GLUTATHIONE RELEASE FROM ASTROCYTES; CHARACTERIZATION AND IMPLICATIONS FOR NEURODEGENERATION

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Abstract.

Reduced glutathione (GSH) plays a major intracellular role in the protection against oxidizing species. Within the brain, the neuronal GSH concentration may be dependent on the release of this antioxidant from neighbouring cells such as astrocytes. The aim of this thesis was to investigate the characteristics of GSH release from astrocytes, using primary rat astrocyte cultures or glial cell line cultures.

GSH release from astrocytes was positively linear with respect to time and cell density. GSH was released predominantly in the reduced form, and the increase in GSH concentration was not due to extracellular reduction of oxidized glutathione (GSSG). This suggested that the preservation of released GSH with time and cell density was due to the presence of an astrocyte-released factor preventing the oxidation of released GSH.

The astrocyte-released factor had similar characteristics to the extracellular isoform of superoxide dismutase (EcSOD), previously shown to be released by astrocytes. The molecular mass of the factor was determined to be between 50-100 kDa, which is comparable to the molecular mass previously determined for EcSOD in rats. In addition, SOD activity was determined in medium previously exposed to astrocytes.

Excessive generation of nitric oxide (NO') and GSH depletion have been proposed to contribute towards neurodegeneration. It was therefore investigated, whether GSH release by astrocytes was affected by NO' exposure. The ability of astrocytes to release GSH was preserved after exposure to NO', and after the induction of NO' synthesis. Thus GSH release by astrocytes was maintained in the presence of NO'.

In summary, the release of an EcSOD-like factor by astrocytes preserves GSH released by astrocytes. This may be important in limiting neuronal damage from astrocyte-derived oxidizing species such as NO'.
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Abbreviations.

Aβ  β-amyloid
AD  Alzheimer’s disease
ALS  Amyotrophic lateral sclerosis
ATP  Adenosine triphosphate
BBB  Blood brain barrier
BSA  Bovine serum albumin
cytc  Cytochrome c
Cu⁺  Reduced copper
Cu²⁺  Oxidized copper
CuZnSOD  Copper zinc superoxide dismutase
CysGly  Cysteinylglycine
Deta-NO'  Diethylenetriamine nitric oxide adduct
DETC  Diethyldithiocarbamic acid
DMEM  Dulbecco’s modified Eagle’s Medium
DNA  Deoxyribonucleic acid
D-Val MEM  D-Valine based Minimal Essential Medium
EBSS  Earl’s balanced salt solution
EcSOD  Extracellular superoxide dismutase
eNOS  Endothelial nitric oxide synthase
FBS  Fetal bovine serum
Fe²⁺  Ferrous iron
Fe³⁺  Ferric iron
FITC  Fluorescein isothiocyanate
γ-GCS  γ-glutamyl cysteine synthetase
γ-GT  γ-glutamyl transpeptidase
GFAP  Gliial fibrillary acidic protein
GPx  Glutathione peroxidase
GR  Glutathione reductase
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<td>GS&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Glutathiol radical</td>
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<td>GSH</td>
<td>Reduced glutathione</td>
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<td>GSSG</td>
<td>Oxidized glutathione</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<tr>
<td>IFNγ</td>
<td>Interferon-γ</td>
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<tr>
<td>iGSH</td>
<td>Intracellular reduced glutathione</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<tr>
<td>L-NAME</td>
<td>N-nitro-L-arginine methyl ester</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>L-Val MEM</td>
<td>L-Valine based Minimal Essential Medium</td>
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<td>MAO</td>
<td>Monoamine oxidase</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal Essential Medium</td>
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<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
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<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
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<tr>
<td>NADH</td>
<td>Reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitroblue tetrazolium</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>OPA</td>
<td>Orthophosphoric acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>Q</td>
<td>Coenzyme Q</td>
</tr>
<tr>
<td>RO&lt;sup&gt;·&lt;/sup&gt;</td>
<td>Tert-butyl alkoxy radical</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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<tr>
<td>TO&lt;sup&gt;·&lt;/sup&gt;</td>
<td>Tocopheroxyl radical</td>
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<tr>
<td>X-Xo</td>
<td>Xanthine-xanthine oxidase</td>
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Chapter 1.

Introduction.
Chapter 1. Introduction.

1.1. GENERATION AND CONSEQUENCES OF REACTIVE OXYGEN SPECIES IN THE NERVOUS SYSTEM.

1.1.1. Mitochondrial sources of reactive oxygen species.

More than 90% of the oxygen (O₂) utilized by the human body is consumed by the mitochondrial electron transport chain, which adds four electrons onto each O₂ molecule to generate two molecules of water. (Figure 1.1.) This is called oxidative phosphorylation, and is the process by which the transfer of reducing equivalents to oxygen is coupled to the synthesis of ATP. The mitochondrial electron transport chain consists of four polypeptide complexes: complex I (reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase ubiquinone reductase; EC 1.6.99.3.), complex II (succinate-ubiquinone reductase; EC 1.3.5.1.)-complex III (ubiquinol-cytochrome c reductase; EC 1.10.2.2.), and complex IV (cytochrome c oxidase; 1.9.3.1.). Complexes I, III and IV act as oxidation-reduction-driven proton pumps, and catalyze the transfer of reducing equivalents in the form of 2H⁺ and 2 electrons from NADH + H⁺ and succinate to coenzyme Q (ubiquinone). Coenzyme Q can diffuse freely in the membrane, and passes the electrons to a series of 5 different cytochrome molecules (cytochromes b, c₁, c, a and a₃), whereby the electrons are ultimately passed directly to O₂. This transfer of reducing equivalents along the electron transport chain is coupled to the pumping of protons across the inner mitochondrial membrane, generating a proton gradient (Mitchell, 1961) subsequently utilized by ATP synthase (Complex V; EC 4.1.3.7.) for the phosphorylation of ADP to ATP (Bolanos et al, 1997) (Figure 1.1.).

However, about 5% of the electrons flowing through the electron transport chain leak directly from intermediate electron carriers onto O₂ (Boveris et al, 1972). As O₂ accepts one electron at a time, the superoxide anion (O₂⁻) is formed, (equation 1), and therefore reactive species are generated in vivo even under normal biological
Figure 1.1. Mitochondrial ATP synthesis.

The transfer of reducing equivalents is coupled to the pumping of protons across the inner mitochondrial membrane, generating an electrochemical gradient of protons, which consists of a membrane potential and a pH gradient. The electrochemical gradient across the membrane provides the driving force for phosphorylation of ADP to ATP. The flow of protons through ATP synthase back into the mitochondria alters the active site of the enzyme, leading to the synthesis of ATP. Q = coenzyme Q, cyt c = cytochrome c, FMN = flavin mononucleotide, FAD = flavin adenine dinucleotide. Adapted from Bolanos et al, 1997.
conditions (Halliwell et al, 1984; Wagner et al, 1992). \(O_2^-\) can be converted into hydrogen peroxide (\(H_2O_2\)) by superoxide dismutase (SOD) and the addition of two hydrogen ions (equation 11) (Halliwell et al, 1985). Even though \(H_2O_2\) does not initiate redox reactions, it contributes towards the degeneration of oxidative stress. Under conditions of excess reduced iron (ferrous iron, \(Fe^{2+}\)) or reduced copper (\(Cu^+\)), \(H_2O_2\) can lead to the formation of the hydroxyl radical ('OH) and hydroxyl anion (\(OH^-\)) by the Fenton reaction (equation 2 and 3) (Halliwell et al, 1985, 1992b). This reaction may continue further by the reduction of oxidized iron (ferric iron, \(Fe^{3+}\)) or oxidized copper (\(Cu^{2+}\)) by excess \(O_2^-\), through the Haber-Weiss reaction (equation 4 and 5) (Halliwell et al, 1985, 1992a). In addition, the interaction of \(O_2^-\) with nitric oxide (NO) leads to the formation of peroxynitrite (ONOO') (equation 6) (Lipton et al, 1993). After the addition of a hydrogen ion and the formation of the peroxynitroso acid (ONOOH) (equation 7), ONOOH can decompose to 'OH and nitrous oxide (\(NO_2^-\)) (equation 8) (Olanow, 1993). Figure 1.2. summarizes the possible formation of reactive species during the reduction of oxygen.

**1.1.2. Other sources of reactive species in the nervous system.**

Further sources of reactive species can be generated in response to a rise in cytosolic free calcium, and are summarized in Figure 1.3. A rise in free calcium can activate nitric oxide synthase (NOS), which converts arginine into citrulline and NO' (Olanow, 1993). Increased intracellular free calcium can also initiate the irreversible conversion of xanthine dehydrogenase to xanthine oxidase by a calcium activated protease. The enzyme xanthine oxidase can then catalyze the oxidation of xanthine, thereby producing \(O_2^-\). This generation of \(O_2^-\) can then react with NO' to produce a further source of 'OH (Olanow, 1993).

In addition, the oxidation of dopamine, produced by dopaminergic neurones in the substantia nigra pars compacta, can also generate the production of free radicals (Jenner et al, 1992). Dopamine can be oxidized by either monoamine oxidase (MAO) or auto-oxidation, and results in the generation of \(H_2O_2\) (equation 9 and 10).
Summary of equations of the formation of reactive species during the reduction of oxygen.

\[
\begin{align*}
O_2 + e^- & \rightarrow O_2^- \quad [1] \\
H_2O_2 + Fe^{2+} & \rightarrow Fe^{3+} + 'OH + OH^- \quad [2] \\
H_2O_2 + Cu^+ & \rightarrow Cu^{2+} + 'OH + OH^- \quad [3] \\
O_2^- + H_2O_2 & \rightarrow O_2 + 'OH + OH^- \quad [4] \\
O_2^- + H_2O_2 & \rightarrow O_2 + 'OH + OH^- \quad [5] \\
O_2^- + NO^- & \rightarrow ONOO^- \quad [6] \\
ONOO^- + H^+ & \rightarrow ONOOH \quad [7] \\
ONOOH & \rightarrow 'OH + NO_2^- \quad [8]
\end{align*}
\]

Other sources of reactive species formation in the nervous system.

**Enzymatic oxidation of Dopamine**

\[
\text{Dopamine} + O_2 + H_2O \xrightarrow{\text{MAO}} 3,4 \text{dihydroxyphenyl-acetaldehyde} + NH_3 + H_2O_2 \quad [9]
\]

**Auto-oxidation of Dopamine**

\[
\begin{align*}
\text{Dopamine} + O_2 & \rightarrow \text{semiquinone} + O_2^- + H^+ \quad [10] \\
\text{Dopamine} + O_2^- + 2H^+ & \rightarrow \text{semiquinone} + H_2O_2
\end{align*}
\]
Figure 1.2. Formation of reactive species during the reduction of oxygen.
Figure 1.3. The formation of NO$^\cdot$, O_2$^-$ and OH$^-$, after a rise in cytosolic free calcium and the activation of NOS and xanthine oxidase.
1.1.3. Consequences of reactive species within the nervous system.

As the human brain comprises only 2% of the total body weight, but consumes up to 20% of the oxygen utilized by the body (Clarke et al., 1999), it is subject to a high exposure of reactive species that may be generated during oxidative metabolism as described above. As reactive species can undergo redox reactions with other molecules, they can damage a variety of biological molecules. For example, experiments have shown that, in vitro, O$_2^-$ inactivates complex I of the mitochondrial transport chain (Zhang et al., 1990). As H$_2$O$_2$ can easily cross cell membranes, it rapidly reaches distant sites within the cell or surrounding cells. An increased generation of both O$_2^-$ and H$_2$O$_2$ has been shown to damage biological materials by oxidizing membrane lipid and fragmenting deoxyribonucleic acid (DNA) (Halliwell et al., 1991; Wagner et al., 1992). H$_2$O$_2$ has been shown to degrade heme proteins to release iron, thereby providing free iron for the formation of the highly reactive OH$^·$ (Gutteridge, 1986; Puppo et al., 1988a, b). Indeed, O$_2^-$ and H$_2$O$_2$ cause the most damage to biological molecules by the generation of the highly reactive OH$^·$, which reacts at great speed with a great number of molecules such as DNA, causing DNA strand breakage and chemical modifications of the deoxyribose and purine and pyrimidine bases (Brawn et al., 1981; Mello Filho et al., 1984). OH$^·$ also attacks membrane lipids by converting polyunsaturated fatty acid side chains of membrane lipids into lipid peroxides, and initiating lipid peroxidation (Halliwell, 1992a). As lipid peroxidation reduces membrane fluidity and membrane potential, it causes an increase in the permeability of ions such as Ca$^{2+}$ and a loss of membrane integrity.

NO$^·$ has been described to promote Ca$^{2+}$ efflux from the mitochondria, possibly involving the mitochondrial transition pore (Packer et al., 1994, 1996). NO$^·$ has also been shown to inactivate complexes I, II, III and IV of the mitochondrial transport chain (Bolanos et al., 1996). Similarly, ONOO$^·$ has also been demonstrated to inhibit mitochondrial complexes I, II-III and IV (Bolanos et al., 1995). The inactivation of the
mitochondrial complexes by ONOO• ultimately resulted in cell death, and inhibition of these enzymes by ONOO• was suggested to be irreversible (Bolanos et al., 1995). Although the inhibition of the mitochondrial electron transport chain by NO• also appears irreversible, (Bolanos et al., 1994), NO• has been shown to compete with oxygen at complex IV, and was therefore suggested to reversibly inhibit the mitochondrial electron transport chain (Brown et al., 1994). This reversible inhibition was later confirmed by a study demonstrating that the inhibition of respiration by NO• was reversed by inhibiting nitric oxide synthase, or by binding the NO• with haemoglobin (Brown et al., 1995b). This rapid and reversible inhibition of complex IV by NO• is proposed to be a physiological mechanism for controlling cellular respiration (Brown, 1995a).

Certain cells such as astrocytes, have been suggested to have the ability to switch to an increased glycolysis during periods of NO• induced-inhibition of the electron transport chain, maintaining their energy homeostasis (Bolanos et al., 1994). This may be compensatory to mitochondrial respiratory chain inhibition, as an increased glycolysis has been shown to maintain cellular ATP levels (Pauwels et al., 1985), and thereby prevent a possible cell death due to ATP depletion. However, the combined damage to the variety of biological molecules by oxidative stress-induced reactive species, can result in both apoptotic or necrotic cell death (Ratan et al., 1994).

1.2. ANTIOXIDANTS WORK IN SYNERGY TO PREVENT OXIDATIVE STRESS.

1.2.1. Antioxidant protection against reactive species generated in the nervous system.
Under normal conditions, a cell’s oxidative state is maintained by an equilibrium between the generation of reactive species and antioxidant defenses. A primary role of antioxidants is to detoxify reactive species, and protect biological molecules against
oxidative stress. Antioxidant defense mechanisms may be enzymatic (glutathione peroxidase (GPx), SOD, catalase), or non-enzymatic (reduced glutathione (GSH), ascorbic acid, α-tocopherol) in nature.

The enzyme superoxide dismutase (SOD) catalyzes the conversion of \( \text{O}_2^- \) into \( \text{H}_2\text{O}_2 \) by adding a hydrogen atom (equation 11). There are three different isoforms of SOD, where one exists in the cytosol, one within the mitochondria, and another in the extracellular spaces of cells. SOD activity is present within all areas of the human and rat brain (Marklund et al., 1983).

The enzyme GPx reduces \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \), utilizing GSH as a substrate and electron donor (equation 12). In addition, the GSH molecule itself can reduce ·OH to water by donating an electron, thereby participating as an antioxidant directly (equation 13). This direct, nonenzymatic antioxidant defense of GSH is possibly one of the most important reactions involving GSH in the nervous system, as there are no enzymatic defenses against the highly reactive ·OH species. Astrocytes have been shown to have a considerably higher content of GSH than neurones (Raps et al., 1989; Devesa et al., 1993; Makar et al., 1994; Bolanos et al., 1995; Dringen et al., 1999b).

Catalase is an enzyme that decomposes \( \text{H}_2\text{O}_2 \) directly (equation 14). However only little activity of catalase is found in the brain (Halliwell et al., 1985). Astrocytes have been estimated to have approximately a 4-5-fold higher catalase activity compared to neurones (Desagher et al., 1996).

Ascorbic acid (Vitamin C) can also reduce free radicals, and therefore high concentrations of ascorbic acid also have antioxidant potential (Buettner et al., 1997; Pazdernik et al., 1994). Ascorbic acid can reduce ·OH, the tert-butyl alkoxy radical (RO'), the glutathyl radical (GS'), urate and the tocopheroxyl radical (TO') (equation 15). However, ascorbic acid may also have a dual effect, as it can also promote redox reactions and lipid peroxidation when at low concentrations and in the presence of
Summary of reactions between antioxidants and reactive species.

\[
\begin{align*}
SOD & : \text{O}_2^- + \text{HO}_2 + \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \\
2 \text{GSH} + \text{H}_2\text{O}_2 & \rightarrow \text{GSSG} + 2 \text{H}_2\text{O} \\
2 \text{GSH} + 2 \cdot \text{OH} & \rightarrow \text{GSSG} + 2 \text{H}_2\text{O} \\
2 \text{H}_2\text{O}_2 & \rightarrow \text{2 H}_2\text{O} + \text{O}_2 \\
\text{Ascorbic acid} + X^\cdot & \rightarrow \text{Ascorbic acid}^\cdot + \text{XH} \\
\text{where } X^\cdot = \text{OH}^\cdot, \text{RO}^\cdot, \text{GS}^\cdot, \text{urate radical or TO}^\cdot \\
\cdot \text{OH} + \text{H}^+ + \alpha-\text{tocopherol} & \rightarrow \text{H}_2\text{O} + \text{TO}^\cdot
\end{align*}
\]
iron (Muakkassah-Kelly et al, 1983; Rowley et al, 1983). Little is known about the pro-oxidant role of ascorbate in vivo, and as it is present at high concentrations in the grey and white matter of the brain ascorbic acid probably acts as an antioxidant rather than a pro-oxidant under normal conditions. Ascorbic acid is present at a concentration of around 100-500μM in extracellular fluid (Svensson et al, 1993) and around 2mM in the intracellular compartment of fresh brain tissue (Grunewald, 1993). However, it has been shown to be highly concentrated within astrocytes, at a concentration of 7mM (Siushansian et al, 1995). Astrocytes have been described to take up ascorbate by a high-affinity sodium-ascorbate co-transport mechanism (Wilson, 1989; Siushansian et al, 1995), and this may explain the increased concentration of ascorbate determined within these cells, when compared to the concentration of ascorbate within the intracellular compartment of fresh brain tissue (Grunewald, 1993).

Finally, α-tocopherol (Vitamin E) acts as a lipid soluble antioxidant by donating hydrogen atoms to reactive species (equation 16), and astrocytes have been shown to have a 3-fold higher concentration of α-tocopherol as compared with neurones cultured under comparable conditions (Makar et al, 1994).

1.2.2. Synergistic interaction of antioxidants to combat oxidative stress.
Antioxidants function in a sequential series, working in synergy to detoxify reactive species. SOD is the first line of defense against the generation of reactive species, as the removal of O$_2^-$ prevents its direct toxic effects as well as its interaction with NO or metal ions and the subsequent formation of ONOO$^-$ and 'OH. SOD enzymes then co-operate with antioxidants that destroy H$_2$O$_2$, such as catalase and GPx. Finally, α-tocopherol is a lipid-soluble antioxidant (Slater, 1984), and is located in the hydrophobic interior of membranes. Therefore α-tocopherol works in synergy with other antioxidants, by protecting the hydrophobic areas in which water soluble antioxidants such as SOD are repelled, against reactive species. Indeed, the efficiency and importance of a synergistic interaction of a variety of antioxidants is illustrated in
the paper of Chen et al. (1994), where an increased diversity and magnitude of antioxidants in diets proved to provide a maximal protection against oxidative damage to heme proteins. 

The synergistic interaction of antioxidants is also important for the regeneration of antioxidants. For example, GSH has been suggested to be involved in the recycling of dehydroascorbate (Meister, 1994). Although a specific dehydroascorbate reductase has not yet been obtained, it is speculated that dehydroascorbate may be oriented in vivo at sites favorable for reduction by GSH (Meister, 1994). Similarly, the α-tocopheryloxyl radical has also been shown to be reduced back to α-tocopherol by GSH (Meister et al., 1994). Furthermore, ascorbic acid is also thought to provide reducing equivalents to regenerate GSH and the α-tocopheryloxyl radical (Niki et al., 1984; Rose et al., 1993). Indeed, ascorbic acid has been shown to prevent the oxidation of GSH in brain cell cultures incubated with the pro-oxidant tert-butyl hydroperoxide (O’Connor et al., 1995). 

1.3. GLUTATHIONE IN THE BRAIN. 

1.3.1. The synthesis and metabolism of glutathione. 

Glutathione [L-γ-glutamyl-L-cysteinyl-glycine] is a tripeptide consisting of the amino acids glutamate, cysteine and glycine, and is synthesized by two ATP-dependent enzymes (reviewed by Meister et al., 1983). γ-glutamyl cysteine synthetase (γ-GCS) uses cysteine and glutamate to form the dipeptide γ-glutamylcysteine. Glutathione synthetase then forms GSH from γ-glutamylcysteine and glycine. Levels of GSH generated are regulated by feedback inhibition of γ-GCS, as this enzyme is inhibited by a negative feedback loop by GSH (Richman et al., 1975; Wirth et al., 1978). (Figure 1.4.) In addition, γ-GCS activity has also been shown to be increasingly inhibited by a greater phosphorylation by the activation of protein kinase A, protein kinase C or the calcium/calmodulin-dependent kinase II (Sun et al., 1996). The
Figure 1.4. Synthesis and metabolism of glutathione.
Adapted from Dringen et al, 2000.
regulation of the activity of γ-GCS and GSH synthesis, results in a steady balance between the consumption and synthesis of GSH.

Glutathione exists in both a reduced (GSH) and an oxidized (GSSG) form (Figure 1.5). Less than 0.5% is usually found in the disulfide form under normal conditions (Cooper, 1997). GSH and GSSG are interconvertible, where one molecule of GSSG can be converted to two molecules of GSH and vice versa (reviewed by Meister et al, 1983). The enzyme GPx transfers electrons from GSH to an electron acceptor such as H₂O₂, thereby resulting in the oxidation of GSH to GSSG and the subsequent reduction of H₂O₂ to H₂O. GSH is then regenerated again from GSSG by the enzyme GR, which transfers electrons from reduced nicotinamide adenine dinucleotide phosphate (NADPH) to GSSG (Figure 1.4). Therefore the conversion of glutathione between its oxidized and reduced state is a process during which GSH is not consumed but recycled.

1.3.2. Origin of glutathione in the brain.

The concentration of GSH in the whole brain from a variety of species has been reported to range between 1 to 3 mM (Cooper et al, 1997). GSH in the brain may be maintained by the transport of GSH or its constituent amino acids across the blood brain barrier (BBB). For example, several groups have suggested that the brain may be able to take up GSH precursors such as glutamate (in the form of glutamine) and cysteine at the BBB via specific transport systems (Wade et al, 1981; Ennis et al, 1998). Furthermore the tripeptide GSH has also been shown to be transported across the BBB (Zlokovic et al, 1994), which more recently has been demonstrated to be due to a Na⁺-dependent transport system (Kannan et al, 2000). The extent to which transport of GSH into the brain contributes towards maintaining GSH concentration within the brain, is unclear. In addition, GSH may be synthesized de novo within the brain, as γ-glutamyl transpeptidase (γ-GT) has been shown to be expressed within the brain (Ghandour et al, 1980; Shine et al, 1981a, Frey et al, 1991).
Figure 1.5. Reduced (a) and oxidized (b) glutathione
1.3.3. Metabolic compartmentalization and cellular localization of glutathione in the brain.

The brain has been shown to have two metabolically distinct pools of glutamate. One pool consists of a small compartment of rapidly turning-over glutamate, and is distinct from a larger, more slowly-turning-over glutamate compartment (Berl et al, 1961, 1962). Experiments suggested that GSH in the brain may be preferentially synthesized in the smaller compartment (Berl et al, 1961), and it has since been established that the small compartment is associated with astrocytes, and the larger compartment to neurones (Cooper et al, 1987). Indeed, cell culture studies have shown that astrocytes have generally been reported to have a higher GSH content than neurones (Raps et al, 1989; Devesa et al, 1993; Makar et al, 1994; Bolanos et al, 1995; Dringen et al, 1999b), where the GSH content of astrocytes has been reported to range between 16–50 nmol/mg protein, (Raps et al, 1989; Devesa et al, 1993) and between less than 1nmol/mg protein (Raps et al, 1989) to 40nmol/mg protein (Pileblad et al, 1991) in neurones. Tracer studies and histochemical studies also support that GSH is primarily synthesized by non-neuronal, glial cells (Slivka et al, 1987; Philbert et al, 1991). Furthermore, γ-glutamylcysteine synthetase and GSH synthetase are enzymes necessary for GSH synthesis. The finding that astrocytes have an 8-fold higher activity of γ-glutamylcysteine synthetase and GSH synthetase than neurones (Makar et al, 1994), as well as a higher activity of GR than other cells (Huang et al, 1995), may explain why astrocytes have a higher concentration of GSH than other cells such as neurones.

