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Phosphorylation status of liver by ^{31}P-n.m.r. spectroscopy, and its implications for metabolic control

A comparison of ^{31}P-n.m.r. spectroscopy (*in vivo* and *in vitro*) with chemical and enzymic determinations of ATP, ADP and P_i

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1. An investigation into the measurement of P_i and ADP in rat liver *in vivo* and in freeze-clamped extracts by ^{31}P-n.m.r. spectroscopy was carried out. 2. The concentration of P_i estimated *in vivo* is less than 25% [1 mM (μmol/ml of cell water)] of the value obtained from freeze-clamped liver (4mM), whereas ADP *in vivo* is undetectable (1.4mM *in vitro*). 3. At 5min after infusion of 750mg of fructose/kg, the P_i content of liver extracts fell to 1.3mM, whereas P_i is undetectable *in vivo* under these conditions [Griffiths, Stevens, Gadian, Iles & Porteous (1980) *Biochem. Soc. Trans.* **8**, 641]. 4. The results indicate that the lower P_i and ADP concentrations found *in vivo* may be due to compartmentation or binding rather than to degradation of labile organic phosphates during extraction. 5. The results are discussed with reference to previous measurements of liver phosphates and investigations of compartmentation in the liver, as are some of the possible consequences for metabolic control in the liver of low ADP and P_i concentrations.

The phosphorylation potential (ΔG_{ATP}), i.e. the energy required for the synthesis of ATP from ADP and P_i under the prevailing conditions, is an important quantity that has frequently been estimated both *in vitro* and *in vivo* (Wilson *et al.*, 1974; Slater, 1976; Veech *et al.*, 1979; Wilson *et al.*, 1981). It is calculated from the relationship:

$$\Delta G_{ATP} = \Delta G_0 + \boldsymbol{RT} \cdot \ln\left(\frac{[ATP]}{[ADP][P_i]}\right) \quad (1)$$

where ΔG_0 is the standard free energy of ATP synthesis.

If ΔG_{ATP} is to be estimated correctly, the concentration terms in eqn. (1) should refer to chemical activities of ATP, ADP and P_i in a single cell compartment, not to the total amounts in all cell compartments. In addition, they should refer only to the free concentrations (assuming these to represent activities) of the appropriate ionized species in cell water and not to protein-bound nucleotides or P_i. Knowledge of the value of ΔG_{ATP} *in vivo* is extremely important, since it enables the efficiency of oxidative phosphorylation to be estimated (Wilson *et al.*, 1974, 1981). The free intracellular concentrations of ATP, ADP and P_i are equally important in their own right, since these metabolites are substrates and effectors for a large number of enzymes. Indeed, considerable controversy has arisen over whether control of oxidative phosphorylation (and hence respiration) is exerted by the cytosolic [ATP]/[ADP][P_i] ratio (Wilson *et al.*, 1974) or by the rate of ADP/ATP exchange across the mitochondrial inner membrane (Heldt & Klingenberg, 1968; Klingenberg & Heldt, 1982; Sestoft & Bartels, 1981) with only a passive role for P_i. Both ATP and ADP can be measured after freeze-clamping and acid extraction of the tissues, by well-established enzymic assays coupled to NAD^+/NADH (Jaworek *et al.*, 1974) or by h.p.l.c. It is generally thought that the ATP concentration measured in this way approximates to the concentration present in cell water. ADP concentrations, on the other hand, are quite high in such assays when compared with 'free' ADP concentrations in the cell water based on the

equilibrium position of kinase-catalysed reactions (Veech *et al.*, 1979), sometimes with the use of data from n.m.r. studies (Dawson *et al.*, 1978; Gadian, 1982; Iles *et al.*, 1982; Cohen, 1983), which give values at least an order of magnitude lower. Clearly, if these lower concentrations represent the 'free' values, they should be used when the phosphorylation potential is calculated from the above equation.

A qualitatively similar difference is found in measurements of P_i. P_i has usually been estimated by chemical analysis, in particular by the acid/molybdate procedure of Fiske & SubbaRow (1925). More recently, however, it has been possible to determine phosphate-containing compounds in intact tissue *in vivo* in a non-destructive manner by using ^{31}P n.m.r. (Ackerman *et al.*, 1980). N.m.r. determinations of intracellular ADP and P_i in a variety of tissues *in vivo* give much lower values than those obtained by enzymic or chemical analyses, ADP often being undetectable (Ackerman *et al.*, 1980; Iles & Griffiths, 1982; Iles *et al.*, 1982; Freeman *et al.*, 1983).

In this paper we present our own findings on the rat liver with the use of ^{31}P n.m.r., and discuss reasons for the apparent discrepancies between these results and those obtained with enzymic or chemical methods and the possible consequences for current concepts of control of energy metabolism. Preliminary reports of this work have been presented (Iles *et al.*, 1982, 1983, 1984).

In a ^{31}P-n.m.r. study on the kidney, Stubbs *et al.* (1984) have reached broadly similar conclusions from their findings of 'n.m.r.-invisible' ADP in the rat kidney. However, in contrast with their findings of a persistent lack of 'n.m.r.-visible' ADP in ischaemia, we have detected up to 1 μmol of ADP/g wet wt. in ischaemic perfused livers and in livers *in vivo* by ^{31}P n.m.r. (Griffiths & Iles, 1980; Iles *et al.*, 1982, 1985).

