Biochem. J. (1979) **184**, 547–554
Printed in Great Britain

Studies of Acidosis in the Ischaemic Heart by Phosphorus Nuclear Magnetic Resonance

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(*Received* 12 *April* 1979)

1. Phosphorus-nuclear-magnetic-resonance measurements were made on perfused rat hearts at 37°C. 2. With the improved sensitivity obtained by using a wide-bore 4.3 T superconducting magnet, spectra could be recorded in 1 min. 3. The concentrations of ATP, phosphocreatine and P_i and, from the position of the P_i resonance, the intracellular pH (pH_i) were measured under a variety of conditions. 4. In a normal perfused heart $pH_i = 7.05 \pm 0.02$ (mean ± S.E.M. for seven hearts). 5. During global ischaemia pH_i drops to 6.2 ± 0.06 (mean ± S.E.M.) in 13 min in a pseudoexponential decay with a rate constant of $0.25\,min^{-1}$. 6. The relation between glycogen content and acidosis in ischaemia is studied in glycogen-depleted hearts. 7. Perfusion of hearts with a buffer containing 100 mM-Hepes before ischaemia gives a significant protective effect on the ischaemic myocardium. Intracellular pH and ATP and phosphocreatine concentrations decline more slowly under these conditions and metabolic recovery is observed on reperfusion after 30 min of ischaemia at 37°C. 8. The relation between acidosis and the export of protons is discussed and the significance of glycogenolysis in ischaemic acid production is evaluated.

Hoult *et al.* (1974) demonstrated that ^{31}P n.m.r. spectra could be recorded from muscle. The major signals were identified as those of ATP, phosphocreatine, P_i and sugar phosphates. From such spectra the intracellular pH (Moon & Richards, 1973), the interaction of ATP with metal ions and slow metabolic changes during ischaemia could be measured. Since that time many reports have appeared on the use of ^{31}P n.m.r. in studies of skeletal muscle (Burt *et al.*, 1976; Seeley *et al.*, 1976; Dawson *et al.*, 1977; Busby *et al.*, 1978), cardiac muscle (Gadian *et al.*, 1976; Jacobus *et al.*, 1977; Garlick *et al.*, 1977, 1978) and kidney (Sehr *et al.*, 1977). Spectra of brain *in situ* have also been recorded from the anaesthetized mouse (Chance *et al.*, 1978). Much of this work has been recently reviewed (Radda & Seeley, 1979; Gadian *et al.*, 1979). The n.m.r. technique, when applied to whole organs, has several advantages over conventional methods: (i) it provides a simultaneous and non-destructive observation of several metabolites and therefore can be used to follow rates of changes in these when conditions are altered; (ii) rates of enzyme-catalysed reactions in the steady state can be derived by using 'saturation-transfer' methods (Brown *et al.*, 1978); (iii) the distribution of intracellular pH, arising either out of compartmentation (Seeley *et al.*, 1976) or from tissue heterogeneity (Hollis *et al.*, 1977) can be observed.

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

There is much interest in the effects of acidosis on heart contractility (Steenbergen *et al.*, 1977), the recovery of cardiac function from such acidosis (Bing *et al.*, 1973) and the control of excitation by pH effects (Vogel & Sperelakis, 1977). Despite this interest there is little information about the mechanisms of proton production and pH homoeostasis in ischaemia. More recently some questions have been raised about the relationship between the glycolytic rate and proton production (Gevers, 1977; Wilkie, 1979). Work on pH homoeostasis in the heart has, to date, been restricted to non-contracting muscle (Ellis & Thomas, 1976).

We describe in the present paper the physiological techniques we have developed to maintain isolated perfused rat hearts in a functional state inside the n.m.r. spectrometer, and we examine the role of H^+ ions in ischaemic cardiac tissue.

Methods

Perfusion

Since the heart must be cannulated outside the spectrometer magnet and subsequently moved into it, our Langendorff perfusion apparatus (Fig. 1) must have spatial flexibility. For this reason, the length of the perfusion circuit (from oxygenated reservoir to the heart) is approximately three times that of a conventional system. Temperature and O_2 content of the perfusion fluid are maintained by

