Studies of Metabolism in Human Astrocytes and Astrocytomas Using NMR Spectroscopy and Other Analytical Techniques

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by

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Abstract

The object of this research was to investigate profiles of human astrocyte and astrocytoma metabolism using NMR spectroscopy and other analytical techniques such as high performance liquid chromatography (HPLC), mass spectrometry and radiation scintillation counting. Using these techniques species differences and transformation-related differences in metabolism were assessed in the human and rodent astrocytic lineage.

In vitro and *in vivo* comparisons of astrocytomas with normal human astrocytes and normal brain revealed that certain characteristic aspects of the transformed cells' metabolism may be related to the normal metabolic features of their lineage. However, several transformation-specific changes were also observed in the astrocytomas both in culture and *in vivo* which implicate the metabolic pathways involved in glutaminolysis and the hydrolysis of the membrane phospholipid, phosphatidylcholine, for generating the unique ^{1}H-NMR profile for astrocytomas which may distinguish them from other CNS tumours. Furthermore, the conservation *in vivo* of the ^{1}H-NMR astrocytoma profile seen in cell culture serves as a strong validation for the development of tumour-specific profiles based on cell culture extracts, where conditions can be carefully controlled and a large number of metabolites simultaneously investigated.

Based on the similarities between the astrocytomas and their normal cell lineage, and the theory that the progression of transformation in malignant cells involves a de-differentiation to an earlier developmental state, the ability of astrocytomas to metabolise fatty acid in a similar manner known to occur in normal astrocytes from the developing brain was investigated. The astrocytomas were found to β-oxidise fatty acids at rates comparable to those reported in the literature for normal astrocytes. This metabolic pathway was capable of producing 37-50% of cellular ATP under different conditions. Exogenously supplied oleic acid was also shown to be readily incorporated into cell membrane phospholipids and possibly into intracellular lipid droplets where they may serve as an energy-reserve.

This has led to the proposal that astrocytomas only partially utilise several metabolic pathways (glycolysis, glutaminolysis, and partial fatty acid β-oxidation) which may be advantageous to adapt to variable energy substrate supplies and energy demands.

For Neil

LIST OF ABBREVIATIONS

3-NPA	3-nitroproprionic acid
α	critical value for significance
ACC	acetyl-CoA carboxylase
ACM	astrocyte-conditioned medium
ADC	apparent diffusion coefficient
ADP	adenosine diphosphate
ADRP	adipose differentiation related protein
Ala	alanine
ALCAR	acetyl-L-carnitine
AMP	adenosine monophosphate
ANOVA	analysis of variance
Asp	aspartate
ATP	adenosine triphosphate
BBB	blood-brain barrier
BHB	β-hydroxybutyrate
BSA	bovine serum albumin
BT	biopsy tissue
CC	cell culture
Cho	choline
Chols	choline-containing compounds
CoA	co-enzyme A
CPT	carnitine palmitoyltransferase
Cr	creatine
CNS	central nervous system
CT	computerized tomography
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
ES	electrospray
FCS	fetal calf serum
FMRI	functional magnetic resonance imaging
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acidic protein
Gln	glutamine
Glu	glutamate
Gluc	glucose
Gly	glycine
GM	glioblastoma multiforme
GPC	glycerophosphocholine
GPE	glycerophosphoethanolamine
HMBC	heteronuclear multiple bond correlation
HPLC	high-performance liquid chromatography
h-Tau	hypotaurine
Ino	*myo*-inositol
Lac	lactate
LPS	lipopolysaccharide
Lys	lysine
M/C	methanol/chloroform
mHS	3-hydroxy-3-methylglutaryl-CoA synthase
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
MS	mass spectrometry
MUFA	monounsaturated fatty acid
NAA	N-acetylaspartate
NMR	nuclear magnetic resoance
NTP	nucleoside triphosphates
O-2A	oligodendrocyte-type 2 astrocyte
OPA	o-phthaldialdehyde
P#	passage number
PBS	phosphate buffered saline
PC	phosphocholine
PCA	perchloric acid
PCr	phosphocreatine
PDE	phosphodiesters
PE	phosphoethanolamine
PET	positron emission tomography
Pi	inorganic phosphate
P_n	postnatal day n
PLC	phospholipase C
PLL	poly-L-lysine
PME	phosphomonoesters
PNET	primitive neuroectodermal tumour
PNS	peripheral nervous system
ppm	parts per million
PtdCho	phosphatidylcholine
PtdEth	phosphatidylethanolamine
PtdIno	phosphatidylinositol
PtdSer	phosphatidylserine
PUFA	polyunsaturated fatty acid
RPM	revolutions per minute
Ser	serine
ST	spontaneously transformed
T1	spin-lattice relaxation time
T2	spin-spin relaxation time
Tau	taurine
TCA	tri-carboxylic acid
Thr	threonine
TSP	trimethylsilylpropionate
Val	valine
VLDL	very low density lipoprotein

TABLES AND FIGURES

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Contents

Chapter 1: Background and Introduction

1.1 General Introduction

Nuclear Magnetic Resonance (NMR) imaging and spectroscopy has been used extensively as a scientific and clinical tool in cancer research. NMR has proved to be particularly valuable as a non-invasive technique to study cancers of the brain where surgical removal of malignant tissues is most difficult. The ultimate goal for the development of NMR in this field is the non-invasive diagnosis and monitoring of cancer treatment. Through the pursuit of this goal, NMR may also further our understanding of CNS cancer metabolism.

Although NMR has already been demonstrated to be able to distinguish between different CNS tumour types *in vitro*, there is still much more information required in order to circumvent the need for biopsy analysis and to understand how the altered metabolism of CNS tumour cells observed by NMR spectroscopy relates to transformation. Astrocytomas are not only the most common CNS tumour-type but they also have the poorest prognosis. Therefore, this thesis has concentrated on cultures of primary human astrocytes and astrocytoma cells to examine several aspects of their lineage-specific and transformation-related metabolism.

1.2 Astrocyte Culture

Primary cultures enriched in glial cells were originally developed by Shein, *et al.* (1965) and Varon, *et al.* (1969). However, Booher, *et al.* (1972) were the first to use CNS tissues of varying developmental stages to prepare highly enriched cultures of astroglial cells separate from neurons. Most glial preparations used today are based on this technique of obtaining cultures from specific regions of perinatal mouse or rat brain. The key to the success of this technique is choosing a developmental stage at which the majority of neurons have been formed and most of the proliferative cells are astroglial precursor cells (see Hertz *et al.,* 1989; Juurlink and Hertz, 1991; Goldman *et al.*, 1996 for reviews).

Cultures established from actively proliferating glial precursors undergo spontaneous differentiation in standard culture conditions (i.e., with serum). Studies of these cultures from various different brain regions have shown that there are several distinct glial lineages. Raff, *et al.* (1983), demonstrated that two

types of glial cells, designated type 1 and type 2, are present in optic nerve cultures. More recently, additional glial types have been identified in forebrain and spinal cord cultures (Hirano, *et al.*, 1988; Goldman and Vaysse, 1991; Miller and Szigeti, 1991; Chvatal, *et al.*, 1995). Type 2 glia were defined by Raff, *et al.* (1983) as having a process-bearing morphology and having positive immunoreactivity with both A2B5 and GFAP antibodies. They also defined type 1 astrocytes by their flat, polygonal morphology and immunoreactivity with GFAP, but not A2B5. There are now a number of additional cell markers to distinguish type 2 from type 1 astrocytes. The origin of the type 2 glial lineage was found to be derived from a bipotential progenitor cell called the O-2A progenitor (Williams, *et al.*, 1985; Miller *et al.*, 1985). In a chemically defined cell medium, the O-2A progenitor cells differentiate into oligodendrocyte cells but in medium supplemented with fetal calf serum they become type 2 astrocytes (Raff, 1983). Type 1 astrocytes develop from an entirely different precursor cell, radial glia (Culican, *et al.*, 1990). A third glial type has been identified *in vitro* by Vaysse and Goldman (1992) which antigenically resembles type 2 glia but are clonally different from type 1 and O-2A lineages. This third glial lineage comprise a tiny percentage of the cultured cells which do not proliferate significantly. It has not been determined if this lineage exists *in vivo* or if it is a byproduct of the culture environment.

In the past, astrocytes were thought of as merely passive support cells to the structure of the CNS. However, with the development of methods for the culture of purified populations of CNS cells, their complex and multifunctional role in brain metabolism is beginning to emerge.

In addition to providing growth factors, synthesising several neurotransmitters, and maintaining ionic homeostasis, astrocytes are now thought to perform the following important metabolic functions from which the other cells of the CNS benefit: (1) delivery of glycogen-derived glucose and lactate to other CNS cells, (2) glutamine synthesis and ammonia clearance, (3) ketone body synthesis and release, (4) glutathione synthesis and release, (5) glutamate-glutamine cycling with neurons, (6) fatty acid β-oxidation.

The fact that these metabolic abilities are possessed solely by astrocytes suggests that astrocytes exert considerable control over the metabolic and functional state of the brain as a whole. The brain may depend on the ability of

astrocytes to adapt to a variety of environments for protection from insults ranging from changes in substrate availability to oxidative stress.

1.3 Energy Metabolism in Astrocytes

1.3.1 Energy metabolism *in vivo*

Under normal circumstances, the adult brain uses glucose exclusively for oxidative energy metabolism. There have been many positron emission tomography (PET) studies and more recently functional magnetic resonance imaging (fMRI)-magnetic resonance spectroscopy (MRS) studies demonstrating a significant coupling of regional cerebral blood flow and regional metabolic rates for glucose and oxygen (for review see Sokoloff, 1977, 1978; Baron *et al.*, 1982; Marchal *et al.*, 1992; fMRI see Hyder, *et al.*, 1997).

Glucose is converted to pyruvate which provides acetyl-CoA for oxidation in the tricarboxylic acid (TCA) cycle. The TCA cycle, in turn, produces the coenzymes NADH and $FADH_2$ for the generation of ATP in the electron transport chain. Additionally, TCA cycle intermediates are involved in the biosynthesis of amino acids and other important cellular components. The brain in particular has a very high rate of transaminase activity, which interconverts TCA cycle intermediates and amino acids for use in neurotransmitter synthesis.

The two major cell types in the brain, neurons and glia (proportionally 1:10), differ significantly in their metabolic rates. Glial cells are known to have higher rates of TCA cycle activity related to their important role in the biosynthesis of neurotransmitters and K^+ homeostasis (Westergaard, *et al.*, 1994; Largo, *et al.*, 1996; Amedee, *et al.*,1997). It has been established that astrocytes play an active role in the regulation of brain glucose metabolism (Magistretti and Pellerin, 1996). Yager, *et al.* (1994) have demonstrated a lower metabolic rate together with lower phosphocreatine levels in astrocytes derived from younger animals compared to those derived from older animals. This is in keeping with *in vivo* data that shows a maturational increase in brain energy metabolism.

1.3.2 Alternative substrates *in vivo*

Although the adult brain depends on glucose as its sole energy source under normal circumstances, it is capable of using ketone bodies during severe starvation which can provide 60-70% of the brain's energy requirements with

synthesised glucose providing the rest (Newsholme and Leech, 1985). Brain glycogen stores can also be utilised for very short-term (approximately 5 minutes) energy supply during insults such as ischemia (Watanabe and Ishii, 1976; Benzi, *et al.*, 1978; Swanson, *et al.*, 1989). Other substrates such as lactate or pyruvate and malate can maintain required ATP and PCr concentrations in whole brain during starvation. However, they can not maintain brain electrical activity on their own (Cox and Bachelard, 1988). The adult brain *in vivo* does not oxidise fatty acids to any significant degree even though they are able to cross the blood brain barrier (Pardridge, *et al.*, 1983). The adult brain maintains a tightly controlled and highly specific pathway of energy production and biosynthesis. However, the developing brain is known to utilise a number of different energy substrates, including fatty acids (Edmond, *et al.*, 1987; Auestad, *et al.*, 1991).

1.3.3 Differences between *in vivo* and *in vitro* brain metabolism

It should be noted that most cell culture studies of CNS metabolism are performed on cells originating from developing rodent brain. Even when differentiated in culture, these cells do not necessarily reflect the same biochemistry as adult cells *in vivo* and often display a greater degree of metabolic multifunctionality than adult brain. This may be due to the lack of blood-brain barrier substrate restriction, the artificial homogeneity and therefore altered metabolic interactions of the cells in association in purified cultures, or a lack of true *in vivo* adult differentiation. Therefore, purified cell cultures may represent a *potential* metabolism for a cell type given the specific conditions of their culture. However, cell culture offers the advantages of being able to investigate individual cell populations in a completely controlled environment and to offer insight into the compartmentation of metabolism in the brain.

1.3.4 Energy metabolism *in vitro*

It has been estimated that the rate of oxidative metabolism of glucose is relatively low in normal cultured astrocytes compared to other substrates such as ketone bodies, fatty acids, and amino acids (Edmond *et al.*, 1987; Alves *et al.*, 1995; Lopez-Cardozo *et al.*, 1986; Martin *et al.*, 1997). These studies have shown that 30-50% of media glucose is instead metabolised to lactate which is then exported out of the astrocytes.

Purified astrocyte cultures from neonatal rat brain can adapt to changes in their environment by using alternative substrates for aerobic metabolism (Edmond, *et al.*, 1987). Although neurons and oligodendrocytes were also demonstrated by this study to utilise glucose, glucose-derived products, and ketone bodies for respiration and lipid synthesis, only the astrocytes could utilise fatty acids and glycogen. Cultured astrocytes, generally originating from developing brain, also utilise ketone bodies, glucose, and glucose-derived substrates (lactate, malate, glutamine, glutamate) (McKenna *et al.*, 1993; Yu and Hertz, 1983) or branched-chain amino acids (Bixel, *et al.*, 1995) for energy metabolism, but they appear to oxidise fatty acids in preference to all other substrates (Edmond, *et al.* 1987). Austead *et al.*, 1991, provided evidence that ketone bodies are the main products of this pathway in rodent brain. Oxidation of leucine, a branched-chain amino acid, in cultured astrocytes can also produce ketone bodies (Bixel, *et al.*, 1995).

Although the ability of cultured astrocytes to both produce and consume these substrates appears counterproductive it has been hypothesised by several groups that astrocytes interact metabolically with other neural cells (Auested, *et al.*, 1991; Arenander and de Vellis, 1982; Federoff, *et al.*, 1986). Through the oxidation of the different fuels available, astrocytes may support energy metabolism in neighboring cells. They may provide alternative substrates for neurons and oligodendrocytes through the production and release of water-soluble fuels such as glucose, lactate, glutamine, and ketone bodies (Bossi., *et al.*,1982; Schurr, *et al.*, 1988; Bittar, 1996; Edmond, 1987; Dringen *et al.*, 1993).

1.3.4.1 Lactate

One of the major products of glucose metabolism in cultured astrocytes is lactate which may be delivered to neurons for use as a substrate for anabolic and catabolic pathways (Magistretti, *et al.*, 1993, 1996; Wiesinger, *et al.*, 1997; Dringen *et al.*, 1993). Bittar, *et al.* (1996), have shown that lactate dehydrogenase-5, an isoform that is optimised for lactate production, is found in cultured astrocytes, whereas, lactate dehydrogenase-1, an isoform which is optimal for lactate use, is found in cultured neurons. Therefore, a net glycolytic flux of lactate from astrocytes to neurons for utilisation is assumed to exist in brain during synaptic activation (Magistretti, *et al.*, 1996). There is also some evidence that glycogen stores in astrocytes contribute significantly to lactate

production rather than to glucose during substrate deprivation (Dringen *et al.*, 1993). Pellerin *et al.* (1997) have shown lactate to be the major product of glycogen breakdown in astrocytes and is thought to be the fuel used by neurons, rather than glucose. Additionally, Pellerin, *et al.*, (1998) have demonstrated the existence of an activity-dependent, glutamate-mediated supply of lactate from astrocytes delivered to neurons for use as an energy substrate.

Glucose utilisation by astrocytes in primary culture has been measured by the 2-deoxyglucose method to be 8-12 nmol/min/mg protein (Brookes and Yarowsky, 1985). Up to half of the glucose entering the glycolytic pathway is converted to lactate and released into the culture medium. During the first several hours following a media change lactate accumulates in the media but it is then oxidised as a substrate (Alves *et al.*, 1995; Hertz, *et al.*, 1986). Most cells do not produce large amounts of lactate under aerobic conditions due to the Pasteur effect, whereby the rate of glycolysis is inhibited in the presence of oxygen. Thus, normal astrocytes would appear to be capable of "aerobic glycolysis", a characteristic of transformed cells. However, high rates of aerobic glycolysis are also known to occur in several other non-tumour, rapidly-dividing cells such as lymphocytes, fibroblasts, and intestinal mucosa cells (Newsholme *et al.*, 1985; Roos and Loos, 1973; Ardawi and Newsholme, 1985). Studies of astrocyte glycolytic rates in culture are almost exclusively performed on preparations from neonatal rodent brain where the cells are rapidly dividing, which is the advantage of the culture method but may result in the observation of a metabolism more typical of developing brain.

Alves, *et al.* (1995) have shown that lactate production from glycolysis in cultured astrocytes is unaffected by the extracellular concentration of lactate and that the total amount of lactate in the media decreases over time, demonstrating that lactate is also used as a substrate by astrocytes. Thus, aerobic glycolysis, which produces only 5% of the available energy per glucose molecule, is not likely to be the major source of cellular energy in rapidly dividing cells. However, glycolysis is important for providing glucose-6-phosphate to fuel the pentose phosphate pathway and thus supplys reducing equivalents (NADPH) and ribose units to support biosynthesis necessary for cell division. Additionally, the glycolytically produced lactate can be utilised by the astrocytes themselves but also by their neighboring cells *in vivo*.

1.3.4.2 Glycogen

Glycogen metabolism is considered a unique property of astrocytes because they are the only CNS cell type to contain the required enzymes such as glycogen phosphorylase to breakdown glycogen to glucose (Reinhart, *et al.* 1990). Glycogen is the largest energy reserve of the brain and it is mainly localised in astrocytes (Magistretti, *et al.*, 1996; Wiesinger, *et al.*, 1997). However, glycogen turnover in the brain is very rapid and under conditions such as ischemia would provide a mere 5-minute energy reserve in non-hypothermic conditions. It is thought that astrocyte glycogen stores may contribute to neuronal survival from insults such as ischemia and hypoxia. However, the source of this protection may be from either (1) the astrocytic release of glycogen metabolic intermediates such as pyruvate and lactate for use by other cells in the CNS or (2) the uptake by astrocytes of excess extracellular aspartate and glutamate for renewal of glycogen stores, which subsequently redues NMDA receptor over-stimulation in neurons (Sugiyama, *et al.*, 1989; Swanson and Choi, 1993).

1.3.4.3 Ketone bodies

Astrocytes in culture can synthesise ketone bodies from both fatty acids (Auestad, *et al.*, 1991; Blazquez *et al.*, 1998; Edmond, *et al.*, 1992) and branched-chain amino acids (Bixel, *et al.*, 1995). It is suspected that, like lactate, astrocytes may provide neurons with ketone bodies as a glucose-replacement (Blazquez, *et al.*, 1998) since astrocytes are primarily ketone-producing cells and neurons are ketone-consuming cells. Cultured neurons have been shown to oxidise acetoacetate to CO_2 at three times the rate at which astrocytes oxidize the substrate (Edmond *et al.*, 1987; Lopez-Cardozo *et al.*, 1986). Bixel, *et al.* (1995) demonstrated that two out of three molecules of ketone bodies produced by astrocytes are oxidised completely via the TCA cycle for ATP production with excess ketone bodies being released into the culture media. This is in agreement with other reports that astrocytes can also use acetoacetate and D-β-hydroxybutyrate for cellular energy (Bossi, *et al.*, 1982; Edmond, *et al.*, 1987; Lopez-Cardozo, *et al.*, 1986).

Ketone body synthesis utilises three main enzymes located in the mitochondria: acetoacetyl-CoA thiolase, 3-hydroxy-3-methylglutaryl-CoA synthase (mHS), and 3-hydroxy-3-methylglutaryl-CoA lyase. Only mHS functions

exclusively for the conversion of acetyl-CoA to acetoacetate. The other enzymes also have functions related to ketone body catabolism and amino-acid turnover (Mitchell, *et al.* 1995; for review see Cullingford, *et al.* 1999). Whole-brain expression of mHS, however, is quite low compared to liver (Thumelin, *et al.* 1993; Cullingford, *et al.* 1998a). Thus, whole-brain is not thought of as highly ketogenic but the different cell types within the CNS are known to exhibit variable mHS expression. Cortical astrocytes and meningeal fibroblasts have mHS expression 6-7 times higher than whole brain but this is still only 8% of liver (Cullingford, *et al.* 1998b). However, several studies have shown that this can be increased dramatically (up to 25% of liver expression) by insulin, hydrocortisone, glucagon, or glucocorticoids, all of which are under some control by fatty acid-activated transcription factors (Gil-Gomez *et al.* 1993; Casals, *et al.* 1992; Cullingford, *et al.* 1999). Thus, astrocytes can be considered ketogenic even though the brain as a whole is not.

Blazquez, *et al.* (1998) suggest that astrocyte ketone bodies may be the main source of carbon for neuronal oxidative metabolism in adult brain during starvation or enhanced synaptic activity, or for neuronal biosynthesis in developing brain (Lopez-Cardozo, *et al.*, 1986; Edmond, *et al.*, 1992). Evidence from studies on substrate deprivation support this theory by demonstrating that neuronal survival is significantly higher in co-cultures with astrocytes than on their own (Swanson, *et al.*, 1993). However, the extent to which ketone body formation by astrocytes occurs *in vivo* remains to be determined.

1.3.4.4 Amino acids as energy substrates

Amino acids such as glutamate and glutamine have also been shown to serve as metabolic substrates for astrocytes (Schousboe, *et al.*, 1993). In particular, glutamine levels are very high in cultured rat astrocytes (Patel and Hunt, 1985). There is evidence that the brain has a net uptake of glutamine which is used for fuel *in vivo* (Tilden, *et al.*, 1983). Glutamine has also been shown to be a major fuel for rapidly dividing cultured cells in general (Zielke, *et al.*, 1984; Krebs, 1980) and to an even greater extent for astrocytes (Yu, *et al.*, 1983). Glutamine utilisation provides nitrogen for a number of important precursors for the synthesis of macromolecules including some nucleotides and amino acids.

Bixel, *et al.* (1995) demonstrated that the branched-chain amino acids (leucine, isoleucine, and valine) play a major role in brain energy metabolism. They found that while radio-labeled leucine was used to form ketone bodies and some other unidentified metabolites, the majority of the available leucine was oxidised completely in the TCA cycle for the generation of cellular energy.

1.3.4.5 Fatty acids

Lipids can be divided into two functional classes: (1) those that are ultimately oxidised to provide ATP (i.e. ketone bodies, fatty acids, and triglycerides) and (2) those that serve a structural function (phospholipids and other lipid compounds). This distinction is supported by the location of the lipids within the cell. The structural lipids are mostly components of membranes but oxidisable lipids are located in intracellular lipid stores (adipose tissue, cytoplasmic droplets, etc.) and blood plasma.

The importance of fatty acids as circulating lipid-fuel was indicated by the observation that their concentration increased during starvation and decreased on feeding which demonstrated the mobilisation of lipid stores when required (Gordon, *et al.*, 1956). Plasma fatty acids are known to arise from the hydrolysis of triglyceride within the adipose tissue and release into the blood stream. Fatty acids are oxidised by most tissues. The liver, kidney, heart, brown adipose tissue, and the aerobic muscles have high capacities for fatty acid oxidation but the rate is considerably lower in developing brain, white adipose tissue and anaerobic muscle fibres. The adult brain is not known to significantly utilise fatty acids as an energy substrate despite the presence of the enzymes for β-oxidation (Spitzer, 1973; Carey, 1975; Horrocks, 1985) but β-oxidation is considered an important source of energy for the developing brain (Cunnane *et al.*, 1994). The long-chain fatty acids present in human blood are the saturated and monounsaturated fatty acids: oleic (43%), palmitic (24%), stearic (13%), linoleic (10%), and palmitoleic (5%) (Havel, *et al.*, 1964). Their transport in blood is facilitated by albumin, a soluble protein which has a high affinity for binding long-chain fatty acids.

The main factor regulating cellular uptake of fatty acids *in vitro* is the molar ratio of fatty acid to albumin (Mackenzie, *et al.* 1970; Spector, 1972; Chambaz, *et al.* 1986; Thomas, *et al.* 1988). The mechanism of fatty acid uptake by cells is not fully understood but the three main theories involve: (1) the

dissociation of fatty acids from albumin followed by their passive diffusion into the cell (Noy, *et al.* 1986; Hamilton, 1989), (2) an uptake mechanism involving the interaction of albumin with the membrane (Chambaz, *et al.* 1986), (3) a receptor-mediated uptake mechanism based on membrane proteins which bind fatty acids and catalyse their transfer across the cell membrane (Stremmel and Theilmann,1986). Regardless of the mechanism of uptake, there does not seem to be a cellular mechanism to limit free fatty acid accumulation by cells. It is well documented that when cells are exposed to an excess amount of fat in the culture medium that they accumulate these as triglyceride in the form of cytoplasmic droplets which are usually visible by electron microscopy (Murphy, *et al.* 1999; Smith *et al.*, 1982; Rosenthal, 1981; Miller *et al.*, 1980).

Long-chain fatty acids (chain length C12-24) are taken up by cells and activated to acyl-CoA esters (outside the mitochondria). Once activated, the fatty acid group can be either incorporated into phospholipids and triglyceride, oxidised in the mitochondria, desaturated, elongated, shortened, or saturated. In the mitochondria, the fatty acids are broken down by β-oxidation to acetyl-CoA, which is converted to CO_2 and water in the TCA cycle. Acetyl-CoA can also be converted to ketone bodies. The fatty acyl-CoA formed outside the mitochondia must first be transported across the inner mitochondrial membrane where the enzymes of β-oxidation are located. The membrane, however, is impermeable to coenzyme A and its derivatives. This transport requires the formation of an ester of the fatty acid with carnitine, acetyl-L-carnitine.

The membrane-bound carnitine transferase enzymes, generally indicated as carnitine palmitoyltransferases (CPT), are widely distributed in organs, including the brain, and subcellular organelles (Bieber, 1988; McGarry *et al.*, 1992). The well-established role of CPT is the transfer of acyl compounds (e.g., long-chain fatty acids) from the cytosol into the mitochondria for β-oxidation (reviewed in Nalecz and Nalecz, 1993). Carnitine is also known to facilitate the removal of short and medium chain fatty acids out of the mitochondria which accumulate in normal and abnormal metabolism (Bremer, 1983; Nalecz, 1993). In this way, CPT plays an important role in fatty acid oxidation. CPT is also strongly correlated with membrane phospholipid turnover (Arduini *et al.*, 1992, 1997). A very active fatty acid turnover has also been shown in the triglyceride pool which may play a role in "buffering" or storing long-chain fatty acids as well (Chakravarthy *et al.*, 1986). In addition to this involvement in fatty acid transport,

carnitine acyl derivatives are known to have other metabolic effects on cells such as the inhibition of protein kinase C in several different tissues including brain (Nakadate & Blumberg, 1987; for review see Nalecz & Nalecz, 1993). Since protein kinase C is known to have an important role in intracellular regulation of proliferation, carnitine may have broader effects on brain metabolism than just those involved with fatty acid transport and β-oxidation.

There are a number of reports of congenital carnitine deficiency but it was first described by Karpati, *et al.* (1975) in which abnormalities of the central nervous system, liver, myocardium, and muscles, are present. There is some evidence that increasing carnitine concentrations in the brain *in vivo* can increase fatty acid oxidation, even in adult brain. Aureli, *et al.* (1990 &1994), have shown that acetyl-L-carnitine (ALCAR) administered intraperitoneally to rats after an ischemia-reperfusion insult enhanced the recovery rate of brain ATP and PCr and potentiated aerobic energy metabolism and subsequent lactic acid build-up during reperfusion by enhancing/stimulating the utilisation of substrates other than glucose (i.e., fatty acids and ketone bodies) to produce energy. Studies of rat astrocyte cultures have demonstrated that fatty acid oxidation rates can be increased up to 3-fold higher than control upon administration of L-carnitine (Esfandiari, *et al.* 1997; Edmond, *et al.* 1987).

In addition to showing that treatment of astrocyte cultures with 2mM L-carnitine increased the rate of [9,10-^{3}H]palmitate oxidation by 23%, Esfandiari, *et al.*, (1997) also showed that the addition of carnitine completely attenuated the loss of β-oxidation activity induced by 3-nitropropionic acid (3-NPA) at low concentrations (<0.5 mM). Additionally, Forloni, *et al.* (1994) have shown chronic treatment of neuronal cell cultures with ALCAR can significantly reduce the cell mortality induced by several neurotoxic conditions including: serum deprivation, NMDA, kainic acid, and toxic glutamate concentrations. They showed that the effect of ALCAR on energy metabolism was most likely the basis of the neuroprotection by carnitine. ALCAR may also prevent the accumulation of acetyl-CoA in the mitochondria and facilitate the transfer of medium and short-chain fatty acids out of the mitochondria. This incomplete fatty acid oxidation increases the free CoA available for activating long-chain fatty acids as well as providing precursors for membrane synthesis and triglyceride stores (Bremer, 1983).

Two CPT isoenzymes, CPT I on the outside and CPT II on the inner mitochondrial membrane, catalyse long-chain fatty acid transport into the mitochondrial matrix (Zammit and Moir, 1994; McGarry and Brown, 1997). Interestingly, when carnitine/acylcarnitine translocase was purified from rat brain mitochondria, the activity of the carrier was dependent on animal age and is twice as high in suckling vs. adult rats (Kaminska *et al.*, 1993). This finding may explain the ability of neonatal but not adult rat brain to β-oxidise fatty acids. CPT-I activity is inhibited by malonyl-CoA, the product of the reaction catabolised by acetyl-CoA carboxylase (ACC). ACC is a key enzyme in fatty acid synthesis and thus allows control over the partition of fatty acid esterification and oxidation in the cell (McGarry, *et al.*, 1989, 1997).

Once inside the mitochondrion fatty acid oxidation involves the progressive removal of two-carbon units from the carboxyl end of the acyl-CoA. This process is called β-oxidation. The complete oxidation of a saturated acyl-CoA (with an even number of carbons) uses four enzymes (acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydrocyacyl-CoA dehydrogenase, and acetyl-CoA acyltransferase) sequentially and repeatedly. The products of β-oxidation are acetyl-CoAs, flavoproteins, and NADH. Oxidation of unsaturated fatty acids requires several isomerase enzymes to shift the double bonds between carbon atoms 2 and 3, as is required in β-oxidation of a saturated acyl-CoA.

There have been many studies in human infants and rat pups which have demonstrated that both glucose and ketone bodies are taken up by the developing brain and used for energy production and as a source of carbon for lipid synthesis. However, the products of fat metabolism (fatty acids and glycerol) are dominant in the metabolic pools during early development because of the high fat content of breast milk (Edmond, *et al.*, 1992; Cunnane and Chen,1992). The findings of Reichmann *et al.* (1988), confirm this observation by demonstrating significant amounts of fatty acid-enzyme activity in developing rat and human brain and in cultured cells of neural origin for all catalytically-dependent reactions involved in the β-oxidation pathway. Warshaw *et al.*, (1976) demonstrated in rat brain homogenates that the activity of CPT and the capacity to oxidise fatty acids to CO_2 increase during development to a peak at 10-15 days of age. This rate of fatty acid oxidation in developing brain is approximately 20% of that observed in liver (Warshaw, 1979). Bossi, *et al.* (1982) have also

shown that whole-brain cell cultures from neonatal mice produce high rates of $^{14}CO_2$ from uniformly-labeled ^{14}C-oleic acid. Furthermore, they demonstrated that lowering the media glucose concentrations below 1mM could significantly increase the rate of $^{14}CO_2$ production. The same cells cultured for a further 7 days required even lower glucose concentrations (<0.5 mM) to produce a significant increase in oleic acid oxidation. This effect is thought by the authors to be due to the *in vitro* maturation of the cells. However, Murphy, *et al.*, 1992, have shown no significant difference in the oxidation rate of [1-^{14}C]palmitate by mouse astrocyte cultures from 10 to 36 days in culture. Thus, the effects of maturation on the ability to β-oxidise fatty acids are unclear.

Miller, *et al.* (1987), used radio-labeled ^{14}C-palmitate to demonstrate that embryonic rat brain *in vivo* oxidises palmitic acid. Approximately 50% of the fatty acid was incorporated into stable protein and lipid compartments (membrane) and the rest underwent β-oxidation. Approximately 65-75% of the water-soluble radioactivity (33-38% of the total pool) was recovered in the glutamate and glutamine pools from 5 minutes to 4 hours after intravenous injection of the ^{14}C-palmitate. However, Auestad, *et al.*, 1991, have shown in rat astrocyte cultures that approximately 50% of the total pool of radioactivity is found in the water-soluble metabolites which consist primarily of labeled ketone bodies, rather than labeled glutamate and glutamine. Thus, there may be a significant difference between *in vitro* and *in vivo* metabolism of fatty acids.

Edmond *et al.* (1998) have suggested that the methodology of *in vivo* studies of brain fatty acid metabolism where labeled fatty acids are introduced directly into the plasma results in an artificial pattern of uptake in developing brain. They have shown that when the labeled fatty acids are introduced in the diet and processed physiologically that the brain does not take up saturated and monounsaturated fatty acids but only the essential polyunsaturated acids are taken up. They concluded that the high accumulation of saturated and monounsaturated fatty acids in the developing brain was independent of the dietary source and consequently serves as evidence for a highly active *de novo* synthesis of these types of fatty acids.

In cell culture, where BBB restriction is not an issue, astrocytes are the only CNS cell from developing brain shown to have the ability to β-oxidise fatty acids, which they will use in preference to all other substrates (Edmond, *et al.*, 1992). Several studies on rodent astrocyte cultures have shown that the

oxidation of labeled palmitate increases as a function of palmitate concentration and reaches a maximum rate at 200μM substrate (Murphy, *et al.*, 1992; Esfandiori, *et al.*, 1997). In addition, Auestad, *et al.* (1991) have shown that incubation of rat astrocytes with ^{14}C-labeled palmitate resulted in more label appearing in water-soluble metabolites than as $^{14}CO_2$, indicating the prominence of partial-oxidation of the substrate.

Cunnane *et al.* (1994) studied the importance of polyunsaturated fatty acids (PUFA) (i.e., arachidonic acid) and more saturated fatty acids (palmitate, oleate, stearate) on brain development in rat pups. They found that arachidonic acid was incorporated into brain lipids but the saturated and monounsaturated fatty acids were more highly labeled than arachidonic acid. This is believed to occur via β-oxidation of the saturated and monounsaturated fatty acids to acetyl-CoA and subsequent recycling of the carbons in *de novo* fatty acid synthesis. However, since the fatty acids in the study were introduced via absorption in the gut it is not known if the β-oxidation or *de novo* synthesis occurred in the liver or brain.

1.3.5 Cellular substrate preferences

Although glucose is considered the most important substrate for brain metabolism, it may not have first priority for respiration (Lai, 1992). A primary fuel defined by Edmond *et al.* (1992), is a fuel which supplies mitochondria with acetyl-CoA directly. Therefore acetoacetate, free fatty acids, and glucose-derived pyruvate are considered primary fuels. Although glucose can not be considered a primary fuel, it is highly regulated so that blood glucose concentrations are very constant, whereas blood concentrations of ketone bodies and fatty acids vary widely in response to nutrition and disease. Consequently, these substrates only significantly contribute to brain energy *in vivo* when there are specific conditions leading to their production. Glucose also importantly provides ribose units and reducing equivalents such as NADPH through oxidation by the pentose phosphate shunt which can not be provided directly by fatty acid catabolism (Edmond, *et al.*, 1992).

Edmond *et al.*, (1987) compared the capacity for the utilisation of various energy substrates in cell cultures of neurons, astrocytes, and oligodendrocytes from developing rodent brain and found that all three types of cells contain pyruvate dehydrogenase and 3-oxoacid-CoA transferase for the oxidation of

carbohydrate (glucose) and ketone bodies, respectively. Additionally, all of the cells, but particularly astrocytes and oligodendrocytes, actively process glucose via the pentose phosphate pathway. Astrocytes, however, were found to be the only cells capable of the oxidation of fatty acids. In fact, astrocytes were found to use fatty acid in preference to other substrates followed by ketone bodies and then carbohydrate. Neurons and oligodendrocytes used ketone bodies in preference to carbohydrate. It has not been established if these substrate preferences change with maturation. Since the adult brain does not significantly oxidise fatty acid this suggests that the astrocyte preference for this substrate reflects the immature metabolic phenotype of the cultured cells.

