Monitoring Real-time Metabolite Trafficking in the Brain using Microelectrochemical Biosensors

John P. Lowry (John.Lowry@may.ie)

Neurochemistry Research Unit, Bioelectroanalysis Laboratory, Department of Chemistry, National University of Ireland, Maynooth, Co. Kildare, Ireland

Abstract

In a first series of experiments a glucose oxidase-based biosensor implanted in the striatum of freely moving rats was used to determine the concentration of brain extracellular (ECF) glucose in two distinct ways. With a modification of the zero-net-flux method, in which different concentrations of glucose are infused through a dialysis probe glued to the biosensor, the concentration at which there was no change in glucose current was calculated by regression analysis; this gave an ECF concentration of 351 ± 16 μM. The concentration calculated from the basal current and the *in vitro* calibration of the biosensor was not significantly different from this. The basal extracellular glucose concentration determined by either method remained constant over a period of several days. In a second series of experiments rats were implanted in the striatum with a Pt/Ir electrode for measurement of regional cerebral blood flow (rCBF, H_2 clearance technique), a carbon paste electrode for monitoring tissue oxygen, and a glucose biosensor for monitoring extracellular glucose. There was a parallel increase in rCBF and oxygen in response to neuronal activation (5 min tail pinch). During the neuronal activation there was a decrease in ECF glucose which was followed by a slow rise that took 30 min to return to basal levels. Finally, a group of rats implanted with a combined glucose biosensor and dialysis probe were given a 5 min tail pinch while the dialysis probe was perfused with either artificial cerebrospinal fluid (aCSF) alone or aCSF with the addition of the β-adrenoceptor antagonist propranolol. Perfusion with aCSF once again produced an initial reduction in extracellular glucose, which was co-extensive with the period of stimulation, followed by a delayed and long-lasting increase in glucose. Propranolol had no effect on basal levels of glucose but suppressed the delayed increase. These results suggests that extracellular glucose in the brain is not derived directly from the blood vascular system but from some other source which is most likely to be astrocytes.

1 INTRODUCTION

According to the classical model of brain metabolism increased energy requirements are met by an increase in blood flow that delivers oxygen and glucose to the extracellular compartment. In this model there is close coupling between blood flow and the delivery and utilisation of glucose and oxygen. This has been replaced by a model in which energy metabolism is compartmentalised; increased energy requirements of activated neurones are met by neuronal utilisation of lactate not glucose, the lactate being provided by astrocytic glycolysis stimulated by the uptake of glutamate. Evidence in support of this model has principally come from *in vitro* experiments using primary cell cultures [1], brain slices [2], and more recently from NMR studies [3]. These techniques, however, cannot provide the temporal relationships of the various processes.

With the development of *in vivo* methods for monitoring changes in metabolic substrates in unanaesthetised animals it has become possible to relate changes in neuronal activity, in selected brain regions, with changes in brain metabolism. The use of microdialysis with enzyme-based assays for glucose and lactate has demonstrated a stimulation-dependent increase in lactate linked to glutamate uptake [4, 5], as well as a dissociation between increases in rCBF and increases in ECF glucose [4]. While these results support the use of lactate as an energy substrate they also suggest that glucose in the extracellular compartment is not derived directly from the blood vascular compartment. This has led to some controversy among proponents of the new compartment model concerning the immediate source of extracellular glucose, since in *in vitro* experiments astrocytes in culture export lactate but not glucose [6], which has led to the suggestion that glucose enters the extracellular space directly from the blood stream [7].

Microdialysis however does have several limitations including a time resolution of 2-3 min and the fact that the concentration of analytes in the dialysate depends on a variable *in vivo* recovery [8]. A growing number of new methodologies are being developed to study neurochemical phenomena in the living brain with high time resolution. One such set of techniques focuses on the detection of substances using amperometric electrodes and voltammetric techniques *in vivo* [9]. By implanting a microvoltammetric electrode (sensor) in a specific brain region, applying a suitable potential profile and recording the resulting faradaic current, changes in the concentration of a variety of substances in the

extracellular fluid can be monitored with a sub-second time resolution over extended periods. This allows investigations of the functions and roles of specific neurochemicals in neuronal signalling, drug actions, and well-defined behaviours, with a time resolution not presently available with other methods.