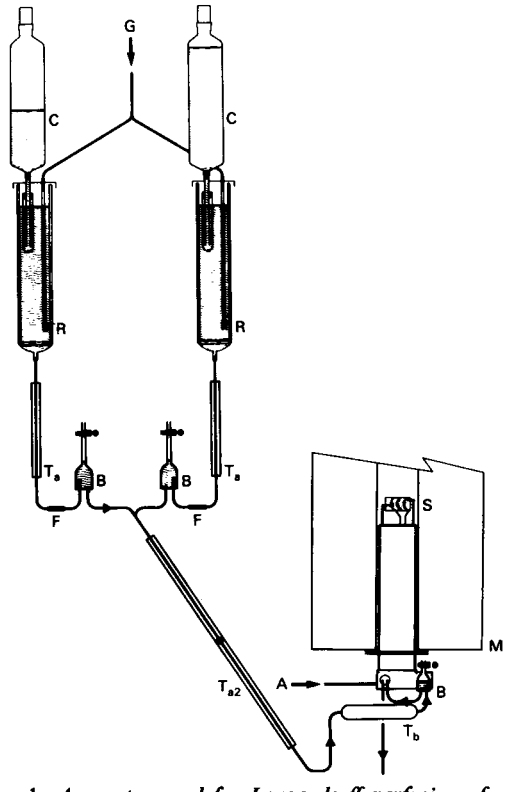

Fig. 1. *Apparatus used for Langendorff perfusion of rat hearts in the n.m.r. spectrometer*

Abbreviations: G, gas supply (O_2/CO_2, 19:1, v/v); C, constant head device; R, thermostatically controlled glass reservoir with sintered glass at base; T_a and T_{a2}, thermostatically controlled glass tubes; B, thermostatically controlled bubble-trap; F, flow-control device; S, sample tube containing heart; M, wide-bore superconducting magnet (4.3T); A, supply of thermostatically controlled air; T_b, thermostatically controlled helical glass tube.

using thermostatically controlled glass units. These are joined by short lengths of flexible Tygon tubing (surgical grade). Slight cooling of fluid (approx. 1°C) is corrected just before its entry to the probe by the heat exchanger (T_b). The narrow Tygon that conveys medium through the probe is warmed, as is the sample tube, by a rapid flow of thermostatically controlled air regulated by a bimetallic sensor and associated electronics. Hearts were perfused at an aortic pressure of 70cm of water giving coronary flow rates around 10ml/min per g fresh wt.

The metal cannula which is customarily used for heart perfusion has been replaced by a glass one to avoid absorption of radiofrequency radiation. Dark paint is fused into the tips of these cannulae to make them visible through the aortic wall and thus eliminate the risk of damaging the aortic valve during cannulation. The heart is immersed in perfusion fluid and maintained free of the walls of the sample tube since hearts even in light contact with the tube have visible ischaemic patches and impaired function. The linewidths of intracellular phosphate signals are reduced by immersion of the heart and this has been shown to cause no additional oedema over periods up to 5h. This experimental setup has enabled us to maintain rat hearts in a steady metabolic state for periods up to 12h, although for experiments reported in this paper hearts were perfused for no more than 2h. Hearts were obtained from male Wistar rats (280–300g). The surgical procedures of Garlick *et al.* (1977) were used.

The basic perfusion medium was Krebs–Henseleit buffer (Krebs & Henseleit, 1932) supplemented with 0.5mM-calcium EDTA and 11mM-glucose. This was gassed with O_2/CO_2 (19:1, v/v). This basic solution was modified when necessary as follows: (i) for phosphate-free Krebs solution, KH_2PO_4 was replaced by an equivalent concentration of KCl; (ii) for Krebs-plus-Hepes solution, NaCl concentration was reduced by 50mM and 100mM-Hepes was added and the pH adjusted with NaOH. The Na^+ concentration in this solution was therefore the same as in 'normal' Krebs–Henseleit buffer; (iii) for Krebs-plus-mannitol solution, 50mM-mannitol was added to Krebs–Henseleit buffer thus giving a solution iso-osmolar with the Krebs-plus-Hepes solution; (iv) for glucose-free Krebs solution, glucose was omitted. All solutions were made with A.R. grade reagents and were filtered through a 0.45μm Millipore or Amicon filter.

pH measurements

pH measurements were made by using a Findip 555A pH meter and a glass combination electrode. The meter was standardized at 37°C with thermostatically controlled samples of electrometrically checked buffers obtained from BDH Chemicals, Poole, Dorset, U.K.

