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N.m.r. Studies of Metabolism in Perfused Organs

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N.m.r. studies of metabolism in perfused organs

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Several metabolites and intracellular pH in intact organs can be studied in a non-destructive manner by phosphorus nuclear magnetic resonance (^{31}P n.m.r.). This possibility was demonstrated by us nearly five years ago. Since then we have developed the appropriate physiological techniques and improved the n.m.r. method for the study of animal hearts and kidneys. Here we describe measurements aimed at clarifying three problems. (1) Having measured the enzyme-catalysed fluxes between phosphocreatine and ATP by the method of saturation transfer n.m.r., we examine the relation between energy supply and heart rate in the isolated perfused rat heart. (2) We describe experiments to establish the validity of the perfusion model. For the first time, we report ^{31}P n.m.r. measurements of an *in vivo* rat heart and compare the results with those obtained for the perfused rat heart. (3) Ischaemia and metabolism in rabbit kidneys is investigated to establish the relation between functional and metabolic recovery after a renal transplant operation.

Introduction

About five years ago, when we completed a series of studies on the regulatory properties of the soluble enzyme glycogen phosphorylase (Busby & Radda 1976), a number of problems arose that suggested that the behaviour of this enzyme is different *in vivo* from that *in vitro*. When we examined a variety of ligands (about ten of them) that modulate the activity of this enzyme and measured their interactions with phosphorylase and the kinetic consequences of these interactions, we predicted that, in resting skeletal muscle, glycogen phosphorylase ought to be many times more active than it is found to be. This is why we started to think about how to look at molecules and molecular interactions in living tissues and organs. We had already been studying enzyme kinetics by phosphorus n.m.r. (Gadian *et al.* 1974) and, combining this with our biochemical interests, we decided to determine whether one could use ^{31}P n.m.r. to detect small molecules in whole tissue (Hoult *et al.* 1974). We chose the phosphorus nucleus for a variety of reasons, including the relative simplicity of the spectra, good resolution, acceptable sensitivity and, not least of all, the biological importance of phosphate compounds (Gadian *et al.* 1979). We showed that one could record the ^{31}P n.m.r. spectra of skeletal muscle from a rat and could readily identify signals from the three phosphate groups of ATP, phosphocreatine, inorganic phosphate and sugar phosphate. From the position of inorganic phosphate signals, we could measure the intracellular pH in these non-perfused muscles and, from the ATP resonances, we were able to conclude that over 95% of the ATP was complexed to divalent cations such as Mg^{2+} (Hoult *et al.* 1974).

It seemed to us that there were three requirements for development of ^{31}P n.m.r. as a valid method for metabolic studies in intact organs. First, one had to modify the appropriate physiological techniques for keeping the tissues alive in the spectrometer. This was first achieved for

frog muscle (Dawson *et al.* 1977). Secondly, further instrumental improvements were needed and these culminated over a year ago in the introduction of a large bore superconducting magnet along with a new series of n.m.r. probes. We can now do a much wider range of experiments than before. Last, but not least of all, it was necessary to ask specific biological questions to which n.m.r. can give unique answers and not just confirm, albeit in a more direct way, what we already know. We felt that an appropriate organ to study, as far as muscle is concerned, is the perfused heart.

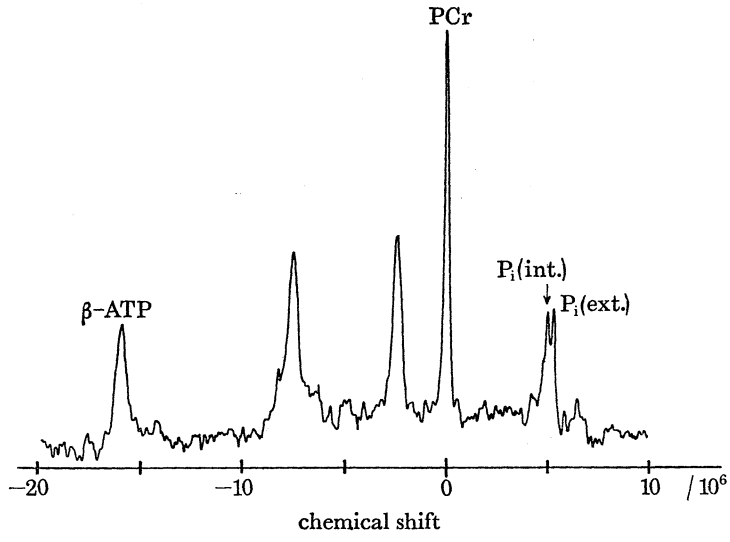

FIGURE 1. High resolution ^{31}P n.m.r. spectrum of Langendorff-perfused rat heart. The spectrum represents the Fourier transform average of 480 sampling pulses of 45° and with a 1 s repetition interval. The spectrum is expanded to display 2.2 KHz. Peak assignments are: β-ATP, β-phosphate of adenosine 5′-triphosphate; PCr, phosphocreatine; P_i(int.), intracellular inorganic phosphate; and P_i(ext.), Krebs–Henseleit buffer inorganic phosphate.