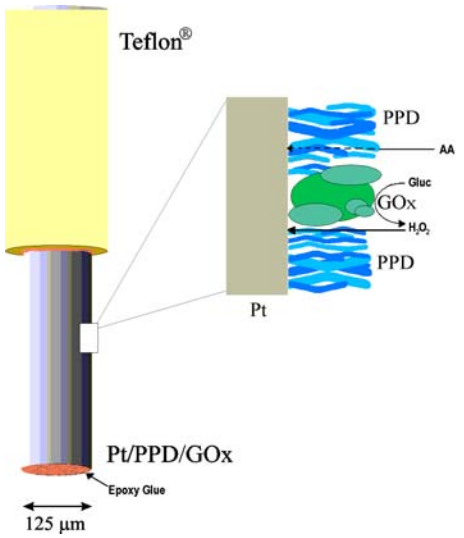

Figure 1. *Schematic illustration of a Pt/PPD/GOx biosensor. The glucose oxidase (GOx) catalysed oxidation of glucose (Gluc) yields H_2O_2. The small H_2O_2 molecules can diffuse easily to the Pt surface to be electro-oxidised with a characteristic fast response time (see Figure 3C). Access to the metal by larger electroactive species, such as ascorbic acid (AA), is selectively restricted by the poly(o-phenylenediamine) (PPD) layer.*

Recently, the development by the author, of a glucose biosensor for real-time voltammetry *in vivo* has provided 100% recovery and a sub-second time resolution [10]. This device is an amperometric biosensor (a device incorporating a biological sensing element, such as an enzyme, that is either intimately connected to or integrated within a transducer, e.g. an electrode) prepared by immobilising glucose oxidase (GOx) onto a platinum microelectrode by trapping the enzyme during electropolymerisation of poly(*o*-phenylenediamine) (PPD) on the electrode surface (*see* Figure 1) [11]. A detailed characterisation of this biosensor was first carried out *in vitro* (Department of Chemistry, University College Dublin) [11,12]. The sensor was modified and fine-tuned, implanted into rat brain (University Laboratory of Physiology, Oxford), and the following characteristics established *in vivo* [13]: (i) high sensitivity to glucose; (ii) freedom from fouling by endogenous matrix macromolecules (lipids and proteins) in the target environment; (iii) insignificant interference from reducing agents, especially ascorbic acid which is normally present in high concentration; and (iv) insensitivity to changes in oxygen partial pressure. This glucose biosensor was subsequently used to study brain energy metabolism *in vivo*, on its own, and in combination with other microelectrochemical sensors: tissue oxygen levels were monitored using a modified carbon paste electrode and a specially designed computer-based voltammetric technique (differential double pulse amperometry) [14]; measurements of regional cerebral blood flow (rCBF) were carried out using a Pt electrode and a modified form of the hydrogen clearance technique designed for use in freely moving animals [15].

In this review paper various results are presented highlighting the application of this new technology as an important approach in the elucidation of the mechanisms of energy supply and utilisation in the mammalian brain.

2 MATERIALS AND METHODS

Details of Materials and Methods can in be found in previous publications: Chemicals and Solutions [10,13]; Working Electrode Preparation [10,13]; Microdialysis Probe Construction [10]; Instrumentation and Software [10,13]; *In Vitro* Conditioning and Characterisation of Pt/PPD/GOx Electrodes [13]; Surgical Procedures [10,13]; Experimental Conditions [13]; Voltammetric Techniques *In Vivo* [13]; Physiological Stimulation [15].

3 RESULTS AND DISCUSSION

3.1. ECF Concentration

As outlined in the Introduction there is some controversy concerning the immediate source of glucose in the extracellular compartment [16]. An accurate determination of the true extracellular concentration is a critical step in the resolution of this controversy. The concentration of brain extracellular glucose has previously been calculated from whole brain glucose and the volume of the extracellular compartment [17]; such measurements have estimated the extracellular glucose concentration as 2.0 mM. More recently, brain extracellular glucose has been monitored *in vivo* using microdialysis. However, it has been shown, using microdialysis, that the calculation of the concentration of glucose in the extracellular compartment from the concentration in the dialysate requires a knowledge of the *in vivo* recovery, which cannot be derived from the *in vitro* recovery of the probe [8]. The zero-net-flux (ZNF) method of Lönnroth [18] provides a figure for both the extracellular concentration and the *in vivo* recovery. Application of the ZNF method has given figures of 0.47 mM [19] and 350 µM [8] in the striatum of unanaesthetised rats and 3.3 mM in the cortex of anaesthetised rats [20].