N.m.r. techniques

(i) *The design of the probe and the sample chamber.* The probe for perfused heart was designed to optimize the signal-to-noise ratio and field homogeneity over the sample. Since conductors positioned close to the radiofrequency coil degrade the signal-to-noise ratio (Hoult & Richards, 1976), it is best to minimize the volume of perfusion medium both surrounding the tissue and in the tubes taking buffer into and out of the sample chamber. Homogeneity of the B_0 field is maintained by: (i) minimizing the size of the perfusion tubes; (ii) arranging for maximum symmetry around the sample; (iii) preventing the formation of bubbles within the sample. For rat

hearts we found that a three-turn solenoidal coil (Hoult & Richards, 1976) (internal diameter 24mm, length 35mm) made of copper wire (standard wire gauge 10) gave optimum performance. Our sample tube was a double-walled glass cylinder (length 45mm, internal diam. 15mm, external diam. 23mm) sealed at both ends with silicone rubber bungs, which are pierced by the inlet and outlet tubes (internal diam. 2.4mm, external diam. 4.0mm). The axis of the coil must be perpendicular to the B_0 field of the superconducting magnet and thus hearts are perperfused in a horizontal position. With the dimensions of our system the heart occupies 70% of the sample volume.

(ii) *N.m.r. measurements.* ^{31}P n.m.r. spectra were recorded at 73.8MHz with a spectrometer as described previously (Hoult & Richards, 1975). An additional new feature of the present instrument is a wide-bore (10cm) superconducting magnet (4.3T) built by the Oxford Instrument Co. The magnet was designed for non-spinning samples and it contains a set of 18 room-temperature-correcting coils. The spectrometer was operated in the Fourier transform mode and was interfaced with a Nicolet B-NC-12 computer. NaCl (100mM)+NaH_2PO_4 (20mM) was used as a standard sample. The 'loaded Q' of the probe was 280 with this solution and the single-scan signal-to-noise ratio was 30 (peak-to-peak noise). Spectra were collected without the use of a field-frequency lock and without proton decoupling. Further details of individual measurements are given in the legends to Figures.

(iii) *pH determination by n.m.r.* In principle, any signal whose frequency is sensitive to proton concentration can be used to measure pH. For intact tissue, the P_i signal is generally the most suitable since it is easily observable and because P_i has a pK_a in the pH region of biological interest. Around neutral pH P_i exists as the ions HPO_4^{2-} and $H_2PO_4^-$. If chemical exchange could be prevented these two species would give rise to two signals separated by approx. 2.3 p.p.m. However, in solution the two species will exchange with each other rapidly (approx. 10^9–$10^{10}s^{-1}$) and, as a result, the observed spectrum is a single peak, the frequency of which is determined by the relative amounts of the two species rather than by the absolute amount of P_i present (Hoult *et al.*, 1974; Gadian *et al.*, 1979). The pH of the solution can then be derived from the frequency of this single peak provided that a calibration curve obtained under similar conditions has been constructed.

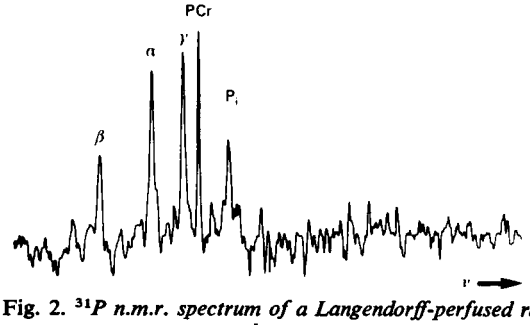

Fig. 2. *^{31}P n.m.r. spectrum of a Langendorff-perfused rat heart*

The spectrum was collected between 20 and 21min of perfusion. Sixty radiofrequency pulses were applied at intervals of 1s. Spectrum width was 6.13kHz. The arrow indicates the direction of increasing frequency. Abbreviations used: PCr, phosphocreatine; α, β and γ indicate the α-, β- and γ-phosphate groups of ATP.

Results

Time resolution

Fig. 2 shows a typical ^{31}P n.m.r. spectrum of a rat heart after 20min perfusion. As shown previously

