³¹P-n.m.r. studies on cerebral energy metabolism under conditions of hypoglycaemia and hypoxia *in vitro*

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

*Department of Biochemistry, St. Thomas's Hospital Medical School, London S.E.1, U.K., and †M.R.C. Biomedical N.M.R. Centre, National Institute for Medical Research, Mill Hill, London N.W.7, U.K.

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1. A system has been developed for performing ³¹P-n.m.r. studies on cerebral tissues superfused *in vitro*, and gives results comparable with those reported from studies *in vivo*. 2. Under optimal superfusion conditions [10 mM-glucose and O₂/CO₂ (19:1)] the tissue concentrations of phosphocreatine and ATP were calculated to be approx. 3.1 and 1.3 μmol/g respectively. 3. When the glucose of the superfusing medium was lowered to 0.5 mM, slightly decreased sugar phosphate peaks were observed, but there was no detectable change in [ATP] or [phosphocreatine]. At 0.2 mM-glucose, significantly decreased concentrations of phosphocreatine and ATP were observed. 4. Substitution of pyruvate plus malate for glucose did not decrease levels of phosphocreatine and ATP. 5. When the superfusing medium was gassed with air/CO₂ (19:1; 'mild hypoxia'), there was an appreciable fall in sugar phosphates and phosphocreatine with no detectable effect on ATP. In the presence of N₂/CO₂ (19:1; 'severe hypoxia', since O₂ was not completely excluded), concentrations of phosphocreatine fell considerably, but with little effect on ATP. 6. The results demonstrate the feasibility of studying cerebral energy metabolism *in vitro* using the non-invasive ³¹P-n.m.r. technique and are discussed in relation to the sensitivity of cerebral tissues to metabolic insults *in vitro* and *in vivo*.

It is generally accepted that the first signs of cerebral dysfunction occur in man or experimental animals when the arterial glucose falls from its normal concentration of 6–7 mM to approx. 2 mM. No cerebral energy deficit can be detected, however, until the arterial glucose concentration falls to below 1 mM, when the electroencephalogram becomes isoelectric and consciousness is lost (Lewis *et al.*, 1974; Ferrendelli, 1975; Bachelard, 1976). Further, in recovery experiments with injections of glucose to animals in deep hypoglycaemic coma, physiological and behavioural functions return rapidly, well before the normal energy state has been restored (Siesjö, 1978). It is therefore argued that neural functioning *in vivo* is more sensitive to decreases in available glucose than is cerebral energy metabolism – that function and overall energy metabolism do not change in parallel (Bachelard, 1976; Siesjö, 1978).

A similar apparent anomaly occurs in mild hypoxia: cerebral function, as seen in 'mountain sickness', becomes impaired at slightly lowered contents of O₂ in the respired air, when here also

there is no detectable change in the energy state (Bachelard *et al.*, 1974; Bachelard, 1978; Siesjö, 1978).

Some of these results have been mimicked *in vitro*. Thus evoked potentials from superfused hippocampal slices are attenuated at glucose concentrations of 2 mM, without detectable energy deficit (Cox & Bachelard, 1982).

In order to characterize in more detail cerebral energy metabolism under such metabolic insults we decided to see if it might be feasible to apply the non-invasive technique of ³¹P-n.m.r. to a superfusion system *in vitro*. This would allow various metabolic manipulations, and pharmacological interventions, to the immediate environment of the tissue, which are difficult to achieve *in vivo*. The potential value of ³¹P-n.m.r. techniques *in vivo* in aiding diagnosis of neurological problems has recently been investigated by others (D. R. Wilkie & O. Reynolds, personal communication); the advantages of the manipulations possible with such an *in-vitro* technique can be expected to contribute much to the interpretation of results obtained *in vivo*. Though ³¹P-n.m.r. techniques have been applied successfully

‡ To whom correspondence and reprint requests should be sent.

in vivo to the heads of small animals (Chance *et al.*, 1978; Ackerman *et al.*, 1980; Shoubridge *et al.*, 1982), no studies appear to have been reported for intact cerebral tissues incubated or superfused *in vitro*.

