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A MICROCALORIMETRIC STUDY OF TURTLE CORTICAL SLICES: INSIGHTS INTO BRAIN METABOLIC DEPRESSION

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Summary

In previous papers, we have examined turtle cortical neurons *in vitro* for mechanisms of anoxic metabolic depression ('channel arrest' and changes in electrical parameters). Negative results prompted the current study with the aim of examining more closely the energy profile and metabolism of turtle cortical slices. Calorimetry is used to measure heat dissipation during normoxia and nitrogen perfusion (120 min) and the results are converted into an ATP utilization rate. These indicate that the control rate of ATP utilization (1.72 μmol ATP g^{-1} min^{-1}) agrees closely with *in vivo* whole-brain metabolic measurements. Both nitrogen perfusion and pharmacologically induced anoxic (cyanide+N_2) groups depressed heat dissipation considerably compared with the control value (nitrogen 37 %; pharmacological anoxia 49 %). The resulting ATP utilization estimates indicate metabolic depressions of 30 % (nitrogen) and 42 % (pharmacological anoxia). The slice preparation did not exhibit a change in any measured adenylate parameter for up to 120 min of anoxia or pharmacological anoxia. Significant changes did occur in [ADP], ATP/ADP ratio and energy charge after 240 min of exposure to anoxic conditions. These results support the idea that the turtle cortical slice preparation has a profound resistance to anoxia, with both nitrogen perfusion and pharmacological anoxia causing a rapid decline in heat dissipation and metabolism.

Introduction

Long-term anoxic survival of the freshwater turtle (*Chrysemys picta*) is well documented in the literature, with individuals surviving forced submergence for up to 6 months at 3 °C (Ultsch, 1985). The high sensitivity of the mammalian brain to anoxia and ischemia (Hansen, 1985) has led to the turtle brain being used as a comparative model for anoxic studies. However, only recently have healthy *in vitro* preparations of turtle brain become available. Three such preparations are the turtle brain stem slice preparation

Key words: turtle, *Chrysemys picta*, anoxia, calorimetry, metabolic depression, adenylates, cortical neurons.

(Jiang *et al.* 1992), the whole cerebellum preparation (Pérez-Pinzón *et al.* 1992) and the cortical slice preparation (Connors and Kreigstein, 1986; Doll *et al.* 1991*a*,*b*).

Several biochemical mechanisms are currently believed to aid the turtle brain in surviving anoxia (see Doll, 1993; Lutz, 1992, for reviews). In particular, two adaptations: (i) a low normoxic metabolic rate, and (ii) the ability to suppress metabolism further during anoxia are believed to be central because they make the largest contributions to acute anoxic survival time.

Metabolic depression was first measured in the turtle using calorimetry. The initial experiments indicated that turtle whole-body heat dissipation decreased by 85 % during anoxia (Jackson, 1968). Whole-body calorimetry has also been used to measure metabolic depression in other anoxia-tolerant animals with similar results. Considering ectothermic vertebrates, both the goldfish (*Carassius auratus*) (van Waversveld *et al.* 1988) and the toad (*Bufo bufo*) (Leivestad, 1960) have shown heat depressions of 70–80 % during anoxia. Although some work on whole-body heat depression in anoxia-tolerant species has been reported, little is known about individual tissues such as the brain.

It is clear that turtle brain preparations *in vitro* are able to survive extended periods of anoxia compared with similar preparations from rats (Doll *et al.* 1991*b*; Jiang *et al.* 1992; Pérez-Pinzón *et al.* 1992). Whether the *in vitro* preparations fully mimic the *in vivo* tissue has not been established with respect to maintaining energy balance and metabolic rate. In previous anoxic studies, we have examined both electrical parameters (Doll *et al.* 1991*b*) and resting ion leakage (Doll *et al.* 1991*a*, 1993) in the turtle cortex as possible mechanism(s) for metabolic depression. Measured parameters did not change, suggesting that turtle cortical metabolism may not be changing with anoxia. In the present study, we utilize microcalorimetry under both aerobic and anaerobic conditions to examine turtle cortical slice metabolism.

