Formation of n.m.r.-invisible ADP during renal ischaemia in rats

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1. Measurement of the adenine nucleotide and inorganic phosphate content of normoxic and ischaemic kidney *in vivo* has been made, comparing enzymic assay (after freeze-clamping and acid extraction) with quantification by ^{31}P -n.m.r. 2. Both methods give similar results for ATP, and n.m.r. quantification of P_i gives a value 25–50% of that obtained by enzymic assay. 3. ADP, which is largely invisible to n.m.r. in the normoxic kidney, remains invisible during ischaemia despite a 2–3-fold rise in enzymically assayed ADP. 4. N.m.r. and enzymic assay of the acid extracts give similar values for all metabolites measured. 5. The question of ADP binding in the kidney is discussed, as are the implications for the metabolic regulation of ADP-dependent reactions.

The concentrations and ratios of phosphorus metabolites that take part in oxidative phosphorylation are obviously a key to the control of cellular energy metabolism (for review see Ereńska & Wilson, 1982). They are also important in the regulation of glycolysis and many other cellular processes. For many years the way in which such measurements have been made has been by HClO_4 extraction and measurement of the phosphorus compounds by chemical or enzymic assay. Since ^{31}P -n.m.r. has become available, it is now possible to identify these metabolites in intact tissues and *in vivo* by this method (Hoult *et al.*, 1974; Gadian, 1982).

In principle, the ^{31}P -n.m.r. spectrum is a simple spectroscopic record, with amplitude (or area) relative to 'metabolic quantity'. In practice, however, quantification of the adenine nucleotides and phosphate by n.m.r. is difficult. There are several reasons for this. N.m.r. is relatively insensitive and many of the phosphorus peaks overlap. ATP has three phosphorus signals, but only one is unique to it (that of its β -phosphate); the frequencies of the α - and β -phosphates of ADP are so close to those of

the α - and γ -phosphate of ATP respectively as to be indistinguishable in n.m.r. experiments *in vivo*. In addition the resonance frequency of the α -phosphate of ATP is shared with that of the nicotinamide nucleotides. It is common practice therefore for ADP and nicotinamide nucleotides to be quantified by subtraction of one peak area from another; e.g. $\text{ADP} = \gamma - \beta$, $\text{NAD}^+ = \alpha - \gamma$. Quantification of P_i is more straightforward in that it has a peak of unique frequency significantly different from those of the adenine nucleotides.

For ATP, the values measured by chemical extraction from the cell or by ^{31}P -n.m.r. in intact cells are similar in all tissues so far examined, i.e. muscle (Dawson *et al.*, 1977), kidney (Freeman *et al.*, 1983, and the present paper) and liver (Iles *et al.*, 1982; Cohen, 1983). In the case of P_i , n.m.r. measurements give a value 25–100% of that found by chemical extraction and assay depending on the tissue in which it is measured (Dawson *et al.*, 1977; Freeman *et al.*, 1983; Meyer *et al.*, 1982).

The biggest discrepancy, however, is in the measurement of ADP. Chemical extraction and assay of ADP in many tissues gives a value one or two orders of magnitude higher than that apparently found by n.m.r. (Gadian, 1982). The suggested origin of this discrepancy and the main difference between the chemical extraction and assay and the ^{31}P -n.m.r. measurement is that the former measures the total (bound plus unbound, mobile plus immobile), whereas the latter measures only 'free' phosphorus compounds.

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On this basis, in muscle there may be a straightforward explanation for the low n.m.r. value of 'free' ADP; a large proportion of the ADP is tightly bound to actin in skeletal muscle (Sachs *et al.*, 1967). This explanation is supported by independent evidence. Because creatine phosphokinase is near to equilibrium in resting muscle, it is possible to calculate the 'free' ADP (Veech *et al.*, 1979). The value obtained in this way is remarkably similar to that suggested by direct n.m.r. measurement (i.e. in the micromolar range; Gadian, 1982).

However, liver and kidney do not have appreciable amounts of creatine phosphokinase, so that the equilibrium constant cannot be used as a check on the ADP measurement by n.m.r. In addition, neither of these tissues contain significant amounts of actin, so that if ADP is 'bound' it must be to other cellular components. The state of n.m.r.-invisible ADP in tissues other than muscle is therefore unclear.