The results show that ^{31}P -n.m.r. techniques can be successfully applied to studies on cerebral tissues superfused *in vitro* and provide some interesting indications on the effects of hypoglycaemia and hypoxia on cerebral energy metabolism. Thus, although the tissue energy state is remarkably resistant to low glucose, it is very sensitive to low oxygen.

Methods

Tissue preparation

Adult male Dunkin–Hartley guinea pigs (300–350 g body wt.) were stunned and exsanguinated, and the brains were quickly removed. The sub-cortical tissues were excised and each cerebral hemisphere was sliced with a McIlwain chopper, either in two directions at right angles with 500 μm spacings, or in one direction to give slices of 350 μm thickness (McIlwain & Bachelard, 1971). The results given below were similar for the two types of preparation. Immediately the slices 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 poly(tetrafluoroethylene) insert carrying perfusion tubing and the shaft of a motor-driven glass stirrer (Fig. 1). The

cerebral cortices from five guinea pigs were required to provide sufficient tissues for each n.m.r. experiment. The incubation medium contained (mM): NaCl (124); KCl (5); KH_2PO_4 (1.2); MgSO_4 (1.2); CaCl_2 (1.2); NaHCO_3 (26); 10 mM-glucose was present for tissue preparation and control experiments. Gassing, for tissue preparation and the control experiments, was with O_2/CO_2 (19:1). The tissues within the n.m.r. tube were superfused with medium at rates of approx. $20\text{ ml} \cdot \text{min}^{-1}$, 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 using a Bruker WM 200 widebore 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 500 8K FIDs using 40° pulses repeated every 0.68 s; in some experiments these were repeated every 8 s. The spectral width was 5.55 kHz. Each block was individually recorded on magnetic disc for subsequent inspection. Spectra from the combined data of five blocks were then analysed. In experiments where the contents of the superfusing medium were varied, the medium was changed without disturbing the tissue or probe, with continuous recording of spectra.

Measurement of $p\text{O}_2$

The oxygen tension of the superfusing fluid, sampled immediately before entry to the n.m.r. tube, was determined by using an Instrumentation Laboratory 413 micro pH/blood gas analyser.

Results

Control spectra

The type of spectrum from the accumulation of one block of signals obtained with an interpulse interval of 8 s in control conditions [10 mM-glucose and O_2/CO_2 (19:1)] is shown in Fig. 2. A considerable proportion (65%) of the P_i peak arises from the phosphate in the superfusing medium, which is constant. The resonances are superimposed on an underlying broad signal similar to that observed *in vivo*, where it was attributed to the phosphate of bone (Shoubridge *et al.*, 1982). However, in view of the absence of bone from the present tissue preparation, it seems likely that their

Fig. 1. Diagram of superfusion apparatus within the n.m.r. magnet

Fig. 2. ^{31}P -n.m.r. spectrum of cerebral tissues superfused in medium containing 10 mM-glucose, gassed with O_2/CO_2 (19:1)

The spectrum represents the combined data from 500 8K FIDS, with interpulse intervals of 8 s, and with a line broadening of 12 Hz. 1, sugar phosphates; 2, P_i ; 3, phosphocreatine; 4, γ -peak; 5, α -peak; 6, β -peak (see the text).

broad signal is partly due to phospholipids that occur in high concentrations in cerebral tissues (McIlwain & Bachelard, 1971).

Since the spectrum of Fig. 2 was obtained with an interpulse interval of 8 s, sufficient to allow virtually complete recovery (over 97% from T_1 values) of the phosphorus nuclei of all tissue metabolites studied and of the medium phosphate (given the medium exchange time of approx. 2 min), the results can be used to estimate the absolute concentrations of the metabolites. The concentrations of phosphocreatine and of ATP were calculated from the peak areas of the spectrum, assuming an unbound tissue P_i content of $3\text{--}4\ \mu\text{mol}\cdot\text{g}^{-1}$ (McIlwain & Bachelard, 1971) superimposed upon a medium phosphate concentration of $1.2\ \mu\text{mol}/\text{ml}$, which contributes some 65% of the total P_i peak. Although it is not possible to measure the proportion of tissue phosphate that is non-covalently bound, which is therefore not measured by n.m.r. spectroscopy, any error is likely to be small in view of the relatively low contribution of the tissue phosphate to the total peak. The superfusion volume of 18 ml present within the receiver coil contained some 4 g of tissue. The calculated approximate tissue contents were $2.9\text{--}3.3\ \mu\text{mol}\cdot\text{g}^{-1}$ for phosphocreatine and $1.2\text{--}1.4\ \mu\text{mol}\cdot\text{g}^{-1}$ for ATP, which compare well with published *in-vitro* values (McIlwain & Bachelard, 1971). The calculated ratio of [phosphocreatine] to [ATP] does not depend on the accuracy of assessment of the tissue phosphate. Our value for this ratio