Materials and methods

Slice preparation

Turtles (*Chrysemys picta*), weighing 150–300 g, were cold-anesthetized before decapitation. The brain was rapidly dissected free and immersed in artificial cerebrospinal fluid (aCSF) which had been precooled and equilibrated with 95 % O_2/5 % CO_2. The cortical tissue was then dissected free as previously described (Connors and Kreigstein, 1986). It should be noted that the cortical slice in this study refers to a cortical sheet peeled away from the brain. These cortical sheets were not sliced and were used whole. Once dissected free, cortical slices were stored at room temperature (22 ˚C) in a recirculating holding chamber until their use in the calorimeter or incubation chamber.

Artificial cerebrospinal fluid

The aCSF for the turtle was a modification (Connors and Kreigstein, 1986) which consisted of (in mmol l^{-1}): 96.5 NaCl, 2.6 KCl, 2.5 $CaCl_2$, 2.0 $MgCl_2$, 2.0 NaH_2PO_4, 10 glucose and 26.5 $NaHCO_3$, with 0.03 Phenol Red as a pH indicator. Final pH of the

solution was 7.4 when saturated with 95 % O_2/5 % CO_2. To mimic anoxia, the aCSF was equilibrated with 95 % N_2/5 % CO_2. Pharmacological anoxia used the same solution as anoxia but, in addition, contained 1 mmol l^{-1} NaCN.

Adenylates

Slices used in the adenylate measurements were incubated (in a recirculating slice chamber) for 2 h in oxygenated aCSF before the experiment was commenced. After incubation, the slices were transferred to 60 ml recirculating semiclosed chambers. All gases flowed through gas-impermeable Viton tubing. A slight but constant positive pressure was maintained in the slice-holding chamber to maximize aCSF gas saturation and minimize contamination from the atmosphere. To ensure anoxia was maintained in the slice chambers, they were tested for oxygen contamination using a Radiometer gas analyzer and O_2 electrode. Upon completion of the incubation, slices were immediately immersed in precooled (−5 °C), preweighed vials of 5 % perchloric acid and weighed (total time approximately 20 s). Tissue preparation for HPLC analysis was similar to techniques previously described (Buck *et al.* 1993).

Chromatography

All adenylates used in these experiments were measured using high performance liquid chromatography (HPLC) using an LKB 2152 HPLC controller and 2150 titanium pump coupled to a 2220 recording integrator as previously described (Schulte *et al.* 1992).

Microcalorimetry

Heat dissipation measurements on turtle cortical slices were performed with an LKB 2277 thermal activity monitor equipped with a 3.5 ml perfusion chamber. The perfusion aCSF was identical to that described above for normoxia, anoxia and pharmacological anoxia. Perfusion aCSF was held in semiclosed glass bottles kept at 25 °C in a circulating water bath. Flow lines for gases and aqueous media were made of Viton and stainless steel, respectively. The flow rate of the perfusion media through the slice and chamber was set at 15.0 ml h^{-1}, and the temperature was maintained at 25 °C for all experiments. Heat dissipation in microwatts was time-corrected by the calibration unit of the calorimeter; typical time constants were t_1=600 s and t_2=17 100 s. Based on a square-wave heat signal for static calibration and a second dynamic calibration, the microprocessor of the calorimeter corrected the raw data to compensate for the instrumental lag. Deconvolution was accurate to 5 % for 2 min intervals (Fig. 1). Heat measurements were taken every 60 s and stored on an IBM XT personal computer. For all experiments, blanks (no slices) were run in parallel and these results were subtracted from the measurements obtained with slices.

Two cortical slices from a single turtle were prepared as described above. After calibration of the instrument, the slices were placed in the perfusion chamber and lowered into the calorimeter. Heat dissipation was then monitored for a minimum of 1 h before data collection was begun. Total time from dissection to collection of data was approximately 2 h. The normoxic solution was switched to nitrogen perfusion or pharmacological anoxia once heat dissipation of the slices had stabilized to less than