STUDIES ON SMALL HEARTS

We first demonstrated that it was possible to observe ^{31}P n.m.r. spectra from rat hearts (Gadian *et al.* 1976), and Pamela Garlick and John Seeley proceeded to develop the perfusion system (Garlick *et al.* 1977). We use a modified Langendorff technique (Garlick *et al.* 1979) in which the heart is perfused with oxygenated Krebs–Henseleit buffer in the retrograde mode. Special attention is paid to avoid oxygen loss, to maintain accurate temperature control (at 37 °C) and to eliminate interference from paramagnetic ions and other impurities. Other important technical details have been summarized elsewhere (Gadian *et al.* 1979). In our initial experiments we were restricted to using an 8 mm n.m.r. tube and could only examine by n.m.r. perfused small rat (Garlick *et al.* 1977) and mouse (Battersby *et al.* 1978; Garlick *et al.* 1978) hearts. Although the collection of good spectra from these small hearts required accumulation times of the order of 4–10 min, we were able to study metabolic events associated with the onset of global ischaemia and the recovery process following this period of oxygen deficiency. For example, we found that a characteristic property of normal buffer-perfused mouse hearts is that of high coronary flow rate and a relatively high phosphocreatine to ATP ratio at the beginning of perfusion. The flow then drops and a steady state situation (in 20–30 min) with a lowered phosphocreatine:ATP ratio is reached. After a period of ischaemia

on reflow, an apparent 'overshoot' in phosphocreatine resynthesis is observed (Battersby *et al.* 1978). While in these early experiments several interesting qualitative observations were made, the small sample size severely restricted the systems one could study, and the time required for spectral accumulation for accurate quantitative measurements was still too long.

Studies of larger organs

In our current magnet, with a room temperature bore of about 9 cm, we can accommodate hearts with sizes ranging from that of a mouse (300 mg) to that of a rabbit (6 g). Depending on the size of the heart we wish to study, we use different kinds of radiofrequency coil arrangements in the n.m.r. probe. For rat hearts weighing around 1 g (wet mass), a solenoidal coil is wrapped around the sample chamber into which the heart is placed in a horizontal direction.

Using this system, we can collect high resolution ^{31}P n.m.r. spectra from rat hearts. Figure 1 shows such a spectrum for a beating heart, together with the peak assignments. Having achieved this kind of sensitivity, we could define the biochemical problems we wanted to study.

Biochemical studies

In the early days of a new technical development, one has to evaluate the validity of the method. We now believe that n.m.r. can take its place in biological studies of tissues (Gadian *et al.* 1979; Radda & Seeley 1979; Garlick & Radda 1979). It is essential to define the problems one wishes to study, making use of those features of the n.m.r. measurement that provide either unique information or better answers than other methods. In this connection, we have studied in detail the relations between high energy phosphates, H^+ production, glycogen content and external buffering during global myocardial ischaemia (Garlick *et al.* 1979; Garlick & Radda 1979). Since, in the next paper, Dr Hollis and his colleagues will be presenting data concerned with ischaemia, we shall concentrate on other aspects of our work.

In situ *rate measurements*

In skeletal muscle the phosphocreatine to ATP ratio is about 6 : 1 and it is generally accepted that phosphocreatine acts as a 'back-up pool' of high energy phosphate, through the reaction catalysed by creatine kinase, in a 'near equilibrium process'. In cardiac muscle the situation is different and more complex. First, the phosphocreatine : ATP ratio is *ca.* 1.2–1.6 (although occasionally much higher values have been observed). Secondly, in addition to the cytoplasmic creatine kinase, a separate enzyme is found in the extramitochondrial space. It has been suggested that the mitochondrial creatine kinase has the role of providing a mechanism for transferring 'energy' from newly synthesized mitochondrial ATP to the cytoplasm, through the production of phosphocreatine (Saks *et al.* 1975). The idea that mitochondrial ATP has 'privileged access' to this creatine kinase is, however, not generally accepted.

In situ rate measurements in relation to the energetic demands of a functioning heart should enable us to study this problem.

N.m.r. allows us to measure reaction rates in a unique way. The method of saturation transfer has already been mentioned by Dr Shulman, and Dr Brown will explain some of the details of the technique. Let us now look at a beating heart positioned within the magnet.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Creatine kinase is catalysing a reversible reaction shuttling 'energy' between ATP and phosphocreatine (equation 1):

$$\text{phosphocreatine} + \text{Mg.ADP} + \text{H}^+ \rightleftharpoons \text{Mg.ATP} + \text{creatine}. \quad (1)$$

ATP is used in numerous energy-requiring reactions, such as muscle contraction, ion and substrate transport and substrate phosphorylation, and is resynthesized primarily by oxidative phosphorylation and by glycolysis. This whole system is maintained in a steady state.

