Glycerol 3-phosphate and lactate as indicators of the cerebral cytoplasmic redox state in severe and mild hypoxia respectively: a ^{13}C - and ^{31}P -n.m.r. study

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The incorporation of ^{13}C from $[1-^{13}\text{C}]\text{glucose}$ and $[2-^{13}\text{C}]\text{acetate}$ into selected intermediary metabolites in extracts prepared from incubated cerebral-cortex slices was monitored by using ^{13}C -n.m.r. spectroscopy under conditions of mild and severe hypoxia. Mild hypoxia had little effect on labelling of tricarboxylic-acid-cycle-related amino acids [glutamate, glutamine and γ -aminobutyrate (GABA)], although the pool sizes of glutamate and glutamine decreased. There were large increases in the labelling of lactate and of alanine, and an increase in the pool size of lactate. In severe hypoxia, the resonances of lactate and alanine remained high, whereas those of the other intermediates decreased greatly. The pool size of GABA increased. Calculation of percentage ^{13}C enrichments and total label incorporated showed that lactate was not further affected by severe hypoxia, but the total label in alanine and its pool size were further

increased. A new resonance appeared in the phosphomonoester region of the ^{13}C -n.m.r. spectrum only in severe hypoxia. This was unambiguously assigned to glycerol 3-phosphate from a combination of ^{31}P - and ^{13}C -n.m.r. spectroscopy. The percentage ^{13}C -enrichment was calculated from the ^{13}C -n.m.r. spectrum, and the total label incorporated was measured by g.l.c./m.s. The results are discussed in terms of the ability of lactate dehydrogenase to maintain normal levels of NADH in mild hypoxia, but not in severe hypoxia. The pyruvate which accumulates under the latter condition is channelled into alanine, and the increased NADH is reflected by the increase in glycerol 3-phosphate. We conclude that glycerol 3-phosphate and alanine may provide novel means of monitoring severe hypoxia, whereas lactate is a reliable indicator only of mild hypoxia.

INTRODUCTION

The first effects observed in hypoxia are a disruption of synaptic activity, and changes in the synthesis and/or release of neurotransmitters, before any effect on the energy state [phosphocreatine (PCr) and ATP] can be detected (for review, see Bachelard, 1990). Prolonged hypoxia is known to cause swelling of cells, with decreased extracellular space and structural damage to mitochondria analogous to the effects observed in ischaemia. Associated with these changes are increased rates of glycolysis with increased production of lactate and decreased pH. Alanine has also been reported to increase in hypoxia (Tews et al., 1963).

ATP is maintained at the expense of PCr, and the pseudo-first-order rate constant of the creatine kinase reaction which regenerates ATP is increased (Morris et al., 1985; Cox et al., 1988). The neurotransmitter systems affected by hypoxia include the amino acid transmitters [glutamate and γ -aminobutyrate (GABA)], and more recently the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor has been suggested to be implicated in the cellular damage which occurs in hypoxia and ischaemia (Olney, 1983).

The relative contributions of neurons and glial cells to the metabolism of glutamate, glutamine and GABA can be measured from their labelling from ^{13}C -labelled glucose and acetate, using ^{13}C -n.m.r. spectroscopy (Badar-Goffer et al., 1990, 1992). We have applied this technique to studies on the metabolism of these intermediates during mild and severe hypoxic conditions. The relatively low sensitivity of ^{13}C -n.m.r. spectroscopy only allows accurate measurement of intermediates of relatively high concentration ($> 0.1 \text{ mM}$).

The results show that a marked decrease in flux through the tricarboxylic acid cycle (TCA) occurs in severe, but not in mild, hypoxia. An unexpected result was that, although we saw the expected large increase in flux through glycolysis to lactate and to alanine in mild hypoxia, severe hypoxia increased the effect on alanine, but not on lactate. A new resonance appeared in severe, but not in mild, hypoxia, which we were able to confirm unequivocally as glycerol 3-phosphate. The observations are discussed in terms of the ability of lactate dehydrogenase to regulate intermediary metabolism in mild, but not in severe, hypoxia. The results suggest that lactate is a reliable indicator only of mild hypoxia, whereas glycerol 3-phosphate is a better indicator than lactate of changes in the cytoplasmic redox state in severe hypoxia.