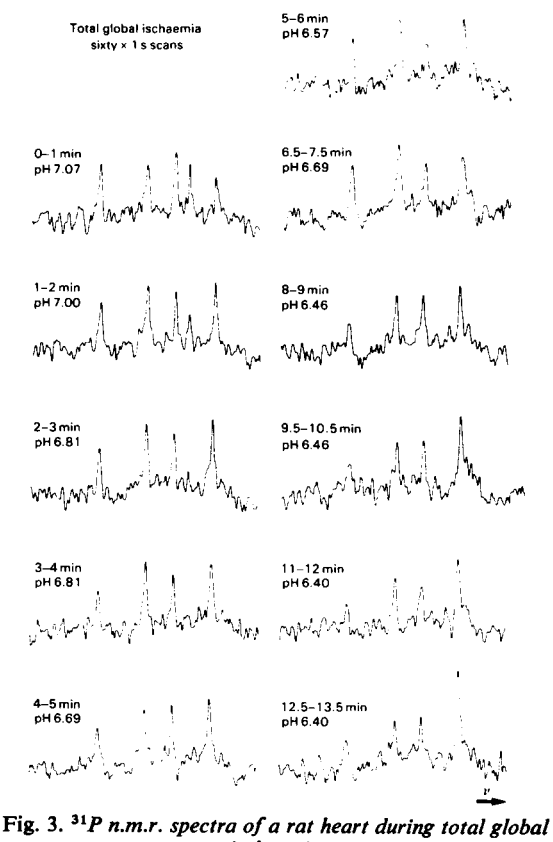

Fig. 3. *^{31}P n.m.r. spectra of a rat heart during total global ischaemia*

Individual spectra were recorded over 1min under the same conditions as in Fig. 2, but the spectrum width is 3.06kHz. Times against individual spectra are with reference to the induction of ischaemia at zero time.

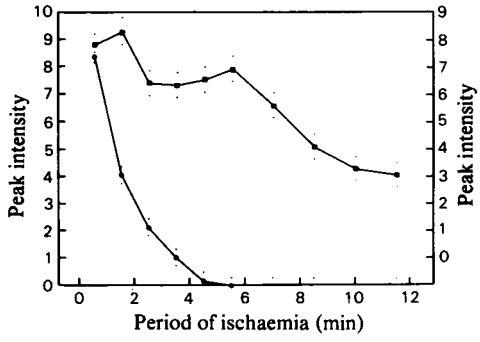

Fig. 4. *Relative changes in ATP and phosphocreatine concentrations in rat hearts during total global ischaemia*
The points are averages for three hearts and the standard deviations are indicated by the dots above and below each point. Symbols: ●, phosphocreatine signals; ■, signals for β-phosphate group of ATP. The left-hand axis is the scale of peak intensities for phosphocreatine signals and the right-hand axis is the scale of peak intensities for the signals of the β-phosphate group of ATP.

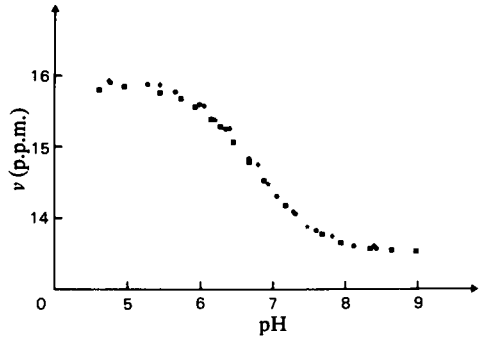

Fig. 5. *Variation of P_i chemical shift with solution pH*
Solutions containing 10mM-P_i and 10mM-phosphocreatine were adjusted to various ionic strengths by addition of KCl or NaCl and titrated at 37°C by addition of HCl and KOH or NaOH. Symbols: ◆, $\mu = 0.12$M, KCl; ■, $\mu = 0.16$M, KCl; ●, $\mu = 0.2$M, KCl; ✱, $\mu = 0.16$M, NaCl. The chemical shift of P_i is expressed with respect to an internal standard (methylenediphosphonate).

(Gadian *et al.*, 1976; Garlick *et al.*, 1977; Hollis *et al.*, 1977) the resonances can be assigned to the β-, α- and γ-phosphate groups of ATP, and to phosphocreatine and P_i. The spectrum is the sum of signals generated by applying 60 radiofrequency pulses separated by intervals of 1s. It therefore represents the average situation over 1min. These parameters of data collection were chosen to optimize the signal-to-noise ratio in any given time. Under these conditions partial 'saturation' of the peaks occurs (Gadian *et al.*, 1976; Garlick *et al.*, 1977) and consequently their areas must be corrected to obtain the true concentrations of metabolites. The time resolution of this method is illustrated in Fig. 3, which gives a series of spectra taken during the first 14min of total global ischaemia. Relative changes in ATP and phosphocreatine concentrations are shown in Fig. 4. The results, which are averages for three separate experiments, show a slight initial increase in ATP and a very rapid decrease in phosphocreatine. In the rest of this paper we will focus on the intracellular pH in ischaemia, calculated from the frequency of the P_i peak.

