³¹P-saturation-transfer nuclear-magnetic-resonance measurements of phosphocreatine turnover in guinea-pig brain slices

Peter G. MORRIS,* James FEENEY,* David W. G. COX† and Herman S. BACHELARD†‡

*M.R.C. Biomedical N.M.R. Centre, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K., and †Division of Biochemistry (U.M.D.S.), St. Thomas's Hospital Medical School, London SE1 7EH, U.K.

(Received 24 September 1984/30 November 1984; accepted 24 January 1985)

1. The technique of ³¹P saturation-transfer n.m.r. was used to determine the forward and the reverse rate constants of creatine phosphotransferase in superfused guinea-pig cerebral tissues *in vitro*. 2. The calculated forward rate constant of $0.22 \pm 0.03 \text{ s}^{-1}$ compared well with a previously reported value for rat brain *in vivo* [Shoubridge, Briggs & Radda (1982) *FEBS Lett.* **140**, 288–292]. The reverse rate constant was found to be $0.55 \pm 0.10 \text{ s}^{-1}$. 3. By using concentrations of ATP and phosphocreatine estimated previously for this superfused preparation [Cox, Morris, Feeney & Bachelard (1983) *Biochem. J.* **212**, 365–370], forward and reverse flux rates were calculated to be 0.68 and $0.72 \mu\text{mol} \cdot \text{s}^{-1} \cdot \text{g}^{-1}$ respectively. The concordance of forward and reverse fluxes contrasts with the situation observed *in vitro* in other tissues, and suggests that the creatine phosphotransferase reaction is at equilibrium under the conditions used here. 4. Lowering the concentration of glucose in the superfusing medium from 10 mM to 0.5 mM had no significant effect on phosphocreatine concentration or on the forward (ATP-generating) flux through creatine phosphotransferase. The results indicate that a normal phosphocreatine content in the presence of lowered glucose availability is reflected by an unchanged turnover rate.

We previously described a system for performing ³¹P-n.m.r. studies on cerebral tissues superfused *in vitro*, which provided calculated tissue concentrations of phosphocreatine and ATP comparable with those obtained from studies conducted *in vivo* (Cox *et al.*, 1983). In that investigation it was found that these concentrations were unchanged when the glucose content of the superfusing medium was lowered from a control concentration of 10 mM to 0.5 mM. At 0.2 mM-glucose, however, the cerebral phosphocreatine concentration fell significantly by 22% and that of ATP by 20%. These results were in marked contrast with the glucose threshold of over 2 mM required to maintain evoked potentials in experiments performed *in vitro* (Cox & Bachelard, 1982; Bachelard *et al.*, 1984), and a normal electroencephalogram and behaviour *in vivo* (Siesjö, 1978). We made the rather obvious point that steady-state concentrations of such energy intermediates as phosphocreatine and ATP may not adequately reflect any

effects on metabolism, since the turnover rates of the metabolites could change without detectable change in steady-state concentrations (Cox *et al.*, 1983).

Saturation-transfer techniques (Forsén & Hoffman, 1963; Gadian *et al.*, 1981) have now been applied to our '*in-vitro*' superfusion system, and turnover rates for the creatine phosphotransferase reaction in forward and reverse directions are reported. The method was used to assess the effects of mildly hypoglycaemic conditions on these turnover rates.

Methods

Tissue preparation

Adult male Dunkin–Hartley guinea pigs (250–350 g body wt.) were stunned and exsanguinated, and the brains were quickly removed. The sub-cortical tissues were excised, each cerebral hemisphere was cut in half with a scalpel and the two halves were sliced at right-angles to this cut in one direction to give slices of 350 μm thickness (McIlwain & Bachelard, 1985). Immediately the slices

‡ To whom correspondence and reprint requests should be sent.

