PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Measurement of cytochrome oxidase and mitochondrial energetics by near –infrared spectroscopy

Chris E. Cooper and Roger Springett

Phil. Trans. R. Soc. Lond. B 1997 **352**, 669-676
doi: 10.1098/rstb.1997.0048

Measurement of cytochrome oxidase and mitochondrial energetics by near-infrared spectroscopy

CHRIS E. COOPER[1] AND ROGER SPRINGETT[2]

[1] *Department of Biological and Chemical Sciences, Central Campus, University of Essex, Colchester CO4 3SQ, UK*
[2] *Department of Medical Physics and Bioengineering, Shropshire House, 11–20 Capper Street, University College London, London WC1E 6JA, UK*

SUMMARY

Cytochrome oxidase is the terminal electron acceptor of the mitochondrial respiratory chain. It is responsible for the vast majority of oxygen consumption in the body and essential for the efficient generation of cellular ATP. The enzyme contains four redox active metal centres; one of these, the binuclear Cu_A centre, has a strong absorbance in the near-infrared that enables it to be detectable *in vivo* by near-infrared spectroscopy. However, the fact that the concentration of this centre is less than 10% of that of haemoglobin means that its detection is not a trivial matter.

Unlike the case with deoxyhaemoglobin and oxyhaemoglobin, concentration changes of the total cytochrome oxidase protein occur very slowly (over days) and are therefore not easily detectable by near-infrared spectroscopy. However, the copper centre rapidly accepts and donates an electron, and can thus change its redox state quickly; this redox change is detectable by near-infrared spectroscopy. Many factors can affect the Cu_A redox state *in vivo* (Cooper *et al.* 1994), but the most significant is likely to be the molecular oxygen concentration (at low oxygen tensions, electrons build up on Cu_A as reduction of oxygen by the enzyme starts to limit the steady-state rate of electron transfer).

The factors underlying haemoglobin oxygenation, deoxygenation and blood volume changes are, in general, well understood by the clinicians and physiologists who perform near-infrared spectroscopy measurements. In contrast, the factors that control the steady-state redox level of Cu_A in cytochrome oxidase are still a matter of active debate, even amongst biochemists studying the isolated enzyme and mitochondria. Coupled with the difficulties of accurate *in vivo* measurements it is perhaps not surprising that the field of cytochrome oxidase near-infrared spectroscopy has a somewhat chequered past. Too often papers have been written with insufficient information to enable the measurements to be repeated and few attempts have been made to test the algorithms *in vivo*.

In recent years a number of research groups and commercial spectrometer manufacturers have made a concerted attempt to not only say how they are attempting to measure cytochrome oxidase by near-infrared spectroscopy but also to demonstrate that they are really doing so. We applaud these attempts, which in general fall into three areas: first, modelling of data can be performed to determine what problems are likely to derail cytochrome oxidase detection algorithms (Matcher *et al.* 1995); secondly haemoglobin concentration changes can be made by haemodilution (using saline or artificial blood substitutes) in animals (Tamura 1993) or patients (Skov & Greisen 1994); and thirdly, the cytochrome oxidase redox state can be fixed by the use of mitochondrial inhibitors and then attempts made to cause spurious cytochrome changes by dramatically varying haemoglobin oxygenation, haemoglobin concentration and light scattering (Cooper *et al.* 1997).

We have previously written reviews covering the difficulties of measuring the cytochrome oxidase near-infrared spectroscopy signal *in vivo* (Cooper *et al.* 1997) and the factors affecting the oxidation state of cytochrome oxidase Cu_A (Cooper *et al.* 1994). In this article we would like to strike a somewhat more optimistic note—we will stress the usefulness this measurement may have in the clinical environment, as well as describing conditions under which we can have confidence that we are measuring real changes in the Cu_A redox state.

1. ALGORITHMS FOR CYTOCHROME OXIDASE DETERMINATION AND THEIR VALIDATION

We have recently performed tests of the various methods for measuring the cytochrome oxidase redox state (Matcher *et al.* 1995). Algorithms for measuring Cu_A redox state changes *in vivo* rely on converting optical density changes into the three chromophore concentration changes—deoxyhaemoglobin (Hb), oxyhaemoglobin (HbO_2) and Cu_A (the other major tissue chromophore in this region, water, is presumed to maintain a constant concentration). Although the purified spectra of these compounds are well characterized, problems arise in allowing for tissue-specific effects, such as the wavelength dependence of the

optical path length (Essenpreis *et al*. 1993) and possible changes in light scattering. Three algorithms, those developed at University College London (UCL) (Wray *et al*. 1988), Keele University (Wickramasinghe *et al*. 1990) and that used in the commercial Critikon NIRS 2000 are based on similar assumptions and would be expected to yield similar results (Matcher *et al*. 1995). They assume that no scattering changes occur and that the wavelength dependence of path length is constant throughout the measurement procedure. A derivation of the UCL algorithm that can theoretically correct for variations in the latter property has also been published (Cope *et al*. 1991). However, the data presented in this paper are based on the basic version of this technique with multiwavelength detection (UCLn as designated by (Matcher *et al*. 1995)).

One algorithm, that published by Tamura and co-workers, attempts to correct for scattering changes by using a fourth reference wavelength (750 nm) where it is stated that there is no absorbance change due to cytochrome oxidase redox state changes (Hazeki *et al*. 1987). As can be seen from some of the problems described later this is a laudable aim, and we certainly hope that in future algorithms will be able to correct for scattering changes. The problem we have is with the statements these authors make that below 780 nm there are no absorbance change attributable to cytochrome oxidase. This is not true for the purified enzyme (Cooper *et al*. 1994), nor for *in vivo* near-infrared spectroscopy (NIRS) measurements by other groups (Wray *et al*. 1988; Cope 1991; Miyake *et al*. 1991). The clearest *in vivo* data showing this is that from Chance's group in the fluorocarbon-perfused haemoglobin-free rat brain (Miyake *et al*. 1991). The lack of an isosbestic point in this region is as expected because no fewer than three chromophores contribute significantly to the mitochondrial absorption spectrum between 700 and 780 nm. As well as the Cu_A centre of cytochrome oxidase, there are the long wavelength tails of the significant absorption due both to the 695 nm band of the cytochrome *c* iron–methionine charge-transfer band and the 655 nm band of the high-spin haem a_3-Cu_B centre of cytochrome oxidase. It is possible to simulate the absorbance changes seen by the group of Chance (Miyake *et al*. 1991) and the UCL group (Cope 1991) using the purified components, whereas it is not easy to see how it is possible to do this with the spectra published by Tamura and co-workers. In this case we feel the onus is on the latter group to explain the physical basis behind their unusual spectra. Nevertheless, this criticism notwithstanding, we found that, in general, cytochrome changes observed by the algorithms of the Tamura and the UCL group showed similar trends both in modelling studies and when comparing the *in vivo* data.