1.3.4. Other functions of glutathione.

Apart from the role of GSH as an antioxidant, GSH also has a variety of other functions. GSH has been suggested to play a role in transport pathways. As γ-GT is located on the cell surface, it has been theorized that GSH and the γ-glutamyl cycle may be involved in the transport of amino acids across the cell membrane (Orlowski et al, 1974). However, the energy required for the transport of amino acids by the cycle has been suggested to be too high and therefore biologically unfavorable,
(Cooper et al, 1997), and the role of amino acid transport by the γ-glutamyl cycle has since been discarded.

A further function of GSH is as an essential cofactor for several enzymatic reactions (reviewed by Meister et al, 1983), such as the glyoxylase reaction in which methylglyoxal is converted to S-lactyl GSH by glyoxylase-1 (Marmstal et al, 1979). GSH also participates as a coenzyme in the cis-trans isomerisation of maleylacetoacetate to fumarylacetoacetate (Edwards et al, 1956), as well as in the dehydrochlorination of 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (Sternburg et al, 1954;). Finally, the reactions catalyzed by prostaglandin endoperoxide D-isomerase and E-isomerase also require GSH as a cofactor (Nugteren et al, 1973).

GSH has also been described to be involved in protective functions, such as the detoxification of harmful xenobiotics through the mercapturate pathway (Cooper et al, 1997a). A further protective function of GSH is as a non-toxic storage form of cysteine. The sulphhydryl of GSH is less reactive than that of cysteine. With the utilization of cysteine for the synthesis of GSH, cysteine concentrations in mammalian tissues are permitted to be 10-100 fold higher, thereby providing a nontoxic storage form of cysteine (reviewed by Cooper, 1997b). Furthermore, high concentrations of glutamate have also been shown to be excitotoxic as a result of N-methyl-D-aspartate/quisqualate glutamate receptor activation (Beal et al, 1993). It has been suggested that under physiological conditions the uptake of extracellular glutamate for the synthesis of GSH may be a way of disposing potentially toxic glutamate (Dringen et al, 1996).

Finally, there is also evidence to suggest that GSH may also have a role as a neurotransmitter in the nervous system, by acting as a selective agonist of glutamate receptors (reviewed by Janaky et al, 1999).
1.4. OXIDATIVE STRESS AND NEURODEGENERATION.

The brain and nervous system may be particularly susceptible to damage by reactive species, as the polyunsaturated fatty acids of membrane lipids of nerve cells are highly sensitive to free radical attack (Stocks et al, 1974). Furthermore, the brain is rich in iron content, but has a relatively low antioxidant capacity (Halliwell, 1985, 1992a; Evans, 1993). The generation of reactive species within the nervous system therefore probably has detrimental effects on the integrity and functioning of nervous system, and oxidative stress may be a contributing factor in the pathology of neurodegenerative diseases.

Evidence for oxidative stress in Parkinson’s Disease (PD), can be drawn from NADPH diaphorase (a marker for NOS activity) positive glial cells found in the substantia nigra of patients with Parkinson’s Disease (PD) (Hunot et al, 1996). Increased neuronal NOS (nNOS) activity and NOS-positive cells (Hunot et al, 1996) provides further evidence of oxidative stress in PD, as does the observation of higher levels of H₂O₂ (Jenner et al, 1992), increased iron concentrations (Riederer et al, 1989; Sofic et al, 1991; Good et al, 1992) and lipid peroxidation in the substantia nigra (Jenner et al, 1992). Furthermore, up to a 40% loss of GSH in the substantia nigra has been reported to be an early and important event in the progression of PD (Sian et al, 1994a).

Evidence for a role for oxidative stress contributing towards the progression of Alzheimer’s Disease (AD), can be drawn from recent findings suggesting that β-amyloid (Aβ) itself may induce oxidative stress by generating reactive species such as H₂O₂, which can damage neurones (Behl et al, 1992, 1994; Butterfield et al, 1994; Hensley et al, 1994). In addition the over-expressed glial-derived factor S100β, has been shown to induce iNOS in astrocytes (Hu et al, 1996). Furthermore, there is evidence of increased lipid peroxidation (Subbarao et al, 1990; Palmer et al, 1994) and DNA oxidation (Mecocci et al, 1994) in AD brains, further supporting a role for
oxidative stress in the condition.

Oxidative damage has also been shown to be a potential factor in multiple sclerosis (MS). Samples of cerebrospinal fluid have shown a marked increase in the NO$^\cdot}$/ONOO$^\cdot$ degradation products nitrate/nitrite (Johnson et al, 1995), suggesting an increase in NO$^\cdot$ production. Post mortem studies have found increases in messenger ribonucleic acid levels coding for inducible nitric oxide synthase as well as a presence of nitrotyrosine residues indicative of ONOO$^\cdot$ formation (Bo et al, 1994; Bagasra et al, 1995). Finally, increased levels of pro-inflammatory cytokines known to induce NOS, have also been shown in MS (Merrill, 1987), PD (Boka et al, 1994) and AD (Griffin et al, 1989; Mrak et al, 1995). This therefore suggests a role for glial-derived NO$^\cdot$ in the neurotoxicity of neurodegenerative diseases.

The energy deficient state occurring during conditions of ischaemia (Chan et al, 1996), is accompanied by increased extracellular concentrations of glutamate (Lincoln et al, 1997). Stimulation of glutamate receptors can lead to elevated cytosolic calcium concentrations (Lincoln et al, 1997), leading to the generation of NO$^\cdot$ as depicted in Figure 1.3, and oxidative stress. Reactive astrocytes in the formation of a gliotic scar have been identified following ischaemia (Rieschke et al, 1990), and as reactive astrocytes have been shown to express inducible NOS, the generation of NO$^\cdot$/ONOO$^\cdot$ by these cells may provide a further source of oxidative stress during this condition. Reperfusion following ischaemia may then provide a further source of oxidative stress, by the generation of O$_2^\cdot$ (Chan et al, 1996).

Finally, oxidative stress may also contribute towards the condition of amyotrophic lateral sclerosis (ALS). In the familial form of the disease, mutations in the copper-zinc subtype of SOD have been identified (Rosen et al, 1993). Such mutations may lead to increased ONOO$^\cdot$ formation as a result of decreased O$_2^\cdot$ scavenging, and initiate reactive species-induced oxidative stress (Beckman et al, 1993). Increased nitrotyrosine (impairing neurofilament assembly) immunoreactivity has also been
shown in motor neurones of both familial and sporadic ALS (Beal et al, 1997), further indicating a role for ONOO'-mediated oxidative damage in these diseases.

1.5. A ROLE FOR ASTROCYTIC GLUTATHIONE IN NEUROPROTECTION.

Astrocytes have been shown to be relatively resistant to oxidative stress. For example, cultured astrocytes have been shown to be resistant to acute exposures of ONOO' of up to concentrations of 2mM, whereas neurones were selectively damaged at much lower concentrations of 0.1mM (Bolanos et al, 1995). In these studies, it was also shown that the neuronal GSH concentration was about half that of astrocytes, and that astrocytes in contrast to neurones, were able to maintain their intracellular GSH concentrations throughout the period of ONOO' exposure (Bolanos et al, 1995). These findings suggest that astrocytes are more resistant to oxidative stress than neurones.

It is the ability of astrocytes to maintain a high intracellular GSH concentration throughout periods of oxidative stress, that probably contributes most towards their resistance to oxidative stress. This is confirmed with the finding that astrocytes become susceptible to the action of ONOO', and mitochondrial complexes I and II+III become severely damaged, if cellular GSH is depleted (Barker et al, 1996).

There is evidence that astrocytes, in contrast to neurones, are able to maintain their energy homeostasis even under extreme conditions of oxidative stress, by switching to an increased glycolysis. (Bolanos et al, 1994; Pauwels et al, 1985). This ability of astrocytes to increase glycolysis and maintain cellular ATP levels even under conditions of oxidative stress, may help to sustain intracellular GSH concentrations. Furthermore, as astrocytes also have higher activities of the enzymes involved in GSH synthesis as well as higher activities of GPx and GR than other cells (as discussed
earlier), astrocytes seem to be particularly well developed to maintain their intracellular GSH levels and general antioxidant status, and to combat oxidative stress.

The above findings suggest, that because of their high intracellular GSH concentration and efficiency to deal with oxidative stress, one of the roles of astrocytes may be to provide a cellular line of defense against oxidative stress in the brain, and to create an extracellular antioxidant environment and protect surrounding cells. Indeed, astrocytes have been shown to protect neurones (Langeveld et al, 1995; Desagher et al, 1996), and oligodendrocytes (Noble et al, 1994) against H$_2$O$_2$ toxicity, where neuronal survival was increased at a ratio of 1 astrocyte to 20 neurones (Desagher et al, 1996). Further evidence for the neuroprotective potential of astrocytes, comes from studies where the coincubation of neurones with astrocytes appeared to limit NO'-induced damage to the neuronal mitochondrial electron transport chain (Bolanos et al, 1996; Stewart et al, 1998).

This neuroprotective potential of astrocytes, may be due to astrocytes maintaining neuronal GSH levels (Sagara et al, 1993a; Dringen et al, 1999a). Indeed, neurones cocultured with astrocytes have been shown to have a significantly higher content of intracellular GSH (Sagara et al 1993; Bolanos et al 1996; Dringen et al 1999a). Several groups have shown that cultured astrocytes release GSH (Yudkoff et al, 1990; Juurlink et al, 1996; Sagara et al, 1996; Dringen et al, 1997a; Wang et al, 2000), and it has been suggested that the release of GSH by astrocytes may provide neurones with GSH precursors such as cysteine (Dringen et al, 1999a; Wang et al, 2000).

Cysteine is one of the amino acid precursors necessary for the synthesis of GSH (Dringen et al, 1996; 1997c). However in stock culture medium, cysteine is usually undetectable due to auto-oxidation (Sagara et al, 1993b). Therefore a much higher proportion of cystine than cysteine may be present in tissue culture medium. Neurones, in contrast to astrocytes, are not able to take up and utilize cystine as a substrate for GSH synthesis (Sagara et al, 1993a; Kranich et al, 1996), and therefore
depend on astrocytes for their supply of cysteine (Sagara et al., 1993a). GSH released by astrocytes has been shown to be a substrate for γ-GT (Dringen et al., 1997a), which is located on the extracellular surface of a number of brain cells (Ghandour et al., 1980; Shine et al., 1981a; Frey et al., 1991). γ-GT transfers the γ-glutamyl segment of GSH onto an acceptor molecule, thereby forming the dipeptide cysteinylglycine (CysGly) (Meister et al., 1981), which is efficiently utilized by neurones as a precursor for neuronal GSH (Dringen et al., 1999a) (Figure 1.6). Astrocyte-induced increase in neuronal GSH has been shown to be suppressed by the inhibition of γ-GT (Dringen et al., 1999a), supporting the hypothesis that CysGly generated from GSH released by astrocytes is a neuronal precursor for GSH synthesis. Moreover, excess CysGly or oxidized CysGly can be taken back up by astrocytes for GSH synthesis (Dringen et al., 1997c).

Furthermore, neurones prefer to utilize glutamine for intracellular generation of glutamate used for GSH synthesis (Kranich et al., 1996). Astrocytes however prefer to utilize glutamate for the intracellular synthesis of GSH, and are able to transport glutamate more efficiently than neurones (Kranich et al., 1996). Astrocytes generate glutamine from glutamate, and release glutamine (Dringen et al., 1999a; Hertz et al., 1999), which can then by utilized by neurones for GSH synthesis. With the release of glutamine by astrocytes, and the extracellular generation of CysGly from released GSH, astrocytes therefore provide precursors for all three constituting amino acids of GSH to neurones (reviewed by Dringen et al., 1999a) (Figure 1.6.).

However, although astrocytes provide protection against reactive species by the release of antioxidants, astrocytes may also contribute towards the production of reactive species in conditions of oxidative stress. Within the brain astrocytes may be a major source of NO' as they have the highest concentration of the NO' precursor L-arginine (Aoki et al., 1991), and contain both the constitutive (Murphy et al., 1990) and inducible form of NOS (iNOS) (Galea et al., 1992). Despite the physiological roles of NO', excess formation in the central nervous system may result in damage to neural
Figure 1.6. Hypothesis of the metabolic interaction between astrocytes and neurones in glutathione metabolism. (Adapted from Dringen et al, 2000). The provision of precursors for all three constituting amino acids of neuronal GSH by astrocytes. 'Glu' denotes glutamate; 'Gln' denotes glutamine; 'Cys' denotes cysteine; 'γGluCys' denotes γ-glutamylcysteine; 'Gly' denotes glycine; 'CysGly' denotes cysteinyl-glycine; 'CysGlyox' denotes the oxidized form of cysteinyl-glycine, 'X' denotes an amino acid acceptor.
cells (Lipton et al, 1993; Bolanos et al, 1994, 1995) Cytokines have been shown to induce the activity of iNOS in astrocytes (Bolanos et al, 1994), and levels of elevated cytokines as well as the induction of NOS has been found in pathological conditions such as MS (Merrill, 1987; Bo et al, 1994), PD (Hunot et al, 1996) and AD (Hu et al, 1996), suggesting that astrocyte-derived NO may be responsible for some of the phenomena observed in these neuropathological conditions. In view of the diffusible nature of NO' and ONOO^−, and the apparent resistance of astrocytes but fragility of neurones to NO' and ONOO^− (Bolanos et al, 1994, 1995), astrocyte-derived NO' may diffuse to susceptible neighbouring neurones and initiate oxidative stress-induced neurotoxicity (Barker et al, 1996). However, this may be prevented initially, as the release of GSH and neuronal GSH precursors by astrocytes could minimize neuronal damage induced by astrocyte-released NO'.

1.6. AIMS.

In summary, there is considerable evidence to suggest that astrocytes release GSH in order to provide surrounding cells, such as neurones, with precursors for GSH synthesis. It is therefore likely that astrocytes are capable of maintaining a steady release of GSH, to provide surrounding cells with GSH precursors over a defined length of time. In addition, astrocytes may also preserve their intracellular GSH concentration during GSH release, and protect released GSH until it is utilized by γ-GT. The aim of this thesis was to further characterize astrocytic GSH release by:

- Evaluating the effect sustained GSH release has upon intracellular GSH status
- Ascertain whether GSH remains in the reduced or oxidized form following release into the extracellular medium
- Determine whether additional factors are released, by astrocytes, to prevent GSH oxidation.
• Document the effect of nitrosative stress on the ability of astrocytes to release GSH.
Chapter 2.

Materials and Methods.


All chemicals were of AnalaR grade, and unless otherwise stated, were purchased from BDH Laboratory Supplies Ltd., Poole, Dorset, U.K. and Sigma Chemical Company, Poole, Dorset, U.K. In particular:

Deoxyribonuclease I Type IV (from bovine pancreas, EC 3.1.21.1), Earle’s Balanced Salt Solution, Hank’s Balanced Salt Solution, bovine serum albumin (from Fraction V albumin, bovine) L-glutamine, antibiotic antimycotic solution (100x), reduced glutathione, oxidized glutathione, glutathione reductase (from bovine intestinal mucosa, EC 1.6.4.2), L-glutamate dehydrogenase (from bovine liver, EC 1.4.1.3.), nitrate reductase (from aspergillus, EC 1.6.6.2.), α-ketoglutaric acid, L-α-amino-3-chloro-4,5-dihydro-5-isoxazole acetic acid, diethyldithiocarbamic acid, triton X-100, primary glial fibrillary acidic protein antibody (monoclonal clone no.: G-A-5), secondary fluorescein isothiocyanate antibody, 100 nominal molecular mass limitation Ultrafree-CL Microcentrifuge filters, lipopolysaccharide (from Escherichia coli), interferon-γ (rat, recombinant), N-nitro-L-arginine methyl ester, diethylenetriamine nitric oxide adduct, bathocuproinedisulfonic acid and copper zinc superoxide dismutase (from bovine erythrocytes, EC 1.15.1.1) were all purchased from Sigma Chemical Company, Poole, Dorset, U.K.

10, 30 and 50 nominal molecular mass limitation Centricon Centrifugal filter devices were obtained from Amicon Bioseparations, Millipore, Watford, U.K.

OX-42 (IgG1) primary antibody was acquired from Dako, Ely, U.K.

25cm², 80cm² and 175cm² tissue culture flasks, multidish 6-well plates, fetal bovine serum heat inactivated, D-Valine based Minimal Essential Medium, L-Valine based Minimal Essential Medium, Phenol-red free Minimal Essential Medium, Dulbecco’s
modified Eagle’s Medium with 4.5g/L glucose, Medium 199, as well as further tissue culture plastics were obtained from Life Technologies, Paisley, U.K.

Reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) and trypsin (from bovine pancreas, EC 3.4.21.4) was bought from Boehringer Mannheim, Lewes, U.K.

Orthophosphoric acid (HPLC Grade) was obtained from Fisher Scientific U.K. Ltd., Loughborough, U.K.

Manganese oxide was acquired from Fluka Chemi. Ag., Neu-Ulm, Switzerland.

Hydrogen peroxide (8.08M) was bought from Aldrich Chemical Company Ltd., Gillingham, Dorset, U.K.

The Bio-Rad DC Protein Assay kit was acquired from Bio-Rad Laboratories, Hercules, California, U.S.A.

All materials used for electron microscopy, such as sodium cacodylate, aqueous osmium tetroxide, agar 100, dodecenyl succinic anhydride, methyl nadic anhydride, 2,4,6-tri (dimethyl aminomethyl) phenol uranyl acetate, Reynold’s lead citrate, borax and toluidine blue were bought from Agar Scientific, Stansted, U.K.

2.2. Cell Culture.

2.2.1. Cell Culture Media Composition.

Solution A consisted of Earle’s Balanced Salt Solution containing deoxyribonuclease I Type IV (75 KU; 20μg/ml), penicillin-streptomycin (1%, vol/vol) and bovine serum albumin (3mg/ml).

Solution B was composed of 20ml Earle’s Balanced Salt Solution supplemented with
deoxyribonuclease I Type IV (826 KU; 220μg/ml), trypsin (0.025%; 25μg/ml), penicillin-streptomycin (1%, vol/vol) and bovine serum albumin (3mg/ml).

Dulbecco’s modified Eagle’s Medium with 4.5g/L glucose (DMEM), and D-Valine or L-Valine based Minimal Essential Medium were all supplemented with fetal bovine serum (10%, vol/vol) (FBS), penicillin-streptomycin (1%, vol/vol) and 2mM L-glutamine.

Medium 199 consisted of N-2-hydroxylpiperazine-N-2-ethanesulphonic acid (HEPES)-buffered Medium 199 containing FBS (10%, vol/vol), penicillin-streptomycin (50 U/ml; 50 μg/ml), amphotericin B (Fungizone; 2.5 μg/ml) and L-glutamine (0.01%, 0.7mM).

Minimal Medium consisted of 44mM NaHCO₃, 110mM NaCl, 1.8mM CaCl₂, 5.4mM KCl, 0.8mM MgSO₄, 0.92mM NaH₂PO₄, 5mM D-glucose, and was adjusted to pH 7.4 with CO₂.

Buffered Hank’s solution consisted of 5.26mM KCl, 0.43mM KH₂PO₄, 134.2mM NaCl, 4.09mM NaHCO₃, 0.33mM Na₂HPO₄, 5.44mM glucose, 2mM CaCl₂ and 20mM HEPES, pH 7.15.

2.2.2. Animals.

Pregnant Wistar rats were bought from A. J. Tuck and Sons Ltd. (Rayleigh, Essex, U.K.), fed ad libitum on a stock laboratory diet, and were maintained on a 12 hour light/dark cycle.

2.2.3. Primary Rat Astrocyte Culture.

2.2.3.1. Cell isolation.

Primary astrocyte cultures were isolated using sterile technique, following a method adapted from Tabenero et al (1993) and Vicario et al (1993). All solutions used were
sterile and heated to 37°C, unless otherwise stated. 0 to 2 day old neonatal Wistar rats were killed by cervical dislocation and decapitated, and the cerebral cortex was removed. Multiple (ranging between 3-12) cerebral cortices were placed into solution A (as described in section 2.2.1.), were mechanically dissociated by trituration, and centrifuged at 2000 rpm for 5 minutes at 4°C. The pellet was then placed into 20ml of solution B (as described in section 2.2.1.), and was incubated at 37°C under a humidified atmosphere containing 5% CO₂ / 95% air for a 15 minute period. Trypsinization was terminated by the addition of FBS (5%, vol/vol), prior to centrifugation as described above. The pellet was re-suspended in solution A, and was further centrifuged as described before. The pellet was then re-suspended in D-Valine based Minimal Essential Medium (as described in section 2.2.1.) (D-Val MEM), before passing through a cell strainer (100μm). The equivalent of two cerebral cortices were distributed into 80cm² tissue culture flasks containing 12ml of D-Val MEM, before incubating at 37°C under a humidified atmosphere containing 5% CO₂/95% air. 24 hours after isolation, and every three days thereafter, the D-Val MEM was replaced with fresh D-Val MEM.

2.2.3.2. Cell trypsinization.

Upon reaching confluency after six days *in vitro*, the cells were passaged by the following procedure: The culture medium was removed and the cells were washed with Hank’s Balanced Salt Solution (HBSS), after which they were dissociated by incubating for a five minute period with trypsin (0.05%, vol/vol). Trypsinization was terminated by the addition of FBS (5%, vol/vol), prior to centrifugation as described above. The cells were then re-suspended in culture medium (L-Valine based Minimal Essential Medium, as described in section 2.2.1.) (L-Val MEM), and distributed into twice the original number of 80cm² tissue culture flasks, before incubating as under previous conditions. The culture medium was replaced every three days. As the cells were split half-way through the culture period, the final cells utilized from these isolations should not be called primary, but secondary astrocytes. However, in this study, astrocytes derived from fresh tissue isolations will be referred to as primary.
2.2.3.3. Cell plating.

Upon reaching confluency after a total of 13 days in culture, the cells were plated onto six-well plates by the following procedure: The culture medium was removed, and astrocytes were washed with HBSS prior to incubating for a five minute period with trypsin (0.05%, vol/vol). Trypsinization was terminated as described above (section 2.2.3.2.), and cells were centrifuged as before (2.2.3.1.). Cells were then resuspended in the culture medium, counted, and plated at a density of either $0.8 \times 10^5$ cells or $1.2 \times 10^5$ cells/cm$^2$ in 6-well plates. Cells were incubated for exactly 24 hours, after which the incubation media was replaced with the respective media to be used for experiments. This time-point marked experimental initiation, and is considered “Time 0” for all experiments described.

2.2.4. Cell Culture of the Human Astrocytoma and the C6 Rat Glioma Cell Line.

The human astrocytoma cell line 1321N1 (ECACC no.: 86030402) and the C6 rat glioma cell line (ECACC no.: 92090409) were provided by the European Collection of Animal Cell Cultures. The C6 rat glioma cell line was kindly provided and maintained by Dr M. Dobbie (Department of Neurochemistry, Institute of Neurology, London, UK) in Medium 199 (as described in section 2.2.1.). The human astrocytoma cell line was cultured in Dulbecco’s modified Eagle’s Medium with 4.5g/L glucose (as described in section 2.2.1.). Cells were cultured in 80cm$^2$ tissue culture flasks at 37°C in a humidified atmosphere consisting of 5% CO$_2$/95% O$_2$, and the culture medium was replaced every 3 days. Each time a respective cell line reached confluency, it was split and plated using the same procedure as described for primary astrocytes (as in section 2.2.3.2. and 2.2.3.3.). 24 hours after cell plating, the incubation media was replaced with the respective media to be used for experiments. Again, this time-point marked experimental initiation, and is considered “Time 0” for all experiments using the human astrocytoma or the C6 rat glioma cell line.
2.3. Reduced Glutathione Analysis.