had been prepared, they were transferred to a test tube containing gassed incubation medium, briefly dispersed with a vortex mixer and decanted into a conical flask containing incubation medium constantly gassed with O_2/CO_2 (19:1). The froth that had accumulated was aspirated away, and the slices were then rinsed five times with fresh gassed medium. They were transferred into a standard 25 mm-diameter n.m.r. tube fitted with a polytetrafluoroethylene insert carrying perfusion tubing and the shaft of a motor-driven glass stirrer (Cox *et al.*, 1983). The cerebral cortices from four guinea pigs were required to provide sufficient tissues for each n.m.r. experiment. The incubation medium contained NaCl (124 mM), KCl (5 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.2 mM), $CaCl_2$ (1.2 mM) and $NaHCO_3$ (26 mM); 10 mM-glucose was present for tissue preparation and control experiments. Gassing was with O_2/CO_2 (19:1). The tissues within the n.m.r. tube were superfused with medium at rates of 20–25 ml/min, giving a medium-exchange time of approx. 2 min. The motor-driven paddle stirrer was adjusted to ensure continuous gentle agitation of the tissues before the tube was placed inside the n.m.r. probe.

N.m.r. spectroscopy

N.m.r. spectra were obtained by using a Bruker WM 200 wide-bore spectrometer, operating at a frequency of 81 MHz for phosphorus, without a deuterium lock. The stability of the superconducting magnet is sufficient to ensure that no n.m.r.-detectable drift occurred during the 6–7 h of each experiment. Magnet shimming was accomplished by using the free induction decay (FID) from the protons in the water of the circulating perfusate. A line-width of 5–10 Hz was normally achieved. Spectra were usually accumulated in blocks of $128 \times$ or 256×8000 FIDs. The spectral width was 5.55 kHz. Each block was individually recorded on magnetic disc for subsequent inspection and analysis. In experiments where the contents of the superfusing medium were varied, the medium was changed without disturbing the tissue or probe (Cox *et al.*, 1983). Data collection was resumed after 22 min superfusion with altered medium.

Saturation transfer

In the case of the creatine phosphotransferase equilibrium:

the forward and reverse fluxes can be expressed as $k_{+1}[\text{phosphocreatine}]$ and $k_{-1}[\text{ATP}]$ respectively, where k_{+1} and k_{-1} are pseudo-first-order rate constants. These rate constants can be determined by the method of ^{31}P saturation-transfer n.m.r.,

which requires the selective irradiation of the resonance of one of the species participating in the equilibrium of interest. For monitoring the forward reaction of the creatine kinase equilibrium, the ATP resonance is saturated for a time t . (This destroys the net ATP magnetization by equalizing the two spin energy levels: the chemical equilibrium is not perturbed.) Since ATP undergoes exchange with phosphocreatine, the amplitude of the latter's resonance is also diminished. Thus:

$$M_t = \frac{M_0}{1 + k_{+1}T_1} \left\{ 1 + k_{+1}T_1 \cdot \exp \left[- \left(k_{+1} + \frac{1}{T_1} \right) t \right] \right\} \quad (1)$$

where M_t is the phosphocreatine magnetization at time t , M_0 is the equilibrium phosphocreatine magnetization (i.e. in the absence of irradiation) and T_1 is the intrinsic spin-lattice relaxation time of phosphocreatine (i.e. in the absence of a chemical exchange process).

As t tends to infinity, this expression approaches the asymptotic limit:

$$M_\infty = \frac{M_0}{1 + k_{+1}T_1} \quad (2)$$

which reflects the balance between the two competing effects, namely the exchange with the saturated ATP tending to diminish the magnetization, and the restoring spin-lattice relaxation process. Fortunately, for the creatine kinase equilibrium, $k_{+1} \approx 1/T_1$, and the diminished steady-state magnetization M_∞ is easily measured. Eqn. (2) can be used to determine k_{+1} if T_1 is known or is measured in a separate experiment. One simply needs to measure the phosphocreatine magnetization at the two limits $t \rightarrow 0$, ($M_t \rightarrow M_0$), and $t \rightarrow \infty$, ($M_t \rightarrow M_0$)/(1 + $k_{+1}T_1$). In our case we have used t values of 0.005 s and 8 s respectively.

Alternatively, if intermediate t values are also recorded, both T_1 and k_{+1} can be determined by fitting eqn. (1): the steady-state value is again $M_\infty = M_0/(1 + k_{+1}T_1)$ and the rate with which it is approached is $k_{+1} + 1/T_1$.

Analogous equations apply for the reverse reaction in which the ATP resonance is measured while the phosphocreatine resonance is saturated.