1.3.6 Glucose-fatty acid-ketone body cycle

Competition between substrates for respiration in animal tissue is a well-established interaction. One of the best characterised metabolic interactions is between glucose and fatty acids (Randle *et al.*, 1963). The glucose-fatty acid cycle, or Randle cycle, involves a reciprocal inhibition based on relative substrate abundance. For example, elevated fatty acid availability inhibits carbohydrate metabolism while stimulating fatty acid oxidation and vice versa. However, the acetyl CoA formed in fatty acid oxidation can enter the TCA cycle only if fatty acid and carbohydrate degradation are appropriately balanced because sufficient TCA-cycle oxaloacetate must be available for condensation with fatty acetyl-CoA. If fatty acid breakdown predominates, acetyl-CoA undergoes a different fate, ketogenesis. Acetyl-CoA is diverted to the formation of the ketone bodies, acetoacetate and β-hydroxybutryate.

The oxidation of ketone bodies can inhibit the oxidation of glucose and fatty acids by increasing the ratio of acetyl-CoA : CoA in the mitochondria which inhibits pyruvate dehydrogenase activity. Octanoate, a medium-chain fatty acid, can enter the mitochondria without prior esterification to carnitine and is oxidised at the same rate in fed, starved, and diabetic rat liver (i.e. with different concentrations of glucose and ketone bodies available) (Stanley, 1981). Thus, given the known effects of substrate interactions, the octanoate example indicates that the transport of fatty acids across the mitochondrial membrane by carnitine is an important mechanism in the regulation of substrate utilisation in the glucose-fatty acid-ketone body cycle.

1.3.7 Effects of substrate deprivation

The superior ability of cultured astrocytes to survive substrate deprivation compared to neurons is thought to be due to the localisation of brain glycogen in astrocytes (Sochocka, *et al.*, 1994). In fact, there is evidence that astrocyte glycogen stores may be converted to lactate and pyruvate for use as an energy substrate by neurons *in vivo*. Swanson, *et al.* (1993) have shown that neuronal degeneration due to glucose deprivation is attenuated by co-culture with astrocytes with enhanced glycogen stores. The protective effect was blocked if astrocyte glycogen levels were reduced. However, brain glycogen *in vivo* should only provide short-term energy to the cells unless cellular metabolic rates are reduced. There is evidence that this occurs because there is decreased cerebral oxygen consumption *in vivo* during starvation (Sokoloff, 1977). It is not clear, however, to what extent neuronal cell death is attributable to energy failure due to lack of substrate or to the toxic effects of extracellular aspartate and glutamate accumulating in the media of hypoxic cultures. Goldberg, *et al.* (1987) and Priestley, *et al.* (1990) have shown that neuronal cell death from hypoxia is reduced by glutamate antagonists. Although astrocyte glycogen may be made available to neuronal cells as an energy substrate, it is possible that an equally significant contribution to neuronal survival by astrocytes in culture and *in vivo* is in the uptake of high concentrations of extracellular glutamate for conversion to glutamine (Rosenberg, *et al.*, 1992). Another possibility that has not, to my knowledge, been fully examined is the contribution of astrocyte triglyceride- or phospholipid-derived fatty acids to support cell survival during substrate deprivation.

Juurlink, *et al.* (1992) demonstrated that immature astrocytes can survive almost twice as long as mature astrocytes from substrate and oxygen deprivation but the reverse is true in the presence of oxygen. It is suggested that the cause of the increased immature cell death in the presence of oxygen is not due to energy failure from substrate deprivation but due to a lack of endogenous antioxidant defenses in immature cells. The ability of the immature cells to utilise a wider range of alternative substrates may account for their ability to withstand deprivation for longer periods of time than their adult counterparts. During both hypoxia and stimulated ischemia, astrocyte cell death does not occur until ATP has dropped to 20-30% of the initial levels, at which time lactate dehydrogenase release occurs (Gregory, *et al.*, 1990; Yager, *et al.*, 1994). However, astrocytes

are able to survive substrate deprevation alone with even lower concentrations of intracellular ATP. Cell death from substrate deprivation begins when ATP has fallen to 10% of initial levels in mature astrocytes and to 5% in immature astrocytes (Hertz, *et al.*, 1995).

1.4 Amino Acid Metabolism in Astrocytes

Astrocytes play a unique role in the synthesis or degradation of several amino acids.

1.4.1 Taurine

Astrocytes are thought to synthesise and export hypotaurine, an intermediate of taurine synthesis, for neurons because neurons lack the essential enzyme cysteine sulfinate decarboxylase to synthesise taurine directly from cysteine themselves. Both taurine and hypotaurine are accumulated by neurons in culture via uptake mechanisms (Brand, *et al.*, 1993). Taurine has inhibitory neuroactive properties, neuroprotective functions, and acts as an important brain osmolyte (Huxtable, 1992). It is one of the most abundant amino acids in cultured rodent astrocytes, but its concentration is considerably lower in human brain (Holopainen, *et al.*, 1986; Perry, *et al.*, 1981). Immunohistochemical studies have shown that taurine is selectively localised in astrocytes of some brain regions (Storm-Mathisen, *et al.*, 1986; Lake and Verdone-Smith, 1990). Although evidence exists to support the role of taurine in all of the functions mentioned above, it is still undetermined what the main function of this highly concentrated amino acid may be in rodent brain.

1.4.2 Aspartate

Astrocytes are known to metabolise aspartate to lactate for release into cell media and also through the TCA cycle to synthesise glutamate and glutamine via oxaloacetate (Bakken, *et al.*, 1998). Several studies have reported that a considerable proportion of the glutamate and glutamine taken up by astrocytes is metabolised via the TCA cycle and converted to deaminated products such as aspartate (Bakken, *et al.*, 1998; Yu *et al.*, 1982; Yudkoff *et al.*, 1988; Sonnewald *et al.*, 1993; McKenna *et al.*, 1996). Aspartate is also involved in the transport of electrons from the cytosolic to the mitochondrial compartment

in the malate-aspartate shuttle (Safer, 1975) and in regeneration of the adenine nucleotide pool through purine nucleotide synthesis (Kovacevic *et al.*, 1988).

1.4.3 Alanine

Westergaard *et al.* (1993) have shown that cultured astrocytes preferentially produce and release alanine (at rates of 40 nmol/hr/mg), but that they also oxidise alanine to CO_2, although to a lesser extent (18 nmol/hr/mg). Glutamate/glutamine utilisation by astrocytes can also result in production of alanine if alanine aminotrasferase activity is high. However, McKenna, *et al.* (1996 & 1998) showed that in normal astrocytes there is a compartmentation of cytosolic pyruvate produced from glycolysis which gives rise to cellular alanine and does not mix with the pool of pyruvate from the mitochondrial metabolism of glutamine/glutamate.

1.4.4 Glutamate and glutamine

Although cultured astrocytes have been demonstrated to have the ability to utilise glutamate and glutamine for energy, they are mainly considered to be glutamine-producing cells.

One of the best known interactions between astrocytes and neurons is the cycling of glutamate and glutamine. When neurons *in vivo* release the neurotransmitter glutamate, it is taken up by astrocytes and converted to glutamine. The glutamine is then released by the astrocytes for uptake by neurons, which in turn convert the glutamine back to glutamate. Glutamine synthetase, which converts glutamate to glutamine is only present in astrocytes. Astrocyte-synthesised glutamine is also thought to play a crucial role in protein synthesis and nitrogen homeostasis when its incomplete oxidation replenishes TCA cycle intermediates for subsequent synthesis of a variety of metabolites. This role for astrocytes in supplying neurons with TCA cycle intermediates or their precursors is supported by the fact that neurons do not have an anaplerotic pathway (Sonnewald, *et al.* 1994). Pyruvate carboxylase, the enzyme necessary for replenishing the TCA cycle, is present exclusively in astrocytes (Shank, *et al.* 1985). It is also believed that the synthesis of glutamine via glutamine synthetase is an important astrocytic mechanism for removing excess brain ammonia (Norenberg and Martinez, 1979).

It has been established that at least two glutamate compartments (designated large and small) exist within the brain. In particular, the TCA cycle associated with the small pool of glutamate is actively channeled toward the synthesis of glutamine and is thought to be located in the astrocytes. The large pool is thought to be used for oxidative degradation and synthesis of other amino acids and glutathione in neurons (reviewed in Sonnewald *et al.*, 1998). Several reports have demonstrated that astrocytes in culture will produce glutamine when it is absent from the culture medium but consume glutamine when it is present at high concentrations. Consumed glutamine can be oxidised completely to CO_2, or partially for the synthesis of aspartate, alanine, leucine, tyrosine, lactate and phenylalanine (Yudkoff, *et al.*, 1988; McKenna *et al.*, 1998). Schousboe, *et al.* (1993) have shown that in astrocyte cultures 70% of media glutamate is also oxidised through the TCA cycle and a lesser amount (30%) metabolised directly to glutamine.

1.4.5 Glycine

There are two high-affinity glycine transporters that have been identified in brain. Both isoforms are found exclusively in different CNS lineages. Glycinergic neurons express the GLYT2 isoform which is thought to be related to transmitter reuptake from the synaptic cleft (Luque *et al.*, 1995) while the GLYT1 isoform is mainly expressed in astrocytes (Zafra *et al.*, 1995). This suggests that astrocytes may have a role in glycine clearance from brain extracellular space. Astroglial cells have been shown to remove glycine from culture medium (Bixel *et al.*, 1993) and labeled intracellular creatine, serine, and glutathione were produced when cultured astrocytes were incubated with media containing [2-^{13}C]glycine (Dringen *et al.*, 1993). Immunocytochemical identification of a protein involved in brain glycine cleavage has also been shown to be localised in astrocytes (Sato *et al.*, 1991).

1.5 Astrocytomas

Astrocytes give rise to more primary brain tumours than any other cell-type and the high-grade astrocytoma is one of the most malignant tumours. For this reason, understanding astrocyte metabolism is of central interest for examining the metabolic changes seen in transformation. A great deal of research on malignant astrocytomas is ongoing in a number of different fields

such as molecular biology, biochemistry, cell surface characterisation, and immunology. Biochemical research is focused on amino acid metabolism, identifying substrate, cofactor, and energy requirements, and protein, enzyme, and structural components that are key factors in transformation.

There are several general characteristics of cancer cells that apply to astrocytomas. Autonomy is considered the chief characteristic of cancer cells. For example, transformed cells are able to overcome cellular controls that limit the growth of normal cells. Thus, they are able to independently grow without stimulation or inhibition from other cells. Secondly, transformed cells can develop the ability to invade adjacent tissues and to metastasise, i.e. to travel to distant parts of the body via the vascular system. However, in primary brain tumours such as the astrocytoma, only invasiveness and autonomy of growth are required to indicate malignancy. Metastasis of brain tumours is rare.

Genetic alterations in transformed tumour cells are known to affect metabolism through the expression of inappropriate isoenzyme forms. Expression of fetal rather than adult isoforms of the enzymes of intermediary metabolism (hexokinase, glucokinase, aldolase, pyruvate kinase, pyruvate dehydrogenase, and glutaminase) has been demonstrated in several tumour-types (Mangiardi & Yodice, 1990). Malignant astrocytomas in particular have been shown to have the fetal-type isoform of pyruvate kinase, hexokinase, and lactate dehydrogenase which are more active forms of the glycolytic enzymes enabling the tumour cells to survive with or without oxygen (Mangiardi, *et al.* 1990 & 1996). Some tumours have also been observed to lose the morphological characteristics of differentiated, adult tissues. This evidence has led to the development of a theory of de-differentiation in tumour development where the transformed cells regress to an undifferentiated state resembling a stem cell (Gabbert *et al.*, 1985; Meis, 1991; Ramon-y-Cajal, *et al.*, 1995). Thus, by regressing to this state, cellular mechanisms for high rates of proliferation, energy consumption, and mobility seen in normal cells during development are made active again in the transformed cells. Although many researchers have shown that human brain tumours in general have much lower metabolic rates than normal brain, Lowry, *et al.* (1977) demonstrated that most human gliomas, on the contrary, maintained a very high energy status with total adenine nucleotides being higher than for brain.

1.6 Clinical Diagnosis and Treatment of Astrocytomas

Astrocytomas are derived from pliomorphic populations of astrocytic cells and are one of the main groups included in the major class of tumours of the neuroepithelial tissue. They have been classified into four subtypes or grades based on histological features by the World Health Organization (Kleihues, *et al.* 1993). Grade I and II astrocytomas are slow-growing, benign tumours. Grade III and IV astrocytomas are malignant. Grade IV astrocytomas are also known as glioblastoma multiforme. All four astrocytoma grades share a high incidence of p53 gene mutations (Bruner, *et al.*, 1991).

Glioblastoma multiforme (GM) usually develop from low grade or anaplastic (grade III) astrocytomas but may also arise on their own. More than half of all primary intercranial tumours are the rapidly growing, highly infiltrative glioblastoma multiforme. Prognosis for GM tumours remains very poor.

1.6.1 The diagnosis of tumours

The diagnosis and assessment of the degree of malignancy of tumours is still reliant on microscopic examination of tissue. Tissue is obtained either as a sample of the tumour (biopsy) or by resection of the tumour. After removal the tissue is then either frozen or fixed before being sectioned, stained, and examined. The function of the pathologist is to determine whether the structure of the cells in the tissue sections is sufficiently different to normal tissue to diagnose neoplasia and whether the tumour is likely to be benign or malignant, its probable cell of origin, its degree of malignancy, and its extent of spread. This process is divided into tumour grading and tumour staging. Tumour grading attempts to measure the degree of de-differentiation in a tumour and is based on histological and cytological criteria. Histological differentiation is concerned with alterations in the structure of tissue and cytological grading is based on the application of similar criteria to the structure of the individual tumour cells. Tumour staging assesses the extent of spread of a tumour. Although many researchers are working to identify markers for each stage of carcinogenesis such as tumour specific or associated proteins, abnormal hormone production, or chromosome changes, their success has been limited and for the moment the most commonly used method of diagnosis is still dependent on histology.

1.6.1.1 Benign tumours

Benign tumours resemble their tissue of origin but often the cells do not function normally. Benign tumours arise in most tissues and increase in size but do not invade surrounding tissues. They usually maintain a distinct tumour border separating them from normal tissue. Cytologically the specific tumour cells do not differ substantially from the structure of the normal organ cells. Uncontrolled growth is their main feature which can still be life-threatening, particularly in the brain.

1.6.1.2 Malignant tumours

Malignant tumours show two characteristic features: cellular abnormalities and invasion of surrounding tissues. The standard cellular criteria include a local increase in cell number, loss of the normal regular arrangement of cells, variations in cell shape and size, increase in nuclear size and density, increased mitosis, and the presence of abnormal chromosomes. Malignant tumours have no well-defined borders and the cells grow in a much more disorganised form than in benign tumours.

1.6.1.3 Tumours of the nervous system

Tumours of neuronal cells only appear in the embryo or very shortly after birth. These are called neuroblastomas or, if they arise from the retina, retinoblastomas. Primitive neuroectodermal tumours (PNET), or medulloblastomas, arise from a primitive multipotent cell that has the capacity to differentiate into any of the different cell types in the CNS (Rorke *et al.*, 1985). PNETs are the malignant counterparts of multipotential neural progenitors or stem cells. PNET tumours are most frequently found in the cerebellum but may also occur in other parts of the CNS (Kleihues *et al.*, 1993). The most common tumours in the brain and spinal cord, apart from metastases from other parts of the body, arise from astrocytes (giving rise to astrocytomas) or from the meninges (giving rise to meningiomas). Less common glial tumours include oligodendrogliomas and ependymomas. As with tumours from other sites, tumours of the CNS are graded by assessing the degree of differentiation. Very rare tumours which contain a range of different tissues, called teratomas, may be found outside of the CNS and these are thought to develop from primitive cells of embryonic origin which contain embryonic stem cells capable of differentiating

into abnormal cells of any origin, including CNS cells. These tumours are usually found in the testis or ovary and often contain a mixture of tissues ranging from skin, teeth, heart muscle, and CNS tissue all in the same tumour. They are sometimes benign but very often malignant change occurs in one component tissue.

1.6.1.4 Tumour staging

Tumour staging is used to give an assessment of the extent of spread of tumours. One of the more commonly used systems is the TNM system which is based on an assessment of the primary tumour (T), the regional lymph nodes (N), and the presence or absence of metastases (M).

1.6.2 Treatment

Cancer treatment depends on the type, grade, and location of a tumour but in general terms usually includes a combined treatment of surgery and radiotherapy, frequently accompanied by chemotherapy using either cytotoxic drugs or endocrine agents.

1.6.2.1 En bloc resection

This remains the dominant and fundamental treatment for cancer. The aim of en bloc resection is to remove the entire tumour with its draining lymph nodes and thus remove the entire tumour-bearing field. The underlying principle of this radical surgery is to eliminate the risk of the progression of tumours from local tissue infiltration into local lymph systems and blood vessels with subsequent distant metastasis.

1.6.2.2 Radiotherapy

Originally radiotherapy was used as a treatment for inoperable or incurable cancers but it is now used as a partner in treatment along with surgery. Advances in both technology and technique have resulted in radiotherapy being accurately applied to the specific region of the cancer with minimal damage of surrounding tissues. Ionising radiation kills cancer cells by causing breaks in the strands of DNA but all dividing cells are particularly sensitive to radiation damage and consequently rapidly proliferating tumour cells are especially vulnerable. Thus, one of the drawbacks of radiation therapy is that dividing cells in normal

tissues may also be killed but the localised treatment hopefully keeps this to a minimum. Radiation is delivered either as an external beam or as short range radiation from an implanted radioactive source. The accurate delivery of radiation requires localisation of the tumour by CT or NMR imaging. The therapy is limited by the poor tolerance of the nervous system to radiation and the resistance to radiotherapy in some CNS tumours (Kleihues, *et al.*, 1993).

1.6.2.3 Chemotherapy

Increasingly, combined approaches towards cancer treatment using surgery, radiotherapy, and chemotherapy are being used effectively. Various different agents are used in chemotherapy including alkylating drugs, anti-metabolites, and mitotic poisons. The dose and route of administration of drugs used in this therapy must be carefully tested and controlled because the difference between efficacy against the tumour and toxicity to the host is often quite small. To be an effective chemotherapeutic agent a drug must reach its target at sufficient concentration and target tumour cells or related metabolic processes. Most chemotherapeutic drugs can be divided into those that are active only on dividing cells, those that affect a very particular phase of cell division, and those that affect several of the phases of the cell cycle. Unfortunately, this means that the drugs are also toxic to normal tissues and the only reason that chemotherapy is feasible is that normal tissues may recover, sometimes quicker than the tumour cells and in combination with localised radiotherapy the tumour cells may be more susceptible to the further insult of toxicity. Furthermore, in the adult CNS neuronal cells are not dividing and are therefore not targeted by the drugs. Chemotherapy has been successful in curing several types of malignancies such as childhood leukaemia, lymphomas, and soft tissue sarcomas. In some tumours, however, a cure is not possible and chemotherapy may be used to extend the duration of survival and improve the quality of life. The occurrence of drug resistance is one of the greatest obstacles in curing cancer by chemotherapy. Many common cancers show little response to drugs and many tumours which respond initially acquire resistance to the drugs after a few months. Resistance can be induced by serial exposure to the drugs resulting in altered cell biochemistry and gene products. Among the biochemical changes that enhance resistance are: an increase in the repair

enzymes for drug-damaged DNA, increased drug degradation, and increased levels of target enzymes.

1.6.2.4 Immunotherapy

The goal of antibody immunotherapy is to treat tumours with anti-tumour antibodies that will selectively bind to the tumours and cause their destruction either directly or through activation of the natural immune system defense mechanisms of the host. Although there is no lymphatic system within the CNS, there can be a host reaction at the site of tumour growth most likely stimulated by antigens associated with brain tumour cells. Human astrocytomas are known to secrete proteins leading to T-cell immunosuppression (Mahaley *et al.*, 1985). Monoclonal antibodies against malignant brain tumour cells can also be used as carriers of chemotherapeutic and radiotherapeutic agents. In addition, immunomodulating agents such as interferons, interleukins, and tumour necrosis factor can be used to enhance or stimulate the immune response of patients with brain malignancies (Leach *et al.*, 1992).

1.6.2.5 Anti-angiogenesis therapy

One of the most exciting new treatments to be developed is anti-angiogenesis therapy that is based on the hypothesis that killing off the blood vessels that supply a tumour or stopping the tumour from recruiting or growing new blood vessels will kill the tumour by 'starvation'. Initial studies with angiostatin in animals were very promising but not consistently reproducible. However, studies on wound repair have recently shown that the anti-angiogenesis effect of angiostatin is achieved by binding and turning off ATP-synthase located on the surface of the cells that line blood vessels. This offers the opportunity to develop more targeted drugs than angiostatin (Moser, *et al.* 1999). Thus, in the future an ATP-synthase binding protein may be used as a target for new drug therapies. These drugs will obviously be most effective against highly vascularised tumours but many tumours such as astrocytomas which have extremely variable and heterogeneous vascularisation may be more resistant to treatment.

1.6.3 Monitoring therapy

Neurological symptoms in patients undergoing brain tumour therapy are difficult to interpret because their clinical status may be affected by side-effects of treatment unrelated to the tumour burden itself such as cerebral edema, seizures, and brain tissue necrosis. Thus, tumour status and response to therapy is best evaluated by imaging techniques such as CT or MRI. Alterations in the size of a tumour monitored non-invasively by imaging has been shown to be a useful indicator of the success or failure of a treatment to slow or halt tumour growth *in vivo*. MR imaging has the advantage as an imaging technique of being non-ionising and having the ability to distinguish between areas of normal tissue, necrosis, and the actual tumour. Despite the advantages of using MRI for locating the tumour and monitoring size following therapy, the heterogenous nature of the tumour response to treatment is not addressed.

NMR spectroscopy (MRS) offers a non-invasive technique for monitoring the progress of tumour therapy through changes in spectral metabolite peaks and ratios of peak areas. The changes observed in the biochemical profile of the tumours can indicate an early biochemical response to treatment, which may occur hours or days prior to other tumour modifications that can be detected by histology or imaging techniques. Accessible nuclei include ^{31}P, ^{1}H, ^{13}C, ^{23}Na, and ^{19}F but most studies have been performed using ^{31}P & ^{1}H spectroscopy.

^{31}P-NMR spectroscopy *in vivo* can detect the phosphorylated compounds, including phosphocreatine (PCr) and the high-energy phosphate breakdown product, inorganic phosphate (Pi). Additionally, intracellular pH and phospholipid metabolites can be measured (see de Certaines, *et al.*, 1993 for review). Altered levels of these bioenergetic metabolites can indicate hypoxia or ischemia in tumours. Untreated tumours that outgrow their blood supply become hypoxic and display a decline in the levels of high-energy phosphate compounds and an increase in phosphomonoesters (PME) and Pi (Steen, 1989). In treated tumours, ^{31}P-NMR has been used to observe a decrease in PME and increasing PCr in regressing neuroblastomas and intracranial lymphoma (Maris *et al.*, 1985; Segebarth *et al.*, 1987). ^{31}P-NMR of untreated high-grade gliomas have shown an elevation of the PME peak and a relatively alkaline intracellular pH (Cadoux-Hudson *et al.*,1989) and like low-grade gliomas they also have a reduced phosphodiester (PDE) peak compared to normal brain (Arnold *et al.*, 1991). Hubesch, *et al.* (1990) used *in vivo* ^{31}P-NMR to monitor the response of an

anaplastic astrocytoma to chemotherapy. A reduction in phosphocreatine and the alkalisation of tumour pH indicated that the tumour was not responding to therapy. NMR imaging of the tumour 9 weeks after therapy confirmed this by showing that the tumour size had not been reduced and the tumour had to then be removed surgically.

^{1}H-NMR spectroscopy *in vivo* can detect hydrogen-containing metabolites present in the brain and given the large natural abundance of hydrogen in many important metabolic compounds ^{1}H-NMR can provide information on a large number of biochemical changes taking place following tumour treatment. Some of the metabolites detected by ^{1}H-NMR reflect general processes such as glucose metabolism and TCA cycle activity whereas others reflect specific pathways or may only be present in certain cell types. Thus, changes in tumour metabolism or reductions in a particular cell type in a localised tumour or tumour region can be monitored before and after therapy. Due to proportionally large quantity of water present in human tissues, the signal from water must be suppressed in order to detect the signal from the other compounds present.

The use of ^{13}C-NMR spectroscopy and substrates enriched with carbon-13 has proved to be a valuable method for investigating metabolism both *in vivo* and *in vitro* (Cerdan & Seelig, 1990; Scott & Baxter, 1981; Lundberg *et al.*, 1990). Carbon NMR is an advantageous technique for monitoring the regulation of intermediary metabolism or metabolic dysfunction in pathological conditions because, unlike carbon-14 methods, ^{13}C-NMR is non-specific in metabolite detection and therefore gives access to a large amount of information from a single spectrum of a complex mixture. ^{13}C-NMR studies have been reported for a variety of metabolic pathways including the TCA cycle, fatty acid metabolism, ketogenesis, gluconeogenesis, and phospholipid metabolism (reviewed in Cerdan & Seelig, 1990).

1.7 Energy Metabolism in Astrocytomas

1.7.1 Glycolysis

One of the characteristic changes seen in transformed cells is a greatly increased rate of glycolysis in both aerobic and anaerobic conditions which coincides with a relatively low respiratory capacity (Warburg, *et al.*, 1956; Nakashima *et al.*, 1984). The reason for this remains unclear but a strong correlation has been established between the increased glycolytic flux and the

degree of malignancy of a tumour (Weinhouse, 1972). Glycolysis is an important process for producing biosynthetic precursors and lactate in tumour cells (Lazo, *et al.*, 1980, 1981). This transformation-based reliance on glycolysis for cellular energy requirements is relatively inefficient (2 moles ATP/ mole glucose) compared to the complete oxidation of glucose in normal cells (36 moles ATP / mole glucose). Normal cells are known to utilise this pathway during anaerobic pathological conditions such as ischemia but the Pasteur effect inhibits glycolysis under aerobic conditions. In transformed cells, however, this pathway is highly active even in the presence of oxygen.

Although there are other normal cells that are capable of aerobic glycolysis, they all share the characteristic of being rapidly-dividing cells. Normal astrocytes have been shown to use 50% of consumed glucose toward lactate production under aerobic conditions, but this is likely to be due to their preparation from developing brain and their high rate of proliferation. Additionally, it is not thought that this serves as the primary energy source for the normal astrocytes. They are thought to oxidise the lactate itself and other amino acids as fuel in culture (Alves, 1995). Despite high lactate production, tumours are known to maintain an intracellular alkaline pH (Vaupel, *et al.* 1989; Griffiths, *et al.* 1981; Stubbs, *et al.* 1999). Human gliomas have been shown to be significantly more alkaline than normal brain (Negendank, 1992). This is thought to be important for various cellular activities including growth and proliferation.

1.7.2 Amino Acid Metabolism

Despite their high rate of glycolysis, tumour cells in general *in vitro* have been demonstrated to utilise more glutamine than glucose. The rate of glutamine utilisation can also be related to the degree of malignancy (Kovacevic and Morris, 1972; Board, 1990). This has led to the theory that glutamine is the main energy substrate in tumour cells (Reitzer, *et al.*, 1979; Lazo, 1981; Mckeehan, 1982; Kovacevic, *et al.*, 1972). Glutamine can be catabolised to produce energy in three possible pathways: (1) complete oxidation to CO_2 providing 21 moles ATP/ mole glutamine, (2) incomplete oxidation to aspartate (a pathway known as glutaminolysis) providing 12 moles ATP/ mole glutamine, or (3) to lactate (6 moles ATP/ mole glutamine). Despite the importance of glutamine as an energy source for cultured cells, it is thought that incomplete oxidation greatly exceeds complete oxidation to CO_2. Although there is evidence that tumour cells

maintain a full compliment of TCA cycle enzymes, complete oxidation of acetyl units does not always occur through the entire TCA cycle (Board, *et al.* 1990). For many tumour cells, as well as normal lymphocytes and macrophages (Newsholme, *et al.* 1987, 1989), there is evidence that the flux through the left side of the the TCA cycle (α-ketoglutarate to oxaloacetate) is much more active than the right side (acetyl-CoA to α-ketoglutarate) and glutamine is only partially oxidised via the left side to aspartate (Coleman & Lavietes, 1981; Kovacevic & Morris, 1972; Kovacevic *et al.*, 1988). This apparent inhibition of the right side remains poorly understood but at least two theories have been proposed: (1) this is due to a high demand for acetyl-CoA/citrate for cholesterol synthesis and (2) intermediates of glutaminolysis and glycolysis, for example aspartate and glucose-6-phosphate, are required for purine and pyrimidine neucleotide and ribose-5-phosphate formation during critical periods in the cell cycle of rapidly dividing cells (Newsholme, *et al.* 1985).

Glycine, which is the simplest amino acid and is involved in a number of metabolic pathways, has been observed to be present at particularly high concentrations in glioblastoma multiforme and experimental gliomas (Kinoshita and Yokota, 1997; Gyngell, *et al.* 1992). High taurine has also been found in some fibrosarcomas, meningiomas, malignant astrocytomas, and experimental gliomas and may be related to increased proliferation and tumour aggressiveness (Peeling & Sutherland, 1992; Evanochko *et al.*, 1984; Moreno *et al.*, 1993).

1.7.3 Fatty Acid Metabolism

A variety of human tumours have been reported to utilise fatty acids and ketone bodies to varying degrees but fatty acid oxidation has not been generally considered to be a significant energy source for transformed cells (Sauer and Dauchy, 1992; Fields, *et al.* 1981; Cederbaum and Rubin, 1976; Stanisz, *et al.* 1983; Mares-Perlman and Shrago, 1988; Tisdale and Brennan, 1986). Tumour cells have been reported to oxidise fatty acids at low rates despite the presence of high CPT activities comparable to those seen in normal lipid-oxidising tissues (Colquhoun and Curi, 1995, 1996 & 1997a). However, there may be a very important distinction in the effects that certain fatty acids have on tumour metabolism and growth.

Lipids are known to promote the development of several human tumours (Buckman & Erickson, 1991; Hubbard & Erickson, 1987, 1991). However, there is increasing evidence that the polyunsaturated fatty acids (PUFA) inhibit tumour growth and metastasis and can trigger apoptosis (Colquhoun, 1998; Colquhoun *et al.*, 1998; Colquhoun and Curi, 1998; Surette, *et al.* 1999; Vartak, *et al.* 1997, 1998; Awad, *et al.* 1995; Hawkins *et al.*, 1998; Das *et al.*, 1995; Jiang, *et al.* 1998; Banni, *et al.* 1999; Falconer, *et al.*1994; Fugiwara *et al.* 1983; Begin, *et al.* 1986). There is a very strong correlation between the degree of unsaturation of the fatty acid and the degree of cytotoxicity experienced by the tumour (Colquhoun, 1998; Hawkins *et al.*, 1998; Anel *et al.*, 1992). Several hypotheses have been put forward to explain why PUFA might be tumour-suppressive. PUFA are thought to act deleteriously through inhibition of CPT (I & II) and through high rates of lipid free-radical production (Colquhoun, 1998; Colquhoun *et al.*, 1998; Begin, *et al.* 1987, 1988). PUFA have been shown to regulate CPT activity via prostaglandin production which is known to cause inhibition of CPT I & II. This in turn reduces mitochondrial β-oxidation of the saturated and mono-unsaturated fatty acids, causing inhibition of cell proliferation (Colquhoun, *et al.* 1998c). CPT I inhibition has been shown to significantly increase apoptosis in a human tumour cell line (Colquhoun, 1998a). Although enhanced lipid peroxidation has also been proposed as a mechanism by which PUFA inhibits tumour growth (Begin, *et al.* 1986), others have shown that the addition of oleic acid in combination with PUFA eliminates the anti-proliferative effect of the PUFA on pancreatic tumour cells even though levels of lipid peroxides remain high (Falconer, *et al.* 1994). There is also evidence that PUFA may act by reducing sensitivity to tumour promoters as a consequence of changes in membrane lipid composition (Narisawa, *et al.* 1991). In addition to anti-proliferative effects, PUFA has also been shown to up-regulate expression of nm-23, a metastasis-suppressor gene, in two highly invasive human cancer cell lines (Jiang, *et al.* 1998).

There is clinical evidence that PUFA can increase the efficacy of anti-tumour drugs (Das, *et al.* 1998; Sircar, *et al.* 1990; Begin,1989; Weber, *et al.* 1994) and radiation-kill even in previously resistant tumours (Vartak, *et al.* 1997) as well as reducing tumour recurrence (Reddy, *et al.* 1998). Thus, PUFA may not only have a potential role in cancer prevention, but there is considerable

interest in including the dietary effects of PUFA as an adjunct to conventional cancer therapy (Spector *et al.*,1994).

On the other hand, the saturated and mono-unsaturated fatty acids (SFA and MUFA), such as palmitate and oleate, have been shown to have the opposite effect, stimulating tumour growth (Vignikin, *et al.* 1989; Buckman & Erickson, 1991; Hubbard & Erickson, 1991). Fetal calf serum, an important, growth-promoting component of cell culture medium, has been shown to contain mainly saturated fatty acids (54-65%) whereas less than 12% of the free fatty acid content were PUFA (Soma, *et al.* 1992; Visioli *et al.*, 2000). However, the effects of these groups of fatty acids are not always so clearly delineated (i.e., PUFA = inhibitory; SFA/MUFA = stimulatory) and there are cases where PUFA can also stimulate cell proliferation. The balance of PUFA to more saturated fatty acids may be a critical factor in the control of cell growth in both normal and transformed cells.

A comparison of dividing and non-dividing normal blood lymphocytes demonstrated that mitogenesis is accompanied by a rapid and sustained increase in plasma membrane PUFA (Shires *et al.*, 1989). An obligatory role for PUFA in cell division was further supported by Lernhardt (1990) who showed that human lymphocytes grown in serum-free defined medium required linoleic acid to support proliferation. However, a dose-dependent effect was observed for linoleic acid with an anti-proliferative effect over 1.8 μg/ml. Herzberg (1991), also demonstrated the importance of the titration of fatty acid supplement combinations and concentrations to support cell proliferation in lymphocytes. However, in these cases the PUFA was affecting proliferation based on membrane incorporation rather than through β-oxidation and use in energy metabolism. Tumours are known to release lipolytic peptides that promote the mobilisation of host lipid stores with the possible function of providing an increased lipid supply to the tumour which may have a stimulating effect on tumour growth (Beck & Tisdale, 1991; Sauer & Dauchy, 1988). Plasma from tumour-bearing patients has a particularly large increase in oleic acid concentrations compared to healthy controls while PUFA concentrations are low (Baro, *et al.* 1998). This is also true of the fatty acid compositions of maternal breast milk (Crawford *et al.* 1981). Thus, it is this highly saturated composition useful for β-oxidation with a small, possibly essential, PUFA content for membrane composition which may support cell growth *in vivo*. Therefore, the

effect of PUFA on CPT I & II may reflect a regulatory role over the β-oxidation of other, more saturated fatty acids.

Although the inhibitory effects of PUFA on CNS tumours has been demonstrated, there have not to my knowledge been any studies on saturated or mono-unsaturated fatty acid utilisation by transformed CNS cells. The extent to which a fatty acid is oxidised may depend on several factors including the type of fatty acid since there is considerable evidence that PUFA and its derivatives may be conserved for their more vital membrane functions. Ultimately, the energy-providing substrate for tumour cells may depend on a combination of the available fuel, the tumour-type (i.e. lineage-specific metabolism), and its degree of malignancy (i.e., dedifferentiated expression of fetal isoenzymes favouring the utilisation of particular substrates).

1.8 NMR of Astrocytomas

In order to determine prognosis and appropriate treatment for CNS tumours it is vital to predict whether the tumour will behave in a benign or aggressive manner. Histological classification from biopsy specimens is generally relied upon for this diagnosis which makes distinctions between low grade (I and II) and high grade (III and IV) tumours. Generally, there is a correlation between tumour grade and prognosis. However, this correlation is not always correct for a number of reasons: (1) sampling errors where the biopsy area is not representative of other areas of the tumour, (2) a change in the grade of the tumour after biopsy or (3) a general lack of the histological appearance in the biopsy to predict tumour behavior. Additionally, the surgical procedure of tumour biopsy is a traumatic invasive procedure which carries with it a certain amount of risk of morbidity and mortality.

Thus the development of a non-invasive, non-ionising method of establishing clinical tumour grade would be an important development in the diagnosis and treatment of CNS cancers. Ideally, NMR imaging and spectroscopy would be developed to identify the location of a tumour in the brain and provide vital chemical information that would allow the tumour type and grade to be established in a single scanning session.