(2.3) agrees well with the value of 2.1 reported by Shoubridge *et al.* (1982) for rat brain *in vivo*.

Such experiments, with the long interpulse interval, are too time-consuming to allow much experimental variation during the time (6–7 h) the tissues remain viable. The studies described below, on the effects on the spectra of modifications to the medium constituents, were conducted with interpulse intervals of 0.68 s, which does not allow full relaxation of the phosphorus nuclei. Absolute metabolite levels cannot therefore be estimated directly but measurement of relative changes of an individual metabolite can be performed (Ackerman *et al.*, 1980). Under such conditions, with differential relaxation times for the various constituent phosphorus nuclei, the calculated [phosphocreatine]/[ATP] ratio was lower, at 1.37 (see Fig. 3a). The results were consistent and had the low standard deviations shown in Tables 1 and 2.

In comparing different metabolic conditions, the intensities of each peak in a given spectrum were measured and calculated as ratios relative to the P_i peak for that spectrum. The ratios for each experimental condition are expressed as percentages of the control values obtained for the same tissue superfused under control conditions. The results presented in Tables 1 and 2 are derived in this way from the FIDs collected over periods of about 30 min, usually started at least 17 min after changing the medium. The spectra used for measurement of peak intensities represent five blocks of combined

Fig. 3. *Effects of low [glucose] and low [oxygen] on ^{31}P -n.m.r. spectra of superfused cerebral tissues*
Spectra are as described for Fig. 2, except that the interpulse interval was 0.68 s (see the text). (a) and (c); control spectra (10 mM-glucose; O_2/CO_2 , 19:1), obtained during a 28 min period before changing superfusion medium. (b), Spectrum obtained over the time period of 40 to 68 min after changing the medium of (a) to 0.2 mM-glucose (O_2/CO_2 , 19:1). (d), Spectrum obtained over the time period of 23–51 min after changing the medium of (c) to 10 mM-glucose with air/ CO_2 (19:1); $p\text{O}_2$ was 16.4 kPa (see Table 2).

Table 1. *Effect of low [glucose] on ^{31}P -n.m.r. responses*

Tissue was superfused with medium gassed with O_2/CO_2 (19:1); experimental details are as described in the text. Results are height ratios based on the P_i peak, expressed as percentages of control (10 mM-glucose), and are means $\pm$ s.d. Where the number of experiments was <3 , the ranges are shown in parentheses. Concentrations of pyruvate plus malate were 2 and 0.2 mM or 10 and 1 mM respectively and results are means of the combined values from the two pairs of concentrations. Values were significantly different from control values at: $\dagger$, $P < 0.05$; $\ddagger$, $P < 0.005$.

Substrate (mM)	n	Peak-height ratio (% of control)				
		Sugar phosphates	Phospho-creatine	γ	α	β
Glucose (0.5)	3	$79 \pm 14\dagger$	98 ± 20	109 ± 46	83 ± 21	104 ± 27
Glucose (0.2)	5	$76 \pm 12\dagger$	$78 \pm 16\dagger$	80 ± 25	$80 \pm 23\dagger$	$74 \pm 25\dagger$
Glucose (0.1)	2	63 (58–68)	74 (65–84)	75 (69–81)	88 (69–108)	82 (80–83)
Pyruvate + malate	3	$84 \pm 11\dagger$	103 ± 6	102 ± 3	$107 \pm 5\dagger$	$108 \pm 6\dagger$

Table 2. *Effect of low [oxygen] on ^{31}P -n.m.r. responses*

Tissue was superfused as described in the text. $p\text{O}_2$ is the oxygen tension in the superfusing fluid immediately available to the tissue in the n.m.r. tube (see the Methods section). The $p\text{O}_2$ of control medium (gassed with O_2/CO_2 , 19:1) was 60.5 ± 0.4 kPa. Presentation of results is as described in the legend to Table 1.