One can use the n.m.r. method to measure reaction rates at equilibrium or in a steady state as follows. If one irradiates the γ-phosphate peak of ATP (this is essentially a form of magnetic labelling), the peak disappears because the two spin states are now equally populated. If the γ-phosphate peak of ATP is then transferred to creatine to form phosphocreatine, the 'saturation effect' should be seen on the phosphocreatine signal. We observed that the intensity of the phosphocreatine resonance is reduced by 70%, compared to the original intensity (Brown *et al.* 1978). Using the T_1 relaxation time for phosphocreatine, one can work out the rate of interconversion of phosphate between phosphocreatine and ATP. If one does the reverse experiment, in which the phosphocreatine peak is irradiated, and looks at the decrease in γ-ATP peak, the rate of the process from ATP to phosphocreatine can be obtained.

The results of these experiments have been described elsewhere (Brown *et al.* 1978). We have not yet measured directly the rate of ATP hydrolysis, but we can estimate this from the rate of oxygen consumption (as in studies up till now), assuming a P:O ratio of 3:1. We use the following simplified scheme to describe the steady-state situation:

$$\text{phosphocreatine} \underset{V_2}{\overset{V_1}{\rightleftharpoons}} \text{ATP} \underset{V_4}{\overset{V_3}{\rightleftharpoons}} \text{ADP} + \text{P}_\text{i}.$$

In this, V_n represents the unidirectional flux through the various paths.

From the n.m.r. experiment, V_1 is *ca.* 2–4 mM s^{-1} and V_3, from O_2 consumption, is *ca.* 1.0–1.5 mM s^{-1}. There are two contributions to V_2: conversion of cytoplasmic ATP to phosphocreatine and, possibly, direct conversion of mitochondrial ATP to phosphocreatine (Saks *et al.* 1975). Although we have measured V_2 by n.m.r. (Brown *et al.* 1978), we do not yet know to what extent the observed rate depends on the compartmentation of ATP or on the contribution of some of the competing processes (i.e. ATP hydrolysis and oxidative phosphorylation). For the present discussion it is sufficient to note that V_1 is not very much larger than V_3 and that it is therefore conceivable that under conditions of increasing work load (increased V_3) V_1 becomes rate limiting. We have, therefore, set out to examine the question of whether the creatine kinase can keep up with the demand for energy when the rate of ATP utilization is increased. It was for this reason that we initiated a series of experiments on paced hearts.

Heart rate and high energy phosphates

We have studied the metabolic responses of isolated perfused rat hearts to tachycardia-induced work overload by following the ^{31}P n.m.r. spectra as a function of heart rate. Figure 2 shows a typical experiment in which we pace the heart at 600 beats/min. It can be seen that, within the time resolution of our measurement, the ATP and phosphocreatine levels decrease in response to the increased heart rate, showing a slight 'recovery' in phosphocreatine after about 1 h. The quantitative features of such experiments are summarized in figure 3. The phosphocreatine level hardly changes between 300 and 700 beats/min; inorganic phosphate

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

slightly increases between 300 and 500 beats/min, shows a marked increase between 500 and 600 beats/min and then recovers at 700 beats/min. In contrast, the ATP concentrations steadily fall as a function of heart rate and decrease more markedly between 500 and 600 beats/min. It is interesting that the contractility of the heart under similar conditions also decreases between 500 and 600 beats/min (Taylor & Cerny 1976) and that the physiological upper range for a living rat is in the region of 600 beats/min (Barnard *et al.* 1974). Evidently the ATP is 'sensing' the demand for energy much more than is phosphocreatine. This is seen even more clearly if, in addition to increasing the heart rate, we increase the perfusion pressure from 70 to 140 cm of water (figure 4). Both the phosphocreatine and the ATP levels drop; ATP almost disappears, while phosphocreatine only drops by about 50%. It is clear that a situation in which the creatine kinase reaction is rate limiting has been reached, but, of course, we will need to measure the fluxes as a function of heart rate to prove this.