During the last several years a number of groups have reported metabolic abnormalities in human brain tumours *in vivo* by ^{1}H-NMR spectroscopy (Gill, *et al.*, 1989; Segebarth, *et al.*, 1990; Bruhn, *et al.*, 1989; Bernsen *et al.*, 1992).

From this information there has been a great deal of interest in developing ^{1}H-NMR spectroscopy to distinguish not only between neoplastic and normal CNS tissue but also between tumours of different lineage and grades.

1.8.1 Clinical studies of Human Astrocytomas

Several studies of human CNS tumours using *in vivo* NMR spectroscopy have demonstrated that most tumours have the general metabolic characteristics of decreased N-acetylaspartate (NAA), increased choline-containing compounds (Chols) and lactate (Lac), and an alkaline pH compared to normal brain (Negendank *et al.*, 1996; Bruhn *et al.*, 1989, 1992; Frahm *et al.*, 1991; Hubesch *et al.*, 1990; Arnold *et al.*, 1990; Hagberg *et al.*, 1995). Astrocytomas in particular have been identified *in vivo* as having decreased total creatine, increased *myo*-inositol, and frequent presence of mobile lipid resonances in addition to the general characteristics of a CNS tumour (Frahm *et al.*, 1991; Bruhn *et al.*, 1992, Negendank *et al.*, 1996). However, most of these early studies used single-patient comparisons with different tumours. A multi-center study by Negendank, *et al.* (1996) compared quantitative *in vivo* ^{1}H-NMR data from a large number of glial brain tumours which were found to have characteristically elevated Chols and decreased creatine and NAA. The chols were highest in the lower grade astrocytomas and creatine was lowest in glioblastoma multiforme tumours. Intratumoural lipid signals were detected in 41% of astrocytomas but were not detected in oligodendrogliomas, ependymomas, or meningiomas and were evident in only 1 of 6 primitive neuroectodermal tumours with the highest mean amount of mobile lipid found in glioblastomas (Negendank *et al.*, 1996; Negendank and Sauter, 1996). However, these metabolic characteristics had large variations within each type of glial tumour and the resulting overlap of profiles produced poor diagnostic accuracy in distinguishing low and high grade tumours. Thus, more specific markers are required.

Despite the wealth of information potentially available from ^{1}H-NMR, *in vivo* spectroscopy has not yet evolved into a routine diagnostic tool clinically. This is mainly due to the rather limited number of metabolite resonances currently detectable *in vivo*. At long echo times NAA, creatine, chols, and lactate are the main visible metabolites. At shorter echo times *myo*-inositol, glycine, and mobile lipid resonances can also be detected. Although these metabolites are relatively informative, it has not been sufficient so far to form tumour-specific

diagnoses. However, recent studies have shown that instead of trying to grade glial brain tumours by analysing these single resonances from the limited *in vivo* spectrum that multiparametric statistical analysis and linear discrimination analysis of patterns can at least successfully classify tumours as high or low grade and also distinguish them from normal tissue and other types of brain abnormalities such as cysts, edema, and necrosis (Hagberg *et al.*, 1995; Preul *et al.*, 1998).

Due to the difficult nature of interpreting *in vivo* spectra (due to large voxel sizes, tissue heterogeneity, partial volume effects, etc.) tissue and cell extracts have become a widely used methodology for investigating altered tumour metabolism by NMR spectroscopy. Although culturing of tumour cells can lead to homogeneous populations not seen *in vivo*, it has been shown that extracts from cultured cells maintain very similar chemical compositions to those observed *in vivo* (Sze and Jardetzky, 1994; Remy, *et al.*, 1994). Furthermore, progress is continuously being made in improving the spatial resolution and metabolic specificity of *in vivo* NMR. Therefore, the biochemical markers identified today in high-resolution NMR spectroscopy of cell and tissue extracts may be important for *in vivo* diagnosis in the future.

1.8.2 *In Vitro* Studies of Human Astrocytomas

Several studies using *in vitro* ^{1}H-NMR spectroscopy of extracts of either tumour biopsy tissue or extracts of primary cell cultures derived from biopsy tissue have identified more tumour-specific markers than those identified *in vivo* (Peeling and Sutherland, 1992; Sonnewald *et al.*, 1993; Kinoshita *et al.*, 1994, 1997; Florian *et al.*, 1996; Usenius *et al.*, 1994). These extract studies allow more accurate identification and quantitation (using an internal standard) of otherwise overlapping or poorly resolved peaks. The general differences observed between CNS tumours and normal brain are similar to those seen *in vivo*: decreased NAA and Cr and increased Chols. However, the extract studies have also revealed a decrease in GABA, an increase in glutamine (although in most tumours, like normal brain, glutamate is still more concentrated than glutamine despite this increase), an increase in *myo*-inositol in neuroectodermal and most non-neuroectodermal CNS tumours, and that the increase in Chols is largely due to a relative enrichment in phosphocholine (PC) over glycerophosphocholine (GPC) and choline (Cho). Although increased PC has

been shown to be inducible directly through oncogene activation, it can also be elevated in highly proliferative normal cells (Bhakoo, *et al.* 1996). Furthermore, since GABA is known to be enriched in rat neurons (Sonnewald *et al.*, 1993), the low levels of NAA and GABA in tumours may generally reflect a loss of functioning neuronal cells in tumours.

Despite this general profile for tumours compared to normal brain tissue or cells, there is a great deal of variation in quantitative concentrations among these studies. For example, the concentration of *myo*-inositol reported for normal brain ranges from 174 $\pm$ 17 in Kinoshita & Yokota (1997) to 555 $\pm$ 20 in Peeling & Sutherland (1992) (mean $\pm$ SEM; μmol / 100g wet weight). Although the Peeling & Sutherland study did not find elevated inositol in astrocytoma extracts, a comparison to the concentration of inositol in normal brain reported in the Kinoshita & Yokota study would have indicated elevated inositol in the tumour. Reports of inositol in other studies of glial tumours are also difficult to compare because Ino may be expressed as relative peak intensities or as concentrations in different units (Nadal *et al.*, 1997; Bruhn, *et al.*, 1992; Kinoshita *et al.*, 1994, 1997; Peeling and Sutherland, 1992; Kotitschke *et al.*, 1994; Carpinelli *et al.*, 1996). These types of problems with comparisons between studies persist for most metabolites attributed to tumours in general because of: (1) differences in the sources of 'normal' brain tissue, (2) differences in tissue storage or extraction techniques, or (3) differences between fresh biopsy tissue and tissue or cells exposed to different culture media compositions. Thus, the general tumour characteristics compared to normal brain are based on increases or decreases in the metabolites within individual studies where methodological differences are largely eliminated. This is also true for studies which aim to distinguish between different tumour types and grades.

In vitro NMR studies have also revealed that in addition to distinguishing between normal and tumour tissue, qualitative and quantitative metabolic differences exist between different tumour types and grades. However, given the effects of methodological differences mentioned above the tumour-specific metabolic profiles are also best considered within individual studies. Thus, most studies have identified tumour-specific markers relative to the particular tumour types they are comparing in their study. A number of these studies on human CNS tumours are summarised in table 1-1.

PAPER	TUMOUR	SMPL TYPE	^{1}H-NMR CHARACTERISTICS
Carpinelli, *et al.*, 1996	astrocytoma grade II	BT	no significant difference
	astrocytoma grade III	BT	between grades I and II
	glioblastoma multiforme	BT	↑ Chols/Cr ratio & ↑ Gly/Cr ratio
	oligodendroglioma	BT	↑ Chols/Cr ratio
Kotitschke, *et al.* 1994 *lipid in all samples	normal glial cells	CC	↑ Asp (Glu> Gln)
	astrocytoma grade II	CC	↑ Lys, ↑ Glu, ↓ Ino (Glu > Gln)
	astrocytoma grade III	CC	↑ Lys, ↓Ino (Glu > Gln)
	glioblastoma multiforme	CC	↑ Ino, ↓ Glu, ↑ Gln (Gln > Glu)
	malignant melanoma	CC	↑ Val (Gln > Glu)
Peeling *et al.*, 1992	normal brain	BT	↑ NAA, ↑ Cr, ↑ Glu, ↑ Ino, ↑ Asp, ↑ GABA, ↓ Ala (Glu > Gln)
	malignant astrocytoma	BT	↑ Gly, ↑ Gln, ↑Chols (Gln > Glu)
	benign astrocytoma	BT	↓ Ala, ↓ Glu, ↓ Tau (Gln > Glu)
	meningioma	BT	↓ Ino, ↑ Ala, ↑ Tau (Glu = Gln)
	metastatic tumour	BT	↑ Ala, (Glu = Gln)
	schwannoma	BT	↓ Cr, ↑ Gluc (Glu > Gln)
	oligodendroglioma	BT	↑ Chols, ↑ GABA, ↑ Tau, (Gln > Glu)
	metastatic tumour	BT	↑ Ala (Glu = Gln)
Kinoshita, *et al.*,1997	normal brain	BT	↑ NAA, ↑ Cr, ↓ Ino
	astrocytoma grade II	BT	↑ Ino
	astrocytoma grade III	BT	↑ Chols, ↓ Ala, ↑ Ino, ↑ PE
	glioblastoma multiforme	BT	↑ Gly, ↑ Ala, ↑ PE
	ependymoma	BT	↑ Gly, ↑ Ala
	medulloblastoma	BT	↑ Chols, ↑ Gly, ↑ Tau, ↑ PE
	meningioma	BT	↑ Tau, ↑ Ala, ↑ PE
	neurinoma	BT	↓ Gly, ↑ Ino
	craniopharyngioma	BT	↓ Chols, ↓ Ino, ↓ PE
	chordoma	BT	↑ PE
	malignant lymphoma	BT	↑ PE
	pituitary adenoma	BT	↑ Chols, ↑ Tau, ↑ Ala, ↑ PE
	metastatic tumour	BT	↑ Chols, ↑ Tau
Florian, *et al.*, 1996	glioblastoma multiforme	CC	↓ Cr, ↓ PC, Ino not detected (Glu>Gln)
	meningioma	CC	↑Ala, ↑Glu, ↑Ser, ↑Thr, ↓Tau (Glu>Gln)
	neuroblastoma	CC	↑ Cr, ↑ GABA, ↑ Tau, ↑ PC (Glu > Gln)
Nadal, *et al.*, 1997	meningioma	BT	↑ Gly, ↓ Ino, ↑ Gln, ↑ Ala
	glioblastoma multiforme	BT	↓ Gly, ↑ Ino, ↓ Gln, ↓ Ala
Hoehn-Berlage *et al.* , 1998	normal brain	BT	↓ Chols/Cr, ↑ Asp, ↑ NAA, ↑ Glu
	meningioma	BT	↑ Gly, ↓ Cr, Ino not detected
	astrocytomas (II & III)	BT	↓ Gly, ↓ Glu, ↓ Ala
	glioblastoma multiforme	BT	↑ Gly, ↑ Glu, ↑ Ala, ↓ Ino
Sonnewald *et al.*, 1993	normal brain	BT	↑NAA, ↑Cr, ↓Chols (Gln > Glu)
	astrocytomas (II & III)	BT	↑Chols, ↑Gly (Gln > Glu)
	glioblastoma multiforme	BT	↑Ala, ↑Lac, ↑Gly, ↓Cr, ↓NAA (Gln>Glu)

Table 1-1. **Qualitative Tumour Characteristics from the Literature**

The literature data summarised in table 1-1 consists of biopsy tissue (BT) or cell culture (CC) extracts of human CNS tumours. The increases or decreases in particular metabolites are relative to the concentrations or peak heights of other tumour-types within each individual study. The following abbreviations were used: choline-containing compounds (Chols), total creatine (Cr), glycine (Gly), lysine (Lys), glutamate (Glu), glutamine (Gln), glucose (Gluc), valine (Val), myo-inositol (Ino), N-acetylaspartate (NAA), aspartate (Asp), alanine (Ala), γ-aminobutyric acid (GABA), taurine (Tau), phosphoethanolamine (PE), serine (Ser), and threonine (Thr).

Thus, depending on which tumours are being compared, the characteristic metabolic profile for a specific tumour can vary. One of the more comprehensive comparisons of different CNS tumour types was undertaken by Kinoshita *et al.* (1994 & 1997) which revealed that glioblastoma multiforme (GM) tumours can be distinguished from metastatic tumour by the presence of high glycine, medulloblastoma from epindymoma by the taurine peak, and neurinoma from meningioma by the inositol peak. They also reported three clincal case studies in which the pre-operative diagnosis based on these ^{1}H-NMR characteristics was considerably accurate at ruling out certain tumour types. For example, tumours with a location suggestive of a meningioma tumour had in one case high glycine and low creatine which are characteristic of astrocytomas and in another case high inositol suggestive of a neurinoma rather than a meningioma, which has characteristically high alanine. The final histological diagnosis was astrocytoma grade III in the first case and schwannoma in the other tumour. So, although the metabolic information gave mixed results at diagnosing the tumour types, it was very effective at ruling out a meningioma on the basis of alanine not being characteristically elevated.

The most common distinguishing metabolic characteristics between astrocytoma tumour grades reported in the literature are: GM tumours have the lowest Cr and NAA and the highest Gly and Ala of the astrocytoma grades, whereas astrocytomas grade I & II have the highest Cr and NAA and the lowest Chols and Gly, and astrocytomas grade III have the highest Tau and Ino (Kinoshita *et al.*, 1994 & 1997; Kotitschke, *et al.*, 1994; Peeling & Sutherland, 1992). A summary of the different quantitative metabolite concentrations for the astrocytomas grades I – IV and normal brain is presented in table 1-2.

	BRAIN	ASTROCYTOMA GRADES I & II	ASTROCYTOMA GRADE III	GLIOBLASTOMA MULTIFORME
Ala	26 - 92	37 - 101	52	92 - 326
NAA	490 - 556	58 - 181	110	21 - 41
GABA	70	0		17
Glu	775	129		171
Gln		216		358
Asp	125	24		67
Gly	67 - 82	91 - 126	270	153 - 502
Tau	125 - 165	33 - 169	228	177 - 215
Cr	690 - 1320	494 - 781	565	185 - 366
Chols	60 - 160	69 – 101	133	119 - 343
Ino	555 - 700	348 - 365	480	334 - 343

Table 1-2. **Mean Metabolite Concentrations from the Literature**

All metabolite concentrations are expressed as μmol / 100g tissue wet weight ± SEM. See table 1 for abbreviations. This table is composed from the following papers: Perry et al., 1971; Lowry et al., 1977; Petroff et al., 1989, 1995; Peeling & Sutherland, 1992; Kinoshita & Yokota, 1997.

In addition to the aqueous metabolites, several studies have demonstrated that CNS tumours contain a higher ratio of phosphatidylcholine (PtdC) to phosphatidylethanolamine (PtdE) in lipid extracts (Christensen *et al.*, 1965; Poduslo *et al.*, 1983; Merchant *et al.*, 1994). More recently Sonnewald, *et al.* (1993) have reported that there was a large variability in the lipid compositions and fatty acid chain lengths from low- and high-grade astrocytomas and normal brain but glioblastoma multiforme could be distinguished by the presence of cholesteryl ester (CholE). The fact that CholE and PtdC are increased in fetal brain supports the idea that tumour cells revert to a low degree of cellular differentiation (Sonnewald *et al.*, 1993).

1.8.3 Experimental glioma models

Several experimental animal models of glial tumours have been used to characterise tumour metabolism with NMR spectroscopy. These models can be divided into *in vitro* (cell and tissue extracts), *ex vivo* (intact cells or tissue), and *in vivo* (implanted intracranial tumours) studies. Human astrocytoma cells implanted into the brain of immuno-compromised rats have produced *in vivo* ^{1}H-NMR spectra resembling clinical spectra from tumours. In particular, increased

Lac and Chols and decreased Cr and NAA were observed compared to contralateral brain (Bernsen *et al.*, 1992; van Vaals *et al.*, 1991). *In vivo* studies of implanted rat gliomas (C6 and F98 cell lines) showed the same metabolic characteristics as the implanted human astrocytomas with the addition of increased Ino observed in the F98 tumours (Gyngell *et al.*, 1992; Bourgeois *et al.*, 1991; Remy *et al.*, 1994).

An *ex vivo* analysis of excised rat C6 glioma by Remy *et al.* (1994) showed the characteristic loss of NAA and Cr in the tumour tissue compared to normal brain but the loss of GABA and the presence of Tau and mobile fatty acids were additionally detected only in the tumour tissues. *In vitro* studies of PCA extracts of rat gliomas (C6 & F98) have revealed significantly higher Ala, Gly, Lac, Tau, and PC but lower Succ, Cho, NAA, Cr, and Ino in tumour tissues compared to normal rat brain (Remy *et al.*, 1994; Gyngell *et al.*, 1992). Hypotaurine (hTau), PE, and Gly were only detected in the tumour extracts (Remy *et al.*, 1994). Interestingly, in the study by Gyngell, *et al.* (1992) they observed that extracting the same F98 gliomas studied *in vivo* revealed that increased Gly in the tumour tissue was the dominant source of a corresponding increase in the less-well resolved *in vivo* resonance signal attributed to *myo*-inositol (3.56 ppm). Thus, increased Ino observed *in vivo* in many studies on astrocytomas may also be due to the overlapping Gly resonance.

In conclusion, the comparison of *in vivo* tumours and extracts from heterogeneous tumour tissues and cell cultures has revealed some general trends but the large variability in metabolic characteristics remains a difficulty for differential diagnosis. One approach towards the development of an NMR-based tumour diagnosis is to study both purified cell cultures of CNS lineages and their transformed counterparts to determine how tumour metabolism and its representative NMR spectra might differ from normal cells. The advantage of this approach for diagnostic purposes is the identification of the specific metabolic changes in transformed cells compared to their normal lineage rather than the development of tumour profiles that are highly dependent on the particular tumour types being compared. A number of studies have been carried out over the last several years in the Unit of Biophysics at the Institute of Child Health in London which have addressed this and provide the background to the work described in this thesis.

1.8.4 Metabolic Fingerprints

Urenjak *et al.*, (1993) demonstrated that it is possible to distinguish between purified populations of normal brain cells (cerebellar granule neurons, cortical astrocytes, meningeal cells, O-2A progenitor cells, and oligodendrocytes) based on ^{1}H-NMR spectra obtained from cell extracts from each cell type. On the basis of these data, it was apparent that spectra obtained from tumour cells of different lineages might also differ from each other.

NMR profiles of cell extracts from meningioma cell lines derived from both primary human brain and spinal cord meningiomas were compared with those from normal rat meningeal cell cultures by Florian, *et al.*, (1995). The data showed that the metabolite peaks characteristic of meningeal cells which distinguish them from other purified populations of brain cells, such as the relative amounts of succinate, β-hydroxybutyrate, creatine, and alanine were also present in the spectra from meningioma cell lines. These ^{1}H-NMR spectral features clearly relate meningioma cells to their tissue of origin. However, there were additional features of the spectra which may differentiate the transformed from normal cells, such as the concentrations of choline-containing compounds (Chols) and glutamate. These findings demonstrate that transformed cells, while being distinguishable from normal brain cells, also share some metabolic features characteristic of their tissue of origin. However, any differences between the human tumour cells and their normal rat counterparts could not be attributed definitively to the transformation process because species differences were an unknown factor in any metabolically distinguishing features.

Florian *et al.*, (1996) also identified distinctive metabolic characteristics of primary human meningioma, neuroblastoma and glioblastoma cell cultures determined by ^{1}H-NMR spectroscopy. Although there were some similarities among the high resolution ^{1}H-NMR spectra obtained from the tumour cells, there were also several obvious differences in the composition and ratios of metabolites (Florian, *et al.*, 1996). These qualitative, discriminating features included the presence of β-hydroxybutyrate resonances only in spectra from meningiomas, the absence of creatine signals in spectra from glioblastomas, and the absence of alanine peaks in spectra from neuroblastomas. Quantitative determinations by both NMR and HPLC showed that there were also statistically significant differences in the concentrations of glutamate, threonine, phosphocholine, serine, γ-aminobutyric acid, and taurine among preparations of

meningiomas, neuroblastomas, and glioblastomas. These findings demonstrated that it is possible to identify unambiguously between these tumour types on the basis of their characteristic ^{1}H-NMR metabolite profile.

However, understanding how these individual metabolic differences are related to transformation or lineage remains to be determined. In order to learn more about the specific effects of transformation in each tumour the NMR profile for each tumour must be compared with normal cells of the same lineage. Due to the difficult nature of obtaining normal human cells these types of comparisons are often made between human tumour cells and normal rodent cells of the same lineage. Although similarities between a human tumour cell and normal rat cells may identify lineage-specific characteristics, any differences could equally be due to transformation or species-differences. Therefore, the definitive comparison must be made to normal human cells grown in identical conditions. To the best of my knowledge, this has not been reported for any of the CNS cell types and their transformed counterparts.

1.8.5 Oncogene activation

In order to examine whether oncogene expression is associated with metabolic alterations that are recognisable with ^{1}H-NMR spectroscopy, Bhakoo, *et al.*, (1996) prepared cell extracts from an *in vitro* model of tumour progression in Schwann cells. NMR spectra from normal Schwann cells were compared with those from clonal cell lines expressing either the thermolabile tsA58 mutant of SV40 large T antigen (tsTAg) or tsTAg together with a transforming mutant of H-*ras*. The cell populations were cultured at either 33°C or 39°C, the permissive and non-permissive temperatures for tsTAg expression, respectively. Normal Schwann cells expressed a distinctive range of NMR-visible metabolites that distinguished them from the other CNS and PNS cell types (Bhakoo, 1996). Specifically, normal Schwann cells had higher concentrations of glycerophosphocholine (GPC) relative to phosphorylcholine (PC). Schwann cells expressing the immortalised phenotype (tsTAg at 33°C) had equal concentrations of GP and PC while cells expressing the fully transformed phenotype, where there is oncogene co-operation between tsTAg and H-*ras* at 33°C, displayed a marked increase in the concentration of PCho only. However, when the same cells expressing tsTAg and H-*ras* were switched to 39°C, the non-permissive temperature for tsTAg, the PC levels were markedly reduced

and GPC was expressed at levels higher than those seen in normal cells. The GPC/PC ratio of the fully transformed cell line was similar to the ratio found in human tumors, whereas the ratio in the normal cells was similar to normal human brain, where GPC > PC. Furthermore, they demonstrated that these oncogene-initiated alterations in Chols metabolism are not necessarily correlated with rates of cell division. Thus, this demonstrated the ability of NMR to characterise changes in cellular metabolism that are directly associated with the transformation process from normal to tumor cell.

1.8.6 Preservation of *in vivo* characteristics

The ^{1}H-NMR profiles of cell populations derived from human meningiomas obtained by Florian, *et al.* (1995) were very similar to ^{1}H-NMR spectra that have been reported for extracts of biopsies from meningiomas (Gill, *et al.*, 1990) and *in vivo* ^{1}H-NMR spectra from patients with meningeal tumours (Bruhn, *et al.*, 1989). These similarities include high amounts of alanine and choline, a reduction in the amount of creatine, and low to undetectable levels of neuroactive amino acids such as γ-aminobutyric acid (GABA), N-acetylaspartylglutamate (NAAG), and the neuronal marker N-acetylaspartate (NAA). The absence of these neuroactive compounds in meninges is consistent with NMR findings that these compounds are enriched in neuronal cells although it is now known that NAA may also be present in mature oligodendrocytes (Bhakoo & Pearce, 2000). Models of *in vivo* tumours using human cells transplanted into immunocompromised rodent hosts have also been shown to maintain important histological and genetic characteristics of the original tumour (Povlsen *et al.*, 1975; Saris *et al.*, 1984; Verzat *et al.*, 1990). The concurrence of these findings with data derived from *in vivo* NMR investigations on human tumours and on extracts of human biopsies demonstrates the reliability of *in vitro* studies performed on cell cultures in carefully controlled conditions. However, to my knowledge there have not been any published studies comparing CNS tumour cells implanted in rodent CNS and grown *in vivo* to the same cells grown only in culture in order to determine how *in vivo* conditions may alter the specific metabolic patterns observed from cell culture extracts.

These metabolic profiles specific to each tumour type obtained by MRS can potentially be used for *in vivo* clinical diagnosis. However, more information,

such as tumour grade, is required from a non-invasive technique in order to circumvent the need for biopsy.

1.8.7 Tumour grade assessment

Knowledge of the tumour grade is also vital for determining the best course of treatment for the patient. A potential MRS-visible marker for the stage of malignancy may be the broad lipid signal commonly seen *in vivo* in patients with high-grade astrocytomas. Several studies have successfully correlated altered levels of NMR-detected lipids with tumour development and progression in cervical and colorectal tissues (Delikatny *et al.*, 1993; Smith *et al.*, 1990; Lean *et al.*, 1993; Mackinnon *et al.*, 1994). The next step toward non-invasive diagnosis would be to determine the components of the lipid signal and to identify the metabolic pathways associated with free lipid and the progression of malignant transformation.

In vivo proton MRS studies have shown that human tumours of advanced grade display lipid resonances in the 1.3 ppm region (Sijens, *et al.*, 1995, 1996; Kuesel, *et al.*, 1994a, 1994b, 1996; Posse, *et al.*, 1993; Ott, *et al.*, 1993; Remy, *et al.*, 1989,1994; Mountford, *et al.*, 1986). In the human CNS, only astrocytomas and primitive neuroectodermal tumours (which are multipotent and may contain immature astrocytic cells) have been shown to give rise to ^{1}H-NMR lipid signals, although the amount of lipid varied from patient to patient (Kuesel *et al.*, 1994). Stimulated and embryonic cultured cells also present this lipid resonance (Mountford and Wright, 1988; May *et al.*, 1986; Holmes *et al.*, 1990; Mountford and McKinnon, 1994; Dingley *et al.*, 1992). The 1.3 ppm resonance is thought to be due to mobile $-(CH_2)_n-$ protons of neutral lipids (mainly triglycerides) with long T_2 relaxation times (200 ms) and MRS detection of the lipids was found to correlate with cellular proliferation, cellular stimulation, acquisition of resistance to anti-cancer drugs, and metastasis (Mountford, *et al.*, 1982, 1987, 1988; Le Moyec *et al.*, 1996). Remy, *et al.* (1994) demonstrated that *ex vivo* ^{1}H-NMR lipid signals in C6-glioma tissue slices arise mainly from the saturated and monounsaturated fatty acids with a very small contribution from polyunsaturated fatty acids (PUFA) such as linolenic acid.

Several cell culture studies have demonstrated that neutral lipid accumulates in tumour cells as cytoplasmic lipid droplets from increased exogenous fatty acid concentrations (Callies, *et al.*, 1993; Remy, *et al.*, 1997;

Gillham and Brindle, 1996). All fatty acids, including PUFA, are known to be taken up exogenously and to induce lipid droplet formation in a wide range of transformed and normal cells (Rosenthal, 1981; Gavino, *et al.* 1981; Edelstein, *et al.* 1994; Le Moyec, *et al.* 1997; Remy, *et al.* 1997; Negendank, *et al.* 1996; Londos, *et al.* 1999; Barba, *et al.*, 1999; Seelaender, *et al.* 1996; Dokko,*et al.* 1998). Lipid droplets form in stimulated neutrophils (leukocytes) from incubation with lipopolysaccharide (LPS), PUFA, and saturated fatty acid but only the saturated fatty acid and LPS also resulted in increased signal intensities of NMR-visible lipid signals (May, *et al.* 1994).

Exogenous lipid appears to be the source of the 1.3 ppm resonance in malignant cells because the lipid signal is diminished when the cells are grown in a lipid-free medium (Mountford, *et al.*, 1988). The lipid resonances are not detected in proton spectra of non-transformed CNS cells and tissues (Mountford, *et al.*, 1988) although they are present in other highly proliferative cells such as fibroblasts and lymphocytes (Ferretti *et al.*, 1999; Dingley *et al.*, 1992). In fact, in the study by Ferretti, *et al.* (1999), NIH-3T3 fibroblasts were found to have higher mobile lipid signals than their transformed counterparts ($3T3^{ras}$). This is in contradiction to clinical studies which have shown that *in vivo* mobile lipid signals are most commonly associated with higher grade (i.e., more transformed) tumours. However, the results from the Ferretti paper are very interesting in terms of understanding astrocytoma metabolism in particular because normal fibroblasts, like astrocytes from developing brain, are a cell type that is capable of high rates of fatty acid β-oxidation. Hypothetically, if ras-transformation was responsible for an up-regulation in the use of this metabolic pathway in the $3T3^{ras}$ fibroblasts, then it may be possible to interpret the lower lipid content in these cells as evidence that the fatty acid is being more efficiently metabolised (without any supplemented media fatty acids to replace the used substrate). In this case, the cells would be expected to have a lower stored fatty acid and/or triglyceride content.

Furthermore, the high oleate concentration (400 μM) used to induce the lipid droplet formation in myeloma and chinese hamster ovary cultures is comparable to the concentration observed in the plasma of patients with cancer (Callies *et al.*, 1993; Gillham & Brindle, 1996; Peeling *et al.*, 1988). Droplets may form when exogenous fatty acid supply is sufficient for uptake to exceed maximum oxidation rates (maximum rate of β-oxidation in rat astrocytes is

reached at 200 μM palmitate (Murphy *et al.*, 1992)). There are numerous reports which indicate that growing tumours are able to mobilise fatty acids from their host's adipose tissue stores and to elevate serum levels of fatty acid (Kitada, *et al.*, 1980; Tijburg, *et al.*, 1991; Beck and Tisdal, 1991). This possibly increases the lipid supply to the tumour, which may have a stimulatory effect on tumour growth. A study by Sauer and Dauchy (1992) investigated the relationship between lipid supply in the arterial blood, tumour uptake, and the requirements for growth in rat hepatic tumours *in vivo*. They showed that the rate of lipid uptake was determined by the supply of lipid available to the tumour, and the efficiency of uptake depended on the type of lipid and tumour. It was determined that the tumours were able to obtain all of the fatty acid needed for daily growth from host arterial blood.

It has been demonstrated in transformed fibroblasts that the accumulation of MR-visible lipid is not transformation or cell cycle-dependent but cell density-, pH-, and nutrient-dependent (Delikatny *et al.*, 1996). Attenuation of proliferation by confluence (high cell density), serum-free medium, or low pH induced the MR-visible lipid signals in this study. It is suggested by the authors that the increased lipid signals at high density or low pH are due to the *de novo* synthesis of triglyceride stores formed from fatty acid released by the catabolism of membrane phosphatidylcholine (PtdCho). Thus, this evidence suggests a scenario where nutrient-deprivation or high cell densities (a condition where media-nutrients may be more limited) activates a cell 'hibernation' or protective mode where high energy-demanding activities such as proliferation are shut down and cell energy stores (triglyceride droplets) are created from the fatty acids available in cell membranes. Barba, *et al.* (1999) have also shown in rat C6 glioma cultures that confluence and low pH can induce cytoplasmic lipid droplet formation.

There is a body of evidence that indicates that at least partial contribution to the production of NMR-detected mobile lipids in tumours is associated with phospholipase C (PLC) hydrolysis of cell membrane PtdCho (Gillham & Brindle, 1996; Delikatney *et al.*, 1996). Ferretti, *et al.* (1999) examined this as the source of mobile lipids in a fibroblast cell line (NIH-3T3) and transformed $3T3^{ras}$ cells. Using a PLC inhibitor, D609, they demonstrated a decrease in mobile lipids in both normal and transformed cells compared to control and an increase in mobile lipids upon addition of a bacteria-derived PLC. However, it should be

noted that the effects of PLC inhibition or stimulation were reported to predominantly affect the polyunsaturated fatty acids from the cell extracts. The authors also report that an analysis of the cells' fatty acid composition without any manipulation of cellular PLC demonstrated a lower degree of unsaturation in the fatty acids from the transformed cells. Remy, *et al.* (1994) also report that mobile lipids in C6 gliomas arise primarily from the saturated and monounsaturated fatty acids. Since unsaturated fatty acids are not thought to be useful as an oxidisable fuel (see section 1.7.3), I would suggest that the normally more saturated nature of transformed cell fatty acid makes it more likely that these fatty acids are being used as an energy substrate to support increased cellular proliferation than would be the case for PLC-liberated PUFA. Furthermore, they assessed the effects of quiescence by serum withdrawl and re-stimulation and found very little change in the mobile lipids in the 3T3 cells but the $3T3^{ras}$ cells had a 2-3 fold increase in mobile lipids with stimulation by FCS (which contains 65% saturated fatty acid and >12% PUFA (Soma *et al.*, 1992)). Interestingly, this is in contrast to the studies by Delikatney, *et al.* (1996), Barba, *et al.* (1999), and Rosi, *et al.* (1999) on transformed fibroblasts, rat C6 gliomas, and HeLa cells, respectively, where mobile lipid is not linked to cells in log-phase growth. However, Delikatny, *et al.* (1996) also showed mobile lipids could be induced in log-phase cultures with low pH conditions. Thus there is considerable conflicting evidence on the correlation between cell proliferation and mobile lipid accumulation. It is still not fully understood, therefore, if mobile lipids are accumulated as an energy store (possibly generated from cell membrane phospholipids) when (1) cells perceive a possible decrease in nutrient supplies, or (2) when cells require the uptake of extra substrate to support high rates of proliferation, or (3) if both scenarios may exist.

Transmission electron microscopy of freeze-fractured sections of the 3T3 and $3T3^{ras}$ cells revealed spherical lipid bodies enveloped by a membrane in the cell membranes and cytoplasm only in cells from cultures with high NMR-detected lipid signals (Ferretti *et al.*, 1999). Furthermore, it was the cells exposed to PLC that were associated with a large increase in the appearance of cytoplasmic lipid bodies. One possible explanation for this effect in proliferating cells is that PLC-liberated PUFA from membrane PtdCho must be isolated via incorporation into membrane-bound lipid droplets otherwise they may have an inhibitory effect on cell proliferation or induce apoptosis (see section 1.7.3).

Thus, if tumours take up considerable amounts of plasma oleic acid *in vivo*, which is known to be both elevated in tumour-bearing patients (Baro *et al.*, 1998) and to activate membrane PLC (Giliham & Brindle, 1996), then lipid droplets may also, in part, be a side-effect of oleic acid metabolism by the cells if the droplets contain mainly PLC-liberated PUFA. Therefore, lipid droplets would not reflect a store of energy-substrate in this scenario but would result from the metabolism of more saturated fatty acids.

However, even the cellular location of the ^{1}H-NMR lipid signal observed *in vivo* is still a point of some debate. In addition to the *in vitro* studies discussed above which attribute the signal to the formation of cytoplasmic lipid droplets, there is evidence for other potential sources such as a fluid cell membrane domain or the membrane breakdown products in necrotic and apoptotic tumour cells.

Several studies have examined the theory that the narrow ^{1}H-NMR lipid signal comes from a fluid membrane layer of triglyceride and cholesterol esters (Mountford *et al.*, 1982, 1988, 1993; Mackinnon *et al.*, 1992). They propose that the triglycerides exist in an isotropically tumbling protein-lipid complex in the plasma membrane of malignant cells since membrane-bound lipids would give very broad resonances. It is suggested that these membrane changes detected by ^{1}H-NMR in activated immune cells and metastatic malignant cells may be associated with their capacity for motility (Dingley, *et al.*, 1992; Holmes, *et al.*, 1990; King, *et al.*, 1991). This is in keeping with evidence of increased fatty acid concentrations in the plasma of cancer patients and the fact that unsaturated fatty acids control the fluidity of biological membranes and hence a number of their activities (Bourre, *et al.*, 1993; Tijburg *et al.*, 1991; Peeling *et al.*, 1988). In fact, even in normal astrocytes, 50% of endogenous fatty acid uptake in culture is directly incorporated into stable protein and membrane phospholipids. However, the other 50% is used by the cells for other processes, including energy metabolism (Miller *et al.*, 1987).

The studies which have attributed the ^{1}H-NMR lipid signal to cell membrane domains have generally used the perchloric acid (PCA) extraction technique on cells or lysed the cells in aqueous solutions (with membrane, i.e., non-soluble components, removed via centrifugation) and conclude that the lack of ^{1}H-lipid signal from these fractions provides evidence that no cytosolic lipid contributes to spectra from the intact cell or tumour (Remy, *et al.*, 1994; Dingley,

et al., 1992; May, *et al.*, 1986). However, cytosolic lipid droplets would not be extracted in PCA nor would they be found in aqueous solutions from lysed cells because they are not water-soluble. Standard lipid extracts of cells or tissue would also not be able distinguish between lipid from the cytosol or cell membrane because the lipid droplets (and the phospholipid membrane surrounding them) would be partitioned with the cellular membrane when extracted or separated. Furthermore, many studies on cell membranes have not grown or incubated the intact cells with supplemented fatty acids. For example, Knijn, *et al.* (1997) have shown a lower lipid content in transformed compared to non-tumourgenic parental cells but did not supplement the normal media with exogenous fatty acid. In this case, if the cells were capable of oxidising fatty acid, then the lower lipid levels may simply reflect their utilisation without an exogenous source to replenish intracellular stores. Cultured tumour cells are generally only known to form cytoplasmic lipid droplets when media fatty acid is supplemented at high concentrations similar to the elevated levels seen in the plasma of cancer patients. Therefore, the conditions under which the relative contribution of cytosolic and membrane lipid are compared may not closely approximate what is seen *in vivo*.