The latter is not true for the algorithm published by Piantadosi (1993), which we found, in most circumstances, had identical time courses for HbO_2 decline and Cu_A reduction (Matcher *et al*. 1995). It has recently been suggested that the published algorithm may have been miscalculated (Macnab & Gagnon 1996). Although we await clarification by the original authors on this issue, it should be noted that they consistently find a much closer *in vivo* correlation between haemoglobin desaturation and cytochrome oxidase reduction than other groups (Hampson *et al*. 1990; Piantadosi 1993) and the resolution of this discrepancy is likely to go deeper than a simple miscalculation in a table. Their paper (Piantadosi 1993) shows two spectra (figures 2 and 3) both claiming to be *in vivo* Cu_A oxidized minus reduced difference spectra. One has a large ΔOD at 770 nm and a peak at 810 nm; the other has no ΔOD at 770 nm and a peak at 830 nm (along with the interesting oxygen-dependent signal at 860 nm that is only detected by this group). The UCL and Chance groups *in vivo* spectra split the difference between these two spectra showing a significant ΔOD at 770 nm and a peak at 830 nm (Wray *et al*. 1988; Cope 1991; Miyake *et al*. 1991). It is therefore not as easy as some have suggested (Macnab & Gagnon 1996) to reconstruct the Piantadosi algorithm from the raw data in this paper. In their defence it should be stated that this group have performed internal validations of the sort that we recommend to determine that, in their hands, there is no obvious 'cross-talk' between their HbO_2 and Cu_A signals (Piantadosi 1993). Nevertheless readers should be aware that, even more so than in the case with the Tamura algorithm, using the published algorithms from this paper will yield vastly different results to those obtained by using the Keele, Critikon or UCL algorithms described above, i.e. with the Piantadosi algorithm, reduction of Cu_A will occur at a much higher PO_2.

We propose the following criteria for authors claiming that they are measuring changes in the Cu_A redox state: (i) the algorithm used for deconvolution should, if possible, be explainable in terms of the known optical properties of the biological tissue and chromophores measured; (ii) there should be no large change in optical path length during the measurement, or if there is the algorithm should account for this; (iii) if possible the fitting technique should contain significantly more wavelengths than chromophores and the residuals of the fit should be random; (iv) the absorbance change observed should be reasonable for the amount of cytochrome oxidase in the tissue (when known); and (v) Cu_A redox state variations should not be a simple function of haemoglobin oxygenation or concentration changes, i.e. there should be limited 'cross-talk' in the system.

Our algorithm for cytochrome oxidase detection (Wray *et al*. 1988; Matcher *et al*. 1995) was designed for the adult rat brain in transmittance mode. We were concerned about transferring it to the neonatal brain where there are clear differences in the optical geometry of the measurement, the tissue studied (amount of skull) and the total cytochrome oxidase concentration (lower in the neonate). We tested our algorithm by determining that the cytochrome oxidase 'oxidized minus reduced' NIR spectrum in the perfluorocarbon-perfused piglet brain was identical to that of the adult rat (Cooper *et al*. 1997) and that the wavelength dependence of the optical path length in the blooded brain were also identical (point (i) above). Changes in the optical path length during short-term

hypoxia or alterations in arterial CO_2 were measured by the water path length and found to be small (point (ii)), and the three chromophores were a good fit to the optical density changes (point (iii)). The reduction of Cu_A upon anoxia in the blooded piglet was consistent with *in vitro* measurements of total cytochrome oxidase content (point (iv)). Finally we tested point (v) by causing large changes in haemoglobin concentration and oxygenation under conditions (following a 5 mg kg^{1} cyanide bolus i.v.) where the cytochrome oxidase redox state should not change and, as in the rat (Cooper *et al*. 1997), found no significant 'cross-talk' in the system. We were thus confident that the algorithm, in this case, could be transferred across the species and developmental age barrier, an important finding, as point (v), the most critical one, is difficult to determine in humans. Despite this 'success' it is important to remember that the points listed above may not hold for all the measurements in a particular system (see later for a case where some, but not all, of the observed 'cytochrome oxidase NIRS signal' can be attributed to a Cu_A redox state change).

2. OXYGEN DEPENDENCE OF THE Cu_A NIRS SIGNAL

As stated previously there has been controversy over how readily the Cu_A centre becomes reduced following a drop in oxygen tension. With the problems of measuring this signal in the blooded brain, perhaps this is not surprising. More worrying is that two groups have recently presented diametrically opposing views as to the ease of reducibility of Cu_A in the blood-free brain. In one case it is claimed that the Cu_A is extremely difficult to reduce and remains oxidized even after significant haem *a* reduction (Hoshi *et al*. 1993; Inagaki & Tamura 1993). Another group, which had more conventional views (Ferrari *et al*. 1996) now suggests that Cu_A reduction occurs very early in the hypoxic process (Stingele *et al*. 1996). In the purified enzyme we have repeated and agree with previous work (Nicholls & Chanady 1982) showing that Cu_A occurs late in the hypoxic process and simultaneously with haem *a* reduction. One possibility for the discrepancy between these groups might be that in one case rapid anoxia is initiated and in the other slow hypoxia; the latter has been suggested to induce a change in the nature of the cytochrome oxidase molecule (Chandel *et al*. 1995). However, our own findings show identical kinetics of haem *a* and Cu_A reduction in the bloodless brain during rapid anoxia (Cope 1991) and those of the Chance group show a similar result for slow-graded hypoxia (Miyake *et al*. 1991). Whilst the resolution of this controversy awaits further developments we are happy to sit in the middle of the debate with *in vivo* data that are readily interpretable by the *in vitro* characteristics of the enzyme.