Calibration of intracellular pH

A calibration curve of the pH against P_i chemical shift was constructed by using a solution whose ionic strength was matched to that of the cell (Fig. 5). The chemical shift is measured from the frequency of the maximum ordinate. The solution also contained phosphocreatine (10mM) as an internal frequency standard. Measurement of P_i frequency with respect to phosphocreatine provides an automatic correction in tissue studies for any magnetic susceptibility differences between the intracellular and extracellular environments. Solutions of different ionic strength ($I = 0.12$–0.20M) and composition (Na^+ or K^+ as cation) were titrated to define the limits of accuracy of the pH measurements. For optimum performance of the heart in the long term, the perfusion medium must contain P_i. The concentration of P_i in the Krebs–Henseleit buffer is 1.2mM and it is therefore necessary to show that this extracellular phosphate does not interfere with the measurement of the intracellular pH.

Fig. 6 shows the P_i frequency region at high resolution of spectra from a single heart perfused in the presence and absence of P_i. Two signals are resolved in spectrum (*a*) by using normal Krebs–Henseleit solution, whereas there is only one signal with phosphate-free solution (spectrum *b*). Similar spectra were obtained from seven other hearts.

On the basis of the calibration curve shown in Fig. 5 and other control experiments described elsewhere (Gadian *et al.*, 1979) we can convert the chemical shift difference between P_i and phosphocreatine in spectra of perfused hearts into intracellular pH. By using this method, the intracellular pH of the perfused rat heart was found to be 7.05 ± 0.02 (mean ± S.E.M. for seven hearts). The range was 7.01–7.07. The pH of the extracellular phosphate was found to be 7.38. In phosphate-free buffer the intracellular pH was 7.04 ± 0.01 (mean ± S.E.M. for eight hearts).

pH in total global ischaemia

We can now examine changes in intracellular pH in total global ischaemia, i.e. no flow of perfusion

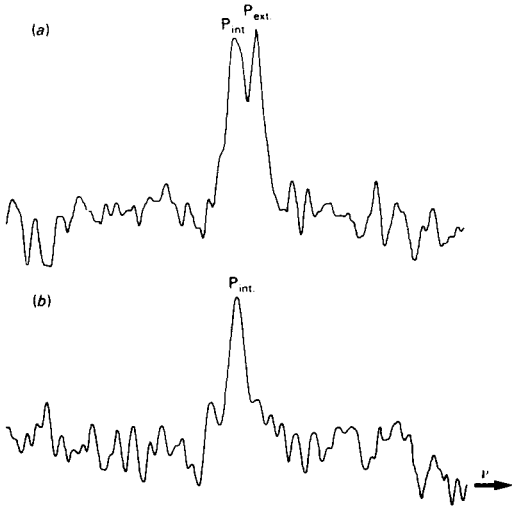

Fig. 6. *High-resolution ^{31}P n.m.r. spectra of the P_i region for perfused rat hearts*

(*a*) Heart perfused with normal Krebs–Henseleit buffer; (*b*) the same heart perfused with phosphate-free Krebs–Henseleit buffer. The $P_{int.}$ signal is assigned to intracellular P_i and corresponds to a pH of 7.05 and $P_{ext.}$ is assigned to extracellular P_i and corresponds to a pH of 7.38.

fluid through the heart. These are shown in Fig. 7. By 13min of global ischaemia the pH had dropped to 6.2±0.06 (mean±S.E.M. for seven hearts). The variation from heart to heart (6–6.4) is larger than in the steady-state situation. The curve can be approximately fitted to a decaying exponential with a first-order rate constant of 0.25min^{-1}. Several hearts were perfused with phosphate-free Krebs–Henseleit buffer for 10min before ischaemia. Their pH–time course is also shown in Fig. 7. The close similarity between this and the control curve indicates the correctness of our contention that the buffer P_i does not interfere with the pH measurement.

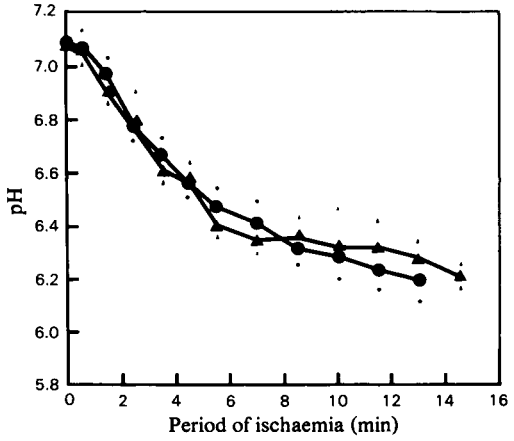

Fig. 7. *Time course of changes in intracellular pH during total global ischaemia*