Nevertheless, there is still compelling evidence from the isolated cell membranes that a fluid layer of triglyceride exists and contributes to *the in vivo* ^{1}H-NMR signals. Mountford, *et al.*, (1982) have shown a great similarity between the proton spectra from intact malignant cells and their isolated cell membranes. Furthermore, they have demonstrated that when a paramagnetic agent which does not cross the intact cell membrane, gadolinium, was used, ^{1}H-NMR lipid signals were significantly broadened (reviewed in Mountford & MacKinnon, 1994). However, Remy *et al.* (1997) have shown that an NMR measurement of tortuosity, the apparent diffusion coefficient (ADC), was four times greater for the ^{1}H-NMR-detected lipids than would be expected if mobile lipids were inside a fluid plasma membrane domain. Although conflicting evidence exists, the membrane compartments identified by Mountford and others may significantly contribute to the *in vivo* lipid signals detected by proton NMR. However, these studies have not ruled out the possibility that cytoplasmic lipid droplets (formed from hydrolysis of membrane phospholipids or uptake from plasma fatty acid supplies) may also significantly contribute to the lipid signals *in vivo*.

Additionally, there is evidence presented by Kuesel, *et al.* (1994b) that the lipid signal *in vivo* arises primarily from the necrotic core of a tumour because the amount of mobile lipid was found to increase with the extent of necrosis and therefore the mobile lipid could be the result of membrane phospholipid breakdown. Similarly, apoptotic cells have been shown to accumulate cytosolic triglyceride (Blankenberg, 1996; Hakumaki,1998). In contrast, Nariai, *et al.*, (1991), have shown significant incorporation of intravenously administered [9,10-^{3}H]palmitate into rat carcinoma tumours implanted into the rat brain compared to normal brain. Comparisons of autoradiographs and histological sections show that tumour areas with the greatest radioactivity corresponded to areas of the highest tumour cell density. Significantly, the areas of low cell density and necrotic areas of the tumour had considerably lower radioactivity incorporation. Thus, the 6-fold higher incorporation of [9,10-^{3}H]palmitate into rat carcinoma can be attributed to the viable tumour cells implanted in the brain *in vivo*. The cellular location (i.e., membrane, cytosol, etc.) of the fatty acid incorporation was not identified.

1.9 Lipid Droplet Formation in Astrocytomas

Most organisms form lipid droplets at some point in their life cycle which is an important mechanism for energy storage and/or transport in cells (Murphy and Vance, 1999). In mammalian liver and intestine, triglyceride and cholesterol esters are either stored as cytosolic lipid droplets or released into the circulation as very low-density lipoproteins (VLDLs) from the liver or as chylomicrons from the intestine. Lipid droplets consist of a core of triglyceride and/or cholesterol esters surrounded by a surface monolayer of phospholipids and proteins (reviewed in Murphy and Vance, 1999). Adipocytes, the major cellular energy store in the body, contain large (10-100μm diameter) and small (2-10 μm) cytoplasmic lipid droplets which contain a surface-bound acylated protein, adipose differentiation related protein (ADRP) (Jiang, *et al.* 1992). It is now known that ADRP is widely distributed and most, if not all, animal tissues contain cells that accumulate lipids for short-term storage with particularly high levels of ADRP expression found in the lung, *brain*, testes, and mammary gland (Brasaemle, *et al.* 1997)

Despite the ongoing debate on the relative contribution of lipid-sources to the ^{1}H-NMR observed resonances, the observation that transformed cells in

culture take up large amounts of exogenously supplied fatty acid and form intracellular lipid droplets is itself very intriguing. Thus, determining how these cells use the lipid (i.e., incorporation into membrane, cellular energy, or intermediary metabolite synthesis) could provide important evidence for determining which cellular activity is dominant in its contribution to transformed and NMR-observed metabolism *in vivo*. Astrocytomas in particular, which are (1) derived from a normal cell-type capable of β-oxidation in developing brain and (2) the only human CNS tumour type (apart from a few cases in human PNETs) known to display ^{1}H-NMR mobile lipid resonances, may utilise fatty acids for specific metabolic purposes that could be targeted as a tumour therapy.

Given all of the evidence collected thus far concerning the formation of lipid droplets in tumour cells, there are two opposing hypotheses which could explain its cause and/or use:

(1) lipid droplets form because of transformation-related dysfunction in either fatty acid transport into the mitochondria or in mitochondrial β-oxidation

(2) lipid droplets form as a substrate store in an adaptive response by transformed cells to increased proliferation and/or energy demands in which a de-differentiated metabolism based on the utilisation of fatty acids for both energy and membrane synthesis is active (i.e., when exogenous fatty acid concentrations exceed the maximum rate for utilisation by the cells, excess fatty acid is stored as lipid droplets until required).

Since Warburg (1930) first suggested that the high rates of aerobic glycolysis seen in tumour cells were due to an impaired respiration, much research has been done on the respiratory properties of tumour cells and their mitochondria. Studies on isolated mitochondria do not, however, support the theory of a generalised impairment of the mitochondrial electron transport chain. Some tumour mitochondria may respire poorly on a particular substrate but the same mitochondria may respire normally with another substrate (Pedersen, 1978). Thus, in order for hypothesis (1) to be assessed it should be demonstrated whether or not the tumour-types that form lipid droplets readily utilise fatty acids as an oxidisable fuel.

Normal rat astrocytes from developing brain have been shown to increase their rate of fatty acid oxidation with increasing exogenous fatty acid concentrations and to reach their maximum rate of oxidation at 200μM of

substrate (Murphy, *et al.* 1992; Esfandiori, *et al.* 1997). Lipid droplets have been shown to form in transformed cell cultures at similarly high exogenous fatty acid concentrations (Callies *et al.*, 1993). Although it has not been reported in astrocytes, if hypothesis (2) is correct, lipid droplets may also form in normal astrocytes from developing brain if exogenous fatty acids in culture or *in vivo* were to exceed the concentration at which their maximum rate of oxidation is achieved (i.e., 200 μM). Based on the established ability of astrocytes from developing rat brain to utilise fatty acids as an energy substrate and the fact that embryonic cells have also been shown to form cytoplasmic lipid droplets, I hypothesise that tumours of the astrocytic lineage in particular may also have this ability. To my knowledge, the ability of astrocytomas to β-oxidise fatty acids and its relative contribution to cellular energy, intermediary metabolism, and membrane synthesis have not been established. Therefore, following the metabolism of labeled fatty acids by high-grade astrocytoma cells may provide important clues to: (1) whether it is a mitochondrial dysfunction or a fetal-like metabolism which causes lipid droplet formation and based on this information (2) whether cytoplasmic lipid accumulation, membrane lipid changes, or necrosis is most likely dominant in contributing to the NMR-observable signal.

1.10 Membrane Phospholipids

Several *in vivo* studies on normal rat brain have shown that intravenously administered ^{14}C palmitate rapidly labels phospholipids (mainly phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEth) (Dhopeshwarkar, *et al.*, 1969, 1971, 1973; Sun and Horrocks, 1971, 1973; Arduini *et al.*, 1994). Miller, *et al.*, 1987, have shown that 50% of plasma ^{14}C palmitate is incorporated into brain lipid and protein. There are a large number of studies that have shown the phospholipid composition and the fatty acid composition of phospholipids differ between brain tumours and normal brain. CNS tumours frequently contained decreased long-chain PUFA, increased linoleic and arachidonic acid, decreased PtdEth and PtdSer, and increased PtdCho (Sun and Leung, 1974; Hattori, *et al.* 1987; Montaudon, *et al.* 1981). Merchant *et al.*, (1994) showed GM tumour biopsy samples had significantly decreased sphingomyelin, phosphatidic acid, and PtdEth and increased PtdCho relative to other intracranial tumours.

Membrane phospholipids from normal brain (with the exception of myelin) are known to contain larger amounts of PUFA than membrane phospholipids from other organs (Clausen, 1969). Robert *et al.* (1978 &1983) demonstrated that an increase in the PUFA content of rat astrocyte, rat C6-glioma, and neuroblastoma membrane phospholipids corresponded to an increase in the fluidity of the cell membrane. In all cell types, the incorporation of the radioactive fatty acids into cell membrane was lower for saturated or monounsaturated fatty acids than for PUFA. Furthermore, the low rate of PUFA oxidation is thought to be due to the low activity of CPT towards PUFA-CoA (Gavino and Gavino, 1991). Thus, the observation that higher amounts of PUFA are incorporated into membrane in fetal and transformed cells than normal adult cells of the same lineage (Yavin and Menkes, 1974; Daniel, *et al.* 1980; Robert, *et al.* 1983) may reflect the low oxidation rate and the advantages of sequestration of these potentially inhibitory and more 'fluid' fatty acids into the membrane.

1.10.1 Phospholipid Metabolism

Phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEth) are the major phospholipids in mammalian cell membranes, representing 40-50% and 20-35% of total phospholipids, respectively (White, *et al.* 1973). The metabolites of membrane PtdCho and PtdEth synthesis and breakdown have been shown to be important markers for distinguishing tumour from normal tissue and monitoring therapy response using *in vivo* NMR spectroscopy (Negandank, 1992; de Certaines, 1996). There are two main phospholipid-related peaks in ^{31}P-NMR spectra due to the phosphomonoesters (PME) and phosphodiesters (PDE) (Evanochoko, *et al.* 1982 & 1983, Ng, *et al.* 1982; Griffiths, *et al.* 1982). The PME region of the spectrum was later found to contain mainly phosphoethanolamine (PE) and phosphocholine (PC) with a small contribution from sugar phosphates (Evanochko *et al.*, 1984a &1984b). The PDE region contains phospholipid catabolites such as glycerophosphocholine (GPC) and glycerophosphoethanolamine (GPE) with mobile membrane macromolecules (including phospholipid headgroups) also making a contribution (Cerdan, *et al.* 1986; Burt, *et al.*, 1979; Murphy, *et al.* 1989; Bates, *et al.* 1989). Cell stimulation by either growth factors, hormones, or tumour promotors are known to activate key synthesis enzymes with consequent enhancement of PC and PtdCho (Negandank, 1992). However, there is also evidence for elevations

in PC due to PtdCho breakdown by phospholipase C and/or D in a number of cells following agonist stimulation or oncogene trasformation (Billah & Anthes, 1990; Exton, 1990). Thus, high PME may reflect a demand for increased phospholipid synthesis for making new membranes for dividing cells, phospholipid hydrolysis for signal transduction by activating protein kinase C, or changes to cell membrane composition involved in invasion, metastasis, or receptor expression (reviewed in Negandank, 1992). Receptor-mediated signal transduction may involve activation of phospholipase-A2 to release the second messenger arachidonic acid or activation of phospholipase-C to release diacylglycerol and inositol 1,4,5-triphosphate, which ultimately leads to proliferation of the activated cells. However, the changes in membrane phospholipids and the quantities of lipid present in lipid droplets in transformed cells are generally in significant excess of the amount required for cell signalling purposes.

A large body of research has reported an increase in PME metabolites in a variety of neoplasms of the CNS, lung, breast, skin, liver, and bone (for reviews see Ruiz-Cabello, 1992; Negendank, 1992; de Certaines, *et al.* 1993). Changes in PME and PDE signal intensities and concentrations have been found to exhibit significant alterations during tumour growth, after effective anti-tumour therapy, and during cell differentiation or maturation. The concentrations of the individual phospholipid metabolites detected vary a great deal according to tumour type, grade, and location. Despite the large variations observed in the concentrations of the phospholipid metabolites, there is strong evidence of a correlation between cellular growth and increased PME and decreased PDE levels in gliomas although this does not hold true for all tumour types, nor is it a unique property of transformed cells (Cadoux-Hudson *et al.*, 1989; Arnold *et al.*, 1991; Ng *et al.*, 1982). These changes in cellular phospholipid metabolism are also seen in other highly proliferative tissues such as neonatal liver (Iles, *et al.* 1990; Murphy, *et al.* 1992), neonatal brain (Williams, *et al.* 1989; Younkin, *et al.* 1984; Peden, *et al.* 1990), and stimulated lymphocytes (Dingley, *et al.* 1992). However, the correlation between increased PME (or ratio of PE to PC) and cellular growth is not absolute. For example, the regenerating liver, which is known to have a large proportion of rapidly proliferating cells, has PME and PDE levels similar to those seen in normal liver (Morikawa *et al.*, 1992). Additionally, Bhakoo, *et al.* (1996) demonstrated that changes in phospholipid metabolites in

conditionally immortalised and transformed Schwann cells (grown in identical culture conditions) were not related to rates of cell division but directly to the activation of specific transforming oncogenes in these cells.

1.10.1.1 *In vivo* observations

There are two important considerations to be made when considering the changes observed in PME and PDE resonances *in vivo*. Firstly, the data is not usually quantitative and metabolite ratios can sometimes be misleading since no particular metabolite is known to be stable in all tissue and tumour types. For example, ratios with total creatine are fairly common but this is known to be significantly decreased in human gliomas (Florian *et al.* 1996). Thus, the PME/Cr ratio in gliomas could indicate a potentially erroneous increase in PME. Other common ratios made with nucleotides (ATP, NTP), which have been shown to be elevated in gliomas (de Certaines, 1993), could potentially produce the opposite error in PME estimation. Furthermore, *in vivo* ^{31}P NMR of human tumours is not commonly proton-decoupled. Therefore, the individual components of the PME and PDE resonances are not resolved. Dramatic increases in PC with concomitant decreases in PE would not be recognised since the total PME would stay the same. Changes seen in PME and PDE *in vivo* could also be due to changes in several phospholipid metabolites or just one metabolite but this could not be detected. *In vivo* ^{1}H-NMR commonly detects a single resonance from the choline-containing compounds (with some contribution also from PE, taurine, and *myo*-inositol). Thus, changes in this resonance are also difficult to interpret without resolution of the individual components.

In vivo ^{31}P metabolite ratios from human gliomas do not differ from normal brain consistently but a large proportion of them do have high PME, low PDE, and low pH (Negendank, 1992; de Certaines, 1993). The variability in the phospholipid metabolites observed in these tumours is thought to be due to the large cellular heterogeneity and the amount of necrosis, hemorrhage, and edema seen in intact gliomas. Thus, given the difficulties in resolving the individual components of the PME and PDE peaks from *in vivo* ^{31}P- & ^{1}H-NMR, studies of biopsy and cell culture extracts have been useful in identifying the changes in these components. However, *in vitro* studies have their own limitations due to the high sensitivity of PME and PDE compounds to

experimental conditions such as temperature, pH, growth phase, and media composition.

1.10.1.2 *In vitro* observations

Extract studies of biopsy specimens have been useful in demonstrating the usefulness of *in vitro* analysis by showing quantitative increases in PME compared to normal tissue. Usensius, *et al.* (1994) showed that although there was no significant difference between normal white matter and extracted human astrocytoma tissue (grades I-IV) for the total choline-containing compounds, the concentration of the PC component was almost two-fold higher in grade IV astrocytomas. Gillies, *et al.* (1994) found rat C6 and 9L gliomas to have lower PME levels compared to other tumours in the literature (Negandank, 1992) but they also demonstrated that cultured cells can not synthesise choline or ethanolamine *de novo* and therefore culture medium concentrations of these precursor metabolites would have a significant effect on PME levels. They further observed that in identical culture conditions PC is elevated during log-phase growth but PC decreases and PE increases upon cessation of cell growth. It should be noted that Bhakoo, *et al.* (1996) have shown that elevated PC can be induced through transformation unrelated to cell growth in rat Schwann cells. Thus, different experimental conditions may lead to changes in PME metabolite concentrations.

Studies using human tumour cell lines have demonstrated significant differences in phospholipid metabolites occurring between cultured cells and *in vivo* xenografts (Hull et al, 1990, 1992; Franks et al, 1996). In particular, Franks, *et al.* (1996) found the largest difference in tumours of various cell lines was high PE and low PDE *in vivo* and little or no PE *in vitro*. This study also showed that changes in the concentration of specific media nutrients such as glucose and choline significantly altered the metabolic profiles and intracelluar PC in particular. Ethanolamine supplementation also reduced PC while increasing PE and GPE. When choline and ethanolamine supplements were adjusted to obtain a PE/PC ratio in the cultured cells resembling what is seen *in vivo*, this also resulted in higher than normal PDE levels. Changes in extracellular pH can also influence phospholipid metabolism in several cell lines. Cell adhesion, growth rate, and PC levels have been shown to decrease with decreasing pH while PE and GPC increase (Kuesel *et al.*, 1990). Media supplementation with

monounsaturated fatty acids has also been shown to directly influence phospholipid metabolism. Gillham & Brindle (1996) supplemented oleic and linoleic acid to the culture medium of three mammalian cell lines and found PE and GPC/NTP to be elevated. These changes are most likely due to fatty acid-induced activation of phospholipases, although the media composition with choline present and ethanolamine absent may have contributed to the observed trend. It is suggested that increased PME and PDE commonly observed *in vivo* in tumours may, therefore, be due to the uptake of plasma fatty acids and subsequent activation of membrane phospholipases. This could explain why Franks, *et al.* (1996) found high PE *in vivo* but little or no PE *in vitro* from the same tumour cells since standard culture media does not contain the high fatty acid concentrations seen in plasma from tumour-bearing patients. However, the fact that oleic and linoleic acid, which are thought to be stimulatory to cell growth, produced elevated PE, which is generally associated with decreased growth rates, appears to be a contradictory observation.

Thus, although PME and PDE increases are generally considered to be an indicator of tumour progression and there are many tumour-related factors that can lead to their accumulation, phospholipid metabolism in tumours is not fully understood and correlations with growth rates are not consistent.

1.11 Aims of the thesis

Thus, this thesis has the following aims:

Aim I: *To determine whether the metabolic profile for human astrocytomas established by ^{1}H-NMR spectroscopy of cell culture extracts is an accurate reflection of in vivo metabolism in the transformed cells.*

Hypothesis: The ^{1}H-NMR metabolic profiles established from cell culture extracts of several human CNS tumour-types are in good agreement with *in vivo* clinical data on the same tumour types. However, due to the lower sensitivity and resolution available from clinical NMR spectroscopy compared to high resolution spectroscopy performed on extracts this agreement can only be ascertained for a limited number of metabolites currently detectable *in vivo.* Therefore, extracts of tumour cells grown *in vivo* compared to extracts of the same cells grown in culture will allow the identification of any differences in a greater number of

metabolites. Since the clinical data is in good agreement with extracts so far, I hypothesised that this would hold true for other metabolic markers detected in the extracts.

To test this hypothesis: Primary cultures of human astrocytoma cells were implanted into the caudate nucleus of immuno-deficient rats and the resulting *in vivo* tumour excised and extracted. The same primary astrocytoma cultures from which the *in vivo* tumours were obtained were also extracted but without implantation into the brain. ^{1}H-NMR spectroscopy of the aqueous metabolites and mass spectrometry of membrane phospholipids were compared to determine whether the metabolic profiles of cells grown *in vivo* differ significantly from the same cells grown in monolayer culture.

Aim II: *To determine whether the characteristic metabolite profiles of astrocytomas are a reflection of their cell type of origin or of an altered metabolism due to the process of transformation.*

Hypothesis: The ^{1}H-NMR metabolite markers that distinguish one tumour type from another may be due to transformation or to their lineage of origin since normal CNS cells can also be distinguished based on lineage-specific metabolic markers. Although the cellular composition of a tumour is heterogeneous (including connective tissues and blood vessels), the main constituent of the mass of cells may originate from a particular dominant lineage that can be identified by ^{1}H-NMR on the basis of lineage or transformation specific changes in cellular metabolism. Some general metabolic characteristics of transformed cells should also be evident in ^{1}H-NMR tumour-profiles, such as high lactate concentrations. Thus, I hypothesized that there may be several metabolic characteristics of astrocytomas related to the normal metabolism of the astrocytic lineage but that comparison to normal astrocytes of the same species would also identify metabolic changes in the astrocytomas related specificially to their transformation.

To test this hypothesis: Primary human astrocyte and astrocytoma cultures were grown in identical conditions and extracted for analysis by ^{1}H-NMR spectroscopy and mass spectrometry. Metabolic profiles for the aqueous metabolites and membrane phospholipids were compared between the normal and transformed human cells.

Aim III: *To determine how lipid resonances commonly detected by in vivo ^{1}H-NMR in malignant astrocytomas and the associated formation of cytosolic lipid droplets may relate to fatty acid metabolism in these high-grade cells.*

Hypothesis: Astrocytoma cells of high grade may have reverted to or re-activated metabolism from an earlier stage of development in which fatty acid metabolism is an important part of maintaining energy and metabolic precursors for high rates of proliferation and mobility. Since astrocytes from developing brain are the only CNS cells capable of fatty acid β-oxidation and the only primary CNS tumours known to have *in vivo* ^{1}H-NMR lipid signals are astrocytomas and tumours of early embryonic brain stem-cell origin, I hypothesised that high grade astrocytomas (glioblastoma multiforme) are metabolising fatty acids like their developmental counterparts for cellular energy and membrane synthesis. Therefore, lipid droplets that have been shown to form *in vivo* may be used as an energy substrate store when fatty acid uptake (which is dependent on extracellular fatty acid concentration) is greater than maximum (developmental-like) rates of oxidation.

Alternative hypothesis: The other possibility, however, is that lipid droplets form because of a dysfunction (rather than a reactivating of developmental metabolism) in either the transport of fatty acid into the mitochondria or in fatty acid β-oxidation by the astrocytoma cells.

To test these hypotheses: The following experiments were performed to determine whether human astrocytomas are capable of oxidising fatty acids when presented at concentrations known to induce lipid droplets in other cultured cell-types and the extent to which the fatty acids contribute to (i) cellular energy, (ii) the generation of intermediary metabolic products, and (iii) cell membrane synthesis:

(1) β-oxidation flux of [1-^{14}C]oleic and palmitic acid was measured in high-grade astrocytoma cultures
(2) The extent to which oleic acid oxidation can contribute to cellular ATP synthesis under normal and alternative-substrate deprivation culture conditions was determined in high-grade astrocytoma cultures

(3) The quantity and pattern of exogenous [1-^{13}C]oleic acid incorporation into cell membrane phospholipids and into specific intracellular and extracellular aqueous products was determined in high-grade astrocytoma cultures.

(4) The extent to which the utilisation of [1-^{13}C]oleic acid by high-grade astrocytoma cultures is altered by the presence or absence of other energy substrates was measured.

(5) The effects of oleic acid metabolism in high-grade astrocytoma cultures on intermediary metabolism.

From these studies it is hoped that a more complete picture will emerge of the metabolic effects of transformation on the astrocytic lineage in human cells and the ability of NMR to non-invasively identify these tumour-specific characteristics for the diagnosis of astrocytic tumours and their grade.

Chapter 2: **Materials and Methods**

2.1 Tissue Culture

Purified populations of rat astrocytes, human astrocytes, and human astrocytomas (grade IV, glioblastoma multiforme) were subcultured in standard tissue culture conditions. Cells were cultured in sterile conditions, in tissue culture flasks (Nunc, filter-top) maintained in a 37°C incubator with humidified atmosphere containing 7.5% CO_2. Cells were subcultured an average of six times in order to obtain the number (10^7-10^8 cells) necessary for high-resolution NMR spectroscopy. An inverted microscope (Carl Zeiss, Oberkochen, Germany) magnification x10-x40) was used for cell observation and counting. Cell counting was carried out on a Neubauer modified haemocytometer. Primary astrocytoma (grade IV) cells were provided by the Institute of Neurology, London. Human astrocytes were obtained from Biowhittaker, UK. Primary rat astrocyte cultures were obtained from rat brain following the method of Wolswijk and Noble, 1989. Immunocytochemical characterisation of parallel cell cultures were performed on cells plated onto glass coverslips, using antibodies and staining techniques appropriate to the cell type as described previously (Noble *et al.*, 1984; Wolswijk and Noble, 1989).

2.1.1 Astrocytomas

Cultures were grown from a stock (bank) of human tumour cells provided by the Institute of Neurology. The cells were derived from surgically resected human grade IV astrocytomas. The starting passage number (P) of the cells studied ranged from P2-P6 but the majority of cells were P2. The diagnosis of the astrocytoma and degree of malignancy was obtained by the Institute of Neurology by histological analysis of the tissue. The tumour bulk had been subjected to enzymatic digestion and a cell suspension was obtained and stored cryogenically.

2.1.2 Preparation of Primary Rat Astrocyte Cultures

Enriched astrocytes were prepared by a modification of the method of Noble and Murray (1984). Cortices from 7 day-old rat pups were removed and dissected free of meninges, chopped finely, and incubated at 37°C for 45 minutes in L-15 medium containing 400 units/ml of collagenase. To this

incubation 6000 units/ml of trypsin were added and reaction allowed for a further 15 minutes. The cell suspension was centrifuged at 200G for 5 minutes. The pellet was suspended in EDTA solution (200 μg/ml solution of EDTA (Sigma UK)) in Ca^{2+}/Mg^{2+}-free media containing 8500 units/ml trypsin and incubated at 37°C for 15 minutes. DNase was added at a ratio of 4 ml for every 7 ml of cortical cell suspension and incubated for a further 5 minutes before being centrifuged at 200G for 5 minutes. The tissue was suspended in DMEM-FCS containing 10% FCS, 4mM glutamine, and 25 μg/ml gentamycin and dissociated by repeated tituration through 21- and 25-gauge needles. The dissociated cells were centrifuged at 200G for 10 minutes. The cells were suspended in subculture media (see 2.1.4.1), and seeded into poly-L-lysine-coated flasks at a density of 10^6 cells/175 cm^2 flask. The cultures were grown for 6 days, and cells on the top of the monolayer were removed by vigorous shaking in serum-free medium. After 24 hours the cells were pulsed with 2 x 10^{-5} M cytosine arabinoside for 4 days. This procedure routinely produced astrocyte cultures of >95% purity as assessed by staining with a polyclonal antiserum against glial fibrillary acidic protein (GFAP).

2.1.3 Preparation and culture of human astrocytes and astrocytomas

Human astrocytes from Biowhittaker, UK and astrocytomas from the Institute of Neurology, London were obtained as cell suspensions (approximately 10,000 cells per sample) frozen in hibernation media containing 5% DMSO. Cells were thawed, suspended in 10ml Dulbecco's Modified Eagle Medium (DMEM), and centrifuged at 200G for 5 minutes. The cells were then re-suspended in fresh media and plated on 75 cm^2 flasks. 75% media changes were carried out every other day. Cells were continuously passaged at confluence until sufficient cell numbers were obtained for analysis.

2.1.4 Cell Media Compositions

Rat and human astrocytes and human astrocytomas were maintained in DMEM (Gibco, UK) containing 10% fetal calf serum (FCS) (Shapiro and Schrier, 1973; Vernadakis, *et al.*, 1984). In this media the normal cells undergo spontaneous morphological and biochemical maturation very similar to the *in vivo* situation (Holopainen, *et al.*, 1986; Schousboe, 1980; Hansson, *et al.*, 1982). The human astrocytomas are also maintained at a relatively high growth

rate through many passages in this media without differentiation. However, based on immunostaining GFAP positive cells may be selected over several passages (Florian *et al.*, 1996). Another modification to cell media composition that was used with the human and rat astrocyte cultures is astrocyte-conditioned media (ACM). When "used" media is taken off cultures of astrocytes and replaced with new media ACM is made by centrifuging, filtering, and supplementing the old media with 2 mM glutamine before being used again with new media (25% ACM + 75% new media).

A deficient media composition was also used to study the effects of metabolic deprivation and utilisation of alternative substrates on the astrocytoma cultures. DMEM medium deficient in glucose and amino acids was obtained from Life Technologies (Gibco), UK. The deficient media used in these studies was deficient in glucose, glutamine, methionine, cystine, arginine, leucine, and inositol. Fetal calf serum (10%) and 2-deoxyglucose were supplemented into the medium. The 2-deoxyglucose was used to inhibit glycolysis as a source of ATP from serum-derived carbohydrate. At low concentrations 2-deoxyglucose is routinely used to measure glycolytic rates because it competes with glucose on the same uptake mechanism and the rate of phosphorylation can be measured. However, at high concentrations, such as that used in the deficient media, the 2-deoxyglucose can be toxic to the cells because of the high dependence on glycolysis for energy production in cultured tumour cells. This "toxic" deprivation media was used to assess the ability of fatty acid oxidation to substitute for glycolysis in maintaining viable ATP levels in astrocytomas. Preliminary experiments determined that the deficient medium with 2-deoxyglucose is toxic to astrocytoma cultures even in the presence of fatty acids, unless carnitine was also available in the media. Carnitine is an important molecule responsible for the transport of fatty acids across the mitochondrial inner membrane where β-oxidation occurs. The rate of fatty acid oxidation in the presence of a range of carnitine concentrations was also determined using ^{14}C-fatty acids (Chapter 7). The highest concentration of carnitine (3mM) produced the highest rate of oxidation and was therefore used in the deficient media with ^{13}C-oleic acid for the study in Chapter 8.

Fetal calf serum (10%) was used even in the deficient media because it contains many important growth factors for the cells and it is believed that the absence of these factors may cause normal cell metabolism to shut down.

Therefore, even if the cells are capable of using alternative substrates in the media, their normal metabolic capacity may be compromised from lack of serum factors. The high concentration of 2-deoxyglucose used in the deficient media should be sufficient to essentially block glycolysis of serum-derived glucose. This was confirmed by the fact that most of the cells incubated in the deficient medium (with 10% FCS and 2-deoxyglucose) were dead (confirmed by a lack of trypan-blue dye exclusion) following 24 and 48 hour incubations (Chapter 7). Thus, fetal calf serum supplementation does not provide adequate energy substrate for cell survival in the deficient media and therefore should not interfere with our studies of alternative substrate utilisation in this media. The serum also contains varying concentrations (by batch) of fatty acids. Although I did not determine the concentration of fatty acid present in the batch of fetal calf serum used in my studies, the contribution of the serum fatty acid to cell energy supplies was not sufficient for keeping the cells alive in the deficient media and is probably negligible compared to the high concentrations (400 μM) of the supplemented fatty acids. Furthermore, the same batch of serum was used for all of the cell culture experiments in this thesis.

2.1.4.1 Subculture Media

Dulbecco's modified Eagle's medium containing 1g/L glucose (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS; Imperial Laboratories, UK), 4 mM glutamine (Sigma), and 25μg/ml gentamicin (Gibco) was the standard media composition used for the subculture of the astrocyte and astrocytoma cells. A 75% media renewal was carried out every two days for all cultures. The new media contained 25% ACM in the rodent and human astrocyte cultures.

COMPONENT	**DMEM** (mg/L)	**DEFICIENT DMEM** (mg/L)
Inorganic salts:		
$CaCl_2$ *$2H_2O$	264.00	264.00
$Fe(NO_3)$ *$9H_2O$	0.10	0.10
KCl	400.00	400.00
MgSO4 *7H2O	200.00	200.00
NaCl	6400.00	6400.00
$NaHCO_3$	3700.00	3700.00
NaH_2PO_4 *$2H_2O$	141.00	---
Amino Acids:		
L-arginine	84.00	---
L-cystine	48.00	---
L-glutamine	580.00	---
Glycine	30.00	30.00
L-histidine	42.00	42.00
L-isoleucine	105.00	105.00
L-leucine	105.00	---
L-lysine	146.00	146.00
L-methionine	30.00	---
L-phenylalanine	66.00	66.00
L-serine	42.00	42.00
L-threonine	95.00	95.00
L-tryptophan	16.00	16.00
L-tyrosine	72.00	72.00
L-valine	94.00	94.00
Vitamins:		
D-Ca pantothenate	4.00	4.00
Choline chloride	4.00	4.00
Folic acid	4.00	4.00
L-inositol	7.20	---
Nicotinamide	4.00	4.00
Pyridoxal HCl	4.00	4.00
Riboflavin	0.40	0.40
Thiamine HCl	4.00	4.00
Other:		
D-glucose	1000.00	---
Phenol Red	15.00	15.00

Table 2-1. **Media compositions for DMEM and deficient DMEM used in these studies.** *Information from Gibco, Life Technologies, UK.*

COMPONENT	AVERAGE **FCS** CONTENT	UNITS
pH	7.07	
Osmolality	315.82	mosm/kg
Total protein	4.08	g/dL
Endotoxin	1.20	EU/ml
Albumin	2.52	g/dL
Bicarbonate	10.00	meq/L
Calcium	14.32	mg/dL
Chloride	97.77	meq/L
Cholesterol	31.21	mg/dL
Creatine	2.77	mg/dL
Globulin:		
Alpha	1.19	g/dL
Beta	0.38	g/dL
Gamma	0.09	g/dL
L-glucose	102.32	mg/dL
High density lipoproteins	10.82	mg/dL
Low density lipoproteins	30.19	mg/dL
Inorganic phosphorus	9.65	mg/dL
Potassium	12.22	meq/L
Sodium	133.59	meq/L
Transferrin	190.22	mg/dL
Triglyceride	65.91	mg/dL
Uric acid	2.22	mg/dL
Hemoglobin	13.11	mg/dL
Lactate dehydrogenase	637.77	mg/dL
Vitronectin	41.50	ug/dL
Cortisol	0.82	ug/dL
Estradiol	18.67	pg/dL
FSH	51.10	ng/dL
Growth hormones	180.80	ng/dL
Insulin	5.96	IU/ml
Luteinizing hormone	0.71	ng/dL
Progesterone	0.08	ng/dL
Prolactin	14.04	ng/dL
Prostaglandin E	0.60	pg/dL
Prostaglandin F	1.85	pg/dL
Testosterone	0.10	ng/dL
TSH	0.83	uIU/ml
Thyroxine	15.18	ug/dL
Total iron binding capacity	264.95	g/dL

Table 2-2. **Typical fetal calf serum composition**. *Gibco, UK.*

2.1.4.2 Metabolic studies 48-hour incubation media

A. Deficient Medium (DefDMEM):

DMEM without amino acids, glucose, or glutamine supplemented with 10% FCS and 25μg/ml gentamicin (Gibco).

Glycolysis blocked by addition of 6mM 2-deoxyglucose

B. Oleic Acid supplement to DMEM and DefDMEM (DOA & DDOA):

400 μM oleic acid was complexed with 30 μM fatty acid-free bovine serum albumin (BSA). NMR studies used [1-^{13}C] labeled oleic acid (Goss, UK); ATP measurements used an unlabeled oleic acid-BSA solution (Sigma, UK); radiolabeled studies use [1-^{14}C] oleic acid and palmitic acid + BSA (New England Nuclear, USA). The preparation of the fatty acid-albumin complex was as follows: 30 μM BSA was prepared in distilled water, the fatty acid was first solubilised in ethanol (1/10, v/v) and then added to water (1/1, v/v). The pH of this solution was neutralised with NaOH before being added to the BSA solution. The final solution was filtered for sterility before use.

C. Carnitine supplemented:

1-3 mM L-carnitine (Sigma, UK) was added to fatty acid-supplemented medium; (^{14}C-palmitate studies (Chapter 7) determined that maximal β-oxidation occurred at 3 mM)

2.1.5 Subculture (passage) of cells

Both the astrocyte and astrocytoma cultures are anchorage-dependent and were grown as monolayer cultures. Frozen cells (human astrocytes and astrocytomas) were initially plated in 75 cm^3 tissue culture flasks (Nunc, UK) and grown to confluence and rat astrocytes were seeded in 175 cm^3 flasks. The first passage was used to freeze one half of the cells and the remaining half were grown to confluence again. The cells were then passaged to 175 cm^3 flasks and subcultured 3-4 times. The cells in the flasks were then transferred to tissue culture plates (175 cm^3, Falcon) at four plates per flask and grown to confluence. Eight plates were used per NMR/mass spectrometry sample comprising approximately 10^{10} - 10^{12} cells. Instead of using large plates, the cells for the

HPLC (ATP & glutathione) and ^{14}C-radiolabeled studies were grown in 6-well plates (Falcon, UK) comprising approximately 10^5 cells per well.