Saturation-transfer conditions

The ATP (forward rate) or phosphocreatine (reverse rate) resonances were saturated for times between 5 ms and 8 s, and data were acquired for a period of 0.68 s following a 90° pulse. The inter-pulse interval was 8 s plus the variable saturation time. In order to avoid the possibility of changes in steady-state concentrations producing artifact changes in turnover rates during the course of the experiments, the saturation times were randomly scrambled. In the experiments where turnover

rates were determined with low glucose concentration, data were obtained only for the 5 ms and 8 s end-points. These were collected in interleaved sub-blocks of either 8 or 16 scans and were preceded and followed by end-point determinations in control media on the same tissue preparation.

The possibility of an artifact decrease in the phosphocreatine peak, due to an inadvertent direct irradiation of its resonance when the γ -peak was being saturated, was excluded by control experiments in which the irradiation was applied on the downfield side of the phosphocreatine peak. No change in the phosphocreatine resonance relative to the non-irradiated case was observed (Fig. 1). No effects on heights or areas of the metabolite

peaks were observed at any of the irradiation time intervals used in these experiments, other than those due to the transfer of saturated nuclei between ATP and phosphocreatine.

The ' γ -peak' includes resonances of the γ -phosphate groups of nucleoside triphosphates and the β -phosphate groups of nucleoside diphosphates (Gadian, 1982). When the γ -peak is used as a measure of the ATP, the only contaminant that could interfere in the context of these studies is GTP. Our results on ^{31}P n.m.r. of cerebral tissue extracts (P. G. Morris, J. Feeney, D. W. G. Cox & H. S. Bachelard, unpublished work) show that the GTP is less than 10% of the ATP, which is similar to the proportion derived from direct chemical and enzymic analysis (McIlwain & Bachelard, 1985).

Data analysis

Where data were available for a range of periods of saturation (t), an exponential function was fitted to the values of the phosphocreatine or ATP magnetization, M_t , by using an iterative three-parameter (intercept, asymptote and rate) computer fit. For accurate determination of the reverse pseudo-first-order rate constant it was found necessary to combine the data for ATP magnetization from seven separate experiments. This required the data to be normalized, which was achieved by expressing the height of the ATP resonance at time t as a percentage of the intensity of the resonance of the ATP γ -phosphate group observed under control conditions in the absence of saturating irradiation.

Results and discussion

Measurement of rate constants

Forward reaction:

Figs. 2(a) and 2(b) show the spectra obtained with irradiation of the γ -peak for 5 ms (Fig. 2a) and 8 s (Fig. 2b), which are the end-points of the time curve of Fig. 2(c). A fortuitous observation was that the irradiation removed the underlying broad signal, which we had previously suggested to be due to membrane phospholipids (Cox *et al.*, 1983). Removal of this broad signal enables more accurate quantitative measurement of changes in smaller peaks such as the γ -peak (see Fig. 1). No effect due to the removal of the broad signal was observed on measured heights or areas of the metabolite peaks, as noted above. Measurement of the area of the phosphocreatine peak after irradiating the γ -peak for various times gave the experimental points of Fig. 2(c). The curve was fitted by using a computerized iterative line-fitting method (see the Methods section); the error bars give the 95%

Fig. 1. Effect of control irradiation on phosphocreatine resonance in superfused cerebral tissue

(a) Non-irradiated spectrum. (b) Irradiated spectrum. Each spectrum represents the combined data of 256×8000 FIDs, with interpulse intervals as described in the Methods section, and with a line broadening of 12 Hz. The irradiation time in (b) was 1.71 s at the frequency shown ($\blacktriangledown$). Peak 1, phosphocreatine; peak 2, γ -peak of nucleoside triphosphates and diphosphates. For assignments of other peaks see Cox *et al.* (1983).

Fig. 2. Determination of the forward rate of creatine phosphotransferase in superfused cerebral tissue (a) and (b) Spectra of cerebral tissues superfused in medium containing 10 mM-glucose obtained after irradiation of the tissue for 0.005 s (a) or 8 s (b). The frequency of the irradiation is indicated in (b). (Other details are given in the Methods section and Fig. 1 legend.) (c) Amplitude of the phosphocreatine resonance plotted as a function of the duration of the saturation pulse; duration times were randomly scrambled throughout the experiment. Individual data points are plotted and the bars represent the 95% confidence limits of the fitted exponential function (see the text for further details).

