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Phosphorus nuclear magnetic resonance studies on normoxic and ischemic cardiac tissue

(NADH fluorescence/intracellular acidity/whole rat heart preparations)

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ABSTRACT **The intact heart of a young rat was excised rapidly and cooled to 0°C; its energy-rich compounds were examined by ^{31}P Fourier Transform nuclear magnetic resonance. The heart showed the characteristic spectrum of sugar phosphates, inorganic phosphate, phosphocreatine, and magnesium ATP, characteristics of the energizing state of the nonbeating tissue. Warming to 30°C imposes an energy load upon the heart consistent with short-term resumption of beating, concomitant intracellular acidosis, and decomposition of all detectable energy-rich compounds. The intracellular acidity causes a shift from pH 7.0 to 6.0. The effects of possible interferences with this pH measurement are considered. The method appears to have wide usefulness in cardiac infarct models for detecting the fraction of the total volume occupied by the infarct and for studying the effect of various proposed therapies upon this infarcted volume.**

A current problem of cardiovascular disease is the identification of oxygen-deficient volumes of cardiac tissue. Ischemic cardiac tissue is characterized by high lactate/pyruvate ratios (about 40) (1) and a 2-fold increase of the NADH fluorescence over the normoxic level (2). Neither of these data indicates the volume of ischemic tissue. For example, failure of perfusion of ischemic tissue volumes causes the measured lactate/pyruvate ratios to be such that the degree and extent of anoxia are underestimated. NADH surface fluorescence precisely indicates the area involved in the infarct, but requires freeze-trapping and tissue sectioning to determine the infarcted volume (3, 4).

The possibility that phosphorus nuclear magnetic resonance (^{31}P NMR) could be used to follow and quantitate events associated with cardiac ischemia follows logically from the application by the Oxford Group (5) of NMR spectroscopy in living tissues, together with a more recent report by Burt, Glonek, and Barany (6). Of particular promise is the observation that the positions of some of the resonances derived from phosphates reflect the intracellular pH (5, 6) and, under some conditions in skeletal muscle, variations in intracellular acidity of different compartments (7, 8).

In this paper, we report on the feasibility of carrying out ^{31}P NMR measurements on preparations of whole rat hearts and examine a simple model system for comparing normoxic and anoxic cardiac tissue.

EXPERIMENTAL

In the absence of having perfected the technique for perfusion of small rat heart in an 8 mm diameter NMR sample tube, in our initial experiments the heart was not perfused. Normoxia, or near normoxia, was maintained by cardiac arrest that occurs at 4°C. At this temperature, energy utilization and oxygen demand are expected to be minimal as a result of decreased metabolic rate and inhibition of mitochondrial adenine nucleotide translocase. Thus, the tissue should have established a "resting" metabolic state. This resting aerobic/ischemic condition is compared with an active hypoxic/ischemic condition established by raising the temperature to 25–30°C, where intermittent spontaneous contractions take place, together with increased mitochondrial respiration. In a few minutes severe tissue anoxia occurs and a progressive depletion of energy reserves simulates the hypoxic ischemia of an infarcted tissue. Progress towards acidosis and depletion of energy reserves reaches a steady state in about 10 min. No attempt was made to establish the reversibility of depletion of the energy levels.

Preparations. Young rats weighing about 70 g were anesthetized by the intraperitoneal injection of 0.15 ml of Nembutal (sodium pentobarbital, 50 mg/ml, B.Vet.C.), and allowed to rest until no response to a tail pinch could be observed. The skin and rib-cage were removed; the heart was grasped in the forefingers, rapidly cut free of blood vessels, and dropped into ice-cold Ringer's solution. The normal beat of the heart ceased in a few seconds. After 1–15 min the heart was transferred to a standard 8-mm diameter sample tube filled with ice-cold aerated Ringer's solution with 10% of H_2O being replaced by D_2O in this buffer. Recordings were first made at 4°C and afterwards at 25°C or 30°C. After the NMR measurements were completed, the heart was dried in a paper towel and weighed.