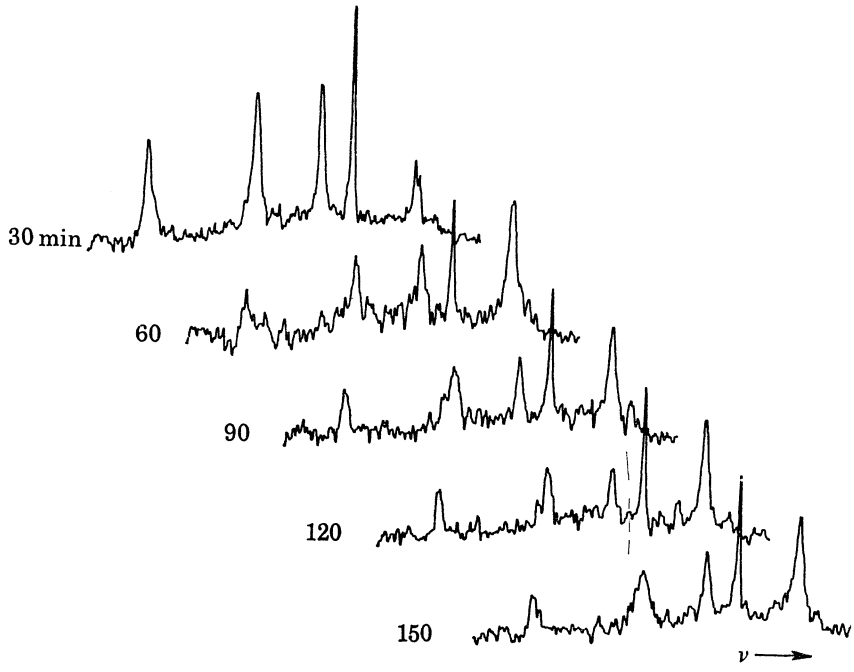

FIGURE 2. Time series of ^{31}P n.m.r. of a Langendorff-perfused rat heart paced at 600 beats/min. The aortic pressure was maintained at 70 cm H_2O. The 30 min spectrum is the baseline reference and was obtained when the heart was beating spontaneously at 300 beats/min. After this spectrum was collected, the heart was paced at 600 beats/min and the ^{31}P n.m.r. spectrum subsequently monitored. The times given are the mid-points of each accumulation and indicate perfusion time. Each spectrum represents the Fourier transform average of 480 sampling pulses and required 8 min for total collection. Sweep width, 4 kHz. The arrow indicates the direction of increasing frequency.

An in vivo *model*

Although the isolated perfused heart is a convenient and wisely used model, it has several disadvantages in terms of viability, stability, oxygen delivery, work load and so on. We have therefore developed a technique for studying the heart of a live animal by n.m.r. (Grove *et al.* 1980). The trachea of an anaesthetized rat is intubated via a tracheostomy and the animal is mechanically ventilated (70–80 breaths/min) with 50% nitrous oxide and 50% oxygen. The abdomen is opened and the receiver coil is introduced through the abdominal incision and across the diaphragm and is positioned around the ventricles. The animal is secured in the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

n.m.r. probe. The ^{31}P n.m.r. spectrum of an *in vivo* heart is compared, in figure 5, with that of a buffer-perfused one. The two spectra are remarkably similar, with comparable sensitivity and resolution. The *in vivo* spectrum remained essentially unchanged for 3–4 h (longer time periods have not been attempted). The assignment of the resonances is given in the legend to figure 5; the low level of 2,3-diphosphoglycerate indicates that blood within the heart contributes an insignificant amount to the ATP signal. (Fresh blood contains only a small amount of ATP and no phosphocreatine relative to 2,3-diphosphoglycerate.) In the spectrum from the