2.1.5.1 Trypsinisation

Cells were detached from the culture flasks for passaging through the enzymatic procedure, trypsinisation. Culture medium was removed from the flasks containing the cell cultures and then rinsed twice with 10 ml of versine (Mg^{2+}-, Ca^{2+}-, FCS –free medium; ICRF, London). Flasks were then rinsed with a trypsin solution (2.5 mg/ml bovine pancreas, type III trypsin (Sigma, UK) in versine). After the trypsin solution was removed the cultures were left for 5 minutes during which time most of the cells detached from the flasks. The flasks were tapped vigorously to detach any remaining cells. The detached cells were collected in a corner of the flask by rinsing with 10 ml of DMEM + 10% FCS which also inactivates the trypsin. The cells were taken up by sterile serological pipette and placed into 15 ml polypropylene centrifuge tubes (Falcon, UK) and centrifuged at 1000 RPM for 5 minutes.

2.1.5.2 Re-plating (re-seeding) Cells

After centrifugation the supernatant was removed and discarded. The cell pellet was then re-suspended in 5ml fresh medium (in order to calculate the number of cells in the pellet). The number of cells was calculated after counting viable cells on a hemocytometer. A further volume of cell medium was then added to obtain a suspension of approximately 10^6 cells/ml. The pellet was dissociated to a single cell suspension by triturating repeatedly through a 2ml serological pipette. The suspension was then added to the appropriate tissue culture flask(s) containing cell media and returned to the incubator. Cells that were frozen were re-suspended in freezing media which contains 5% DMSO (Gibco, UK) instead of normal DMEM medium and placed in a –20°C freezer for 2 hours, then in a –80°C freezer overnight, and finally stored in liquid nitrogen.

2.1.6 Harvesting Cells

Confluent plates of cells were harvested for extraction after either a 48-hour incubation in the culture medium for the appropriate study or at least 24 hours after feeding normal subculture medium. Plates were rinsed 3 times with 25ml of phosphate buffered saline (PBS). After the final rinse the cells were

mechanically scraped to the bottom of the plate, taken up by pipette and placed into micro-centrifuge tubes. The cells were then centrifuged at 13,000 RPM for 20 seconds, the remaining PBS supernatant removed, and the cell pellet placed immediately into liquid nitrogen.

2.2 Indirect Immunofluorescence Staining Protocol

Immunostaining was performed according to the methods of Wolswijk and Noble, *et al.*, (1989). Cells were plated on glass microscope slide coverslips overnight. The next day the cells were fixed with either ice-cold methanol (for GFAP, fibronectin, or vimentin) or 4% paraformaldehyde (for GalC) and then treated with a primary unconjugated monoclonal or polyclonal antibody (anti-human GFAP, fibronectin, vimentin, or GalC) followed by a second antibody conjugated to fluorescein or rhodamine. After immunolabeling the coverslips were washed and mounted face down onto a drop of anti-fade solution on glass slides. Stained cells were then analysed by immunofluorescence microscopy using a Zeiss Universal microsope with phase contrast and rhodamine and fluorescein optics.

2.3 Brain Funnel Freezing

Adult male Sprague-Dawley rats (n=21) were anaesthetised with a halothane/N_2O/O_2 mixture (induced on 4% halothane, 1.5 % during surgery, and 1% during funnel freezing). Brain tissue was frozen *in situ* by pouring liquid nitrogen through a funnel directly onto the exposed skull of the rat (Lust, *et al.*, 1989). The brain tissue was removed intact by chiseling away skin and bone with the brain submerged in a reservoir of liquid nitrogen to prevent thawing.

2.4 Tumour cell implantation

Human astrocytomas were implanted into the brains of 40g *rnu-rnu* nude rats supplied by Harlan, UK based on the methods of Bernsen, *et al.*, (1992) with some modification. Approximately 100,000 cells in a 2µl of cell media were injected into the left caudate nucleus of anesthetised rats directly through the skin and skull. Stereotactic coordinates for injections were determined using zero ear bars and pilot measurements based on dye injections through the skin and skull on identical weight rats in separate terminal experiments. Rats with tumour cell transplantations were allowed to recover and tumour growth was followed by

weekly *in vivo* NMR imaging. Rats with tumour growth were kept for approximately 3 weeks post-transplantation and killed by anaesthesia (isoflourane) overdose. The tumour and a similar size sample of normal contralateral brain tissue were rapidly excised by blunt dissection and immediately frozen in liquid nitrogen prior to extraction.

2.5 Tissue and Cell Extraction

Frozen tumour and brain tissues were kept under liquid nitrogen and ground to a fine powder with a mortar and pestle. Frozen cells were kept in liquid nitrogen until ice-cold solvents were added. The cell-solvent mixture was sonicated until thoroughly broken up.

Methanol-Chloroform-Water (M/C) extraction: Cold, reagent-grade methanol and chloroform (Sigma, UK) in a ratio of 2:1 (3 ml/g tissue or 250 μl/cell pellet) were added to frozen, ground tissue and to the frozen cell pellets. The tissue-solvent mixture was allowed to thaw and was transferred into Teflon centrifuge tubes (Oakridge). The cell pellet-solvent mixture was sonicated. After approximately 15 minutes in contact with the first solvents, chloroform and distilled water were added to the samples in a ratio of 1:1 (1ml/g tissue each or 250 μl/cell pellet) to form an emulsion. The samples were then centrifuged at 13,000 rpm for 20 minutes. The upper phase (methanol and water) was separated from the lower (organic) phase using glass syringes and the protein pellet was retained for further analysis. The two phases were dried at room temperature under a stream of nitrogen gas. The protein pellets from the tissue extract comparison (Chapter 3) were re-extracted and dried separately. The pellets from the cells, tumour tissue, and normal contralateral brain extractions were re-extracted once and the separated fractions were pooled with the original extraction fractions before drying.

Perchloric Acid (PCA) extraction: Cold 12% perchloric acid (3ml/g tissue or 250 μl/pellet) was added to the ground tissue under liquid nitrogen or to the frozen cell pellet. The tissue-solvent mixture was allowed to thaw and was transferred into centrifuge tubes. The cell pellet-solvent mixture was sonicated. The samples were then centrifuged at 13,000 RPM for 20 minutes. The supernatant was removed and the pellet was re-extracted using either M/C (tissue) or PCA (cells). The first and second PCA supernatants of the cell extracts were pooled and neutralised with 1M NaOH and the protein pellet kept

for further analysis. The precipitated salt was removed by centrifugation and the supernatant was freeze-dried overnight.

2.5.1 Cell Media extraction

The cell media used in the studies of [1-^{13}C]oleic acid utilisation (Chapter 8) was decanted off prior to cell harvesting and kept for further analysis. The used media and the same volume (20 ml) of fresh (natural abundance) media were freeze-dried overnight. The dried media was extracted using the M/C technique and the aqueous and lipid fractions were analysed separately for ^{13}C-labeled aqueous metabolites released by the cells into the media during incubation and for the amount of [1-^{13}C]oleic acid remaining in the lipid fraction of the media extract.

2.6 ^{1}H-NMR Spectroscopy

The separated and dried methanol fraction from the M/C extractions and the freeze-dried PCA extracts (30mg/ml) were reconstituted in D_2O and adjusted to pH 7.0. One μmol of an internal standard, trimethylsilylpropionate (TSP) was added to each sample. Spectroscopy was performed at 25 °C with a Varian Unity Plus spectrometer operating at 500 MHz. Fully relaxed spectra were acquired for all studies. The T1 relaxation time was determined for all metabolites being investigated and for TSP on a representative brain extract sample.

2.7 ^{13}C-NMR Spectroscopy

The dried aqueous and lipid fractions from the cell media extracts in the [1-^{13}C]oleic acid incubation study were reconstituted in D_2O or $CDCl_3$ and adjusted to pH 7.0 (Chapter 8). One hundred μmol of TSP was added as an internal standard. Direct-detected ^{13}C-NMR was performed at 25 °C with a Varian Unity Plus spectrometer operating at 500 MHz. Spectra from control media (i.e. media that had not been incubated with cells) was subtracted from the incubation media using standard Varian software and the remaining ^{13}C-labeled resonances were assigned according to the literature and by comparison with standards purchased from Sigma, UK for comparison. ^{13}C-enrichment was determined by comparison of metabolite concentrations calculated from ^{1}H- and

^{13}C-NMR spectra of the same sample and by comparison with natural abundance media of the same composition.

2.8 2D-nmr: HMBC

The ^{13}C label in the aqueous metabolites of the cell extracts in Chapter 8 was most likely to be found in the carboxyl carbons of TCA cycle intermediates and amino acid products. Therefore, a heteronuclear multiple bond correlation (HMBC) sequence was used to detect long-range ^{1}H-^{13}C couplings. The HMBC sequence is a 2D method in which the coupling of ^{13}C label to protons on remote carbons (2 or more bonds) gives rise to crosspeaks in a ^{1}H-detected experiment. A gradient-enhanced sequence was used. Neither one-bond couplings nor directly-detected ^{13}C were observed in any of the samples due to the dilute concentration in cellular extracts. Therefore, any HMBC-detected label could be considered enriched from the labeled oleic acid since no natural abundance signal could be detected from the other carbons in the sample. Due to the low concentration of metabolites in the cell extracts, the samples from each group were pooled, freeze-dried, reconstituted in 40 μl of D_20 and analysed in a micro-NMR probe at the University of Manchester. Due to the very small volume of the sample, pH adjustment was not possible and the 1D ^{1}H-NMR spectra from the HMBC experiments were difficult to assign. Therefore, standards of glutamate, glutamine, and aspartate were run using the HMBC sequence at various pHs in order to identify the metabolite which most likely contributed to the ^{1}H-^{13}C cross-peaks detected in the extract samples.

2.9 Metabolite Identification and Quantitation

Resonance assignments were made based on published chemical shifts and coupling patterns of known compounds (Behar and Ogino, 1991; Sappy-Marinier, 1990; Henke, *et al.*, 1996; Halliday, 1988). Peak areas were integrated using standard Varian software. Tissue extract metabolite concentrations are expressed as μmol/g wet weight and as nmol/mg protein for cell extracts.

2.10 Protein Determination

Protein pellets were solublised in 2ml/g tissue or 200 ml/pellet of 1 molar NaOH and analysed for protein content against a set of bovine serum albumin

protein standards using the Bio-Rad (UK) reagent kit and an LKB Ultrospec II spectrophotometer (wavelength, 595 nm).

2.11 HPLC

2.11.1 Amino acids and glutathione

The aqueous fraction of extracts were reconstituted in D_2O and adjusted to pH 7.0 for NMR and were then diluted for HPLC analysis. Glutathione samples were extracted in the mobile phase and run without adjustment. Analysis of the samples was performed according to Lindroth and Mopper (1979) with some modification. Samples were derivatised with o-phthaldialdehyde (OPA) and concentrations were calculated against standards. A LiChroGraph HPLC system (Merck-Hitachi, Darmstadt, Germany) with a LiChrospher 100HP-18 separation column was used. The OPA derivatives were detected by a UV/VIS fluorometer (LiChroGraph F1050). A 1000-fold dilution with ultrapure water was used for samples from tissue extractions and a 100-fold dilution was used for the cell extract samples. One ml of the diluted sample was mixed with 1 ml of the reagent containing 0.8 mg/ml of OPA. The compounds were allowed to react for 3 minutes before 80 μl of the mixture was injected into the column. Elution temperature was 40°C with a three step gradient of methanol in phosphate buffer (50 mmol/L in ultrapure water, pH6) starting at 20% methanol, increasing to 35% methanol in 5 minutes, decreasing to 30% methanol over 30 minutes, and ending at 20% methanol.

2.11.2 ATP

ATP, ADP, and AMP standards were purchased from Sigma, UK. Samples were extracted using either M/C or PCA extracts. Astrocytomas grown in 6-well plates were rinsed three times with 5ml of phosphate buffered saline (Chapter 7). Cells were extracted using either M/C or PCA extractions. The aqueous phase of M/C extracts were dried and reconstituted in 200 mM phosphate buffer (pH 6.0). PCA extracts were prepared by adjusting to pH 7.0 and precipitated salts centrifuged out of the sample. Freeze-drying was not required and the samples were run following pH adjustment. The mobile phase for HPLC consisted of 200 mM phosphate, pH 6.0 with a gradient of methanol from 0-98% over 30 minutes. The solvent mixture was pumped at a constant flow rate of 0.7 ml/min. ATP was detected by monitoring the effluent at 370 nm.

Quantitation was performed using a standard curve obtained from multiple external standards. Several samples were spiked with a known concentration of ATP prior to extraction in order to evaluate the recovery efficiency of the M/C and PCA extraction techniques.

2.12 Mass Spectrometry

All phospholipid standards were obtained from Sigma, UK. Mass spectrometry (MS) analyses were performed with a triple quadrupole instrument (Quattro One) equipped with a nanoelectrospray source operating at a flow rate of 20-50 nl/min. For each spectrum between 20 and 100 repetitive scans of 4 sec duration were averaged. All tandem MS experiments were performed with argon as collision gas at nominal pressure of 2mTorr. Quantitative determination of the phospholipid classes (phosphatidyl-serine, -choline, -inositol, and -ethanolamine) required the addition of internal standards for each phospholipid because the ionisation efficiency between different phospholipid classes may differ significantly with respect to experimental conditions. Synthetic phospholipids with nonnatural fatty acid compositions were purchased from Sigma, UK. Fragmentation patterns for each phospholipid were also established using standards. ^{13}C-label enrichment was determined by comparing the phospholipid acyl group (mass**+1**) to natural abundance (normal mass) samples. PtdEth was detected in positive electrospray (ES) as the neutral loss of 141; PtdCho was detected in positive ES as the parents of 184; PtdSer was detected in negative ES as the neutral loss of 88; PtdIno was detected in negative ES as the parents of 241. Representative spectra are presented in appendix I.

2.13 Radio-label studies

For radio-labeled experiments cells were grown in 6-well plates under normal culture conditions (Chapter 7). When confluent, the cells were incubated in deficient DMEM supplemented with a 400 μM [1-^{14}C] fatty acid-30 μM BSA complex with a specific activity of 0.4 μCi/μmol palmitate or 5.0 μCi/μmol oleate. $^{14}CO_2$ was trapped in filter paper saturated with 250 μl of 3.5 M NaOH which was secured above each well. The incubation was quenched with 100 ml of 20% PCA at the time points indicated in Chapter 7. Since long-chain fatty acids are not soluble in the acid, all the remaining radioactive palmitate was precipitated and removed from the sample by centrifugation. Thus, the radioactivity

remaining in the supernatant has been incorporated into non-specific acid-soluble products that may include acetyl-L-carnitine, TCA cycle intermediates, amino acids, ketone bodies, etc. The samples were then prepared for scintillation counting by placing the filter papers and the aqueous supernatant into polypropylene containers. The filter papers were then dried and Optisorb 'S' added to the container. Ecoscint A was added to the aqueous component. Samples were then placed in a scintilation (radioactivity) counter. Protein determination was made in parallel cell cultures.

2.14 Statistical Analysis

Statistical analysis of the data in this thesis was carried out using paired t-tests assuming unequal variance and one-way analysis of variance (ANOVA) to determine significant differences among group means. P-values are quoted without correction for multiple comparisons. The critical values for assessing significance levels were obtained after performing a Bonferroni correction for multiple comparisons as a function of the number of metabolites being compared.

Chapter 3: **Cell and Tissue Extraction**

3.1 Introduction

Cell and tissue extraction is an important and widely used technique in NMR spectroscopy studies of many diseases. A number of different extraction techniques exist for this purpose but the most ubiquitous is the perchloric acid (PCA) method which extracts the water-soluble metabolites.

A less widely used technique is the methanol-chloroform-water extraction (M/C) which facilitates the simultaneous extraction of both the water-soluble metabolites and the organic-soluble lipid components from the same tissue sample. *In vivo* proton NMR spectroscopy has shown significant changes in lipids as well as amino acids in many different CNS diseases such as multiple sclerosis (Davie, *et al.*, 1993) and high-grade brain tumours (Remy, *et al.*, 1994; Sijens, *et al.*, 1996). Therefore, it may be very useful to extract and investigate both pools of metabolites *in vitro* as well.

The important requirements of an extraction technique are that it is capable of extracting a reasonably high amount of the total tissue metabolites (efficiency) and that it is highly reproducible (low variability). It is well established that PCA extraction fulfills these criteria for the water-soluble metabolites (Evanochko, *et al.*, 1984a; Peeling and Sutherland, 1992; Glonek, *et al.*, 1982; Burri, *et al.*, 1990; Bates, *et al.*, 1989; Fan, *et al.*, 1986; Sze and Jardetzky, 1990a) and the M/C extraction fulfills these criteria for lipids (Tyagi,*et al.*, 1996; Henke, *et al.*, 1996; Jones, *et al.*, 1994; Katyal, *et al.*, 1985; Edzes, *et al.*, 1992; Sappey-Marinier, *et al.*, 1990; Capuani, *et al.*, 1992; Sze and Jardetzky,1990b). However, the M/C extraction has not yet been established as a reliable technique for proton NMR spectroscopy of the water-soluble metabolites. In this study the quantitative aqueous metabolite yields and protein pellet content were compared for both the PCA and the M/C extraction techniques on both rat brain tissue and cellular preparations of purified astrocytes.

3.2 Methods

In order to compare PCA and M/C techniques both funnel frozen rat brain and purified rat astrocyte cultures were extracted according to the methods outlined in Chapter 2. Cell and tissue extracts were analysed by ^{1}H-NMR spectroscopy and the concentrations of seven different metabolites were calculated. These metabolites represent several important to ^{1}H-NMR, and

include non-polar (alanine), polar (glutamine), and acidic (glutamate) amino acids, other anions (lactate and N-acetyl-aspartate), cations (choline-containing compounds), and zwitterions (creatine). In the astrocyte extracts, hypotaurine was analysed in place of NAA, which is not present in astrocytes (Urenjak, *et al.*, 1993). Cell purity of astrocyte cultures was determined by GFAP immunostaining. Protein content was determined according to the methods in Chapter 2 and protein yield per gram of wet-weight tissue was also compared between extraction techniques.

3.2.1 Cell and Tissue Groups

The frozen brain tissue was divided into two halves for extraction by either PCA or M/C. The samples from this first extraction were analysed by high-resolution ^{1}H-NMR spectroscopy (n=21/group). Six tissue pellets from each extraction group were then chosen at random for protein content determination (n=12). The metabolite yields were greater using the M/C technique, indicating that it was superior in terms of extraction efficiency for the aqueous metabolites analysed. Therefore, the remaining tissue pellets from the original M/C and PCA extracts (n=15/group) were then re-extracted using the M/C technique. The re-extraction was analysed separately from the first extraction using ^{1}H-NMR spectroscopy to determine the metabolite yield left behind in the pellets from the first extraction.

Primary rat astrocyte cultures were grown to sufficient numbers for 12 separate NMR samples (approximately 10^{7} cells/ NMR sample) for extraction by either PCA or M/C (n= 6/group). Each group of cells was extracted twice using a single extraction technique and the supernatants were pooled before drying and analysis by high-resolution ^{1}H-NMR spectroscopy. See figure 3-1 for summary.

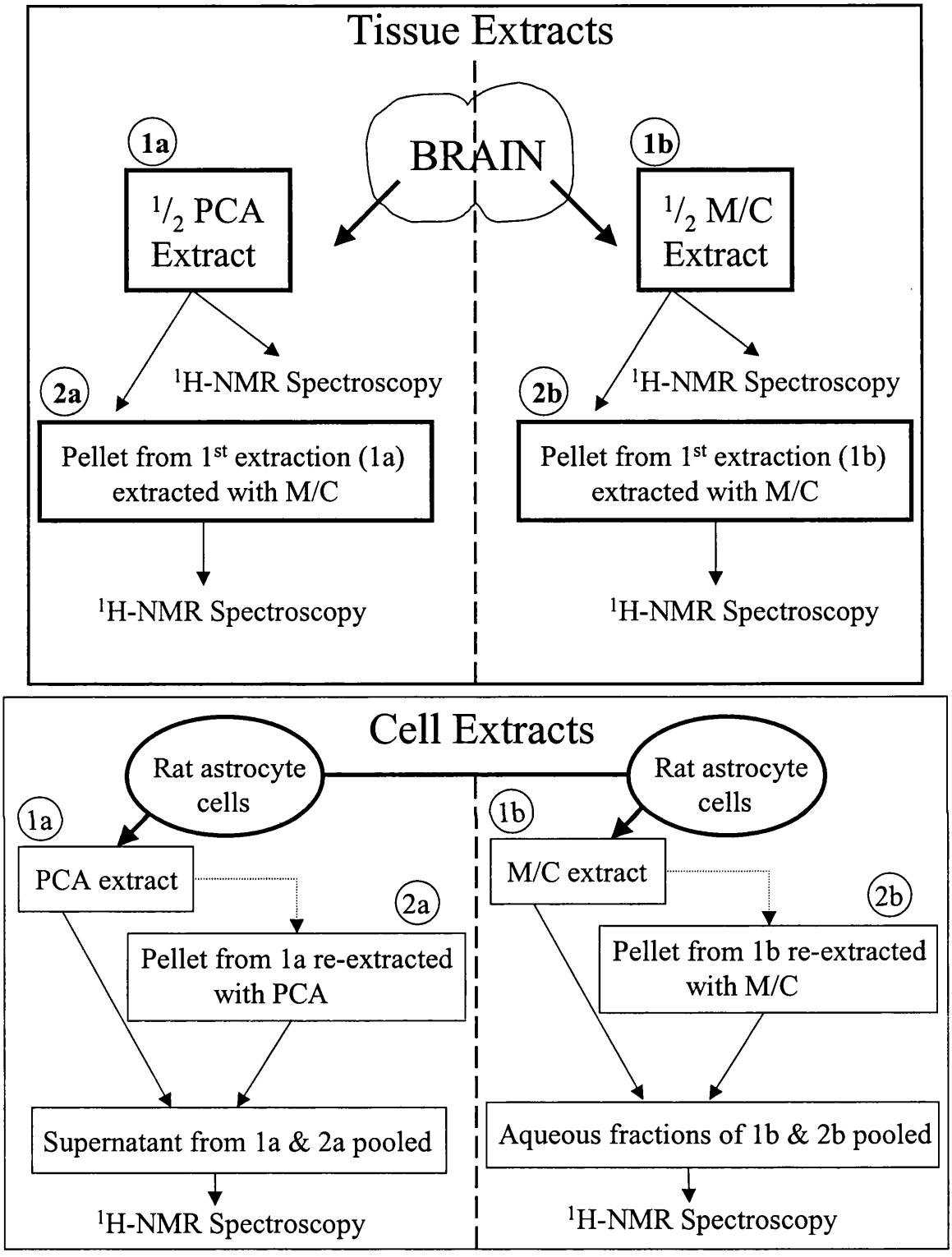


Figure 3-1. **Summary of experimental design.**

3.2.2 Phosphatidylcholine Extraction

After analysis of the tissue extractions, where an unusually large amount of choline was extracted from the PCA tissue pellets when they were re-extracted using M/C (table 3-2), it proved necessary to also investigate the

effects of PCA extraction followed by M/C extraction on the membrane phospholipid, phosphatidylcholine (PtdCho). One mmol of PtdCho standard (Sigma, UK) was exposed to 12% PCA for time periods of 10 minutes, 24 hours, 48 hours, and 2 weeks (n=3 for each time group) followed by M/C extraction. ^{1}H-NMR spectroscopy was used to determine the concentration of choline, a breakdown-product of PtdCho, in the aqueous fraction of the extract.

3.2.3 Statistical Analysis

Paired t-tests assuming unequal variance were used to compare the concentrations of seven different metabolites and the protein content in the two groups corresponding to the two extraction techniques.

3.3 Results

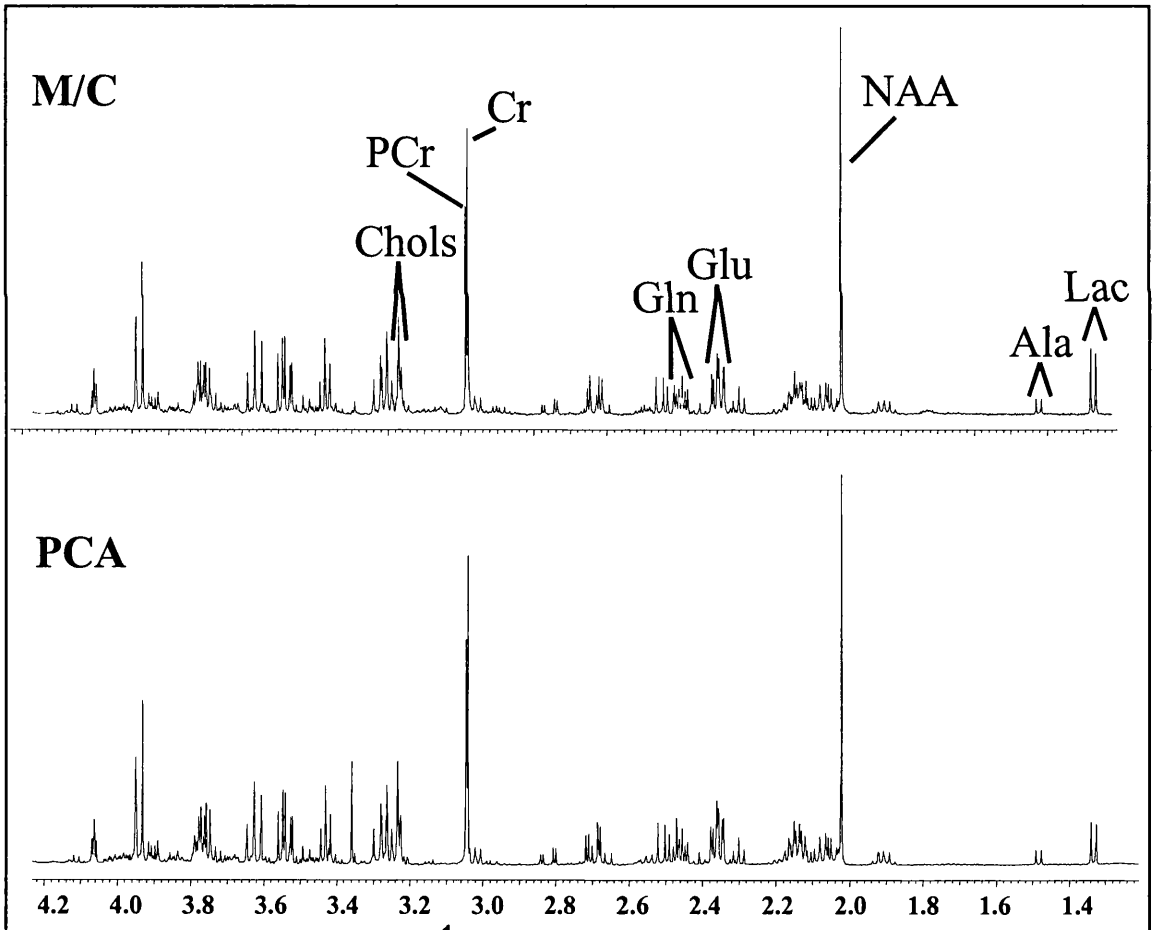


Figure 3-2. **Representative ^{1}H-NMR spectra from the first M/C (top) and PCA (bottom) extracts of whole rat brain frozen *in situ*.** *Abbreviations are as follows: Lac = lactate; Ala = alanine; NAA = N-acetylaspartate; Glu = glutamate; Gln = glutamine; PCr = phosphocreatine; Cr = creatine; Chols = choline-containing compounds.*

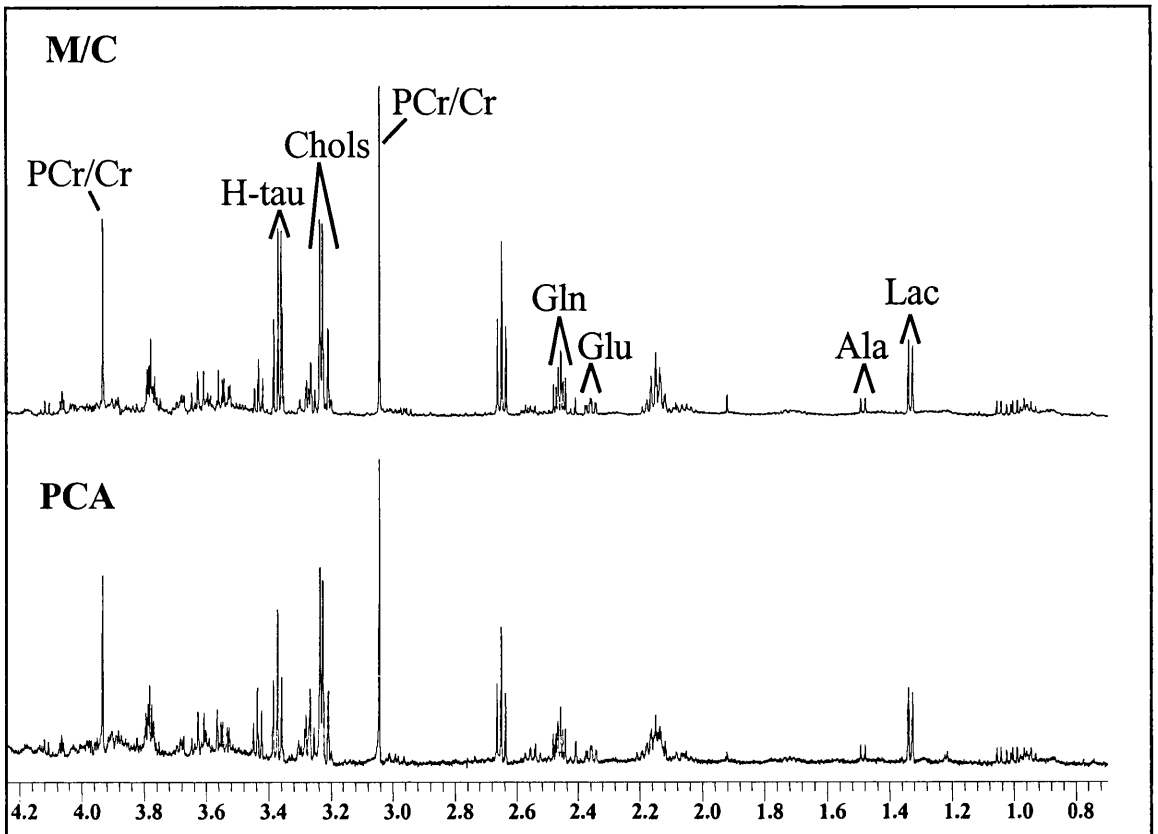


Figure 3-3. **Representative ^{1}H-NMR spectra from the M/C (top) and PCA (bottom) extracts of rat astrocyte cultures.** *See Figure 3-2 for abbreviations; H-tau = hypotaurine.*

3.3.1 Tissue Extractions

The first tissue extractions compared a single extraction by either the PCA or M/C technique of frozen rat brain tissue divided in half. The metabolite yields from the M/C extraction were greater than the PCA extraction (table 3-1), with significant differences in total Cr, Ala, and Lac yields which were higher in the M/C group (p<0.007). In addition, the coefficient of variation was consistently lower using the M/C technique (5 - 9% as compared to 14 - 19% in PCA, with the exception of lactate). The variation in protein content was similar for both techniques (table 3-1), which is an important consideration for studies of cell extracts in which metabolite concentrations are expressed relative to protein. Although the absolute protein content is different between extraction methods, this is due to sample variation in the wet weight of tissue in the extracted sample rather than methodological differences. Therefore, there was no significant difference seen between extraction methods (p=0.24; α=0.05) when the protein content is expressed as a percentage of the wet weight of the tissue extracted.

	M/C	CEV	PCA	CEV	P-VALUES
Total Cr	8.75 ± 0.15	8	7.74 ± 0.24	14	**0.001**
Chols	1.36 ± 0.03	9	1.28 ± 0.04	14	0.091
Glutamine	5.37 ± 0.07	6	5.18 ± 0.20	18	0.396
Glutamate	9.40 ± 0.11	5	8.64 ± 0.32	17	0.033
NAA	7.09 ± 0.13	8	6.51 ± 0.22	16	0.030
Alanine	0.65 ± 0.01	9	0.57 ± 0.02	19	**0.0003**
Lactate	1.98 ± 0.07	15	1.46 ± 0.05	17	**3×10^{-6}**
Protein	9.73 ± 1.30	33	15.10 ± 1.29	21	0.012

Table 3-1. **Summary of metabolite yields from the first set of extractions of rat brain samples using either the methanol-cholorform (M/C) or perchloric acid (PCA) extraction techniques.** *Metabolites expressed as μmol/g wet weight and mean ± SEM. Abbreviations are as follows: Total Cr = total creatine (creatine + phosphocreatine); Chols = choline-containing compounds (glycerophosphocholine + phosphocholine + choline); NAA = N-acetylaspartate; CEV = coefficient of variation (SD x 100/mean). The critical value α, adjusted for the number of comparisons, is 0.007 (0.05/7) for p-values to achieve significance.*

3.3.2 Tissue Re-extractions with M/C

Subsequent M/C re-extraction of the tissue pellets obtained from both original extractions resulted in a substantially higher amount of all of the metabolites (with the exception of lactate) being extracted from the PCA-pellets than the M/C-pellets, demonstrating the superior efficiency of the original M/C extraction (table 3-2). However, some metabolite was also re-extracted from the M/C pellets indicating the need for at least one re-extraction of the tissue pellet in order to maximise the metabolite yield for analysis. Comparison of the total metabolite yield (original extraction plus M/C re-extraction) revealed no significant difference between the methods except for choline-containing compounds and lactate which were significantly lower ($p=8\times10^{-8}$) and higher ($p=1\times10^{-6}$; $\alpha=0.05/7$) from the M/C pellets, respectively. Thus, the M/C re-extraction of the original extract pellets removed the remaining metabolites left behind by the original PCA and M/C extracts (table 3-2).

RE-EXTRACTION					
	M/C	CEV	PCA	CEV	P-values
Total Cr	0.74 ± 0.05	24	1.04 ± 0.08	30	0.005
Chols	0.10 ± 0.01	31	0.91 ± 0.05	22	1.2×10^{-10}
Glutamine	0.49 ± 0.02	12	0.81 ± 0.05	25	5.9×10^{-6}
Glutamate	0.80 ± 0.07	33	1.55 ± 0.08	19	3.1×10^{-7}
NAA	0.55 ± 0.04	26	1.06 ± 0.04	15	7.9×10^{-7}
Alanine	0.11 ± 0.01	28	0.18 ± 0.01	30	0.005
Lactate	0.35 ± 0.01	15	0.26 ± 0.02	35	0.011
TOTAL YIELD					
	M/C	% first extraction	PCA	% first extraction	P-values
Total Cr	9.49 ± 0.20	92	8.78 ± 0.34	88	0.027
Chols	1.46 ± 0.04	93	2.19 ± 0.11	58	8.0×10^{-8}
Glutamine	5.86 ± 0.12	92	6.00 ± 0.27	86	0.605
Glutamate	10.20 ± 0.19	92	10.19 ± 0.40	85	0.897
NAA	7.64 ± 0.17	93	7.56 ± 0.27	86	0.872
Alanine	0.76 ± 0.03	86	0.72 ± 0.04	79	0.223
Lactate	2.33 ± 0.10	85	1.73 ± 0.09	84	1.1×10^{-6}

Table 3-2. **Summary of metabolite yields from the M/C re-extraction of the tissue-pellets and the total yields (original extraction + re-extraction).** *Metabolites are expressed as μmol/g wet weight and mean ± SEM. See table 3-1 for abbreviations The critical value α, adjusted for the number of comparisons, is 0.007 (0.05/7) for p-values to achieve significance. The % first extraction represents the percentage of the total metabolite yield that was extracted in the original extraction by either M/C or PCA.*

3.3.3 Cell Extractions

Due to the results from the tissue extractions, where a significant amount of the total metabolite yield was left behind after the first extraction but was sufficiently recouped by a second extraction of the tissue pellet, the cell samples were extracted twice by either the M/C or the PCA technique. The supernatants from the original extraction and re-extraction of the cells were pooled prior to analysis.

The cellular metabolite yields from the M/C extraction were once again greater than the PCA extraction (table 3-3), with highly significant differences detected between the extraction techniques for all of the metabolites measured.

In addition, the coefficient of variation was consistently lower using the M/C technique (5 - 12% as compared to 12 - 45% in PCA).