The following experiments utilised a recent modification of the ZNF method in which an implanted glucose

biosensor was combined with a dialysis probe [21] to determine the extracellular concentration of glucose in the striatum of freely moving rats. This value was then compared with that derived from calculations using the *in vitro* calibration of the biosensor.

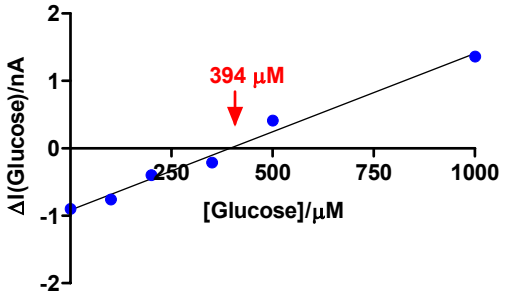

Figure 2. *A typical regression curve constructed from a zero-net-flux experiment during which glucose solutions ranging from 100 to 1,000 µM were perfused in random order through a dialysis probe (combined with a glucose biosensor) at a flow rate of 2 µL/min.*

Combined Pt/PPD/GOx sensors and microdialysis probes were implanted in the right striatum of freely moving rats and the modified Lönnroth ZNF method used to determine the true extracellular concentration of glucose *in situ*. The experimental procedure has been described in a previous publication [10]. Perfusion of glucose concentrations lower or higher than that in the ECF resulted in a net loss or gain from the tissue into the dialysis probe and was characterised by a decrease or increase in the Pt/PPD/GOx current. Random perfusion of concentrations above and below the point of ZNF resulted in quite rapid changes in the glucose current which reached a steady-state usually within 10-15 min. However, once perfusion was stopped the return to baseline or control values was longer for the higher concentrations: 26 ± 3 min for 1,000 µM glucose (n = 4, 6 determinations) and 6 ± 1 min for aCSF (n = 4, 6 determinations). With certain concentrations there was little or no change in the glucose current, indicating that these concentrations were close or equal to the ECF concentration. As the animals were freely-moving there were often periods during the perfusion of glucose when the animals were quite active (e.g. feeding) and the glucose current required longer periods of time to reach steady-state conditions. Linear regression analysis of a plot of the observed change in current (ΔI) versus perfused concentration yielded a straight line (r^2 = 0.98 ± 0.01) and a mean ZNF point of 351 ± 16 µM (n = 4, 11 determinations) which represents the extracellular concentration of glucose (*see* Figure 2). In ZNF experiments carried out on two consecutive days (1 and 2) in four animals the calculated extracellular concentrations were 373 ± 12 µM for day 1 and 358 ± 19 µM for day 2, a difference which was not statistically significant (P > 0.57). This is in contrast to earlier microdialysis data, where dialysate glucose levels showed a progressive decrease [19].

In the alternative approach basal glucose levels were calculated from the baseline currents recorded *in vivo* and the *in vitro* calibration data. These values were compared with those obtained using the ZNF method. The *in vitro* sensitivity of the Pt/PPD/GOx electrodes for glucose was 61 ± 25 nA/mM (n = 4). The difference in glucose concentration calculated from the *in vitro* calibration of the four biosensors and the value calculated from ZNF experiments carried out 24 h after implantation was 48 ± 86 µM, a difference not different from 0 (n = 4, 4 determinations, P = 0.61). When the comparison was made between basal concentrations determined by these two methods on days 1 and 2 after implantation the difference was 19 ± 49 µM (n = 4, 8 determinations, P = 0.74). Similarly, there was no significant difference (P > 0.38) in the extracellular concentration calculated from *in vitro* calibrations for day 1 (422 ± 81 µM, n = 4) and day 2 (398 ± 69 µM, n = 4).

These findings have several implications. The first is that there is little or no change in the sensitivity of the biosensor following implantation, supporting previous results [11]. The second is that the close agreement indicates that *in vivo* the biosensor current represents only glucose. The third is that in contrast to microdialysis, measurements with the implanted biosensor provide a direct, real-time measure of the extracellular concentration of glucose. In microdialysis the delivery of glucose to the dialysate is by diffusion along a concentration gradient. In addition to the dialysis membrane the narrow and tortuous clefts of the extracellular compartment offer barriers to free diffusion and hence restrict the flux to the probe. Processes of supply and utilisation increase the concentration gradient and hence the flux from the brain [22]. The removal of glucose by microdialysis varies with flow rate, and even at 2 µL min^{-1} is 41 times greater than the removal by the enzyme-based biosensor. The much smaller removal flux of the biosensor is much more easily met by the tissue processes surrounding the probe. Consequently, the local extracellular concentration is not perturbed by the electrode which records the true extracellular concentration which can be calculated from the *in vitro* calibration.