However, some observations by Ross *et al.* (1978) and Chan (1983) offer a means of studying and identifying the pool of invisible ADP. These workers have shown that up to 6 μmol of ADP/g dry wt. (measured by chemical extraction and assay) can be formed during ischaemia in rat kidney. This is two or three times more ADP than is observed in a normoxic kidney, and seemed a good starting point from which to investigate the quantification and localization of ADP and other phosphorus metabolites in the kidney. In the present study the surprising result was obtained that even the relatively large amounts of ADP formed during renal ischaemia remain invisible to n.m.r. An explanation based upon compartmentation at a cellular level is discussed, as are the implications for metabolic regulation in the kidney of a micromolar cellular concentration of ADP.

Experimental

Animals

Male Wistar rats of 300–350 g body wt. maintained on Purina laboratory diet and with free access to water were used. The kidneys were made ischaemic by ligating the renal artery immediately prior to insertion in the magnet for n.m.r. measurements. At the end of the ischaemic period (30 min) the kidney was freeze clamped, extracted with HClO_4 and neutralized for metabolite measurements.

Metabolite measurements

ATP, ADP and AMP were measured by methods described in Bergmeyer (1974). P_i was measured by the method of Lowry & Lopez (1946) as modified by Chandra Rajan & Klein (1976).

N.m.r. measurements

The method for collection of spectra *in vivo* is described in Chan *et al.* (1982). A 4.2 T superconducting magnet with a 10 cm bore was used. In order to obtain quantitative spectra it is necessary to have delay times of at least four times the spin lattice relaxation (T_1) times. The adenine nucleotides and phosphate have T_1 times of the order of 1 s (Freeman *et al.*, 1983). Spectra from ischaemic kidneys were collected with 4 s delay times for 152 scans starting 20 min after the onset of ischaemia until immediately prior to freeze clamping (about 30 min). The control spectra were collected with 12 s delay for 200 scans to ensure optimum signal intensities for all metabolites.

Handling of n.m.r. data

Spectra were stored on magnetic discs and were quantified by comparison of the methylene diphosphonate peak with those corresponding to the recognized phosphate resonances. ^{31}P spectra of kidneys show a broad asymmetrical peak (or hump) probably due to phospholipids, which underlies the signals. Areas obtained by cutting and weighing expanded traces of the relevant peak areas were used for this comparison, assuming that the peaks originate from the top of the hump. ATP was determined from the β -phosphate peak that is unique to ATP, and ADP from its β -phosphate peak (in the absence of ATP) and from ATP γ -phosphate minus ATP β -phosphate when ATP was present. Measurements were also made of the ADP α -phosphate/NAD peak and the P_i peak.

Results

In control kidneys the three peaks of ATP are clearly seen (Fig. 1, solid line) and represent about 6 $\mu\text{mol/g}$ dry wt. (see Fig. 2a). In a series of nine experiments the ADP value obtained by peak integral measurements (see the Experimental section) of the ATP γ - and β -phosphates and subtraction of the β - from the γ - gave the following result: $0.07 \pm 0.14 \mu\text{mol/g}$ dry wt. The s.d. value shows the potential error in such measurements due to the poor signal-to-noise ratio, but also makes it clear that the n.m.r.-measured ADP is much lower than the chemical extraction and assay value for kidney ADP, 2.5 ± 0.4 ($n = 4$) $\mu\text{mol/g}$ dry wt. (mean $\pm$ s.d.).

In renal ischaemia, when most of the renal ATP disappears and appears as ADP (see Fig. 2b), the corresponding n.m.r. spectra (see Fig. 1, broken line) show no peaks at either the β - or the γ -positions, and only a small peak at the α -position which is due to nicotinamide nucleotides. The quantification of the n.m.r. scans in Fig. 1 is detailed in Table 1, where direct comparisons are

Fig. 1. ³¹P n.m.r. spectra of control (—) and ischaemic (---) rat kidney *in vivo*
Abbreviations used: MDP, methylene diphosphonate; GPC, glycerophosphocholine.