	M/C	CEV	**PCA**	CEV	P-VALUES
Total Cr	21.72 ± 0.63	7	11.16 ± 2.53	45	0.006
Chols	40.72 ± 1.34	8	26.80 ± 1.55	12	4×10^{-5}
Glutamine	29.09 ± 0.89	7	15.19 ± 3.15	41	0.001
Glutamate	12.01 ± 0.24	5	7.25 ± 0.50	14	2×10^{-5}
hypotaurine	39.99 ± 2.02	12	19.25 ± 3.08	32	5×10^{-5}
Alanine	3.90 ± 0.18	11	1.98 ± 0.26	26	0.0004
Lactate	14.82 ± 0.49	8	8.68 ± 1.05	24	5×10^{-5}

Table 3-3. **Summary of metabolite yields from the rat astrocyte cultures using either the methanol-cholorform (M/C) or perchloric acid (PCA) extraction techniques**. *Metabolites are expressed as nmol/mg protein and mean ± SEM. N = 6 samples/ extraction group. Abbreviations are as follows: Total Cr = total creatine (creatine + phosphocreatine); Chols = choline-containing compounds (glycerophosphocholine + phosphocholine + choline); CEV = coefficient of variation (SD x 100/mean). The critical value α, adjusted for the number of comparisons, is 0.007 (0.05/7) to achieve significance.*

3.3.4 Phosphatidylcholine Extraction

Exposing a standard amount of phosphatidylcholine to PCA for varying times before subsequent extraction with M/C resulted in the recovery of small amounts of choline in the aqueous fraction which increased with the time of PCA exposure (table 3-4). Choline was not detected in the aqueous fraction of the M/C extract when the PtdCho standard was not previously exposed to PCA.

	CHOLINE
10 min	0.027 ± 0.002
24 hours	0.035 ± 0.004
48 hours	0.037 ± 0.003
2 weeks	0.054 ± 0.005

Table 3-4. **The yield of choline extracted by M/C from 1mmol of phosphatidylcholine standard with varying prior exposure to PCA.** *Choline concentrations are expressed as μmoles and mean ± SEM. N = 3 per time group.*

3.4 Discussion

3.4.1 Tissue Extraction

The aqueous metabolite concentrations obtained by the M/C extraction are in good agreement with those obtained by Aureli, *et al.* (1990 & 1994) in control rat brain using the M/C technique. In addition, the PCA brain extracts are in good agreement with literature values (Evanochko, *et al.*, 1984a; Peeling and Sutherland, 1992; Glonek, *et al.*, 1982; Burri, *et al.*, 1990; Bates, *et al.*, 1989; Fan, *et al.*, 1986; Sze and Jardetzky, 1990a; Florian *et al.*, 1996).

Once it had been established from the first extractions that the M/C technique was more efficient and less variable than PCA, the tissue pellets from both original extractions were all extracted for a second time with the M/C technique and analysed separately for metabolite yield. As expected, a higher amount of tissue metabolites were re-extracted from the PCA pellets because a larger amount of metabolites were left behind by the original extraction than in the M/C pellets. Therefore, the total yields from the M/C and PCA pellets were similar. The exceptions were the choline-containing compounds and lactate. Calculating the PCA and M/C metabolite concentrations from the first extractions as a percentage of the total extraction demonstrated that the M/C re-extractions maximised the total metabolite extracted from each tissue sample (table 3-2).

The very high yields of choline-containing compounds from PCA pellets re-extracted with M/C were an unexpected result. However, I believe this may be an artifact resulting from the combination of PCA and M/C extraction on the same tissue pellet, which was not being investigated as a technique *per se*. Bruhn, *et al.* (1992) also observed that considerable amounts of choline can be recovered when PCA extractions of human tumour biopsy tissue were followed by a chloroform/ethanol extraction on the tissue. They hypothesised that the hydrophilic membrane choline headgroups are separated by a chlorform/alcohol extraction and may be responsible for the higher Cho/Cr ratios seen *in vivo* compared to the lower ratios found in *in vitro* proton spectra from PCA extracts alone. This hypothesis was investigated further in the present study by analysing the choline-containing compounds individually and by extracting phophatidylcholine standards with PCA followed by M/C which demonstrated that some choline is released from the phospholipid (see section 3.4.2).

In addition to cholines, the total yield for lactate was found to be different between techniques. Lactate was found to be higher in the first and subsequent

M/C extractions which may indicate that the technique does not inactivate tissue enzymes as efficiently as PCA. On the other hand, the relatively high PCr/Cr ratio would suggest that enzyme inactivation has been relatively efficient. It is more likely that the source of this discrepancy is the difficulty with lactate peak integration due to an overlapping broad signal from contaminating protein or lipid in the M/C extracts.

3.4.2 Phosphatidylcholine Extraction

In response to the unusually high yield of total choline-containing compounds (Chols) in the M/C re-extraction of PCA tissue pellets, I investigated the individual Chols and how the membrane phospholipid, phosphatidylcholine, was affected by exposure to PCA followed by a M/C extraction. When the Chols were analysed individually it was found that the main source of the significant increase in choline signals from the re-extraction of the PCA pellet was choline and not glycerophosophocholine (GPC), phosophocholine (PC) (table 3-5).

	GPC	PC	CHO
1st M/C extract	0.87 ± 0.02	0.39 ± 0.01	0.10 ± 0.002
1st PCA extract	0.82 ± 0.03	0.39 ± 0.01	0.07 ± 0.002
M/C pellet re-extraction	0.07 ± 0.01	0.02 ± 0.002	0.01 ± 0.001
PCA pellet re-extraction	0.16 ± 0.01	0.07 ± 0.003	0.65 ± 0.04

Table 3-5. **Summary of the individual choline-containing compounds extracted from whole rat brain using the M/C and PCA techniques for the first extraction and M/C only for the re-extraction of the protein pellets.** *Abbreviations are as follows: GPC = glycerophosphocholine; PC = phosphocholine; Cho = choline. Metabolites are expressed as μmol/g wet weight and as mean ± SEM. GPC>PC>Cho, except for the PCA pellet re-extraction by M/C which yielded: Cho>GPC>Cho.*

When the phosphatidylcholine (Ptdcho) standards were exposed to PCA for varying times before extraction by M/C, increasing concentrations of choline, a breakdown product of Ptdcho, were extracted with increasing time of contact with PCA (table 3-4). However, choline is not normally extracted from Ptdcho by the M/C technique alone and the intact PtdCho was partitioned in the lipid fraction of the extract. Thus, I hypothesise that in the case of the high choline in

the M/C re-extraction of the PCA-pellets, the small amount of PCA left with the pellets from the original tissue extraction may have hydrolysed the C-O-P phosphoester linkages in membrane Ptdcho molecules, releasing choline into the aqueous fraction of the subsequent M/C re-extractions performed on the PCA pellets. This may explain the significant difference between techniques for total Chols (original extraction plus the re-extractions). Thus, such high choline concentrations would not be obtained from either single extraction method but only in the artificial case of PCA followed by M/C, which was not being evaluated as a technique. Comparison of the yields for the choline-containing compounds in the original extraction demonstrates the normal efficiency of the techniques and that M/C extraction is superior to PCA.

3.4.3 Cell Extraction

The comparison of M/C and PCA extraction of purified rat astrocytes yielded similar results to those observed in tissue extractions: the M/C extraction proved to be a less variable and more efficient technique (Table 3-3). The M/C technique may be ideally suited to cellular extract studies where very small amounts of biological material are used. This is due to the entire aqueous fraction being redissolved in D_2O for NMR analysis. In PCA extracts, the neutralised supernatant is freeze-dried but there are complications associated with the reconstitution of the dried extract in D_2O. The maximum concentration of dried extract that will dissolve fully in D_2O was determined to be 30 mg/ml. However, the total dry weight of the extracts was often two to three times greater. Thus, the reason for the much higher variability in the PCA extraction may be due to the difficulties caused by precipitated salt which were still present in the dried extract despite neutralization and centrifugation prior to drying. One possible solution is to use only 30 mg of the dried extract and to account for the fractionation of the total sample in metabolite concentration calculations. However, cell extract samples contain such small amounts of metabolites for NMR analysis that the entire sample must be used for adequate sensitivity. Therefore, if the whole sample were reconstituted in D_2O, the remaining undissolved salt must be centrifuged and removed again prior to NMR analysis. Although the salt pellet is washed, this extra step undoubtedly adds to the experimental error of the technique.

Aqueous metabolite yields for rat astrocyte extracts are difficult to compare with published values due to variations in experimental techniques. The only papers known to me which provide quantitative concentrations of metabolites from PCA extractions of rat astrocytes (Urenjak, *et al.*,1993; Serkova, *et al.*, 1996) used different cell preparation and harvesting protocols which may produce metabolic differences. However, since the cells in both extraction groups in the present study were grown and harvested in exactly the same way, any differences in metabolite yield can be attributed to the extraction techniques alone. To my knowledge, these are the first reported quantitative values of the aqueous metabolites from M/C extraction. ^{1}H, ^{13}C, and ^{31}P NMR, HPLC, and gas-chromotography studies have been used to analyse lipids but the quantitative analysis by proton NMR of the water-soluble metabolites from the dual phase extraction has not been reported. Tyagi, *et al.* (1996) reported the use of the M/C technique to extract murine melanoma cell cultures and the analysis of both the aqueous and organic fraction by ^{31}P and ^{13}C NMR. They compared the aqueous fraction from M/C and PCA extractions with ^{31}P NMR and found that M/C was more consistent in recovering the phosphate metabolites. Henke, *et al.* (1996) have reported the use of PCA extraction to obtain water-soluble metabolites followed by M/C extraction to obtain the lipid metabolites. Two separate techniques are used on the same tissue sample because the Folch method of M/C extraction used alone gives unsatisfactory recovery of the aqueous metabolites. However, the results of the present study demonstrated that modifications made by Bligh-Dyer to the original Folch technique (eliminating the presence of excessive salts used to maximise the extraction of acidic lipids) allow for very good recovery of aqueous metabolites as well as total lipid. Although Henke, *et al.* (1996) have demonstrated PCA followed by M/C to be a successful method for the analysis of both aqueous and organic metabolites, the present study has demonstrated that a single extraction procedure can produce similar results. In addition, Katyal, *et al.* (1985) have reported that there is a significant loss of total phospholipids and cholesterol during extraction with perchloric acid and formic acid/acetone due to lipid hydrolysis compared with yields from a M/C extraction.

3.4.4 The M/C procedure

Folch, *et al.* (1957) introduced the use of a methanol and chloroform solvent system for the extraction of total lipids from animal tissues. Although many different solvent-systems existed for the extraction of particular lipid species, the Folch method offered the advantage of an efficient *total* lipid extraction. However, it was a time-consuming method which required washing procedures with salt solutions that render the aqueous fraction unsuitable for analysis. This method was later improved by Bligh and Dyer (1959) by carrying out the extraction and separation of layers almost simultaneously, retaining the precipitated protein between phases. Although the Bligh-Dyer method was only designed to extract the lipid species, the technique eliminated the addition of salts to the aqueous layer and used significantly smaller volumes of solvents. The Bligh-Dyer paper compared the lipid yields using their method and the methods of Folch, *et al.* and others and found their method to yield comparable lipid quantities. The Bligh-Dyer method also established the proportions of solvents (methanol : chloroform : water) based on the water content of the specific tissues being extracted and the use of smaller solvent volumes made the method applicable to dilute cell suspensions as well as to tissue samples.

The use of salt solutions in the Folch M/C method rather than water was thought to give a better recovery of acidic lipids in the organic fraction. Although the Bligh-Dyer M/C method was shown to extract a similar amount of total lipids they did not compare the yields of individual lipid species. However, Tyagi, *et al.* (1996) have compared the individual phospholipids extracted by the Folch method to a dual-phase extraction method based on the Bligh-Dyer technique using ^{31}P NMR. The extracts performed on murine melanoma cells demonstrated that phospholipid yields from the dual-phase extraction compared well with the Folch method. They found no significant difference between the methods for any of the phospholipids extracted, including the acidic lipids.

The methods of Folch and Bligh-Dyer have been widely used, though often in modified form, in both NMR and non-NMR studies of tissue lipid composition and changes in disease states (Pollesello, *et al.*, 1996; Capuani, *et al.*, 1992; Meneses and Glonek, 1988; Sappey-Marinier, *et al.*, 1988). Meneses and Glonek (1988) have previously analysed phospholipid mixtures by NMR from animal and plant extracts and found the qualitative and quantitative lipid yields to be comparable to those obtained by chromatographic techniques.

Sappey-Marinier, *et al.* (1988) used the M/C technique in a quantitative ^{31}P-NMR study of the lipid composition of human white matter. Pollesello, *et al.* (1996) used the M/C technique to analyse, by ^{1}H- and ^{13}C-NMR, the lipid composition of liver tissue from normal and diseased liver tissue. The values obtained for normal liver from M/C extraction agreed with literature data on lipid composition obtained by other techniques. Thus, the use of M/C extraction for the analysis of cell and tissue lipids is well established. However, the information from the aqueous phase of the M/C extraction has not been utilised, until now.

3.5 Conclusions

The metabolite yields for PCA extractions from brain tissue and astrocyte cells are in good agreement with data from the literature. This work is the first to report quantitative data on the metabolite yields from the aqueous fraction of a M/C extraction. Most previous work on cell and tissue extraction has considered either the lipid or aqueous metabolites separately or involved two separate extraction techniques to yield the lipid and aqueous metabolites from a single sample. The present study has demonstrated the M/C method is an efficient and reproducible technique for extracting the aqueous metabolites and previous work by other groups on the lipid fraction alone has shown it to also be a successful method of total lipid extraction.

In conclusion, we have shown that the M/C extraction technique, based on the Bligh-Dyer method, which obtains both the lipid and aqueous compounds from a single sample, is well suited for NMR investigation and is superior to either PCA extraction alone, or PCA extraction followed by lipid extraction.

Chapter 4: **Metabolic Differences Between Rat and Human Astrocytes Detected by ^{1}H-NMR Spectroscopy**

4.1 Introduction

Normal rat CNS cells are often used as a model for comparison with abnormal human cellular metabolism because of the difficulty in obtaining normal human CNS tissue for cell culture. However, only tentative conclusions can be drawn about human cellular metabolism because any species differences between experimental and control rat cells can not be completely ruled out. For example, Florian, *et al.*, (1995) have shown that transformed human meningioma cells share many ^{1}H-NMR observable characteristics with rat meningeal cells which demonstrated that certain lineage characteristics are carried over between species. However, conclusions about any differences between these cells are not possible to make unless it can be demonstrated that there are no metabolic differences between their normal human and rat cell counterparts. In this regard, it is necessary to compare normal human and rat cells of every CNS cell lineage to validate any metabolic comparisons made to abnormal human cells. I have, therefore, undertaken a quantitative comparison of the aqueous metabolites and membrane phospholipids in both normal human and rat astrocyte cultures from developing brain using high-resolution ^{1}H-NMR spectroscopy and mass spectroscopy.

4.2 METHODS

Highly purified (98%) rat astrocyte cultures were prepared from the whole brain of 1-day-old rat pups as previously described in Chapter 2. Purified (99%) human astrocytes (fetal) were obtained from BioWhittaker, UK. Both rat and human astrocytes were subcultured to obtain approximately 10^7 cells per NMR sample. Cell culture purity was determined by GFAP immunostaining. Cells were harvested and extracted using a methanol-chloroform-water extraction. The aqueous fraction of the extract was analysed by ^{1}H-NMR spectroscopy and the lipid fraction was analysed by gas ionisation tandem mass spectrometry for phospholipid concentrations and composition. Protein content was determined using the method of Smith, *et al.* (1985). Two-sample student's t-Tests without correction were used to test for statistical differences between species for each metabolite measured. The critical value for significance was adjusted for the

number of comparisons (α = 0.005 at 5% and 0.001 at 1% for the comparison of 10 different metabolites).

4.3 RESULTS

4.3.1 ^{1}H-NMR results

Human and rat astrocytes contained significantly different total creatine (Creatine = creatine + phosphocreatine), glutamine (Gln), and glycerophosphocholine (GPC) concentrations ($p=2x10^{-8}$, $2x10^{-7}$, and $2x10^{-4}$ respectively), although the total choline containing compounds were not significantly different ($p=0.007$; $\alpha=0.005$). Additionally, the human astrocytes did not contain detectable quantities (ND = not detected) of either Tau or h-tau.

Metabolites (nmol/mg protein)	Rat Astrocytes (n=6)	Human Astrocytes (n=4)	p value
Myo-inositol	13.67 ± 0.4	8.27 ± 1.2	0.013
Creatine	21.72 ± 0.6	3.92 ± 0.5	**2.0E-08**
Glycine	4.44 ± 0.2	5.12 + 0.8	0.463
Taurine	16.13 ± 0.7	ND	**
GPC	17.12 ± 0.7	7.20 ± 0.9	**0.0002**
PC	17.20 ± 0.8	10.49 ± 2.3	0.048
Choline	6.40 ± 0.3	4.53 ± 1.4	0.295
Hypotaurine	39.99 ± 2.0	ND	**
Glutamine	29.09 ± 0.9	15.01 ± 0.71	**2.0E-07**
Glutamate	12.01 ± 0.2	21.28 ± 2.2	0.025
Alanine	3.90 ± 0.2	3.43 ± 0.3	0.193
Lactate	14.82 ± 0.5	10.83 ± 1.4	0.052

Table 4-1. **Comparison of metabolites quantified from ^{1}H-NMR spectra of human and rat astrocyte extracts.** *Metabolite concentrations (nmol/mg protein) are expressed as mean ± SEM. P-values are given without correction for the number of comparisons and the results for the astrocyte groups are considered significantly different if p<0.05/10 (0.005; 5% level) or p<0.01/10 (0.001; 1% level). Abbreviations are as follows: GPC = glycerophosphocholine, PC = phosphocholine; Creatine = creatine + phosphocreatine.*

Representative ^{1}H-NMR Spectra:

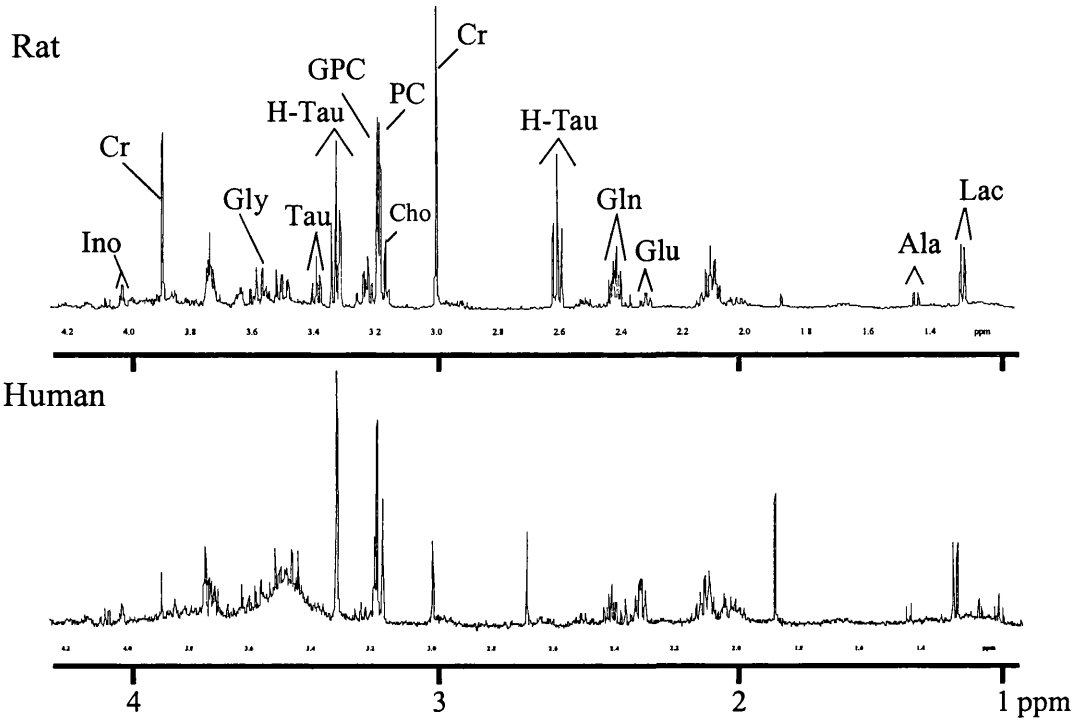


Figure 4-1. **Fully relaxed proton spectra from rat and human astrocyte M/C extracts.** *NMR spectroscopic analysis was performed at pH 7.0 with 2,000 scans and tr=22 sec. Spectra are referenced to TSP (0 ppm). Abbreviations are as follows: Ino = inositol; Cr = creatine + phosphocreatine; Gly = glycine; Tau = taurine; GPC = glycerophosphocholine, PC = phosphocholine, Cho = choline; h-Tau = hypotaurine; Gln = glutamine; Glu = glutamate; Ala = alanine; Lac = lactate.*

4.3.2 Mass Spectrometry

Mass spectrometry analysis of membrane phospholipids demonstrated that there were no significant quantitative differences between species (table 4-2). Furthermore, the qualitative analysis of fatty acid side-chains revealed that the species have very similar phospholipid compositions (table 4-3). Although the human astrocytes have slightly more unsaturated PtdEth and more saturated PtdIno than the rat cells, the overall ratio of saturated to unsaturated fatty acids is very similar (human = 0.75; rat = 0.79).

	HUMAN ASTROCYTES	RAT ASTROCYTES	P-VALUES
PtdCho	97.67 ± 4.94	92.17 ± 5.16	0.463
PtdEth	5.43 ± 0.69	5.12 ± 0.49	0.727
PtdSer	12.37 ± 3.34	11.28 ± 1.92	0.790
PtdIno	10.84 ± 1.68	9.94 ± 1.48	0.700
Total	126.31 ± 10.65	118.51 ± 9.05	0.610

Table 4-2. **Comparison of membrane phospholipids between human and rat astrocytes**. *All metabolites are expressed as nmol/mg protein and as mean + SEM. Abbreviations are as follows: PtdCho = phosphatidylcholine; PtdEth = phosphatidylethanolamine; PtdSer = phosphatidylserine; PtdIno = phosphatidylinositol.*

	HUMAN ASTROCYTES				RAT ASTROCYTES			
	PtdCho	PtdEth	PtdSer	PtdIno	PtdCho	PtdEth	PtdSer	PtdIno
16:0	**37.9** ± 1.5	**8.5** ± 0.6	**7.3** ± 0.4	**8.0** ± 0.2	**41.3** ± 2.6	**18.3** ± 1.3	**8.9** ± 0.4	**10.4** ± 0.5
18:0	**7.0** ± 0.4	**31.1** ± 1.0	**33.5** ± 2.0	**36.7** ± 1.7	**9.3** ± 1.0	**33.6** ± 1.3	**31.4** ± 0.9	**24.6** ± 1.9
18:1	**43.1** ± 1.6	**28.4** ± 2.3	**26.1** ± 2.8	**12.3** ± 0.2	**38.9** ± 1.9	**29.7** ± 0.6	**27.4** ± 0.7	**23.3** ± 2.1
18:2	**1.0** ± 0.1	**0.7** ± 0.1	**1.1** ± 0.4	**1.1** ± 0.2	**1.1** ± 0.1	**1.1** ± 0.1	**1.0** ± 0.2	**0.9** ± 0.1
20:4	**9.0** ± 0.4	**19.6** ± 2.6	**23.9** ± 1.6	**41.1** ± 1.5	**7.1** ± 0.8	**12.3** ± 1.5	**21.8** ± 1.2	**39.9** ± 1.2
22:6	**2.0** ± 0.4	**11.5** ± 1.21	**8.1** ± 0.2	**0.9** ± 0.2	**2.1** ± 0.5	**5.0** ± 1.4	**9.7** ± 0.9	**1.0** ± 0.2

Table. 4-3 **Phospholipid acyl group compositions.** *The fatty acid acyl groups are expressed as a percentage of the total of each phospholipid analysed. See table 4-2 for abbreviations.*

4.4 Discussion and Conclusions

4.4.1 Creatine

Creatine, which is enriched in rat astrocytes relative to neurons (Urenjak, 1993), was strikingly low in the human astrocytes in this study. However, this may be a consequence of the cell culture environment rather than a true reflection of the creatine content of human astrocytes *in vivo*, since it seems unlikely that the high creatine concentration in human brain would be predominantly non-astrocytic in origin. Creatine kinase mRNA has been shown

to be 15 times higher in rat astrocytes and oligodendrocytes than neurons and led to the theory that the PCr content of rat brain is mainly localised in glial cells (Molloy, *et al.* 1992; Brand, *et al.* 1993). Nevertheless, this result does indicate a real difference between rat and human astrocytes in the way they handle creatine even though it may have resulted from an "artificial" culture environment if human astrocytes rely more heavily on creatine uptake than synthesis since the media concentrations of creatine were low. This may be an important consideration for cell-culture studies of human astrocytomas since low concentrations of creatine have until now been thought to be a characteristic of the transformed astrocyte. It is also possible that the low creatine in the human astrocytes *is* a true reflection of their *in vivo* metabolism since the studies suggesting that brain-creatine is mainly localised in glia were performed using only rodent cells/brain. Therefore, the low concentration of creatine commonly seen in high-grade astrocytic tumours *in vivo* may be a reflection of astrocytic phenotype. Low amounts of creatine compared to normal surrounding brain tissue would, therefore, remain a distinguishing characteristic of the astrocytoma compared to other CNS tumours and normal tissue. The fact that astrocytomas may be identifiable on the basis of a metabolic aspect of the dominant cell lineage in the tumour mass rather than a specific characteristic of its transformed metabolism would not preclude the use of low total creatine in ^{1}H MRS for differential diagnosis.

4.4.2 Taurine

The most striking differences detected between species were the lack of taurine and hypotaurine in human astrocytes. This is consistent with data from whole brain extracts in which rat brain contains relatively high taurine concentrations (Bates *et al.*, 1989; Florian *et al.*, 1996b) compared to its low presence in human brain (Perry *et al.*, 1981). Taurine is at its highest concentration in brain postnatally but decreases to low adult concentrations in both rats and humans (Bates, *et al.* 1989; Nakada *et al.* 1991; Peeling and Sutherland, 1992). The utilisation of taurine by the brain is not fully understood, although there is evidence that it can act as a free radical scavenger (Huxtable, 1989) and as an organic osmolyte (Pasantes, et al 1988; Nagelhus, *et al.* 1993).

Hypotaurine (h-tau) concentrations in whole rat brain have been reported to be only 1% of those of taurine (Perry and Hansen, 1973). However,

hypotaurine is almost twice as concentrated in cultured rat astrocytes compared to neurons while in co-cultures of astrocytes and neurons h-tau concentrations are similar to what is seen in whole brain (Brand, *et al.* 1997 & 1998). This has led to the theory that hypotaurine and taurine are synthesised in astrocytes and then transferred to neurons.

Studies of cultured rat astrocytes and neurons have indicated that the high hypotaurine concentration in astrocytes corresponds to the fact that the enzymes catalysing taurine and hypotaurine synthesis from cysteine are found in rat astrocytes but not in neurons (Almarghini, et. al. 1991; Brand, *et al.* 1993; Tappaz et al, 1994). Hypotaurine has also been identified in rat oligodendrocytes and O-2A progenitor cells (Urenjak, *et al.* 1993). Taurine and hypotaurine produced in rodent astrocyte cultures have been shown to be exported into cell culture medium and subsequently taken up by neurons, where uptake mechanisms for both metabolites have been demonstrated (Pasantes, et al 1988; Nagelhus, *et al.* 1993; Brand *et al.* 1998).

Cysteine is a metabolic precursor for the synthesis of hypotaurine, taurine, and glutathione and hypotaurine is considered an intermediate in taurine synthesis (Brand, *et al.* 1998). The developing rat, but not the human neonate, is able to use this metabolic pathway to synthesise part of its brain-taurine requirement even though the rate of taurine uptake in fetal rat brain is much higher than in the adult (Sturman *et al.* 1977). Studies on infant milk formula discovered that taurine is an *essential* amino acid during human development which is thought to improve fat absorption (Erbersdobler, *et al.* 1984; Galeano, *et al.* 1987; Benson, *et al.* 1994). Therefore, there may be significant differences between species in the metabolism of taurine with evidence pointing towards the dominance of a low uptake mechanism in human brain and *de novo* synthesis on the other hand in rat brain. Thus, the lack of taurine and hypotaurine in human astrocytes in the present study, despite the presence of cysteine in the cell media, may indicate the importance of direct taurine uptake in the human cells. Given the apparent lack of taurine synthesis in human astrocytes and the very low concentrations of taurine and the absence of hypotaurine detectable in human brain, this amino acid may not play the same important roles in antioxidant defense and osmotic regulation that have been hypothesised in rodent brain.

4.4.3 Choline-containing compounds

Although the total choline-containing compounds (Chols) were not significantly different between species, the GPC constituent was significantly higher in the rodent astrocytes. However, despite this, the ratio of the average choline metabolite concentrations was GPC=PC>Cho in the rodent cells and GPC=PC=Cho in the human astrocytes. Thus, the ratio of means is slightly ambiguous given the sample variances. If taken individually though, most of the rat astrocyte samples had the Chols ratio GPC>PC>Cho. The human astrocytes also had GPC>PC>Cho in half of the samples but had PC>GPC>Cho in the other half. However, it should be noted that individually the absolute GPC and PC concentrations were similar in most of the rat and human samples. Therefore, in each sample the ratio of GPC=PC>Cho was a strong trend in both species which may become significant on average if more human astrocyte samples were available (figure 4-2).

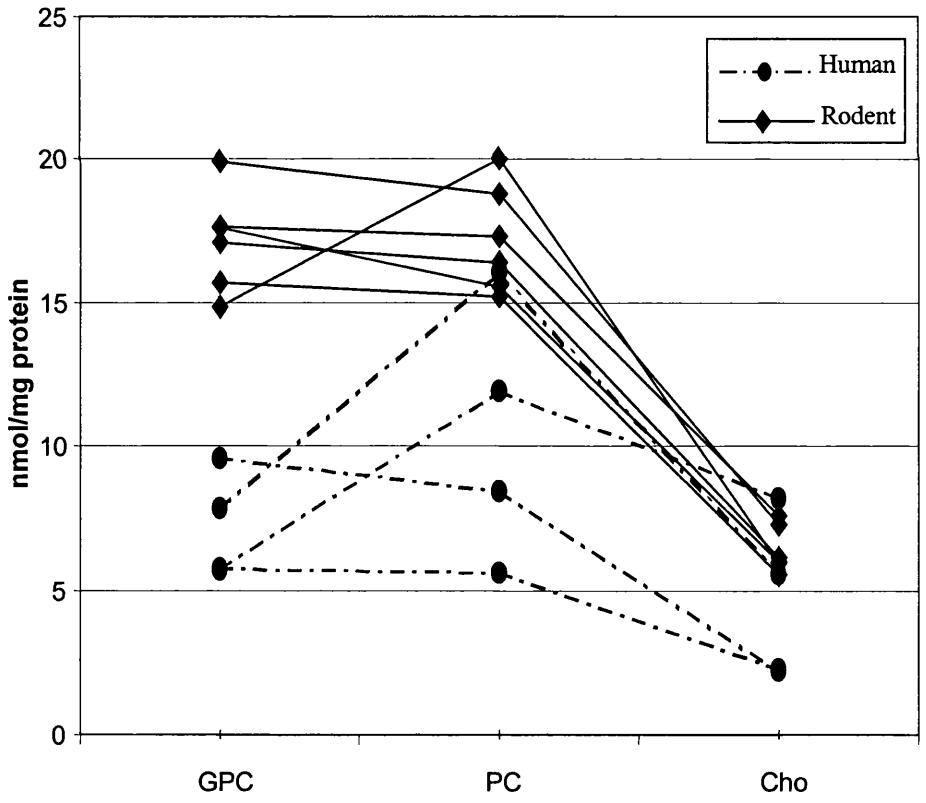


Figure 4-2. **Individual choline-containing compounds from each rat and human astrocyte extract sample.** *The concentrations are expressed in nmol/mg protein. The human cells are more variable than the rat cells but GPC and PC are very close in concentration in most of the samples from both species with choline being generally at the lowest concentration.*

Although the trend for choline-containing compounds (GPC=PC>Cho) within individual samples was similar in astrocytes from both species, this ratio is different from that seen in whole-brain extracts (GPC>PC>Cho) where GPC is quite significantly higher than PC (see Fig. 4-3 & 4-4). The observation could indicate that relatively enriched PC is a specific characteristic of astrocytes but this seems unlikely since astrocytes are the most abundant cell type in the CNS and this ratio is not observed in whole-brain extracts. There is also evidence from Usenius, *et al.* (1994) that, like the whole brain, human white matter is enriched in GPC relative to PC. Furthermore, they demonstrated that astrocytomas grade I-III have GPC>PC but only the malignant astrocytomas (grade IV) have PC>GPC. Therefore, elevated PC in normal astrocytes is an unexpected finding.

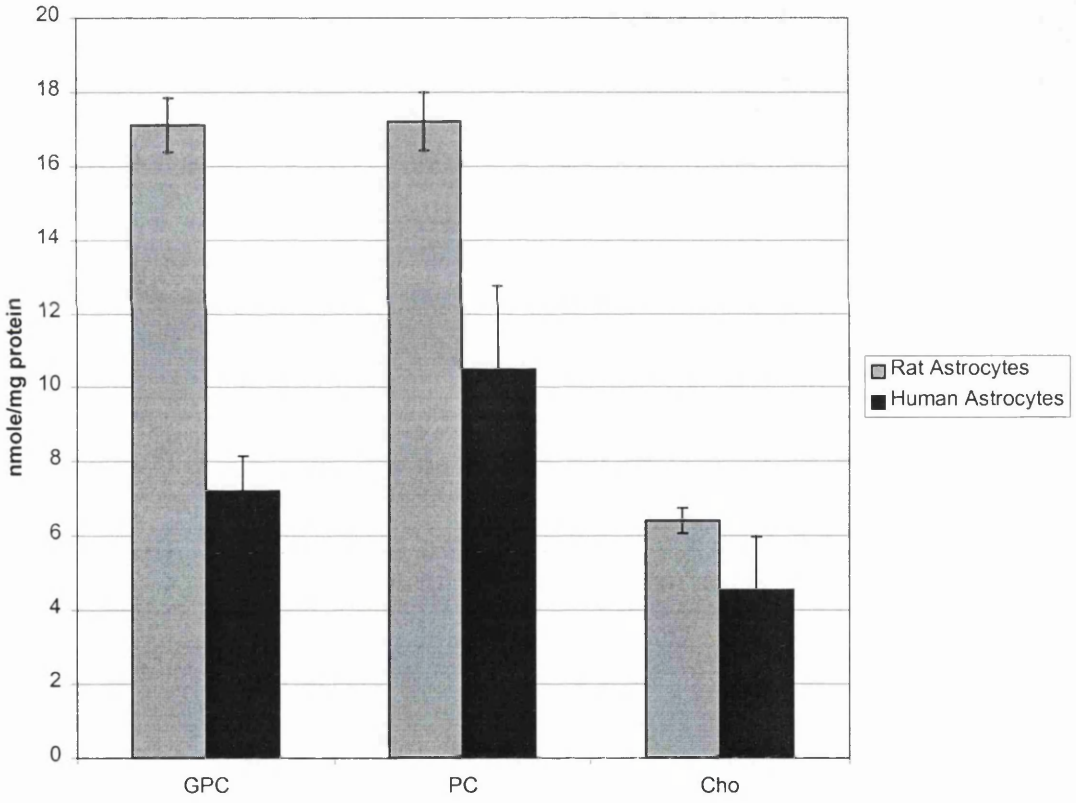


Figure 4-3. **Comparison of the average choline-containing compounds measured from ^{1}H-NMR spectra of extracts from human and rat astrocyte cultures.** *Concentrations are expressed as nmol/mg protein. The average ratio in rat astrocyte extracts is GPC=PC>Cho and in human astrocyte extracts is GPC=PC=Cho. Analysis of individual samples, however, revealed a common trend of similar GPC and PC concentrations in astrocyte cultures but this is different to what is seen in whole brain (see figure 4-4).*

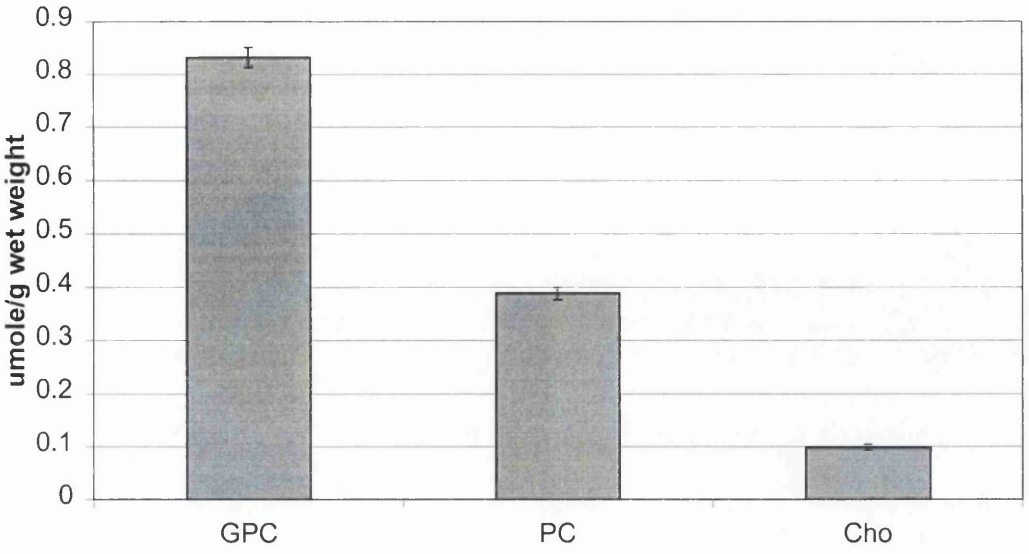


Figure 4-4. Comparison of individual choline-containing compounds from ^{1}H-NMR spectra: M/C extracts of whole rat brain. *This data is from the study in Chapter 3 comparing extraction techniques. Concentrations expressed as μmol/g tissue wet weight. Abbreviations: GPC= glycerophosphocholine, PC = phosphocholine, and Cho = choline. The ratio of the compounds observed for whole brain in the rat is: GPC>PC>Cho. However, this is different from what is seen in M/C extracts from rat astrocyte cultures (GPC=PC>Cho) (see figure 4-3).*

^{31}P-NMR studies of tumours and tumour cells have shown growth-related increases in the phosphomonoesters (PME) which include PC and phosphoethanolamine (PE). Increases in PMEs have been shown by a number of studies to be associated with tumour progression whilst decreases are seen with successful treatments (Hirakawa, *et al.* 1988; Smith, *et al.* 1991; Dixon, *et al.* 1991; for reviews see De Certaines, *et al.* 1993; Negendank, 1992; Ruiz-Cabello and Cohen, 1992). These changes in cellular phospholipid metabolism are also seen in other highly proliferative tissues such as regenerating neonatal liver (Iles, *et al.* 1990; Murphy, *et al.* 1992), neonatal brain (Williams, *et al.* 1985), and stimulated lymphocytes (Dingley, *et al.* 1992). However, Bhakoo, *et al.* (1996) have demonstrated that elevated PC does not always correlate with high rates of proliferation in a range of normal and transfomed cell cultures.