Condition	Glucose (mM)	$p\text{O}_2$ (kPa)	n	Peak-height ratio (% of control)				
				Sugar phosphates	Phospho-creatine	γ	α	β
Air/ CO_2 (19:1)	10	16.4	4	$79 \pm 10\dagger$	$68 \pm 11\dagger$	107 ± 22	106 ± 14	96 ± 16
N_2/CO_2 (19:1)	10	6.0	3	99 ± 7	$46 \pm 12\dagger$	91 ± 13	109 ± 12	103 ± 15
Air/ CO_2 (19:1)	1	16.4	2	68 (53–83)	38 (30–47)	54 (40–68)	64 (45–84)	54 (36–72)

data (see the Methods section). Recovery experiments were performed by returning the tissues to control conditions; only in the case of 'severe hypoxia' (N₂/CO₂, 19:1) was restoration incomplete.

In the Figures and Tables, the 'γ-peak' represents resonances of the γ-phosphates of nucleoside triphosphates and the β-phosphates of nucleoside diphosphates. The 'α-peak' represents the resonances of the α-phosphate groups of the nucleoside diphosphates and triphosphates and the diester phosphate groups of the nicotinamide nucleotides (often seen as a shoulder); only the 'β-peak' solely reflects the resonance of the phosphate of a nucleoside triphosphate, largely ATP (Gadian, 1982).

Effects of low [glucose] and alternative substrates

Lowering the [glucose] of the superfusing medium from 10 mM to 0.5 mM significantly decreased sugar phosphates but had no effect on phosphocreatine or nucleotide peaks (Table 1). However, a further decrease of the [glucose] to 0.2 mM (Table 1; Fig. 3b) gave significant decreases in the intensities of all of the peaks, except for the γ-peak. Thus under these conditions, no change in energy state appears until the external glucose concentration is well below 0.5 mM. Examination of the time course of change in intensity of the phosphocreatine peak indicated that a new steady-state level had been obtained, rather than a gradual disappearance of the peak with time.

The combination of pyruvate plus malate is known to replace glucose adequately *in vitro*, to the extent that respiration and oxidative phosphorylation are unimpaired (McIlwain & Bachelard, 1971). In our experiments, when the cerebral tissues were superfused with pyruvate plus malate in the absence of glucose, there was no detectable change in [phosphocreatine], even after 1 h, but a significant decrease in the [sugar phosphates] and a very small statistically significant increase in [ATP] were noted (Table 1).

Effects of low [oxygen]

When the tissues were superfused with medium containing normal concentrations (10 mM) of glucose, but gassed with air/CO₂ (19:1; 'mild hypoxia'), the peaks of sugar phosphates and phosphocreatine decreased significantly (Fig. 3d) compared with those in the control spectra obtained with the same tissue preparation (Fig. 3c). As observed with low [glucose] (see above), from the time course of change the [phosphocreatine] was judged to have reached a new steady state. There was no detectable effect on [ATP] (Table 2).

In the severely hypoxic conditions obtained by gassing the superfusing medium with N₂/CO₂ (19:1) the [phosphocreatine] fell within 30 min to below 50% of control values (Table 2). Again there was

little detectable effect on [ATP]. The P_i peak was distorted, indicating an intracellular pH shift attributable to the lactic acidosis known to occur in hypoxia (Bachelard *et al.*, 1974; Siesjö, 1978). In contrast with the other conditions employed in these studies (low [glucose] or mild hypoxia), severe hypoxia caused the [phosphocreatine] to fall slowly and continuously throughout the period of exposure to the N₂/CO₂ (19:1). The medium was therefore replaced with control medium within 30 min of first exposure to the N₂. Recovery was abnormal in that the level of sugar phosphates decreased significantly to 72% of control values, whereas restoration of [phosphocreatine] occurred.