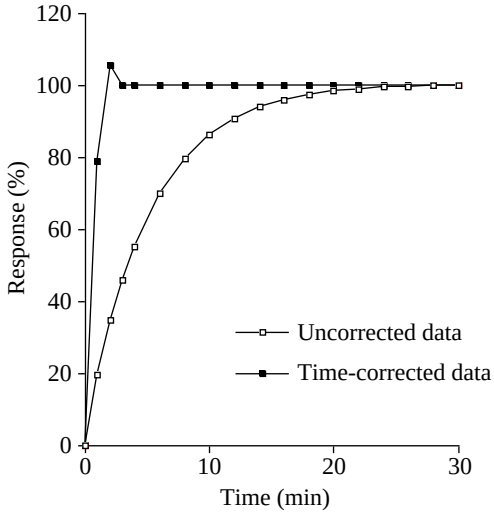

Fig. 1. Accuracy of the time resolution for the calorimetry signal. At time zero, the internal calibration heater generated a 100 μW, square-wave signal. Values are means ± 1 S.D. (N=8). No error bars are given because the standard deviation was within the size of the symbol.

0.2 μW min^{-1}. During perfusion with nitrogen-saturated medium, previous measurements indicated that a period of 60–90 min was needed at similar flow rates to reduce oxygen content below 0.5% air saturation in the excurrent flow (Hand and Gnaiger, 1988). For nitrogen perfusion experiments, nitrogen-equilibrated aCSF was switched to normoxic aCSF to monitor recovery. The experiment was terminated once heat dissipation had returned to predicted levels or stabilized.

Calorimetric data analysis

Data collected with an IBM XT were imported into CA-Cricket Graph III for the Macintosh and corrected for baseline drift. ATP turnover estimates were derived using a conversion factor of 0.76 μmol ATP g^{-1} min^{-1} mW^{-1} for aerobic heat dissipation and 0.86 μmol ATP g^{-1} min^{-1} mW^{-1} for anaerobic heat dissipation with the following assumptions and conversion factors.

Aerobic conditions

(1) The turtle slice is using only glucose or glycogen as a fuel (Clark and Miller, 1973; Doll *et al.* 1991*b*; Pérez-Pinzón *et al.* 1992). (2) The aerobic catabolism of glucose to CO_2 and H_2O yields −469 to −476 kJ mol^{-1} O_2 (Gnaiger and Kemp, 1990). (3) For glucose, 6 moles of ATP are produced for every mole of O_2 consumed.

Anaerobic conditions

(1) The turtle slice is using only glucose as a fuel (Clark and Miller, 1973; Doll *et al.* 1991*b*; Pérez-Pinzón *et al.* 1992). (2) Lactate is the only anaerobic end product produced (Robin *et al.* 1979). (3) The catabolism of glucose to lactate yields −70 kJ mol^{-1} lactate

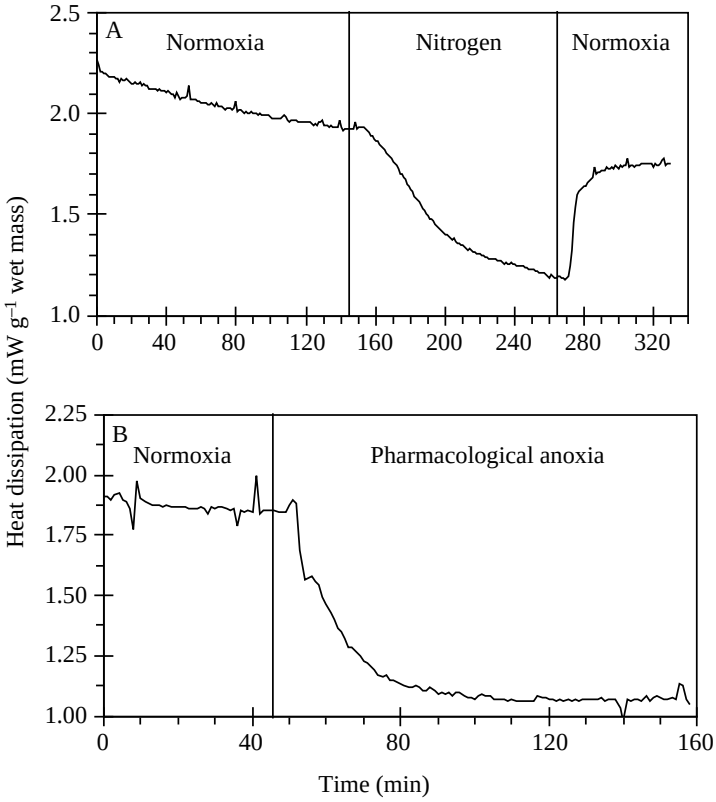

Fig. 2. Representative chart recordings of heat dissipation during nitrogen perfusion (A) and pharmacological anoxia (B) for turtle cortical slices (25 ˚C). Vertical lines indicate the time at which the indicated solution entered the calorimeter slice chamber.