3.2. Neuronal Activation

Measurement of changes in rCBF were carried out at 5 min intervals using a method described in detail in a previous publication [15]. The mean basal rCBF was 86.5 ± 7.9 mL/100g/min (n = 9). Figure 3A shows the rCBF monitored over a period of 75 min at a Pt electrode implanted in the left striatum. During this period two tail pinches were administered by attaching a paper clip to the

rat's tail. The 5 min tail pinch caused an increase in rCBF which was maximal in the first measurement after the onset of the tail pinch and returned to baseline levels within the next 1 or 2 measurements after the end of the tail pinch. The mean increase in rCBF was 73.4 ± 8.4 mL/100mg/min (n = 9), which is an increase of 88 ± 11% above basal.

Figure 3B shows the simultaneous measurement of tissue oxygen measured as the oxygen current at a carbon paste electrode implanted in the right striatum. The application of the tail pinch produced an immediate increase in oxygen current that reached a maximum at the end of the tail pinch and began to decrease as soon as the paper clip was removed. The mean baseline current was 194 ± 65 nA and the mean increase 10.4 ± 1.7 nA (n = 9). These figures cannot be used to calculate the percentage change, since the carbon paste electrode has a substantial background current [15]. However, using the *in vitro* calibration, carried out after the removal of the electrode, the increases in oxygen current were converted to oxygen concentration and gave a mean increase of 39 ± 5.5 µM; taking basal oxygen concentrations as 50 µM [23] this gives a percentage increase of 78 ± 11% above basal. The time for return to basal of the oxygen current was 9.8 ± 3 min; at no time was there a decrease below basal levels.

In a separate group of rats changes in ECF glucose were measured at a glucose biosensor implanted in the right striatum. In contrast to rCBF and tissue oxygen, the application of the tail pinch produced a decrease in the glucose current of 0.86 ± 0.21 from a mean basal value of 15.3 ± 2.1 nA (n = 11), which represents a decrease of 5.2 ± 0.9% (*see* Figure 3C). On removal of the paper clip the glucose current began to rise and reached a peak of 1.8 ± 0.3 nA above basal 10 ± 3 min after the end of the tail pinch, which represents a peak increase of 11.3 ± 1.1%. The time of return to basal glucose current was 30 ± 3 min, and occasionally glucose levels fell below basal. Using the *in vitro* calibration curve the changes in glucose can be converted to concentrations; this gives a mean decrease of 16.2 ± 2.5 µM and a mean increase of 34.1 ± 4.3 µM.

Another group of rats implanted with a combined glucose biosensor and dialysis probe were given a 5 min tail pinch while the dialysis probe was perfused with aCSF. This, once again, produced an initial reduction in extracellular glucose, which was co-extensive with the period of stimulation, followed by a delayed and long-lasting increase in glucose (*see* Figure 4A). The tail pinch stimulus was repeated after the addition to the perfusion fluid of the β-adrenoceptor antagonist propranolol to a final concentration of 50 µM. This produced the changes in glucose illustrated in Figure 4B. Using the calibration of the glucose electrode, current values were converted to glucose concentrations. There was no effect on basal glucose (*see* Figure 4). The initial decrease in glucose during neuronal activation, in the presence of aCSF, was –10.5 ± 2.4 µM (n = 8) or 3.4 ± 0.7% of basal. This was increased to –16.6 ± 3.2 µM (n = 8) or 5.3 ± 0.6% in the presence of propranolol (P = 0.08). The delayed increase in glucose following activation, in the presence of aCSF, was +44.6 ± 4.4 µM (n = 9) or 13.7 ± 1.4% of basal. This was reduced to +1.2 ± 6.4 µM (n = 9) or 4.5 ± 0.6% of basal in the presence of propranolol (P = 0.0015). The early decrease in glucose during neuronal stimulation is probably partly masked by the onset of the delayed increase; when this is depressed the early decrease is enhanced.