MATERIALS AND METHODS

Materials

N-Methyl-*N*-t-butyltrimethylsilyltrifluoroacetamide ('MTBST-FA') was from Aldrich Chemical Co. $[1-^{13}\text{C}]\text{glucose}$ and $[2-^{13}\text{C}]\text{acetate}$ were from Omicron Biochemical Inc., Ithaca, NY, U.S.A. $^2\text{H}_2\text{O}$ was from Norsk Hydro, Oslo, Norway. Other chemicals and enzymes were from Sigma Chemical Co., Poole, Dorset, U.K.

Tissues

Tissues were prepared as previously described (Badar-Goffer et al., 1990). After the preincubation period the slices were transferred to labelled media, gassed with either air/ CO_2 (19:1) for mild hypoxia, or N_2/CO_2 (19:1) for severe hypoxia (Cox et al.,

1983), for 45 min. Neutralized HClO_4 extracts were prepared as previously described (Badar-Goffer et al., 1990). Labelled media contained 5 mM $[1-^{13}\text{C}]\text{glucose}$ (single label) or 5 mM $[1-^{13}\text{C}]\text{glucose}$ plus 5 mM $[2-^{13}\text{C}]\text{acetate}$ (double label).

N.m.r. spectroscopy

All studies were performed on a Bruker AM 400 wide-bore spectrometer operating at 100 MHz for carbon, 160 MHz for phosphorus and 400 MHz for protons, except for three mild-hypoxia experiments, which were carried out at the laboratories of Bruker Spectrospin Ltd., Coventry, U.K., with a Bruker AMX 400 narrow-bore spectrometer.

^{13}C -n.m.r. spectroscopy

^{13}C spectra were obtained by using 90° radiofrequency pulses and an interpulse interval of 4 s in a 10 mm ^{13}C probe head. Spectra were broad-band decoupled at 10 W, only during acquisition, in order to avoid differential nuclear Overhauser effects and to minimize sample heating. Blocks of 4000 scans were summated and multiplied by an exponential function, giving a line broadening of 4 Hz. Chemical shifts are given relative to an internal dioxan standard set at 67.4 p.p.m.

^{31}P -n.m.r. spectroscopy

^{31}P spectra were obtained by using 60° radiofrequency pulses and an interpulse interval of 2 s in a 15 mm broad-band probe head. Heteronuclear P-H coupling was eliminated by broad-band decoupling at 2 W. Chemical shifts of phosphorus resonances are given relative to phosphocreatine set at 0 p.p.m. Blocks of 2000 scans were summated and multiplied by an exponential function, giving a line broadening of 3 Hz.

^1H -n.m.r. spectroscopy

^1H spectra were obtained by using 90° radiofrequency pulses and an interpulse interval of 3.8 s in a 5 mm ^1H probe head. The intensity of the water signal was decreased by using a pre-saturation sequence at 2 W. Blocks of 500 scans were summated and multiplied by an exponential function, giving a line broadening of 0.25 Hz. Chemical shifts are given relative to the water signal set at 4.7 p.p.m.

G.l.c./m.s. of glycerol 3-phosphate

Derivative formation and quantification

Samples (100 μl) were taken from each n.m.r. sample and the pH was adjusted to 2.8 with HCl . *t*-Butylmethylsilyl derivatives of glycerol 3-phosphate were prepared as described by Mawhinney et al. (1986). Analysis was carried out on a Hewlett-Packard g.l.c./m.s. instrument: g.l.c. model 5891, m.s. model 5970 mass-selective detector. The molecular ion, m/z 571–575 ($M-57$), was monitored for glycerol 3-phosphate. Isotope abundance was determined from the abundance of each peak according to the formula of Biemann (1962).