In the literature, glutathione analysis has previously been performed in a number of ways. For example, total glutathione concentration (the amount of GSH plus GSSG concentration) has been quantified using an enzymatic recycling assay (Tietze et al, 1969; Baker et al, 1990). In the method of Baker et al, the amount of total glutathione was quantified by the rate of 5,5'-dithiobis (2-nitro-benzoic acid) reduction, detected by an increase in absorbance using a microtiter plate reader. After the conjugation of GSH with an addition of excess 2-vinylpyridine, this method could also determine the amount of GSSG in a sample. GSH has also been detected by a variety of different high performance liquid chromatography (HPLC) methods, whereby eluting compounds can either be separated using a reverse phase column (Riederer et al, 1989), or an ion-paired reverse phase column (Harvey et al, 1989). A number of differing detection modes also exist for GSH detection by HPLC. For example, electrochemical detection of GSH has been operated on gold-mercury amalgam electrodes (Dupury et al, 1987; Killa et al, 1989) or porous graphite electrodes (Carro-Ciampi et al, 1988). In addition, GSH has also been quantified by HPLC using fluorimetric detection, whereby a fluorescent signal forms with, and thereby detects the concentration of GSH. For example, a number GSH-selective fluorescent reagents have been reported for the detection of GSH, such as monobromobimane (Svardal et al, 1990), monochlorobimane (Fernandez-Checa et al, 1990), pyrenemaleimide (Johansson et al, 1988), 4-(aminosulphonyl)-7-fluoro-2,1,3-benzoxadi-azole and 7-fluoro-2,1,3-benzoxadiazole-4-sulphonate (Toyo’oka et al, 1986), dansyl chloride (Martin et al, 1991) and o-phthalaldehyde (Neuschwander-Tetri et al, 1989). Flow cytometry has also been applied for the quantification of GSH. This technique involved the staining of GSH with monochlorobimane (Scott et al, 1990) or monobromobimane (Chow et al, 1995), and the GSH concentration was determined by the measurement of fluorescence with a flow cytometer.

The method of GSH analysis used in the present study was adapted from a method by Riederer et al (1989), and was performed using reverse phase HPLC coupled to a
coulometric dual-electrode electrochemical detector (Figure 2.1.). This method was chosen for its sensitivity, as GSH could be detected down to a concentration of 4nM. Furthermore, the ease of sample preparation followed for GSH analysis (as described below), results in a minimal occurrence of artefacts, further ensuring accurate detection of GSH. The chromatographic system consisted of a pump (Beckman Model 110A), a sample injection system (HPLC 360 Autosampler, Kontron Instruments, Watford, U.K.), a HPLC Technology Techsphere reverse phase guard column (Macclesfield U.K.) (1cm x 3mm; octadecasilyl) and a HPLC Technology Techsphere system reverse phase column (Macclesfield U.K.) (25cm x 4.6mm; octadecyasilyl). Detection was by a dual electrode screen model ESA coulometric electrochemical analytical cell used in series, Model 5010 (ESA, Aylesbury, U.K.). The mobile phase was prepared using reverse osmosis de-ionized water, filtered prior to use by a Millipore filter system (Milli-Q Purification System, 18 megaohm-cm resistivity), and consisted of 17mM HPLC grade orthophosphoric acid (OPA). The mobile phase was degassed using a DEG-1033 degasser (Kontron Instruments, Watford, U.K.). Separation was performed while maintaining the column temperature at 30°C by a column heater (Jones Chromatography, Hengoed, U.K.; Omron E5CS), with a flow rate of 0.5ml/min. Peak height was detected using an ESA Model 5100A detector (ESA, Aylesbury, U.K.) and a programmable Thermo Separation Products Chrom Jet Integrator (Manchester, U.K.).

Samples were injected onto a reverse phase column, where separation occurred. The polarity of the octadecyasilyl column was less than that of the mobile phase, and solutes were therefore separated and eluted in order of polarity, the most polar being eluted first. Detection of GSH was performed in the oxidation mode, with a dual electrode coulometric electrochemical analytical cell used in series. The first electrode was used as a screening electrode, where compounds oxidizing at lower potentials than GSH were eliminated from reacting at the second electrode. GSH was oxidized at the second electrode. The magnitude of current generated at the second electrode was recorded as a peak on the integrator, and was proportional to the amount of GSH.
Figure 2.1. High Performance liquid chromatography system for the analysis of reduced glutathione.
To determine the appropriate potentials for the two electrodes to obtain a maximal oxidation of GSH, a Current-Voltage curve was generated. This involved altering the applied potential of both electrodes until optimum peak height of GSH was obtained. The optimal potentials for both electrodes to obtain an optimum peak height for the oxidation of GSH were established at 0.1V for the screening electrode, and 0.6V for the second electrode (Figure 2.2).

Standards were prepared of 0.2, 0.5, 1, 2, 5 and 10μM GSH in 17mM OPA, and used for calibration. 20μl of each standard was injected onto the system, and a standard curve showed that detection of GSH was linear over a range of 0.2 to 10mM (Figure 2.3.). Samples frozen for GSH measurement were thawed and homogenized 1:4 in 17mM OPA, and spun at 14000g for 4 minutes to remove protein. 20μl of the supernatant was then injected onto the system for the measurement of GSH. The stability of GSH during freezing and storing of frozen samples was tested, and was shown not to effect the GSH concentration of samples. Figure 2.4. and 2.5. shows a typical HPLC trace from a 5μM standard and a sample respectively.

2.4. Oxidized Glutathione Analysis.
GSSG was measured after enzymatic conversion to GSH. To 100μl of sample, one unit of GR dissolved in 0.1M potassium phosphate buffer at pH 7.6, and 80μM NADPH dissolved in 0.5% NaHCO₃ was added. The final volume of 1ml of the reaction mixture was then incubated at 37°C under a humidified atmosphere containing 5% CO₂ / 95% air for 10 minutes. GSSG was detected by HPLC as a further increase of GSH in the sample compared to before the conversion. Using this protocol, an excess of 95% of GSSG was converted and detected as GSH, where one molecule of GSSG was converted to 2 molecules of GSH (Figure 2.6.). Control of 10μM GSSG in the presence of both, or the absence of either GR and NADPH were also evaluated. These controls showed a consistency of the assay in converting 10μM GSSG to 20μM GSH when in the presence of both GR and NADPH, but not when in the absence of either.
Figure 2.2. Current-Voltage curve for reduced glutathione.
Figure 2.3. Standard curve for reduced glutathione.
Figure 2.4. Chromatogram from a 5μM standard.
Figure 2.5. Chromatogram from a sample of cell culture medium exposed to astrocytes for 4 hours.
2.5. Protein Analysis.

The determination of protein concentrations in samples was based on the method of Lowry et al. (1951), and was performed using the Bio-Rad DC Protein Assay kit. In brief, cell pellets were resuspended in HBSS, and freeze-thawed three times to lyse cell membranes. Bovine serum albumin (BSA) was used to prepare a standard curve across the range of 0 to 200μg of protein/ml. To 200μl of sample or BSA standard, 100μl of alkaline copper tartrate solution A and 800μl of Folin-Ciocalteu phenol reagent solution B was added. These were then vortexed and left for a 30 minute period in a dark environment. Protein concentrations of the standard curve and unknown samples were then determined by monitoring a change in absorbance at a wavelength of 750nm using a Uvikon model 940 spectrophotometer (Kontron Instruments Ltd., Watford, U.K.).

2.6. Lactate dehydrogenase analysis.

For results obtained from cell cultures, it was essential to establish cell viability. This was important, to ensure that cells remained healthy throughout experimental conditions. In addition, as GSH release from cultured cells was also investigated in this study, it was necessary to confirm that cytosolic contents of cells were not liberated freely as a result of membrane disruption. Lactate dehydrogenase (EC 1.1.1.27) (LDH) is a cytosolic enzyme, and its liberation was therefore used as an index of cell death to determine cell viability. LDH activity was measured in the medium as described by Vassault (1983). 50μl of cell culture medium was taken from
cultured cells both before and after the addition of Triton X-100 (1.0%, 20µl/ml). Triton X-100 was added to the cell culture medium and was left for a 2 minute period, causing a maximum disruption of membranes. The cell culture medium was taken and centrifuged at 14000 g for 4 minutes, to ensure the supernatant was free of protein that could interfere with the assay. The supernatant was then utilized for the assessment of total LDH activity in the cells and the media. The decrease in absorbance of a reference sample containing 950µl phosphate pyruvate buffer at pH 7.5 (containing 38mM K$_2$HPO$_4$, 6.5mM KH$_2$PO$_4$, 0.34mM Na$^+$-pyruvate) and 150µM NADH-Na$^+$ was measured at 30°C by a Uvikon 940 Spectrophotometer (Kontron Instruments Ltd., Watford, U.K.) at a wavelength of 340nm. This was compared to the absorbance of buffer, NADH-Na$^+$ and the addition of 50µl unknown samples of culture medium. The percentage of LDH released into the media was then calculated by the following formula: (LDH activity in the media / total LDH activity) x 100, where total LDH activity represents LDH activity in cells and media.

2.7. Nitrate/Nitrite assay.

Nitrate and nitrite are stable end-products of nitric oxide synthesis, and concentrations of nitrate and nitrite were therefore quantified in culture medium as markers of nitric oxide synthase induction and nitric oxide production and release (Castillo et al, 1993; Ignarro et al, 1993). Nitrate and nitrite concentrations were determined by a colorimetric assay based on the Griess reaction (Green et al, 1982), which relies on a colorimetric reaction between nitrite, sulphanilamide and N-(1-naphthyl) ethylenediamine, measured at an absorbance of 543nm. Nitrate/nitrite concentrations were determined after converting all nitrate to nitrite using nitrate reductase (EC 1.6.6.2.), and therefore this assay measures nitrate and nitrite concentrations as total nitrite concentrations. Nitrate and nitrite calibration standards (concentrations ranging from 0 to 50µmol/L) were prepared by diluting sodium nitrate and sodium nitrite. The assay was performed in a standard 96-well microtitre plate, containing 50µL/well of standard or previously centrifuged (at 14000g for 4 minutes) unknown sample. For the conversion of nitrate in samples to nitrite, 100U/L nitrate reductase (and 25µM
NADPH were added to each well, and the plate was incubated at room temperature for 3 hours. Excess NADPH was consumed by the addition of 500U/L L-glutamate dehydrogenase, 4mM α-ketoglutaric acid and 100mM NH₄Cl followed by a 10 minute incubation at 37°C. The nitrite concentration was then measured by the addition of 50μl of each Griess reagent 1 (1% sulphanilamide in 5% concentrated phosphoric acid) and Griess reagent 2 (0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in water), and the absorbance was read at 540nm using a plate reader after a 10 minute incubation at room temperature.


Peroxynitrite (ONOO⁻) was synthesized using the method described by Hughes et al, 1968: A solution of 0.6M NaNO₂ was simultaneously added with a solution consisting of 8% of 8.08M H₂O₂ and 5.1% of concentrated HCl, into a solution of 1M NaOH. All solutions were of the same volume, and were previously cooled on ice. Excess H₂O₂ was consumed by the addition of sufficient manganese oxide, whereafter the solution was passed through a cell strainer (100μm) and frozen until solid. The concentrated volume of ONOO⁻ (bright yellow in appearance), which forms upon freezing and separates from the remaining solution, was then utilized in experiments. The concentration of ONOO⁻ was determined by the following procedure: 10μl ONOO⁻ was added to 990μl NaOH (1M), and the absorbance was measured at 302nm using a Uvikon 940 Spectrophotometer (Kontron Instruments Ltd., Watford, U.K.). The concentration of ONOO⁻ was then determined using the molar extinction coefficient of 1670 M⁻¹ cm⁻¹.

2.9. Statistical Analysis.

Measurements from individual cultures or experimental preparations were performed in duplicate, and results were expressed as the Mean ± SEM for the number of experimental or culture preparations indicated in legends. Multiple comparisons were made by one-way analysis of variance followed by the least significant difference multiple range test. In all cases, p<0.05 was considered significant.
Chapter 3.

Astrocyte Characterization: Electron Microscopy and Immunohistochemistry.

3.1. INTRODUCTION.

An important consideration when characterizing the release of GSH from primary rat astrocytes, is the purity of cultures. It is essential to verify that the procedure followed for tissue isolation and cell culture results in the growth of a high purity of the required cell type. At present, various laboratories isolating rat astrocytes, have achieved primary cultures ranging from 90-98% purity (Yudkoff et al, 1990; Simmons et al, 1992; Devesa et al, 1993; Bolanos et al, 1995, 1996; Huang et al, 1995; Druckarch et al, 1997; Iwata-Ichikawa et al, 1999; Koyama et al, 2000). The first aim of this study was therefore to confirm that the isolation protocol followed in this present study, resulted in cultures consisting primarily of astrocytes.

Glial fibrillary acidic protein (GFAP) was first described in 1971 to be an acidic protein that was a component of the intermediate filaments found in the cytoplasm of astrocytes (Eng et al, 1971). GFAP was shown to be selectively expressed by astrocytes both in situ (Bignami et al, 1972) and in culture (Bock et al, 1975, 1977), and was described as a defined marker for astrocytes in the central nervous system (Bignami et al, 1972). However, GFAP has also been found in cells of non-neuronal origin such as lens epithelial cells (Hatfield et al, 1984), salivary gland tumors (Nakazato et al, 1982), perisinusoidal stellate cells of liver (Gard et al, 1985; Buniatian et al, 1996) and chondrocytes of elastic cartilage (Kepes et al, 1990). Although the precise function of the GFAP molecule remains unclear, it has been suggested to contribute towards the formation of stable glial processes (Abd-el-Basset et al, 1989). Indeed a suppression of GFAP synthesis has been shown to inhibit the formation of astrocytic processes (Weinstein et al, 1991; Chen et al, 1994), whereas an increased expression of GFAP resulted in the formation of longer cell processes (Rutka et al, 1993; Toda et al, 1994). However a study has also recently reported that
GFAP-deficient astrocytes in primary cerebellar cultures could form normal processes in response to neurones, and therefore GFAP may not be a sole prerequisite for the formation of astrocytic processes (Pekny et al, 1998).

Intermediate filaments containing GFAP can be detected with electron microscopy. As GFAP is primarily localized within the central nervous system to astrocytes, these cell types can be therefore be identified by electron microscopy by their presence of intermediate filaments. In addition, with the use of specific antibodies against GFAP, GFAP-positive cells can also be identified using immunohistochemical techniques. This chapter therefore identifies astrocytes in primary cultures utilized in this study, using electron microscopy and immunohistochemistry. In addition, the percentage of microglial growth in primary cultures of this present study was also estimated, using an anti CR3 complement receptor antibody using OX-42 (Robinson et al, 1986). Microglia express CR3 complement receptors throughout development, and can therefore be identified by OX-42 (Robinson et al, 1986; Graeber et al, 1988).

3.2. METHODS.

3.2.1. Light Microscope Photography.
To investigate the appearance of rat astrocytes during a period of tissue culture, astrocytes were observed and photographed under low magnification. Rat astrocytes were cultured as described in Chapter 2, section 2.2. After one week of culture, astrocytes were photographed using a Nikon digital camera (Coolpix 950, Nikon UK Ltd., Kingston, Surrey, UK) under a magnification of x100 by a Nikon TMS light microscope (Nikon UK Ltd., Kingston, Surrey, UK). This was performed with the kind help of C. Casley, (Department of Neurochemistry, Institute of Neurology, London, U.K.).
3.2.2. Electron Microscopy.

Isolated primary cultures were investigated using electron microscopy, and identified as astrocyte cultures by the presence of filaments representing histochemically detected GFAP in isolated cells. The cells were kindly prepared with the assistance of B.C. Young (Inter-Departmental Electron Microscope Unit, Institute of Neurology, London, U;K.) and examined with the help of Professor D. N. Landon (Inter-Departmental Electron Microscope Unit, Institute of Neurology, London, U;K.) In brief, primary rat astrocytes were plated at a density of $0.5 \times 10^6$ cells/cm$^2$ in L-Val MEM in six-well plates after 13 days in culture, as described previously in Chapter 2, section 2.2. The cells were then washed with HBSS, and fixed by the addition of 3% glutaraldehyde in buffer (the latter consisting of 0.1mM sodium cacodylate, 5mM CaCl$_2$) at pH 7.4 over a 2-hour period. Thereafter, the cells were washed with distilled water, and submersed in 1% aqueous osmium tetroxide for a 2-hour period, as a secondary fixative and a cell 'stain'. After further rinsing in distilled water, specimens were dehydrated in ascending grades of ethanol (70%, 90% and 100%). A mixture of 100% ethanol and epoxy resin (the latter consisting of agar 100, dodecenyl succinic anhydride, methyl nadic anhydride and 2,4,6-tri(dimethyl aminomethyl) phenol) (at a ratio of 1:1) was then added to the cells for a 30 minute period. This was replaced with 100% epoxy resin, and left for a further 3 hours at room temperature. Thereafter, the resin was incubated at 60°C over-night, for polymerization to take place. Areas for electron microscope study were selected from samples of 1μm survey sections, stained with toluidine blue/1% borax. 70nm-thin sections of cells contained within the resin were then cut on an RMC MT6000 ultramicrotome, using a Diatome diamond knife. Cut sections were collected on 3mm mesh copper grids, and stained with 25% uranyl acetate in 50% methyl alcohol over a 20 minute period. The sections were washed in methanol, and transferred to aqueous Reynold's lead citrate for a 20 minute period. After further washing in distilled water, the stained cells were examined in a JOEL 1200EX electron microscope.
3.2.3. Immunohistochemistry.

The purity of the isolated primary astrocyte cultures was evaluated immunohistochemically with an anti-glial fibrillary acidic protein (monoclonal clone no: G-A-5) antibody. Astrocyte cultures were also stained with the microglial marker OX-42, to determine the presence of microglia. Briefly, after 13 days in culture, primary rat astrocytes were plated as described previously in Chapter 2, section 2.2, at a density of 0.5x10^6 cells/cm^2 in L-Val MEM onto glass coverslips (13mm x 0.17mm), which had previously been washed in ethanol. To allow the cells to attach to the coverslips, they were incubated for a 24 hour time period under conditions as described before (section 2.2.3.3.). The cells were then washed with HBSS, and fixed by placing into ice-cold acetone solution over a 15 minute period. After further washing with HBSS, the moisture from the coverslips was carefully removed, and the cells were placed into a dry dish. A blocking solution consisting of 1% BSA/Phosphate Buffered Saline (PBS) solution, (wght/vol) was then added for 1 hour at room temperature. After washing with PBS, the cells were incubated with either the OX-42 primary antibody or the GFAP primary antibody (both diluted 1:200) in a moist environment at room temperature for 1 hour. To control for autofluorescence as a result of staining, a selection of astrocytes were incubated under the same conditions without the addition of a primary antibody. The primary antibody-labeled cells were then washed with 1% BSA/PBS (wght/vol) and the secondary fluorescein isothiocyanate (FITC) labeled anti-mouse IgG antibody (diluted 1:20) was added, and left in a dark, moist environment at room temperature for 1 hour. To control for nonspecific staining by the secondary antibody, the FITC labeled antibody was also added to cells in which no primary antibody had been added to. After extensive washing with 1% BSA/PBS, the coverslips were mounted onto glass slides with setting mount, and left to dry in a dark environment for two days. Control slides, GFAP and OX-42 labeled astrocytes were then visualized by an Olympus IX 70 inverted fluorescence microscope. Images were captured using a Life Sciences Resources Mira-Cal Imaging System. Fluorescent responses to either GFAP or OX-42 positive cells were obtained at 380nm excitation wavelengths, with emission above
505nm wavelengths. The cells were also photographed using the same exposure time. The cells were kindly prepared and examined with the help of Dr. J. Pocock (Department of Neurochemistry, Institute of Neurology, London, U.K.).

3.3. RESULTS.

3.3.1. Astrocyte Morphology during Tissue Culture.
Figure 3.1. shows a photograph of rat astrocytes taken under low magnification during a period of tissue culture. The photograph depicts a healthy appearance and an even morphology of cells with respect to size and shape. Extending processes can be identified at the stellate-like projections of cells. The uniform spreading of cells shows even proliferation during culture conditions.

3.3.2 Electron Microscopy of Cultures.
A high distribution of filaments most likely representing histochemically detected GFAP (represented as arrow A on the electron micrographs) were found in the cultured cells (Figure 3.2. and 3.3.). In addition, a possibly higher distribution of GFAP was found in the extending processes (Figure 3.3.) than in the cell body (Figure 3.4.). Organelles such as ribosomes (arrow B), rough endoplasmic reticulum (arrow M), lysosomes (arrow D), the internal lumen of endoplasmic reticulum (arrow E) secondary lysosomes (arrow F), mitochondria (arrow G) and empty vesicles (arrow H) can also be detected on the electron micrographs shown in Figure 3.2., 3.3. and 3.4. In addition, the electron micrograph depicted in Figure 3.4. shows two separate cells, which can be seen to be separated by an indefinite line (arrow L). Both cells contain a nucleus (arrow K) and the above cell can be seen to contain a nucleoli (arrow J). The nucleus of the bottom cell depicted, can be seen to contain patchy increases in internal density, representing the last remains of segregated chromosomes. Therefore the bottom cell could be described to have recently divided.
3.3.3. Immunohistochemistry of Cultures.
After the evaluation of 11 separate fields of cells, 94% of cells were found to be positively labeled by the primary anti-GFAP antibody and secondary antibody, and identified as astrocytes (Figure 3.5.A.). In comparison, only 6% of cultured cells were positively labeled after incubation with the primary antibody OX-42 and secondary antibody, and determined to be microglia (Figure 3.6.A). However cells incubated with the secondary antibody only, did not result in a positive labeling of cells (Figure 3.5.B. and 3.6.B.), eliminating the possibility of autofluorescence as a result of staining.

3.4. DISCUSSION.

The even morphology of cells in culture with respect to size and shape observed under low magnification, suggests a uniform growth of the culture. The morphology of astrocytes in vivo has previously been described as predominantly stellate in the white matter, and mostly flat/polygonal in the grey matter (Walz et al, 2000). This is in agreement with the morphology observed in the present study, as the cells, originally isolated from the white matter, presented extending processes and were stellate-like in shape.

The likely determination of the presence of GFAP-positive cells by both electron microscopic and immunohistochemical methods, confirms the primary cell culture to be of a glial nature. Furthermore the observation of a higher distribution of GFAP in the cellular extensions than the cell body may support the suggestion of the function of GFAP contributing towards the formation of stable glial processes (Abd-el-Basset et al, 1989; Weinstein et al, 1991; Chen et al, 1994). The low proportion of positive labeling of cells after incubation with the microglial marker OX-42, confirms a minimal presence of microglia and high purity of the astroglial cell culture. This confirmation of a high purity of astroglial cell culture reduces the possibility of data
contamination by additional cell types.

Contamination with 6% of microglial cells in the cell culture, may be a reflection of the difficulty in achieving a 100% pure primary cell culture from a cell isolation from the brain. As the brain is characterized by the close interaction and co-localization of numerous brain cell types, a sample of brain tissue isolated for the culture of primary cells will inevitably contain a variety of different cell types.

Depending on the isolation and culture technique for purifying the cell culture, a high percentage of cell purity may be achieved in the cell culture. For example the culture method adapted for the culture of rat astrocytes in this current study, involves cell trypsinization half way through the culture period and before cell plating. Trypsinization of cells during the culture of rat astrocytes has been confirmed to reduce the number of contaminating microglia by 80% (Simmons et al, 1992). Therefore the process of trypsinization during the culture period of rat astrocytes in this present study contributes towards the purity of the astrocyte culture.

The use of a D-Valine containing medium has been shown to retard fibroblast growth, and result in a greatly purified astroglial cell culture (Cholewinski et al, 1989). Indeed, fibroblasts have been reported to lack the enzyme D-amino acid oxidase and are therefore unable to convert and utilize D-Valine for growth (Gilbert et al, 1975). In this latter paper, astroglial cell cultures grown in D-Valine containing medium were shown to have a 92% purity of astrocytes, and contain less than 3% fibroblasts, less than 5% oligodendrocytes, no neurones, and less than 5% macrophages and/or microglia. Therefore the utilization of D-Valine based MEM throughout the first half of the culture period in this current study also contributes towards the purification of the astrocyte culture.

Current techniques utilized by various laboratories for isolating rat astrocytes, have been shown to produce cultures that vary between 90-98% in their content of
astrocytes (Yudkoff et al, 1990; Simmons et al, 1992; Devesa et al, 1993; Bolanos et al, 1995,1996; Huang et al, 1995; Druckarch et al, 1997; Iwata-Ichikawa et al, 1999; Koyama et al, 2000), which is in agreement with the percentage of astrocytes determined in the cultures of this present study. As in this present study, these laboratories therefore also see a low percentage of cells other than GFAP-positive cells in their astrocyte cultures.

The technique followed for the culture of rat astrocyte in this present study was adapted from a procedure followed by Tabemero et al (1993) and Vicario et al (1993). These two authors observed a 90-95% immunopositivity against GFAP in their cultures, and this is identical to the percentage of GFAP-positive cells determined in the primary cultures of this present study.