NMR Measurements. ^{31}P NMR spectra were recorded at 129 MHz on a spectrometer constructed in Oxford (9). The spectrometer was operated in the Fourier Transform mode and was interfaced with a Nicolet B-NC 12 computer. The magnetic field was locked to the deuterium NMR signal from heavy water present in the Ringer's solution. The validity of using an internal rather than external deuterium lock (D_2O) has been studied in detail before (10), and in this work the temperature dependence of the lock frequency was studied separately. Thus, the measured chemical shifts (and consequently the measured tissue pH) have all been corrected for the changes in the lock frequency. Spectra were collected by applying 60° pulses of radiation at intervals of 2 sec, and the accumulated free induction decays were stored on a magnetic disk. Metabolite levels can be obtained directly from the signal areas if the radiofrequency pulses are applied at time intervals much greater than the spin-lattice relaxation time, T_1, of the resonances. In this work, in order to optimize the signal-to-noise ratio of the spectra, we applied pulses at intervals that approximate the T_1 values of the resonances. Under these conditions, the areas of the resonances are reduced, for a given number of pulses, by factors determined by their T_1 values. In skeletal muscle preparations we have studied the spectra at several pulse repetition rates and concluded that for pulse intervals of 2 sec, the true quantities of metabolites can be obtained if the ATP, creatine phosphate, and inorganic phosphate resonance areas are multiplied by factors of 1.0, 1.5, and 1.5, respectively (Gadian,

Abbreviation: ^{31}P NMR, phosphorus nuclear magnetic resonance.

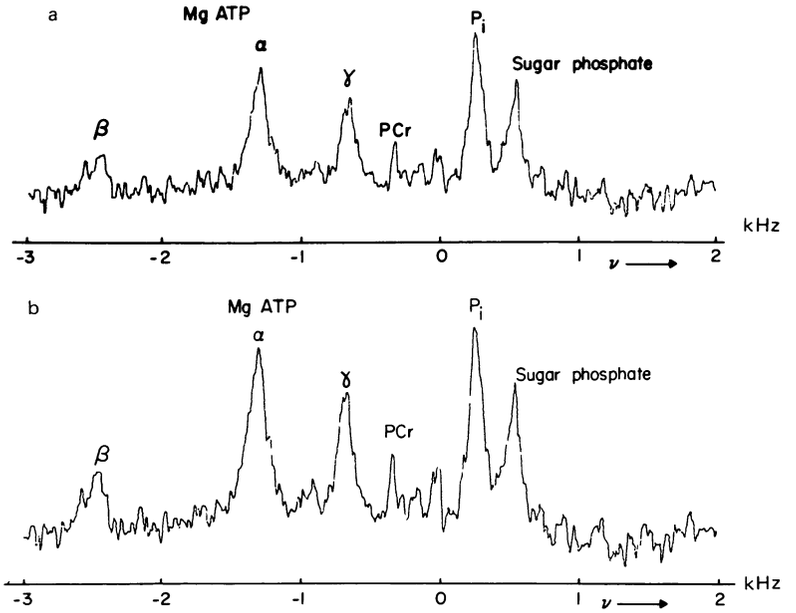

FIG. 1. (a) ^{31}P NMR spectrum of a 170-mg rat heart recorded at 129 MHz without proton irradiation. Temperature, 4°C. The pulse interval was 2 sec, and the pulse angle 60°. Sweep width, 5 kHz. 1040 accumulations. The heart was immersed in aerated Locke-Ringer's solution containing 10% D_2O at pH 7.0. PCr, phosphocreatine; P_i, inorganic phosphate. (b) The sum of the ^{31}P NMR spectra of two separate hearts, 2240 scans, collected under the conditions given for panel a. Abbreviations as in panel a.

Radda, Richards, and Seeley, unpublished observations). These corrections are likely to apply to cardiac muscle as a first approximation, although we have not yet carried out such measurements for this tissue. In any case, at this stage the relative levels of a given metabolite at different tissue states can be accurately compared. Calculations of pH were based upon the frequencies of inorganic phosphate buffers of known pH (5). KCl concentrations up to 100 mM and Ca^{2+} and Mg^{2+} concentrations up to 20 mM showed negligible effects. No correction for specific binding of rapidly exchanging phosphates was possible. Signals were assigned on the basis of our previous work on skeletal muscle (5). The chemical shifts were measured relative to 85% H_3PO_4 at 20°C.

RESULTS AND DISCUSSION

Figure 1a shows the ^{31}P NMR spectrum of a 170-mg rat heart at 4°C obtained after transformation of 1040 free induction decays. The spectrum was also observed during the accumulation after 200, 400, and 800 scans. Since only small time-dependent changes were observed, it seemed justifiable to average the NMR data over periods of approximately half an hour. The peaks can be assigned to the resonances of ATP, phosphocreatine, inorganic phosphate, and sugar phosphate (likely to be mainly glucose 6-phosphate). A nearly identical spectrum was obtained from a 200-mg heart preparation, and indeed the two spectra could be summed (Fig. 1b) to obtain a better signal-to-noise ratio. The values of the integrals are given in Fig. 1b. The most notable feature of these is that the three signals corresponding to ATP are in the ratio: $\beta:\alpha:\gamma$ = 0.5: 1.5:0.9. This implies that the γ peak contains a contribution from ADP and that the α peak, in addition to ADP, also contains resonances from other pyrophosphates, possibly NAD and re-