3. CYTOCHROME OXIDASE NIRS—WHY BOTHER?

(*a*) *Tissue dysoxia*

It is easy to measure concentration changes in HbO_2 and Hb and, as shown above, it is difficult to do the same for cytochrome oxidase. It is also likely that in the not-too-distant future absolute quantitation of haemoglobin oxygenation will be available. Bioenergeticists are interested in the characteristics and behaviour of cytochrome oxidase *in vivo* in its own right, but the clinician is likely to require more convincing in order to feel the need to struggle through the literature of mitochondrial redox-state measurements.

At least two uses for this signal are apparent. Firstly as many (Bashford *et al*. 1980; Hazeki *et al*. 1987; Ferrari *et al*. 1995; Matcher *et al*. 1995), but not all (Hampson *et al*. 1990; Jöbsis-Vandervliet 1991; Stingele *et al*. 1996) groups contend, it requires low oxygen tensions before cytochrome oxidase Cu_A is reduced, whereas haemoglobin deoxygenates much more readily. This is because cytochrome oxidase has both a low K_m (Michaelis constant) for oxygen and is sensitive to mitochondrial, not tissue or cytosolic PO_2. Therefore cytochrome oxidase may be a useful marker of actual, rather than impending, tissue dysoxia. Two recent clinical studies stand out in this regard. One (Nollert *et al*. 1995) measured a number of parameters, including NIRS, during cardiac surgery and found that the only parameter correlating with subsequent neurological dysfunction was the reduction of cytochrome oxidase Cu_A. Another study on neonates (McCormick *et al*. 1993) showed that the standard treatment of patent ductus arteriosus, indomethacin, caused a significant unwanted side-effect, namely a drop in the Cu_A redox state. Alternative treatments with ibuprofen appear not to have this problem (Patel *et al*. 1995) and this, to our knowledge, is likely to become the first example of clinical practice being altered, at least in part due to a cytochrome oxidase NIRS measurement.

Although the Cu_A reduction was the best marker for mitochondrial dysoxia in these cases there were also significant haemodynamic changes and decreased haemoglobin oxygenation. Therefore one might argue that as improved measurements of absolute haemoglobin oxygenation become available, measurements of cytochrome oxidase NIRS will become less useful. It would be interesting to find instances where changes in the cytochrome oxidase NIRS signal correlate with energy failure, but haemoglobin oxygenation does not decrease significantly. We believe that such a situation arises in neonatal hypoxia–ischaemia.

(*b*) *Neonatal hypoxia–ischaemia*

Magnetic resonance spectroscopy of the newborn human infant, following birth asphyxia, reveals little change in brain energy metabolites on the first day of life (Hope *et al*. 1984; Azzopardi *et al*. 1989). However, NIRS reveals haemodynamic changes, including an increased cerebral blood flow (CBF) and cerebral blood volume (CBV), and a much-reduced CBV

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

response to changes in P_aCO_2 (CBVR) (P_a is arterial CO_2 tension). By day 2 many infants have impaired energy metabolism, resulting in a reduced cerebral PCr/P_i ratio (where PCr stands for phosphocreatine) (Hope *et al*. 1984; Azzopardi *et al*. 1989), the level of which is predictive of the subsequent neurological outcome (Azzopardi *et al*. 1989; Roth *et al*. 1992).

We have modelled the nuclear magnetic resonance (NMR) changes in a piglet model of hypoxia–ischaemia (Cady *et al*. 1993, 1994; Lorek *et al*. 1994; Mehmet *et al*. 1994; Thoreson *et al*. 1995) and shown that the primary cerebral energy failure in the insult provokes the same secondary (delayed) energy failure observed in the human. Most significantly lactate is also seen to increase in the secondary phase (Cady *et al*. 1993), strongly suggesting that an impairment in mitochondrial energy metabolism is directly responsible for the secondary fall in PCr and ATP levels. We have shown that there is an increase in apoptosis during secondary energy failure (Mehmet *et al*. 1994) and that mild hypothermia both prevents this rise in apoptosis (Edwards *et al*. 1995) and is cerebroprotective in this model (Thoreson *et al*. 1995).

Our current working hypothesis for the mechanism of this delayed energy failure is that there is an induction of nitric oxide synthase as occurs in adult models of hypoxia–ischaemia (Iadecola *et al*. 1995). Nitric oxide (NO) is now a well-established intercellular messenger, produced from L-arginine and oxygen by nitric oxide synthase(s) (Knowles & Moncada 1994). However, NO is also cytotoxic and has been implicated in neurotoxicity (Lipton *et al*. 1993; Iadecola *et al*. 1994). In the brain NO is responsible for the maintenance of cerebrovascular tone (Iadecola *et al*. 1994) and probably mediates the increased CBF and CBV following hypercapnia (Iadecola *et al*. 1994).

NO inhibits mitochondrial respiration (Stadler *et al*. 1991; Bolanos *et al*. 1994; Brown & Cooper 1994; Cleeter *et al*. 1994). It has generally been assumed that this is due to inhibition of mitochondrial iron–sulphur enzymes (Henry *et al*. 1991). However, iron–sulphur enzymes are very resistant to NO (Vanin *et al*. 1992), although they are sensitive to peroxynitrite (Heiss *et al*. 1994; Pantopoulous *et al*. 1994). We have recently shown that physiological levels of NO reversibly inhibit cytochrome oxidase activity by competing for oxygen at the active site (Brown & Cooper 1994). This is clearly the primary mechanism for NO inhibition of cellular respiration. Long-term damage may be caused by the increase in superoxide ions generated as cytochrome oxidase is inhibited, reacting with NO to generate peroxynitrite (Castro *et al*. 1994; Cleeter *et al*. 1994; Hausladen & Fridovich 1994). A similar sequence of events has recently been proposed (Cleeter *et al*. 1994) to explain the link between mitochondrial impairment and long-term neurological disorders, e.g. Huntingdon's disease (Jenkins *et al*. 1993) and Parkinson's disease (Hantraye *et al*. 1996).

