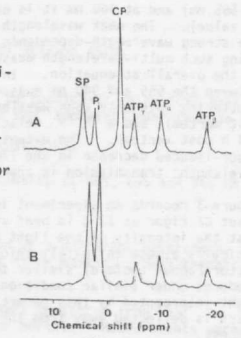

Fig. 5. ^{31}P-NMR spectra of non-stimulated (A) and electrically stimulated (B) muscles from the same carcass at 1h after slaughter.

From the cross-over plot of mean values from five bovine carcasses a decrease of the CP content of more than 50% can be seen as an immediate response to electrical stimulation. The difference increased during the first hours post mortem. This was much in line with the previously reported findings that were based on biochemical analysis (Fabiansson & Laser Reuterswärd, 1984). Sugar phosphates and related compounds as well as inorganic phosphate increased due to electrical stimulation. The sugar phosphates showed a biphasic pattern. The initial increase was more pronounced in the electrically stimulated muscles and started to fall off earlier than in the non-stimulated muscles. The difference in the amount of inorganic phosphates levelled off at about 9 hours after slaughter. At this time the pH was the same in both parts of the carcasses and only minute amounts of CP and ATP were present. Half of the ATP present was consumed in 4 hours in the electrically stimulated muscles compared to 6 hours in the non-stimulated ones. The immediate pH drop caused by electrical stimulation was only 0.07 μunits, but the difference increased to 0.20 μunits at about 6 hours after slaughter (Figure 7).

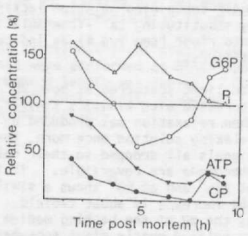

Fig. 6. Cross-over plot of the concentrations of phosphorous compounds as analyzed by ^{31}P-NMR in electrically stimulated muscles in relation to the concentrations in the non-stimulated counterpart (100%) at different times post mortem (n=5).

This is a very low response to electrical stimulation. Normally a pH drop of 0.3-1.1 units is reported early post mortem, depending on the conditions (George et al., 1980; Bouton et al., 1980; Mojto et al., 1983). In the present investigation, however, the difference between animals in response to electrical stimulation was considerable, with a variation in the immediate pH drop from no response up to 0.77 units. There was also a considerable variation between carcasses when pH was measured electrochemically, but not as pronounced as that observed using ^{31}P-NMR. No explanation for this finding can be given. We noted in the course of our studies, however, that electrical stimulation exerted a great effect on muscles containing a high CP/ATP ratio, whereas the effect on muscles with low CP levels was very small.

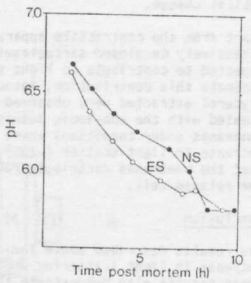

Fig. 7. pH fall as followed by changes in the chemical shifts of the P_i resonance as determined by ^{31}P-NMR during 10h after slaughter in electrically stimulated (ES) (n=5) and non-stimulated (NS) (n=5) muscles.

Pig and lamb

Spectra from pig and lamb showed the same general pattern as for cattle. Post mortem metabolism in both species tended to be faster than in bovine muscles. Results were obtained at two different temperatures. Both for pig and lamb samples we observed the faster post mortem metabolism at 25°C than at 16°C.

The effect of mechanical damage on muscle metabolism was tested by grinding a lamb muscle sample for a very short time in a food processor. The effect was most pronounced with ATP being depleted within 2 h and final pH reached within 4 h at 16°C. This effect is important in two ways. First, mechanical damage to muscle is unavoidable when sampling for all kinds of measurements including pH measurements on intact muscles and could give erroneous results. Second, when using hot boned meat for sausage production attempts are sometimes made to preserve the ATP level by grinding and freezing or salting. In practice this is very hard to achieve since the time for processing is too long to leave enough ATP to really influence the binding properties of the emulsion, especially if the meat is preground and preblended.

Thaw rigor

Attempts were made to follow the metabolism during thawing of pre rigor frozen samples of bovine longissimus dorsi. Since ^{31}P-NMR only detects freely diffusable compounds, useful spectra are not obtained in the frozen state. At 0°C about -5°C all six peaks in the ordinary spectrum had appeared. At about -5°C good spectra were obtained. Upon further heating a very fast metabolic activity appeared, CP showing the fastest decrease followed by ATP. G6P decreased more slowly as was the case with the P_i increase and both showed the typical pH shift. The intensity of the P_i peak relative to other peaks showed a small decrease just before the level of CP and ATP started to fall off. The most likely explanation for this behaviour is that phosphate in fact is consumed during a short period of intense glycogenolysis.

Conclusion

Summarizing, we feel that ^{31}P-NMR is a valuable complement to existing methods of analysing post mortem metabolism in muscles from slaughter carcasses.

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