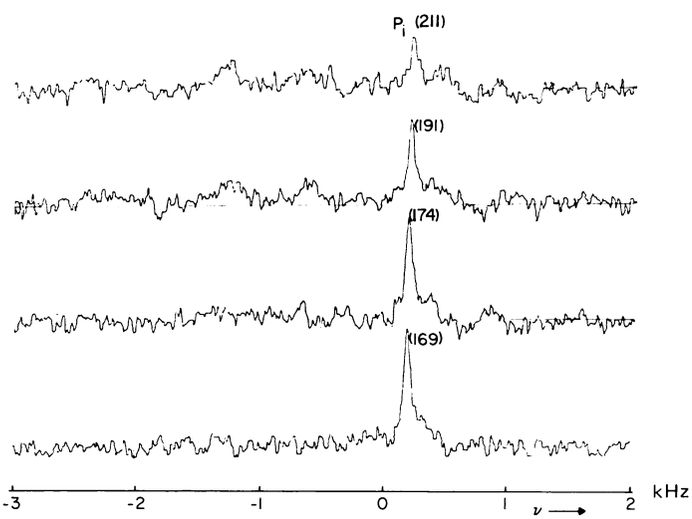

FIG. 2. ^{31}P NMR spectra of a 200-mg rat heart at various times after warming to 30°C. The time for warming and equilibration was 10 min. Other conditions are as given in the legend of Fig. 1a, except that each spectrum represents 200 accumulations. Direct superposition of the spectra of Figs. 1 and 2 is not valid because the deuterium oxide lock frequency depends upon the temperature. Numbers in parentheses are Hz.

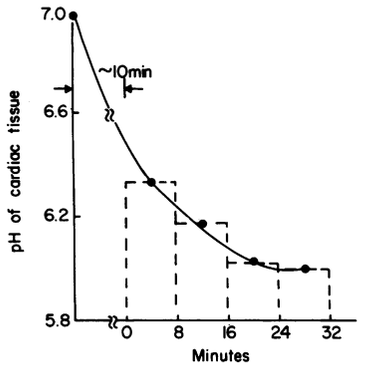

FIG. 3. Variation of the pH of cardiac tissue with time after warming to 30°C. Conditions as given in the legend of Fig. 2. The data are corrected for the temperature dependence of the lock frequency.

lated molecules. The frequency of the inorganic phosphate resonance shows that the intracellular pH of the resting muscle is 7.0. At this pH the three ATP resonances coincide with those of Mg^{++} ATP and not those of uncomplexed ATP, showing that almost all ATP is complexed to a divalent cation, most likely to be Mg^{++}. Similar observations were made for skeletal muscle (5, 6).

After the temperature of the NMR sample cavity was raised to 30°C and about 10 min was allowed for equilibration and the stoppage of intermittent contractions of the heart, spectra were recorded by accumulating sets of 200 scans successively (Fig. 2). These spectra show a steady increase in the inorganic phosphate level and a rundown on the energy store of the cardiac tissue. (The broad shoulder appearing on the high frequency side of the inorganic phosphate signal is incompletely identified, but probably corresponds in frequency to glucose 6-phosphate or AMP.) The significant result is that the inorganic phosphate resonance progressively shifts to lower frequency, indicating binding of protons by phosphate and thus acidosis of the heart.

The tissue pH as a function of time is plotted in Fig. 3. The difference in pH between the initial resting state at 4°C and the final acidic state (after about 30 min) is approximately one pH unit, with one-half the change occurring during the warming interval.

The inorganic phosphate signals from the ischemic hypoxic states are significantly shifted from those observed in normoxic tissue, so that portions of the heart that are in ischemic hypoxia (pH about 6.0) could be readily distinguished from the normoxic portions (pH $\geq$ 7.0). Thus, the fraction of cardiac tissue that is in the ischemic hypoxic state can be evaluated by comparing the areas under the high and low pH phosphate peaks. In this way, ^{31}P NMR would provide a diagnostic tool for tissue metabolic states and can be used to monitor ischemic hypoxic tissue volumes. In particular, our preliminary data suggest that the NMR method can be used for the perfused heart, using the Langendorff or working heart models under normal conditions for comparison with hearts in which the coronary circulation has been occluded to simulate infarcted tissue. The volume of the infarct may be directly and continuously evaluated.

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