(Gnaiger and Kemp, 1990). (4) Production of 1 mole of lactate from glucose provides 1 mole of ATP.

Suppliers

All chemicals were purchased from Sigma, St Louis, MI; turtles were purchased from Lemberger, Oshkosh, WI; statistics were performed using Systat, Jandel Inc., Evanston, IL, and StatView SE+Graphics, Abacus Concepts, Inc., Berkeley, CA; graphs were produced using CA-Cricket Graph III, Computer Associates, San Jose, CA. The LKB thermal activity monitor was purchased from LKB-Thermonmetic AB, Jarfalla, Sweden. Viton tubing was purchased through Cole Palmer, Chicago, IL.

Results

Typical calorimetry recordings are illustrated in Fig. 2A,B. The results demonstrated that cortical cells depress heat flow under both treatments (Fig. 3A,B). Both nitrogen perfusion and pharmacological anoxia produced rapid declines in heat dissipation, but

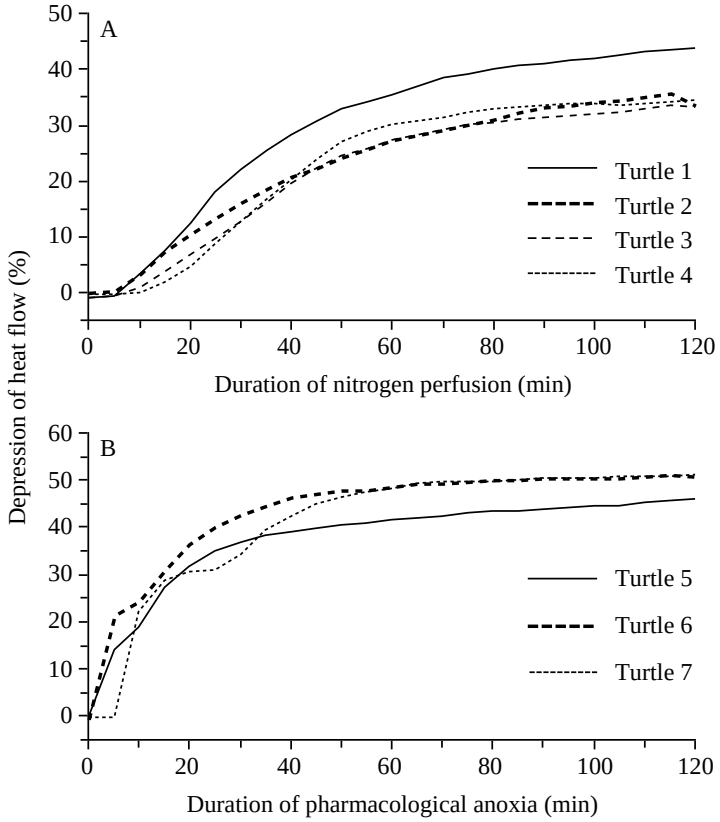

Fig. 3. The individual percentage heat depression from the predicted corresponding control value during nitrogen perfusion (A) and pharmacological anoxia (B) for all tested slices. Time zero indicates the point at which the test solution first entered the calorimeter chamber.

pharmacological anoxia produced a significantly ($P \leqslant 0.05$) more rapid effect (Table 1), because of the slow washout of oxygen. After 60 min of treatment, the rates of change were $6.3 \pm 0.8\,\% \,h^{-1}$ for nitrogen perfusion and $3.1 \pm 0.8\,\% \,h^{-1}$ (mean ± S.E.M.) for pharmacological anoxia. Nitrogen perfusion followed by normoxia returned heat flow to the predicted control level in all trials; an example is shown in Fig. 2A. Average heat depression (expressed as a percentage of control) by the end of the treatment was significantly smaller in the nitrogen perfusion group than in the pharmacological anoxia group (Table 1).