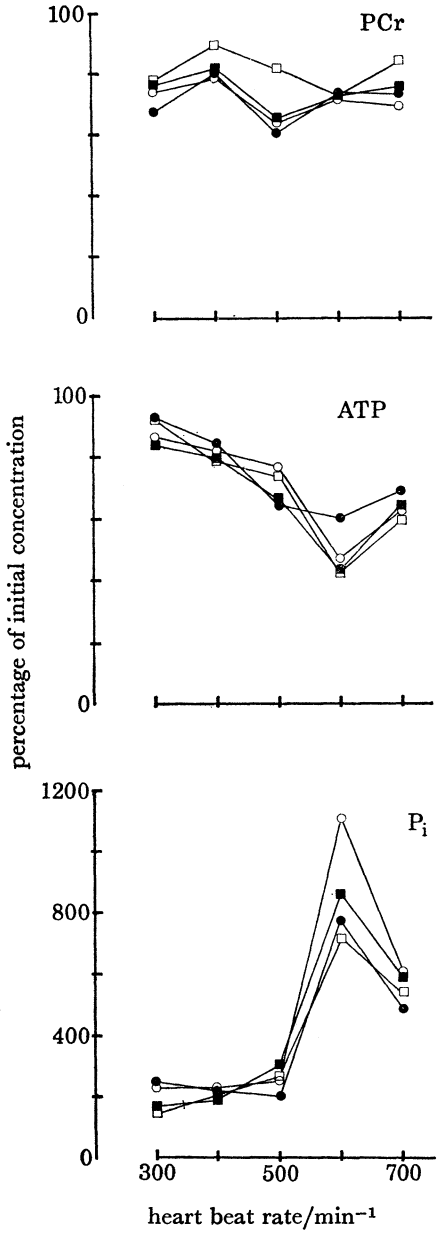

FIGURE 3. Relation between phosphate content and heart rate in the Lagendorff-perfused rat heart. Concentrations were assessed by measuring peak areas. The 100% concentration is that for the 30 min pre-pace spectrum of each heart; pacing was initiated immediately after this spectrum was collected. Each symbol represents the average of four hearts and refers to perfusion time: ●, 60 min; ○, 90 min; ■, 120 min; and □, 150 min.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

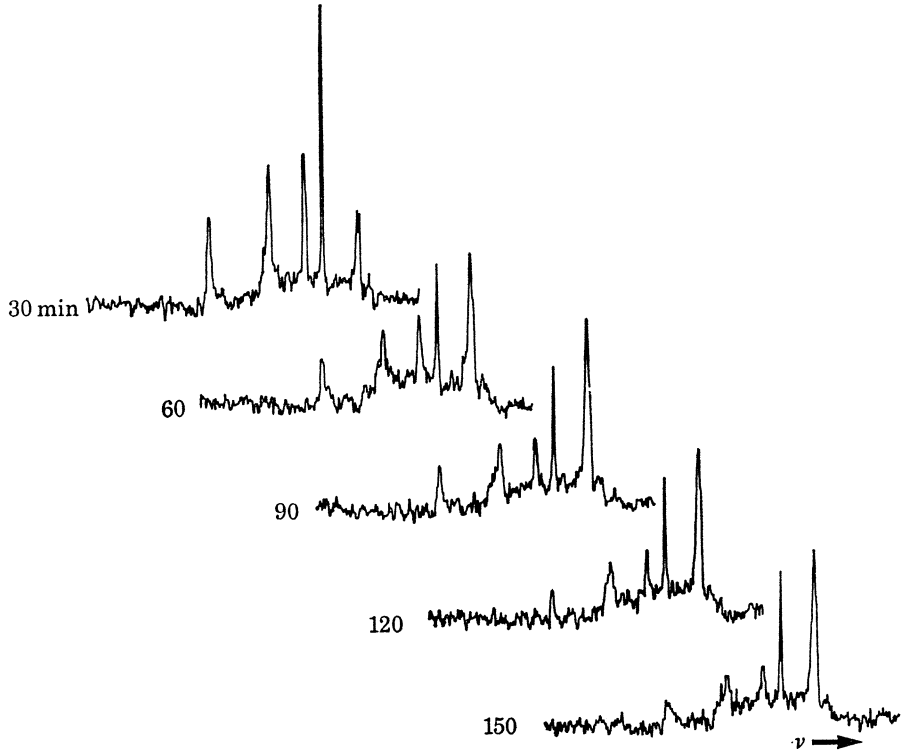

FIGURE 4. Time series of ^{31}P n.m.r. spectra of a Langendorff-perfused rat heart paced at 600 beats/min and subjected to an aortic pressure of 140 cm H_2O. The details of the procedure are the same as in the legend for figure 2, except that the spontaneous heart rate was 285 beats/min and the aortic pressure was increased from 70 to 140 cm H_2O with the initiation of pacing.

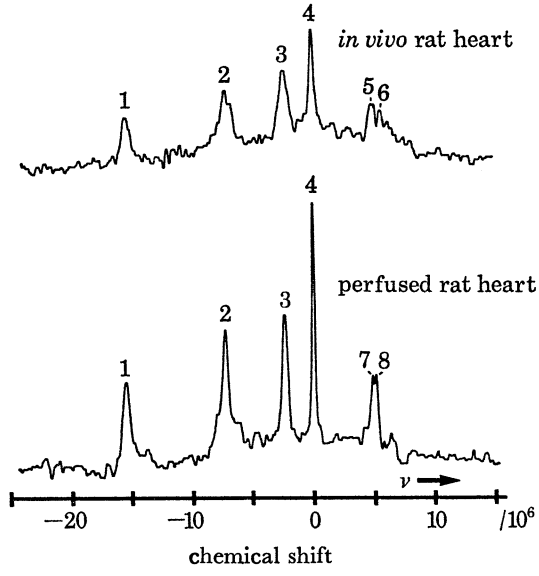

FIGURE 5. High-resolution ^{31}P n.m.r. spectra of *in vivo* and perfused rat hearts. Both spectra are of hearts from 300 g animals. Each spectrum represents the Fourier transform average of 480 sampling pulses and required 8 min for total collection. Sweep width, 3 kHz. Peak assignments: 1, β-ATP; 2, α-ATP + α-ADP + phosphodiesters; 3, γ-ATP + β-ADP; 4, phosphocreatine; 5, P_i + 2-phosphate of 2,3-diphosphoglycerate; 6, 3-phosphate of 2,3-diphosphoglycerate; 7, intracellular P_i; and 8, P_i from Krebs–Henseleit perfusion buffer.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