Materials and methods

Animals

Male Wistar rats (200–250g) in either the fed or the 48h-starved state were used throughout.

Materials

ATP and phosphocreatine were purchased from Sigma Chemical Co., Poole, Dorset, U.K.

Tissue preparation

Rats were anaesthetized with an intraperitoneal injection of sodium pentobarbitone (50mg/l). An abdominal incision was made just below the diaphragm. Livers were freeze-clamped with aluminium clamps pre-cooled in liquid N_2. In some experiments fructose (750mg/kg body wt.) was infused via the jugular vein as a 30% (w/v) solution over a period of 1min. Livers were freeze-clamped 5min after the start of infusion. For ATP-recovery studies, some samples were then broken into two approximately equal portions (2–3g) and 0.1ml of 100mM-ATP was added to one portion. Each portion was then treated identically. The frozen tissue was ground under liquid N_2 and homogenized with 2–3vol. of 10% (w/v) $HClO_4$ (pre-cooled to 0°C). The extracts were then stored in solid CO_2 (−80°C). The samples were thawed to 4°C and neutralized with 20% (w/v) KOH up to 1h before n.m.r. spectroscopy and centrifuged.

Studies on livers in vivo

Rats were anaesthetized and mounted in a Perspex [poly(methyl methacrylate)] cradle attached to the top of an n.m.r. probe (Gadian, 1982, Fig. 8.10). An abdominal incision was made to expose the liver, which was then covered in plastic film and a two-turn surface coil (15mm diameter) was placed on its surface (Griffiths *et al.*, 1980). The probe was then inserted into the magnet.

N.m.r. spectroscopy

^{31}P-n.m.r. spectra were obtained at 81MHz with a Brucker WM 200 wide-bore spectrometer. Spectra from the liver extracts were acquired from the neutralized supernatants in a standard 15mm-diameter n.m.r. tube at 4°C. To each sample was added 0.1ml of 100mM-phosphocreatine to act both as a chemical-shift reference and also as a reference for absolute quantification. No change in the relative peak areas of phosphocreatine or P_i present in the extracts occurred during the experiments, showing that no significant phosphocreatine hydrolysis took place. The homogeneity of the B_0 field was then optimized by adjusting the shim coils while observing the free-induction decay of the water protons. For liver *in vivo* shimming was carried out similarly, and the chemical shift of the water peak was also used as a reference for the ^{31}P-n.m.r. studies (Ackerman *et al.*, 1981). ^{31}P-n.m.r. spectra were accumulated in blocks of 200–240 scans by using a 10 μs (30°) pulse repeated every 4s (experiments *in vivo*) or 8s (*in vitro*). The spectral width was $\pm$2500Hz.

Quantification of resonances in the liver *in vivo* is hampered by the presence of underlying broad signals, which are thought to arise from the phospholipids and high-M_r phosphate compounds subject to chemical-shift anisotropy. In a separate group of experiments we have been able to minimize this problem by selectively saturating the phospholipid resonance. This was achieved by irradiating with 1.7W of radiofrequency power for 1–2s before acquisition. The irradiation frequency

was 1 p.p.m. downfield from the phosphocreatine resonance position (0 p.p.m.), though this is not critical: the only requirement is that the irradiation frequency falls somewhere within the broad phospholipid resonance.

Calculations

The concentrations of phosphorus-containing metabolites in the liver extracts were calculated by comparison of their peak areas with the peak area of the added phosphocreatine standard and from the known cocentration of the latter. The three 'ATP' peaks also contain resonances from other triphosphates, e.g. CTP, GTP, UTP and ITP, which are normally indistinguishable from each other by ^{31}P n.m.r. The GTP concentration in this strain of rats measured by h.p.l.c. was approx. 10% of the ATP concentration, which agrees with previous enzymic measurements (Gruber *et al.*, 1974). The sum of hepatic ITP, UTP and CTP is negligible, compared with the ATP concentration. We have therefore corrected our n.m.r.-measured ATP concentrations by 10%.

Direct absolute quantification of the metabolites from n.m.r. experiments *in vivo* was not possible, as the exact volume of the liver from which n.m.r. signals are obtained cannot be determined with the use of surface coils (Gadian, 1982). Alternatively, results can be given in terms of percentages of the β-ATP peak area (which is proportional to the ATP concentration) provided that the delay between pulses is sufficient to avoid partial saturation of resonances. However, from previous n.m.r. work with the isolated perfused liver, where it was possible to contain the whole liver within a more conventional solenoidal radiofrequency coil, we were able to define, more accurately, the volume from which the n.m.r. signals originated (Iles *et al.*, 1980; R. A. Iles, D. G. Gadian, A. N. Stevens & J. R. Griffiths, unpublished work). We concluded that 90–100% of the total cell ATP contributed signal to the β-ATP peak. Thus we have 'calibrated' our present surface-coil measurements *in vivo* of ATP by assuming that the β-ATP records the same amount of nucleoside triphosphate as measured in the liver extracts (Table 1) and expressed our results in absolute units.