Observations of heat flow depression indicated highly significant differences between the control rate of heat dissipation and both treatment groups by 120 min (Table 1). Similarly, the ATP utilization rates based on these heat dissipation values indicated large differences between control and treatment groups by the end of the treatment (Table 1). Because heat output under aerobic conditions tended to drop slowly over the experiment, an initial control ATP turnover rate (Table 1) was calculated for comparison with other experimental preparations.

Adenylate concentrations (ATP, ADP and AMP) showed no significant changes under

Table 1. *Comparison of heat dissipation, ATP utilization and percentage depression of heat flow in turtle cortical slices*

	Initial[a,b] *N*=8	Nitrogen[a] *N*=4	Pharmacological anoxia[a] *N*=3
Heat dissipation (mW g^{-1})			
Control[c]	2.26±0.08	1.79±0.03	1.87±0.04
120 min	–	1.14±0.05*	0.95±0.05*
ATP utilization (μmol g^{-1} min^{-1})			
Control[c]	1.72±0.06	1.36±0.03	1.42±0.03
120 min	–	0.97±0.04*	0.82±0.04*
Percentage heat depression (of control)[c]			
20 min	–	8.6±1.8	32.8±1.8
120 min	–	36.5±2.6†	49.3±1.7†

[a]All values are means ± S.E.M.

[b]Initial values represent the first accurately recorded heat values of the tissue.

[c]Control values are predicted normoxic values at 120 min based on the curve-fitting procedures described in Materials and methods.

*Significantly different from the corresponding control value ($P \leqslant 0.001$; paired *t*-test).

†Significantly different for the pharmacological anoxia treatment group ($P \leqslant 0.05$; unpaired *t*-test).

control conditions ($P \leqslant 0.05$; Fig. 4A–C). The 240 min ADP levels for both treatment groups were significantly higher than the corresponding control values ($P \leqslant 0.05$; Fig. 4B). [IMP] did not change significantly under control or treatment conditions ($P > 0.05$; Fig. 4D). Differences among adenylates with anoxia and pharmacological anoxia were more apparent when expressed as energy charge (EC) or adenylate (ATP/ADP) ratios. Both anoxia and pharmacological anoxia produced significant differences in EC and ATP/ADP ratios at 240 min compared with equivalent control values ($P \leqslant 0.05$; Fig. 4E,F).

Discussion

The goals of this study were twofold: (i) to measure adenylate pools during anoxia and (ii) to establish whether there is a metabolic depression in the turtle cortical slice during oxygen deprivation. Adenylates were measured in this experiment for two reasons. First, although some studies with these metabolites have been carried out on the whole turtle brain, very little is known about individual tissue compartments in the brain and their changes during anoxia. Second, one assumption made in calculating ATP turnover directly from heat dissipation was that energy intermediates remain in a steady state (Shick *et al.* 1983). The control and anoxic [ATP] in the present study are similar to normoxic [ATP] reported in cortical slices (Bickler, 1992). Additionally, both control and anoxic concentrations for all adenylates reported here are similar to adenylate concentrations reported in whole brain studies *in vivo* (Kelly and Storey, 1988; Lutz *et al.* 1984). Lutz *et al.* (1984) reported an initial drop in the levels of all adenylates; however, both Lutz *et al.* (1984) and Kelly and Storey (1988) reported that adenylate levels were