It is therefore possible that a rise in cerebral NO production, subsequent to the hypoxic–ischaemic insult, is implicated in the haemodynamic changes and/or the secondary energy failure observed in the neonate. In this picture NO generated secondary to the insult would inhibit mitochondrial respiration initially at cytochrome oxidase; generation of peroxynitrite would then cause long-term mitochondrial damage and secondary energy failure primarily via an increase in apoptotic cell death. The increased levels of NO immediately subsequent to the insult would cause the observed increases in CBF, CBV and the absent CBVR (this response would already be saturated with NO). Hypothermia is cerebroprotective in our piglet model; intriguingly hyperthermia has recently been shown to stimulate NO production *in vivo* (Hall *et al*. 1994). Also, the close links recently shown between nitric oxide, mitochondrial damage and apoptosis are also consistent with our model (Shimaoka *et al*. 1995; Liu *et al*. 1996).

How is this model testable? A strong prediction is that, in contrast to the indomethacin and cardiac surgery situations described above, energy failure should be accompanied by an increase in haemoglobin oxygenation. As we (Brown & Cooper 1994) have shown, the highly effective inhibition of cytochrome oxidase by nitric oxide *in vitro* takes place at the oxygen reduction site of cytochrome oxidase, the haem a_3-Cu_B centre. NO inhibition therefore causes an increase in the redox state of the electron donor prior to this site, haem *a*, despite adequate oxygenation of the medium. Cu_A would also be expected to become more reduced.

Our prediction is therefore that in neonatal hypoxia–ischaemia, in the primary insult haemoglobin oxygenation will fall and Cu_A will become reduced. However, in the delayed energy failure haemoglobin oxygenation will not fall (indeed HbO_2 should increase as cerebral blood volume and flow rise) and yet cytochrome oxidase Cu_A will become more reduced. This latter event should correlate with other measures of energy failure. Results consistent with part of this hypothesis have come from studies involving ischaemic insults on the foetal sheep brain. A delayed rise in cerebral blood volume was detected, along with an increase in Cu_A reduction (Marks *et al*. 1996*a*). The latter correlated with histological damage and a decline in electrocortical activity. The addition of a nitric oxide synthase inhibitor decreased the amplitude of the rise in blood volume, although the histological damage was worse (Marks *et al*. 1996*b*). Unfortunately the effect of NO synthase inhibition on the Cu_A signal during secondary energy failure was not reported. Measurements of cytochrome oxidase redox state have been attempted in the neonatal human following hypoxia–ischaemia (Van Bel *et al*. 1993)—Cu_A reduction increased somewhat earlier than would be predicted from the animal models and, in contrast to other clinical finding, no delayed rise in cerebral blood volume was observed (Wyatt 1993).

It is useful to compare changes in the cytochrome oxidase NIRS signal with other non-invasive measurements of brain energy function. Correlations have previously been shown with cortical impedance (Marks *et al*. 1996*a*), [ATP] (Tsuji *et al*. 1996) and EEG & Tamura 1993). We therefore used our neonatal piglet model (Lorek *et al*. 1994) to perform simultaneous NIRS and magnetic resonance spec-

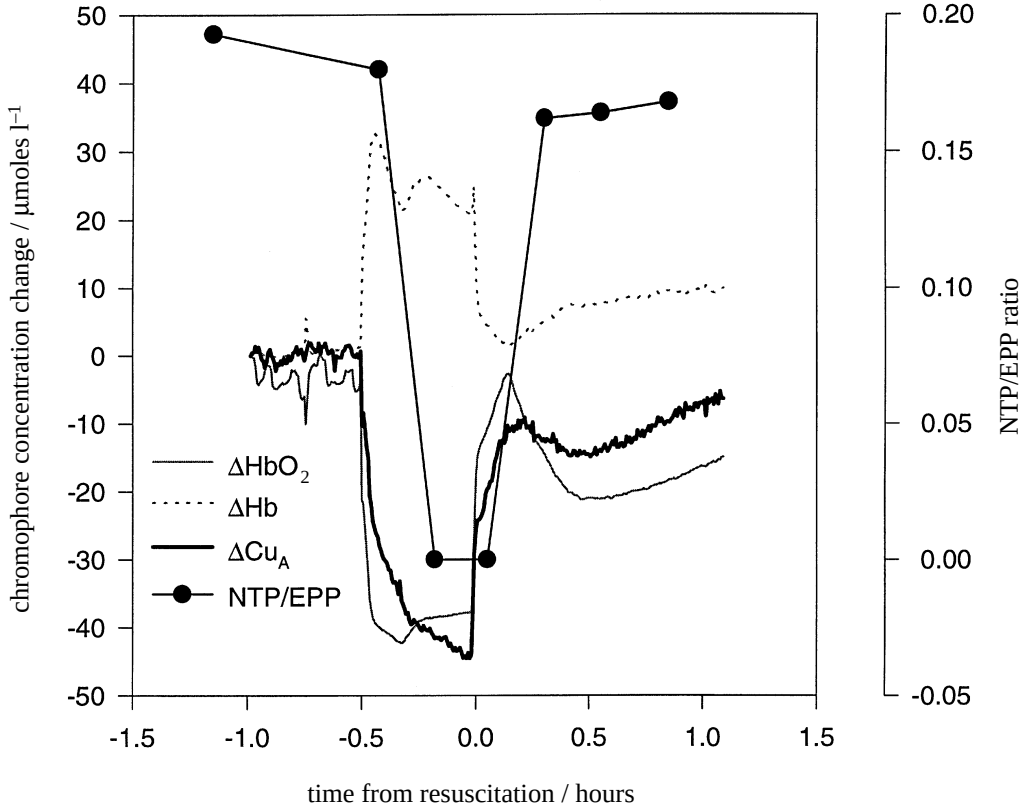

Figure 1. The effect of hypoxia–ischaemia on the haemodynamic and energetic properties of the neonatal brain. Simultaneous NIRS and MRS measurements were made on the neonatal piglet head. At time zero the carotid artery was occluded and F_iO_2 reduced to 11%. Changes in Hb, HbO_2 and the cytochrome oxidase Cu_A redox state were calculated as described previously (UCLn algorithm). NTP (nucleotide triphosphate) was measured by MRS as a fraction of the total exchangeable phosphate pool (EPP), i.e. [NTP]/([P_i]+[PCr]+[NTP]) (Lorek *et al.* 1994). Cytochrome oxidase Cu_A redox state changes are displayed on a ×10 expanded scale.

troscopy (MRS) measurements. We used a broadband source and a multiwavelength detection system. This system allows more accurate measurements of the cytochrome oxidase redox state (Matcher *et al.* 1995) and has the added advantage of enabling continuous optical path-length measurements to be made via second differential spectroscopy (Matcher & Cooper 1994; Matcher *et al.* 1994).