Symbols: ●, control hearts (points are means for seven hearts and the small symbols represent standard errors in the mean); ▲, hearts pre-perfused with phosphate-free buffer (three hearts). These and subsequent time courses are based on spectra collected over 1min.

pH changes in glycogen-depleted hearts

The major pathway that produces protons in ischaemia is glycogenolysis. As a first step towards quantifying the processes responsible for acidosis in 'control' ischaemic hearts, we have therefore examined the behaviour of intracellular pH in ischaemic hearts depleted of their glycogen. Hearts were equilibrated for 30min with normal Krebs–Henseleit buffer. They were then glycogen-depleted by 70%, by a 45min perfusion with glucose-free buffer (Dhalla *et al.*, 1972). The mean pH curve (Fig. 8) shows that the initial intracellular pH is the same as in the control hearts, but that after 14min global ischaemia the pH has only dropped to 6.6±0.08 (mean±S.E.M.).

Let us consider the large variation in the pH against time curves for individual hearts (Fig. 9). We suggest that the hearts that showed a large decrease in pH had a higher glycogen content at the beginning of ischaemia than those that showed a small pH decrease. Unfortunately we cannot measure glycogen

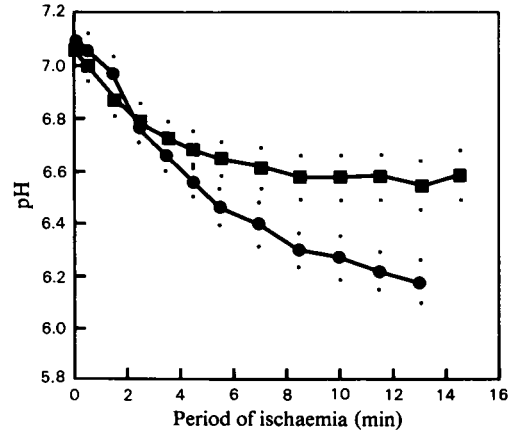

Fig. 8. *Time course of changes in intracellular pH in total global ischaemia*

Symbols: ●, control hearts (seven hearts); ■, glycogen-depleted hearts (six hearts). The small symbols represent S.E.M. values.

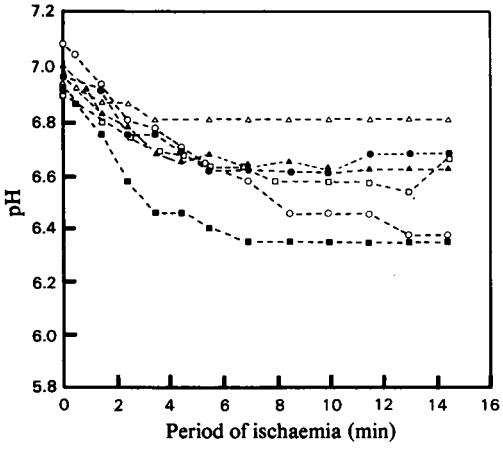

Fig. 9. *Time course of changes in intracellular pH during total global ischaemia for individual glycogen-depleted hearts*

non-destructively, but we can compare changes in the ATP contents of the individual glycogen-depleted hearts with their pH changes in ischaemia (Fig. 10). The time at which the ATP concentration reaches zero (which we presume coincides with total glycogen depletion) is contemporaneous with that at which the pH value levels off. This observation suggests that at least in glycogen-depleted hearts there is a relationship between the final ischaemic pH and glycogen content. The situation in normal hearts is more complicated. Glycogenolysis may stop because of total glycogen depletion or possibly because phosphofructokinase is inhibited at low pH and thus glycolysis cannot continue.

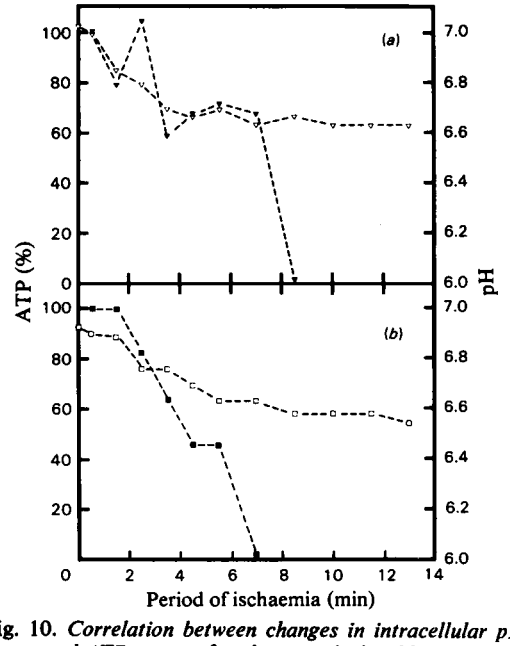

Fig. 10. *Correlation between changes in intracellular pH and ATP content for glycogen-depleted hearts*
The ATP content is expressed as a percentage of its value in the first minute of ischaemia. The results for two different hearts are shown. Open symbols are used for pH and closed symbols for ATP content. Similar observations were made for the other hearts of Fig. 9.