Peak areas were measured either by computer integration or by the 'cut and weigh' method, which gave identical results. In experiments where the selective irradiation pulse had been used to diminish the phospholipid signal the γ-ATP peak area did not change, The γ-ATP resonance at −2.7 p.p.m. is closest to the central frequency of irradiation (1 p.p.m.) and is not diminished by the presaturating pulse. We therefore conclude that the P_i peak would be unaffected. Tissue-biopsy water content was assumed to be 80% (Krebs & Veech, 1969; Iles *et al.*, 1979). To obtain estimates of the intracellular contents of metabolites [(*c*) and (*d*) in Table 2] from total tissue values a value of 27% was used for extracellular water (R. A. Iles, P. G. Baron & R. D. Cohen, unpublished work), and 2 mM for extracellular P_i concentration (Sestoft & Bartels, 1981). The contributions of extracellular ATP and ADP (which would come from the small erythrocyte content) were considered negligible.

We have previously found that the longitudinal relaxation times (T_1 values) for ATP and P_i in the liver at 72 MHz are approx. 100 and 300 ms respectively, whereas a second P_i component, which was assigned to the extracellular space, had a T_1 of 3000 ms (Iles *et al.*, 1980). In this earlier work we used a pulse repetition of 0.5 s to eliminate signals from this long-T_1 P_i component, but in the present study, with a 4 s pulse delay, we have assumed that this component is present. If for any reason the signal from extracellular P_i is lower than our estimate, which is a maximum then the intracellular P_i concentration corrected for extracellular P_i concentration [(*c*) in Table 2] will be underestimated, since the concentration outside (2 mM) is higher than that inside (1 mM).

The α-, β- and γ-phosphorus nuclei of ATP resonate at different frequencies, as do the α- and β-phosphorus nuclei of ADP. Because of their similar molecular environments, the γ-ATP peak resonates close to the β-ADP peak, and the α-ATP peak resonates close to the α-ADP peak. In n.m.r. spectra of intact tissues these signals coalesce as a result of line broadening. In extracts, however, the γ-ATP and β-ADP peaks can usually be distinguished and their areas determined separately (Fig. 2). The β-ATP peak resonates alone, and therefore the ADP concentration is calculated in spectra of intact tissue by subtracting the β-ATP peak area from the γ-ATP + β-ADP peak areas (Fig. 1).

Results

Figs. 1 and 2 show spectra from a fed-rat liver *in vivo* and a liver extract from a fed rat respectively. The broad phospholipid peak is absent from the spectrum of the extract, but two distinctive peaks are seen at 3.1 and 3.61 p.p.m., which we have previously assigned to glycerophosphocholine and glycerophosphoethanolamine respectively (Iles *et al.*, 1982). It is also apparent that the largest contributor to the 'sugar phosphate' peak in Figs. 1 and 2 is phosphocholine. Figs. 3(*a*) and 3(*b*) show spectra from (*a*) a fed-rat liver *in vivo* and (*b*) the same liver after application of a selective irradiation pulse at 1 p.p.m.

Table 1 shows values for the concentrations of ATP, ADP and P_i in liver extracts and livers *in vivo*

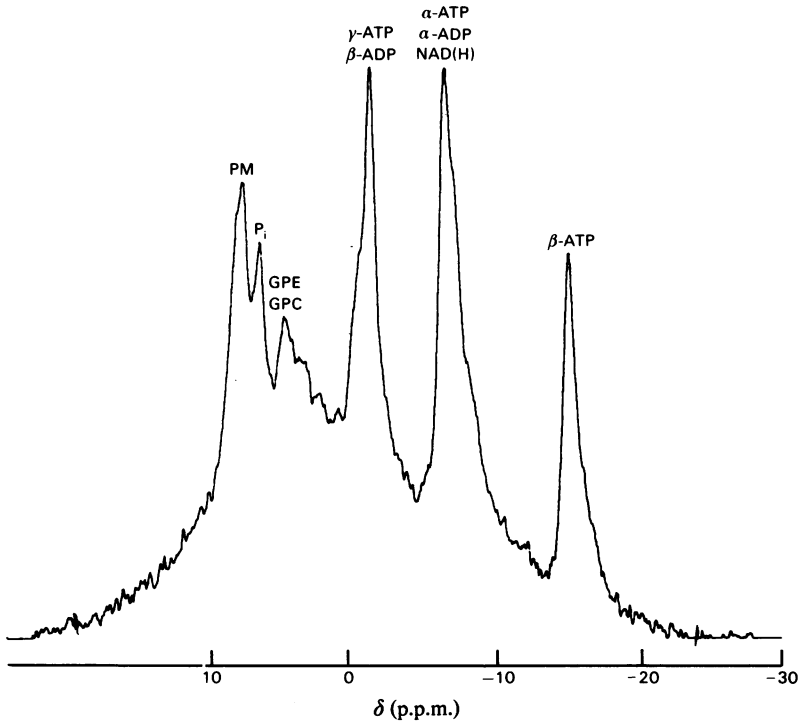

Fig. 1. *^{31}P-n.m.r. spectrum of the liver of an anaesthetized fed rat*
The spectrum was recorded at 81 MHz and is the sum of 1000 data accumulations over a period of 34 min. Abbreviations: PM, phosphomonoesters, mainly phosphocholine with small amounts of phosphorylated monosaccharides, e.g. glycerol 1-phosphate: GPE, glycerophosphoethanolamine; GPC, glycerophosphocholine.