Amino acid analysis

Samples for amino acid analyses were taken from the n.m.r. samples. The analysis was carried out on an LKB 4400 amino acid analyser.

Enzymic assays

Lactate and glycerol 3-phosphate was measured by the enzymic spectrophotometric methods described by Lowry and Passonneau (1972).

Percentage ^{13}C enrichment of metabolites was calculated as previously described by Badar-Goffer et al. (1990). Measurement of the T_1 for C-3 of glycerol 3-phosphate in tissue extracts gave a value of 1.46 s, which is within the range of the CH_2 - groups of the amino acids (Badar-Goffer et al., 1990), so the same method of calculation of its percentage ^{13}C enrichment was used.

RESULTS

Throughout these studies, routine ^{31}P -n.m.r. spectra were performed to monitor the extent of hypoxia, to confirm that mild hypoxia caused a decrease in PCr to approx. 70% of control with no change in ATP, and that severe hypoxia produced a further decrease in PCr to less than 50% of control (Cox et al., 1983).

The spectra obtained from slices incubated with 5 mM $[1-^{13}\text{C}]\text{glucose}$ are shown in Figure 1. From a comparison of Figures 1(a) and 1(b), it is clear that there is little, if any, effect of mild hypoxia on the labelling of TCA-related amino acids, including GABA. There is a considerable increase in the CH_3 resonance of lactate, and the appearance of a distinct resonance of the CH_3 of alanine (Figure 1b) which is not detected in the control, normoxic, spectrum (Figure 1a). A spectrum obtained (Figure 2) of an extract of severely hypoxic tissue is quite different in that, whereas the resonances of lactate and alanine remain high, all of the other resonances are greatly decreased. ^1H -n.m.r. observation of the ^{13}C -labelled lactate in the severely hypoxic extract (Figure 3) shows the triplet reflecting the protons attached to the ^{12}C and ^{13}C atoms, from which the percentage ^{13}C enrichment of the lactate can be accurately determined (Badar-Goffer and Bachelard, 1991). In this study it was close to the theoretical maximum, at 46%. The pool sizes (Table 1) of the TCA-related amino acids are changed only slightly in mild

Figure 1 ^{13}C -n.m.r. spectra of extracts prepared from tissues incubated for 45 min with 5 mM $[1-^{13}\text{C}]\text{glucose}$

(a) Control; (b) mild hypoxia. Peaks: 1, glutamate C2; 2, aspartate C2; 3, GABA C4; 4, aspartate C3; 5, GABA C2; 6, glutamate C4; 7, glutamate C3; 8, lactate C3; 9, alanine C3. The values shown in Table 2 were calculated from internal dioxan standards (Badar-Goffer et al., 1990).

Figure 2 ^{13}C -n.m.r. spectra of extracts prepared from tissues incubated for 45 min with 5 mM $[1-^{13}\text{C}]$ glucose under severely hypoxic conditions

Peaks: 1, glycerol 3-phosphate; 2, glutamate C2; 3, GABA C2; 4, glutamate C4; 5, glutamate C3; 6, lactate C3; 7, alanine C3. The spectra are not plotted to the same scale as for Figure 1, having been performed on different facilities (see the acknowledgements), so the heights of the resonances in Figures 1 and 2 cannot be compared directly. The values shown in Table 2 were calculated from internal dioxan standards (Badar-Goffer et al., 1990).