External buffering power and intracellular pH

Are the protons that are produced within the ischaemic-heart cell transported to the extracellular space? Such an export process would slow down as the extracellular pH decreased. It is likely that if the external pH were kept up, e.g. by increasing the extracellular buffering capacity, this proton export could continue longer, thus reducing the fall in internal pH. To test this, we perfused hearts for 12min before ischaemia with a Krebs–Henseleit buffer supplemented with 100mM-Hepes. Both the rate and the extent of cellular acidification are significantly reduced compared with the controls and the final pH value is 6.8 (Fig. 11). This is not a result of the increased external osmolarity, as is shown by the observation that the pH-against-time curves from hearts perfused with Krebs–Henseleit solution plus 50mM-mannitol are superimposable over those of the controls.

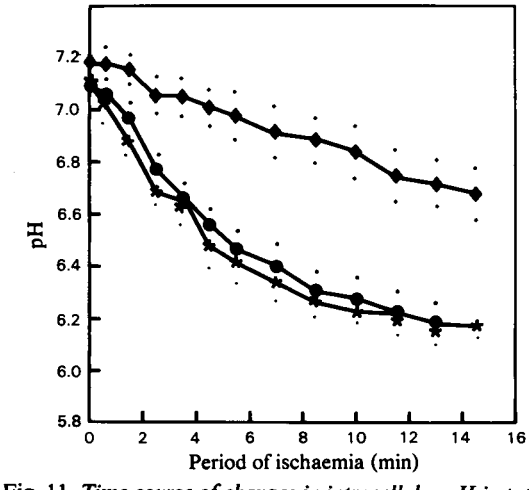

Fig. 11. *Time course of changes in intracellular pH in total global ischaemia*
Symbols: ●, control hearts (seven hearts); ✱, hearts pre-perfused with Krebs–Henseleit buffer+50mM-mannitol (three hearts); ◆, hearts pre-perfused with Krebs–Henseleit buffer+100mM-Hepes (seven hearts). The small symbols represent S.E.M. values.

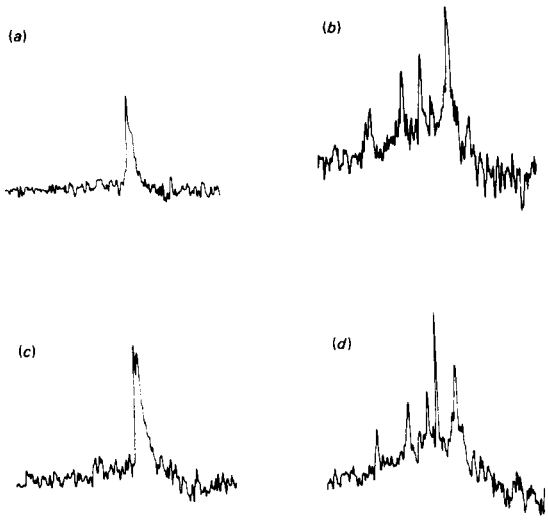

Fig. 12. *^{31}P n.m.r. spectra of rat hearts during total global ischaemia and after reflow*

Spectra (*a*) and (*b*) were recorded over 14–15min of ischaemia; spectra (*c*) and (*d*) were recorded over 5–6min reflow after 30min of ischaemia. The heart in spectra (*a*) and (*c*) was perfused with Krebs–Henseleit buffer both before and after ischaemia, whereas the heart in spectra (*b*) and (*d*) was perfused both pre- and post-ischaemia with Krebs–Henseleit buffer+100mM-Hepes.