Figure 1 shows that as expected in the initial hypoxic–ischaemic period there is an initial drop in HbO_2 and rise in Hb, accompanied by a small fall in the total cerebral haemoglobin concentration (CHC). Consistent with our previous findings (Matcher *et al.* 1995), subsequent to the fall in HbO_2 there is an increase in Cu_A reduction. The latter has the same time course as the fall in cerebral [ATP]. Upon reversal of the hypoxia–ischaemia the NIRS and MRS parameters return to close to baseline values. Note that in all the observed figures the Cu_A signal is shown on a ×10 expanded scale with respect to the haemoglobin signals.

What happens during delayed energy failure (figure 2)? In this case there is a clear rise in HbO_2 and CHC, consistent with previous findings from the neonatal human (Wyatt 1993). However, there is a significant fall in the 'cytochrome oxidase NIRS signal' that, again, occurs over a similar time scale as the fall in [ATP]. An excellent correlation of the cytochrome oxidase NIRS signal with cerebral [ATP], although interestingly not with PCr/P_i, is maintained throughout the period of delayed energy failure (figure 3).

One should always beware of results that appear to agree too readily with a cherished hypothesis. In this case, although the data appear on the surface to be exactly as expected if nitric oxide is inhibiting cytochrome oxidase *in vivo*, problems lurk in the details. In effect the results are too good. Our algorithm, when coupled to the continuous optical path length measurements, enables us to measure quantitative redox-state changes. The redox-state change in cytochrome oxidase measured in the delayed energy failure of up to 8 μM is over double that observed in the primary insult. We have used the method developed by Brown to measure the total amount of cytochrome oxidase in the neonatal pig brain homogenates (Brown *et al.* 1991)—in no case have we found a value higher than 4 μM and we are therefore sceptical of redox-state changes larger than this value. We have independent evidence that the optical properties of the brain are changing during the energy failure—there is an increase in the mean optical path length and the residuals of the multilinear least-squares fits of the pure chromophores to the optical density changes become less random (this path length variation could be interesting in its own right as it might be due to a change in cell volume, consistent with our simultaneous measurements of changes in the cerebral water diffusion rate by magnetic resonance

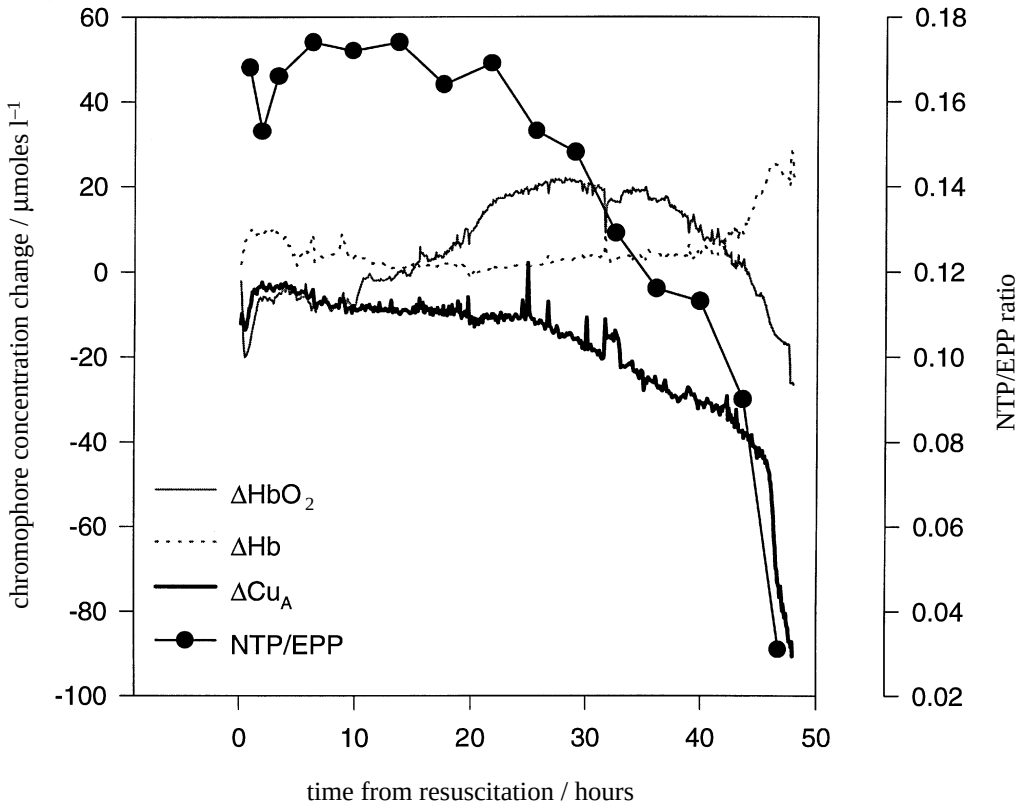

Figure 2. Delayed energy failure in the neonatal brain as monitored by NIRS and MRS. Simultaneous NIRS (Matcher *et al*. 1995) and MRS (Lorek *et al*. 1994) measurements were made on the neonatal piglet for 48 hours following the hypoxic–ischaemic insult described in figure 1. Cytochrome oxidase Cu_A redox state changes are displayed on a ×10 expanded scale. Abbreviations as per figure 1.