Finally the limitations of utilizing a primary culture with a high percentage of cell purity must be recognized, when making implications about this cell type within the intact central nervous system. The intact central nervous system is characterized by the close interaction of various cell types by both physical contact and the secretion of factors. Therefore cells such as astrocytes in an almost pure primary culture may have different morphologies or physiological responses from astrocytes interacting with and in the presence of other cell types such as neurones (Hattan et al, 1985; Corvalan et al, 1990). In addition, cells in vivo are not subjected to the numerous limitations and variables under cell culture conditions, which most likely also has a dramatic affect on the physiology of cells in culture. However although there are limitations in the conditions of cells grown in an in vitro primary culture, observations can still be interpreted, with the recognition of the limitations of this system.
Figure 3.1. Astrocyte morphology during tissue culture. Final magnification x 100.
Figure 3.2. Electron micrograph of a section of a primary cultured cell. Photograph shows a high distribution of filaments representing histochemically detected GFAP (arrow A). In addition, photograph shows ribosomes (arrow B), lysosomes (arrow D), rough endoplasmic reticulum (arrow M) and the internal lumen of endoplasmic reticulum (arrow E). Final magnification x 15000.
Figure 3.3. Electron micrograph of an extending process of a primary cultured cell. Photograph shows a high distribution of filaments representing histochemically detected GFAP (arrow A). In addition, photograph shows ribosomes (arrow B), mitochondria (arrow G) and empty vesicles (arrow H). Final magnification x 15000.
Figure 3.4. Electron micrograph of the cell body of primary cultured cells. Photograph shows rough endoplasmic reticulum (arrow M), secondary lysosomes (arrow F), mitochondria (arrow G), empty vesicles (arrow H), nucleus (arrow K) and nucleoli (arrow J). The two separate cells are separated by an indefinite line (arrow L). Final magnification x 7500.
Figure 3.5. Histochemical identification of astrocytes in primary cultures. Primary cultured cells labeled with the primary anti-GFAP antibody and the secondary antibody (A), or the second antibody only (B).
Figure 3.6. Histochemical identification of microglia in primary cultures. Primary cultured cells labeled with the primary antibody OX-42 and the secondary antibody (A), or the second antibody only (B).
Chapter 4.

The Release and Preservation of Reduced Glutathione by Astrocytes.
4.1. INTRODUCTION.

A primary role of GSH in the nervous system is as an antioxidant (Cooper et al, 1997; Shaw et al, 1998). Neurones have been suggested to be particularly vulnerable to free radical damage (Olanow, 1993; Halliwell, 1992, 1996; Evans, 1993; Bolanos et al, 1995, 1996). Astrocytes are reported to have a higher GSH content than neurones (Slivka et al, 1987; Raps et al, 1989; Devesa et al, 1993), and this may contribute towards their increased resistance to reactive species as compared to neurones in vitro (Bolanos et al, 1994, 1995; Iwata-Ichikawa et al, 1999). Therefore the release of GSH by astrocytes may have neuroprotective potential. For example, GSH released by astrocytes may provide neighbouring cells, such as neurones, with precursors for GSH synthesis. Dringen et al, (1999a) and Wang et al, (2000) have suggested that the release of GSH by astrocytes provides neurones with GSH precursors such as cysteine. Indeed, neurones cultured with primary rat astrocytes have been shown to have a significantly higher content of intracellular GSH (Sagara et al, 1993a; Bolanos et al, 1996; Dringen et al, 1999a). Neurones in coculture with astrocytes have also been shown to have a lower susceptibility to oxidative stress possibly as a result of their increased intracellular GSH concentration (Langeveld, 1995; Bolanos et al, 1996; Iwata-Ichikawa, 1999). Therefore the release of GSH or GSH precursors by astrocytes may also contribute towards the protection of neurones against oxidative stress.

Although various groups have shown a release of GSH from primary rat astrocytes, there is little consistency between the data with respect to the concentration of GSH released. For example, Yudkoff et al (1990) measured approximately 3µM GSH after 3 hours of release from 1x10^6 primary rat astrocytes/ml of medium. Similarly, Wang et al (2000) also measured a concentration of 3µM of released GSH. However, this was released by 1.5x10^6 primary rat astrocytes/ml of medium after a 12 hour...
incubation period. Therefore, the increased cell density and incubation period in the paper of Wang et al as compared to Yudkoff et al, did not result in a higher concentration of GSH release. Furthermore, in contrast with the above papers, another study could only establish a release of GSH from primary astrocytes as a result of cell death (Juurlink et al, 1996).

A primary aim of this current chapter was to further characterize the release of GSH by cultured rat astrocytes. This was achieved by initially investigating the relationship between cell density and GSH release. The release of GSH by other glial cells, such as the human astrocytoma cell line 1321N1 (ECACC no.: 86030402) and the C6 rat glioma cell line (ECACC no.: 92090409), was also compared to the release of GSH by rat astrocytes. GSH release from rat astrocytes was also studied in the presence of the γ-GT inhibitor, acivicin (α-amino-3-chloro-4,5-dihydro-5-isoxazole acetic acid), (Figure 4.1.).

![Acivicin structure](attachment:image)

Figure 4.1. Structure of acivicin (Stole et al, 1994).

Acivicin transforms to an inhibitory species, after binding to a specific hydroxyl group close to the catalytic site of γ-GT, and rapidly and reversibly inhibits the enzyme up to 99% (Stole et al, 1994). Dringen et al (1997a) showed that GSH released by rat astrocytes was apparently increased following an inhibition of γ-GT.
To further characterize the release of GSH from rat astrocytes, GSH released in the presence of increasing concentrations of acivicin was investigated. The intracellular GSH (iGSH) concentration was also monitored, to establish if the iGSH concentration varied during a period of GSH release. To ensure that any GSH measured was released via an efflux mechanism, and not due to leakage from the cell following damage to the cell membrane, LDH release was measured to establish membrane integrity and hence cell viability. The final objective for the characterization of the release of GSH by rat astrocytes, was to establish if glutathione was released in the reduced or the oxidized form.

Oxygen is a strong oxidizing agent, which can form reactive species (Halliwell et al., 1985). Medium used for the culture of cells invariably contains O$_2$. Thus reactive species can potentially be generated within culture medium. GSH can react both directly and indirectly with reactive species such as the OH radical, and is oxidized in the process (Shaw, 1998). Therefore the stability of GSH in a solution may vary, depending on the rate of the generation of reactive species.

As a pre-requisite for experiments investigating the release of GSH by astrocytes into the extracellular culture solution, a principal aim of the current chapter was to establish the stability of GSH in different solutions. This was to ensure that a medium chosen for release experiments was not a medium in which released GSH would rapidly decay, and therefore not be detected. Minimal Essential Medium (MEM) supplemented with L-glutamine and fetal bovine serum, is the medium routinely used in our laboratory for the culture of primary rat astrocytes. Thus, the stability of GSH in supplemented MEM was investigated to establish the suitability of this medium for performing GSH release experiments. Minimal Medium is a medium previously used by Dringen et al. (1997a), for experiments determining the release of GSH by astrocytes. Therefore, the stability of GSH in Minimal Medium was also investigated. Finally, the stability of GSH in the presence of double distilled water (dd H$_2$O) was also monitored, as a comparison to the stability of GSH in either Minimal Medium or
Although GSH may be oxidized by reactive oxidizing species present in tissue culture medium, several groups have measured a cumulative increase of released GSH from cultured rat astrocytes into the extracellular culture medium (Yudkoff et al., 1990; Sagara et al., 1996; Dringen et al., 1997a; Wang et al., 2000). Therefore, the presence of astrocytes may prevent GSH oxidation by free radicals in tissue culture medium. This possible preservation of GSH by astrocytes was considered. Consequently the last section of this current chapter compares the stability of GSH in medium previously conditioned by astrocytes to the stability of GSH in unconditioned medium.

In summary, the aims of this chapter were to select a suitable medium for GSH release experiments, to further characterize the release of GSH by astrocytes, and to investigate if astrocytes preserve released GSH.

4.2. METHODS.

4.2.1. Cell Culture:
Primary rat astrocytes, the human astrocytoma cell line 1321N1 (ECACC no.: 86030402) and the C6 rat glioma cell line (ECACC no.: 92090409), were cultured as previously described in Chapter 2, section 2.2. Upon reaching confluency, the cells were plated at a density of either 0.8 or 1.2 x 10^6 cells per well onto six-well plates as described in Chapter 2, section 2.2.3.3. 24 hours after cell plating, the culture medium in wells with 0.8 x 10^6 cells per well was replaced with either 1.5 or 3 ml of medium. The application of these two volumes of media to 0.8 x 10^6 cells per well, will be referred to as either 0.25 or 0.5 x 10^6 cells per ml of media in the remainder of this chapter. In addition, cells plated at a density of 1.2 x 10^6 cells / well were replaced with 1 ml of culture medium. This replacement of media marked the start of experiments.
4.2.2. Preparation of conditioned Minimal Medium:
24 hours after plating rat astrocytes or the human astrocytoma cell line at a density of either 0.8 x 10^6 cells or 1.2 x 10^6 cells per well onto six-well plates, the culture medium was replaced with 1.5 or 1ml of Minimal Medium respectively. Minimal Medium was left for a period of 4 hours in the presence of the primary astrocytes or the human astrocytoma cell line, and was then recovered and utilized in experiments investigating the stability of GSH in conditioned Minimal Medium.

4.2.3. Storage of samples:
Media utilized to determine GSH release, GSH stability or GSSG formation was immediately frozen in liquid nitrogen and stored at -80°C. Samples taken for the determination of the iGSH and protein concentration were resuspended in HBSS, and also immediately frozen in liquid nitrogen and stored at -80°C.

4.2.4. Biochemical analysis:
GSH analysis and protein concentrations were determined as described in Chapter 2, section 2.3. and 2.5. respectively. GSSG was measured after conversion to GSH as described in Chapter 2, section 2.4., and the percentage LDH released by cells was determined as previously described in Chapter 2, section 2.6.

4.2.5. Experimental protocols:

GSH stability in different solutions: GSH was added to MEM (supplemented as described in Chapter 2, section 2.2.1.), Minimal Medium (prepared as described in Chapter 2, section 2.2.1.), or to dd H2O, to a final concentration of 50μM. Measurements of the GSH concentration in the above solutions were taken at several time points after GSH addition over a 6 hour incubation period at 37 °C.

iGSH concentration of rat astrocytes in the presence of MEM and Minimal Medium: The iGSH concentration of rat astrocytes in the presence of either MEM or
Minimal Medium was determined immediately following, and 2 hours after medium replacement.

**GSH release from the various cell types:** GSH release from primary rat astrocytes, the C6 rat glioma cell line and the human astrocytoma cell line was evaluated in the presence of Minimal Medium at a number of time points over a 4 hour incubation period at 37 °C.

**Determination of the proportion of released glutathione in the reduced form:** During the 4 hour time period of release from rat astrocytes, the concentration of released GSH and GSSG was determined as described above (section 4.2.4.), and the proportion of glutathione present in the reduced form was calculated.

**Determination of the ability of conditioned medium to reduce GSSG:** Minimal Medium was conditioned by rat astrocytes as described above (section 4.2.2.). GSSG (5μM) was then added to the conditioned medium, and the concentration of GSH was observed over a 6 hour incubation period at 37 °C.

**Determination of the iGSH concentration and release of LDH during GSH release experiments:** The iGSH concentration and release of LDH was determined in primary rat astrocytes, the C6 rat glioma cell line and the human astrocytoma cell line at various time points, during a 4 hour time period of GSH release.

**GSH release in the presence of acivicin:** The release of GSH by rat astrocytes into Minimal Medium containing increasing concentrations of acivicin (0 - 20μM, diluted from a stock of 10mM acivicin dissolved in 10mM HCl), or HCl (to a final concentration of 20μM) was evaluated at several time points over an 8 hour incubation period at 37 °C. GSH release was investigated over an extended time period of 8 hours, and a density of only 0.25 x 10⁶ cells per ml of media was used for this study, to replicate conditions of the paper of Dringen *et al* (1997a).
The stability of GSH in conditioned Minimal Medium: Minimal Medium was conditioned by rat astrocytes or the human astrocytoma cell line as described above (section 4.2.2.). GSH (5μM or 20μM) was added to the conditioned Minimal Medium, and its stability was determined at various time points over a 5 hour incubation period at 37 °C. Furthermore, GSSG formation was followed in these experiments utilizing 20μM GSH.

4.2.6. Statistical analysis and data evaluation:
Results were expressed as the Mean ± SEM. Statistical analysis was performed as described in Chapter 2, section 2.9.

The concentration of GSH released by rat astrocytes or the human astrocytoma cell line into Minimal Medium during a period of conditioning (ranging from approximately 1-3μM), was subtracted from the final concentrations of GSH determined in experiments observing the stability of GSH in conditioned Minimal Medium.

4.3. RESULTS.

4.3.1. GSH Stability in Water, MEM or Minimal Medium.

Although GSH was found to be stable in dd H₂O over the 6 hour time period investigated (Figure 4.2.), dd H₂O was not considered a suitable medium for the culture of cells, as osmotic rupturing of the cells would occur. The concentration of GSH in MEM declined rapidly to undetectable levels over the 6 hour incubation period, with an estimated half-life of less than 0.25 hours (Figure 4.2.). However, the half-life of GSH in Minimal Medium was estimated to be 4.5 hours (Figure 4.2.). As GSH was found to decay rapidly in MEM but was relatively stable in Minimal Medium, and Minimal Medium has also been utilized by Dringen et al for release
experiments (1997a), Minimal Medium was selected as a medium for further experiments investigating the release of GSH by astrocytes into the extracellular culture solution.

MEM is the solution routinely used by our laboratory for the culture of cells, as well as throughout the duration of an experiment. To ensure that the use of Minimal Medium in future GSH release experiments, rather than the routinely used MEM, did not compromise the GSH concentration of primary rat astrocytes, the iGSH concentration of primary rat astrocytes cultured in MEM and Minimal Medium was compared after a 2 hour incubation period. No significant difference between the iGSH concentration of primary rat astrocytes cultured in MEM or Minimal Medium was found (Table 4.1.).

Data summary of section 4.3.1.:
♦ GSH was relatively stable in Minimal Medium when compared to MEM. Minimal Medium was therefore selected as a suitable medium for further experiments investigating the release of GSH by astrocytes into the extracellular culture solution.
♦ No significant difference in the iGSH concentration was observed in primary rat astrocytes, when cultured in MEM or Minimal Medium.

4.3.2. Characterization of the Release of GSH by Astrocytes.

For the initial characterization of the release of GSH by astrocytes, GSH release from rat astrocytes plated at increasing densities was monitored (Figure 4.3.). Rat astrocytes were found to release GSH, and a higher cell density of astrocytes resulted in an increased release of GSH (Figure 4.3.). In addition there was a direct correlation between cell density and GSH release from astrocytes (Figure 4.4.).
The release of GSH from rat astrocytes was compared to that of other glial cells, i.e. the human astrocytoma cell line 1321N1 and the C6 rat glioma cell line. Whilst the human astrocytoma cell line was found to release GSH, there appeared to be no GSH released from the C6 rat glioma cell line (Figure 4.5. and 4.6.). Although there was no significant difference, there was a trend for the human astrocytoma cell line to release a lower concentration of GSH when compared to rat astrocytes (Figure 4.5. and 4.6.). More specifically, human astrocytoma released GSH at a rate of 0.2μM per hour as compared to 0.25μM per hour released by rat astrocytes, when plated at a density of 0.5 x 10⁶ cells/ml of medium (Figure 4.5.). When plated at a higher density of 1.2 x 10⁶ cells/ml of medium, human astrocytoma released GSH at a rate of 0.3μM per hour as compared to 1μM per hour released by rat astrocytes (Figure 4.6.). However there appeared to be an undetectable rate of GSH release from the C6 rat glioma cell line (Figure 4.5. and 4.6.).

GSH release from rat astrocytes was monitored during inhibition of γ-GT by acivicin. Increasing concentrations of acivicin were found to display a ‘bell-shape’ effect with regards to GSH release (Figure 4.7.). A significant increase in GSH release from rat astrocytes was observed in the presence of 2μM acivicin, as compared to the release of GSH in the absence of acivicin (Figure 4.7.). No significant increases in detectable GSH were apparent in the presence of 1, 5, 10 or 20μM acivicin (Figure 4.7.). As acivicin was dissolved in HCl, the addition of increasing concentrations of acivicin also resulted in the addition of increasing concentrations of HCl (up to a final concentration of 20μM). Therefore, to ensure that the effect of acivicin on GSH release was not due to the presence of HCl, GSH release by rat astrocytes was also determined in Minimal Medium containing 20μM HCl. No significant difference in GSH release could be determined in Minimal Medium in either the absence or presence of added HCl (where 0.2, 0.5, 0.9 and 1.2μM GSH was released at 2, 4, 6 and 8 hours respectively in the absence of HCl, and 0.3, 0.5, 1.0 and 1.3μM GSH was released at 2, 4, 6 and 8 hours respectively in the presence of HCl).
In order to establish if the release of GSH corresponded to a decline in the iGSH concentration, the iGSH concentration of rat astrocytes, human astrocytoma and C6 rat glioma cell line was monitored during the period of GSH release. The iGSH concentration of rat astrocytes was significantly lower after 4 hours of GSH release (Table 4.2.). However, no significant decrease in the iGSH concentration was apparent in the human astrocytoma or the C6 rat glioma cell line (Table 4.2.). In addition, a significantly lower iGSH concentration was found in the C6 rat glioma cell line throughout the 4 hour period of GSH release, as compared to rat astrocytes or the human astrocytoma cell line (Table 4.2).

The amount of GSH released from the various cell types during a period of GSH release was calculated as a percentage of their iGSH concentration (Table 4.3.). Rat astrocytes were found to release a considerably higher percentage of their iGSH concentration, compared to the human astrocytoma or the C6 rat glioma cell line (Table 4.3.).

To ensure cell viability during the period of GSH release, LDH release from primary rat astrocytes, the C6 rat glioma or the human astrocytoma cell line was evaluated. No more than approximately 3.5% of LDH was released from the respective cell types throughout the 4 hours (Table 4.4.).

The final objective for the characterization of the release of GSH by rat astrocytes, was to document the proportion of the total glutathione present in the extracellular medium that is present in the reduced form, i.e. as GSH. Figure 4.8. shows the percentage of the total glutathione present as GSH as a function of time. The proportion in the reduced form was found to increase, with both an increase in time and cell density (Figure 4.8.).

To determine if this increase in the proportion of released glutathione in the reduced form was due to a conversion of released GSSG to GSH, GSSG was added to rat
astrocyte-conditioned Minimal Medium and the concentration of GSH was observed. No further increase in the GSH concentration was detected after a 6 hour incubation.

**Data summary of section 4.3.2.:**

- A linear relationship between cell density and GSH release was established for primary rat astrocytes.
- GSH was not released by the C6 rat glioma cell line. Although there was not a significant difference, there was a trend for the human astrocytoma cell line to release a lower concentration of GSH compared to rat astrocytes.
- The iGSH concentration of rat astrocytes significantly declined during incubations where release occurred.
- A significantly lower iGSH concentration was found in the C6 rat glioma cells when compared to rat astrocytes or the human astrocytoma cell line.
- Rat astrocytes released a considerably higher percentage of their iGSH concentration, when compared to the human astrocytoma and C6 rat glioma cell line.
- Increasing concentrations of acivicin (0-20μM) resulted in a ‘bell shaped’ dose response curve with regards to GSH release from rat astrocytes; a maximal enhancement of extracellular GSH concentration was determined in the presence of 2μM acivicin.
- No significant release of LDH from rat astrocytes, the human astrocytoma or the C6 rat glioma cell line was determined over the periods of this study.
- The percentage of total glutathione present in the extracellular medium in the reduced, GSH form, increased with incubation time and cell density.

4.3.3. Preservation of Reduced Glutathione by Astrocytes.

The observation that the percentage of total released glutathione present as GSH increased with time and cell density (Figure 4.8.), may suggest that a factor is released
by astrocytes that preserves GSH. To investigate this suggestion further, the stability of GSH in Minimal Medium was compared to its stability in Minimal Medium previously conditioned by rat astrocytes or the human astrocytoma cell line 1321N1.

The stability of 5μM GSH was increased in Minimal Medium previously conditioned for 4 hours by rat astrocytes, when compared to ‘unconditioned’ Minimal Medium (Figure 4.9.). The concentration of GSH in Minimal Medium declined by more than 90% within a 2 hour time period, i.e. a half life of less than 1 hour (Figure 4.9.). In comparison, the concentration of GSH in Minimal Medium conditioned by rat astrocytes declined by 66% after 4 hours, with an estimated half life of approximately 3.5 hours (Figure 4.9.).

20μM GSH also displayed increased stability in Minimal Medium conditioned by rat astrocytes (Figure 4.10). In unconditioned Minimal Medium, the concentration of GSH decreased with an estimated half-life of approximately 3 hours. In comparison, in the presence of Minimal Medium conditioned by either 0.5 or 1.2 x 10^6 cells/ml, the estimated half life was approximately 5 hours or above (Figure 4.10). Furthermore, 20μM GSH was also more stable in Minimal Medium conditioned by the human astrocytoma cell line, than in unconditioned Minimal Medium (Figure 4.11).

Although the loss of GSH in conditioned medium was retarded, the protection was not 100%. Consequently, at the end of a defined incubation period, GR and NADPH were added. Following such an incubation, a marked increase in GSH was noted (Table 4.5.), suggesting that oxidation of GSH occurred.

Data summary of section 4.3.3.:
♦ The stability of GSH was significantly increased in the presence of Minimal Medium conditioned by rat astrocytes, as compared to Minimal Medium.
♦ Minimal Medium conditioned by the human astrocytoma cell also preserved GSH.
♦ Although GSH stability was increased by conditioned Minimal Medium, oxidation to GSSG still occurred.

4.4. DISCUSSION.

4.4.1. Selection of a Medium for Experiments Investigating Glutathione Release.

Although GSH was stable in dd H₂O₂, it was not considered a suitable solution for cell culture as osmotic rupturing of the cells would occur, and was only used as a comparison to the stability of GSH in MEM and Minimal Medium. As the concentration of GSH in MEM declined rapidly with a half life of less than 0.25 hours, this medium was also not considered suitable for future experiments investigating the release of GSH by astrocytes into the extracellular culture solution. GSH was found to be relatively stable in Minimal Medium, with a half-life of approximately 4.5 hours. Minimal Medium has also previously been used for experiments determining the release of GSH by astroglial cells (Dringen et al., 1997a). Therefore some comparisons with previous observations can be made, and Minimal Medium was utilized in all further studies monitoring the release of GSH by astrocytes.

A possible explanation for the relative stability of GSH in Minimal Medium compared to MEM, may be that the stability of GSH in a particular tissue culture medium depends on the potential generation of reactive species generated within a given medium. Tissue culture medium invariably contains O₂ and metal ions. O₂ is a strong oxidizing agent (Halliwell et al., 1985). The acceptance of a single electron by O₂ forms the superoxide radical (equation 1). O₂⁻ itself has a limited reactivity (Halliwell, 1992), but has been found to damage particular biomolecules (Tyler et al, 1975). Furthermore, in aqueous solutions, O₂⁻ is converted to hydrogen peroxide
(H$_2$O$_2$) (Halliwell et al, 1985) (equation 2). H$_2$O$_2$ itself is unreactive, but can react with certain metal ions such as iron (Fe$^{2+}$) ions to form the hydroxyl radical ‘OH by the Fenton reaction (equation 3). ‘OH is a highly oxidizing species, damaging DNA, proteins, carbohydrates and lipids (Halliwell et al, 1985).

\[
\begin{align*}
\text{O}_2 + e^- & \rightarrow \text{O}_2^- \quad \text{equation 1} \\
2 \text{O}_2^- + 2\text{H}^+ & \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad \text{equation 2} \\
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^- \quad \text{equation 3}
\end{align*}
\]

Glucose has been shown to oxidize rapidly when in the presence of transition metal ions, causing the formation of O$_2^-$ in the process (Wolff et al, 1987), and the subsequent initiation of the formation of other reactive species as mentioned above. GSH can react both directly and indirectly with free radicals such as OH and H$_2$O$_2$, and is oxidized in the process (Shaw, 1998). Therefore the stability of GSH in a solution containing glucose may be limited. Glucose contained within MEM may undergo oxidation in the presence of transition metal ions, therefore causing a subsequent formation of O$_2^-$, H$_2$O$_2$ and ‘OH. However, Minimal Medium and MEM contain the same concentration of glucose (5mM), and GSH was found to be considerably more stable in Minimal Medium than in MEM. Therefore the generation of free radicals, and the resulting subsequent decay of GSH within a medium containing glucose, does not explain the rapid decay of GSH observed in MEM. However it is possible that the concentration of transition metals within MEM may be higher than in Minimal Medium, and a higher concentration of transition metals in MEM could lead to an increased oxidation of glucose and free radical formation, as compared to Minimal Medium.

The stability of GSH in Minimal Medium also appears to be related to the initial
concentration of GSH added to the medium: An initial GSH concentration of 50µM added to Minimal Medium had an estimated half-life of 4.5 hours; in comparison initial concentrations of 20 and 5µM GSH added to Minimal Medium had estimated half-lives of approximately 3 hours and less than 1 hour respectively. This finding suggests that at lower GSH concentrations, rapid loss of GSH will occur; i.e. the ratio of GSH : oxidants present in the medium appears to be an important factor in influencing GSH stability.