Table 1. Comparison of n.m.r. and chemical extract measurements of adenine nucleotides and phosphate in control and ischaemic kidney

Results are expressed as $\mu\text{mol/g}$ dry wt. and are the summed data from three kidneys. N.m.r. spectra from which the n.m.r. data are calculated can be seen in Fig. 1.

Metabolite	Amount ($\mu\text{mol/g}$ dry wt.) found by:			
	Quantification of n.m.r. spectra		Chemical extraction and assay	
	Control	Ischaemic	Control	Ischaemic
ATP	6.19	<1.0	5.8	0.43
ADP	<1.0	<1.0	2.5	5.72
AMP	—	—	0.4	1.96
P _i	2.09	26.5	9.0	52.8
ADP α -phosphate/NAD	9.1	8.6	—	—

made with chemical extraction and assay of the same kidneys. The ATP measurements made by n.m.r. and by chemical extraction and assay are very similar (6.19 and $5.8 \mu\text{mol/g}$ dry wt.), whereas the n.m.r. assayed ADP value is unmeasurable compared with $5.72 \mu\text{mol/g}$ dry wt. found by chemical extraction. The ADP α -phosphate/NAD peak is about the same in ischaemia as in the control. It is not possible to measure AMP by n.m.r. *in vivo* since this resonance comprises AMP, sugar

phosphate and an additional, as yet unidentified, component (Freeman *et al.*, 1983).
The P_i measured by n.m.r. in the kidney is between 23 and 50% of the value observed by chemical extraction. A large amount of phosphate [50 ± 7.3 ($n = 8$) $\mu\text{mol/g}$ dry wt.] is generated in renal ischaemia. It is not clear where all of it comes from; degradation of ATP accounts for about 20% of the P_i in chemical extracts; this value is about 75% when quantified by n.m.r. Much of the P_i

Fig. 2. Measurements of ATP and ADP in control and ischaemic kidneys

(a) Quantitative n.m.r. measurements. The peak area measurements on the ischaemic kidneys were made from spectra collected between 20 and 30 min after the onset of ischaemia. (b) Chemical extracts of the same kidneys measured by freeze clamping and extraction after 30 min ischaemia.

formed must be 'invisible' to n.m.r., while much of the ATP is hydrolysed only to 'invisible' ADP.

Comparison of n.m.r. assay and enzymic assay of chemical extracts of ischaemic kidney

When chemical extracts of the ischaemic kidneys were assayed by n.m.r., similar results to those obtained by enzymic assay were observed (see Fig. 3 and Table 2). The results confirm that in aqueous extracts similar values are obtained by both methods, and in particular that ADP becomes visible to n.m.r. during the process of HClO_4 extraction.

Discussion

Quantification of ATP and P_i by n.m.r. in intact tissues such as the kidney is accurate to $\pm 7\%$ of peak areas (Freeman *et al.*, 1983) because the concentrations of ATP and P_i are relatively high. However, the quantification of ADP in an aerobic kidney is virtually impossible, especially since the calculation of ADP demands subtraction of one

Fig. 3. N.m.r. spectrum of a neutralized HClO_4 extract of ischaemic kidneys

Abbreviations used: MDP, methylene diphosphonate; GPC, glycerophosphocholine.

Table 2. Comparison of n.m.r. and enzymic assay of chemical extract of ischaemic rat kidney

Neutralized HClO_4 extracts of kidneys freeze-clamped after 30 min ischaemia were either assayed enzymically or assayed by n.m.r. in a Helmholtz coil, after which the relevant portions of the expanded spectra were cut out and weighed. The results are expressed as $\mu\text{mol/g}$ dry wt. and are the mean of two experiments, but are representative of several others.