Fig. 3. Determination of the reverse rate of creatine phosphotransferase in superfused cerebral tissue (a) and (b) Spectra obtained after irradiation of the tissue for 0.005 s (a) or 8 s (b) (see Fig. 1 legend). (c) Amplitude of the ATP resonance plotted as a function of the duration of the saturation pulse; duration times were randomly scrambled throughout the experiment. The points are the means of the numbers of individual normalized values shown (see the text for further details).

confidence limits of the fitted curve at each experimental point. The results for the forward reaction rate are shown in Table 1: the mean rate constant was $0.22 \pm 0.03 \text{ s}^{-1}$, which compares very well with the value of 0.26 s^{-1} reported for rat

brain *in vivo* (Shoubridge *et al.*, 1982). Our studies confirmed that the same turnover rate can be derived with confidence from end-point measurements (i.e. irradiation for 5 ms and 8 s respectively) as from the full time course (Table 1). The

Table 1. *Turnover rates of phosphocreatine in superfused cerebral tissue*
Results are means $\pm$ S.D.; n is the number of separate experiments. Rates are:

Methods of determination are given in the text. Concentrations are from Cox *et al.* (1983).

Direction	Determination	n	Rate (s^{-1})	T_1 (s)	Concentration (range in parentheses) ($\mu\text{mol/g}$)	Flux rate (range in parentheses) ($\mu\text{mol} \cdot \text{s}^{-1} \cdot \text{g}^{-1}$)
Forward	Time curve	2	0.20, 0.21	4.14, 4.22	—	—
	End-point	8	0.22 ± 0.03	—	3.1 (2.9–3.3)	0.68 (0.64–0.73)
Reverse	Combined data	7	0.55 ± 0.10	1.9	1.3 (1.2–1.4)	0.72 (0.66–0.77)

concurrence between end-point and full-time course determinations of k_{+1} also indicates that the simple kinetic model used (see the Methods section) is appropriate.

Reverse reaction:

Figs. 3(a) and 3(b) show the spectra obtained with irradiation of the phosphocreatine peak for 5 ms (Fig. 3a) and 8 s (Fig. 3b). Owing to the relatively small size and the signal/noise ratio of the γ -peak, measurement of this rate was less accurate than that of the forward rate (above), although the removal of the broad 'phospholipid' signal was beneficial. The curve, fitted to the combined normalized experimental points as described in the Methods section, is shown in Fig. 3(c). The results (Table 1) showed reasonable consistency: the calculated mean rate constant was $0.55 \pm 0.10 \text{ s}^{-1}$.

Forward and reverse fluxes. Flux rates for the forward and reverse reactions were estimated by using values for the tissue concentrations (Cox *et al.*, 1983) of $3.1 \mu\text{mol/g}$ for phosphocreatine and $1.3 \mu\text{mol/g}$ for ATP. The results (Table 1) gave a forward flux rate of $0.68 \mu\text{mol} \cdot \text{s}^{-1} \cdot \text{g}^{-1}$ and a very similar reverse rate of $0.72 \mu\text{mol} \cdot \text{s}^{-1} \cdot \text{g}^{-1}$. The apparent equality, within the limits of experimental error, of forward and reverse flux rates through creatine phosphotransferase has not been generally observed in studies on muscle *in vivo*, yet the present result is in agreement with the enzymological studies by Veech *et al.* (1979). The discrepancy between the flux rates *in vivo* has been attributed to the participation of ATP in other reactions, or compartmentation of the ATP pool, leading to an apparent lowering of k_{-1} as measured by saturation-transfer n.m.r. (Nunnally & Hollis, 1979; Ackerman *et al.*, 1980; Gadian *et al.*, 1981; Shoubridge *et al.*, 1982). Also relevant to this problem is the absence from cerebral tissues

incubated *in vitro* of the sensory input that is constantly occurring *in vivo*. Thus *in vitro* the utilization of ATP in supporting some cellular functions (e.g. cation-dependent ATPases) will be decreased. On the other hand, unlike our studies conducted *in vitro*, the experiments conducted *in vivo* were performed on anaesthetized animals, and anaesthesia is known to have a depressant effect on cerebral oxidative metabolism (McIlwain & Bachelard, 1985; Siesjö, 1978).