Figure 3 ^1H -n.m.r. spectrum of the lactate resonance of Figure 2

hypoxia; in severe hypoxia there are also only small changes, except for GABA, which is increased as previously reported (Wood, 1967). Lactate is increased in mild hypoxia, but not

Figure 4 ^{13}C -n.m.r. spectra of extracts prepared from tissues incubated for 45 min with 5 mM $[2-^{13}\text{C}]$ acetate plus 5 mM $[1-^{13}\text{C}]$ glucose

(a) Control; (b) severe hypoxia. Peaks: 1, glycerol 3-phosphate; 2, aspartate C2; 3, glutamate C2; 4, citrate C2/C4; 5, aspartate C3; 6, GABA C2; 7, glutamate C4; 8, glutamine C4; 9, glutamate C3; 10, acetate C2 (artefact from medium); 11, lactate C3; 12, alanine C3.

further increased in severe hypoxia, whereas alanine levels are only increased in severe hypoxia (Table 1).

Labelling of glutamine from $[1-^{13}\text{C}]$ glucose is difficult to detect at the relatively short time intervals (45 min) used here; however, glutamine is clearly labelled when $[2-^{13}\text{C}]$ acetate is the precursor (Badar-Goffer et al., 1990). The spectra of Figure 4, obtained from slices incubated in the presence of a combination of 5 mM $[2-^{13}\text{C}]$ acetate with 5 mM $[1-^{13}\text{C}]$ glucose, show that the effects of hypoxia on labelling of glutamine follow a similar pattern, in that a large decrease in labelling of glutamine was observed in severe hypoxia.

The percentage ^{13}C enrichments of the individual metabolites are shown in Table 2. The μmol of ^{13}C in each metabolite/100 mg of protein were calculated from the percentage ^{13}C enrichments and the pool sizes. These confirm the lack of effect of mild hypoxia on the TCA-related amino acids; there was a massive decrease in labelling of glutamate and of glutamine in severe hypoxia, but the decrease in labelling of GABA was less, approx 50% of control. The values also demonstrate the increases in labelling of lactate and alanine in mild hypoxia; however, whereas the pool size and total label in alanine increase in severe hypoxia, there was no further change in lactate.

The spectra of Figures 2 and 4 show an unexpected resonance at 65.7 p.p.m. in severe hypoxia, which was not observed in mild hypoxia or normoxia. This resonance was tentatively assigned as

Table 1 Pool sizes

Values are means $\pm$ S.D. for the numbers of individual experiments shown in parentheses, determined by amino acid analysis or enzymic assay (see the Materials and section): * $P < 0.01$.

	Metabolite pool size ($\mu\text{mol}/100$ mg of protein)					
	Glutamate	GABA	Glutamine	Aspartate	Alanine	Lactate
Control (6)	8.6 ± 1.4	1.2 ± 0.2	2.3 ± 0.6	1.9 ± 0.3	0.15 ± 0.1	1.5 ± 0.6
Mild (3)	5.8 ± 0.9	1.4 ± 0.1	$1.4 \pm 0.1^*$	1.2 ± 0.1	0.2 ± 0.0	$2.8 \pm 0.9^*$
Severe (6)	6.7 ± 1.3	$2.3 \pm 0.2^*$	1.6 ± 0.7	1.5 ± 0.3	$0.5 \pm 0.1^*$	2.9 ± 0.7

Table 2 % ^{13}C enrichments from n.m.r. spectra

Tissues were incubated for 45 min in the presence of 5 mM $[1-^{13}\text{C}]$ glucose (single label) or 5 mM $[1-^{13}\text{C}]$ glucose plus 5 mM $[2-^{13}\text{C}]$ acetate (double label) as described in the Materials and methods section. Values are means $\pm$ S.D. for numbers of individual experiments shown in parentheses. Total label (μmol of ^{13}C in each metabolite/100 mg of protein) is calculated from the % enrichment and the pool size. The percentage values are total label as % of control. Abbreviation: U.D., undetectable in the spectra.

	^{13}C enrichment (%)				
	Glutamate C4	GABA C2	Glutamine C4	Alanine C3	Lactate C3
Single label					
Control (4)					
Enrichment (%)	12.5 ± 3.4	17.0 ± 5.6	U.D.	U.D.	22.0 ± 0.3
Total label	107.5	20.4	—	—	33.0
Mild hypoxia (3)					
Enrichment (%)	14.0 ± 3.7	13.7 ± 3.1	U.D.	24.7 ± 4.6	25.2 ± 5.2
Total label	81.2 (75%)	19.2 (94%)	—	5.0 ?	70.5 (214%)
Severe hypoxia (3)					
Enrichment (%)	0.43 ± 0.07	3.7 ± 3.5	U.D.	17.4 ± 4.3	25.5 ± 7.8
Total label	2.9 (2.7%)	8.5 (42%)	—	8.7 ?	74 (229%)
Double label					
Control (4)					
Enrichment (%)	14.1 ± 1.6	13.7 ± 2.2	8.2 ± 0.8	5.6	21.5 ± 3.2
Total label	121.3	16.5	18.9	0.85	32.3
Severe hypoxia (3)					
Enrichment (%)	1.2 ± 0.7	3.7 ± 3.5	U.D.	16.4 ± 5.1	31.1 ± 13.8
Total label	8.0 (6.6%)	8.5 (52%)	—	8.2 (965%)	90.2 (279%)

Figure 5 ^{31}P -n.m.r. spectrum of the extract (severe hypoxia) of Figure 2

Peaks: 1, 2, phosphomonoesters; 3, P_i; 4, 5, phosphodiesters; 6, PCr; 7, γ -ATP; 8, β -ADP; 9, α -ADP; 10, α -ATP; 11, NAD/NADP; 12, β -ATP. The arrowed resonance at 7.48 p.p.m. (a) is that which we assigned as glycerol 3-phosphate. The inset (b) shows an expanded view of the 6–8 p.p.m. region collected without broad-band decoupling, where the resonance appears as a triplet with a heteronuclear J_{PH} constant of 7 Hz.

glycerol 3-phosphate, in view of the high activity of glycerol 3-phosphate dehydrogenase in the brain (McIlwain and Bachelard, 1985). A ^{31}P -n.m.r. spectrum of the same extract was therefore performed, since if it was a phosphorylated intermediate it might be detectable in a ^{31}P -n.m.r. spectrum. The new resonance in the phosphomonoester ester region at 7.48 p.p.m. of Figure 5 occurs with a chemical shift consistent with glycerol 3-phosphate. The

sample was then re-run without proton broad-band decoupling (see the Materials and methods section) and showed in the ^{31}P -n.m.r. spectrum as a triplet (inset, Figure 5) with a heteronuclear coupling constant (J_{PH}) of 7 Hz. Enzymic analysis confirmed that the concentration of glycerol 3-phosphate was increased in severe hypoxia. The calculated ^{13}C enrichments of glycerol 3-phosphate in hypoxia are shown in Table 3.

Table 3 Labelling of glycerol 3-phosphate

Values are means $\pm$ S.D. for the numbers of individual experiments shown in parentheses. Pool sizes are $\mu\text{mol}/100$ mg of protein, from enzymic assay. Abbreviation: U.D., undetectable in n.m.r. spectra.

		Pool size	^{13}C n.m.r.*	G.l.c./m.s.†	Total label‡
Control	(9)	0.028 ± 0.015	U.D.	7.9 ± 1.6	0.24
Mild hypoxia	(6)	0.078 ± 0.036	U.D.	15.5 ± 3.0	1.24
Severe hypoxia	(8)	0.76 ± 0.21	13.4 ± 2.4	31.6 ± 4.4	24.0

* % ^{13}C enrichment.

† Atoms % excess (APE).

‡ Total label (μmol of ^{13}C in each metabolite/100 mg of protein) is calculated from the pool size and the APE.

Table 4 NADH and glycerol 3-phosphate in hypoxia

Values (means $\pm$ S.D.) for NADH and NAD^+ are nmol/100 mg of protein, determined in a previous study (Garafalo et al., 1988) and those for glycerol 3-phosphate are mmol/100 mg of protein, estimated enzymically† (see Table 3) and from the ^{31}P -n.m.r. spectrum of Figure 5‡. (n) is the number of individual experiments; * $P < 0.01$. N.D., not detectable in the n.m.r. spectra.