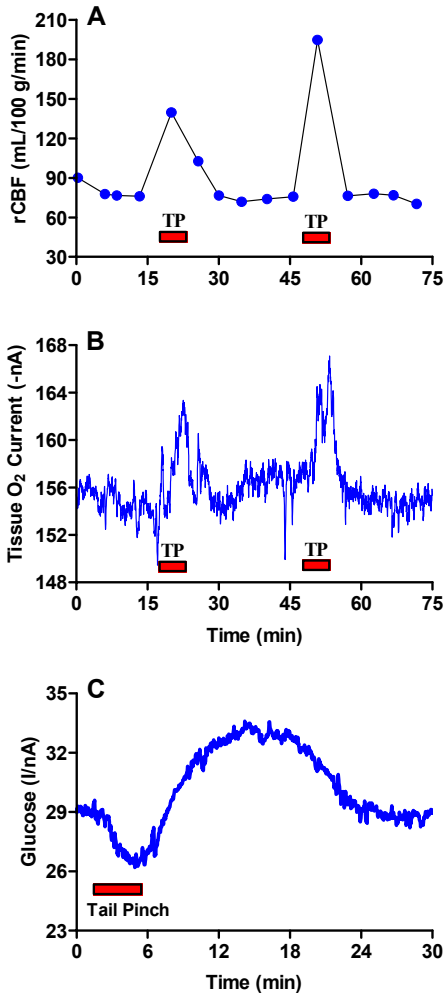

Figure 3. A & B: *Simultaneous measurement of regional cerebral blood flow (rCBF, A) using the H_2 clearance technique (Pt electrode), and tissue O_2 (B) using a carbon paste electrode, in rat striatum in response to neuronal stimulation (5 min tail pinch, TP).*
C: *The effect of a 5 min tail pinch on brain extracellular glucose levels recorded in real time in the striatum of a freely moving rat using a microelectrochemical glucose biosensor.*
Note the dissociation between the glucose and rCBF/O_2 responses during the period of behavioural activation.

Tail pinch increases neuronal activation in the striatum as

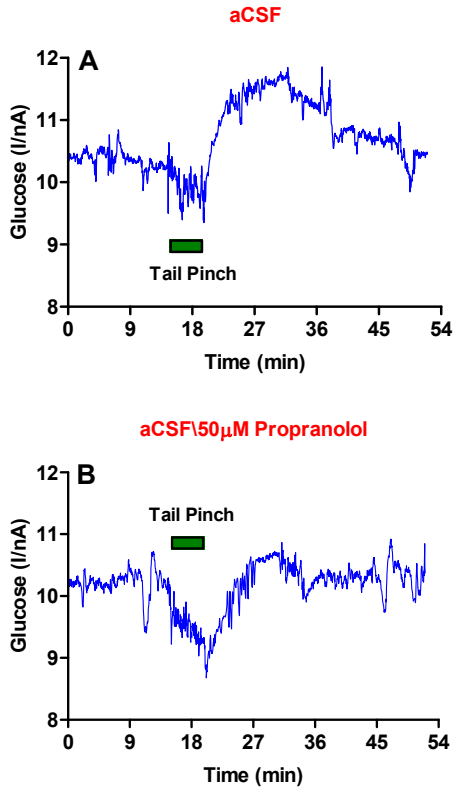

Figure 4. *The effect of a 5-min tail pinch on brain extracellular glucose levels recorded in the striatum of a freely moving rat using a Pt/PPD/GOx glucose biosensor combined with a microdialysis probe.*
A: *Glucose changes during perfusion with aCSF. Mean basal concentration, 329 ± 15 µM*
B: *Glucose changes during perfusion with 50 µM propranolol. Mean basal concentration, 350 ± 27 µM.*

shown by the increased release of dopamine and ascorbic acid [24]. Co-extensive with the increased neuronal activity there was an increase in rCBF. The H_2 clearance measurements show that the increase in rCBF was rapid and began to decline as soon as motor activity ceased. The carbon paste electrode gives a continuous measure of tissue oxygen, which is a balance between supply and utilisation. The close correspondence in the size and time course of the increase in rCBF and oxygen, measured as a percentage of basal, suggests that the changes in oxygen current are simply a reflection of changes in rCBF; there appears to be an increase in supply with no change in oxygen utilisation. Since at no time does the oxygen current decrease below basal level, there appears to be no increased oxygen utilisation either before or after the increase in rCBF.