Hepes (100mM) also has a significant protective effect on the ischaemic myocardium. After 14min of ischaemia, spectra of hearts perfused with Krebs–Henseleit buffer have only P_i and sugar phosphate signals (Fig. 12*a*), whereas both phosphocreatine and ATP can be observed in hearts perfused with Krebs-plus-Hepes solution (Fig. 12*b*). Reperfusion of the hearts for 5min with the original buffers after 30min of total ischaemia resulted in complete recovery of phosphocreatine and ATP concentrations in hearts perfused with Krebs-plus-Hepes solution (Fig. 12*d*), but resulted in no recovery in the control (Fig. 12*c*).

Discussion

Poole-Wilson (1978) reviewed the methods that have been used to measure myocardial intracellular pH in normal and pathological states. He concluded that, although current methods give a qualitative indication of pH changes, they cannot be used to measure the internal pH quantitatively in ischaemia or hypoxia. ^{31}P n.m.r. was included in this statement, as a result of the report by Jacobus *et al.* (1977).

Subsequent n.m.r. measurements by the same authors (Taylor *et al.*, 1977) and by ourselves (Garlick *et al.*, 1977, 1978) suggested that the conditions used by Jacobus *et al.* (1977) were not optimal, as has already been discussed in detail previously (Radda & Seeley, 1979; Gadian *et al.*, 1979).

The measurement of intracellular pH from the resonance position of P_i requires knowledge of the pK_a of this molecule under conditions that exist in the cell. Our calibrations show that cations have very little specific effects on the P_i resonances. Binding of P_i to macromolecules may give rise to systematic errors in the measurements, but these are likely to be small for the following reasons. (i) In systems where cellular pH can be measured by both microelectrodes and ^{31}P n.m.r. the pH values are in close agreement. (ii) Binding of P_i to macromolecules must be limited in extent because relatively narrow signals are observed for this compound in the n.m.r. spectra of heart (10–20Hz). (iii) Relatively small chemical-shift differences have been observed between phosphorus-containing molecules free in solution and in their enzyme complexes, although the generality of this is not known. For example, in a solution containing 6mg of creatine kinase/ml we did not observe any significant change in chemical shift for P_i as a result of addition of the enzyme (D. G. Gadian & G. K. Radda, unpublished work). (iv) Since several molecules have pK_a values in the range of interest, it is possible to cross-check pH measurements by observing several n.m.r. signals. Although such studies have not been carried out for cardiac tissue, Moon & Richards (1973) measured the pH in the erythrocyte by using P_i and bis(phospho)glycerate signals and Brown *et al.* (1977) used ^1H signals from the histidine residues of haemoglobin in the same system. All the values agree closely.

Any rational treatment of the acidosis produced in ischaemia depends on information on proton-producing and -consuming reactions in the cell. Gevers (1977) has catalogued several metabolic events involving H^+ ions but the relative importance of the various proton-producing reactions was not evaluated. The significance of glycogenolysis in total global ischaemia in the rat heart is indicated by the calculations presented in Table 1. Calculations for glycogen-depleted and the Hepes-buffer cases are also included. In all three cases, the close agreement between the calculated and measured amounts of H^+ ions produced indicates that the breakdown of glycogen to lactate is the major proton-producing process. We have no evidence as to whether or not Hepes enters the cells, but have assumed in the calculations that it remains in the extracellular space. It is noteworthy, however, that Hepes has a significant protective effect on the ischaemic myocardium. In contrast, although glycogen-depleted hearts also show a much smaller extent of acidification than the controls, they do not recover on reflow. Thus decreasing the pH change in itself is not beneficial in ischaemia unless accompanied by maintenance of

Table 1. *Calculation of pH changes in the rat heart during the first 10 min of total global ischaemia*
All calculations are made for a 1 g heart.

Condition	Calculated proton production* (μ-equiv.)	Buffering capacity of heart and extracellular space‡ (μ-equiv./pH unit)	Predicted pH decrease	Observed pH decrease
Control	35	31–42	0.8–1.1	0.94
Glycogen-depleted	15.4†	31–42	0.4–0.5	0.55
Hepes	35	55	0.63	0.50

* Assuming that the numbers of protons produced from anaerobic glycolysis or hydrolysis of the following are: glycogen, +2; ATP, +1; phosphocreatine, −1, and using the metabolite contents in ischaemia as given by Hearse *et al.* (1977).
† Results of Dhalla *et al.* (1972).
‡ Results of Ellis & Thomas (1976), Polimeni (1974) and Morgan *et al.* (1959).

ATP and phosphocreatine concentrations. Thus the effect of Hepes in maintaining internal pH is to decrease the inhibition of glycolysis by H^+ accumulation.

This work was supported by the Science Research Council, British Heart Foundation and N.I.H. (grant HL 18708-02S1).

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