As Minimal Medium was found to be a suitable medium for further experiments investigating the release of GSH by astrocytes, it was important to establish that this medium, rather than the routinely used MEM, did not affect the iGSH concentration of primary rat astrocytes. However, the iGSH concentration of primary rat astrocytes determined after a 2 hour time period when cultured in Minimal Medium or MEM was found to be comparable, and further confirmed that Minimal Medium would be suitable for GSH release experiments.

4.4.2. Release of Glutathione by Astrocytes.

After establishing a suitable medium for GSH release experiments, GSH release from rat astrocytes was characterized. The data reported here suggest a linear release, with regard to time, of GSH. Furthermore, the concentration of GSH, present in the extracellular medium appears proportional to cell density.

This demonstration of release of GSH by rat astrocytes is in agreement with several groups (Yudkoff et al, 1990; Sagara et al, 1996; Juurlink et al, 1996; Yonezawa et al, 1996; Dringen et al, 1997a; Wallin et al, 1999; Kannan et al, 2000; Wang et al, 2000). Yudkoff et al (1990) showed a release of 3µM GSH from 1x10⁶ rat astrocytes/ml of medium after a 3 hour time period. This is comparable to the concentration of GSH released from rat astrocytes observed in the present study. In
addition, several groups have also shown a linear increase in the concentration of GSH released from rat astrocytes with time (Sagara et al, 1996; Dringen et al, 1997a; Wang et al, 2000). In contrast however, Yudkoff et al did not establish a linear increase in the concentration of released GSH with time. The GSH release observed by Yudkoff et al was found to reach a maximum concentration and plateau after one hour of release.

A possible reason for the different rates of GSH release from rat astrocytes determined by Yudkoff et al and the current study may be due to different experimental conditions. GSH was released from rat astrocytes into Minimal Medium in the present study, but into Ham’s F-12 medium in the study of Yudkoff et al. As demonstrated earlier in this chapter, the stability of GSH in a solution may vary, depending on the extracellular medium used. It may be that the concentration of transition metals within Ham’s F-12 medium is higher than in Minimal Medium, therefore resulting in an increased formation of free radicals (Wolff et al, 1987) and higher rate of oxidation of GSH when released into Ham’s F-12 medium.

LDH leakage was evaluated, to ensure cell viability throughout experiments monitoring GSH release. The low level of LDH leakage obtained indicates that the data was not compromised due to perturbation of the plasma membrane. This observation suggests that a coincidental release of GSH, as a result of the liberation of cytosolic contents, is not occurring. This is in direct contradiction to the conclusions by the study of Juurlink et al (1996), where a release of GSH from astrocytes is described as a result of cell death, as measured by an increase of LDH release. It may be possible that the Earle’s salt solution utilized in the study of Juurlink et al did not provide cultured astrocytes with sufficient precursors for GSH synthesis. This may have resulted in a lower rate of intracellular GSH synthesis of the cultured astrocytes, and subsequently have compromised the concentration of GSH release. In addition, the subjection of astrocytes to an O2-free environment in the study of Juurlink et al may have resulted in decreased cell viability. The onset of cell death would have
resulted in the liberation of cytosolic contents, and this may explain why Juurlink et al (1996) could only establish a release of GSH from astrocytes as a result of cell death.

Furthermore, although the studies of Yudkoff et al (1990), Kannan et al (2000) and Wang et al (2000) demonstrated a release of GSH from astrocytes, cell viability was not determined. Therefore, the possibility of GSH release from astrocytes as a result of cell death, cannot be discarded in these papers. However in the studies of Sagara et al (1996), Dringen et al (1997a, 1999a) and Yonezawa et al (1996), both the release of GSH and a high percentage of cell viability was determined in rat astrocytes utilized. These studies therefore support the findings of the current study that GSH is released by rat astrocytes, and that GSH release is not as a result of cell death as proposed by Juurlink et al (1996).

GSH release from rat astrocytes was compared to other glial cells such as the human astrocytoma cell line and the C6 rat glioma cell line. GSH release has not previously been reported for human astrocytes, and the demonstration of GSH release by a human-derived cell line in the current study may imply that astrocytes in the human nervous system also release GSH. In contrast to rat astrocytes and the human astrocytoma cell line, the C6 rat glioma cells do not appear to release significant amounts of GSH. This may be as a result of an inferior intracellular GSH concentration. The reason for this difference is not clear but may be a reflection of the transformed nature of this cell line. Consequently, studies utilizing this cell line should, in view of this observation, be interpreted with caution.

For further characterization of GSH release, GSH release was investigated during an inhibition of γ-GT. The increased concentration of released GSH observed in the presence of acivicin, may be due to the prevention of its degradation as a result of the inhibition of γ-GT. As previously described by Dringen et al (1997a), GSH released by rat astrocytes was found to be a substrate for γ-GT. Dringen et al (1999a) suggests that γ-GT utilizes GSH released by astrocytes for the generation of the dipeptide
cysteinyl-glycine, which is subsequently used as a neuronal GSH precursor.

In this current study, concentrations of acivicin above 2\(\mu\)M caused a concentration-decline in the apparent release of GSH from rat astrocytes. However, Dringen \textit{et al.} (1997a) found a concentration-dependent increase in the release of GSH from rat astrocytes with increasing concentrations of acivicin, where a maximal release of GSH was observed in the presence of 20\(\mu\)M acivicin. This discrepancy of data could be due to many factors. One possible explanation may be the differences in the age of rat astrocyte-cultures utilized. Rat astrocytes in this current study were utilized at 14 days \textit{in vitro}; however Dringen \textit{et al} performed experiments on rat astrocytes of variable ages of 14 to 21 days \textit{in vitro}. It may be possible that high concentrations of acivicin are potentially toxic to younger astrocytes. As astrocytes utilized by Dringen \textit{et al} have had up to an extra 50\% of time in culture, their detoxification systems may be more developed than astrocytes in this current study. It may be possible that no further increase in GSH release from rat astrocytes incubated with increasing concentrations of acivicin above 2\(\mu\)M was detected in this study, as a result of the onset of cell death due to their inability to detoxify higher concentrations of acivicin. However, LDH release was monitored throughout the experiment and cell death was not detected. Therefore, higher concentrations of acivicin did not cause cell death in this current study, but may have compromised GSH metabolism and release without affecting cell viability.

The iGSH concentration during a period of GSH release was investigated. The iGSH concentration of rat astrocytes, but not the human astrocytoma or the C6 rat glioma cell line, was found to decrease significantly during a period of GSH release. This may be explained by the former observation of this current study, that rat astrocytes tend to release a comparatively higher concentration of GSH than either the human astrocytoma or the C6 rat glioma cell line. This suggests that the release of GSH is as a result of the direct release of GSH from the intracellular compartment. Indeed, Sagara \textit{et al.} (1996) has found that the rate of GSH release was dependent on the
intracellular level of GSH, where the rate of GSH release was found to increase almost linearly with an increase in GSH content. Therefore the significantly lower iGSH concentration of the C6 rat glioma cell line determined as compared to the rat astrocytes and the human astrocytoma cell line, may explain the lower concentration of GSH release established in this cell line. Wang *et al* (2000) also concludes that GSH was released from rat astrocytes from an intracellular reservoir of GSH. Both Sagara *et al* (1996) and Dringen *et al* (1997a) have estimated that 10% of the iGSH concentration is released per hour from rat astrocytes during a period of GSH release, and this is in agreement with the estimated 6-12% of the iGSH concentration released per hour in the current study. The finding that rat astrocytes were not only found to release the highest concentration of GSH, but were also found to release the highest percentage of their iGSH concentration, suggests that rat astrocytes have a higher capacity to release GSH as compared to glial cell lines. This could imply that rat astrocytes may possibly have a greater potential than transformed cells, to protect surrounding cells against oxidative stress. Further studies are clearly required to support this suggestion.

The final objective for the characterization of the release of GSH from rat astrocytes, was to establish if glutathione was present in the extracellular medium in the reduced or the oxidized form. Previous studies have either measured released glutathione from astrocytes in the reduced form (Yudkoff *et al*, 1990; Juurlink *et al*, 1996; Sagara *et al*, 1996; Yonezawa *et al*., 1996; Wang *et al*, 2000), or as total glutathione (Dringen *et al*, 1997a, 1999a). However, the relative proportion of released glutathione that is present in the reduced and oxidized form has not been extensively investigated. In this current study, the proportion of reduced glutathione present in the extracellular medium, was found to increase with time and cell density. In addition, no further increase in the GSH concentration was detected in rat astrocyte-conditioned medium after the addition of GSSG. GSSG was therefore not converted to GSH when in the presence of conditioned medium. The increase of the percentage of glutathione released by rat astrocytes in the reduced form with time and cell density observed in the current study
was therefore not due to a reduction of released GSSG. Therefore the finding of an increase in the proportion of released glutathione in the reduced form, suggests the presence of an astrocyte-derived factor preventing oxidation of released GSH.

4.4.3. Preservation of Reduced Glutathione by Astrocytes.

To investigate if an astrocyte-derived factor may preserve the concentration of released GSH, the stability of GSH in Minimal Medium was compared to the stability of GSH in Minimal Medium previously conditioned by rat astrocytes. Both 5\(\mu\)M and 20\(\mu\)M GSH were considerably more stable in conditioned medium than in Minimal Medium. This supports the hypothesis that an astrocyte-derived factor in conditioned medium may prevent the degradation of GSH. In addition, Minimal Medium conditioned by the human astrocytoma cell line preserved the concentration of GSH to the same degree as rat astrocytes.

Although GSH was preserved by Minimal Medium conditioned by rat astrocytes, a lower concentration of GSH (5\(\mu\)M) was found to decay more rapidly than a comparatively higher one (20\(\mu\)M). Although conditioned Minimal Medium may contain an astrocyte-released factor preventing the oxidation of GSH, conditioned medium may also contain transition metals, which would lead to the oxidation of glucose contained within the medium and the subsequent formation of free radicals (Wolff et al, 1987). A lower concentration of GSH may therefore be oxidized more rapidly than a higher concentration of GSH by a possible generation of free radicals in conditioned Minimal Medium. In addition, the former observation, that the ratio of GSH : solution present in a medium is also an important factor in influencing GSH stability, also supports the determination of a shorter half-life of 5 \(\mu\)M GSH as compared to 20\(\mu\)M GSH.

After establishing that conditioned medium retarded GSH degradation, the loss of
GSH in rat astrocyte-conditioned medium was shown to be due to oxidation to GSSG. Almost 100 percent of the initial GSH concentration could be recovered after GR addition to both rat astrocyte-conditioned medium or Minimal Medium. Thus, the decline of GSH concentration in both conditioned medium and Minimal Medium was as a result of its oxidation into GSSG.

CONCLUSIONS.

In summary, the conclusions of this chapter are that rat astrocytes and the human astrocytoma release reduced glutathione. Furthermore, astrocytes appear to release a factor that retards the oxidation of GSH released by astrocytes.
Figure 4.2. The stability of 50μM glutathione in double distilled water, MEM or Minimal Medium. Data are expressed as μM, and are mean ± SEM for 3 independent experiments.
Figure 4.3: GSH release from rat astrocytes plated at increasing densities. Primary rat astrocytes were plated at a density of 0.25, 0.5 and 1.2 $\times$ 10^6 cells/ml of medium. Data are expressed as μM, and are mean ± SEM for 3 independent culture preparations. a p<0.05 when compared to the same time point for data from 0.25 $\times$ 10^6 cells/ml. b p<0.05 when compared to the same time point for data from 0.5 $\times$ 10^6 cells/ml.
Figure 4.4: Concentration of released GSH after 4 hours of release, by rat astrocytes plated at variable densities. Primary rat astrocytes were plated at increasing densities of 0.25, 0.5 and $1.2 \times 10^6$ cells/ml of medium. Data are expressed as $\mu$M, and are mean ± SEM for 3 independent culture preparations.
Figure 4.5: A comparison of the release of GSH from rat astrocytes, the human astrocytoma cell line, and the C6 rat glioma cell line, when plated at a density of $0.5 \times 10^6$ cells/ml of medium. Data are expressed as μM, and are mean ± SEM for 3 separate experimental preparations. * $p<0.05$ when compared with C6 rat glioma at 4 hours.
Figure 4.6: A comparison of the release of GSH from rat astrocytes, the human astrocytoma cell line and the C6 rat glioma cell line, when plated at a density of $1.2 \times 10^6$ cells/ml of medium. Data are expressed as μM, and are mean ± SEM for 3 separate experimental preparations. * p<0.05 when compared with C6 rat glioma at 4 hours.
Figure 4.7: The effect of increasing concentrations of acivicin on extracellular GSH. Cells were plated at a density of 0.25 x 10^6 primary rat astrocytes/ml of medium. Extracellular GSH was determined after an 8 hour incubation. Data are expressed as μM, and are mean ± SEM for 3 independent culture preparations. * p<0.05 when compared to data in the absence of acivicin.
Figure 4.8: The proportion of total glutathione released by rat astrocytes present in the reduced form. Primary rat astrocytes were plated at a density of 0.5 or 1.2 x 10^6 cells/ml of media. Data are expressed as a percentage, and are mean ± SEM for 3 separate experimental preparations. * p<0.05 when compared to data for 0.5 x 10^6 primary rat astrocytes/ml of media.
Figure 4.9. A comparison between the stability of 5μM GSH in Minimal Medium, and in Minimal Medium conditioned by 0.5 x 10^6 rat astrocytes/ml of medium. Data are expressed as μM, and are mean ± SEM for 3 separate experimental preparations. * p<0.05 when compared with the stability of GSH in Minimal Medium at the same time point.
Figure 4.10: A comparison between the stability of 20μM GSH in Minimal Medium, and in Minimal Medium conditioned by 0.5 or 1.2 x 10^6 rat astrocytes /ml of medium. Data are expressed as μM, and are mean ± SEM for 3 or more separate experimental preparations. * p<0.05 when compared with the stability of GSH in Minimal Medium at the same time point.
Figure 4.11: A comparison between the stability of 20µM GSH in Minimal Medium and in Minimal Medium conditioned by rat astrocytes or the human astrocytoma cell line. Minimal Medium was conditioned by 1.2 x 10^6 primary rat astrocytes or human astrocytoma cells/ml of medium. Data are expressed as µM, and are mean ± SEM for 3 or more separate experimental preparations. * p<0.05 when compared to the same time point for Minimal Medium.
<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>iGSH concentration (nmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minimal Medium</td>
</tr>
<tr>
<td></td>
<td>0.5 x 10^6 cells</td>
</tr>
<tr>
<td>0</td>
<td>17.3 ± 1.6</td>
</tr>
<tr>
<td>2</td>
<td>15.9 ± 1.9</td>
</tr>
</tbody>
</table>

Table 4.1: A comparison of the iGSH concentration of rat astrocytes when cultured in Minimal Medium or MEM. Cells were plated at a density of either 0.5 or 1.2 x 10^6 cells/ml of medium. Data are expressed as nmol/mg of protein, and are mean ± SEM for 3 separate culture preparations.
<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>iGSH content (nmol/mg of protein)</th>
<th>rat astrocytes</th>
<th>human astrocytoma cell line</th>
<th>C6 rat glioma cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 x 10^6 cells</td>
<td>1.2 x 10^6 cells</td>
<td>0.5 x 10^6 cells</td>
<td>1.2 x 10^6 cells</td>
</tr>
<tr>
<td>0.25</td>
<td>18.8 ± 2.6</td>
<td>22.6 ± 1.7</td>
<td>21.6 ± 1.1</td>
<td>21.2 ± 0.4</td>
</tr>
<tr>
<td>2</td>
<td>15.6 ± 1.5</td>
<td>18.9 ± 1.9</td>
<td>18.9 ± 1.8</td>
<td>19.3 ± 1.0</td>
</tr>
<tr>
<td>4</td>
<td>13.4 ± 1.1 *</td>
<td>14.9 ± 0.8 *</td>
<td>18.0 ± 2.0</td>
<td>19.3 ± 0.2</td>
</tr>
</tbody>
</table>

Table 4.2: The effect of a 4 hour incubation period on the iGSH concentration of rat astrocytes, the human astrocytoma cell line and the C6 rat glioma cell line. Cells were plated at a density of either 0.5 or 1.2 x 10^6 cells/ml of medium. Data are expressed as μM, and are mean ± SEM for 3 or more separate culture preparations. *p<0.05 when compared to 0.25 hours, for the same cell density of primary rat astrocytes.
<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Percentage of total iGSH concentration released</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rat astrocytes</td>
</tr>
<tr>
<td></td>
<td>0.5 x 10^6 cells</td>
</tr>
<tr>
<td>0.25</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>11.1 ± 1.1</td>
</tr>
<tr>
<td>4</td>
<td>28.9 ± 2.1</td>
</tr>
</tbody>
</table>

Table 4.3. The percentage of total intracellular GSH released from rat astrocytes, the human astrocytoma cell line and the C6 rat glioma cell line, during a period of GSH release. Cells were plated at a density of either 0.5 or 1.2 x 10^6/ml of medium. Data are expressed as a percentage of the iGSH concentration released, and are mean from 3 or more separate experimental preparations.
Table 4.4: LDH release from rat astrocytes, the human astrocytoma cell line and the C6 rat glioma cell line, during a period of GSH release. Cells were plated at a density of either 0.5 or 1.2 x 10^6 cells/ml of medium. Data are expressed as a percentage of the total LDH release, and are mean ± SEM for 3 or more separate culture preparations.
Table 4.5: The concentration of exogenously added GSH (20µM) in Minimal Medium and in conditioned medium. The effect of glutathione reductase (GR) addition (1 Unit). Minimal Medium was conditioned by 0.5 or 1.2 x 10^6 rat astrocytes over a 5 hour time period. Data is expressed as µM, and are mean ± SEM for 3 or more separate experimental preparations. * p<0.05 when compared to the same time point for Minimal Medium conditioned by the same cell density, before GR incubation.
Chapter 5.

Characterization of the Astrocyte-Derived Factor; - A role for EcSOD?
Chapter 5. Characterization of the Astrocyte-Derived Factor; -A role for EcSOD?

5.1. INTRODUCTION.

In the previous chapter, it was shown that conditioned medium conveyed protection towards GSH degradation. In addition, the proportion of released glutathione present in the reduced form was found to increase with both an increase in conditioning time and cell density. It was therefore concluded that astrocytes may release a factor which limits GSH degradation. The aim of this present chapter was to characterize this factor by using rat astrocyte cultures.

In the previous chapter, it was hypothesized that because medium invariably contains O$_2$ and metal ions, reactive species such as O$_2^-$, H$_2$O$_2$ and 'OH may be generated. This generation of O$_2^-$ in Minimal Medium may lead to the breakdown of GSH, by various reactions proposed by Winterbourn et al, (1995) (Figure 5.1., reactions 1-3). Furthermore during these stages of GSH decay, mixed thiol radicals are generated. In the presence of O$_2$, thiol radicals may lead to the regeneration of O$_2^-$ (Figure 5.1., reactions 4-5), thereby propagating an autocatalytic O$_2^-$ dependent cycle of GSH decay (Winterbourn et al, 1983). These reactions may contribute towards the degradation of GSH in Minimal Medium reported in the previous chapter.

Superoxide dismutase (SOD) is the major enzymatic antioxidant defense against O$_2^-$. In aqueous solutions, O$_2^-$ is converted into H$_2$O$_2$:

\[
2 \text{O}_2^- + 2 \text{H}^+ \xrightarrow{\text{SOD}} \text{H}_2\text{O}_2 + \text{O}_2
\]

However, SOD greatly accelerates this reaction (Halliwell et al, 1985). The removal of O$_2^-$ by SOD would prevent its interaction with GSH and other thiols, and inhibit the autocatalytic O$_2^-$ dependent cycle of GSH decay illustrated in Figure 5.1. Thus a possible candidate for the astrocyte-released factor limiting GSH degradation, may be
Figure 5.1. The autocatalytic cycle of GSH decay by the reaction of $O_2^-$ with GSH and mixed thiols.
a SOD-like molecule.

There are three mammalian isoforms of SOD, and the first described was the copper and zinc-containing SOD (CuZnSOD) (Type I SOD). CuZnSOD was discovered in 1969 when an enzyme was purified from bovine erythrocytes, and was found to catalyze the dismutation of $O_2^-$ (McCord et al., 1969). In that paper, CuZnSOD was shown to be a dimer, and was found to have a molecular mass of 32.6 kDa per subunit (McCord et al., 1969). It was later described to have 0.9 atoms of copper and 0.8 atoms of zinc per subunit (Weisiger et al., 1973). Due to its wide intracellular distribution, specifically in the cell cytosol, cell nucleus and peroxisomes of cells (Crapo et al., 1992), CuZnSOD has an important role as a bulk scavenger of $O_2^-$ produced in these locations.

The second isoform of SOD discovered was the manganese-containing SOD (MnSOD) (Type II SOD), and was first described in 1973 during an investigation of the SOD of chicken liver (Weisiger et al., 1973). In this study, MnSOD, which was found to be localized to the mitochondria, had a total molecular mass of 80 kDa and consisted of four subunits, each containing 2.3 atoms of manganese per molecule. MnSOD plays an important role as an antioxidant in the mitochondria, as this organelle is capable of generating significant amounts of $O_2^-$ and $H_2O_2$ (Boveris et al., 1972).

The third isoform of SOD discovered was extracellular SOD (EcSOD) (Type III SOD). EcSOD was first described in 1980 when SOD-like activity sensitive to cyanide was identified in serum (Marklund, 1980). However, the activity of this cyanide-sensitive SOD could not be attributed to CuZnSOD, as this serum protein was shown to be immunologically distinct from the intracellular CuZnSOD (Marklund, 1982a). EcSOD was then isolated and purified from human lung tissue, and determined to have a total molecular mass of approximately 135 kDa. It was found to be a secretory, tetrameric glycoprotein composed of 4 identical subunits,
each containing a copper and a zinc atom in its active site (Marklund, 1982a). EcSOD was later described to be evolutionarily related to CuZnSOD, as a 50% sequence identity of the EcSOD sequence has been shown to have a strong homology with the sequence defining the active site of CuZnSOD (Hjalmarsson et al, 1987). EcSOD was shown to be the major SOD in the extracellular space (Marklund, 1982b). Studies investigating which cell types synthesize EcSOD, have shown that a number of cells such as fibroblasts, glial cells, macrophages, endothelial cells, and chondrocytes produce EcSOD (Marklund, 1990a; Su et al, 1996). It has been implied that the extracellular localization corresponds closely to the cells producing EcSOD (Oury et al, 1994), and this suggests that EcSOD remains in the extracellular matrix close to its site of its production.

EcSOD has been shown to have an affinity to heparin (Karlsson et al, 1987, 1988). This may indicate an affinity for membrane lipids and heparin sulphate on cell surfaces, and may allow EcSOD to exist in high concentrations in particular regions of the extracellular matrix or cell surfaces of selected tissues (Marklund, 1984a, b; Sandstrom et al, 1993; Oury et al, 1994). This extracellular localization of EcSOD may support the suggestion that EcSOD may be important in protecting cell surfaces and extracellular surface matrix proteins from $O_2^-$-mediated damage (Oury et al, 1994). Therefore the release of EcSOD from such cells as fibroblasts, glial cells, macrophages, and endothelial cells, may contribute towards protecting these cells from oxidative damage.

In summary, EcSOD is the isoform of SOD that is synthesized and secreted by a number of cells including glial cells, is located in the extracellular surroundings of tissues, and has been suggested to protect the extracellular environment from $O_2^-$-mediated damage. These characteristics of EcSOD make it an ideal candidate for the astrocyte-released factor, which limits GSH degradation in conditioned medium. The aim of this chapter was to characterize the astrocyte-derived factor, and to determine
if it had similar characteristics to EcSOD. To establish this, the following was investigated:

- The stability of GSH in medium containing CuZnSOD was investigated. EcSOD is evolutionarily related and has a strong sequence homology to CuZnSOD, and both EcSOD and CuZnSOD have a copper and a zinc atom in their active site. Therefore a resulting limitation of GSH degradation by the presence of exogenous CuZnSOD, may imply that a presence of EcSOD in conditioned medium could also limit GSH oxidation.

- The concentration of GSH in conditioned medium was monitored in the presence of a SOD inhibitor. This was observed, to determine if the presence of a SOD inhibitor resulted in a decline in the preservation of GSH degradation by conditioned medium. Diethyldithiocarbamic acid (DETC) has been shown to inhibit EcSOD and CuZnSOD (Marklund, 1984c; Siwik et al, 1999) by removing the copper atom from the active site of the enzyme (Cocco et al, 1981), and was therefore chosen as a SOD inhibitor in this study.

- The stability of the astrocyte-derived factor was investigated. The concentration of exogenously added GSH was monitored in conditioned medium, which had previously been heated, frozen, or left to decay over a period of time.

