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^{31}P-NMR SPECTROSCOPY OF PHOSPHATE COMPARTMENTATION DURING ISCHAEMIA IN HEARTS PROTECTED BY CARDIOPLEGIC TREATMENT

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Four tissue compartments, differing in proton and inorganic phosphate concentration, were resolved by ^{31}P-NMR spectroscopy in samples from dog hearts after cardioplegic treatment with HTK solution. Inversion of the physiological cytoplasmic-mitochondrial pH gradient was observed. The considerable ensuing acidosis of the matrix is discussed with regard to a possible delocalisation of ferrous ions.

KEY WORDS: Cardioplegia, acidosis, pH-homeostasis, tissue compartmentation, inner mitochondrial membrane.

ABBREVIATIONS: Pi = inorganic phosphate, GPC = α-glycerophosphorylcholine

INTRODUCTION

Acidosis, a result of lactate accumulation from anaerobic glycolysis, is a challenge to cellular pH homeostasis.[1] Membrane-associated pumps and transporters normally regulate cytoplasmic pH and maintain pH gradients of physiological significance between subcellular compartments. Oxidative phosphorylation, for instance, is driven by a gradient over the inner mitochondrial membrane which sets the matrix alkaline relative to the cytoplasm. Lysosomes and endosomes are other examples of subcellular pH compartmentation. Disturbance of the structure and function of cellular membranes is therefore critical in determining the revivability of tissue after global or local ischaemia.

The major principle of the HTK solution used in cardioplegia to prolong cardiac tolerance to global ischaemia, is buffering of tissue acidosis, so delaying the processes which lead to irreversible cell damage.[2] A welcome side-effect is that this gives us more time to investigate disturbances of energy metabolism and cellular pH homeostasis. ^{31}P-NMR spectroscopy is the technique of choice for such investigations as it is non-invasive and registers a considerable number of experimental parameters simultaneously.[3,4] This is demonstrated here by the detection of four tissue compartments with different pH in samples from dog hearts after cardioplegic treatment with HTK solution. The observed acidification of the mitochondrial matrix will be discussed in relation to the release of ferrous ions assumed to be involved in reperfusion injury.

TABLE I
Composition of the HTK-solution

	[mM]
NaCl	15
KCl	9
$MgCl_2$	4
histidine	180
histidine-HCl	18
tryptophan	4
ketoglutarate	1
mannitol	30
pH at 25°C	7.1

MATERIALS AND METHODS

Cardioplegia

Retrograde coronary perfusion of dog hearts was performed in situ for 11 min with HTK solution (8°C; for composition see Table I) under neurolept analgesia (N_2O, continuous infusion of a piritramide and fentanyl) after initiation with thiopental.[5] Samples excised from the right ventricle were kept in HTK solution at 6–8°C for 35 min before being warmed up to the measuring temperature.

^{31}P-NMR spectroscopy

Spectra (1600 scans) were recorded with a Bruker MSL300 Fourier transform spectrometer at 121.49 MHz using 40° pulses at 500 ms intervals and continuous broad band proton decoupling in a 10 mm probe-head. The samples comprised about 3 g of heart muscle immersed in HTK solution in a 10 mm diameter tube and a tube-insert containing α-glycerophosphorylcholine (GPC; cadmium complex, pure, Serva, Heidelberg) as external standard (chemical shift = 0.552 ppm relative to 85% phosphoric acid) in D_2O for the field frequency lock. Before measurement a 5 min delay to equilibrate the samples at the measuring temperature (25°C) was observed. The sample tube was spun during the recording. All chemical shift data are given relative to GPC. For tissue extracts and standard curves the same measuring conditions were used.