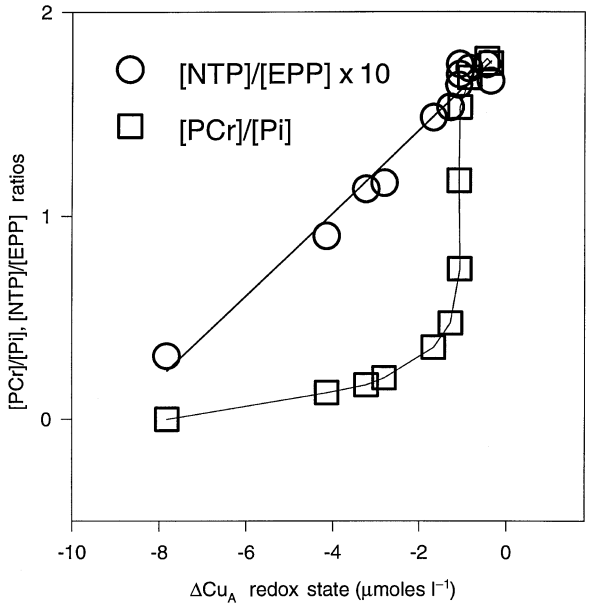

Figure 3. Correlation of energy failure measured by MRS and NIRS. From the data of figure 2, correlations were drawn between the cytochrome oxidase Cu_A redox state, PCr/P_i and NTP/EPP (as defined in figure 1).

imaging; an immediate effect upon cellular depolarization or a more general effect following apoptotic cell death might be expected to yield such volume changes). We have, however, been able to overcome the problems of scattering changes by directly testing the amount of oxidized cytochrome oxidase present in the brain at the end of the study. Prior to the end of the study a new reference spectrum is taken and the NIRS algorithm is therefore re-zeroed on the current optical properties of the brain. The inspired oxygen fraction F_iO_2 is then reduced to zero, allowing the Cu_A to become fully reduced. In six piglets the amount of reduction observed was always less than that during the primary insult, demonstrating that significant reduction of cytochrome oxidase had indeed occurred during the delayed energy failure, i.e. the starting redox state of cytochrome oxidase prior to the F_iO_2 drop at 48 h was significantly more reduced than that prior to the hypoxic–ischaemic insult at 0 h. Therefore our data are consistent with a role for cytochrome oxidase inhibition playing a major part in cell death during neonatal hypoxia–ischaemia. We are currently investigating whether nitric oxide is the ultimate source of this inhibition.

These studies clearly demonstrate the usefulness of NIRS measurements of the cytochrome oxidase redox state. A clinician viewing a moderately raised haemoglobin oxygenation would probably conclude that the brain was not damaged—the fall in the 'cytochrome oxidase NIRS signal' would clearly show that it was. They also suggest that we should be cautious in overinterpreting the biochemical and physiological mechanisms behind measurements of the cytochrome oxidase redox state. Perhaps we should utilize a new terminology. References to the 'cytochrome NIRS signal' would be used in cases where, although related to energy failure, there was still doubt as to the

biochemical process being monitored—references to the Cu_A redox state would be used when we were clear that the signal observed was shown to be due to redox-state changes in this centre by the methods described previously.

4. CONCLUSIONS

Large changes in near-infrared spectroscopy measurements of 'cytochrome oxidase' correlate with energy failure in a wide variety of systems. In some cases, but not all, these can be directly attributed to redox-state changes of the Cu_A centre in the enzyme.

We thank the Wellcome Trust, Medical Research Council, the Royal Society and Hamamatsu Photonics for financial support. We are grateful for discussions with colleagues at University College London, in particular Professor David Delpy and Dr John Wyatt.

REFERENCES

Azzopardi, D., Wyatt, J. S., Cady, E. B. *et al.* 1989 Prognosis of newborn infants with hypoxic–ischaemic brain injury assessed by phosphorus magnetic resonance spectroscopy. *Pediatr. Res.* **2**, 366–370.

Bashford, C. L., Barlow, C. H., Chance, B. & Haselgrove, J. 1980 *FEBS Lett.* **113**, 78–80.

Bolanos, J. P., Peuchen, S., Heales, S. J. R., Land, J. M. & Clark, J. B. 1994 Nitric oxide-mediated inhibition of the mitochondrial respiratory chain in cultured astrocytes. *J. Neurochem.* **63**, 910–916.

Brown, G. C. & Cooper, C. E. 1994 Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal cytochrome oxidase respiration by competing with oxygen at cytochrome oxidase. *FEBS Lett.* **356**, 295–298.

Brown, G. C., Crompton, M. & Wray, S. 1991 Cytochrome oxidase content of rat brain during development. *Biochim. Biophys. Acta* **1057**, 273–275.

Cady, E. B., Lorek, A., Penrice, J. *et al.* 1994 Brain-metabolite transverse relaxation times in magnetic resonance spectroscopy increase as adenosine triphosphate depletes during secondary energy failure following acute hypoxia–ischaemia in the newborn piglet. *Neurosci. Lett.* **182**, 201–204.

Cady, E. B., Wyatt, J. S., Takei, Y. *et al.* 1993 Cerebral energy failure secondary to acute hypoxia–ischaemia in the newborn pig studied by 1H magnetic resonance spectroscopy. *Proc. Soc. Magn. Reson. Med.* **12**, 1502.

Castro, L., Rodriguez, M. & Radi, R. 1994 Aconitase is readily inactivated by peroxynitrite, but not by its precursor, nitric oxide. *J. Biol. Chem.* **269**, 29409–29415.

Chandel, N., Budinger, G. R., Kemp, R. A. & Schumacker, P. T. 1995 Inhibition of cytochrome-*c* oxidase activity during prolonged hypoxia. *Am. J. Physiol.* L918–L925.

Cleeter, M. W. J., Cooper, J. M., Darley-Usmar, V. M., Moncada, S. & Schapira, A. H. V. 1994 Reversible inhibition of cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases. *FEBS Lett.* **345**, 50–54.

Cooper, C. E., Cope, M., Quaresima, V. *et al.* 1997 Measurement of cytochrome oxidase redox state by near infrared spectroscopy. In *Photon migration and optical spectroscopy* (ed. A. Villringer & U. Dirnagl). Berlin: Springer. (In the press.)