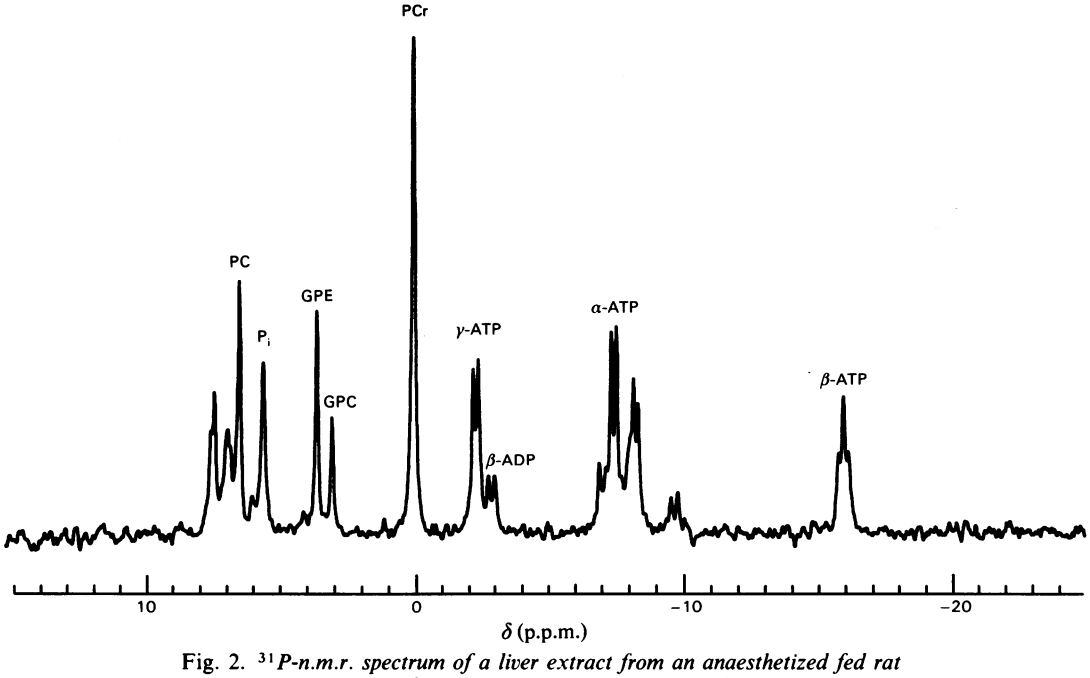

Fig. 2. *^{31}P-n.m.r. spectrum of a liver extract from an anaesthetized fed rat*
The spectrum was recorded at 81 MHz at 4°C and pH 8.2 and is the sum of 400 data accumulations over 54 min. Abbreviations: PC, phosphocholine; PCr, phosphocreatine added as a standard; other assignments as for Fig. 1.