Simulation of ^{31}P-NMR spectra

^{31}P-NMR spectra were simulated by defining the position, halfwidth, intensity and form (Lorentz and/or Gauss) of the various components believed to comprise the experimentally observed spectra and comparing the resulting bands with the experimental data. In some cases the fit was optimised by selective use of the procedure described.[6] For an optimal fit, Lorentzian line shape had to be assumed for the cytoplasmic compartment, a combination of Lorentzian and Gaussian lines for the first five and Gaussian lines for the following spectra for the mitochondrial compartment, and Gaussian lines for the unidentified compartment. Gaussian lines result

from Gaussian distributions of Lorentzian signals as a consequence of pH heterogeneity within a compartment.

The pH of tissue compartments was determined from the chemical shifts of the simulated resonances of inorganic phosphate and the 6-phosphoryl group of D-fructose 1,6-diphosphate by reference to standard curves. These were obtained by measurement of the chemical shift of inorganic phosphate and D-fructose 1,6-diphosphate (sodium salt, Serva, Heidelberg) as a function of pH in solutions containing 10 mM D-fructose 1,6-diphosphate, 10 mM KH_2PO_4, 150 mM KCl, 2 mM $MgCl_2$; conditions which are assumed to simulate the intracellular ionic strength and free magnesium ion concentration.[7]

RESULTS

The energetic state of a sample measured between 40 and 53 min after the end of the cardioplegic treatment is well preserved, as can be seen from the high phosphocreatine content relative to ATP (Figure 1). Phosphocreatine disappears within 100, ATP within 220 min of cardioplegia.

The change in the resonance frequencies of inorganic phosphate (Pi) shown in Figure 2 is of particular interest. With time the chemical shifts move to lower values, indicating a lowering of tissue pH as lactate accumulating from anaerobic glycolysis causes increasing acidosis.[5] Concomitant with this, the Pi signal gradually separates into several bands.

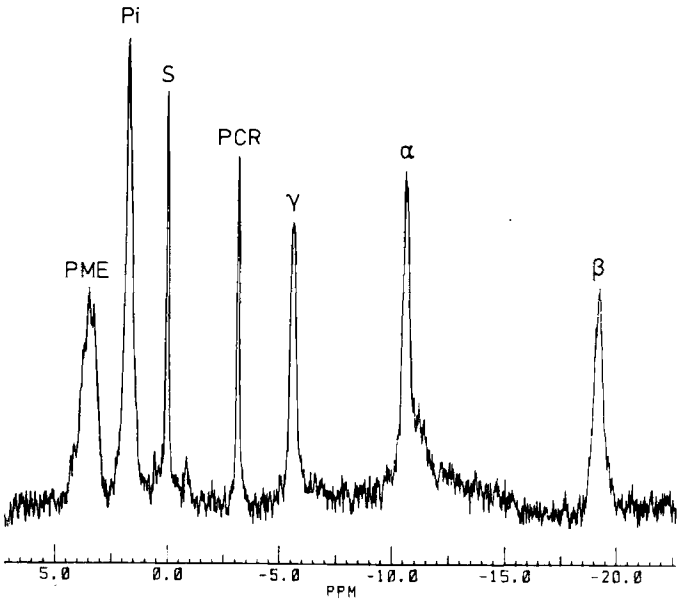

FIGURE 1 ^{31}P-NMR spectrum of a sample of dog heart right ventricle muscle recorded between 40 and 53 min after the end of cardioplegic treatment with HTK solution. PME = phosphomonoesters; Pi = inorganic phosphate; PCR = phosphocreatine; α, β, γ = adenosine triphosphate; S = GPC as external standard.