Cooper, C. E., Matcher, S. J., Wyatt, J. S. *et al.* 1994 Near-infrared spectroscopy of the brain: relevance to cytochrome oxidase bioenergetics. *Biochem. Soc. Trans.* **22**, 974–980.

Cope, M. 1991 The application of near infrared spectroscopy to non-invasive monitoring of cerebral oxygenation in the newborn infant. PhD thesis, University of London.

Cope, M., Van der Zee, P., Essenpreis, M., Arridge, S. R. & Delpy, D. T. 1991 Data analysis methods for near infrared spectroscopy of tissue: problems in determining the relative cytochrome *aa3* concentration. *Proc. SPIE* **1431**, 251–262.

Edwards, A. D., Yue, X. & Squier, M. V. 1995 Specific inhibition of apoptosis after cerebral hypoxia–ischaemia by moderate post-insult hypothermia. *Biochem. Biophys. Res. Commun.* **217**, 1193–1199.

Essenpreis, M., Elwell, C. E., Van der Zee, P., Arridge, S. R. & Delpy, D. T. 1993 Spectral dependance of temporal point spread functions in human tissues. *Appl. Opt.* **32**, 418–425.

Ferrari, M., Williams, M. A., Wilson, D. A., Thakor, N. V., Traystman, R. J. & Hanley, D. F. 1995 Cat brain cytochrome *c* oxidase redox changes induced by hypoxia after blood-fluorocarbon exchange-transfusion. *Am. J. Physiol.* **38**, H417–H424.

Hall, D. M., Buettner, G. R., Matthes, R. D. & Gisolfi, C. V. 1994 Hyperthermia stimulates nitric oxide formation: electron paramagnetic resonance detection of NO-heme in blood. *J. Appl. Physiol.* **77**, 548–553.

Hampson, N. B., Camporesi, E. M., Stolp, B. W. *et al.* 1990 Cerebral oxygen availability by NIR spectroscopy during transient hypoxia in humans. *J. Appl. Physiol.* **69**, 907–913.

Hantraye, P., Brouillet, E., Ferrante, R. *et al.* 1996 Inhibition of neuronal nitric oxide synthase prevents MPTP-induced parkinsonism in baboons. *Nature Med.* **2**, 1017–1021.

Hausladen, A. & Fridovich, I. 1994 Superoxide and peroxynitrite inactivate aconitases, but nitric oxide does not. *J. Biol. Chem.* **47**, 29405–29408.

Hazeki, O., Seiyama, A. & Tamura, M. 1987 Near-infrared spectrophotometric monitoring of haemoglobin and cytochrome *a,a3 in situ*. *Adv. Exp. Med. Biol.* **215**, 283–289.

Heiss, L. N., Lancaster, J. R. Jr, Corbett, J. A. & Goldman, W. E. 1994 Epithelial autotoxicity of nitric oxide: role in the respiratory cytopathology of pertussis. *Proc. Natn. Acad. Sci. USA* **91**, 267–70.

Henry, Y., Ducrocq, C., Drapier, J.-C., Servent, D., Pellat, C. & Guissani, A. 1991 Nitric oxide, a biological effector. *Eur. Biophys. J.* **20**, 1–15.

Hope, P. L., Costello, A. M. d. L., Cady, E. B. *et al.* 1984 Cerebral energy metabolism studied with phosphorus NMR spectroscopy in normal and birth-asphyxiated infants. *Lancet* **2**, 366–370.

Hoshi, Y., Hazeki, O. & Tamura, M. 1993 Oxygen dependence of redox state of copper in cytochrome oxidase *in vitro*. *J. Appl. Physiol.* **74**, 1622–1627.

Iadecola, C., Pelligrino, D. A., Moskowitz, M. A. & Lassen, N. A. 1994 Nitric oxide synthase inhibition and cerebrovascular regulation. *J. Cereb. Blood Flow Metab.* **14**, 175–192.

Iadecola, C., Zhang, F., Xu, S., Casey, R. & Ross, M. E. 1995 Inducible nitric oxide synthase gene expression in brain following ischemia. *J. Cereb. Blood Flow Metab.* **15**, 378–384.

Inagaki, M. & Tamura, M. 1993 Preparation and optical characteristics of hemoglobin-free isolated perfused rat head *in situ*. *J. Biochem.* **113**, 650–657.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Jenkins, B. G., Koroshetz, W. J., Beal, M. F. & Rosen, B. R. 1993 Evidence for impairment of energy metabolism *in vivo* in Huntingdon's disease using localized 1H NMR spectroscopy. *Neurology* **43**, 2689–2695.

Jöbsis-Vandervliet, F. F. 1991 Near infrared monitoring of cerebral cytochrome *c* oxidase: past and present (and future?). In *Fetal and neonatal physiological measurements* (ed. H. N. Lafeber), pp. 41–55. Amsterdam: Elsevier.

Knowles, R. G. & Moncada, S. 1994 Nitric oxide synthases in mammals. *Biochem. J.* **298**, 249–258.

Lipton, S. A., Choi, Y. B., Pan, Z. H. *et al.* 1993 A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* **364**, 626–632.

Liu, X. S., Kim, C. N., Yang, J., Jemmerson, R. & Wang, X. D. 1996 Induction of apoptotic program in cell-free-extracts–requirement for dATP and cytochrome *c*. *Cell* **86**, 147–157.

Lorek, A., Takei, Y., Cady, E. B. *et al.* 1994 Delayed ('secondary') energy failure following acute hypoxia–ischaemia in the newborn piglet: continuous 48 hour studies by phosphorus magnetic resonance spectroscopy. *Pediat. Res.* **36**, 699–706.

Macnab, A. J. & Gagnon, R. E. 1996 Potential sources of discrepancies between living tissue near infrared spectroscopy algorithms. *Anal. Biochem.* **236**, 375–377.

Marks, K., Mallard, C., Roberts, I. *et al.* 1996*a* Delayed vasodilation and altered oxygenation after cerebral ischemia in fetal sheep. *Pediat. Res.* **39**, 48–54.