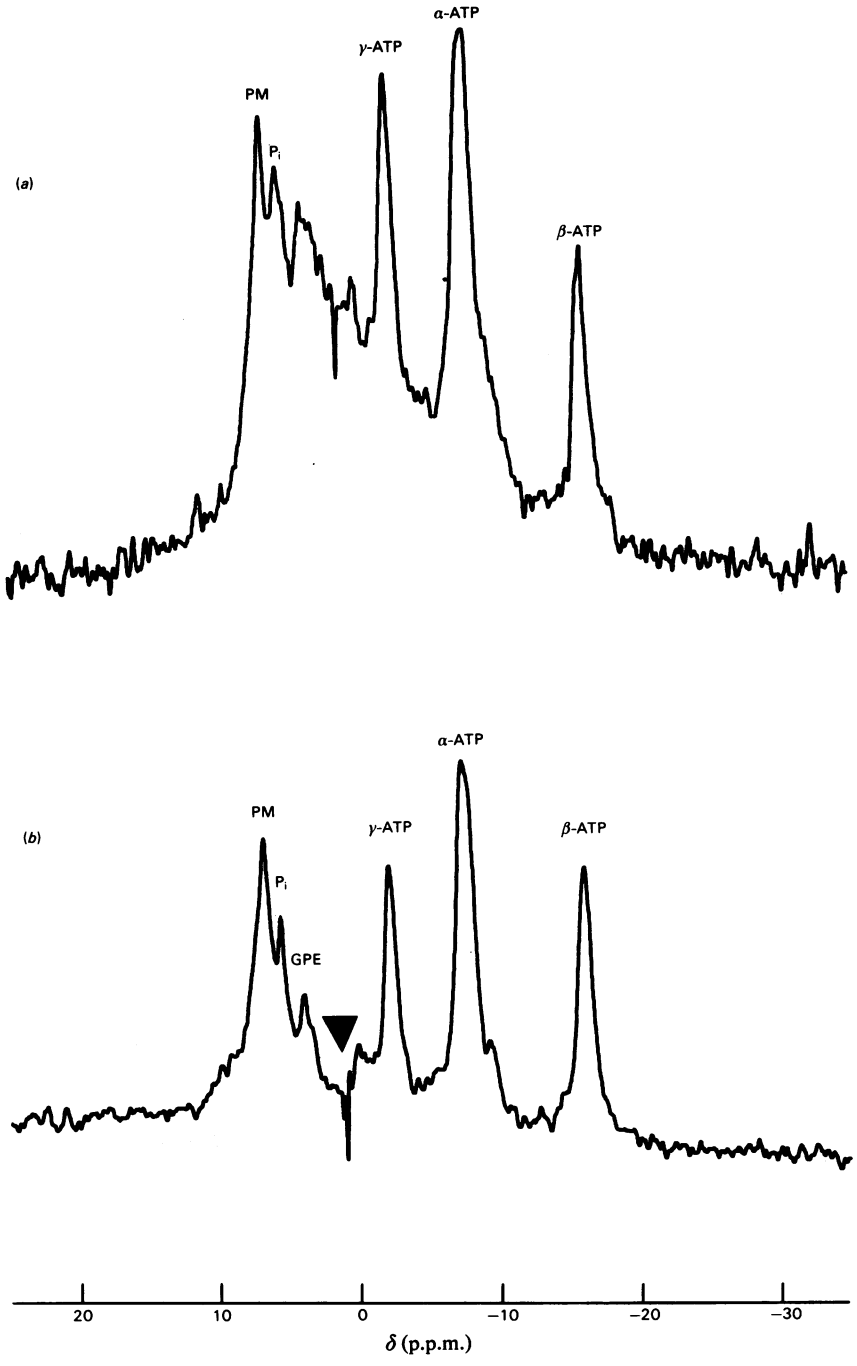

Fig. 3. ^{31}*P-n.m.r. spectra of the liver of an anaesthetized fed rat*
(*a*) Normal spectrum of 280 data accumulations; (*b*) spectrum after the application of a selective irradiation pulse at 1 p.p.m. (500 data accumulations).

calculated from n.m.r. peak areas. The [ATP]/[P_i] ratio is greater than that in the freeze-clamped tissue, whereas ADP in the intact tissue is apparently below the level of detection by n.m.r. There are no significant differences between results for fed and starved rats from the freeze-clamped liver apart from a decrease in glycerophosphoethanolamine in the livers from starved

Table 1. *Concentrations of phosphorus metabolites in freeze-clamped liver measured by ^{31}P n.m.r. from normally fed, 48 h-starved and fructose-challenged rats and in liver of fed rats in vivo*

Conditions are as given in the Materials and methods section. Results are means ± S.E.M. expressed as mM (μmol/ml of total tissue water). Significance of differences from the mean of the fed rats: $*P<0.05$; $***P<0.001$.

	No. of rats	$[P_i]$ (mM)	[ADP] (mM)	[ATP] (mM)	[ATP]/$[P_i]$ ratio	[Glycero-phospho-ethanolamine] (mM)	[Glycero-phospho-choline] (mM)
Freeze-clamped livers							
Fed	8	4.11 ± 0.23	1.40 ± 0.11	2.80 ± 0.15	0.70 ± 0.05	2.94 ± 0.41	1.50 ± 0.12
Starved	7	3.56 ± 0.40	1.54 ± 0.16	2.52 ± 0.21	0.80 ± 0.14	1.90 ± 0.32*	1.28 ± 0.14
Fructose infusion (750mg/kg)	4	1.34 ± 0.18***	0.92 ± 0.05*	1.09 ± 0.12***	0.71 ± 0.08	3.82 ± 0.70	1.48 ± 0.25
Livers *in vivo*							
Fed	5	0.93*	Not detectable	2.80†	3.01 ± 0.27*	—	—
Fed (+ irradiation pulse)	3	1.01*	Not detectable	2.80†	2.53 ± 0.05*	—	—

† Taken from the freeze-clamped liver value (see the text).

Table 2. *Concentrations of ATP, ADP and P_i, $[ATP]/[ADP][P_i]$ ratio and ΔG_{ATP} from enzymic and ^{31}P-n.m.r. measurements*

Results are given as mM (μmol/ml of total tissue water) except for those marked*, which are μmol/g wet wt., and (*c*) and (*d*), which are μmol/ml of intracellular water. For further details see the text.

		[ATP]	[ADP]	$[P_i]$	$10^{-3} \times [ATP]/[ADP][P_i]$	ΔG_{ATP} (kJ/mol)
Liver						
Hems & Brosnan (1970)*		2.74	1.34	3.82	0.54	−48.5
Woods *et al.* (1970)*		2.24	0.74	4.25	0.71	−49.4
Veech *et al.* (1979) (measured)		3.38	1.32	4.76	0.54	−48.5
Veech *et al.* (1979) (calculated cytosolic [ADP])		3.38	0.047	4.76	15.1	−57.3
N.m.r. (extracts)		2.80	1.40	4.11	0.49	−48.5
N.m.r. (*in vivo*) (+irradiation pulse)	(*a*)	2.80	0.05†	0.93	60.2	−61.1
	(*b*)	2.80	0.05†	1.01	55.4	−61.0
N.m.r. (*in vivo*) (corrected for extracellular space)	(*c*)	3.83	0.05†	0.53	145	−63.2
N.m.r. (*in vivo*) (+irradiation pulse; corrected for extracellular space)	(*d*)	3.83	0.05†	0.64	120	−62.8
Muscle						
Gupta & Moore (1980)*		6.2	0.005	2.9	403.4	−65.3
Calculated maximum available free energy available from the electron-transport chain per oxidative phosphorylation site (Clark, 1960)						−73.6

† From Veech *et al.* (1979); calculated value.

rats. In livers from rats given fructose infusion, ATP concentration had fallen to 39%, ADP concentration to 66% and P_i concentration to 33% of the values for control (fed) rat livers. In previous experiments *in vivo* with the same dose of fructose, ATP concentration had fallen to 35% and P_i was undetectable at the corresponding time after infusion (Griffiths *et al.*, 1980; Iles & Griffiths, 1982; R. A. Iles, A. N. Stevens & J. R. Griffiths, unpublished work). In three separate experiments recovery of ATP added to freeze-clamped tissue was 98.7 ± 3.9%; no significant change in ADP or P_i concentrations occurred.