Metabolite	Amount ($\mu\text{mol/g}$ dry wt.) found by	
	N.m.r. assay	Enzymic assay
ATP	<1.0	0.90
ADP	5.36	7.21
AMP	—	10.6
P_i	52.5	47.9
ADP α -phosphate/NAD	14.0	—

large peak from another large peak. Nevertheless, in the anaerobic kidney, when [ADP] is similar in value to that of [ATP] in the aerobic kidney, no corresponding phosphate peaks are seen in n.m.r.

spectra. This implies that the ADP formed during ischaemia is invisible to n.m.r. Extraction of the kidney into aqueous HClO_4 immediately renders ADP visible and similar in quantity to that known to be present from chemical assay. Reasons for this could be one of the following: (1) the ADP may be bound to some protein or paramagnetic substance which renders it invisible to n.m.r., (2) it could be that rapid exchange of ADP across a membrane separating two compartments (e.g. between cytoplasm and mitochondria) of different magnetic environment would so broaden the signal as to render it invisible in the spectrum, or (3) that the ADP is sequestered in a compartment that is invisible to n.m.r. (e.g. mitochondria; see Veech *et al.*, 1979).

Because of the heterogenous nature of the kidney there are many compartments to consider. Burch *et al.* (1980) have shown that ATP+ADP+AMP is about 25% lower in the proximal straight part of the nephron compared with the distal straight and convoluted segments. However, using an all-enveloping solenoid coil both n.m.r. and chemical assay see an average over the whole tissue; anatomical considerations preclude any rapid exchange of nucleotides between nephron segments so that this sort of compartmentation cannot be relevant to the problem.

More important is the intracellular compartmentation, especially since there is some evidence that intramitochondrial ATP and ADP are invisible to n.m.r. (Wong, 1980). In liver the ADP is distributed 1:1 between mitochondria and cytoplasm (Siess & Wieland, 1975), which makes the concentration gradient about 6:1 (Siess & Wieland, 1976). In kidney the distribution has not been accurately assessed (W. G. Guder, personal communication). However, it seems likely that it will be similar to that in liver. Assuming a similar distribution in ischaemia as in the aerobic kidney, this means that of the extra ADP generated in ischaemia about $3 \mu\text{mol/g}$ dry wt. would be in the cytoplasm, i.e. about $0.7 \mu\text{mol/g}$ wet wt., which should be visible to n.m.r. This amount of ATP would certainly be visible (it would be about one-half of that in the aerobic spectrum seen in Fig. 1).

Adler *et al.* (1984) suggest that in renal tubules incubated at 37°C , only cytosolic (i.e. not mitochondrial) P_i is visible. However, other workers (Ogawa *et al.*, 1978) suggest that in liver cell experiments at 0°C intramitochondrial P_i and adenine nucleotides are visible to n.m.r. This is controversial and we can offer no explanation. It is indeed surprising if the location of ADP within mitochondria is the cause of its n.m.r. invisibility, since under the anoxic conditions selected for the present study it might be expected that ADP would leak to the cytosolic compartment.

The concentration of 'free' ADP is a key to the

control of the regulation of metabolism in the cell. In this context 'free' implies both Mg^{2+} -chelated and unchelated ADP. The K_m for the adenine nucleotide translocator is between 1 and $12 \mu\text{M}$ (Duee & Vignais, 1969) and it has been a controversial issue for many years as to why so obvious a point for control should have such a low K_m when the prevailing substrate concentration was apparently one or two orders of magnitude higher (Beis & Newsholme, 1976; Veech *et al.*, 1979; Akerboom *et al.*, 1978; Stubbs *et al.*, 1978; Stubbs, 1979), since this was measured by the then only available technique of chemical extraction and assay. However the n.m.r.-assessed value of ADP is still several times higher than the K_m , and there is still controversy (Erecinska & Wilson, 1982; Groen *et al.*, 1982) about regulation of the adenine nucleotide translocator.

The implications of ADP in the micromolar range extend to several other reactions. The adenylate kinase reaction is assumed to be in equilibrium in the cell, and this would imply that AMP would also be in the micromolar range. AMP is believed to be a powerful regulator of phosphorylase and phosphofructokinase (Newsholme & Start, 1973), and many theories of metabolic control would need to be reassessed since they are all based on ADP and AMP measurements made by chemical extraction and assay (i.e. in the millimolar range). From these experiments in the kidney we conclude, as have others, that ADP must be 'free' to be seen in an n.m.r. spectrum. It is apparent that, in kidney at least, 'free' [ADP] is finely controlled, since even a 2–3-fold rise in intracellular [ADP] results in no change in n.m.r.-visible ADP.