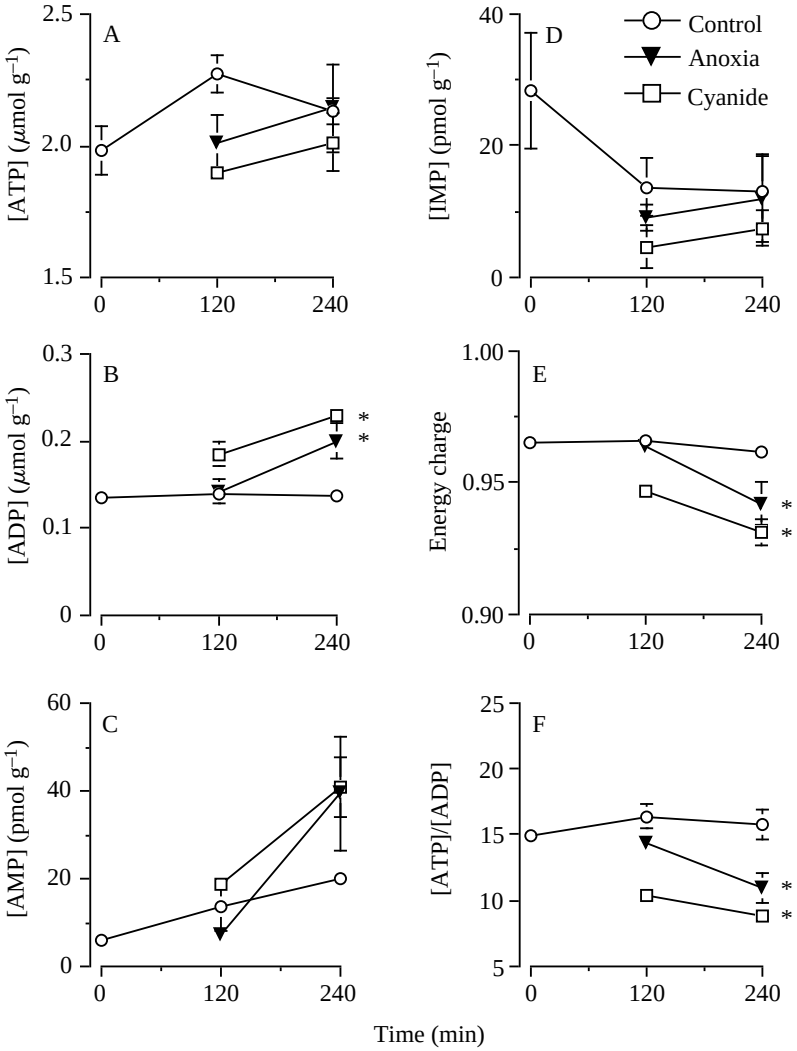

Fig. 4. The effect of anoxia and pharmacological anoxia on adenylate concentrations, energy charge and adenylate ratios for turtle cortical slices (25 °C). An asterisk denotes a significant difference for that particular mean value compared with the corresponding control mean value ($P \leqslant 0.05$; Tukey HSD test). Data (N=4) are mean values ± S.E.M. Concentrations are expressed as μmol g^{-1} wet mass (ATP; ADP) and pmol g^{-1} wet mass (AMP; IMP). Where error bars are absent, the standard error was within the size of the symbol.

similar to control adenylate concentrations by 1 h into anoxia. Our adenylate concentrations are similar to corresponding control concentrations after 120 min. Kelly and Storey (1988) further reported that by 6 h of anoxia all adenylate levels (ATP, ADP, AMP) had surpassed control levels by at least a factor of two. The present study also indicates a possible increasing trend, but at 240 min only [ADP] is significantly different from the corresponding control value (Fig. 4). The reason for the increases in overall adenylate pools is not understood, but it may be due to anoxic tissue dehydration. The

increases in [ADP] and [AMP] compared with the smaller increase in [ATP] resulted in a significant decrease in both energy charge and adenylate ratio at 240 min, which suggests possible tissue degeneration. However, the anoxic [ATP] is not significantly different from the control value, supporting the contention that the tissue remained healthy. Thus, [ATP] may be a better estimate of tissue viability in this preparation than adenylate ratios or energy charge because of overall increases in tissue adenylate concentration.

The neuromodulator adenosine has been implicated as a possible agent causing the initial anoxic electrical arrest observed in the turtle brain (Nilsson, 1991). Microdialysis studies have demonstrated an eightfold increase in extracellular turtle brain adenosine levels, which peaked at 120 min of anoxia (Nilsson and Lutz, 1992). Two possible precursor pools for adenosine production would be IMP and AMP. Interestingly, the present study does not show significant drops in the levels of any of the measured nucleotides after 120 min of anoxia, as might have been expected with an eightfold increase in extracellular adenosine concentration (Fig. 4). Additionally, it is not known whether the rise in extracellular adenosine concentration is reflected by a corresponding intracellular rise.