Table 2 shows results for hepatic ATP, ADP and P_i obtained by a number of authors in previous studies with freeze-clamped tissue extracted by $HClO_4$ and assayed enzymically (for ATP and ADP) or chemically (for P_i), together with our

current n.m.r. results on extracts and *in vivo*. In general, results from other studies are similar to the n.m.r. measurements on liver extracts prepared similarly. For values of ADP *in vivo* we have used a calculated value of 0.05mM taken from the study by Veech *et al.* (1979) (see below). We have also calculated the [ATP]/[ADP][P_i] ratio for comparison and free energy of hydrolysis for ATP (ΔG_{ATP}).

Discussion

Several explanations are possible for the apparent discrepancies between the measurements of ATP, ADP and P_i by n.m.r. on intact livers compared with n.m.r. of liver extracts and between the n.m.r. method on intact livers and chemical/enzymic assay of liver extracts (Tables 1 and 2).

It is difficult to estimate the phosphate peak areas accurately in the presence of the broad underlying phospholipid peak (Fig. 1). This is particularly so for the resonances of the phosphomonoesters and P_i, since they lie on the rising edge of the underlying phospholipid resonance. However, we do not consider that this provides an explanation for the discrepancy in P_i magnitude, since the experiments performed to minimize this difficulty by irradiation of the phospholipid resonance gave a concentration of P_i (1.01mM, $n = 3$) that is only 9% greater than that obtained in the absence of irradiation (Table 1). If the ATP concentration measured by n.m.r. is assumed to be the same as by enzymic assay, then the difference in P_i concentration measured by n.m.r. and chemical analyses, approx. 3mM (Table 1), is too large to be accounted for by errors in baseline measurements.

One explanation for the discrepancy of ADP and P_i measurements could be that there are pools of both ADP and P_i that are invisible to n.m.r. Secondly, the large ADP and P_i concentrations in enzymic or chemical assays might be produced from breakdown of a labile ATP pool during the tissue-extraction procedure. Thirdly, the larger P_i content measured by the chemical assay might also arise from degradation of organic phosphates during the acid/molybdate procedure adopted in most methods (e.g. Fiske & SubbaRow, 1927; Martin & Doty, 1949).

For the first explanation to be correct there would need to be pools of ADP and P_i that are either bound too tightly to protein or compartmented in some other way. Binding to proteins can result in n.m.r. peaks being broadened out to such an extent that they become undetectable. This is caused either by an intermediate exchange rate between the bound and free states (Anderson, 1954) or by the slow motion of the macromolecule, which will tend to shorten the relaxation times of the bound molecule (Daszkiewicz *et al.*, 1963). ADP n.m.r. signals cannot be detected in skeletal muscle (Dawson *et al.*, 1977) and this has been attributed to the binding of most of the intracellular ADP to actin during the formation of F-actin. Since the actin concentration in the liver is only 0.08μmol/g wet wt. (Gordon *et al.*, 1977) and assuming one binding site/molecule of actin, this could only account for a maximum of 0.08μmol of ADP/g. However, there are many ADP-utilizing enzymes within the cell, and it is possible that a significant amount is bound to these. Slater (1976) has calculated that between 25 and 30% of intramitochondrial ADP is bound to mitochondrial ATPase, which binds 2 mol of ADP/mol (Harris *et al.*, 1973).

Compartmentation of ADP in the mitochondrion has been proposed as an explanation for its invisibility to n.m.r. spectroscopy *in vivo* (Wong, 1981). Because of the low water/soluble-protein ratio in the mitochondrial matrix, there has been some speculation that n.m.r. signals emanating from soluble metabolites would be affected adversely (Wong, 1981; Iles *et al.*, 1982). Some ^{31}P-n.m.r. evidence has been obtained that indicates a proportion of mitochondrial ADP is bound (Wong, 1981), and other n.m.r. studies on isolated mitochondria suggest that, although some mitochondrial signals are detectable, they give rise to broad peaks (Ogawa *et al.*, 1978*a*,*b*; Shen *et al.*, 1980). If most of the hepatic ADP is in the mitochondrion, then the amount bound by cytosolic actin might be a significant fraction of the total cytosolic content. For P_i the situation is different because of the much greater magnitude of the discrepancy in concentration (3mM). If this 'missing' P_i were in 'free' solution in the mitochondrion, its concentration would be of the order of 20–30mM, perhaps far too high for such an insoluble anion. Although some workers have estimated mitochondrial concentrations of 15–20mM (Ackerboom *et al.*, 1978; Coty & Pedersen, 1974), others have obtained much lower values (Holian *et al.*, 1977; Soboll *et al.*, 1978).

The second possibility would seem to be unlikely, since ATP added to the freeze-clamped tissue was recovered completely and without any significant increase in P_i (or ADP) (Table 2). However, there still remains a possibility that there is another labile organic phosphate present in sufficient concentration in the liver that liberates 2–3mM-P_i on extraction. Against this is the lack of evidence for a phosphate-containing intermediate of greater than 2mM present in spectra *in vivo* but not in spectra of extracts (Figs. 1 and 2).

The results from the fructose-infusion experiments show that, after 5min exposure to fructose, the P_i concentration had fallen by 2.8mM to within

0.4–0.5mM of the normal n.m.r. derived value *in vivo*. This observation also suggests that the discrepancy in P_i concentration is not due to breakdown of an organic source of phosphate during extraction but represents phosphate that is 'metabolically available', whether compartmented or bound.

The third possibility, overestimation of P_i by degradation of labile organic phosphates during the acid/molybdate procedure used for chemical analysis of P_i (Seraydarian *et al.*, 1961), seems unlikely. The data in Table 2 show little difference between chemically assayed P_i and P_i measured by n.m.r. in extracts. This implies that any hydrolysis of labile phosphate occurs during freeze-clamping or extraction of livers and not during chemical assays. There is extensive evidence of breakdown of phosphocreatine to P_i and creatine during acid/molybdate assays of muscle extracts (Seraydarian *et al.*, 1961; Kretzschmar & Wilkie, 1969), but liver contains no phosphocreatine.

If 'free' ADP and P_i concentrations are much lower than the total concentrations, two questions are pertinent. Firstly, is there any evidence, other than from n.m.r. studies, that 'free' ADP and P_i concentrations are lower than their total concentrations or compartmented in some way? Secondly, what are the consequences for the phosphorylation potential [ATP]/[ADP][P_i] and putative effector function of ADP and P_i in metabolic control?

ADP

A study of cytosolic phosphorylation potential was carried out by Veech *et al.* (1979). They determined a lumped equilibrium constant 'K_{G+G}' for the combined glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase reactions *in vitro* as well as the equilibrium constants for lactate dehydrogenase (K_{LDH}), myokinase (K_{MYK}) and creatine kinase (K_{CK}). They then compared the ratio K_{G+G}/K_{LDH} *in vitro* with the value determined *in vivo* in a variety of tissues. A similar calculation of the equilibrium constants for creatine kinase (K_{CK}) and adenylate kinase (K_{MYK}) was made. There was a large discrepancy between the values of K_{G+G}/K_{LDH} *in vitro* and *in vivo*, for K_{MYK} and K_{CK} in brain and muscle and for K_{G+G}/K_{LDH} and K_{MYK} from the liver. However, for the erythrocyte K_{G+G}/K_{LDH} and K_{MYK} there was good agreement between the values *in vitro* and *in vivo*. They assumed that the creatine kinase reaction must be close to equilibrium *in vivo* and substituted a calculated 'free' cytosolic ADP concentration into the creatine kinase mass-action ratio *in vivo* to bring K_{CK} *in vivo* equal to K_{CK} *in vitro*. This value for ADP, when applied to K_{G+G}/K_{LDH}, also brought the value *in vivo* close to the one *in vitro*. These authors commented that the 'free' cytosolic ADP concentration (which they calculated as 0.05mM) might be considerably lower than the measured whole-cell ADP concentration (1.3mM in liver), owing either to binding or to compartmentation of ADP in the mitochondrion. Thus, if the erythrocyte is assumed to be a simple one-compartment system, one would expect the free ADP concentration to equal total ADP concentration, and little discrepancy in the equilibrium *in vitro* and *in vivo*, as was indeed found to be the case.

If the liver cytosolic or 'free' ADP concentration is as low as this (0.05mM), then it would not be detected by n.m.r. in the presence of ATP at nearly 100-fold greater concentration. Dawson *et al.* (1978) and Dawson (1982) calculated the free ADP concentration in skeletal muscle from the creatine kinase reaction by using n.m.r.-measured phosphocreatine, H^+ and ATP and assuming a phosphocreatine/(phosphocreatine + creatine) ratio of 0.85 and an Mg^{2+} concentration of 2.5mM. They obtained 0.02mM for ADP, a similar value to that for rat muscle calculated by Veech *et al.* (1979) (0.037mM). Gupta & Moore (1980) used ^{31}P n.m.r. to estimate the free Mg^{2+} concentration to be 0.6mM in frog skeletal muscle from the chemical-shift differences of α- and β-ATP peaks. From this they calculated both MgADP and 'free' ADP concentrations to be 3.1 and 2.2μM respectively. Thus their calculated total 'free' (ADP + MgADP) was 5.3μM. Veech *et al.* (1979) suggested that, since their experimentally determined K_{MYK} showed no discrepancy with the predicted value in the erythrocyte but did in tissues containing mitochondria, their results were consistent with ADP compartmentation in mitochondria or association with proteins.

There is evidence for a higher cytosolic [ATP]/[ADP] ratio compared with the corresponding mitochondrial ratio from cell-fractionation studies. Studies on isolated hepatocytes with the digitonin fractionation procedure by Ackerboom *et al.* (1978) and Brocks *et al.* (1980) yielded values of approx. 2:1 for mitochondrial [ATP]/[ADP] and 6.5–9:1 for cytosolic [ATP]/[ADP] ratios. Using the organic-extraction technique in the isolated perfused rat liver, Soboll *et al.* (1978) obtained values for mitochondrial [ATP]/[ADP] ratio of 0.2:1 and cytosolic values of 6:1, whereas in the liver *in vivo* Schwenke *et al.* (1981) obtained 0.9:1 and 7:1 respectively. Qualitatively the results are consistent with a low mitochondrial/cytosolic [ATP]/[ADP] ratio. Quantitatively, the cytosolic [ATP]/[ADP] ratios of all these studies agree well and are approx. 2–3 times the whole-cell values obtained from perfused livers (Hems *et al.*, 1966; Iles *et al.*, 1979), isolated hepatocytes and livers *in vivo* (Hems & Brosnan, 1970).