There is evidence for both an anabolic and catabolic pathway involved in the increase of PMEs in rapidly growing cells:

(1) Cell stimulation by growth factors, hormones, and tumour promoters has been shown to increase choline and ethanolamine kinase activity and subsequent PC, PE, phosphatidylcholine (Ptdcho), and phosphatidylethanolamine (PtdEth) synthesis (Warden and Friedkin, 1985). PC and PE are intermediates in the pathway of membrane synthesis as precursors of the membrane-bound phospholipids, Ptdcho and PtdEth, and their elevation can be interpreted as a reflection of increased membrane synthesis for proliferation. Gillies, *et al.* (1994) have shown in rat glioma cultures that the uptake and phosphorylation of exogenous choline is the only route of net PC synthesis which is increased with proliferation and decreased with quiescence (i.e., confluence). PC decreases were equally shown to be associated with a down-regulation of the uptake/phosphorylation of choline.

(2) Alternatively, elevated PME in rapidly growing cells may be due to elevated phospholipase activity and consequent Ptdcho and PtdEth breakdown with PC, PE, choline and ethanolamine release which is known to be stimulated by a number of factors including transformation (Macara, 1985; Billah, *et al.* 1990). Gillham and Brindle (1996) have shown that stimulation of phospholipases C and D in several tumour cell lines and primary hepatocytes resulted in increased PC in myeloma cells and hepatocytes and increased PE in most of the cell lines. However, the pathways described in (1) and (2) are not mutually exclusive; PC and PE production may involve both pathways.

Therefore, the relatively high PC measured in human and rat astrocytes in the present study compared to relatively lower PC seen in whole brain may be an indication of the higher rates of proliferation observed in culture. Thus, in this case high PC may reflect high rates of membrane cycling rather than being lineage-specific for astrocytes. The variation in the choline compounds in the astrocyte extracts relative to each other may also reflect slight differences in the proliferative state of the cells at the time of harvesting. Confluence was determined visually and this may have led to some variation between the fraction of cells in a particular cell cycle phase. Thus, the lower [PC] relative to [GPC]

seen in whole brain may reflect the low proliferative state of the adult brain. The general enrichment of PC observed in the astrocyte cultures derived from developing brain may reflect the higher proliferation rates seen in cell culture and in fetal/neonatal tissues.

PME and PDE levels in cultured cell systems can exhibit considerable variations due to many environmental factors (Gillies, *et al.* 1994; Shedd, *et al.* 1993; Kuesel, *et al.* 1990; Franks, *et al.* 1996). A number of human cell lines in a variety of media formulations are able to maintain phospholipid metabolite patterns seen in the same cells implanted *in vivo* but other cell lines are more sensitive to the concentrations of specific media components such as choline, ethanolamine, and glucose (Hull, *et al.* 1990 & 1992; Franks, *et al.* 1996). Thus, comparisons to other culture studies in the literature are difficult and experimental factors such as temperature, pH, media and serum composition, cell cycles, harvesting methodology, etc. must be carefully controlled for experimental comparisons. In the present study (and in Chapter 5) most of these factors were controlled since cells were grown in identical media and serum and subject to the same culture and harvesting methodologies. However, the culture media contained free choline, which may have contributed to the high PC observed in the cell extracts from both groups if choline kinase activity was high in the cells.

4.4.4 Glutamate-glutamine

There were also differences in the relative ratios of the amino acids glutamine and glutamate observed in the different species (fig. 4-5). In the rat astrocyte cultures glutamine was more than twice the concentration of glutamate but in the human cultures glutamate was very slightly higher in concentration than glutamine. Astrocytes exclusively contain the enzyme, glutamine synthetase, for the conversion of neuron-exported glutamate to glutamine. Thus, the differences in rat and human astrocyte concentrations of these compounds despite identical culture conditions may indicate that the rates or even the compartmentation for astrocytic glutamate catabolism and glutamine synthesis are different in the two species.

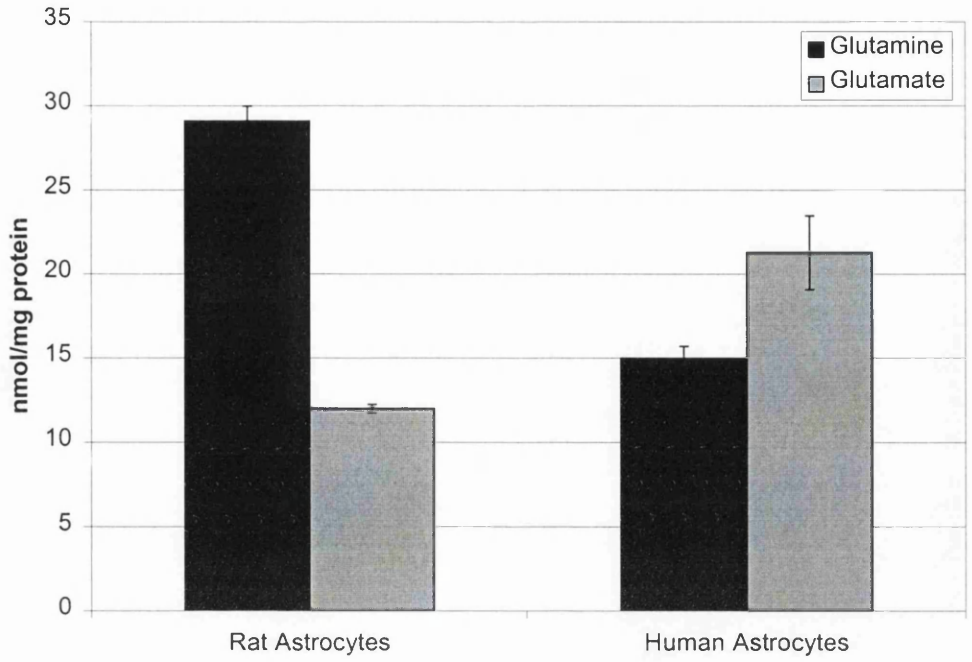


Figure 4-5. **Comparison of the ratios of glutamate and glutamine from ^{1}H-NMR spectra of M/C extracts from both human and rat astrocyte cultures.** *Concentrations are expressed as nmol/mg protein. Rat astrocyte extracts contain the ratio Gln>Glu whereas, the human astrocyte extracts have the opposite ratio Gln<Glu. This indicates that there may be significant species differences in the compartmentation and/or metabolism of these metabolites in the brain.*

To summarise, under identical culture conditions, rat and human astrocytes have several metabolic differences detectable by NMR spectroscopy that may reflect differences in cellular energy and precursor requirements. Thus, the *in vivo* situation may not be represented for both cells in the standard culture conditions thought to support their cell type. This indicates that there must be several species differences in how astrocytes handle substrates and synthesise amino acids. This is of some significance considering that the vast majority of studies on CNS cell metabolism have been performed on rodent cell cultures.

4.4.5 Developmental metabolism

Both rats and humans are neurologically immature at birth and use a mixture of glucose, ketone bodies, and fatty acids for catabolic and anabolic actvities until the development of enzymes for the complete oxidation of glucose is completed (Clark *et al.* 1993). Although these substrates were available to the

astrocytes in this study in the culture media and fetal calf serum, their utilisation might have differed because of differences in the time-course of metabolic maturation between species. If these differences were substantial between the two groups, the NMR-observed differences between the human and rat cell extracts would be more heavily influenced by their developmental stage rather than differences in their innate metabolic pathways. However, there is some evidence that this is not likely to be the case, despite the fact that the human astrocyte cultures were established from fetal tissues while the rat astrocytes were taken from early post-natal rat brain which is known to mature more quickly than human brain. The human cells, therefore, could be developmentally less mature even though both the human and rodent cells are thought to undergo spontaneous differentiation/maturation in culture. Yet it is the human astrocytes that contained no detectable levels of taurine and hypotaurine which should be *higher* in less developed tissue/cells and decrease with maturation (Florian *et al.*, 1996). Therefore, the metabolism of taurine in the human cells (i.e. a lack of synthesis) is more likely a reflection of a species-related metabolism rather than reflecting the early developmental stage related to brain-taurine. The case for creatine and glutamate/glutamine is less clear. There is a known increase in the total creatine concentration in rat brain with age (Bates *et al.* 1989; Lolley *et al.*1961) and also with glutamine synthetase activity (Caldani *et al.* 1982) which could account for the lower creatine and glutamine concentration in the human astrocytes. Vernadkis and Woodbury (1962) reported a progressive increase in the concentrations of glutamate, glutamine, GABA, and aspartate in rat brain up to adult levels over 45 days. However, in the present study the potentially more immature cells, the human astrocytes, had higher glutamate and aspartate than the rat astrocytes. Since the metabolic characteristics do not generally follow the known patterns of development, the differences observed are more likely to arise from species differences. However, it should be considered that the developmental metabolism studies in the literature have been carried out using rodent cells and tissue.

I have extracted one sample of human astrocytes derived from adult autopsy material using the same harvesting and extraction methods as the other astrocytes in the present study (cells provided by Dr. Nick Gutowski, Institute of Neurology, London). The adult human astrocytes appear to have elevated taurine and glutamate and decreased choline-containing compounds and

glutamine compared to the human astrocytes derived from fetal tissues (table 4-4). Since this is only 1 sample, I can only tentatively suggest that the developmental pattern of NMR-observable metabolites may be different in human cells. For example, based on the developmental metabolism established in rodent cultures, taurine would be expected to be lower in the adult cells but this was not the case in the adult human astrocyte sample.

	RAT ASTROCYTES (NEONATAL)	**HUMAN ASTROCYTES** (FETAL)	**HUMAN ASTROCYTES** (ADULT)
Inositol	13.7 ± 0.4	8.3 ± 1.2	11.85
Creatine	21.7 ± 0.6	3.9 ± 0.5	2.09
Glycine	4.4 ± 0.2	5.1 ± 0.8	6.9
Taurine	16.1 ± 0.7	ND	7.46
Chols	40.7 ± 1.8	22.2 ± 4.6	5.77
Hypotaurine	40.0 ± 2.0	ND	ND
Glutamine	29.0 ± 0.9	15.0 ± 0.7	6.81
Glutamate	12.0 ± 0.2	21.3 ± 2.2	38.94
Alanine	3.9 ± 0.2	3.4 ± 0.3	3.12
Lactate	14.8 ± 0.5	10.8 ± 1.4	12.66
	N = 6	*N = 4*	*N = 1*

Table 4-4. **Comparison of rat and human astrocytes derived from neonatal, fetal, and adult brain.** *Metabolite concentrations are expressed as nmol/mg protein and mean ± SEM. Abbreviations are as follows: Chols = choline-containing compounds (glycerophosphocholine + phosphocholine + choline). ND = not detected.*

4.4.6 Membrane phospholipids

The absolute concentrations of the extracted phospholipids from the rat astrocytes are in good agreement with Murphy, *et al.* (1995). This is the only reference I have found which reports the quantitative concentrations of extracted phospholipids rather than reporting the data as percentages of the total lipids. For comparison to all other literature values for rodent phospholipids, I have expressed my data as a percentage of the total phospholipids measured (table 4-5). There has not, to my knowledge, been a report of the phospholipid composition of human astrocytes for comparison.

	PtdCho	PtdEth	PtdSer	PtdIno	REFERENCE
Human Astrocytes	74 - 81	3 - 5	9 - 11	8 - 10	present study
Rat Astrocytes	74 - 82	4	8 - 12	7 - 9	present study
Rat Astrocytes	53 - 64	27 - 34	2 - 10	3 - 10	(1), (2), (3), (4), & (6)
Whole Rat Brain	45 - 52	32 - 38	13 - 14	3 - 4	(4)
Hamster Astrocytes	72 - 75	13 - 19	4	5 - 8	(5)

Table 4-5. **Phospholipid yields in rat and human astrocytes compared to literature values**. *The data from the literature has been adjusted to reflect the percentage of the total phospholipids measured in the present study (PtdCho + PtdEth + PtdSer + PtdIno) and not other lipids such as sphingomyelin, etc. which may also have been reported. The following references were used to compile this table: (1) Murphy et al., 1995; (2) Murphy et al., 1997; (3) Poduslo et al., 1983; (4) Norton & Poduslo, 1971; (5) Eichberg et al., 1976; (6) Robert et al., 1983.*

Most of the previous studies are in good agreement with the present data (table 4-5). However, PtdEth was considerably lower in my extracts. There are several reasons why this may have resulted:

(1) There are many different techniques employed for lipid extraction of cells and tissue (Chapter 3) and previous studies (table 4-5) have used slightly different methods from each other and from the present study for lipid extraction. It is known that certain extraction methods are better at extracting particular lipid classes than other techniques. For example, the Folch method (Folch *et al.*, 1957) is thought to be particularly efficient for extracting acidic lipids from tissue. Therefore, it is possible that the M/C extraction methodology employed in the present study is not as efficient for extracting PtdEth than the extraction techniques utilised in the other studies.

(2) The media in which the cells for the present study were grown contained choline, serine, and inositol which may serve as precursors for the headgroups in phospholipid synthesis, but the media did not contain ethanolamine (see Chapter 2 for detailed media compositions). This media composition may have contributed to the low PtdEth composition of the cell membranes. Most of the studies in the literature have not mentioned the precise composition or commercial source of the media in which they cultured their cells. Therefore, it is difficult to determine whether or not the

media composition caused the low PtdEth in my cells by comparison to the literature values.

Nevertheless, the cells in the present study were exposed to identical culture conditions and media compositions. Therefore, any differences between the phospholipid composition for the rat and human astrocytes should be attributable to species-differences. However, there were no significant differences in phospholipid concentrations between the groups.

The qualitative composition (fatty acid acyl groups) of the individual phospholipids has been expressed as a percentage of the total for each phospholipid. A slightly more unsaturated composition in PtdEth and more saturated PtdIno was demonstrated in the human astrocytes and the data is in broad agreement with other studies on rodent cells in the literature (table 4-6). However, there is essentially no difference in the ratio of saturated to unsaturated fatty acids overall between the species. Taken as a whole, the human astrocytes' phospholipids are dominated by stearic (18:0) and oleic (18:1) acids which are present in equal proportions. Stearic and oleic acids are also the most abundant fatty acid side-chains in the rodent cells but they contain slightly more oleic than stearic acid.

	HUMAN ASTROCYTES					**RAT ASTROCYTES**			
	PtdCho	PtdEth	PtdSer	PtdIno		PtdCho	PtdEth	PtdSer	PtdIno
16:0	38	9	7	8	**16:0**	41	18	9	10
18:0	7	31	34	37	**18:0**	9	34	31	25
18:1	43	28	26	12	**18:1**	39	30	27	23
18:2	1	1	1	1	**18:2**	1	1	1	1
20:4	9	20	24	41	**20:4**	7	12	22	40
22:6	2	12	8	1	**22:6**	2	5	10	1
Ref	present study				Ref	present study			
	HAMSTER ASTROCYTES					**RAT ASTRO-GLIAL CELLS**			
	PtdCho	PtdEth	PtdSer	PtdIno		PtdCho	PtdEth	PtdSer	PtdIno
16:0	17	10	10	13	**16:0**	49	9	8	10
18:0	17	22	32	38	**18:0**	11	31	50	36
18:1	42	33	19	34	**18:1**	29	15	16	10
18:2	8	4	5	3	**18:2**	2	4	2	1
20:4	15	25	32	8	**20:4**	7	24	5	42
22:6	2	6	4	4	**22:6**	2	18	19	2
Ref	Robert *et al.*, 1979				Ref	Kanfer, 1986			

Table 4-6. **Fatty acid acyl groups as a % of total phospholipid in the present study and in the literature.** *See table 4-3 for abbreviations.*

There were only very small differences between the rodent and human astrocytes in qualitative and quantitative membrane compositions. The major differences between species were detected in the aqueous metabolites from the dual-phase extractions. In conclusion, data from primary rodent cell cultures must be treated with caution if they are to be used to model the metabolism of normal human cells since significant species differences in important cellular metabolites and pathways may exist.

Chapter 5: **Metabolic Differences Between Normal Human Astrocyte and Astrocytoma Cells: Evidence from ^{1}H-NMR and Mass Spectrometry for Lineage and Transformation-specific Features**

5.1 Introduction

Analysis of metabolite profiles by ^{1}H NMR is a powerful technique to probe the biochemical differences between normal and transformed cells, and can aid interpretation of clinical spectra. Many studies of human CNS tumours using *in vivo* ^{1}H-NMR spectroscopy have demonstrated that there are general metabolic characteristics common to CNS tumours such as decreased NAA, increased choline-containing compounds and lactate, and an alkaline pH compared to normal brain. Astrocytomas are frequently characterised *in vivo* by low total creatine, increased *myo*-inositol (although this may be due to an overlapping glycine peak), and the frequent presence of mobile lipid resonances (Chapter 1, 1.8 for review). However, differential diagnosis from *in vivo* spectroscopy remains difficult due to large voxel sizes, tissue heterogeneity, and partial volume effects. Thus, biopsy tissue and cell culture extracts have become a widely used methodology for investigating tumour metabolism. Several of these *in vitro* studies have identified more tumour-specific markers than those identified *in vivo* because the improved resolution allows the identification of otherwise overlapping or poorly resolved peaks and the use of internal standards allows more accurate quantitation than is possible *in vivo*.

Unfortunately, there is a great deal of variability in the quantitative concentrations of the metabolites detected by ^{1}H-NMR in the biopsy and cell culture extracts so that comparisons between different studies are difficult (table 1-1). Differences in the source of normal brain tissue, in the storage or extraction techniques, in the use of fresh biopsy tissue or cell cultures, and in the compositions of culture media can contribute to the variability between studies. Therefore, most tumour-specific profiles are developed by comparisons to other tumours within the same study, i.e. under identical experimental conditions. This has led to tumour-specific profiles that are heavily dependent on the particular tumours being compared. For example, in a comparison of glioblastoma multiforme (GM), lower grade astrocytomas, and neurinomas, the GM tumour would be distinguishable by high alanine and glycine but if a meningioma (which

has high alanine) is also compared, then GM tumours are distinguishable only by high glycine (Kinoshita *et al.*, 1997). Thus, depending on which tumours are being compared the characteristic metabolic profile for a tumour can vary. Another approach to overcoming these problems for differential diagnosis is to study purified normal CNS cells and their transformed counterparts in order to base tumour profiles on the differences between normal and transformed cell metabolism rather than on comparisons between other abnormal metabolisms. Furthermore, culture conditions are optimal for high rates of cell metabolism compared to biopsy tissue that may have suffered varying degrees of warm ischemia before extraction. Culture conditions also allow an exact matching of substrate availability and oxygenation which may vary in tissue samples from the brain.

The importance of making these comparisons on cells from the same species has been demonstrated in Chapter 4 in which rat and human astrocytes were shown to have quantitative and qualitative metabolic differences detected by proton MRS. This chapter reports a comparison of normal human astrocytes and grade IV human astrocytoma cells, in order to determine which features of the astrocytoma spectrum may be related to *transformation* and which may be related to *lineage*.

5.2 METHODS

Purified (99%) human astrocytes (fetal) were obtained from BioWhittaker, UK. Human astrocytoma (glioblastoma multiforme) cultures derived from primary tumours were obtained from the tissue bank at the Institute of Neurology, London. Both astrocytes and astrocytomas were subcultured in identical conditions (Chapter 2, 2.1). Cells were extracted using the M/C technique. The aqueous fractions of the cell extracts were prepared for ^{1}H-NMR. Fully relaxed proton spectra were obtained and analysed using a Varian Unity-Plus spectrometer operating at 500MHz (Chapter 2, 2.6). The lipid fractions (in chloroform) were analysed using an electrospray ionisation tandem mass spectrometer (Quattro One). Phospholipids were assessed for fatty acid compositions as well as quantitative concentrations. Protein content was determined using the method of Smith, 1985. Two-sample Student's t-tests were performed to detect differences in metabolite concentrations between the two species, and p values are reported without correction for the number of

comparisons. The results for the astrocyte groups are considered significantly different if $p<0.05/10$ (0.005; 5% level) or $p<0.01/10$ (0.001; 1% level).

5.3 Results

5.3.1 ^{1}H-NMR spectroscopy

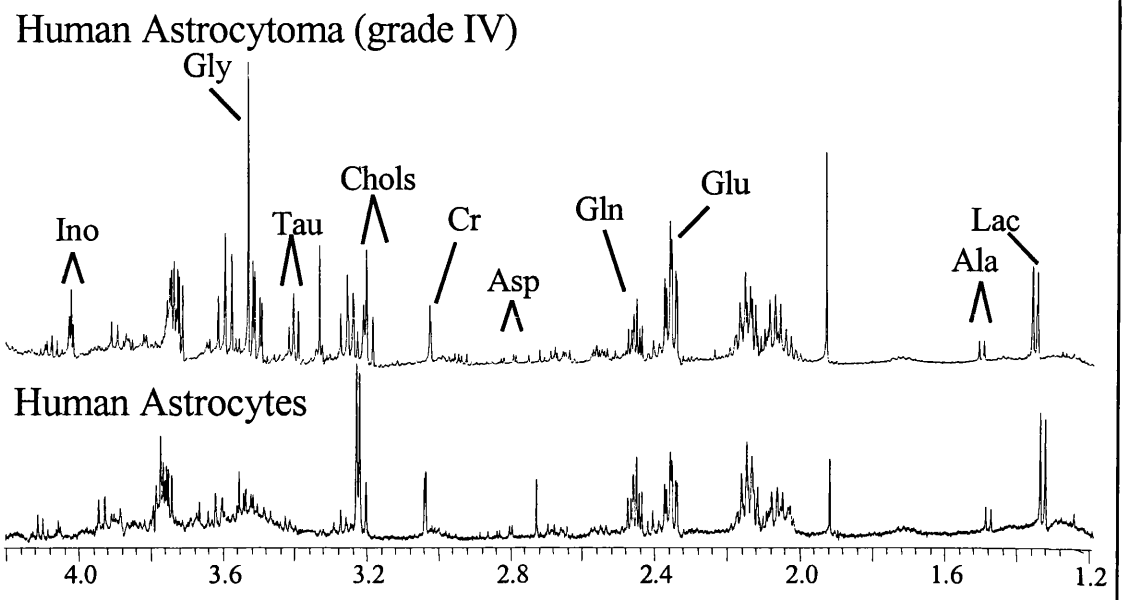


Figure 5-1. **Representative ^{1}H-NMR spectra of the aqueous fraction from the M/C extracts of human astrocytes and astrocytomas grown in identical culture conditions.** *Fully relaxed spectra were collected using 2,000 scans and tr = 22 sec. Spectra are referenced to TSP at 0 ppm (not shown). Abbreviations are as follows: Ino = myo-inositol; Cr = creatine + phosphocreatine; Gly = glycine; Tau = taurine; Chols = choline-containing compounds (glycerophosphocholine, phosphocholine, and choline); Gln = glutamine; Glu = glutamate; Asp = aspartate; Ala = alanine; Lac = lactate.*

Significantly higher amounts of *myo*-inositol, glycine, aspartate, and lactate were found in the human astrocytomas compared to human astrocytes grown in identical culture conditions ($p = 0.0003$, 0.00005, 0.002, and 0.001, respectively; $\alpha=0.005$; table 5-1). In addition, taurine, which was not detected (ND) in the normal cells was found to be present in the high-grade astrocytomas. Hypotaurine, however, was not detected in either the normal or transformed cells.

METABOLITES (NMOL/MG PROTEIN)	ASTROCYTOMA (N=8)	ASTROCYTES (N=4)	P VALUE
Myo-inositol	24.98 ± 2.7	8.27 ± 1.2	**0.0003**
Glycine	20.01 ± 1.9	5.12 ± 0.8	**5e-05**
Taurine	16.34 ± 1.7	ND	**
Chols	28.23 ± 3.2	22.22 ± 3.4	0.238
Creatine	4.25 ± 0.7	3.92 ± 0.5	0.718
Aspartate	17.66 ± 2.9	5.30 ± 1.9	**0.002**
Hypotaurine	ND	ND	
Glutamine	25.56 ± 3.0	15.01 ± 0.71	0.040
Succinate	2.04 ± 0.2	1.17 ± 0.2	0.051
Glutamate	58.93 ± 12.3	21.28 ± 2.2	0.020
Alanine	5.77 ± 0.7	3.43 ± 0.3	0.012
Lactate	22.64 ± 2.0	10.83 ± 1.4	**0.001**

Table 5-1. **Comparison of metabolites quantified from ^{1}H-NMR spectra of human astrocyte and astrocytoma extracts.** *Metabolite concentrations (nmol/mg protein) are expressed as mean ± SEM; Abbreviations are as follows: Chols= glycerophosphocholine + phosphocholine + choline; Creatine = creatine + phosphocreatine.*

5.3.2 Mass spectrometry

Mass spectrometry analysis of the membrane phospholipids demonstrated that there was no significant difference in total membrane phospholipid concentration but there was a significant reduction in phosphatidylcholine ($p = 0.0001$; $\alpha = 0.05/4$) and a significant increase in all other phospholipids (table 5-2). Analysis of phospholipid acyl groups indicated that the tumour cells had a slightly more saturated composition overall (table 5-3). The ratio of the average saturated to unsaturated fatty acid in the astrocytes is 0.75 compared to 0.85 in the astrocytomas. However, PtdCho, the most abundant phospholipid, was considerably more unsaturated in the astrocytoma cells. Furthermore, considering the total phospholipids (PtdCho + PtdEth + PtdSer + PtdIno), oleic (18:1) and stearic acid (18:0) are the dominant fatty acid acyl groups in equal proportions in the astrocytes but in the astrocytomas oleic acid was by far the dominant acyl group. The overall unsaturation is not increased in the astrocytomas despite the high proportion of 18:1 because the longer chain unsaturated fatty acids (18:2, 20:4, & 22:6) are decreased.

	HUMAN ASTROCYTES	HUMAN ASTROCYTOMAS	P-VALUES
PtdCho	97.67 ± 4.94	50.08 ± 4.84	**0.0001**
PtdEth	5.43 ± 0.69	11.11 ± 0.92	**0.0008**
PtdSer	12.37 ± 3.34	25.86 ± 4.33	**0.034**
PtdIno	10.84 ± 1.68	21.26 ± 1.40	**0.002**
Total	126.31 ± 10.65	108.31 ± 11.49	0.304

Table 5-2. **Comparison of membrane phospholipids between human astrocytomas and normal human astrocytes in culture.** *All metabolites are expressed as nmol/mg protein and as mean + SEM. Abbreviations are as follows: PtdCho = phosphatidylcholine; PtdEth = phosphatidylethanolamine; PtdSer = phosphatidylserine; PtdIno = phosphatidylinositol.*

	HUMAN ASTROCYTES				HUMAN ASTROCYTOMA *IN VITRO*			
	PtdCho	PtdEth	PtdSer	PtdIno	PtdCho	PtdEth	PtdSer	PtdIno
16:0	**37.9** ± 1.5	**8.5** ± 0.6	**7.3** ± 0.4	**8.0** ± 0.2	**31.7** ± 1.1	**10.3** ± 0.4	**10.2** ± 1.0	**17.1** ± 1.2
18:0	**7.0** ± 0.4	**31.1** ± 1.0	**33.5** ± 2.0	**36.7** ± 1.7	**4.0** ± 0.1	**39.0** ± 0.8	**35.9** ± 0.8	**33.8** ± 2.0
18:1	**43.1** ± 1.6	**28.4** ± 2.3	**26.1** ± 2.8	**12.3** ± 0.2	**50.9** ± 1.3	**29.9** ± 1.1	**32.1** ± 0.5	**14.3** ± 0.4
18:2	**1.0** ± 0.1	**0.7** ± 0.1	**1.1** ± 0.4	**1.1** ± 0.2	**5.1** ± 0.2	**0.2** ± 0.1	**0**	**0**
20:4	**9.0** ± 0.4	**19.6** ± 2.6	**23.9** ± 1.6	**41.1** ± 1.5	**8.0** ± 0.2	**12.0** ± 0.6	**17.8** ± 0.5	**32.1** ± 1.6
22:6	**2.0** ± 0.4	**11.5** ± 1.2	**8.1** ± 0.2	**0.9** ± 0.2	**0.5** ± 0.04	**9.1** ± 0.6	**3.9** ± 0.2	**3.0** ± 0.5

Table 5-3. **Phospholipid acyl group compositions.** *The fatty acid acyl groups are expressed as a percentage of the total of each phospholipid analysed. See table 5-2 for abbreviations.*

5.4 Discussion and Conclusions

The metabolic profiles of human astrocyte and astrocytoma cultures are different as assessed by ^{1}H-NMR spectroscopy and mass spectrometry. Moreover, several of the metabolites previously thought to be related to transformation may instead, reflect the lineage of the dominant cells of the tumour. The most common metabolic changes seen *in vivo* in human astroctyoma tumours compared to normal brain are: increased glycine, *myo-*

inositol, lactate, and choline-containing compounds (Chols); decreased creatine and N-acetylaspartate (Negendank, *et al.*, 1996; Kinoshita & Yokota, 1997). The astrocytoma cells in the present study showed similarly elevated glycine, *myo*-inositol, and lactate when compared to normal astrocytes. However, the low creatine and the absence of NAA were also characteristics of normal human astrocytes and total choline-containing compounds were also similar between the normal and transformed cells.

5.4.1 Creatine

Creatine, which on the basis of rat primary culture was believed to be enriched in astrocytes relative to neurons (Urenjak, 1993), was shown to be quite low in normal human astrocytes in Chapter 4. Thus, the characteristically low creatine observed clinically in human astrocytomas compared to normal brain may not be a feature of transformation but a reflection of the normal metabolism of the human astrocyte. However, it is possible that the low creatine in human astrocytes and astrocytomas, while reflecting how the human cells handle creatine in culture, resulted from the particular culture conditions used in the experiments. Given the abundance of astrocytes in the human brain (10 : 1, astrocytes : neurons) and the high creatine concentrations know to exist in the intact brain, it seems unlikely that the *in vivo* source of brain-creatine would be primarily a non-astrocytic cell type. The low intracellular creatine observed in both human cell groups in standard culture conditions in the present study suggests that creatine uptake could predominate over synthesis in these cells since the culture media contained significant quantities of the amino acid precursors of creatine, glycine and arginine, while creatine itself was present only at low concentrations from the 10% fetal calf serum supplement (Chapter 2 for media and serum compositions). Thus, the culture media composition may have contributed to the low intracellular creatine concentrations in one or both cell groups. On the other hand, it is possible that this low creatine in the human astrocytic lineage actually does exist *in vivo* and another CNS-cell type contains characteristically high creatine to account for the high creatine in normal brain. This possibility can not be ruled out until purified populations of other human CNS cells are similarly analysed for characteristic metabolic patterns. As mentioned in Chapter 4, this would not, however, rule out low creatine as a potential diagnostic marker of astrocytomas because the concentration of

transformed cells of the astrocytic lineage in the tumour mass may still present an overall lower creatine content than normal brain.

5.4.2 Choline-containing compounds

In addition to the changes in tumour cell glucose and amino acid metabolism, changes in the individual membrane phospholipids have also been shown to be altered in transformation. Phospholipid catabolites and anabolites are represented in the PME and PDE signals from ^{31}P-NMR and in the choline-containing compounds in ^{1}H-NMR. The aqueous metabolites GPC, PC, and free choline in ^{1}H-NMR of cell extracts may represent the precursors of phosphatidylcholine (Ptdcho) synthesis or the catabolites of Ptdcho hydrolysis. The PME resonance mainly comprises signal from PE and PC and the PDE signal is composed of GPC and GPE plus some additional contribution from mobile phospholipid head groups (reviewed in Podo, 1999 and Chapter 1, 10.1).

The most common *in vivo* ^{31}P-NMR feature in human brain cancers is a high PME signal and an elevated choline signal in ^{1}H-NMR. Tumour extracts have confirmed that the elevated PME resonance is due to either elevated PE or PC although a large amount of variability has been reported and there is not a clear relationship between proliferation and relative PC or PE levels (Kinoshita & Yokata, 1997). Elevated PC to GPC ratios have also been reported in a number of malignant brain tumours including meningiomas, glioblastomas, and neuroblastomas (Florian *et al.*, 1996; Bhakoo *et al.*, 1996; Usenius *et al.*, 1994). Several studies on non-CNS tumour cells have also shown that elevated PC can be specifically associated with malignancy (Ting *et al.*, 1996; Aiken *et al.*, 1996, for review see Podo, 1999). However, elevated PC is not exclusive to malignancy since it is known to be a feature of other highly proliferative tissues such as embryonic and fetal organs, regenerating liver, and some benign tumours (Iles *et al.*, 1990; Murphy *et al.*, 1992; Williams *et al.*, 1985). There is considerable evidence that elevated PC (but not PE) is correlated with the S + G1 phase of cell growth and high bromo-deoxyuridine incorporation (i.e., high proliferation) (Smith *et al.*, 1991, 1996). Studies have also shown that the relative amounts of PE and PC may accurately reflect the status of cell growth rates *in vivo* but not *in vitro* where media composition (usually containing free choline but not ethanolamine) influences PC and PE levels (Franks *et al.*, 1996; Hull & Wildenberg, 1998; Jackowski, 1996). Furthermore, Bhakoo *et al.*, (1996) have

shown that PC/GPC increases upon oncogene expression in conditionally transformed Schwann cells but this did not correlate simply with cell proliferation. Thus, the evidence in the literature so far has demonstrated that growth status, cell tumorgenicity, and culture conditions can contribute to elevated PC and PME in normal and transformed cells. Thus, the interpretation of elevated PC in cellular extracts remains unclear.

Even though the total choline-containing compounds (chols) are thought to be elevated in astrocytoma tumours compared to normal brain (Gill *et al.*, 1990; Fulham *et al.*, 1992; Kugel *et al.*, 1992), the total chols were not significantly different between the normal and transformed astrocytes in the present study. The ratios of the individual choline-containing compounds were also very similar between the two groups with a relatively high contribution of phosphocholine compared to that seen in whole brain. This is in agreement with *in vivo* data from Usenius, *et al.* (1994) which showed no difference in absolute total chols in glioblastomas compared to normal white matter and showed in extracts that PC was enriched relative to GPC and choline.

The fact that PC was elevated in both the normal and transformed astrocytes in the present study could indicate that the changes in PC may not represent a transformation-related metabolism in the astrocytomas (figure 5-2). Usenius, *et al.* (1994) showed elevated PC in biopsy extracts from grade IV astrocytomas but reduced PC relative to GPC in the lower grade astrocytomas (I-III) and normal white matter. Given the fact that neither the lower grade astrocytomas (which would have higher rates of proliferation than normal brain) nor the normal white matter had elevated PC, it is surprising that the normal astrocytes in the present study had relatively enriched PC.