Using microdialysis with a sampling time of 2.5 min, it has previously been shown that tail pinch induces a brief decrease followed by an increase in glucose. As discussed above, microdialysis provides an indirect measure of ECF glucose which depends on the *in vivo* recovery which cannot be derived from the *in vitro* probe recovery [25]. The glucose oxidase electrode or biosensor provides a direct and continuous measure of ECF glucose, which is the balance between supply and utilisation. The 5% decrease in glucose during the tail pinch, which is very similar to the size of the decrease measured with microdialysis [4], must represent an increase in utilisation, since it occurs during the period of increased rCBF and hence presumably is not a fall in glucose supply. Furthermore, the absence of an increase in oxygen utilisation suggests neuronal aerobic glycolysis. During this period there is an increase in extracellular lactate, that partly overlaps the decrease in glucose [4]. It is not known whether the lactate resulting from neuronal glycolysis passes into the ECF. Although there is much evidence that lactate is exported by astrocytes and utilised by neurones as a metabolic substrate [1], some of the extracellular lactate in response to tail pinch could also be derived from neuronal glycolysis.

At the end of the tail pinch ECF glucose showed a slow rise which took 30 min before it returned to baseline level. The size of this increase as a percentage of basal is also very similar to that seen using microdialysis [4]. This delayed increase in glucose must represent increased supply rather than decreased utilisation since an increase in glucose utilisation by neuronal activation has been demonstrated by the use of the 2-deoxyglucose technique [26]. This increased supply is severely depressed by propranolol, a β-adrenoceptor antagonist. Since propranolol has no effect on the increase in local cerebral blood flow triggered by neuronal activation [4], and blood flow has returned to basal during this late increase in glucose, the increase in glucose must come from a compartment other than the blood stream. Evidence from a number of sources suggests that astrocytes constitute this compartment [1, 16]. Astrocytes have a small store of glycogen with a rapid turnover rate, which in cultured astrocytes is broken down by stimulation of β-adrenoceptors [7]. It has been shown previously in *in vivo* experiments that local infusion of the β-adrenoceptor agonist isoprenaline into the rat striatum causes an increase in extracellular glucose [4]. The present results suggest that propranolol may be used to distinguish between changes in supply and utilisation of glucose and support the hypothesis that the stimulated increase in glucose in response to neuronal activation is derived from astrocytes rather then directly from the blood stream.

There remains the problem of the apparent absence of an increase in oxygen utilisation. Since there is little or no transport of lactate from brain to blood, the lactate, whether derived from neurones or astrocytes must ultimately undergo oxidative phosphorylation which requires increased oxygen utilisation. At present there is no indication when that occurs. Functional magnetic resonance imaging (fMRI) depends on the dissociation of rCBF and oxygen utilisation in response to neuronal activation resulting in an increase in haemoglobin

saturation. The present results suggest that this applies to activation of the striatum. However, there have been reports that certain forms of stimulation in certain brain regions lead to a parallel increase in rCBF and $rCMO_2$ [27]; this would make it blind to fMRI.

4 CONCLUSIONS

The conclusions of this research work can be summarised as follows: (a) the Pt/PPD/GOx biosensor provides a direct real-time measure of the true extracellular concentration of brain glucose; (b) the disparity between the changes in glucose and the changes in rCBF and oxygen during neuronal activation (tail pinch) suggests that extracellular glucose in the brain is not derived directly from the blood vascular system but from some other source; (c) the fact that proranolol has no effect on glucose utilisation but blocks the delivery of glucose suggests that astrocytes are the most likely source.

Finally, these experiments emphasise that continuous, simultaneous measurement of rCBF, oxygen and glucose, provide an important approach to the elucidation of the problems of energy supply and utilisation in the brain.

5 ACKNOWLEDGEMENTS

J.P.L. was supported by Marie Curie Fellowships (E.U. Grant Nos. ERB CHB ICT94 1353 and ERB FMB ICT96 1319) and is a MCFA member, MN1174. Other funding sources supporting this work include the Health Research Board (HRB, EQN12/2000 and RP/14/2002), Enterprise Ireland (SC/99/149 & SC/2003/331), NUI Maynooth (Dean's Fund), the Wellcome Trust and the RSC/EPSRC. The advice and support of Professor Robert D. O'Neill (Dept. of Chemistry, University College Dublin, Ireland) and Dr. Marianne Fillenz (University Laboratory of Physiology, University of Oxford, UK) is gratefully acknowledged.

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