Marks, K. A., Mallard, E. C., Roberts, I., Williams, C. E., Gluckman, P. D. & Edwards, A. D. 1996*b* Nitric oxide synthase inhibition attenuates delayed vasodilation and increases tissue injury following cerebral ischemia in fetal sheep. *Pediat. Res.* **40**, 185–191.

Matcher, S. J. & Cooper, C. E. 1994 Absolute quantification of deoxyhaemoglobin concentration in tissue near infrared spectroscopy. *Physics Med. Biol.* **39**, 1295–1312.

Matcher, S. J., Cope, M. & Delpy, D. T. 1994 Use of the water absorption spectrum to quantify tissue chromophore concentration changes in near-infrared spectroscopy. *Physics Med. Biol.* **39**, 177–196.

Matcher, S. J., Elwell, C. E., Cooper, C. E., Cope, M. & Delpy, D. T. 1995 Performance comparison of several published tissue near-infrared spectroscopy algorithms. *Anal. Biochem.* **227**, 54–68.

McCormick, D. C., Edwards, A. D., Brown, G. C. *et al.* 1993 Effect of indomethacin on cerebral oxidised cytochrome oxidase in preterm infants. *Pediat. Res.* **33**, 603–608.

Mehmet, H., Yue, X., Squier, M. V. *et al.* 1994 Increased apoptosis in the cingulate sulcus of newborn piglets following transient hypoxia–ischaemia is related to the degree of high energy phosphate depletion during the insult. *Neurosci. Lett.* **181**, 121–125.

Miyake, H., Nioka, S., Zaman, A., Smith, D. S. & Chance, B. 1991 The detection of cytochrome oxidase heme iron and copper absorption in the blood-perfused and blood-free brain in normoxia and hypoxia. *Anal. Biochem.* **192**, 149–155.

Nicholls, P. & Chanady, G. A. 1982 Titration and steady state behaviour of the 830 nm chromophore in cytochrome *c* oxidase. *Biochem. J.* **203**, 541–549.

Nollert, G., Möhnle, P., Tassan-Prell, P. *et al.* 1995 Postoperative neuropsychological dysfunction and cerebral oxygenation during cardiac surgery. *Thorax. Cardiovasc. Surgeon* **43**, 260–264.

Pantopoulous, K., Weiss, G. & Hentze, M. W. 1994 Nitric oxide and the post-translational control of cellular iron traffic. *Trends Cell. Biol.* **4**, 82–85.

Patel, J., Marks, K. A., Roberts, I., Azzopardi, D. & Edwards, A. D. 1995 Ibuprofen treatment of patent ductus arteriosus. *Lancet* **346**, 255.

Piantadosi, C. A. 1993 Absorption spectroscopy for assessment of mitochondrial function *in vivo*. *Methods Toxicol.* **2,** 107–126.

Roth, S. C., Edwards, A. D., Cady, E. B. *et al.* 1992 Relation between cerebral oxidative metabolism following birth asphyxia and neurodevelopmental outcome and brain growth at one year. *Dev. Med. Child Neurol.* **34**, 285–295.

Shimaoka, M., Iida, T., Ohara, A. *et al.* 1995 NOC, a nitric-oxide-releasing compound, induces dose dependent apoptosis in macrophages. *Biochem. Biophys. Res. Commun.* **209**, 519–526.

Skov, L. & Greisen, G. 1994 Apparent cerebral cytochrome *aa*3 reduction during cardiopulmonary bypass in hypoxaemic children with congenital heart disease. A critical analysis of *in vivo* near-infrared spectrophotometric data. *Physiol. Meas.* **15**, 447–457.

Stadler, J., Billiar, T. R., Curran, R. D., Stuehr, D. J., Ochoa, J. B. & Simmons, R. L. 1991 Effect of exogenous and endogenous nitric oxide on mitochondrial respiration of rat hepatocytes. *Am. J. Physiol.* **260**, C910–916.

Stingele, R., Wagner, B., Kameneva, M. V. *et al.* 1996 Reduction of cytochrome-*c*-oxidase copper precedes failing cerebral oxygen utilization in fluorocarbon perfused rats. *Am. J. Physiol.* **40**, H579–H587.

Tamura, M. 1993 Non-invasive monitoring of the redox state of cytochrome oxidase in living tissue using near-infrared laser lights. *Jpn. Circ. J.* **57**, 817–824.

Thoreson, M., Penrice, J., Lorek, A. *et al.* 1995 Mild hypothermia following severe transient hypoxia–ischaemia ameliorates delayed cerebral energy failure in the newborn piglet. *Pediat. Res.* **37**, 667–670.

Tsuji, M., Naruse, H., Volpe, J. & Hotlzman, D. 1996 Reduction of cytochrome *aa*3 measured by near-infrared spectroscopy predicts cerebral energy loss in hypoxic piglets. *Pediat. Res.* **37**, 253–259.

Van Bel, F., Dorrepaal, C. A., Benders, M. J., Zeeuwe, P. E., Van de Bor, M. & Berger, H. M. 1993 Changes in cerebral hemodynamics and oxygenation in the first 24 hours after birth asphyxia. *Pediatrics* **92**, 365–372.

Vanin, A. F., Men'shikov, G. B., Moroz, I. A., Mordvintcev, P. I., Serezhenkov, V. A. & Burbaev, D. S. 1992 The source of non-heme iron that binds nitric oxide in cultivated macrophages. *Biochim. Biophys. Acta* **1135**, 275–279.

Wickramasinghe, Y., Thorniley, M., Rolfe, P., Houston, R., Livera, L. N. & Faris, F. 1990 *Proc. 12th Ann. Int. Conf. IEEE Eng. Biol. Soc.* pp.1545–1554.

Wray, S., Cope, M., Delpy, D. T., Wyatt, J. S. & Reynolds, E. O. R. 1988 Characterisation of the near infrared absorption spectra of cytochrome *aa*3 and haemoglobin for the non-invasive monitoring of cerebral oxygenation. *Biochim. Biophys. Acta* **933**, 184–192.

Wyatt, J. S. 1993 Near-infrared spectroscopy in asphyxial brain injury. *Clin. Perinatol.* **20**, 369–378.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES