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Characterization of the near infrared absorption spectra of cytochrome aa_3 and haemoglobin for the non-invasive monitoring of cerebral oxygenation

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Near infrared (IR) spectroscopy can give continuous, direct information about cerebral oxygenation in vivo by providing signals from oxygenated and deoxygenated haemoglobin and cytochrome aa_3. Due to a lack of precise spectral information and uncertainties about optical path length it has previously been impossible to quantify the data. We have therefore obtained the cytochrome aa_3 spectrum in vivo from the brains of rats after replacing the blood with a fluorocarbon substitute. Near infrared haemoglobin spectra were also obtained, at various oxygenation levels, from cuvette studies of lysed human red blood cells. Estimates of optical path length have been obtained. The data were used to construct an algorithm for calculating the changes in oxygenated and deoxygenated haemoglobin and oxygenated cytochrome aa_3 in tissue from changes in near IR absorption.

Introduction

There are many clinical and physiological situations where direct measurement of cerebral oxygenation and haemodynamics would provide extremely valuable information. The technique of near infrared (IR) spectroscopy shows great promise for these purposes [1]. The principle of the technique is based on the fact that near IR absorption signals which change with oxygenation arise from only two compounds in the mammalian brain, cytochrome aa_3 and haemoglobin. Absorption changes due to these compounds can be obtained at near IR wavelengths (over 700 nm), where light penetrates tissue relatively easily. Svaasand and Ellingsen [2] have measured optical penetration depth in human brain tissue and shown that intensity falls by $1/e$ (approx. 37%) every 0.4 mm at 514 nm and 3.2 mm at 1060 nm. Near IR spectroscopy has recently been applied to the study of the brain in human subjects and seems likely to become an established clinical tool [3–6].

A limitation of previous studies has been the inability to obtain quantitative information from the spectra. It has been possible only to document relative changes in signal intensity, so comparisons of findings between subjects, or from the same subject on different occasions, could not be made. There are two main difficulties to overcome if the near IR signals are to be made quantitative. Firstly the individual signals from cytochrome aa_3 and haemoglobin must be clearly separated from

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one another and from background tissue absorption: this requires accurate quantitative data defining the absorption spectra of these compounds. Spectra from haemoglobin can be obtained quite simply using cuvettes – although few studies of spectra in the near IR region have reported numerical quantitative data [7,8]. Spectra from cytochrome aa_3 are more difficult to obtain. Several investigators have produced curves from in vitro cytochrome aa_3, measured in mitochondria [9] or purified enzyme [10]. The reliability of cuvette studies has been queried because of the difficulty of purification and uncertainties about the replication of conditions in vivo [11].

The second problem to overcome if near IR spectra are to be made quantitative is that the apparent optical (light) path length in the tissue must be defined. Biological tissue is a highly light-scattering medium and little information about its optical properties is available, particularly in the near IR region. As a result it has not been possible to measure the apparent optical path length. Furthermore, most studies have been made in reflectance mode which further complicates such calculations. As light does not take a 'straight through' path in a scattering medium the absorption caused by any absorber present is increased because the optical path length is greater than the physical distance between where light enters and exits the scattering media. This increase in absorption can be expressed as a 'path length factor' modifying the standard relation derived from Beer Lambert law:

$$\text{absorption} = \alpha c\, BL$$

where α is the absorption coefficient $mM^{-1} \cdot cm^{-1}$, c is concentration mM, L is the straight line distance of the scattering medium and B is path length factor increase due to the scattering medium.

The purposes of this study were therefore to define separately the near IR spectra of haemoglobin and cytochrome aa_3 and also to make an estimate, for measurements made in transmission mode, of apparent optical path length. In vivo cytochrome aa_3 difference spectra (oxygenated minus deoxygenated) were obtained from the brains of rats whose blood had been replaced by a fluorocarbon substitute. Oxygenated and deoxygenated haemoglobin spectra were obtained from cuvette studies of lysed human red blood cells. An algorithm was then derived which allowed absorption changes to be related to the signals from oxygenated and deoxygenated haemoglobin and oxygenated minus deoxygenated cytochrome aa_3.

An abstract of some of this work has been published [12].

Methods

Characterization of near IR spectra from cytochrome aa_3

Cytochrome aa_3 is normally attached to the inner mitochondrial membrane, and it has been purified by several investigators. Because of its high affinity for oxygen, cytochrome aa_3 is normally reduced by strong reducing agents such as NADH plus *p*-phenylenediamine or sodium dithionite in in vitro studies. We found that reduction of oxygenated haemoglobin by sodium dithionite alters the absorption spectrum from that seen by equilibrating with nitrogen. Similar problems may well exist for cytochrome aa_3. We have therefore preferred to measure cytochrome aa_3 as a difference spectrum in vivo, as opposed to in intact mitochondria or purified enzyme. This approach also ensures that cytochrome aa_3 is unquestionably fully functional. In order to remove the large contaminating haemoglobin signal, blood was replaced with a fluorocarbon substitute.

Animals

Six male Wistar rats weighing 300–500 g were anaesthetized with urethane (ethyl carbonate, 36% w/v solution, 0.5 ml/100 g body weight, i.p.). They were tracheotomised and a femoral artery and vein were cannulated. The blood vessels to the spleen were tied off. The head was secured in a stereotactic frame and the scalp tissues and temporal muscles were removed and cautery was performed. EEG leads were placed in burr holes made in the skull. The animals were ventilated with 100% oxygen and paralysed with tubocurare (i.v., 0.2 ml, 1.5 mg/ml solution, additional doses being given throughout the experiment if required). Urethane anaesthesia was maintained throughout

by giving 0.1 ml of 24% urethane every 2–3 h, i.v. The animals were kept on a warmed bed and their rectal temperature was maintained at $37 \pm 1°C$. Blood pressure was monitored by a transducer (Elcomatic, U.K.) attached to the arterial catheter.

Blood was replaced as an oxygen carrier by the fluorocarbon perfluorotributylamine (FC43, Green Cross Corp., Japan). This was done by exchange transfusion at a rate of approx. 2 ml/min. Between 150 and 200 ml of fluorocarbon were used and the haematocrit was lowered to 0.01%. (Fluorocarbon FC43 has been shown to deliver oxygen and remove carbon dioxide while maintaining normal cerebral function [13], and while the animal was ventilated with 100% oxygen we recorded normal EEG activity.) Near IR absorption spectra were collected throughout the exchange procedure to monitor the residual haemoglobin absorption. The results showed that at least 150 ml of fluorocarbon had to be exchanged before the haemoglobin absorption fell to a point where absorption changes could no longer be detected. After replacing the blood with FC43 an oxygenated brain spectrum was obtained while the animal breathed 100% O_2 and then the ventilating gas was changed to 100% N_2 so that the deoxygenated spectrum could be obtained.

Near IR spectroscopy

A stabilized 250 W quartz halogen light source was used. Visible light was excluded by a 650 nm cut-off filter and mid and far IR by a water filter. Fibre-optic light guides of 2.25 mm diameter were secured to the parietal bones via the stereotactic frame and good optical contact was maintained by using a thickened, optically transparent, silicone oil as a coupling gel (E308, ICI, U.K.). The light emerging from the skull was guided to a scanning optical spectrum analyser (Rofin, U.K.) which sampled the signal between 700 and 1000 nm in steps of approx. 5 nm. The optical detector was a GaAs photomultiplier tube (Hamamatsu R636, Japan) operating in photon counting mode. Complete spectra with noise of ± 0.01 A per 5 nm sample took 5 min to collect and were saved onto disc via a microprocessor and displayed. (Due to the fall in sensitivity of the photomultiplier tube, the signal-to-noise ratio above 900 nm prevented useful data being collected above this wavelength, in these experiments.) Temperature control of the photomultiplier tube at $-15°C$ ($\pm 0.1°C$) maintained a constant dark count which was subtracted from experimental reading and also prevented photocathode sensitivity changes. At the end of the experiments the spectral sensitivity of the system was measured by comparison to a neutral absorber after the animal was removed.

Characterization of near IR spectra from haemoglobin

As haemoglobin can be easily isolated from other cellular components and maintained in good physiological condition in vitro, near IR data for haemoglobin were obtained from cuvette studies of normal human blood obtained by venepuncture. The red blood cells were washed three times with isotonic saline, lysed with distilled water shaken with carbon tetrachloride and spun. Finally buffered to pH 7.2, the solution was filtered to below 0.2 μm particle size to give a clear haemoglobin solution. Samples (5 ml) of approx. 20 mg/ml haemoglobin solution were reduced by equilibrating with 100% N_2 for 1 h in an IL 413 tonometer (Instrumentation Laboratories. U.K.) before transfer to an anaerobic 1 cm spectrometer cuvette. Fully oxygenated spectra were obtained by bubbling with humidified 100% O_2. Intermediate spectra at various degrees of oxygenation were used to find the isosbestic point near 800 nm. The haemoglobin concentration was measured independently by converting it to the cyano-met form. The Rofin spectrometer wavelength range for these studies was 650–1050 nm using a silicon photodetector with a nominal resolution of 3 nm.

Estimation of path length

These measurements were performed on a separate group of four animals. The preparation was identical to the previous group but no fluorocarbon blood exchange was performed.

A different spectroscopic system was used, the scanning monochromator and photomultiplier detector being replaced by a fixed grating spectrograph and a focal plane array of 1024 silicon detectors, (E.G.&G, P.A.R.C, Princetown). This enabled spectra to be taken between 650 and 1050 nm across the rat's head. The major difference between this and the previously described system

lies in the ability to detect light in the 900–1050 nm region. This enabled observation of the water absorption peaks at 965 nm. The height of this water peak, measured across the 1 cm of brain, is then compared to that of 3.5 cm of pure water, measured in a cuvette, to yield a value for the apparent optical path length. A correction is needed to take into account the fact that the brain is only 80% water [14].

Spectra were recorded in rats at differing levels of oxygenation and immediately after death. The recorded spectra were background-subtracted and normalised to the system response with no absorber or scatterer present.

Results

Cytochrome aa_3 difference spectra

Spectra were obtained continuously from the brains of six animals before, during and after fluorocarbon exchange, while they were breathing 100% O_2 and then while breathing 100% N_2. Fig. 1 shows the results from one such experiment. It can be seen that a marked decrease in absorption took place after the blood was replaced with fluorocarbon (spectra A and B), and that a much smaller change followed when the cytochrome was deoxygenated by ventilating the animal with nitrogen (spectrum C). During the exchange transfusion no significant spectral changes were seen in any of the animals after around 150 ml had been exchanged, indicating that the absorption effects of haemoglobin were by then insignificant compared to the final difference spectrum. In fluorocarbon exchange-transfused animals changes in blood pressure in the range 50–150 mm Hg elicited only a small, wavelength-independent change in absorption, with no apparent effects on the cytochrome aa_3 oxygenation.

The absorption change produced by the reduction of cytochrome aa_3 alone was found to be approx. 10% of the absorption change which occurred upon replacement of the haemoglobin with fluorocarbon. Before changing the ventilating gas to N_2 the p_{O_2} was greater than 40 kPa during the fluorocarbon exchange. The individual cytochrome aa_3 difference spectra from the six animals were almost identical in shape although small individual variations were observed. These variations were wavelength-independent. The spectra were summed and the average cytochrome aa_3 difference spectrum obtained is illustrated in Fig. 2. The absorption changes of this spectrum are given in Table I.

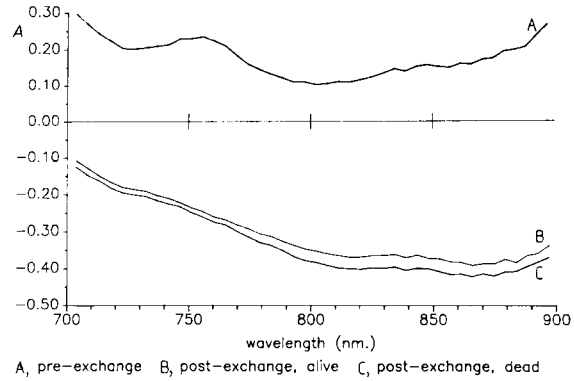

Fig. 1. Near IR absorption spectra obtained across a rat's head showing the change in absorbance in going from control (A) to fluorocarbon-exchanged conditions (B and C). Spectra B and C were obtained with the animal ventilated with 100% O_2 and 100% N_2, respectively. The difference between B and C gives the cytochrome aa_3 difference spectrum.

Oxygenated and reduced haemoglobin spectra

The near IR spectra from fully oxygenated and deoxygenated haemoglobin are shown in Fig. 3. The absorption coefficients for oxygenated and deoxygenated haemoglobin are summarised in Table II. The isosbestic point was at 798 nm ($n = 5$).

Optical path length

The spectrum of a normally oxygenated rat

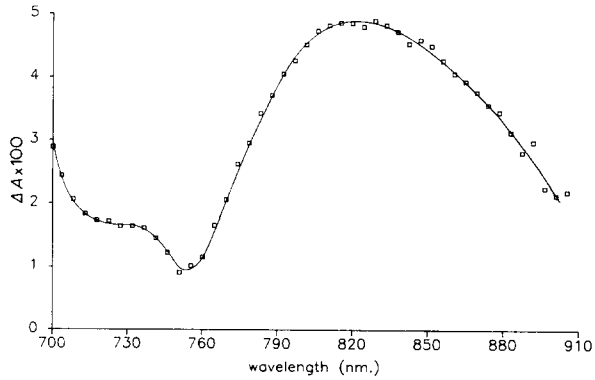

Fig. 2. The in vivo near IR difference spectrum of cytochrome aa_3; mean of six experiments.

TABLE I

THE NEAR IR ABSORPTION CHANGES FOR OXYGENATED-DEOXYGENATED CYTOCHROME aa_3 BETWEEN 700 AND 900 NM

Wavelength (λ) against absorption change (ΔA, $\times 100$) for in vivo cytochrome aa_3 difference spectrum (experimental data points, unsmoothed).

λ	ΔA	λ	ΔA	λ	ΔA	λ	ΔA
698	2.876	756	1.019	811	4.803	866	3.916
704	2.442	760	1.158	816	4.843	870	3.745
708	2.063	765	1.651	821	4.837	875	3.543
713	1.836	770	2.058	825	4.779	879	3.427
718	1.734	774	2.619	830	4.874	884	3.107
723	1.720	779	2.951	834	4.899	888	2.790
727	1.638	784	3.417	839	4.700	893	2.958
732	1.642	788	3.706	843	4.511	897	2.233
737	1.612	793	4.045	848	4.569	901	2.121
742	1.451	798	4.251	852	4.475	906	2.178
746	1.225	802	4.503	857	4.241	910	2.264
751	0.912	807	4.719	861	4.038		

brain is shown in Fig. 4 (solid line). The absorption scale does not start at zero because of the light loss due to scattering (approximately 3 absorption units). It can be seen that this light loss is essentially wavelength-independent. Comparison of this curve with that of 3.5 cm of pure water (dotted line in Fig. 4) shows very similar features, the most obvious of which is the water absorption peak at 965 nm. When the water curve is fitted to the rat brain curve it requires a multiplication factor which is in fact the apparent optical path length.

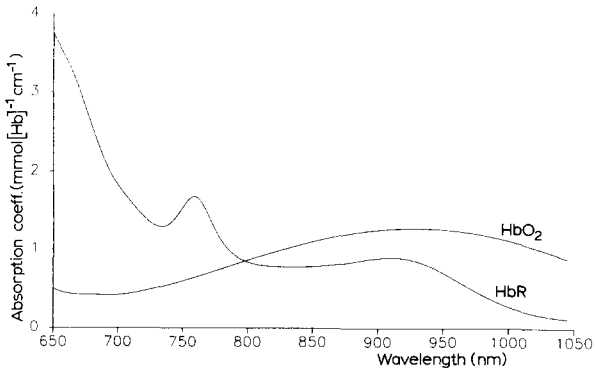

Fig. 3. Haemoglobin near IR absorption spectra from lysed, normal human blood obtained from fully oxygenated and fully deoxygenated haemoglobin.

The mean value for apparent water path was 34.8 ± 0.35 ($n = 4$), corresponding to a path length factor of 3.5, before correction for brain water content. Taking a value of 80% for brain water [14] gives a value of apparent optical path length of 4.34 ± 0.44-times the head diameter.

Interestingly, changes in this value could not be observed with differing levels of oxygenation or following death. This indicates that the path length factor is a constant for small changes in absorption or scattering caused by variations in the concentration of haemoglobin or cellular changes at death.

Algorithm for calculating concentration changes in oxygenated and deoxygenated haemoglobin and oxygenated cytochrome aa_3 in brain tissue

The information derived from the near IR spectral characteristics of oxygenated minus deoxygenated cytochrome aa_3 and oxygenated and deoxygenated haemoglobin were used to convert variations in near IR absorptions into changes in the concentration of these compounds in brain tissue. This was accomplished by measuring absorption changes at as many wavelengths as unknown compounds, in our case three: oxygenated haemoglobin, deoxygenated haemoglobin and oxygenated minus deoxygenated cytochrome aa_3. (For simplicity in the following equations these compounds have been abbreviated as follows; HbO, HbR and C3, respectively.) It is necessary to measure both HbO and HbR since they can vary independently as cerebral blood volume alters whereas total cytochrome aa_3 is assumed to be constant. Assuming that absorption at each wavelength is a linear summation of the effects of these 3 compounds (see Discussion) then:

$$A_1 = \alpha_1^{HbR}[HbR] + \alpha_1^{HbO}[HbO] + \alpha_1^{C3}[C3]$$

$$A_2 = \alpha_2^{HbR}[HbR] + \alpha_2^{HbO}[HbO] + \alpha_2^{C3}[C3]$$

$$A_3 = \alpha_3^{HbR}[HbR] + \alpha_3^{HbO}[HbO] + \alpha_3^{C3}[C3]$$

where A_n are absorption measurements at wavelengths n, and α_n^x is absorption coefficient of compound x at wavelength n and [x] is concentration of compound x.

Then for the three measured absorption changes, the change in concentration of HbR,

TABLE II

THE NEAR IR ABSORPTION COEFFICIENTS FOR OXYGENATED AND DEOXYGENATED HAEMOGLOBIN BETWEEN 650 AND 1050 NM

Wavelength (λ) against absorption coefficients ($mM^{-1} \cdot cm^{-1}$) for fully reduced and oxygenated haemoglobin (HbR and HbO_2, respectively).

λ	HbR	HbO_2	λ	HbR	HbO_2	λ	HbR	HbO_2	λ	HbR	HbO_2
650	3.743	0.506	755	1.644	0.624	857	0.788	1.122	953	0.674	1.244
653	3.644	0.479	758	1.678	0.641	860	0.792	1.134	956	0.645	1.239
657	3.546	0.459	762	1.660	0.658	863	0.797	1.146	959	0.616	1.233
660	3.442	0.445	765	1.590	0.675	866	0.803	1.157	962	0.587	1.226
663	3.326	0.435	768	1.485	0.692	869	0.810	1.167	965	0.558	1.220
667	3.193	0.430	772	1.365	0.710	872	0.817	1.177	968	0.529	1.213
670	3.043	0.427	775	1.250	0.728	876	0.824	1.187	972	0.501	1.205
674	2.879	0.426	778	1.149	0.745	879	0.833	1.196	975	0.473	1.197
677	2.713	0.425	782	1.066	0.763	882	0.841	1.204	978	0.445	1.189
681	2.550	0.423	785	0.999	0.781	885	0.850	1.212	981	0.419	1.180
684	2.392	0.420	788	0.948	0.799	888	0.858	1.219	984	0.394	1.170
687	2.243	0.417	792	0.908	0.817	891	0.866	1.226	986	0.370	1.160
691	2.108	0.415	795	0.877	0.835	895	0.873	1.232	989	0.347	1.149
694	1.990	0.415	798	0.852	0.852	898	0.880	1.238	992	0.325	1.138
698	1.887	0.416	801	0.832	0.869	901	0.885	1.243	995	0.305	1.126
701	1.798	0.421	805	0.816	0.886	904	0.890	1.248	998	0.285	1.114
705	1.720	0.427	808	0.804	0.903	907	0.892	1.252	1001	0.267	1.103
708	1.647	0.435	811	0.796	0.920	910	0.893	1.255	1004	0.250	1.089
711	1.579	0.444	814	0.789	0.936	913	0.892	1.258	1007	0.234	1.076
715	1.513	0.454	818	0.785	0.952	916	0.889	1.260	1010	0.219	1.062
718	1.450	0.466	821	0.782	0.968	920	0.883	1.261	1013	0.206	1.047
721	1.392	0.478	824	0.779	0.983	923	0.875	1.262	1016	0.193	1.033
725	1.343	0.490	828	0.778	0.998	926	0.865	1.263	1019	0.182	1.018
728	1.307	0.503	831	0.778	1.013	929	0.852	1.263	1022	0.171	1.003
732	1.286	0.517	834	0.777	1.028	932	0.836	1.263	1025	0.162	0.988
735	1.286	0.532	837	0.777	1.043	935	0.819	1.262	1028	0.153	0.972
738	1.307	0.546	840	0.777	1.057	938	0.799	1.259	1031	0.145	0.956
742	1.349	0.561	844	0.778	1.070	941	0.778	1.258	1034	0.138	0.940
745	1.412	0.576	847	0.780	1.084	944	0.755	1.255	1036	0.131	0.923
748	1.490	0.592	850	0.781	1.097	947	0.729	1.251	1039	0.125	0.905
752	1.574	0.608	853	0.784	1.110	950	0.702	1.248	1042	0.120	0.889

HbO_2 and cytochrome aa_3 redox can be calculated by solving the three simultaneous equations. We do this using matrix inversion and obtain a multiplying factor, a, (units mM/A) for each wavelength and compound. The concentration changes are then calculated as:

$$[\mathrm{HbR}] = a_1^{\mathrm{HbR}} A_1 + a_2^{\mathrm{HbR}} A_2 + a_3^{\mathrm{HbR}} A_3$$

$$[\mathrm{HbO}] = a_1^{\mathrm{HbO}} A_1 + a_2^{\mathrm{HbO}} A_2 + a_3^{\mathrm{HbO}} A_3$$

$$[\mathrm{C3}] = a_1^{\mathrm{C3}} A_1 + a_2^{\mathrm{C3}} A_2 + a_3^{\mathrm{C3}} A_3$$

As our aim is to apply near IR spectroscopy to the brains of human neonates, we have made our absorption data applicable to the wavelengths used in that system. For studying the brains of newborn infants, which involves transilluminating 6–10 cm of head (cf. 1 cm in the rat), we have built apparatus incorporating four laser diodes operating at 778, 813, 867 and 904 nm which are used as the light sources [15]. From the above absorption data we have derived the following multiplying factors (a), to calculate changes in the concentration of HbO_2, HbR and cytochrome aa_3, using three of the wavelengths.

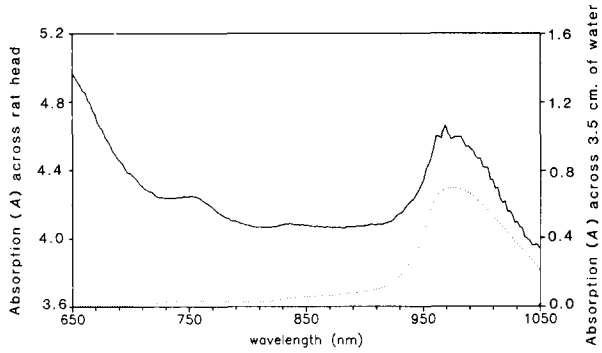

Fig. 4. Absorption spectra across a rat's head (solid line) and 3.5 cm of water (dotted line) between 650 and 1050 nm. The peak at 965 nm is from water in both curves.

	Multiplying factor		
	778 nm	813 nm	867 nm
HbO_2	−0.499	−1.756	+2.577
HbR	+1.768	−0.877	−0.421
Cyt. aa_3 redox	−0.559	+1.659	−0.949

The concentration of each compound is calculated by linear summation of the absorption changes multiplied by these factors. The calculated concentration changes are then expressed in mmol/l multiplied by optical path length (l) in cm, for example, for oxygenated haemoglobin:

$$l \cdot [HbO_2] = -0.499 \cdot A_{778} + -1.756 \cdot A_{813} + 2.577 \cdot A_{867}$$

Discussion

We have characterized the near IR absorption spectra from haemoglobin and cytochrome aa_3, and obtained an estimate of optical path length, across the rat head, so that quantitative data can be obtained from near IR spectroscopy of the brain.

Cytochrome aa_3 and haemoglobin absorption spectra

As expected, fluorocarbon proved to be a satisfactory blood substitute for maintaining the normal electrical activity of the brain, as judged by the EEG. Thus we are confident that while the animals were ventilated with 100% O_2 the in vivo cytochrome aa_3 signal was from healthy, well-oxygenated brain. There were unavoidable changes in many physiological variables associated with a failing circulation when the ventilating gas was changed to 100% N_2. The only way to minimise these changes would be to use an isolated perfused head preparation [16]. We have started preliminary studies with this technique.

We found that changes in near IR signals continued to occur during fluorocarbon exchange transfusion until at least 150 ml (about 6 blood volumes) had been exchanged and the haematocrit had fallen below 0.01%. Earlier investigators attempting to characterise in vivo near IR cytochrome aa_3 spectra have not always performed such complete exchange transfusions, reporting haemoglobin concentrations of up to 53 mg/ml (i.e., approximate haematocrit of 15%) at the end of the exchange, compared to a mean initial value of 174 mg/ml [17]. This residual haemoglobin is likely to have had a substantial effect on the cytochrome aa_3 difference spectra. Any algorithms derived for calculating changes in the cytochrome signal would therefore include large errors. The absorption signal from cytochrome aa_3 proved to be only about one-tenth that of haemoglobin. (For example, at 800 nm, cytochrome aa_3 absorption is 0.04 A (Fig. 2), and the change at this wavelength for haemoglobin is around 0.5 A (Fig. 1).

Baselines have been indicated on our absorption spectra for both cytochrome aa_3 and haemoglobin (Figs. 2 and 3). No previous published near IR data included baselines and sufficient exchange-transfusion with fluorocarbon to enable quantitative algorithms to be derived. We are aware that there will be small errors in our baseline figures, because of the physiological derangements which accompanied ventilation with nitrogen. Since fluorocarbon is a milky, highly light-scattering medium, the inevitable reduction in intracerebral fluorocarbon concentration caused a reduction in scattering and therefore a decrease in absorbance. This decrease was found to be the same at all wavelengths. It was not possible to quantitate exactly the absorption change, but we estimate from our spectra that it is no more than 0.02 A.

Comparison of our in vivo cytochrome aa_3 redox spectrum (Fig. 2) with previously published

in vitro spectra of cytochrome aa_3 [10] revealed certain differences, in the region between 700 and 750 nm. Deoxygenated haemoglobin has an absorption peak in this region, but we do not consider this difference to be due to residual haemoglobin. This is because: (1) in fluorocarbon-exchanged rats, when we extended our observations down to 580 nm we did not see the characteristic haemoglobin peaks (which are an order of magnitude larger than cytochrome absorption); (2) the characteristics of the haemoglobin difference spectrum at other near IR wavelengths are quite different from the cytochrome aa_3 curve shown in fig. 2; (3) a large volume of fluorocarbon compared to blood volume was used in the exchange-transfusion. Damage to the enzyme during preparation for the in vitro studies, or at the time the studies were done (some strong chemical reducing agents were used) seems more probable, especially since spectral differences between the purified enzyme and isolated mitochondria have been shown [11].

Optical path length

The rat brain was consistently about 1 cm across at the points where the optodes were applied to the skull. Our calculations indicated an apparent optical path length of 4.34 ± 0.44 cm, assuming that water constitutes 80% of the brain. This value for path length across the rat head is consistent with the change of 0.4 to 0.5 *A* seen when blood is replaced with fluorocarbon.

In calculating changes in concentration of haemoglobin and cytochrome aa_3 we have assumed that absorption changes are linearly additive. We consider that this assumption is justifiable for three reasons. (1) At near IR wavelengths there are only relatively small absorption differences in the spectra (see Fig. 3 and Ref. 18). By contrast, in the visible region (400–700 nm), where there are relatively intense and narrow absorption bands, non-linear effects are observed [18]. (2) We found that after fluorocarbon exchange when cerebral blood volume varied, the associated near IR spectral changes were wavelength-independent. This indicates that scattering is also wavelength-independent. (3) Calculations based upon a model of light transport in tissue predict a linear variation of intensity with absorption in transmission spectroscopy, whereas in reflection a non-linear response is predicted [19].

Algorithm for quantitating changes in cerebral oxygenation

The spectral data obtained in this study showed strong internal consistency. If the various assumptions made in deriving an algorithm for calculating changes in cerebral oxyhaemoglobin, deoxygenated haemoglobin and oxygenated cytochrome aa_3 are, as argued, valid, then the value of near IR spectroscopy as a method for observing changes in cerebral oxygenation will be much enhanced. We have already applied this algorithm to preliminary studies of newborn infants [5,20,21], and shown how the data can be further analysed to provide important haemodynamic information. For example, cerebral blood volume, venous haemoglobin saturation and cerebral blood flow can be derived [5].

Conclusions

We have obtained the near IR absorption spectra of haemoglobin and cytochrome aa_3. An algorithm was constructed which permits quantitative changes in oxygenated and deoxygenated haemoglobin and cytochrome aa_3 in brain tissue to be calculated.

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