A further possible complication in interpreting the reverse rate is that the resonance of the GTP γ -phosphate group, present at a frequency close to that of the ATP γ -phosphate group, may result in smaller than expected changes in the ' γ -ATP' magnetization on irradiation of phosphocreatine. However, this is not likely to be of importance, as we have found that the GTP is equivalent to less than 10% of the area under the ' γ -ATP' peak (see above).

Turnover rate of phosphocreatine in mildly hypoglycaemic conditions

Lowering the glucose concentration of the superfusing medium to 2 mM has been shown to cause a significant impairment of evoked electrical activity (Cox & Bachelard, 1982; Bachelard *et al.*, 1984), but no effect on the energy state was observed in our previous ^{31}P -n.m.r. studies when the glucose concentration was lowered even further to 0.5 mM (Cox *et al.*, 1983), a result confirmed also by direct chemical and enzymic measurement (Bachelard *et al.*, 1984). When the saturation-transfer technique as described above was repeated in the presence of 0.5 mM-glucose, the turnover rate of the forward reaction was unchanged. The size of the phosphocreatine peak was 98% of control (i.e. in the presence of 10 mM-glucose) and the turnover rate was $111 \pm 9\%$ of control (mean $\pm$ S.D., $n = 3$).

These studies show that saturation-transfer techniques can be applied successfully to measurement of turnover rates of phosphate intermediates in our superfusion preparation of cerebral tissues *in vitro*, under conditions where the immediate environment can be varied. The calculated rate for the forward reaction is similar to that obtained from experiments conducted *in vivo*. Comparison of the forward and reverse rates of flux presented here show that the creatine phosphotransferase system is in equilibrium in the cerebral cortex *in vitro*, which, as noted above, is in agreement with the enzymological studies by Veech *et al.* (1979). The superfusing conditions of the preparation can be readily modified without disturbing the probe and while continuous collection of data can be achieved. The unchanged turnover rate of phosphocreatine in the presence of 0.5 mM-glucose indicates that the lack of effect on the steady-state concentration of this intermediate is reflected in the absence of change in its rate of turnover.

We are grateful to the Medical Research Council (Grant no. 8111613N) for expenses and support for D. W. G. C.

References

- Ackerman, J. J. H., Bore, P. J., Gadian, D. G., Grove, T. H. & Radda, G. K. (1980) *Philos. Trans. R. Soc. London Ser. B* **289**, 425–436
- Bachelard, H. S., Cox, D. W. G. & Drower, J. (1984) *J. Physiol. (London)* **352**, 91–102
- Cox, D. W. G. & Bachelard, H. S. (1982) *Brain Res.* **239**, 527–534
- Cox, D. W. G., Morris, P. G., Feeney, J. & Bachelard, H. S. (1983) *Biochem. J.* **212**, 365–370
- Forsén, S. & Hoffman, R. A. (1963) *J. Chem. Phys.* **39**, 2892–2901
- Gadian, D. G. (1982) *N.M.R. and its Application to Living Systems*, pp. 24–25, Clarendon Press, Oxford
- Gadian, D. G., Radda, G. K., Brown, T. R., Chance, E. M., Dawson, M. J. & Wilkie, D. R. (1981) *Biochem. J.* **194**, 215–228
- McIlwain, H. & Bachelard, H. S. (1985) *Biochemistry and the Central Nervous System*, 5th edn., Churchill-Livingstone, Edinburgh
- Nunnally, R. L. & Hollis, D. P. (1979) *Biochemistry* **18**, 3642–3646
- Shoubridge, E. A., Briggs, R. W. & Radda, G. K. (1982) *FEBS Lett.* **140**, 288–292
- Siesjö, B. K. (1978) *Brain Energy Metabolism*, John Wiley and Sons, Chichester
- Veech, R. L., Lawson, J. W. R., Cornell, N. W. & Krebs, H. A. (1979) *J. Biol. Chem.* **254**, 6538–6547