- The molecular mass of the astrocyte-derived factor was determined and compared to EcSOD. The stability of GSH was observed in conditioned medium which had previously been centrifuged through filters of both above and below the estimated molecular mass of EcSOD.

- SOD activity was directly measured and quantified in conditioned medium, to support the hypothesis of the release of an EcSOD-like factor by astrocytes. To evaluate if any activity of SOD in conditioned medium was due to the activity of MnSOD, the medium was incubated with cyanide. Both EcSOD and CuZnSOD are inhibited by the binding of cyanide to the copper portion of EcSOD and CuZnSOD (Rotilio et al, 1972; Haffner et al, 1973; Marklund, 1984c). Therefore a remaining activity of MnSOD was quantified in conditioned medium after the incubation with cyanide. SOD activity was also measured in Minimal Medium, to
determine if the medium itself contained any SOD activity before being exposed to astrocytes during the conditioning period.

LDH release from astrocytes utilized to condition media, was evaluated to establish cell viability and to indicate if a proportion of the possible SOD activity in the astrocyte-conditioned Minimal Medium may be due to a leakage of the intracellular CuZnSOD into the medium. Furthermore the activity of SOD was also determined in the intracellular compartment of astrocytes.

5.2. METHODS.

5.2.1. Cell Culture:
Primary rat astrocytes were cultured as previously described in Chapter 2, section 2.2. Upon reaching confluency, the cells were plated at a density of 0.8 x 10^6 cells per well onto six-well plates as described in Chapter 2, section 2.2.3.3.

5.2.2. Preparation of conditioned Minimal Medium:
24 hours after plating astrocytes, the culture medium was replaced with 1.5 ml of Minimal Medium and incubated for 4 hours before being recovered and utilized in subsequent experiments.

5.2.3. Storage of samples:
Media taken for the measurement of the concentration of GSH was immediately frozen in liquid nitrogen and stored at -80°C. Samples taken for the determination protein concentration were resuspended in HBSS, and also immediately frozen in liquid nitrogen and stored at -80°C. Samples of medium in which SOD activity was measured, as well as samples of medium and conditioned medium utilized for filtration experiments, were kept on ice, as these media were utilized immediately after the end of the conditioning time period.
5.2.4. Biochemical analysis:
GSH analysis and protein concentrations were determined as described in Chapter 2, section 2.3. and 2.5. respectively. LDH activity in the cell culture medium was determined as previously described in Chapter 2, section 2.6.

Determination of SOD activity:
The measurement of SOD activity was adapted from the method of Hargreaves et al (1999). Each assay cuvette (1ml) contained 50mM potassium phosphate buffer, pH 7.8, 0.05mM bathocuproine disulphonate disodium salt, 560µM NBT, 3mM xanthine, 0.13mg BSA and increasing volumes of sample. Each assay cuvette also contained 1 unit of catalase to remove H$_2$O$_2$, and a sufficient concentration of xanthine-xanthine oxidase to achieve an initial rate of NBT reduction of approximately 0.020 ± 0.005 absorbance/min. All reagents were made up in dd H$_2$O, except for xanthine, where a stock solution of 150mM was dissolved in 0.1M NaOH. The assay was performed at 30°C.

Briefly, xanthine-xanthine oxidase (EC 1.1.3.22, X-Xo) was utilized to generate a O$_2$\textsuperscript{−} flux. Nitroblue tetrazolium (NBT) was added to the assay, and the rate of reduction of NBT by O$_2$\textsuperscript{−} was followed at 560nm using a Uvikon 940 spectrophotometer (Kontron Instruments Ltd., Watford, UK) (Figure 5.2. a and b). The rate of NBT reduction recorded was approximately 0.020 ± 0.005 absorbance/min, and was used as a reference rate when determining SOD activity in culture medium.

As a validation of the assay system, increasing units of CuZnSOD (to a final concentration of 0.5–2 units; dissolved in 0.05M potassium phosphate buffer, pH 7.8) were added to the assay cuvette (Figure 5.2.c.). The addition of increasing units of CuZnSOD resulted in an inhibition of the rate of NBT reduction (Figure 5.3.), thereby confirming the sensitivity of the assay system to SOD activity.

For determination of SOD activity in the culture medium or within the intracellular
Figure 5.2. Illustration of assay system determining SOD activity. 'X-Xo oxidase' = 'xanthine-xanthine oxidase'.
Figure 5.3: Effect of CuZnSOD on the reduction of NBT in the SOD assay. Data are expressed as percentage inhibition of NBT reduction, and are mean ± SEM (n = 3).
compartment of astrocytes, increasing amounts of sample were added to the assay cuvette (Figure 5.2.c.). The percentage inhibition of NBT reduction versus final protein concentration was then plotted. One SOD unit was defined as the "amount" of enzyme capable of inhibiting NBT reduction by 50%. The amount of protein responsible for causing 50% inhibition was then determined, and from this the number of SOD units per milligram of sample protein could then be calculated (Spitz et al., 1989; Hargreaves et al., 1999).

Cyanide inhibits the activity of EcSOD and CuZnSOD (Rotilio et al., 1972; Haffner et al., 1973; Ledig et al., 1982; Marklund, 1984c;). Thus MnSOD activity in the culture medium could be determined after the addition of sodium cyanide (NaCN) to the assay cuvette (5mM, incubated for 45 minutes at 30°C before the addition of xanthine-xanthine oxidase), and was subtracted from the total SOD activity to calculate the remaining activity.

GSH is present in conditioned medium. Therefore, it is possible that if GSH is present in high enough concentrations, it could interfere with the SOD assay, ie. by scavenging $O_2^-$. Consequently, the ability of GSH to affect this assay was determined. Increasing concentrations of GSH (to a final concentration of 0.5-2$\mu$M) were added to the assay cuvette, and the inhibition of NBT reduction was observed.

5.2.5. Experimental protocols:

Effect of CuZnSOD on GSH stability: 20$\mu$M GSH was added to Minimal Medium or to Minimal Medium to which increasing amounts of CuZnSOD (0-175 Units; dissolved in 0.05M potassium phosphate buffer, pH 7.8) had previously been added. The concentration of remaining GSH in both the above solutions was determined after a 5 hour incubation at 37 °C.

Effect of DETC on GSH stability: 20$\mu$M GSH was added either to dd H$_2$O or to
Minimal Medium previously conditioned over a 4 hour time period as described above (section 5.2.2.), in the absence or presence of DETC (10mM). The concentration of remaining GSH in both solutions was examined over a 5 hour incubation period at 37 °C.

**Determination of the stability of the astrocyte-derived factor:** Minimal Medium was conditioned over a 4 hour time period, as described above (section 5.2.2.). 4 samples of conditioned medium were then taken, where one was a control, another was heated for 10 minutes at 60°C, one was freeze-thawed three times in liquid nitrogen, and one was left to stand in the incubator for an extended time period of 21 hours. 20μM GSH was then added to Minimal Medium and the various samples of conditioned Minimal Medium, and its concentration was compared over a 5 hour incubation period at 37 °C.

**Stability of GSH in Minimal Medium, or conditioned Minimal Medium after centrifugation through filters with molecular mass cutoffs:** Minimal Medium was conditioned over a 4 hour time period, as described above (section 5.2.2.). 20μM GSH was added to Minimal Medium or conditioned medium, and the concentration of remaining GSH was measured after a 5 hour incubation period at 37 °C. In addition, 20μM GSH was added to samples of conditioned medium which had previously been centrifuged according to manufacturer’s descriptions, through filters with varying molecular mass cutoffs (10–100 kDa), and the concentration of remaining GSH was then determined after a 5 hour incubation period at 37 °C. 20μM GSH was also added to previously centrifuged (10 kDa filters) conditioned medium, to which the retained protein had been added back into, and the concentration of remaining GSH was observed over a 5 hour incubation period at 37 °C.

**Determination of SOD activity in Minimal Medium, conditioned Minimal Medium and in the intracellular compartment of astrocytes:** To enable the
quantification of SOD activity in conditioned medium, it was necessary to utilize a medium with a high concentration of SOD activity. Therefore Minimal Medium was added to confluent flasks of astrocytes at a lower ratio of 1ml per 1.0 x 10^6 cells, and left for a longer time period of 20 hours before being recovered and utilized in the respective experiments. At the end of the conditioning period, the conditioned medium was collected, and the astrocytes were harvested. The release of LDH from astrocytes was determined as described previously (section 5.2.4.) at the beginning and the end of the conditioning period, to establish cell viability. To further obtain a high concentration of SOD activity within the medium, samples of Minimal Medium and conditioned medium were centrifuged through filters with a molecular mass cutoff of 10 kDa, resulting in the retention of a pellet of protein, and a loss of a high proportion of the volume of medium. SOD activity was then determined in the retained pellets of the filtered media, as described above (section 5.2.4.). SOD activity of the samples of harvested astrocytes was also determined (as described in section 5.2.4.) to evaluate the activity of SOD within the intracellular compartment of astrocytes.

5.2.6. Statistical analysis and data evaluation:
Results were expressed as the Mean ± SEM. Statistical analysis was performed as described in Chapter 2, section 2.9.

The concentration of GSH released by astrocytes into Minimal Medium during a period of conditioning (ranging from approximately 1-3μM), was subtracted from the final concentrations of GSH determined in experiments measuring the concentration of GSH in conditioned Minimal Medium.

5.3. RESULTS.

The stability of GSH (20μM) in Minimal Medium, in the presence of increasing
concentrations of CuZnSOD, was observed. Addition of CuZnSOD, resulted in a greater concentration of GSH being present in the medium after a 5 hour incubation. Furthermore, the effect was dependent on the concentration of enzyme added to the medium (Figure 5.4.). In the absence of CuZnSOD, only 17% of GSH remained. In comparison, the concentration of GSH was almost 4-fold higher in the presence of 175 units of CuZnSOD, where approximately 60% of the GSH concentration remained after a 5 hour incubation. (Figure 5.4.).

The concentration of GSH in conditioned medium was monitored in the presence of the SOD inhibitor DETC, which has been shown to inhibit EcSOD and CuZnSOD (Marklund, 1984c; Siwik et al, 1999). The concentration of exogenously added GSH (20µM) to conditioned medium declined in the presence of DETC (10mM) (Table 5.1.). After a 5 hour incubation period, 50% of the initial GSH concentration remained in conditioned medium. In comparison, the concentration of GSH decayed to undetectable levels after a 2 hour time period when in the presence of 10mM DETC (Table 5.1.). To assess if this decline in GSH concentration was possibly due to a direct reaction of DETC with GSH, the stability of GSH in dd H₂O in the presence or absence of DETC was compared (Table 5.1.). The concentration of GSH in dd H₂O was stable over a 5 hour period. However in the presence of 10mM DETC, the concentration of GSH declined by 65% after a 0.25 hour time period, and to undetectable levels after a 5 hour time period (Table 5.1.). This indicates that the decline in the GSH concentration in conditioned medium in the presence of DETC is as a result of DETC directly reacting with GSH.

The stability of the astrocyte-derived factor was investigated. There was no significant difference throughout the 5 hour incubation period, between the preservation of exogenously added GSH to conditioned medium, and the preservation of exogenously added GSH to conditioned medium which had previously been heated, freeze-thawed, or left for a 21 hour time period (Table 5.2.). Therefore, the treatment of the conditioned medium with either heating, freeze-thawing or prolonged storing did not
affect its ability to preserve GSH.

The stability of GSH in conditioned medium previously centrifuged through filters of varying molecular mass cutoffs was determined. After 5 hours, the remaining GSH concentration was significantly less in medium that had been centrifuged through filters with a cutoff of 50 kDa or below, when compared to unfiltered conditioned medium (Figure 5.5.). However, the remaining concentration of GSH in conditioned medium after centrifugation through filters of 100 kDa was comparable to the remaining concentration of GSH in conditioned medium (Figure 5.5.).

In addition, after the centrifugation of conditioned medium through 10 kDa filters, the protein retained by the filters was added back into the medium in which the stability of GSH was then observed. The stability of GSH in conditioned medium previously centrifuged through 10 kDa filters was significantly decreased after 2 hours, and there was a 30-fold decline in GSH stability after 5 hours, when compared to the stability of GSH in unfiltered conditioned medium (Table 5.3.). However, the addition of the filtered protein to previously filtered conditioned medium fully restored the ability of the medium to preserve GSH (Table 5.3.).

The activity of SOD in conditioned and unconditioned Minimal Medium, and within the intracellular compartment of astrocytes, was investigated (Table 5.4.). No SOD activity could be detected in Minimal Medium. Activity was detectable in conditioned medium. In addition, this activity of SOD was totally inhibited by NaCN (Table 5.4.), indicating that the activity of SOD in conditioned medium is due to the cytosolic or extracellular copper containing isoform of SOD.

Furthermore, no significant increase of LDH release (less than 4%) was determined from astrocytes at the end of the conditioning period (Table 5.5). This suggests that the SOD activity detected in conditioned medium, was not due to the liberation of the cytosolic contents of SOD as a result of cell death. This also supports data suggesting
that the activity of SOD in conditioned medium is due to the extracellular copper containing isoform of SOD.

Finally it was established if the addition of GSH could result in an inhibition of the enzymatic SOD assay. The addition of GSH was found to cause an increased inhibition of NBT reduction (Figure 5.6.), indicating that GSH can mimic the activity of SOD in this assay system. However, after analysis by HPLC, no GSH could be determined in the retained pellets of conditioned medium. GSH can be detected down to a concentration of 4nM with the assay system utilized in this study. Therefore a concentration of less than 4nM GSH was present in the retained pellets of conditioned medium. As concentrations of 0.5 – 2μM caused an inhibition of NBT reduction, it is very unlikely that the minimal concentrations of less than 4nM possibly retained within the pellets of conditioned medium, would inhibit the enzymatic assay of SOD. Therefore a possible inhibition of NBT reduction by a presence of GSH in conditioned medium can be discarded.

5.4. DISCUSSION.

In this current chapter, the astrocyte-derived factor was characterized. The first observation of this chapter was the demonstration that the addition of CuZnSOD to Minimal Medium led to a preservation of GSH. This finding suggests that SOD can limit the degradation of GSH in Minimal Medium, ie. by removing O$_2^-$ generated in Minimal Medium.

Due to the intracellular distribution of CuZnSOD and MnSOD, it is unlikely that these isoforms of SOD would be contained within the extracellular medium conditioned by astrocytes. However, as EcSOD has been shown to be a glycoprotein secreted by glial cells (Marklund, 1990a), is the major isoform of SOD in the extracellular space (Marklund, 1982b), and has been suggested to protect cell surfaces from O$_2^-$-
mediated damage (Oury et al, 1994), the release of EcSOD from astrocytes may contribute towards protecting these and surrounding cells from oxidative damage. It is therefore possible that this isoform of SOD may be contained within medium conditioned by astrocytes. Therefore, the remaining experiments of this chapter were designed to investigate whether the astrocyte-derived factor had similar characteristics to EcSOD.

Addition of the SOD inhibitor DETC (Marklund, 1984c; Siwik et al, 1999) to the conditioned medium appeared to remove the ability of the medium to prevent GSH degradation. However, subsequent studies revealed that DETC directly reacted with GSH. Thus its use, in this study, would create the impression of GSH degradation. In view of this reaction with GSH, studies reporting the effects of DETC in biological systems in general, should be interpreted with caution.

The finding that exogenously added GSH was equally stable in conditioned medium as in conditioned medium which had previously been heated, freeze-thawed, or left for an extended time period, indicates that the astrocyte-derived factor may be of a particularly robust nature. It has previously been shown, that repetitive freeze-thawing of culture medium containing EcSOD, does not effect the activity of EcSOD (Marklund, 1990a). Therefore the stability of EcSOD established in this latter paper, agrees with the stability of the factor observed in this present study.

To further evaluate if the astrocyte-derived factor may have similar characteristics to EcSOD, its molecular mass was estimated using molecular mass filters. Since its first isolation in 1982 from human lung tissue, EcSOD has been studied in several tissues derived from other mammals, including pig, cat, rabbit, guinea pig and mouse, and estimates of its total molecular mass range from 135 up to 170 kDa (Marklund, 1982a; Karlsson et al, 1988; Ookawara et al, 1997). However, EcSOD in rats has been shown to have a lower total molecular mass of between 85 to 97 kDa (Willems et al, 1993; Karlsson et al, 1988). The reason for this atypical molecular mass for
EcSOD in rats when compared to other species is not known. However in a study by Karlsson et al (1998), it was suggested that the plasma EcSOD from all mammals investigated except in rats may be tetrameric, and that the plasma EcSOD from rats may be dimeric. This suggestion was later supported by a study of Willems et al (1993), where rat EcSOD was identified to be a dimer consisting of two polypeptides with a subunit molecular mass of 34 and 36 kDa.

In this current study, centrifugation of conditioned medium through filters of molecular mass cutoffs of 50 kDa or below, resulted in a loss of the ability to retard GSH decay. However, centrifugation through filters with molecular mass cutoffs of 100 kDa did not appear to affect the ability of the conditioned medium to limit GSH oxidation. These data suggest that the factor in the conditioned medium that conveys protection towards GSH, has a total molecular mass between 50 and 100 kDa. Therefore the astrocyte-derived factor preserving the stability of GSH may have a molecular mass similar to EcSOD.

Furthermore, the stability of GSH in conditioned medium previously centrifuged through filters below the molecular mass of EcSOD, was significantly increased upon the addition of the protein retained by the filters. This is likely to be due to the addition of the astrocyte-derived EcSOD-like factor back into the previously filtered conditioned medium.

It is also apparent that the concentration of remaining GSH in conditioned medium after centrifugation through filters with cutoffs between 10 and 50 kDa, resulted in medium that was inferior to unconditioned Minimal Medium with regards to preventing GSH degradation. This may be due to the fact that filters with low molecular mass cutoffs may be capable of retaining other substances, in addition to the astrocyte-derived SOD-like factor in conditioned medium. This may suggest that additional, low molecular mass molecules present in Minimal Medium are capable of retarding GSH degradation.
The next stage in the characterization of the astrocyte-derived factor was to determine whether SOD-like activity could be detected in the conditioned medium. In order to try and obtain a maximum possible activity, the conditioned medium was centrifuged through filters with a molecular mass cutoff of 10 kDa, and the retained protein was assayed. The validity of this approach was confirmed by the observation that resuspension of the pellet in filtered medium restored the ability to preserve GSH. The demonstration of SOD activity in conditioned medium, supports the hypothesis that the preservation of the concentration of GSH by conditioned medium is due to a presence of SOD. The finding that NaCN inhibited SOD activity by 100%, indicates the absence of active MnSOD in conditioned medium, and supports the hypothesis of the extracellular isoform of SOD limiting GSH oxidation.

It is evident, that the addition of 87.5 Units or more of exogenous CuZnSOD led to the preservation of GSH in Minimal Medium. However, this is at variance with the determined activity calculated in the medium, which was also found to convey protection towards GSH; i.e. only 2.4 Units of SOD activity were identified. However, the activity of exogenous CuZnSOD was not determined using Minimal Medium, and it may be possible that exogenous CuZnSOD may convey greater protection towards GSH when in this chosen medium. In addition, EcSOD may have differing properties when assayed than compared to exogenous CuZnSOD; i.e. 2.4 Units of EcSOD may be more efficient in protecting GSH than exogenous CuZnSOD. This may explain why a greater concentration of exogenous CuZnSOD was needed to convey the same degree of protection towards the concentration of GSH then the possible EcSOD-like molecule detected in conditioned Minimal Medium.

The low concentration of LDH leakage determined, suggests a high level of membrane integrity of the cells utilized to condition the medium. Therefore the possibility that the SOD activity determined in conditioned medium is derived from intracellular CuZnSOD by cell lysis is unlikely. The absence of SOD activity in Minimal Medium, further suggests that the activity in conditioned medium is due to a
release of SOD by astrocytes.

The inhibition of NBT reduction by the addition of astrocyte homogenates to the SOD assay confirms, as expected, SOD activity in astrocytes. In agreement with this are previous studies that have shown that glial cells produce EcSOD (Marklund, 1990a). However, this data must be interpreted with caution, as GSH was also shown to mimic SOD activity in the assay system employed. As the GSH content of astrocytes has been reported to range between 16–50 nmol/mg protein, (Chapter 4, Table 4.1 and 4.2.; Raps et al, 1989; Devesa et al, 1993), the inhibition of NBT reduction observed by the addition of astrocyte homogenates, may be due to their intracellular concentration of GSH and not their activity of SOD. Therefore one cannot conclusively conclude that intracellular SOD activity was determined here.

Finally, as GSH was also shown to mimic SOD activity in the assay system employed, this raised the possibility that GSH released by astrocytes could give the impression of SOD activity. However, no GSH was determined in the protein pellets of conditioned medium retained by the 10 kDa filters, and this reduced the possibility of an artefactual result being obtained.

A large interspecies differences in the activity of EcSOD has been documented in the literature (Marklund, 1984a; Karlsson et al, 1988; Stralin et al, 1995; Oury et al, 1996). It is because of this reason, that it is important to compare the data obtained in this current study to other studies investigating EcSOD activity of rat. However, in previous studies investigating EcSOD activity in the kidney, lung or aorta of the rat (Marklund, 1984a; Stralin et al, 1995), much higher levels of activity have been measured when compared to the SOD activity quantified in the present study. A possible reason for this may be that EcSOD has been found to be highly distributed in tissues with abundant smooth muscle consistency (Marklund, 1984b; Sandstrom et al, 1993; Oury et al, 1994). This may explain why a higher activity of EcSOD was determined in the above studies (Marklund, 1984a; Stralin et al, 1995) when
compared to this current study investigating the possible activity of EcSOD secreted by astrocytes. In a paper by Willems et al (1993), the activity of EcSOD released from a rat C6 glioma cell line was quantified. In that study, 3.3 units of EcSOD activity was measured per mg of protein in medium conditioned by rat C6 glioma cells. This determination of EcSOD activity in medium conditioned by a glioma cell line supports the hypothesis of this present study, that the factor released by astrocytes may be EcSOD.

CONCLUSION.

The data presented in this study provide support for the astrocyte-derived factor having similar characteristics to EcSOD. Firstly, the finding that the astrocyte-derived factor had a molecular mass of between 50-100 kDa, agrees with the molecular mass previously determined for EcSOD in rats (Willems et al, 1993; Karlsson et al, 1988). Furthermore, the determination of no significant increase in LDH release by astrocytes by the end of the conditioning period, indicates that the SOD activity determined in conditioned medium was due to an active release of the enzyme. This provides support for an extracellular location of this enzyme, and further indicates that the SOD activity determined in the medium conditioned by astrocytes may be due to the activity of EcSOD. Further support for this, is that EcSOD has been suggested to be the major isoform of SOD in the extracellular environment (Marklund, 1982b). Finally, the determination of the robust nature of the astrocyte-derived factor agrees with a previous determination of the stable nature of EcSOD (Marklund et al, 1990a). Therefore the above findings indicate that the astrocyte-derived factor may have similar characteristics to EcSOD. EcSOD has been shown to be secreted by glial cells (Marklund, 1990a; Willems et al, 1993), and has been suggested to protect against $O_2^-$-mediated oxidative damage (Oury et al, 1994). Therefore, it is possible that EcSOD is released by astrocytes, and prevents GSH degradation by extracellular $O_2^-$. 

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Figure 5.4: The concentration of GSH remaining 5 hours after the addition of exogenous GSH (20μM) to Minimal Medium containing increasing concentrations of CuZnSOD. Data are expressed as μM, and are mean ± SEM (n = 3 to 5 in all cases). * p<0.05 when compared to Minimal Medium in the absence of CuZn SOD.
Figure 5.5: The concentration of GSH remaining 5 hours after the addition of exogenous GSH (20 μM) to Minimal Medium (MM), or to Minimal Medium conditioned by rat astrocytes (con MM) after centrifugation through filters of varying molecular mass cutoffs (kDa). Data are expressed as μM, and are mean ± SEM (n = 3 to 5 in all cases). a p<0.05 when compared to Minimal Medium. b p<0.05 when compared to uncentrifuged conditioned Minimal Medium.
Figure 5.6: Effect of GSH on the reduction of NBT in the SOD assay. Data are expressed as percentage inhibition of NBT reduction, and are mean ± SEM (n = 3).
Table 5.1. The stability of exogenously added GSH (20μM) in conditioned Minimal Medium or dd H₂O, in the absence or presence of 10mM Diethylthiocarbamic acid (DETC). Data are expressed as μM, and are mean ± SEM (n = 3). * p<0.05 when compared to the same time point for the same solution in the absence of DETC. 'ND' = 'not detectable'.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>conditioned Minimal Medium</th>
<th>dd H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- DETC</td>
<td>+ DETC</td>
</tr>
<tr>
<td>0.25</td>
<td>16.9 ± 0.8</td>
<td>13.5 ± 1.7</td>
</tr>
<tr>
<td>2</td>
<td>14.7 ± 1.5</td>
<td>ND *</td>
</tr>
<tr>
<td>4</td>
<td>11.9 ± 1.2</td>
<td>ND *</td>
</tr>
<tr>
<td>5</td>
<td>10.1 ± 1.2</td>
<td>ND *</td>
</tr>
</tbody>
</table>
Table 5.2. Percentage of remaining GSH concentration in conditioned Minimal Medium, or conditioned medium previously heated, freeze-thawed or left for a 21 hour time period, over a 5 hour incubation period. Data are expressed as mean ± SEM (n = 3).