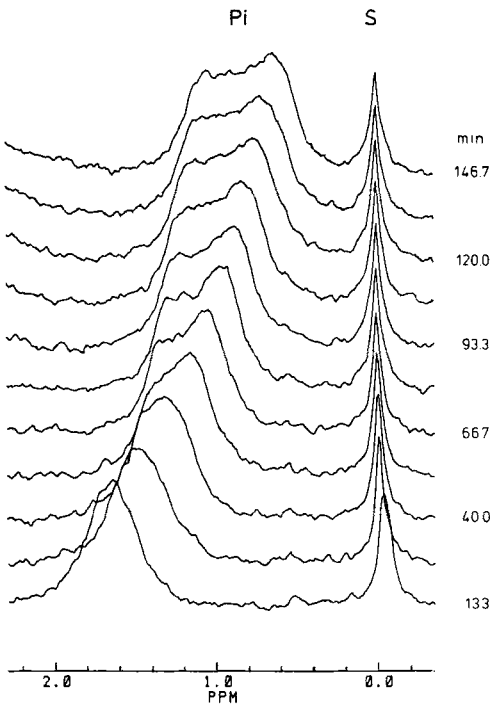

FIGURE 2 Spectra of a sample of dog heart right ventricle muscle acquired up to 146.7 min from the start of the NMR measurement (between 40 and 186 min after the end of cardioplegic treatment) showing inorganic phosphate (Pi) and standard (S) signals (expanded with respect to Figure 1).

Simulation clearly resolves three major bands of differing Pi content, which may be attributed to tissue compartments with pH values of 6.61, 6.47 and 6.27 (see Figure 3) as determined from standard curves of the the pH-dependence of the chemical shift of Pi in solutions simulating the intracellular ionic milieu.

The least acid compartment, that containing the second-largest Pi signal, (the resonance C at 1.09 ppm in Figure 3) can be assigned to the cytoplasm from the following facts. (a) By fortunate chance, D-fructose 1,6-diphosphate was identified in tissue extracts (see Figure 4) and accumulates in the tissue in the early stages of ischaemia. The chemical shift of the resonance of the phosphoryl group at position 6 is sensitive to pH,[8] and by simulation of the resonances in the spectral band attributable to phosphomonoesters, its position in spectra of intact tissue can be accurately determined. Thus, with the aid of a standard curve, the pH value of the tissue compartment can be estimated. (b) The pH values so determined correspond to those determined from the observed chemical shift of the Pi resonance. (c) D-fructose 1,6-diphosphate is known to be produced in the cytoplasm by the glycolytic pathway.

The assignation is confirmed by the correlation of the tissue pH values determined from the chemical shift of the D-fructose 1,6-diphosphate resonance with those determined from the Pi signal during the time that D-fructose 1,6-diphosphate is discernable in the tissue NMR spectra (Figure 5).

We attribute the most acidic of the three major compartments, that with the highest

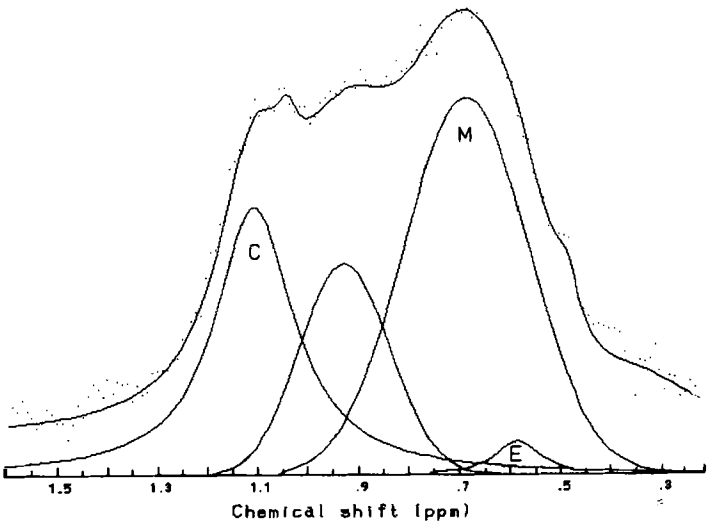

FIGURE 3 Simulation of the inorganic phosphate resonances of a ^{31}P-NMR spectrum recorded 120 min after start of the NMR measurement (160 min after the end of cardioplegic treatment, see also Figure 2) superimposed on the experimental spectrum (dotted line). The major components of the simulation are shown and are assigned C = cytoplasm; M = mitochondria; E = extracellular space. Other components corresponding to base line distortion, overlap with neighbouring bands and a small signal, probably glycerophosphorylethanolamine, are not drawn.

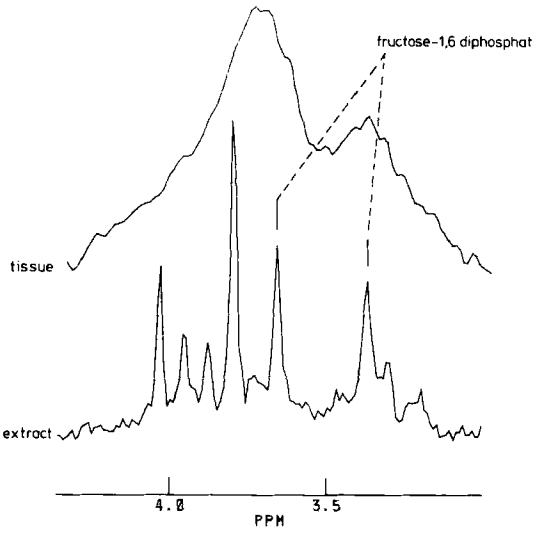

FIGURE 4 ^{31}P-NMR spectra of a dog heart right ventricle muscle sample after cardioplegic treatment with HTK solution, and of the extract of a sample freeze-clamped at the same time. The resonances of D-fructose 1,6-diphosphate are indicated, the phosphoryl group at position 6 corresponds to the resonance at 3.35 ppm. Perchloric acid extracts have been prepared as described[15] and adjusted to pH 7.0.

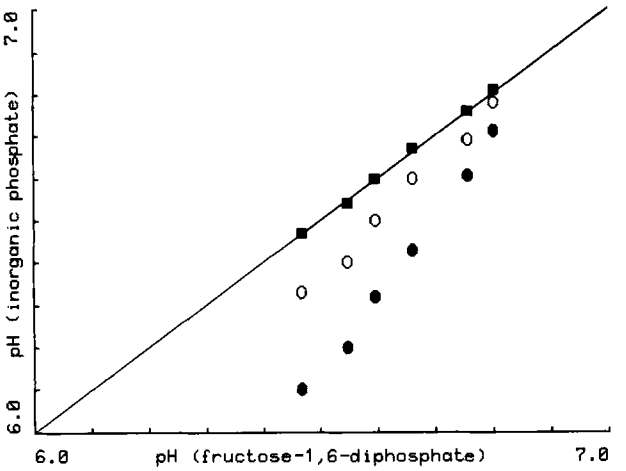

FIGURE 5 The pH values determined from the chemical shift of D-fructose 1,6-diphosphate (phosphoryl group in position 6) from spectra taken from a heart sample are correlated with the pH values determined from the resonances of inorganic phosphate of the least acid compartment (■), but not with the values of either of the other compartments (● = mitochondria, ○ = unidentified compartment).

Pi content (the signal M at 0.68 ppm in Figure 3), to the mitochondrial matrix on the following grounds. (a) The mitochondrial space is the second largest subcellular compartment. The extracellular space can be excluded as HTK solution contains no Pi (see below). (b) A high ratio of mitochondrial to cytoplasmic Pi content has been observed by others[9] and is consistent with this assignation (see Figure 3). (c) It is

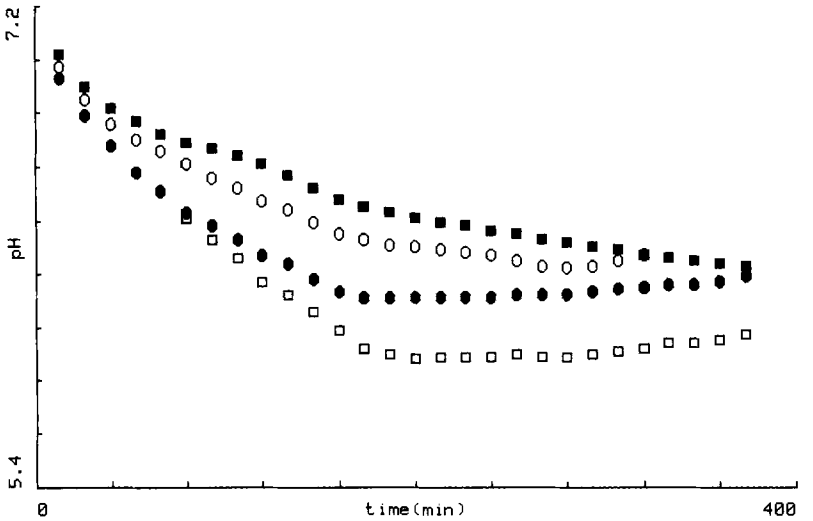

FIGURE 6 Tissue acidosis increases with time of ischaemia at different rates in the four compartments (● = mitochondria; ■ = cytoplasm; ○ = unidentified compartment; □ = extracellular space). pH values were determined by simulation of the inorganic phosphate resonances (compare Figures 2 and 3). The time is given from the start of the NMR experiment.

probable that part of the large increase in cytoplasmic Pi is transferred to the mitochondrial matrix via the Pi/OH^- antiporter with the effect of increasing mitochondrial pH. (d) Initially it is observed that the pH of this compartment moves away from that of the cytoplasm (it becomes more acid – see Figure 6), but this trend is later reversed and the two Pi signals eventually coalesce. This is probably due to breakdown of the boundary function of the inner mitochondrial membrane, at which point the buffering capacity of the larger compartment plays the dominant role in determining the common pH.

The third compartment, containing the third-largest amount of Pi (at 0.92 ppm in Figure 3), which merges with the cytoplasm at 320 min, has not yet been assigned.

A small amount of extracellular Pi becomes visible in the NMR spectra (signal E at 0.58 ppm in Figure 3) at later times of ischaemia. The pH value of this compartment determined by ^{31}P-NMR spectroscopy corresponds to the pH value determined from extracellular pH measurements using electrodes.

The changes in the chemical shifts of the Pi resonances in the four compartments with time (Figure 6) show a net transfer of protons from the cytoplasm to the other three compartments, particularly to the extracellular space, during the 3-hour period following cardioplegia. This is also roughly the time for which ATP remains detectable in the tissue by NMR spectroscopy.

DISCUSSION

The observation of 4 tissue compartments, and of proton transport between them, demonstrates the value of ^{31}P-NMR spectroscopy in investigations of membrane function in an object as complex as heart tissue, and the advantages of organ protection measures in making NMR-spectroscopic investigations of viable tissue samples possible.

The results indicate the importance of tissue acidosis as the main cause of cell damage during ischaemia and emphasise the importance of the buffering capacity of HTK solution.[2]

The inversion of the physiological pH gradient over the inner mitochondrial membrane, with ensuing lowering of the pH to 6.0 in the matrix, is of considerable interest. This value approaches those at which, under physiological conditions, lysosomal hydrolases are activated (pH 4.6–5.0) and ferrous ions dissociate from transferrin in endosomes (pH 5.0–5.5).[10] In view of the importance of decompartmentation of ferrous ions to the generation of OH radicals[11] which, from our knowledge of radical chemistry and biochemistry, have to be considered the chemical species ultimately responsible for reperfusion injury,[12] the question arises whether the pH-drop promotes release of ferrous ions from complexes present in the inner mitochondrial membrane. It has been shown that mobilisation of iron from a pool of "non-heme non-FeS iron" in the inner mitochondrial membrane is maximal when the pH drops below 6.5, and that sulphur proteins become unstable below pH 6.0.[13] Activation of hydrolases, the mitochondrial phospholipase A_2 for instance,[14] could be an oxygen-independent cause of membrane damage. Altogether the results suggest that the inner mitochondrial membrane is a critical target for acidosis-induced cell damage by oxygen-dependent and -independent mechanisms.

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