A higher metabolic rate for cortical slices (Table 1), calculated from heat values of normoxic controls, was obtained at the beginning of each run, because heat dissipation tended to drop over the course of the experiment (Fig. 2A,B). The reason for this decrease is unknown, but it is presumably due to degeneration of unhealthy cells. Initial glucose consumption in guinea pig cortex slices (37 °C) is 16 μmol ATP g^{-1} min^{-1} when glucose consumption values are corrected for glycogen breakdown and lactate production (Rolleston and Newsholme, 1967). This estimate indicates a 9.3-fold difference in metabolic rate between the turtle and mammalian slice preparation, which is consistent with our earlier whole-brain deoxyglucose observation (37 °C rat; 25 °C turtle) of a 12-fold difference (Suarez *et al.* 1989).

The nitrogen perfusion experiments demonstrate a large reduction in heat dissipation in the turtle cortex during anoxia. The resulting ATP utilization estimates indicate that the turtle cortex depresses metabolism by between 30 % (nitrogen) and 42 % (pharmacological anoxia) during O_2 limitation. Estimates from lactate accumulation studies with whole brain suggest a larger (>80 %) metabolic depression (Kelly and Storey, 1988; Lutz *et al.* 1984).

The discrepancies between previous *in situ* studies and the current *in vitro* study may be partially explained by the absence of spontaneous electrical activity in the brain slice preparation. Current evidence suggests that one mechanism for metabolic depression in the turtle brain *in vitro* is reduction of spontaneous electrical activity (Lutz, 1992). Brain slices consume approximately 50 % less oxygen than the intact tissue (Lipton and Whittingham, 1984). This discrepancy has been attributed to the loss of spontaneous electrical activity in the tissue slice (McIlwain and Bachelard, 1971). This hypothesis is supported by the observations that tetrodotoxin (a blocker of voltage-gated Na^+ channels) causes only a 5 % reduction in oxygen consumption *in vitro* (Okamoto and Quastel, 1972), but barbiturate anesthetics (which depress electrical activity) inhibit oxygen consumption by 50 % in the intact brain (Siesjo, 1978). The absence of significant amounts of spontaneous electrical activity in the turtle cortical slice may cause a reduced

metabolic depression compared with that *in vivo*. The lack of electrical activity in the slice together with the large metabolic depression observed in the present study suggest that other metabolic processes are also depressed.

In some organisms, the anoxic heat dissipation does not match indirect measures of metabolism (heat dissipation can exceed measured end-product formation by as much as 50 %) (Gnaiger, 1980; Shick *et al.* 1983). Several lines of evidence suggest a negligible 'anoxic gap' for our turtle cortical slice preparation (Hardewig *et al.* 1991; Shick *et al.* 1983): (i) adenylate levels were fairly constant, (ii) all experiments were of short duration, (iii) the use of cyanide+N_2 minimized any oxidative metabolism, and (iv) the 1:2 matching of glucose consumption and lactate production in whole brain slices of anoxic turtles indicated that no other glucose catabolic pathways were active during anoxia (Robin *et al.* 1979). On the basis of glucose consumption and lactate production, turtle whole brain slices yield an anoxic ATP utilization rate of $0.88\,\mu\text{mol ATP}\,\text{g}^{-1}\,\text{min}^{-1}$ (Robin *et al.* 1979). The rate measured in the earlier study was almost identical to the ATP utilization rates estimated in the present study (anoxia, $0.973\,\mu\text{mol ATP}\,\text{g}^{-1}\,\text{min}^{-1}$; pharmacological anoxia, $0.82\,\mu\text{mol ATP}\,\text{g}^{-1}\,\text{min}^{-1}$), suggesting that no anoxic heat gap exists and that our metabolic calculation is accurate. It is important to note, however, that, if such a gap exists in our preparation, this discrepancy would increase the difference between control and anoxic rates of ATP utilization when corrected.

In the calculation of ATP turnover, we have assumed that the slices are fully aerobic or fully anaerobic. Aerobic brain tissue generally has an anaerobic component (Robin *et al.* 1979; Rolleston and Newsholme, 1967). Additionally, as a tissue enters an anoxic transition, there is a shift from aerobic to anaerobic metabolism, making metabolic calculations difficult (Gnaiger and Kemp, 1990). However, for our tissue, because the theoretical amount of heat dissipated per mole of ATP consumed is similar under aerobic and anoxic conditions (0.76 and $0.86\,\mu\text{mol}\,\text{g}^{-1}\,\text{min}^{-1}\,\text{mW}^{-1}$, respectively; see Materials and methods), the error associated with assuming that the tissue is totally aerobic or totally anaerobic is small (approximately 12 %). Consequently, heat dissipation along the experimental curves (Fig. 2A,B) follows changes in metabolic rate fairly closely.

Because energy status (measured as adenylate levels) during pharmacological anoxia is reasonably constant (indicating that the glycolytic ATP supply is matching cellular energy demands), and because of the immediate and total inhibition of oxidative metabolism by cyanide, the heat dissipation curve for pharmacological anoxia reflects both metabolic and glycolytic changes. On the basis of our chamber perfusion rates ($15\,\text{ml}\,\text{h}^{-1}$), we calculate that the cyanide K_i ($1\,\mu\text{mol}^{-1}$) for cytochrome aa_3 would be achieved in less than 1 s. The mixture of N_2+cyanide with pharmacological anoxia minimizes any oxidative metabolism in the transition phase, giving a picture of anaerobic metabolic transition uncontaminated by aerobic metabolism. As a result, the percentage depression of heat flow after 20 min of pharmacological anoxia is significantly greater compared with that in nitrogen perfusion (Table 1). Thus, the pharmacological anoxic transition curve, unlike the anoxic transition curve, reflects changes in both metabolic rate and glycolysis. A 12 % drop in heat dissipation with pharmacological anoxia would indicate no metabolic depression in the slice because of the difference between aerobic

and anaerobic heat dissipation (see Materials and methods), and would also indicate a large Pasteur effect. Owing to the catabolic heat production from glucose (140 kJ mol^{-1} for anoxia *versus* 2820 kJ mol^{-1} for aerobic conditions), heat flux would have to drop below 5 % (140 is 5 % of 2820) of control values before a reversed Pasteur effect could occur. However, this calculation represents a theoretical maximum value because of the assumption that energy production is 100 % aerobic during normoxia. Kelly and Storey (1988) have suggested that the turtle brain goes through a biphasic transition into anoxia. In the first phase, glycolytic activation occurs to maintain ATP concentrations. As the brain reduces its metabolism, glycolysis can return to a normal or a reduced rate (reversed Pasteur effect). Our pharmacological anoxia results support this contention. Initially, there is a rapid drop in heat dissipation, and this rate remains steady for 10–20 min (Fig. 3B). This steady rate averaged 76 % of the corresponding control heat dissipation measurement for the three experimental turtles, which indicates a large Pasteur effect. However, following this steady phase, there is a gradual drop in heat dissipation, suggesting a gradually declining metabolic and glycolytic rate.

In conclusion, the results reported here support a substantial metabolic depression in the turtle cortical slice preparation during oxygen deprivation. Considerable indirect evidence has accumulated suggesting a reduction in turtle brain electrical activity during anoxia as a mechanism for metabolic depression. However, since slices are considered to be electrically silent, the results reported here support the idea of anoxic suppression of other metabolic process. We have previously examined the reduction of the membrane leakage process in neurons as one mechanism for metabolic depression. Results from these earlier studies indicated that there was no change in ion leakage during anoxia. However, recent evidence in cortical slices suggests that internal ion leakage may be reduced (Bickler, 1992). Additionally, studies on anoxic turtle hepatocytes suggest a substantial (>90 %) anoxic reduction in protein synthesis (Land *et al.* 1993). Further investigation into these other metabolic processes in the turtle cortex are needed before definitive conclusions can be made regarding the mechanisms of metabolic depression in the turtle cortex.

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