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>% remaining GSH concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>conditioned Minimal Medium</td>
</tr>
<tr>
<td>2</td>
<td>73.2 ± 2.3</td>
</tr>
<tr>
<td>4</td>
<td>60.6 ± 1.3</td>
</tr>
<tr>
<td>5</td>
<td>51.5 ± 2.7</td>
</tr>
</tbody>
</table>
Table 5.3. The stability of exogenously applied GSH (20μM) in conditioned Minimal Medium, conditioned medium after centrifugation through 10 kDa filters, and in centrifuged conditioned medium after the addition of the filtered protein. Data are expressed as μM, and are mean ± SEM (n = 3). "p<0.05 when compared with the same time point of unfiltered conditioned Minimal Medium. a p< 0.05 when compared with the same time point of conditioned Minimal Medium centrifuged through 10 kDa filters.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Exogenous GSH concentration in conditioned Minimal Medium (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>not centrifuged</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>0.25</td>
<td>17.7 ± 0.9</td>
</tr>
<tr>
<td>2</td>
<td>14.6 ± 1.1</td>
</tr>
<tr>
<td>4</td>
<td>11.9 ± 0.6</td>
</tr>
<tr>
<td>5</td>
<td>9.1 ± 1.6</td>
</tr>
</tbody>
</table>

*Exogenous GSH concentration in conditioned Minimal Medium (pM)*

Table 5.3. The stability of exogenously applied GSH (20μM) in conditioned Minimal Medium, conditioned medium after centrifugation through 10 kDa filters, and in centrifuged conditioned medium after the addition of the filtered protein. Data are expressed as μM, and are mean ± SEM (n = 3). a p<0.05 when compared with the same time point of unfiltered conditioned Minimal Medium. b p< 0.05 when compared with the same time point of conditioned Minimal Medium centrifuged through 10 kDa filters.
<table>
<thead>
<tr>
<th>Activity</th>
<th>Units/mg of protein</th>
<th>Units/ml of medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimal Medium</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>conditioned medium</td>
<td>2.4 ± 0.2</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>conditioned medium after incubation with NaCN</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>astrocytes</td>
<td>1.9 ± 0.1</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 5.4. SOD activity in Minimal Medium, conditioned Minimal Medium and in rat astrocytes. Data are expressed as mean ± SEM (n=3). ND = not detectable, NA= not applicable.
Table 5.5. Comparison between the percentage of LDH released from rat astrocytes at the start and the end of the conditioning period of Minimal Medium. Data are expressed as mean ± SEM (n=3). ND = not detectable.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>LDH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td>4</td>
<td>3.9 ± 0.6</td>
</tr>
</tbody>
</table>
Chapter 6.

Nitrosative Stress and Glutathione Release.
6.1. INTRODUCTION.

A number of cells within the central nervous system synthesize NO\(^\cdot\) (Murphy et al, 1993; 1996). NO\(^\cdot\) is synthesized by NOS, which catalyzes the \(O_2\) and NADPH-dependent conversion of L-arginine to L-citrulline and NO\(^\cdot\) (Fukuto et al, 1996) (Figure 6.1.). There are 3 known isoforms of NOS: a calcium-dependent neuronal isoform (neuronal NOS, nNOS), a calcium-independent isoform activated following induction (inducible NOS, iNOS), and a third isoform associated with the brain vasculature and mainly expressed by endothelial cells (endothelial NOS, eNOS) (Galea et al, 1992; Simmons et al, 1992; Murphy et al, 1996). NO\(^\cdot\) is a free radical, and reacts rapidly with \(O_2^-\) to form ONOO\(^-\) (Beckman et al, 1993; Lipton et al, 1993). Both NO\(^\cdot\) and ONOO\(^-\) have been shown to inactivate the mitochondrial respiratory chain (Brown et al, 1994, 1995a, b; Bolanos et al, 1994, 1995; Mitrovic et al, 1994). Such mitochondrial damage may contribute towards the pathology observed in a number of neurological disorders such as Alzheimer’s Disease, Parkinson’s Disease, Multiple Sclerosis, Amyotrophic Lateral Sclerosis and ischaemia/reperfusion (as discussed in Chapter 1).

It is apparent that brain cell types may have a differential susceptibility to reactive species-induced oxidative stress. For example, astrocytes have been shown to be highly resistant to ONOO\(^-\)-induced oxidative damage, where an exposure of up to 2mM ONOO\(^-\) did not effect mitochondrial respiratory chain activity or cause cell death (Bolanos et al, 1995). However, in contrast to astrocytes, neurones are particularly susceptible to the effects of ONOO\(^-\), where exposure to much lower concentrations resulted in irreversible mitochondrial damage and cell death (Bolanos et al, 1995). The intracellular GSH concentration has been suggested to be a particularly important determining factor in the susceptibility of a cell towards oxidative stress. As ONOO\(^-\) has been shown to react favorably with thiol-containing
Figure 6.1. The conversion of L-arginine to L-citrulline and NO\(^-\), with the intermediate formation of N-hydroxy-L-arginine. NO\(^-\) is synthesized by nitric oxide synthase, which catalyzes the O\(_2\) and NADPH-dependent conversion of L-arginine to L-citrulline and NO\(^-\). NADPH donates a hydrogen atom to O\(_2\), whereby one atom of O\(_2\) is reduced to H\(_2\)O, and the other atom of O\(_2\) is incorporated into arginine, forming the hydroxy-L-arginine intermediate. Therefore the initial reaction of NOS appears to be the formation of N-hydroxy-L-arginine. Adapted from Fukuto et al, 1996.
compounds (Bolanos et al, 1995; Lizasoain et al, 1996), the intracellular GSH concentration of a cell may be an important factor for preventing ONOO\(^{-}\)-mediated mitochondrial damage. Indeed, the GSH concentration of astrocytes has been shown to be double that of neurones cultured under identical conditions (Bolanos et al, 1995). The intracellular GSH concentration of neurones decreases significantly after exposure of 0.1mM ONOO\(^{-}\), whereas astrocytic GSH concentration is not affected up to 2mM ONOO\(^{-}\) (Bolanos et al, 1995). Therefore, the comparatively high intracellular GSH concentration of astrocytes, and their ability to maintain intracellular concentrations under conditions of oxidative stress, has been suggested to be an underlying factor contributing towards the resistance of these cells against ONOO\(^{-}\)-mediated mitochondrial damage (Bolanos et al, 1995). In addition, the depletion of astrocytic GSH has been shown to result in a higher susceptibility of these cells to ONOO\(^{-}\) (Barker et al, 1996), further supporting the hypothesis that the intracellular GSH concentration of a cell is an important factor in protecting against oxidative stress.

GSH released by astrocytes has been suggested to provide neurones with precursors for GSH synthesis (Dringen et al, 1999a, 2000; Wang et al, 2000), and neurones cocultured with astrocytes have been shown to have a higher GSH concentration (Sagara et al, 1993a; Bolanos et al, 1996; Dringen et al, 1999a). Therefore, if astrocytes could also maintain the release of GSH during conditions of oxidative stress, they could sustain the supply of neuronal GSH precursors. Indeed, it has been suggested that neurones cocultured with NO\(^{-}\)-releasing astrocytes are much less susceptible to NO\(^{-}\)-induced mitochondrial damage, and have approximately a 2-fold higher intracellular GSH concentration (Bolanos et al, 1996). As this latter study reports that the coculture of neurones with activated NO\(^{-}\)-releasing astrocytes increased neuronal GSH concentrations (Bolanos et al, 1996), this suggests that astrocytes are able to maintain the release of GSH under conditions of mitochondrial damage and oxidative stress. Furthermore, this implies that the released GSH is still utilized, via \(\gamma\)-GT, to yield the appropriate precursors for \textit{de-novo} neuronal GSH
synthesis.

As a result of the above observations, this chapter investigates if GSH release by rat astrocytes and the human astrocytoma cell line 1321N1 is maintained, after exposure to substantial amounts of reactive nitrogen species. Therefore, the following were determined:

♦ As NO' and ONOO' have been shown to inactivate the mitochondrial respiratory chain and contribute towards oxidative stress (as discussed above), the release of GSH from rat astrocytes and the human astrocytoma cell line was established after incubation with ONOO', or after incubation with the NO'-donor Diethylenetriamine nitric oxide adduct (Deta-NO'), which produces a controlled release of NO' in solution (Diodati et al, 1993; Hrabie et al, 1993).

♦ The induction of iNOS in astrocytes has been shown to release NO', and contribute towards oxidative stress-induced damage (Bolanos et al, 1994). It was therefore determined if iNOS induction in rat astrocytes compromised GSH release. The exposure of rat astrocytes to lipopolysaccharide (LPS) in combination with interferon-γ (INFγ) has been shown to induce NOS in rat astrocytes (Bolanos et al, 1994), and was therefore used in this study.

6.2. METHODS.

6.2.1. Cell Culture:
Primary rat astrocytes and the human astrocytoma cell line were cultured as previously described in Chapter 2, section 2.2. Upon reaching confluency, the cells were plated at a density of 1 x 10^6 cells per well onto six-well plates as described in Chapter 2, section 2.2.3.3. 24 hours after cell plating, the culture medium was replaced with either L-Val MEM or HBSS used for cell treatments. This subsequently also marked the start of the incubation periods with various chemical additions as described below (section 6.2.4.).
6.2.2. Storage of samples:
Media utilized to determine GSH release or the release of nitrate plus nitrite was immediately frozen in liquid nitrogen and stored at -80°C.

6.2.3. Biochemical analysis:
GSH analysis and nitrate plus nitrite concentrations were determined as described in Chapter 2, section 2.3. and 2.7. respectively. The percentage LDH released by cells was determined as previously described in Chapter 2, section 2.6.

6.2.4. Experimental protocols:

**GSH release in the presence of Deta-NO**: 24 hours after cell plating, the culture medium of rat astrocytes or the human astrocytoma cell line was replaced with fresh L-Val MEM. Deta-NO (100μM, dissolved in culture medium) was then added to the culture medium, and the cells were incubated for a 24 hour period at 37 °C. As a control, identical volumes of Deta-NO which had previously been left to decay over a 5 day period, was also added to the culture medium of rat astrocytes or the human astrocytoma cell line at the start of the incubation period. Similarly, parallel cultures in the absence of additions were also carried out as controls. At the end of the 24 hour incubation period, the culture medium was replaced with 1ml of Minimal Medium, and GSH release was evaluated over a 4 hour incubation period at 37 °C.

**GSH and nitrate plus nitrite release in the presence of cytokines**: 24 hours after cell plating, the culture medium of rat astrocytes was replaced with FBS-free and phenol red-free L-Val MEM. Additions of LPS (1μg/ml) and IFNγ (100U/ml) in combination were then made to the culture medium of rat astrocytes. This combination of cytokines was also added to the culture medium of rat astrocytes to which the NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME; 1mM, dissolved in HBSS) had been added, and additions of L-NAME only were also applied to rat astrocytes. The cells were then incubated for a 24 hour period at 37 °C. As a control,
astrocytes were incubated in the absence of any additions. At the end of the 24 hour incubation period, samples of culture medium were taken for the determination of the concentration of released nitrate plus nitrite, to assess NO\(^-\) synthesis as a result of NOS induction (Ignarro et al, 1993). The culture medium was then replaced with 1ml of Minimal Medium, and GSH release from rat astrocytes was evaluated over a 4 hour incubation period at 37 °C.

**GSH release in the presence of ONOO\(^-\):** ONOO\(^-\) was prepared as described in Chapter 2, section 2.8. 24 hours after cell plating, the culture medium of rat astrocytes or the human astrocytoma cell line was replaced with buffered HBSS, and ONOO\(^-\) was added to a final concentration of 0.5mM. Because stock solutions of ONOO\(^-\) are in 1M NaOH, cells were also incubated with ONOO\(^-\) that had previously been left to decay. This was performed to control for any possible effects of the addition of NaOH or degraded ONOO\(^-\) on cellular GSH release. Therefore, ONOO\(^-\) (0.5mM) was added to buffered HBSS and was left to decay over a ten minute period, before being utilized to replace the culture medium of further wells containing rat astrocytes or human astrocytoma. Cells were incubated in the presence of ONOO\(^-\) or degraded ONOO\(^-\) for 5 minutes, before the culture medium was replaced with L-Val MEM, and the cells were incubated for a further 24 hour time period at 37 °C. As a further control, rat astrocytes or human astrocytoma which had not been exposed to ONOO\(^-\), were also incubated for a 24 hour time period at 37 °C. The culture medium of all incubated cells was then replaced with 1ml of Minimal Medium, and GSH release was evaluated over a 4 hour incubation period at 37 °C.

**Determination of the release of LDH during GSH release experiments:** LDH release was determined from rat astrocytes and the human astrocytoma cell line as described in chapter 2, section 2.6. at various time points, during the four hour time period of GSH release.
6.2.5. Statistical analysis and data evaluation:
Results were expressed as the Mean ± SEM. Statistical analysis was performed as described in Chapter 2, section 2.9.

6.3. RESULTS.

GSH release in the presence of Deta-NO'.
The release of GSH was observed from rat astrocytes and the human astrocytoma cell line previously incubated with the NO' donor, Deta-NO'(100μM; 24 hours). No difference in the concentration of released GSH was detected from either rat astrocytes or the human astrocytoma cell line previously incubated with Deta-NO' or degraded Deta-NO', when compared to control levels of released GSH from the respective cell types which had not been exposed to treatments (Figure 6.2 and 6.3.).

GSH release after incubation with cytokines.
The production of nitrate plus nitrite by rat astrocytes after exposure to L-NAME and/or cytokines was determined after a 24 hour incubation period, and was compared to basal concentrations of released nitrate plus nitrite of untreated cells. Combined treatments of LPS (1μg/ml) + IFNγ (100U/ml) caused approximately a 15-fold increase in nitrate plus nitrite production, as compared with control levels in rat astrocytes (Figure 6.4.), and therefore confirmed the production of NO' in these cells. The presence of L-NAME (1mM) during cytokine treatments, decreased the nitrate plus nitrite production of rat astrocytes by approximately 60% (Figure 6.4.). However, the addition of L-NAME to rat astrocytes that had not been treated with cytokines, did not have an effect (Figure 6.4.).

No difference in the concentration of GSH release from rat astrocytes was detected after combined treatments of LPS + IFNγ, when compared with control levels of GSH release in untreated astrocytes (Figure 6.5.). Similarly, the addition of L-NAME
during cytokine treatments, as well as the addition of L-NAME to untreated rat astrocytes, also had no effect (Figure 6.5.).

**GSH release after ONOO⁻ exposure.**

The release of GSH was monitored from rat astrocytes and the human astrocytoma cell line after previous incubations of ONOO⁻ (0.5mM, 24 hours). No difference in the concentration of released GSH was detected from either rat astrocytes or human astrocytoma previously incubated with ONOO⁻ or degraded ONOO⁻, when compared to control levels of released GSH from either cell types which had not previously been exposed to treatments (Figure 6.6. and 6.7.).

LDH activity released to the culture medium from rat astrocytes or the human astrocytoma cell line was determined for all the treatments in this current chapter, and was not found to be higher than approximately 20%, or significantly increased by any of the treatments when compared to control values.

6.4. DISCUSSION.

NO⁻ has been shown to inactivate the mitochondrial respiratory chain of astrocytes and contribute towards oxidative stress (Bolanos *et al.*, 1994). Furthermore, it has also been suggested, that ONOO⁻ may contribute towards the mitochondrial damage observed in astrocytes after induction of NOS (Bolanos *et al.*, 1994, 1995). The finding of this current study, that the release of GSH from rat astrocytes or the human astrocytoma cell line was not compromised after an incubation period with either ONOO⁻ or a NO⁻-donor, shows that GSH release is maintained from both cell types during conditions of oxidative stress and inhibited mitochondrial function. Furthermore, the determination that GSH release from the human astrocytoma cell line was maintained after exposure to oxidative stress may imply that astrocytes in the
human nervous system can also maintain a release of GSH during conditions of oxidative stress.

The induction of NOS in rat astrocytes has previously been shown to inhibit the mitochondrial electron transport chain and cause an increase in glycolysis and lactate formation (Bolanos et al., 1994). However, in that previous study, the intracellular GSH concentration of these cells was not compromised (Bolanos et al., 1994). This suggests that the ability of astrocytes to maintain their energy homeostasis by switching to an increased glycolysis after an onset of mitochondrial inhibition, may contribute towards maintaining the ATP-dependent synthesis of intracellular GSH despite mitochondrial inhibition. Furthermore, the above observation of this current study, that the induction of NOS in rat astrocytes does not compromise the release of GSH, also indicates that these cells can maintain a release of GSH during conditions which have previously been confirmed to inhibit the mitochondrial electron transport chain and cause oxidative stress.

The determination of this current study, that astrocytes can maintain GSH release during conditions reported to initiate oxidative stress and inhibit mitochondrial function, indicates that astrocytes are particularly well developed in preserving their general antioxidant status and combating oxidative stress. Furthermore, increased cytokine concentrations that have been shown to induce NOS, have been found in neuropathological conditions (Merrill et al., 1987; Banati et al., 1993). Neurones are particularly susceptible to NO' and ONOO' (Bolanos et al., 1995, 1996). As the intracellular GSH concentration of a cell may be particularly significant in determining the susceptibility of a cell towards oxidative stress (Bolanos et al., 1995, 1996; Barker et al., 1996), it may be especially important that astrocytes maintain the release of GSH under conditions of increased cytokine concentrations, as this may enable astrocytes to provide neurones with precursors for GSH synthesis and initially limit the neurodegenerative process. This is supported by a previous paper, reporting that neurones cocultured with NO'-releasing astrocytes had a 2-fold higher
intracellular GSH concentration (Bolanos et al, 1996). The determination of this present study, that rat astrocytes maintained the release of GSH after the induction of NOS, implies that GSH released by astrocytes is not oxidized rapidly by the extracellular presence of released NO', but remains in the reduced form for a sufficient time period to be utilized for neuronal GSH synthesis. Therefore the release of GSH by activated astrocytes has important neuroprotective implications.

It is well documented that NO' can react extremely rapidly with O$_2^-$ to form ONOO' (Beckman et al, 1993; Lipton et al, 1993). It is highly probable that ONOO' is formed in the extracellular medium surrounding activated astrocytes, as culture medium contains O$_2^-$ (as discussed in Chapter 4). Furthermore, the induction of NOS produces NO' continuously in astrocytes (Murphy et al, 1993) over prolonged time periods (Bolanos et al, 1994), and therefore may be a constant source of ONOO' synthesis. As GSH reacts favorably with ONOO' (Bolanos et al, 1995; Lizasoain et al, 1996), the finding that the concentration of GSH release is maintained by activated astrocytes is important. This observation implies that the presence of activated astrocytes prevents released GSH from reacting with ONOO'. The previous chapter proposed that the release of an EcSOD-like factor by astrocytes inhibits the oxidation of released GSH. Similarly, it is possible, that the release of an EcSOD-like factor would prevent the accumulation of O$_2^-$ in the extracellular medium, and therefore its interaction with NO' released by activated astrocytes. This would prevent the subsequent formation of ONOO', and its interaction with released GSH. This would explain why GSH released by activated astrocytes can accumulate in the extracellular medium (as observed in this current chapter), and is not oxidized by the parallel release of NO'.

As discussed above, the intracellular GSH concentration of a cell is postulated to determine its susceptibility towards oxidative stress. It has previously been reported that an increase in neuronal GSH by the coculture of activated astrocytes, prevents neuronal oxidative stress-induced cell death (Bolanos et al, 1996). However, it has also been reported that cytokine-activated astrocytes are neurotoxic, and can cause
NO⁻-induced neuronal cell death (Dawson et al, 1994; Chao et al, 1996; Jeohn et al, 1998). In these latter studies, observations were made from mixed neuronal/glial cell cultures, as opposed to separate cultures of activated astrocytes cocultured with neurones (as in the study of Bolanos et al, 1996). It has been suggested that iNOS may be expressed by most cell types if the appropriate cytokine or LPS stimulation is delivered (Griffith et al, 1995). Therefore the application of cytokines to mixed glial/neuronal cultures, may not only induce NOS in astrocytes, but also in neurones. Therefore an increase in neuronal GSH by activated astrocytes may not be sufficient to prevent neuronal cell death, if neurones are subject to NOS induction as well as extracellular NO⁻ released from activated astrocytes.

In conclusion, the data of this present chapter shows that the release of GSH from rat astrocytes and the human astrocytoma cell line is maintained during conditions of nitrosative oxidative stress, and implies that this released GSH can be utilized to generate neuronal GSH precursors.
Figure 6.2. GSH release from rat astrocytes (1x10^6 cells/ml) previously incubated for 24 hours in the absence (control) or presence of Deta-NO (100µM) or degraded Deta-NO. Data are expressed as µM, and are mean ± SEM for 3 independent culture preparations.
Figure 6.3. GSH release from the human astrocytoma cell line (1x10⁶ cells/ml) previously incubated for 24 hours in the absence (control) or presence of Deta-NO⁺ (100µM) or degraded Deta-NO⁺. Data are expressed as µM, and are mean ± SEM for 3 independent culture preparations.
Figure 6.4. Final concentration of released nitrate plus nitrite from rat astrocytes (1x10^6 cells/ml) after a 24 hour incubation period in the absence (control) or presence of cytokines (100U/ml IFNγ + 1µg/ml LPS), or in the presence of L-NAME (1mM), or cytokines plus L-NAME. Data are expressed as µM, and are mean ± SEM for 3 independent culture preparations.
Figure 6.5. GSH release from rat astrocytes (1x10^6 cells/ml) previously incubated for 24 hours in the absence (control) or presence of cytokines (100U/ml IFNγ + 1μg/ml LPS), or in the presence of L-NAME (1mM), or cytokines plus L-NAME. Data are expressed as μM, and are mean ± SEM for 3 independent culture preparations.
Figure 6.6. GSH release from rat astrocytes (1x10^6 cells/ml) after a 24 hour incubation period in the absence (control) or presence of ONOO' or degraded ONOO' (0.5mM). Data are expressed as μM, and are mean ± SEM for 3 independent culture preparations.
Figure 6.7. GSH release from the human astrocytoma cell line (1x10^6 cells/ml) after a 24 hour incubation period in the absence (control) or presence of ONOO' or degraded ONOO' (0.5mM). Data are expressed as µM, and are mean ± SEM for 3 independent culture preparations.
Chapter 7.

General Discussion.
Chapter 7. General Discussion.

7.1. DISCUSSION.

The release of GSH by astrocytes has been suggested to provide neurones with glutamate (in the form of glutamine), cysteine and glycine for GSH synthesis (Dringen et al, 1999a; Hertz et al, 1999; Wang et al, 2000), and this hypothesis is supported by data showing that coculture of neurones with astrocytes increases neuronal GSH concentrations (Sagara et al, 1993a; Bolanos et al, 1996; Dringen et al, 1999a). GSH release has previously been shown from cultured astrocytes (Yudkoff et al, 1990; Juurlink et al, 1996; Sagara et al, 1996; Dringen et al, 1997a; Wang et al, 2000). However, in order for GSH release by astrocytes to provide neurones with GSH precursors, it was hypothesized that the release of GSH must be maintained over a period of time, and that the GSH should be relatively preserved in the extracellular environment. Therefore the characteristics of astrocytic GSH release, and its stability in the extracellular environment, were investigated.

The initial objective was to select a suitable medium for GSH release experiments. The short half life of GSH of less than 0.25 hours in MEM (Figure 4.2.), was suggested to be due to the potential generation of reactive species within the medium, and was determined to be unsuitable for GSH release experiments. As the concentration of GSH was relatively maintained in Minimal Medium (Figure 4.2.), and this medium had also previously been used for GSH release experiments (Dringen et al, 1997a), Minimal Medium was utilized for all further experiments monitoring GSH release in this study. Furthermore, the use of Minimal Medium as opposed to the routinely used MEM, did not affect the intracellular GSH concentration of primary rat astrocytes (Table 4.1.).

After establishing a suitable medium for GSH release experiments, GSH release from astrocytes was characterized. A linear release of GSH was found from rat astrocytes,
with regard to time (Figure 4.3.). In addition, the concentration of released GSH from rat astrocytes appeared to be proportional to the initial cell density (Figure 4.4.). During this period of GSH release, the intracellular GSH concentration of rat astrocytes was determined to decline significantly (Table 4.2.), suggesting that the release of GSH from these cells was as a result of direct release of GSH from the intracellular compartment. Furthermore, it was estimated, that approximately 6-12% of the intracellular GSH concentration was released per hour from rat astrocytes (Table 4.3.), agreeing with previous estimations of the percentage of intracellular GSH released per hour in the literature (Sagara et al, 1996; Dringen et al, 1997a).