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References

- Adler, S., Shoubridge, E. A. & Radda, G. K. (1984) *Am. J. Physiol.* in the press
- Akerboom, Th. P. M., Bookelman, H., Zuurendonk, P. F., van der Meer, R. & Tager, J. M. (1978) *Eur. J. Biochem.* **84**, 413–420
- Beis, I. & Newsholme, E. A. (1976) *J. Mol. Cell. Cardiol.* **8**, 863–876
- Bergmeyer, H. U. (ed.) (1974) *Methods of Enzymatic Analysis*, 2nd ed., Verlag Chemie, Weinheim
- Burch, H. B., Choi, S., Dence, C. N., Alvey, T. R., Cole, B. R. & Lowry, O. H. (1980) *J. Biol. Chem.* **255**, 8239–8244
- Chan, L. (1983) D.Phil. Thesis, University of Oxford
- Chan, L., Ledingham, J. G. G., Dickson, A. A., Thulborn, K. R., Waterton, J. C., Radda, G. K. & Ross, B. D. (1982) in *Acute Renal Failure* (Eliahou, R. E., ed.), pp. 35–41, John Libbey, London

- Chandra Rajan, J. & Klein, R. le R. (1976) *Anal. Biochem.* **72**, 407–412
- Cohen, S. M. (1983) *J. Biol. Chem.* **258**, 14294–14308
- Dawson, M. J., Gadian, D. G. & Wilkie, D. R. (1977) *J. Physiol. (London)* **267**, 703–735
- Duee, E. D. & Vignais, P. V. (1969) *J. Biol. Chem.* **244**, 3920–3931
- Erecinska, M. & Wilson, D. F. (1982) *J. Membr. Biol.* **70**, 1–14
- Freeman, D., Bartlett, S., Radda, G. & Ross, B. (1983) *Biochim. Biophys. Acta* **762**, 325–336
- Gadian, D. G. (1982) *Nuclear Magnetic Resonance and its Applications to Living Systems*, Clarendon Press, Oxford
- Groen, A. K., Wanders, R. J. A., Westerhoff, H. V., van der Meer, R. & Tager, J. M. (1982) *J. Biol. Chem.* **257**, 2754–2757
- Hoult, D. I., Busby, S. J. W., Gadian, D. G., Radda, G. K., Richards, R. E. & Seeley, P. J. (1974) *Nature (London)* **252**, 285–287
- Iles, R. A., Stephens, A. N. & Griffiths, J. R. (1982) *Prog. Nucl. Magn. Reson. Spectrosc.* **15**, 49–200
- Lowry, O. H. & Lopez, J. A. (1946) *J. Biol. Chem.* **162**, 421–428
- Meyer, R. A., Kushmerick, M. J. & Brown, T. R. (1982) *Am. J. Physiol.* **242**, C1–C11
- Newsholme, E. A. & Start, C. (1973) *Regulation in Metabolism*, John Wiley, London
- Ogawa, S., Rottenberg, H., Brown, T. R., Shulman, R. G., Castillo, C. L. & Glynn, P. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1796–1800
- Ross, B. D., Bishop, M. & Marshall, V. (1978) in *Organ Preservation II* (Pegg, D. E. & Jacobsen, A., eds.), pp. 220–239, Churchill-Livingstone, London
- Sachs, J., Duckett, W. D., Morgan, S. & Schaefer, M. (1967) *Arch. Biochem. Biophys.* **122**, 591–593
- Siess, E. A. & Wieland, O. H. (1975) *FEBS Lett.* **52**, 226–230
- Siess, E. A. & Wieland, O. H. (1976) *Biochem. J.* **156**, 91–102
- Stubbs, M. (1979) *Pharmacol. Ther.* **7**, 329–349
- Stubbs, M., Vignais, P. V. & Krebs, H. A. (1978) *Biochem. J.* **172**, 333–342
- Veech, R. L., Lawson, J. W. R., Cornell, N. W. & Krebs, H. A. (1979) *J. Biol. Chem.* **254**, 6538–6547
- Wong, G. (1980) D.Phil. Thesis, University of Oxford