buffer-perfused heart, peaks 7 and 8 are due to intracellular and extracellular inorganic phosphates, corresponding in position to pH values of 7.11 ($N = 23$; s.e.m., ± 0.01) and 7.32 ($N = 23$; s.e.m., ± 0.01) respectively†. In the *in vivo* spectrum, peaks 5 and 6 are from 2,3-diphosphoglycerate, the increased area of peaks relative to peak 6 resulting from the presence of inorganic phosphate at a pH of 7.38 ($N = 9$; s.e.m., ± 0.05). It is possible that the principal source of the inorganic phosphate signal is blood; this would indicate a relatively small amount of cellular inorganic phosphate *in vivo*. The ATP : phosphocreatine ratio in the buffer-perfused rat heart is 30% higher than in the *in vivo* rat heart (table 1); we believe that this results from a decrease in the amount of phosphocreatine (Grove *et al.* 1980) in the buffer-perfused preparation.

TABLE 1. COMPARISON OF HIGH ENERGY PHOSPHATE COMPOUNDS IN *IN VITRO* AND *IN VIVO* RAT HEART

method	system	N	ATP : PCr (mean ± s.e.m.)	reference
freeze-extraction	perfusion	—	0.84 ± 0.06	taken from the literature
^{31}P n.m.r.	perfusion	20	0.89 ± 0.04	present authors
^{31}P n.m.r.	whole animal	10	0.62 ± 0.07	present authors

Freeze-extraction data are the average of ATP : phosphocreatine ratios from six references. The hearts were freeze-extracted after 15–30 min of perfusion. All of these hearts were perfused in the non-recirculating Langendorff mode, at 37 °C, and were perfused with Krebs–Henseleit; the pressure head varied between 54 and 100 cm of water and the glucose concentration between 5 and 11 mM. The ^{31}P n.m.r. data for the present perfusion system were collected after 30 min of perfusion. The ^{31}P n.m.r. data for the *in vivo* system were collected immediately after the rat was positioned within the magnet. This was approximately 30 min after the start of the operation.

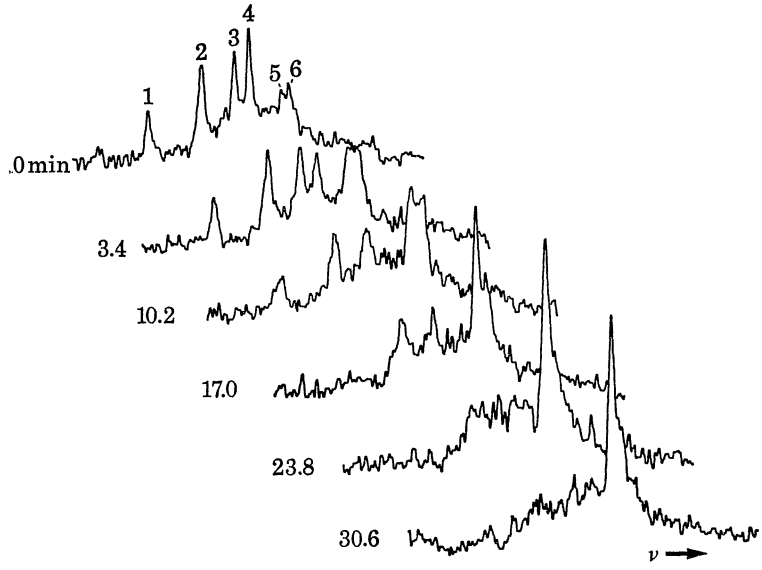

FIGURE 6. Series of ^{31}P n.m.r. spectra of *in vivo* rat heart recorded as a function of time during respiratory arrest. Each spectrum represents the Fourier transform average of 400 sampling pulses and required 6.8 min for total collection. The times given are the midpoints of each accumulation. Sweep width, 4 kHz. Symbols are defined in the legend for figure 5.