The results (Table 2) also show that the energy state of the tissue is more sensitive to low [glucose] in mild hypoxia than in normoxia. Decreasing the [glucose] from 10 mM to 0.5 mM in the presence of O₂/CO₂ (19:1) had no effect on the energy state (Table 1) but in the presence of air/CO₂ (19:1) 1 mM-glucose caused a further decrease in [phosphocreatine] (Table 2).

We regard the N₂/CO₂ (19:1) as severe hypoxia, rather than anoxia, because, as can be seen from the data of Table 2, some O₂ had leaked into the system despite the use of butyl rubber tubing of low gas-permeability.

Discussion

Siesjö (1978) has discussed the estimation of the oxygen tension at the mid-point between cerebral capillaries *in vivo* in normoxia, which is of the order of 50 mm Hg (6.7 kPa); the tension is thought to decrease to some 25 mm Hg (3.3 kPa) or less in arterial hypoxia, thus causing changes in behaviour and in the electroencephalogram. The results of Table 2 show that in air/CO₂ (19:1) the oxygen available to the tissue was 16.4 kPa (120 mm Hg), which would provide some 30–40 mm Hg at the centre of the tissue (see Umbreit *et al.*, 1964; McIlwain & Bachelard, 1971). Thus the changes we observed in [phosphocreatine] in the presence of air/CO₂ (19:1) may be concluded to occur at oxygen tensions that are just subnormal, so indicating a high vulnerability to oxygen. The contribution of dissolved oxygen to T₁ is known to be very small (Morgan & van Wazer, 1975; McCain & Markley, 1980), so our results on different oxygen tensions are unlikely to be due to differential effects on T₁.

This contrasts with our results for lowered [glucose] in O₂/CO₂ (19:1), where the tissue seems to be remarkably resistant. *In vivo*, as noted in the introduction, arterial glucose concentrations of around 2 mM are required to maintain normal neural function. Over 75% of the glucose consumed by the brain is thought to be utilized by the neurons (Siesjö, 1978) and the extracellular glucose immediately

available to the neurons has been suggested to be considerably less than the arterial concentration (Crone, 1980). The brain possesses a high-affinity transport system for glucose (K_m approx. 0.25 mM), shown to be present in isolated nerve-ending preparations (Diamond & Fishman, 1973; Heaton & Bachelard, 1973) and in cerebral cortex slices (Fletcher & Bachelard, 1978), and it may be of relevance that the threshold requirement for glucose in the results presented here (between 0.2 mM and 0.5 mM) is of the same order as the K_m value for this transport system. It is, however, much less than the threshold (above 2 mM) shown for synaptic activity *in vitro* (Cox & Bachelard, 1982), thus providing further evidence that cerebral vulnerability to low glucose is not simply due to its energy requirements. Work on cerebral vulnerability to hypoglycaemia and hypoxia has clearly demonstrated the sensitivity of acetylcholine synthesis to such insults (Gibson & Blass, 1976; Gibson & Duffy, 1981; Ksiezak & Gibson, 1981a,b; Harvey *et al.*, 1982). Thus in mild hypoglycaemia, labelling of acetylcholine from either labelled glucose or labelled choline was impaired under conditions where the energy state was normal. Furthermore it is known from the work of the same investigators that pyruvate (with malate) is a most adequate precursor for the acetyl moiety of acetylcholine *in vitro*; our results (Table 1) indicate that it adequately supports the energy state, yet it cannot support synaptic function (Cox & Bachelard, 1982). Therefore, although impairment of acetylcholine formation clearly occurs in hypoglycaemia and hypoxia, we must look for further effects of these metabolic insults, since not all the phenomena observed so far can be explained on this basis of impaired acetylcholine synthesis.

We are conscious of the fact that we (and others) judge the 'energy state' from steady-state levels of intermediates rather than turnover. A major contribution of the present paper is to demonstrate that sufficiently-high-quality ^{31}P n.m.r. spectra can be obtained from superfused slices to allow application of saturation transfer studies *in vitro* to provide indications of turnover (Brown, 1980). Further work with a view to a non-invasive study of the turnover of phosphocreatine under conditions similar to those reported here is now planned.

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