The 1321N1 human astrocytoma cell line was also confirmed to release GSH. However, the concentrations released from this cell line was found to be lower than that released by primary rat astrocytes (Figure 4.5. and 4.6.), and may be as a result of the lower intracellular GSH concentrations determined in this cell line when compared to primary cells (Table 4.2.). The C6 rat glioma cell line was not found to release GSH (Figure 4.5. and 4.6.). It is possible that the physiological mechanisms within cell lines are altered as a result their transformation, and this may also explain why a lower concentration of GSH was released from these cell lines than from rat astrocytes.

Furthermore, the low level of LDH leakage obtained from rat astrocytes and the human astrocytoma cell line throughout the period of GSH release (Table 4.4.), indicates that the release of GSH determined from these cells was not due to a liberation of cytosolic contents. The finding that GSH was released by astrocytes over an extended period, indicates that GSH release may be maintained for a sufficient time to be utilized as a substrate for neuronal GSH precursors. Moreover, GSH release has not previously been reported for human astrocytes, and its demonstration in this current study may imply that astrocytes in the human nervous system also release GSH.
Putative transporters which could mediate GSH export from astrocytes observed in this current study, could include the multidrug resistance protein MRP1. MRP1 functions as a primary active transporter of structurally diverse organic anions, and is involved in a number of glutathione-related cellular processes (Hipfner et al, 1999). Furthermore, MRP1 has been shown to be expressed in astrocytes, and has been demonstrated to participate in the release of GSH and GSSG (Borst et al, 1999; Hirrlinger et al, 2001).

For further characterization of GSH release, GSH release from rat astrocytes was determined during an inhibition of γ-GT. GSH is a substrate for γ-GT, which uses extracellular concentrations of GSH to generate the dipeptide cysteinylglycine. The increased concentration of released GSH determined in the presence of 2µM acivicin, is possibly due to the preservation of its extracellular concentration, as a result of the inhibition of γ-GT (Figure 4.7). Concentrations of acivicin above 2µM, caused a concentration-dependent decline in the apparent release of GSH from rat astrocytes (Figure 4.7), and were suggested to be as a result of potentially toxic concentrations of acivicin.

The final objective for the characterization of the release of GSH from rat astrocytes was to establish if glutathione was released in the oxidized or the reduced form. Glutathione appeared to be released predominantly in the reduced form, and the proportion of reduced glutathione in the extracellular medium increased both with time and cell density (Figure 4.8). It was confirmed, that this increase in GSH concentration was not due to a reduction of released GSSG, and suggests that the preservation of released GSH with time and cell density is due to the presence of an astrocyte-released factor preventing the oxidation of released GSH.

To investigate if an astrocyte-derived factor may preserve the concentration of released GSH, the stability of the concentration of GSH in Minimal Medium was compared to the GSH concentration in Minimal Medium previously exposed and
conditioned by rat astrocytes. The concentration of GSH was considerably preserved in conditioned medium (Figure 4.9. and 4.10.), supporting the hypothesis of an astrocyte-derived factor preserving GSH. Furthermore, medium conditioned by the human astrocytoma cell line was found to preserve the concentration of GSH to the same degree as medium conditioned by primary rat astrocytes (Figure 4.11.). This suggests that the release of a factor preserving the concentration of GSH, may be of relevance in the human nervous system.

After determining that the concentration of GSH was preserved by an astrocyte-derived factor, the characteristics of the factor were investigated. As the decay of GSH in medium may be due to the generation of $\text{O}_2^{-}$, and SOD is the major enzymatic defense against this reactive species, a possible candidate for the astrocyte-released factor limiting GSH degradation was considered to be a SOD-like molecule. The addition of CuZnSOD to Minimal Medium led to the preservation of GSH (Figure 5.4.), and suggests that SOD could limit the degradation of GSH in Minimal Medium. As EcSOD has been shown to be a glycoprotein secreted by glial cells, and is the major isoform of SOD in the extracellular space, it was investigated whether the astrocyte-derived factor had similar characteristics to EcSOD.

A previous paper has shown that repetitive freeze thawing of culture medium with exogenously added EcSOD did not affect the activity of the enzyme (Marklund, 1990a). In this current study, repetitive freeze-thawing, heating or leaving conditioned medium for an extended time period to decay, did also not affect the ability of the medium to preserve GSH (Table 5.2.), demonstrating a similarity between the robust nature of the factor and EcSOD. The molecular mass of the rat astrocyte-derived factor in conditioned medium was estimated and compared to EcSOD. The data of this current study suggest that the factor has a molecular mass of between 50 to 100 kDa (Figure 5.5.), which is comparable to the molecular mass previously determined for EcSOD in rats (Karlsson et al, 1988; Willems et al, 1993). SOD activity was demonstrated in conditioned medium, but not unconditioned medium, supporting the
hypothesis that the concentration of GSH in conditioned medium is due to the release of SOD (Table 5.4.). In addition, no significant release of LDH was determined from rat astrocytes at the end of the conditioning period (Table 5.5.). This suggests that the release of the SOD activity in conditioned medium was not due to the liberation of cytosolic contents containing CuZnSOD, and implies the activity of SOD in the extracellular medium is due to an extracellular isoform of SOD. Moreover, the activity of SOD determined in conditioned medium, was comparable to the activity of EcSOD released from a rat C6 glioma cell line in a previous paper (Willems et al, 1993). This provides further support for the astrocyte-derived factor having similar characteristics to EcSOD. In addition, the addition of NaCN (which inhibits the activity of CuZnSOD) to the assay system, was found to inhibit the activity of SOD in conditioned medium (Table 5.4.), further supporting the hypothesis of an extracellular isoform of SOD limiting GSH oxidation. Finally, no GSH was determined in the protein pellets utilized to determine the activity of SOD in conditioned medium, eliminating the possibility of a presence of released GSH mimicking the activity of SOD in the assay system utilized.

A recent study reported that compounds exhibiting SOD/catalase activities augmented the life span of hermaphrodite worms, and suggested that this was as a result of the SOD/catalase mimetics augmenting the natural antioxidant defenses of the worm (Melov et al, 2000). This observation that a SOD/catalase mimetic may increase antioxidant concentrations supports the hypothesis that the release of an EcSOD-like factor by astrocytes may preserve the concentration of GSH.

The preservation of released GSH by the possible release of an EcSOD-like factor by astrocytes has neuroprotective implications, as neurones depend on astrocytes for their supply of cysteine for GSH synthesis (Dringen et al, 1999a; Sagara et al, 1993a). GSH released by astrocytes is a substrate for γ–GT (Dringen et al, 1997a), which transfers the γ-glutamyl segment of GSH onto an acceptor molecule and forms the dipeptide cysteinylglycine (Meister et al, 1981). The preservation of extracellular
Molecular mass of rat EcSOD is between 85-97 kDa. Molecular mass of astrocyte-derived factor is between 50-100 kDa.

Demonstration of SOD activity

Repetitive freeze-thawing, heating or leaving conditioned medium to decay over 21 hours, does not effect ability of medium to retard GSH decay.

Cyanide sensitive

Table 7.1. Characteristics of EcSOD and the astrocyte-derived factor.
GSH by an EcSOD-like factor, may result in a higher concentration of substrate availability for γ-GT, and subsequently generate a greater concentration of precursors for neuronal GSH synthesis. The synthesis of GSH precursors may have neuroprotective implications under conditions of oxidative stress, as the scavenging of reactive species by a possible EcSOD-like factor may result in the provision of neuronal GSH precursors under conditions generating reactive species.

Support for astrocytic neuroprotection by the preservation of released GSH by an EcSOD-like astrocyte-derived factor, is a study reporting protection against oligodendroglial death by a diffusible glial factor (Yonezawa et al, 1996). Oligodendrocytes, like neurones, are dependent on cysteine in the extracellular medium for survival (Sagara et al, 1993a; Yonezawa et al, 1996). In this latter study, astrocyte-conditioned medium prevented the toxicity of cystine-depleted medium in oligodendrocytes (Yonezawa et al, 1996). The diffusible factor in the astrocyte-conditioned medium was not determined to be cystine or cysteine, as there was no increase in the concentrations of these amino acids in the conditioned medium. However, a 100-fold increase in the concentration of total glutathione was found in the medium previously conditioned by astrocytes. The addition of GSH or GSSG to cystine-depleted medium had no protective effect against oligodendroglial toxicity of cystine-depleted medium, and the diffusible factor was therefore not GSH or GSSG. It was concluded in this latter study that a diffusible factor was possibly released by astrocytes into conditioned medium, resulting in an increase in total glutathione concentration in the extracellular medium and a protection against oligodendroglial toxicity against cystine depletion. It is possible that in that latter study (Yonezawa et al, 1996), an EcSOD-like factor may have been released by astrocytes into the conditioned medium, thus preserving GSH released by astrocytes. This preservation of released GSH, would increase the substrate availability of γ-GT and therefore the cysteine concentration of the cystine-depleted medium, resulting in the reduction of the toxicity of the cystine-depleted medium for oligodendrocytes.
γ-GT has been determined to be abundant in brain microvessels, with a high activity in capillaries (Orlowski et al, 1974), endothelial cells (Ghandour et al, 1980) and pericytes (Frey et al, 1991). Immunoreactivity studies suggest that γ-GT is largely though not exclusively associated with glial cells, but to a lesser extent in oligodendrocytes or neurones (Shine et al, 1981a, b). Therefore, GSH released by astrocytes would need to participate as a substrate for γ-GT located on the extracellular surface of astrocytes, for the generation of GSH precursors for neurones or oligodendrocytes. This would lead to the generation of GSH precursors in the conditioned medium, before its application to neurones or oligodendrocytes; ie. GSH precursors are contained within astrocyte-conditioned medium, and are not generated by the participation of released GSH with γ-GT on the extracellular surface of neurones or oligodendrocytes. Therefore a presence of astrocytes is necessary for the release of GSH, and the interaction of GSH interaction with γ-GT, for the generation of neuronal and oligodendroglial GSH precursors.

The hypothesis of the maintenance of neuronal GSH concentrations under conditions of oxidative stress, by the preservation of GSH by EcSOD, is supported by the data of this present study: GSH release by rat astrocytes and the human astrocytoma cell line was maintained after the acute exposure to an NO' donor or ONOO' (Figure 6.2., 6.3., 6.6. and 6.7.), which have been shown to inhibit the mitochondrial respiratory chain, and contribute towards oxidative stress (Bolanos et al, 1994, 1995; Mitrovic et al, 1994). Furthermore, GSH release by rat astrocytes was also maintained after the induction of NOS (Figure 6.4. and 6.5.), which has also been shown to contribute towards NO' mediated mitochondrial damage (Bolanos et al, 1994). Therefore, the possible release of an EcSOD-like factor may be a primary mechanism developed and activated by astrocytes during acute conditions of oxidative stress such as iNOS induction. This mechanism may maintain concentrations of released GSH when antioxidant protection is most necessary, such as during acute conditions of mitochondrial inhibition by NO' or ONOO'.
However, during chronic conditions of oxidative stress, such as during neurodegenerative diseases, the prolonged exposure of astrocytes to reactive species may impair their ability to release EcSOD and to retard GSH oxidation. This may contribute towards the loss of up to 40% GSH reported in the substantia nigra of Parkinson’s Disease (PD) patients (Sian et al, 1994a). In addition, the impairment of astrocytes to release EcSOD and preserve GSH concentrations during chronic conditions of oxidative stress, may also possibly result in increased concentrations of H$_2$O$_2$ in PD (Jenner et al, 1992), and postulated to contribute towards the neuropathology of Alzheimer’s Disease (AD) (Behl et al, 1992, 1994; Butterfield et al, 1994; Hensley et al, 1994). Furthermore, there is also evidence for a role of NO$^\cdot$ in the progression of PD, AD and Multiple Sclerosis (MS) (Merrill, 1987; Griffin et al, 1989; Boka et al, 1994; Mrak et al, 1995; Johnson et al, 1995;). NO$^\cdot$ has been shown to inactivate all complexes of the mitochondrial transport chain (Bolanos et al, 1994, 1996; Mitrovic et al, 1994), and decreased mitochondrial activity has been observed in PD (Schapira et al, 1990). GSH has been shown to be an important factor protecting against NO$^\cdot$ or ONOO$^-$/mediated mitochondrial damage (Bolanos et al, 1995, 1996). However, the impairment of astrocytes to release EcSOD and preserve GSH concentrations during chronic conditions of oxidative stress, would result in the loss of protection against NO$^-$/mediated mitochondrial damage observed during neurodegenerative circumstances.

CONCLUSIONS AND IMPLICATIONS FOR NEURODEGENERATION.

The data of this current study shows that GSH is released by astrocytes into the extracellular medium. However, free radicals such as superoxide can potentially be generated within tissue culture medium (Halliwell et al, 1985), and cause the oxidation of GSH released by astrocytes. During this process of GSH oxidation, a thiol radical in the extracellular medium can be formed (Winterbourn et al, 1995), which can readily combine with oxygen in the extracellular medium to form another
superoxide radical (Winterbourn et al, 1995). Thus, superoxide in the extracellular medium may cause an auto-oxidative cycle of GSH oxidation (Figure 7.1.). However, the data of this study also provides evidence that astrocytes may release EcSOD. SOD rapidly removes superoxide (Halliwell et al, 1985), and therefore the release of EcSOD into the extracellular medium could prevent the oxidation of released GSH by \(O_2^-\) within the medium (Figure 7.1.).

Furthermore, the preservation of extracellular GSH by the release of EcSOD, may result in a higher concentration of substrate available for \(\gamma\text{-GT}\), and subsequently may generate a greater concentration of precursors for neuronal GSH synthesis (Figure 7.1.): Cysteine is one of the amino acid precursors necessary for the synthesis of GSH (Dringen et al, 1996; 1997c). However in stock culture medium, cysteine is usually undetectable due to auto-oxidation (Sagara et al, 1993b), and neurones therefore depend on astrocytes for their supply of cysteine (Sagara et al, 1993a; Dringen et al, 1999a). GSH released by astrocytes is a substrate for the \(\gamma\text{-GT}\) (Dringen et al, 1997a), which transfers the \(\gamma\)-glutamyl segment of GSH onto an acceptor molecule, thereby forming the dipeptide cysteinylglycine (CysGly) (Meister et al, 1981). CysGly is then efficiently utilized by neurones as a precursor for neuronal GSH (Dringen et al, 1999a).

This study therefore suggests that an important function of EcSOD, possibly released by astrocytes, may be to remove \(O_2^-\) generated in the extracellular environment and prevent the auto-oxidative decay of GSH. This preservation of released GSH by EcSOD may result in a higher concentration of substrate available for \(\gamma\text{-GT}\), and subsequently generate a greater concentration of precursors for neuronal GSH synthesis.

This study also has possible implications for the treatment of oxidative-stress induced conditions of neurodegeneration. Previous studies have shown that neurones are susceptible to oxidative stress after exposure to ONOO' or NO' (Bolanos et al, 1995,
Figure 7.1. GSH released from astrocytes is prevented from oxidation by the release of EcSOD. This preserves released GSH which can act as an extracellular antioxidant and is also a substrate for γ-glutamyl transeptidase (γ-GT). The cleavage of GSH by γ-GT forms the dipeptides cysteinyl-glycine, which is utilized as a precursor by neurones for the intracellular synthesis of neuronal glutathione. Adapted from Dringen et al (1999a).
However, neurones cocultured with activated astrocytes were protected against oxidative stress-mediated neuronal death (Bolanos et al., 1996). During acute conditions of oxidative stress, such as brief exposures to ONOO· or 24 hour exposures to an NO·-donor or cytokines, astrocytes maintain the release and preservation of extracellular concentrations of GSH (as observed in Chapter 6). Because this system of GSH release and preservation is maintained by astrocytes during acute conditions of oxidative stress, NO·-releasing astrocytes can still sustain neuronal GSH concentrations (Bolanos et al., 1996). Therefore neurones may not initially be vulnerable to oxidative stress. However, during chronic conditions of oxidative stress such as AD, PD and MS, this protective system of GSH release and preservation by astrocytes may fail. Therefore there may be a window of opportunity during the initial phase of neurodegenerative diseases for treatment. By strengthening the potential astrocytic system of the preservation of GSH release by the release of EcSOD during the initial phase of neurodegenerative diseases, it may be possible to limit the oxidative damage observed during more advanced stages.

7.2. FUTURE WORK.

- As previous studies have shown that astrocyte-conditioned medium conveys neuroprotection against oxidative stress due to the release of a diffusible factor (Engele et al., 1991b; O’Malley et al., 1994), the neuroprotective potential of astrocyte-conditioned medium should be investigated. Neurones would be exposed to medium previously conditioned by astrocytes, and subjected to oxidative stress by the addition of either ONOO·, an NO·-donor, or cytokines inducing neuronal NOS-synthesis. The inhibition of the mitochondrial transport chain of these neurones would then be determined, and compared to further neurones which had not been exposed to astrocyte-conditioned medium but also subjected to oxidative stress.
The preservation of the concentration of GSH by cells in addition to astrocytes should be determined, to establish if the retardation of GSH oxidation by the release of a factor is exclusive to astrocytes. Medium would be conditioned by neurones and oligodendrocytes, and the concentration of GSH in this medium would be monitored over an extended time period, and compared to the stability of the concentration of GSH in unconditioned medium.

Mice carrying a targeted disruption of the EcSOD gene have been generated (Carlsson et al, 1995). GSH release could be observed from primary astrocytes cultured from these mice, and compared to GSH release from wild-type mice. A possible decreased protection towards the concentration of released GSH from mice lacking the EcSOD gene would provide further support for the hypothesis of this current study, that the release of an EcSOD-like factor by astrocytes preserves GSH released by astrocytes.

The molecular mass of the astrocyte-derived factor should be further characterized by western blotting. Initial studies determining the molecular weight of the factor in conditioned medium were performed using the technique of PAGE during this current study, but were unsuccessful as a result of the unsatisfactory separation of the protein by the PA gel. Further studies investigating this, are therefore essential.

It should be determined, if the factor preserving GSH stability in conditioned medium could be inhibited by an antibody specific to EcSOD. Treatment of culture medium containing EcSOD released by human glial cell lines, with an immobilized anti-(human EcSOD) antibody, has previously been shown to abolish all SOD activity within the medium (Marklund, 1990a). This antibody could therefore be utilized and added to astrocyte-conditioned medium, and the preservation of the concentration of GSH in this medium could be
observed and compared to the stability of the concentration of GSH in unconditioned medium.
References.
References.


Beal, M.F.; Ferrante, R.J.; Browne, S.E.; Mathews, R.T.; Kowall, N.W.; Brown, R.H. (1997). Increased 3-nitrotyrosine in both sporadic and familial amyotrophic lateral


the National Academy of Sciences USA, 92: 6264-6268


Chen, H.; Tappel, A.L. (1994a). Protection by Vitamin E, selenium, trolox C, ascorbic acid palmitate, acetylcystine, coenzyme Q, beta-carotine, canthaxin, and (+)-catechin against oxidative damage to liver slices measured by oxidized heme proteins. Free Radical Biology and Medicine, 16: 437-444


Dringen, R.; Pfeiffer, B.; Hamprecht, B. (1999a) Synthesis of the antioxidant glutathione in neurones: supply by astrocytes of cysgly as precursor for neuronal

180
glutathione. The Journal of Neuroscience, 19: 562-569


dopaminergic neurones against hydrogen peroxide toxicity independent of their effect on neuronal development. Neuroscience Letters, 192: 13-16


Lizasoain, I.; Moro, M.A.; Knowles, R.G.; Darley-Usmar, V.; Moncada, S. (1996). Nitric oxide and peroxynitrite exert distinct effects on mitochondrial respiration which are differently blocked by glutathione or glucose. Biochemical Journal, 314: 877-880


Neurochemistry, 62: 45-53


Neuschwander-Tetri, B.A.; Roll, F.J. (1989). Glutathione measurement by high-
performance liquid chromatography separation and fluorimetric detection of the glutathione-orthphthalaldehyde adduct. Analytical Biochemistry, 179: 236-241


capillaries: possible site of a blood-brain barrier for amino acids. Science, 184: 66-68


reduced heparin affinity. The Journal of Biological Chemistry, 267: 18205-18209


Stocks, J.; Gutteridge, J.M.C.; Sharp, R.J.; Dormandy, T.L. (1974). The inhibition of
lipid auto-oxidation by human serum and its relation to serum proteins and α-tocopherol. Clinical Science and Molecular Medicine, 47: 223-233


Toyo’oka, (1986). Amino acid composition catalysis of minute amounts of cysteine-containing protein using 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole and 4-fluoro-7-nitro-2,1,3-benzoxadiazole in combination with HPLC. Biomedical Chromatography, 1: 15-20


and kainate: implications in neurotoxicity? Journal of Neurochemistry, 73: 1566-1572


Appendix.

Publications.
Astrocyte Nitric Oxide Causes Neuronal Mitochondrial Damage, but Antioxidant Release Limits Neuronal Cell Death

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INTRODUCTION

Excessive generation of nitric oxide (NO), by astrocytes, has been proposed to be an important cause of the neuronal mitochondrial damage that may occur in conditions such as Parkinson's disease, Alzheimer's disease, and multiple sclerosis. Previous studies have revealed that neurons, when cultured alone, are particularly susceptible to exposure to NO, resulting in marked damage to the mitochondrial respiratory chain and cell death. Using a system whereby NO-generating astrocytes were cocultured with neurons, we recently demonstrated NO-mediated damage to the neuronal mitochondrial respiratory chain. Under these coculture conditions, there was no evidence of neuronal cell death. In view of this observation, we have considered the possibility that neuronal damage is minimized due to the concomitant release, by astrocytes, of antioxidants such as reduced glutathione (GSH). In order to begin to address this hypothesis, we monitored the ability of cultured astrocytes to release GSH.

METHODS

Primary cultures of astrocytes were prepared from neonatal Wistar rats as described by Taberner et al. After 13 days of culture, the cells were seeded at a density of \(2.5 \times 10^5\) cells ml\(^{-1}\). On day 14 the L-valine-containing minimal essential medium was replaced with minimal medium. At set time points (0–8 hours), the experiment was terminated by removal of the astrocyte-conditioned media. This media was then frozen immediately, by immersion in liquid nitrogen, and stored at \(-70^\circ C\) until analysis. For GSH analysis, the media was thawed and mixed (1:1) with 15 mM

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orthophosphoric acid. Following mixing and centrifugation (15,000 \times g for 6 minutes), 20 \mu l of supernatant was injected onto an HPLC. Separation and electrochemical detection were based on the method described by Riederer et al. In order to ascertain whether oxidation of GSH to GSSG had occurred, some of the media was pretreated with glutathione reductase (1.0 units) and NADPH (80 mM) for 10 minutes prior to the addition of ortho-phosphoric acid. GSH stability in both conditioned (previously astrocyte-exposed) and unconditioned minimal medium was also determined by adding GSH (5 \mu M) to both media, sampling at set time points and treating as above. In all cases results are expressed as mean ± SEM.

RESULTS

Astrocytes were found to release reduced GSH. The GSH concentration in the extracellular medium increased in a linear manner over the eight-hour time period, accumulating to 1.13 ± 0.12 \mu M (Fig. 1). GSSG was not formed in the extracellular media at any time point, as incubation with glutathione reductase and NADPH did not alter the amount of GSH detected. The stability of 5 \mu M GSH in both conditioned and unconditioned minimal medium was monitored over a 5-hour time period. GSH was found to be more stable in astrocyte-conditioned minimal medium than in unconditioned minimal medium (Fig. 2). More specifically, after a 5-hour incubation in astrocyte-conditioned minimal medium, 5 \mu M GSH was found to be preserved and was 43% of its initial concentration. However, 5 \mu M GSH added to unconditioned minimal media was very unstable, decaying to 0.4 ± 0.18 \mu M instantaneously, and dropping further after 2 hours to 0.14 ± 0.12 \mu M. This loss of GSH was due to oxidation to GSSG, as incubation with glutathione reductase and NADPH restored the GSH concentration to within 75% of the initial GSH concentration.
CONCLUSIONS

Astrocytes release GSH into the extracellular medium. This observation supports the previous findings of Dringen et al. who monitored total glutathione (GSH + GSSG) release from astrocytes. The data presented here suggest that GSH is released from astrocytes and is not oxidized to GSSG over an incubation period of at least eight hours. In contrast, GSH added to minimal medium (unconditioned and cell free) is rapidly oxidized to GSSG, but in conditioned medium GSH oxidation is impaired. These findings imply that astrocytes, in addition to releasing GSH, release a factor that prevents extracellular GSH from oxidation. The release and preservation of GSH may be important in limiting neuronal damage from astrocyte-derived NO.

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REFERENCES


Abstracts.