† The pH values are quoted to two decimal places to show the statistical variations. We do not claim to have measured the absolute pH value with this accuracy.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The possibility of measuring dynamic changes in the *in vivo* heart was examined by following the ^{31}P n.m.r. changes occurring after respiratory arrest. After the reference spectrum was collected, at 0 min, the ventilator was turned off and the ventilator tubes clamped. The animal then went through an undefined sequence of normoxia, hypoxia and, finally, anoxia. During this sequence the heart is gradually failing and finally arrests. The results are shown in figure 6. As phosphocreatine decreases, over the first 10 min, to an undetectable concentration, P_i concentration increases while ATP concentration remains constant. After phosphocreatine is metabolically consumed, ATP concentration decreases, until it is no longer detected at 17 min. It is instructive to compare this series of measurements with the time course of metabolic changes observed when an isolated buffer-perfused heart is subjected to global ischaemia (Garlick *et al.* 1979). In the latter case, the phosphocreatine disappears in only 4 min (at 37 °C). There are several reasons for this difference. In respiratory arrest, blood circulation and oxygen delivery do not stop immediately after the ventilator is turned off, while, in the isolated heart model, both flow and oxygen supply cease immediately after aortic clamping. In addition, the buffering capacity of blood is higher than that of the normal Krebs–Henseleit medium used in the isolated perfusion system. We have demonstrated that external buffering has a beneficial effect on ischaemic tissue (Garlick *et al.* 1979).

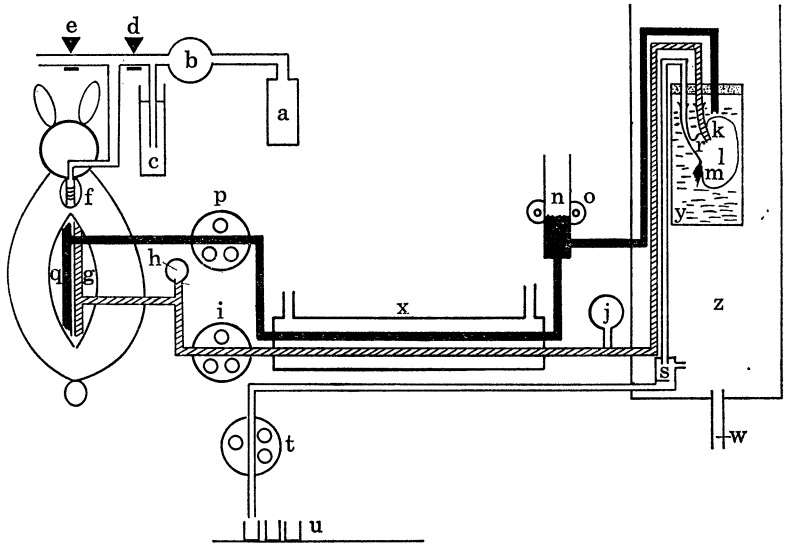

FIGURE 7. Kidney blood perfusion circúit; a, gas cylinder (50 % O_2, 50 % NO_2); b, halothane vaporizer; c, pressure limiting device; d, inspiratory valve; e, expiratory valve; f, tracheotomy; g, aorta; h, pressure gauge; i, arterial pump; j, pressure gauge; k, renal artery; l, kidney; m, renal vein; n, bubble trap/flow gauge; o, photoelectrode level sensor; p, venous pump; q, inferior vena cava; r, ureter; s, urine line, air-bleed; t, urine-pump; u, fraction collector; w, heating air inlet; x, water jacket; y, n.m.r. tube; and z, n.m.r. probe.

RENAL TRANSPLANTATION AND N.M.R.

The most common and successful organ transplantation is that of the kidney. Apart from the immunological problems, tissue preservation and prediction of viability are of both theoretical and practical interest. We have initiated, with the Renal Transplant Unit at Liverpool, a collaborative programme of research using animal models to assess the value of ^{31}P n.m.r. for the study of kidney metabolism in relation to transplantation (Sehr *et al.* 1977).

During the past two years, we (P. Bore, P. Sehr and J. Paptheophanis) have developed a model in which a 'donor' rabbit kidney is placed in a 28 mm n.m.r. tube inside the spectrometer. This kidney derives its renal arterial blood via tubing connected to the aorta of an anaesthetized and ventillated 'assist' (or recipient) rabbit. The venous effluent from the donor kidney drains into the n.m.r. tube and is returned to the assist animal via a cannula in the inferior vena cava. A third cannula is tied into the ureter of the kidney, to collect any urine produced. The circuit, which includes two pumps, a bubble trap, thermostatic control etc., is shown in figure 7 (see also Sehr 1978).