P_i

Cytosolic and mitochondrial P_i concentrations of 3 and 15–20mM respectively were measured by Ackerboom *et al.* (1978) after subcellular fractionation of isolated hepatocytes with the use of digitonin. Coty & Pedersen (1974) obtained similar intramitochondrial values in isolated mitochondrial suspensions. These are of magnitude comparable with the 'missing P_i' calculated above. However, Soboll *et al.* (1978) found higher cytosolic (9.5mM) but lower mitochondrial (9mM) values in the isolated perfused rat liver after non-aqueous fractionation. Sestoft & Bartels (1981) concluded that the cytosolic P_i content was 1.5–2μmol/g of liver on the basis of the high mitochondrial P_i content found by other workers and their own observations on freeze-clamped liver. On the assumption that P_i was distributed across the mitochondrial membrane according to the pH gradient measured by Coty & Pedersen (1974), Ottaway & Mowbray (1977) calculated that, with an overall liver P_i concentration of 5mM, the P_i concentration would be 15mM in the mitochondrion and 2.8mM in the cytosol. From our own observations, assuming that cytosolic P_i concentration is 0.5–1.0mM and total liver P_i concentration is 4.1mM (Table 2), the mitochondrial concentrations (assuming mitochondrial volume is 18% of the total liver cell volume) would be about 22–24mM.

Calculated values for the free energy of ATP hydrolysis, ΔG_{ATP}, are shown in Table 2. It can be seen that, if the calculated liver cytosolic ADP value determined by Veech *et al.* (1979) is combined with the n.m.r.-derived ATP and P_i concentrations. then a large increase in [ATP]/[ADP][P_i] ratio is obtained (Table 2) as well as an increase in ΔG_{ATP} to within 12kJ/mol of the probable maximum energy available from each of the electron-transport sites coupled to oxidative phosphorylation.

There are further implications for the function of ADP and P_i as metabolic regulators and substrates. Both have been implicated in controlling the rate of oxidative phosphorylation either by the rate of ADP translocation (Klingenberg & Heldt, 1982; Heldt & Klingenberg, 1968) or by direct substrate limitation of ADP (Chance & Williams, 1956) or of P_i (Chance, 1959). Chance & Williams (1955) showed that 30μM-ADP was sufficient for half-maximal O_2 uptake by rat liver mitochondria, and Chance (1959) showed that the K_m for P_i for the same process was of the order of 1mM (Table 3). Thus, if the hepatic ADP and P_i concentrations are in the region of 0.05mM and 1mM respectively, mitochondrial oxidation would be very sensitive to any changes in these values. Table 3 also lists values for K_m for P_i and ADP taken from the literature for four important enzymes involved in glycolysis (or gluconeogenesis) in the liver. Two important enzymes using P_i as substrate in liver, glycogen phosphorylase *a* and glyceraldehyde-3-phosphate dehydrogenase, have K_m values in the region of 0.7–1.5mM (Table 3). If these K_m values pertain *in vivo*, our present estimates of cytosolic P_i concentration suggest that both these enzymes may be under substrate control.

The K_m values for ADP for pyruvate kinase and 3-phosphoglycerate kinase are an order of magnitude higher than the calculated cytosolic ADP concentration determined by Veech *et al.* (1979). However, pyruvate kinase appears to be subject to control by a large number of effectors, and these values must therefore be treated with caution. Nevertheless, during ischaemia, hepatic glycolysis shows a rapid increase, and rises in P_i and ADP concentrations occur (Hems & Brosnan, 1970; Iles & Griffiths, 1982; Iles *et al.*, 1985). In this situation a rapid increase in phosphorylase, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase and pyruvate kinase activities may be facilitated by this increase in P_i and ADP.

The concentration of 'free' AMP may also be calculated if it is assumed that the adenylate kinase reaction is at equilibrium (Veech *et al.*, 1979). By using their value for the equilibrium constant for this reaction (1.12) and a 'free' ADP+MgADP concentration of 0.05mM a value for AMP concen-

Table 3. *P_i and ADP as substrates in rat liver*

P_i	Phosphorylase *a*	0.7–1.5mM	Van den Berghe *et al.* (1973)
P_i	Glyceraldehyde-3-phosphate dehydrogenase	1.5mM	Smith & Velick (1972)
P_i	O_2 uptake in mitochondria	1mM	Chance (1959)
ADP	Pyruvate kinase (L isoenzyme)	0.7mM	Middleton & Walker (1972)
	(M isoenzyme)	0.6–0.9mM	
ADP	3-Phosphoglycerate kinase	0.35mM	Krietsch & Bucher (1970); Fritz & White (1974)
ADP	ATP/ADP mitochondrial translocase	1–4μM	Klingenberg (1970)
ADP	O_2 uptake in mitochondria	30μM	Chance & Williams (1955)

tration of 0.65 μM is obtained, nearly three orders of magnitude below the value for total hepatic AMP measured enzymically (Hems & Brosnan, 1970).

Gupta & Moore (1980) have calculated a value of 0.004 μM for frog skeletal-muscle 'free' AMP compared with 0.2 μM obtained by Dawson (1982) for the same tissue.

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