Condition	n	NADH	NAD^+	NADH/ NAD^+	Glycerol 3-phosphate†	Glycerol 3-phosphate‡
Control	41	43 ± 3	301 ± 17	0.14	0.028 ± 0.015	N.D.
Air/ CO_2	9	55 ± 11	336 ± 59	0.16	0.078 ± 0.036	N.D.
N_2/CO_2	5	$89 \pm 13^*$	315 ± 12	0.30^*	$0.76 \pm 0.21^*$	0.77

DISCUSSION

What seems surprising at first sight is that, though we see the expected large increase in pool size and labelling of lactate in mild hypoxia, this is not increased significantly in severe hypoxia. There is also an increase in labelling of alanine in mild hypoxia, but, in contrast with lactate, the total label in alanine is increased further in severe hypoxia, due to the increase in its concentration. There is very little detectable glycerol 3-phosphate in mild hypoxia, but a considerable increase in its level and labelling in severe hypoxia. It is noteworthy that Glonek et al. (1982) observed in their ^{31}P -n.m.r. studies on tissue extracts an increase in glycerol 3-phosphate when intermediary metabolism was inhibited by cyanide.

These results are compatible with previous biochemical results from our laboratories, i.e. we saw no significant increase in the NADH/NAD^+ ratio in mild hypoxia, but a sharp increase in severe hypoxia (Garafalo et al., 1988). These comparisons are summarized in Table 4.

In mild hypoxia, the inhibition of oxidative phosphorylation in the electron-transport system results in a decrease in the rate of pyruvate oxidation by the TCA and a consequent tendency to increase the level of NADH. The build-up of pyruvate and NADH is minimized by lactate dehydrogenase, resulting in an increase in lactate. However, the ability of lactate dehydrogenase to compensate for (or buffer) these changes in the redox state would appear to be limited; at more severe stages of hypoxia,

this buffering ability is overcome, so the accumulation of NADH cannot be controlled by lactate dehydrogenase; an increase in glycerol 3-phosphate thus occurs, due to the NAD^+ -dependent glycerol 3-phosphate dehydrogenase reaction, which has been suggested to be a glial enzyme (Leveille et al., 1980). The tendency to build up pyruvate is then reflected in the accumulation of alanine. It would be of interest to know what happens to the increased alanine: is it exported to the bloodstream or does it interact within the brain? This may be important in the consequences of severe hypoxia. If the increase in glycerol 3-phosphate is occurring essentially in glial cells, and bearing in mind the evidence that has been reported to indicate that lactate is produced mainly by glia (Walz and Mukerji, 1990; R. Dringen, R. Gebhardt and B. Hamprecht, unpublished work), this may provide further evidence for suggestions (Tholey and Ledig, 1990) that glial cells are particularly vulnerable to hypoxia.

Most of the NADH occurs in the cytoplasm rather than in mitochondria (Garofalo et al., 1988). It seems possible that glycerol 3-phosphate can provide a novel means of monitoring changes in the cytoplasmic NADH/NAD^+ redox state in severe hypoxia, whereas lactate may be a more reliable indicator for changes in the cytoplasmic redox state in mild hypoxia. If this tentative interpretation is valid, then a complete assessment of the redox state in various stages of hypoxia might depend on an ability to monitor changes in both lactate and glycerol 3-phosphate.

We are grateful to Alex Gallagher for technical assistance, to Dr. Ruth Dixon for useful discussions on the assignment of glycerol 3-phosphate, to Bruker Spectrospin Coventry for use of their ^{13}C -n.m.r. facilities while our machine was being installed, to Mrs. Juliet Jacoby, Department of Biochemistry, University of Cambridge, for the amino acid analyses, and to the M.R.C. for financial support. We also thank the Biochemical Society for the award of the Krebs Memorial Fellowship to O. B.-Y.

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