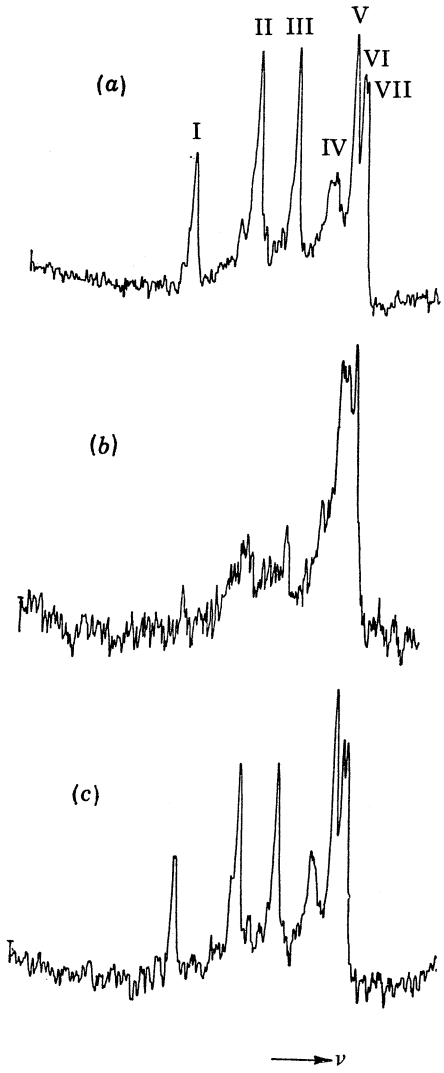

FIGURE 8. High-resolution ^{31}P n.m.r. spectra of a blood-perfused rabbit kidney maintained at 37 °C. The kidney was excised from the 'donor animal' and connected to the blood perfusion circuit after 12 min of warm ischaemia. After 1 h of blood perfusion, spectrum *a* was collected. The kidney was then subjected to 6 min of warm ischaemia after which spectrum *b* was collected. The kidney was subsequently reperfused for 12 min and spectrum *c* was collected. Spectra *a* and *c* are the transform of 200 and spectrum *b* of 50 free induction decays. The spectra were collected at 2 s intervals with 90° sampling pulses and a sweep width of 5 kHz. Peak assignments are: I, β-ATP; II, α-ATP, α-ADP; III, β-ADP, γ-ATP; IV, unidentified compound; V, inorganic phosphate, 2-phosphate of 2,3-diphosphoglycerate; VI, 3-phosphoglycerate; VI, 3-phosphate of 2,3-diphosphoglycerate; and VII, nucleotide monophosphate, sugar phosphates.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Some typical ^{31}P n.m.r. spectra of rabbit kidney are given in figure 8. After 1 h of blood perfusion, the spectrum shown in figure 8*a* is obtained. The unknown signal in the phosphodiester region (peak IV) appears at the same frequency, but is of lower intensity, than for rat kidneys (Sehr *et al.* 1977) and corresponds in position to glycerophosphorylcholine, which has been chemically identified in the spectra of certain muscles (Burt *et al.* 1976; Seeley *et al.* 1976).

Figure 8*b* shows the ^{31}P n.m.r. spectrum after 6 min of warm (37 °C) ischaemia. ATP and ADP have almost entirely disappeared and the inorganic phosphate resonance and signals in the AMP region have correspondingly increased. By comparison of the spectrum of venous blood with that of non-perfused ischaemic kidneys, we can conclude that the remaining small ATP signal in figure 8*b* is a result of the contribution from ATP in blood erythrocytes. We also note that, as for rat kidneys (Sehr *et al.* 1977), the inorganic phosphate signal shifts to lower frequencies after the onset of ischaemia, indicating acidification of the cytoplasm. As a result of this shift, the signal from 2-diphosphoglycerate (in erythrocytes) now appears between those for inorganic phosphate and the AMP region, and the 3-diphosphoglycerate resonance is contained within the AMP region.

After *ca.* 8 min of ischaemia the kidney is reperfused and, after 12 min, the metabolite concentrations very nearly recover to their preischaemic levels (figure 8*c*). At the same time, kidney function is also restored as judged by measurements of urine production, glomerular filtration rate, Na^+ and H_2O reabsorption (Sehr 1978).

Using this model, we have been able to correlate functional and metabolic recovery after varying periods of ischaemia. The intracellular pH drop in ischaemic kidney is much slower than that in the heart and only reaches its limiting value in about 50 min, which also seems to correspond to the 'cut-off' time after which a rabbit kidney will become non-viable. These results will be discussed in detail elsewhere (see also Sehr 1978), but it is clear that n.m.r. provides a non-destructive 'metabolic handle' with which to investigate the biochemical basis of renal viability.

The work described here relies on contributions from many individuals during the past five years. In particular, we wish to acknowledge the work by Dr P. J. Seeley and Miss Pamela Garlick in development of perfused heart n.m.r., the participation of Dr P. Sehr and Dr J. Papatheophanis in the kidney project, and the invaluable contributions of P. Styles and R. Porteus to the n.m.r. developments. Financial support was provided by the British Heart Foundation, the Science Research Council and N.I.H. G.K.R. is a member of the Oxford Enzyme Group. J.J.H.A is a N.I.H. Postdoctoral Fellow, T.H.G. a Nato Fellow in Science, and P.J.B. is a Merseyside Association for Kidney Research Fellow in Transplantation.

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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