The media used in the present study contained free choline and this could contribute to elevated PC synthesis. Gillies, *et al.* (1994) demonstrated that higher PC in log-phase compared to stationary (quiescent) cultures of rat gliomas was specifically related to synthesis of PC from media choline in the proliferating cells. The similarity in relative choline compound compositions between normal and transformed cells in the present study may, therefore, be influenced by the composition of the culture media and not representative of *in vivo* metabolism. This scenario would require a high rate of choline phosphorylation by choline kinase in these cells since intracellular choline is not high compared to PC. High rates of choline kinase activity have been shown to

be related to high rates of cell proliferation and membrane synthesis (Warden & Friedkin, 1984; Kent, 1990). Thus, if both the normal astrocytes and the astrocytomas had high rates of membrane synthesis, both would be expected to have elevated PC. This could be a characteristic of lineage and/or developmental stage in the cells in the sense that high rates of PC synthesis are associated with high cell proliferation rates.

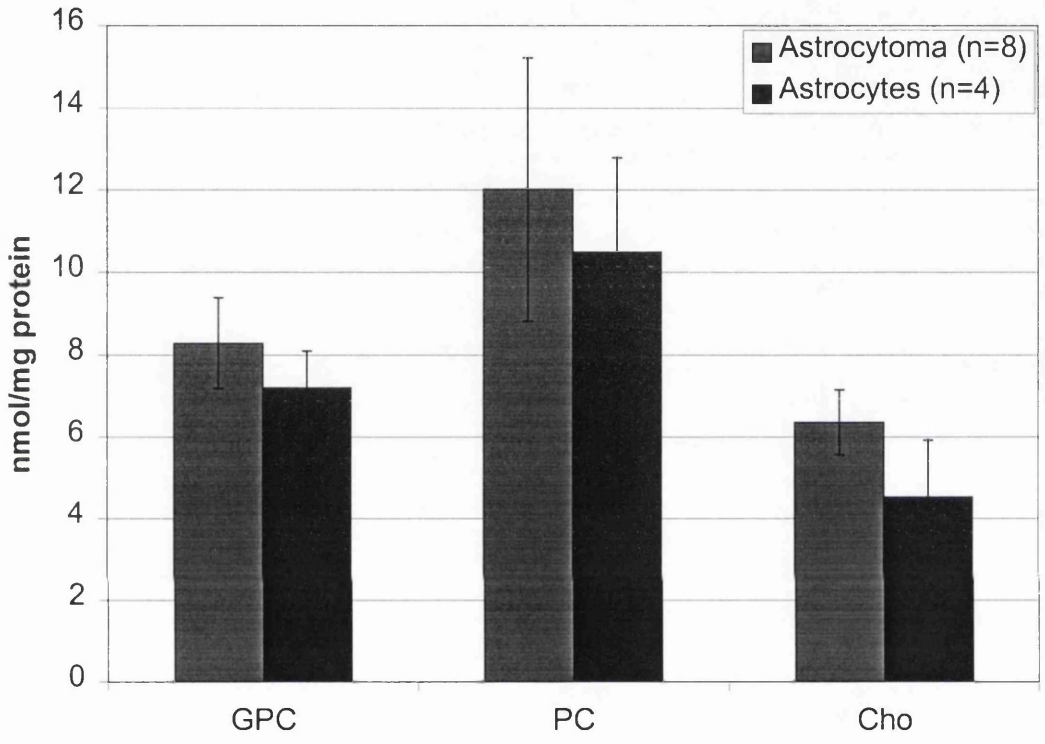


Figure 5-2. **Individual choline-containing compounds from ^{1}H-NMR spectra of human astrocyte and astrocytoma extracts**. *The abbreviations are as follows: GPC = glycerophosphocholine; PC = phosphocholine; Cho = choline. The metabolites are expressed as nmol/mg protein and as mean + SEM. Both normal human astrocytes and the high-grade glioblastomas have PC>GPC>Cho.*

Both growth status and transformation can contribute to elevated PC and these processes may utilise different pathways (PtdCho anabolism vs. catabolism). For example, Podo *et al.*, (1996) have shown that a PtdCho-specific phospholipase C is translocated from the cytoplasm to cell membranes upon oncogene-induced transformation in fibroblasts. The contribution of Ptdcho hydrolysis to intracellular [PC] following mitogen or oncogene stimulation of

membrane phospholipases is another possible source of the elevated PC observed in cellular and tissue extracts. However, both the normal and transformed cells in the present study had the same profile of chols and the standard media in which they were both grown is not known to stimulate PtdCho hydrolysis on its own. Therefore, evaluation of the differences in cell membrane Ptdcho content between human astrocytes and astrocytomas by mass spectrometry in the present study may help clarify whether or not the high PC observed in both groups was differentially influenced by choline uptake and phosphorylation in normal cells and by Ptdcho breakdown in transformed cells.

5.4.3 Aspartate-glutamate

Glutamine, which was present in the cell media at a concentration of 4mM and is 5.8 –9.7 mM in human blood plasma (Hiscock and Mackinnon, 1998), has been shown to be an important fuel for rapidly dividing cells in culture, especially astrocytes (Zielke *et al.*, 1984; Krebs, 1980; Yu and Hertz, 1983). In tumour cells, glutamine is considered to be an even more important substrate than glucose despite high rates of glycolysis, and the rate of glutamine utilisation has been correlated with the degree of malignancy (Kovacevic and Morris, 1972; Board *et al.*, 1990; Reitzer *et al.*, 1979; Lazo, 1981; Mc Keehan, 1982). Despite having a full complement of TCA cycle enzymes many tumours are known to carry out oxidation of glutamine predominantly via the left-hand side of the TCA cycle (i.e., from α-ketoglutarate to oxaloacetate) (Board *et al.*, 1990). This partial oxidation of glutamine, known as glutaminolysis, yields 12 mols ATP/ mol Gln and aspartate and has been shown to be an important energy source in many tumours (Reitzer *et al.*, 1979; Lazo *et al.*, 1981; Mckeehan, 1982; Board *et al.*, 1990). The apparent inhibition of the right side of the TCA cycle is not fully understood but there is some evidence that the high glutaminase activity observed in several tumours is important for cholesterol synthesis (Parlo & Coleman, 1984, 1986) and for producing aspartate from which purine and pyrimidine nucleotides can be formed (Newsholme *et al.*, 1985). By exporting citrate generated from pyruvate out of the TCA cycle for cholesterolgenesis only substrates (e.g., glutamine) entering the TCA cycle beyond citrate (i.e., the left side of the cycle) can be metabolised for ATP-generation and are therefore vital for maintaining cellular energy in cells with high rates of membrane/cholesterol synthesis. Therefore, the elevated concentration of aspartate in the

astrocytomas in the present study may reflect characteristically high rates of glutaminolysis in transformed cells. It is worth noting that the astrocytoma extracts discussed in Chapter 9, where the cells were incubated in a media lacking glutamine, had a concentration of aspartate (7.9 ± 1.4 μmol/mg protein; mean ± sem) that is similar to the normal astrocytes and much lower than the astrocytomas incubated in media with glutamine (30.1 ± 1.9). Thus, it is likely that the high aspartate (and glutamate) concentration in the transformed cells is a reflection of high rates of glutaminolysis and subsequent aspartate accumulation.

5.4.4 Glutamate/glutamine ratio

Both the normal human astrocytes and the astrocytomas had [Glu] > [Gln] whereas in Chapter 4 the rat astrocytes were shown to be enriched in glutamine relative to glutamate. The relative enrichment of glutamate in human astrocytic cells compared to whole brain could suggest that the compartmentation of glutamate and glutamine may be different in the human brain compared to the rodent brain. However, the glutamate concentration was significantly higher in the astrocytoma cells compared to the normal human astrocytes. This abnormally high level of Glu may indicate an increased use of the glutaminolysis pathway by the transformed cells. Similarly, the increased glutamate in the human astrocytes compared to the rodent astrocytes may also reflect glutaminolysis in the human cells or lower glutamine synthase activity. If this were to be the case, many of the studies that have elucidated the important pathways of brain metabolism may require re-evaluation in human cells.

5.4.5 Glycine

High glycine is commonly observed clinically in astrocytomas and in experimental gliomas (Gyngell *et al.*, 1992; Carpinelli *et al.*, 1996; Kinoshita *et al.*, 1994, 1997). This metabolite was significantly higher in the present study in astrocytoma cells compared to their normal counterpart. Rodent astrocytes have been shown to degrade glycine by a glycine cleavage system which is not present in neurons (Sato *et al.*, 1991; Bommakanti *et al.*, 1996). Additionally, astrocytes have been shown to convert serine (which was present in the culture media in the present experiment) into glycine which can be subsequently used for the synthesis of glutathione and creatine (Dringen & Hamprecht, 1996;

Dringen *et al.*, 1998). Thus, if the low creatine concentrations detected in the human astrocytes and astrocytomas were due to a lack of creatine synthesis then the precursor, glycine, may accumulate in astrocytomas if the glycine cleavage system in not functioning normally in the transformed cells. Alternatively, high glycine can arise via the glycolytic intermediate phosphoglycerate in association with the high rate of glycolysis in tumour cells but the purpose of elevated glycine synthesis is not known.

5.4.6 Taurine

Since the human astrocytes do not contain NMR-detectable quantities of taurine, the relatively high concentration detected in the astrocytoma cultures is likely to be a feature of the cancerous cell. The lack of taurine in the normal astrocytes is in agreement with the observation that human brain *in vivo* has low taurine/hypotaurine (Perry *et al.*, 1981). The enzymes catalysing hypotaurine/taurine synthesis from cysteine have been shown to be present only in glial cells (Almarghini *et al.*, 1991). Although the localisation of taurine synthesising enzymes in glial cells was determined in rodent cells, the fact that the human astrocytomas were able to synthesise taurine indicates that the normal human astrocytes may also have the appropriate enzymes even though intracellular taurine was not detected in these normal cells. Taurine levels are known to be highest in the developing brain in both humans and rats and this decreases with age. Therefore, the higher taurine in the astrocytoma cultures may reflect a dedifferentiation to an earlier developmental state/metabolism. Remy *et al.*, (1994) demonstrated in an experimental rat glioma model that taurine is significantly higher than in normal rat brain and this is associated with cellular proliferation and tumour aggressiveness (Moreno *et al.*, 1993). Peeling & Sutherland (1992) also report a higher taurine concentration in malignant human astrocytomas compared to normal brain. Although taurine is known to have roles as neuromodulator and osmolyte in the normal brain, its role in tumour metabolism has not been addressed.

5.4.7 Inositol

Inositol (Ino) was significantly higher in the astrocytoma cultures in the present study. Frahm *et al.*, (1991) have also reported elevated *in vivo* concentrations of Ino in human grade IV astrocytomas. Elevated inositol has

been shown to be a characteristic of the rat C6 glioma (Portais *et al.*, 1992; Merle *et al.*, 1992; Gyngell *et al.*, 1992) and to be specifically associated with a de-differentiated state in human colon adenocarcinomas (Galons *et al.*, 1989). Furthermore, Ino is a precursor for the membrane phospholipid, phosphatidylinositol, and has been suggested to be an important cellular growth requirement in mammalian cells because its derivatives are involved in a signaling pathway important to cell proliferation (Sze & Jardetzky, 1990). Free inositol is a component of the cell media used in the present study and, therefore, the elevated intracellular inositol content may reflect increased uptake by the astrocytoma cells.

5.4.8 Lactate

One of the most common features of transformation is high levels of aerobic glycolysis. Therefore, it was not surprising to observe elevated lactate in the astrocytoma cells and this can be considered a genuine feature of transformation in theses cells. Unfortunately, the common nature of high glycolysis in tumours means that lactate is unlikely to be useful for the differential diagnosis of astrocytomas.

5.4.9 Developmental metabolism

It must be stressed that this comparison has been made between tumour cells and astrocytes derived from *fetal* material, thus some of the common features may be characteristic of immature, partly de-differentiated cells. This would be consistent with the notion that a loss of cell cycle control and reversion to an embryonic phenotype are associated with malignancy. For example, studies on developmental metabolic changes in rat brain have shown that the concentration of creatine increases during brain maturation (Bates *et al.*, 1989; Florian *et al.*, 1996). Therefore, the low creatine in the astrocytes and astrocytomas observed in this study may reflect an immature developmental state. However, the extract (n=1) of adult human astrocytes derived from autopsy material also had low creatine (table 4-4).

Comparison of the astrocytomas in the present study to the metabolic profiles reported by Florian, *et al.* (1996) in primitive neuroectodermal tumours (PNETs) may be useful because PNETs represent developmentally immature, multipotent cells. Although methodological variations (e.g., media compositions)

make direct comparisons of metabolite concentrations difficult, general trends such as elevated glycine, *myo*-inositol, and lactate compared to normal brain do indicate that there are some similarities between the grade IV astrocytomas in the present study and the undifferentiated PNETs, representing an immature metabolism.

5.4.10 Membrane Phospholipids

Mass spectrometry analysis of the membrane phospholipids in the lipid fraction of the M/C cell extracts showed a significant decrease in PtdCho and an increase in the other phospholipids in the astrocytoma cultures such that there was no significant difference in the total quantitative amount of membrane phospholipid. As described in section 5.4.2, this may aid in the interpretation of the aqueous metabolite concentrations since the lipids analysed are from the same samples. Thus, although both the normal human astrocytes and the astrocytoma cells both had enriched PC, the transformed cells had a significantly decreased PtdCho concentration. This suggests that PtdCho hydrolysis may contribute to the elevated PC in the astrocytomas while PC synthesis may predominate in the normal cells. Phospholipase and choline kinase activity would have to be measured in order to investigate this possibility more conclusively. Similarly, the elevated PtdIno in the astrocytoma cells may indicate that the elevated inositol in the aqueous fraction may reflect increased uptake for PtdIno synthesis.

When the data were expressed as a percentage of the total phospholipid (PtdCho + PtdEth + PtdSer + PtdIno) they were in good agreement with the literature (table 5-4) except for PtdEth which is unusually low. As mentioned in Chapter 4, this may be due to a lack of free ethanolamine in the media for PtdEth synthesis or due to a particular inefficiency of the M/C methodology used for PtdEth extraction. Therefore, apart from PtdEth, the astrocytoma phospholipids were similar to those from other primary human astrocytoma extracts (Kostic & Buchheit, 1970) and a human glioma cell line extract (Poduslo *et al.*, 1983). Unfortunately, to the best of my knowledge there is no data on normal human astrocyte phospholipids in the literature for comparison. Nevertheless, even though the extraction technique or cell media composition may have influenced the cell phospholipid profiles in the present study, both the normal and transformed cells were exposed to identical culture conditions and

the same extraction technique. Therefore, the differences between them should reflect true transformation-related alterations in membrane phospholipid concentrations.

	PtdCho	PtdEth	PtdSer	PtdIno	REFERENCE
Human Astrocytes	74 - 81	3 - 5	9 - 11	8 - 10	present study
Human Astrocytoma	42-50	9-11	20-28	19-21	present study
Human Astrocytoma	50	27	17	7	(8)
Human Glioma Line	56	14	20	10	(3)
Rat Astrocytes	53 - 64	27 - 34	2 - 10	3 - 10	(1), (2), (3), (4), & (6)
Rat C6 Glioma	49-81	10-30	1-11	8-9	(5), (6), & (7)
Hamster Astrocytes	72 - 75	13 - 19	4	5 - 8	(5)
Hamster Astrocytoma	51-72	19-29	4-11	5-9	(6) & (7)

Table 5-4. **Comparison between the present study and literature values of phospholipid yields in astrocytes and their transformed counterparts**. *The phospholipid concentrations from the present study are shown as a percentage of total phospholipid for comparison to the literature. The literature data has been re-calculated to reflect the percentage of the total phospholipids measured in the present study (i.e., PtdCho + PtdEth + PtdSer + PtdIno) and not other lipids such as sphingomyelin, etc. which may have been reported. ST hamster astrocytes = spontaneously transformed hamster astrocytes. The following references have been used: (1) Murphy et al., 1995; (2) Murphy et al., 1997; (3) Poduslo et al., 1983; (4) Norton & Podulso, 1971; (5) Eichberg et al., 1976; (6) Robert et al., 1983; (7) Hauser et al., 1976; (8) Kostic & Buccheit, 1970.*

The experimental animal tumour models in the literature do not reveal any consistent transformation-related changes compared to normal cells. For example, although there is a small decrease in PtdCho in rat C6 gliomas compared to normal rat astrocytes (Robert *et al.*, 1983), there is no difference in PtdCho between spontaneously transformed hamster astrocytes (Hauser *et al.*, 1976) and normal hamster astrocytes (Eichberg *et al.*, 1976).

There were also some differences in the fatty acid acyl group composition of the membrane phospholipids between the normal and transformed human cells in the present study. PtdCho, the most abundant and rapidly turning-over phospholipid (Miller *et al.*, 1987; Poduslo *et al.*, 1983; Hugues *et al.*, 1985; Eichberg *et al.*, 1976), was more enriched with oleic acid (18:1) while longer chain unsaturated fatty acids were decreased in the astrocytomas. Increased oleic acid uptake in transformed cells may be related to the known increase in oleic acid concentrations in the plasma of tumour-bearing patients. This may also reflect the fact that oleic acid (a monounsaturated fatty acid) is not only an efficient energy source, but unlike the saturated fatty acids, it is also readily incorporated into structural lipids in cells during growth and tissue repair (Leyton *et al.*, 1987). Polyunsaturated fatty acids, in contrast, have very low rates of oxidation (Coots, 1965). Therefore, oleic acid is a highly useful fatty acid to rapidly proliferating cells since it can be used readily for both energy metabolism and membrane synthesis. I would hypothesise that given the advantageous properties of oleic acid, tumour cells (or any other highly proliferative cell) may have a preference for this fatty acid which is readily available in blood plasma and breast milk *in vivo* and in fetal calf serum in culture. Therefore, the more rapidly turning-over phospholipid, PtdCho, may have an acyl group composition which reflects the increased use of oleic acid by the astrocytoma cells. This is in good agreement with the data from Robert, *et al.* (1983) which showed an increase in monounsaturated fatty acids (MUFA) and a decrease in the polyunsaturated fatty acids (PUFA) in rodent gliomas, particularly in the composition of PtdCho. Differentiation of the glioma cells with DcAMP resulted in a normalisation of membrane lipid composition (decreased MUFA and increased PUFA) (Robert *et al.*, 1983). Based on uptake of radioactively-labeled precursors, the authors concluded that the differences observed in fatty acid composition between the proliferative and DcAMP-differentiated gliomas was due to metabolism of the acyl groups (desaturation and elongation) rather than to differences in precursor uptake from the media. Similarly, I would suggest that in the present study the incorporation of intact oleic acid and the proportionally less long-chain acyl groups in the transformed astrocytes reflects a de-differentiated metabolic state. These data are in good agreement with other astrocyte and glioma extracts in the literature (table 5-5).

	RAT ASTROCYTE				HUMAN ASTROCYTE			
	PtdCho	PtdEth	PtdSer	PtdIno	PtdCho	PtdEth	PtdSer	PtdIno
16:0	41	18	9	10	38	9	7	8
18:0	9	34	31	25	7	31	34	37
18:1	39	30	27	23	43	28	26	12
18:2	1	1	1	1	1	1	1	1
20:4	7	12	22	40	9	20	24	41
22:6	2	5	10	1	2	12	8	1
Ref	Chapter 4				present study			
	HAMSTER ASTROCYTES				HAMSTER GLIOMA			
	PtdCho	PtdEth	PtdSer	PtdIno	PtdCho	PtdEth	PtdSer	PtdIno
16:0	17	10	10	13	20	8	22	6
18:0	17	22	32	38	12	24	33	46
18:1	42	33	19	34	48	37	37	43
18:2	8	4	5	3	10	1	4	1
20:4	15	25	32	8	9	25	4	3
22:6	2	6	4	4	1	4	0	0
Ref	Robert *et al.*, 1976				Robert *et al.*, 1976			
	RAT ASTRO-GLIAL CELLS				C6 GLIOMA			
	PtdCho	PtdEth	PtdSer	PtdIno	PtdCho	PtdEth	PtdSer	PtdIno
16:0	49	9	8	10	20	8	6	23
18:0	11	31	50	36	12	24	47	34
18:1	29	15	16	10	48	37	43	38
18:2	2	4	2	1	10	1	1	1
20:4	7	24	5	42	9	25	3	4
22:6	2	18	19	2	1	4	0	0
Ref	Kanfer *et al.*, 1986				Kanfer *et al.*, 1986			
	HAMSTER GLIOMA				HUMAN ASTROCYTOMA			
	PtdCho	PtdEth	PtdSer	PtdIno	PtdCho	PtdEth	PtdSer	PtdIno
16:0	17	10	13	10	32	10	10	17
18:0	17	22	38	32	4	39	36	34
18:1	42	33	34	17	51	30	32	14
18:2	8	4	3	5	5	0	0	0
20:4	15	25	8	32	8	12	18	32
22:6	2	6	4	4	1	9	4	3
Ref	Kanfer *et al.*, 1986				present study			

Table 5-5. **Fatty acid acyl groups as a % of total phospholipid in the present study and in the literature.** *See table 5-2 for abbreviations.*

The experimental rodent gliomas in the literature (table 5-5) similarly reported a substantial increase in oleic acid (18:1) with a concomitant decrease in longer chain PUFA fatty acids compared to normal rodent glial cells. The role of these

membrane phospholipid composition changes are not well understood but, they could be related to an increased use of oleic acid for oxidative metabolism as well as membrane synthesis in proliferative cells

In conclusion, these data are the first comparisons of normal and transformed CNS cells from the human grown under identical culture conditions. As a result, several lineage- and transformation-related characteristics of the astrocytoma have been identified.

Chapter 6: *In Vivo* vs. *In Vitro* Human Astrocytoma Metabolism

6.1 Introduction

Although there are many advantages to studying purified populations of cells in a completely controlled culture environment, there remains the possibility that the metabolic profiles observed in extracts do not occur *in vivo* where there may be conditions that affect cellular metabolism which have not been accounted for in culture. Experiments designed to investigate the specific metabolic alterations associated with transformation in a particular cell type such as astrocytes are best suited to cell culture investigations because the transformed and normal cells can be examined in experimentally identical environments. However, tumours *in vivo* and extracts of biopsy tissue have heterogeneous conditions of oxygenation, nutrient supply, and exposure to hormones and immunological factors. In culture most of these factors can be controlled and true transformation-related metabolism can be characterised and studied. In Chapter 5 the comparison of grade IV astrocytomas and normal human astrocytes in identical culture conditions identified several metabolic pathways such as increased glutaminolysis, which may be altered in the transformed cells. However, the metabolic characteristics identified by the *in vitro* study may only represent a potential metabolism by the cells rather than what is actually observed *in vivo*. For the purposes of differential diagnosis of intracranial tumours, the validity of *in vitro* metabolite profiles for each type of tumour must be established. As there can be a certain degree of variability between tumour cells obtained from different patient brain tumours, the ideal comparison is between tumour cells grown in culture and the same cells implanted in rodent brain and grown *in vivo*. Extracting the cells in culture and the (excised) tumour from the host-brain allows the maximum number of aqueous and lipid metabolites to be compared. I had originally intended to obtain *in vivo* localised ^{1}H-NMR spectra from the implanted tumours to compliment the high-resolution extract data. Unfortunately, tumour growth *in vivo* was relatively slow and the animals' body size outgrew the bore-size capacity of the NMR magnet before the brain tumours were large enough for localised spectroscopy (27μl volume minimum). Nevertheless, the extracted tumours would be more informative given the low resolution of *in vivo* spectroscopy and the advantage of obtaining data on

the membrane phospholipids in the lipid fraction of the extracts. The astrocytoma tumour extracts described herein are referred to as *in vivo* samples because the cells have been grown *in vivo* as opposed to in culture. If the metabolic pattern identified in the astrocytoma cells remains the same beyond the culture dish, then the real potential for the use of ^{1}H-NMR metabolic profiles can be realised.

6.2 METHODS

Human astrocytoma (glioblastoma multiforme) cultures derived from primary tumours were subcultured to obtain 10^{10} - 10^{12} cells per extract-NMR sample. The same astrocytoma cells were used for injection into immuno-deficient nude rat brain. Cells for injection were harvested using trypsin, centrifuged, and resuspended in normal cell medium (DMEM + 10% FCS). Cells were kept at 4 °C for approximately 10-20 minutes prior to injection. *Rnu-rnu* nude rats weighing approximately 40 grams were injected with 100,000 cells in a 2μl injection into the caudate nucleus according to the methods in Chapter 2. Approximately three weeks after implantation, tumour tissue and a sample of normal contralateral brain were surgically excised from the rat brain following *in vivo* NMR imaging to confirm tumour growth and location. Cell cultures and tumour tissue were extracted using the M/C technique. The aqueous fractions from the extracts were analysed using ^{1}H-NMR spectroscopy and the lipid fractions were analysed by electrospray ionisation tandem mass spectrometry. Phospholipids were assessed for fatty acid compositions as well as quantitative concentrations using internal standards as described in Chapter 2. Two-sample Student's t-tests were performed to detect differences between the metabolites in the tumour extracts and the cell extracts and between the extracts of tumour and contralateral brain tissues. P values are given without correction for the number of comparisons. Therefore, the results are considered statistically significant if $p<0.05/9$ (0.006; 5% level) or $p<0.01/9$ (0.001, 1% level).

6.3 Results

6.3.1 ^{1}H-NMR Spectroscopy

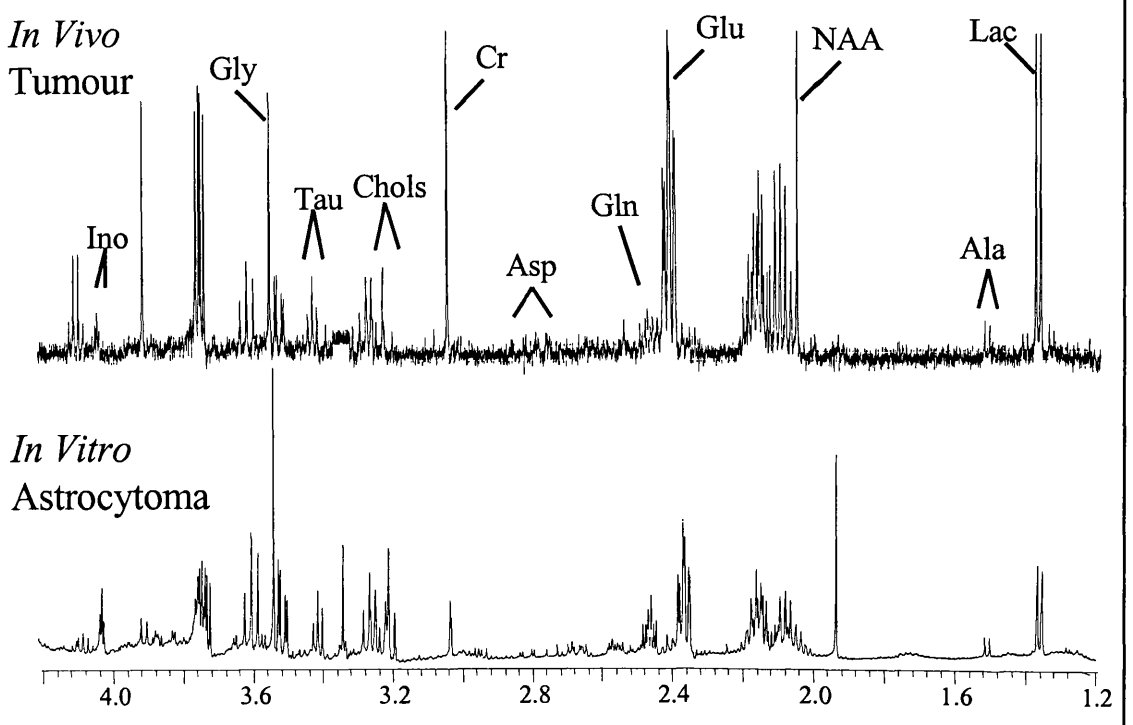


Figure 6-1. **Representative ^{1}H-NMR spectra of the aqueous fraction from the M/C extracts of the same astrocytoma cells grown *in vivo* and in culture.** *Fully relaxed spectra were collected using 2,000 scans and tr = 22 sec. Spectra were referenced to TSP (0 ppm). Abbreviations are as follows: Ino = myo-inositol; Gly = glycine; Tau = taurine; Chols = choline containing-compounds (glycerophosphocholine + phosphocholine + choline); Cr = creatine + phosphocreatine; Asp = aspartate; Gln = glutamine; Glu = glutamate; NAA = N-acetylaspartate; Ala = alanine; Lac = lactate.*

The only differences detected between the astrocytoma cells grown in culture and the excised tumours were higher concentrations of creatine and NAA in the *in vivo* tumour. The cultured cells had significantly lower creatine ($p=0.001$) and undetectable levels of NAA (table 6-1). There were several metabolic differences when the *in vivo* tumour extracts were compared to samples of normal contralateral brain corresponding to the same anatomical brain region as the tumour. The tumour tissue contained significantly higher glycine ($p = 0.002$), aspartate ($p = 0.003$), and glutamate ($p = 0.004$) (table 6-2).

	HUMAN GM TUMOUR *IN VIVO (N = 4)*	HUMAN GM *IN VITRO (N = 4)*	P-VALUES
Ino	18.0 ± 0.9	25.7 ± 3.7	0.137
Cr	22.4 ± 2.2	5.8 ± 0.9	**0.002**
Gly	27.5 ± 2.1	24.3 ± 1.7	0.271
Chols	14.6 ± 3.3	28.9 ± 4.9	0.059
Glu	142.3 ± 19.9	82.2 ± 15.4	0.054
Gln	34.2 ± 19.1	29.5 ± 2.3	0.824
NAA	16.7 ± 2.0	ND	**
Lac	73.9 ± 17.3	26.6 ± 2.2	0.073
Asp	22.6 ± 2.2	13.7 ± 1.8	0.021

Table 6-1. **Comparison of aqueous metabolites from M/C extracts of the same glioblastoma multiforme cells grown *in vitro* and *in vivo*.** *All metabolites are expressed as nmol/mg protein and as mean ± SEM. Abbreviations are as follows: Ino = myo-inositol; Cr = creatine + phosphocreatine; Gly = glycine; Chols = glycerophosphocholine + phosphocholine + choline; Glu= glutamate; Gln = glutamine; NAA = N-acetyl-aspartate; Lac = lactate; Asp = aspartate; GM = glioblastoma multiforme; α = 0.006 at 5% level and 0.001 at 1% level.*

	HUMAN GM *IN VIVO*	CONTRALATERAL BRAIN	P-VALUES
Ino	18.0 ± 0.9	13.0 ± 1.2	0.013
Cr	22.4 ± 2.2	34.9 ± 9.0	0.270
Gly	27.5 ± 2.1	3.6 ± 0.2	**0.002**
Chols	14.6 ± 3.3	8.3 ± 0.6	0.177
Glu	142.3 ± 19.9	16.0 ± 1.3	**0.004**
Gln	34.2 ± 19.1	12.8 ± 0.4	0.344
NAA	16.7 ± 2.0	29.1 ± 2.4	0.008
Lac	73.9 ± 17.3	39.6 ± 2.4	0.144
Asp	22.6 ± 2.2	7.2 ± 0.8	**0.003**

Table 6-2. **Comparison of the aqueous metabolites form M/C extracts of glioblastoma multiforme tumours compared to normal contralateral brain**. *All metabolites are expressed as nmol/mg protein and as mean ± SEM. See table 6-1 for abbreviations. N = 4 in both groups.*

6.3.2 Mass spectrometry

Mass spectrometry analysis of the lipid fraction of the cell and tissue extracts revealed that although the total phospholipid concentrations were not significantly different, phosphatidylethanolamine (PtdEth) was significantly elevated *in vivo* ($p = 0.0008$; $\alpha = 0.05/4$) while phosphatidylinositol (PtdIno) was significantly lower ($p = 0.002$; table 6-3).

	HUMAN ASTROCYTOMAS *IN VITRO*	*IN VIVO* TUMOUR	P VALUES
PtdCho	50.08 ± 4.84	43.16 ± 7.21	0.455
PtdEth	11.11 ± 0.92	25.30 ± 1.74	**0.0008**
PtdSer	25.86 ± 4.33	15.99 ± 4.56	0.155
PtdIno	21.26 ± 1.40	13.68 ± 1.02	**0.002**
Total	108.31 ± 11.49	98.13 ± 14.53	0.511

Table 6-3. **Comparison of membrane phospholipids between human astrocytomas grown *in vivo* and *in vitro*.** *All metabolites are expressed as nmol/mg protein and as mean ± SEM. Abbreviations are as follows: PtdCho = phosphatidylcholine; PtdEth = phosphatidylethanolamine; PtdSer = phosphatidylserine; PtdIno = phosphatidylinositol.*

Comparison of the astrocytoma cells grown *in vivo* with normal contralateral brain demonstrated a significant reduction in membrane phosphatidylcholine ($p = 0.004$; $\alpha = 0.05/4$) and a significant increase in phosphatidylethanolamine ($p = 0.0002$) in the tumour (table 6-4).

	IN VIVO TUMOUR	CONTRALATERAL BRAIN	P-VALUES
PtdCho	43.16 ± 7.21	93.08 ± 8.16	**0.004**
PtdEth	25.30 ± 1.74	5.73 ± 1.15	**0.0002**
PtdSer	15.99 ± 4.56	11.80 ± 3.70	0.504
PtdIno	13.68 ± 1.02	10.90 ± 1.64	0.131
Total	98.13 ± 14.53	121.51 ± 14.65	0.0532

Table 6-4. **Comparison of membrane phospholipids between human astrocytomas grown *in vivo* and normal contralateral brain.** *All metabolites are expressed as nmol/mg protein and as mean ± SEM. See table 6-3 for abbreviations.*

There was very little difference in the acyl group composition of the phospholipids between the astrocytoma cells grown *in vivo* and *in vitro* (table 6-5). The overall ratio of saturated to unsaturated fatty acids was essentially the same (0.8) for the astrocytomas grown in brain or in culture, although there was a trend toward a greater concentration of palmitic acid (16:0) and a lower concentration of stearic acid (18:0) in the *in vivo* tumours.

	HUMAN ASTROCYTOMA *IN VITRO*				HUMAN ASTROYCTOMA *IN VIVO*			
	PtdCho	PtdEth	PtdSer	PtdIno	PtdCho	PtdEth	PtdSer	PtdIno
16:0	**31.7** ± 1.1	**10.3** ± 0.4	**10.2** ± 1.0	**17.1** ± 1.2	**35.2** ± 0.2	**12.1** ± 1.2	**10.4** ± 0.6	**18.2** ± 0.6
18:0	**4.0** ± 0.1	**39.0** ± 0.8	**35.9** ± 0.8	**33.8** ± 2.0	**4.1** ± 0.4	**34.8** ± 1.3	**33.3** ± 1.3	**30.0** ± 1.8
18:1	**50.9** ± 1.3	**29.9** ± 1.1	**32.1** ± 0.5	**14.3** ± 0.4	**47.7** ± 0.4	**32.1** ± 1.2	**28.6** ± 1.1	**18.5** ± 1.0
18:2	**5.1** ± 0.2	**0.2** ± 0.1	**0**	**0**	**1.0** ± 0.1	**0**	**1.0** ± 0.3	**2.2** ± 0.3
20:4	**8.0** ± 0.2	**12.0** ± 0.6	**17.8** ± 0.5	**32.1** ± 1.6	**8.1** ± 0.2	**10.9** ± 0.7	**22.4** ± 2.1	**27.6** ± 1.2
22:6	**0.5** ± 0.04	**9.1** ± 0.6	**3.9** ± 0.2	**3.0** ± 0.5	**4.0** ± 0.5	**9.9** ± 1.0	**4.5** ± 0.5	**3.2** ± 0.6

Table 6-5. **Phospholipid acyl group compositions.** *The fatty acid acyl groups are expressed as a percentage of the total for each phospholipid analysed and mean ± SEM. See table 6-3 for abbreviations.*

6.4 Discussion and Conclusions

The only differences detected between the *in vivo* and *in vitro* astrocytomas in this study were elevated creatine and NAA in the extracted tumour tissue. Since NAA has been detected only in purified cultures of rodent neurons, O-2A progenitor cells, and mature oligodendrocytes (Urenjak *et al.*, 1993; Bhakoo *et al.*, 2000), its presence in the astrocytoma tumour was probably due to contamination by normal rat brain tissue within the extracted tumour mass. This is entirely likely, given the infiltrative nature of high-grade malignant tumours. Thus, the metabolic profile for the human astroctyoma established in culture appears to be, on the whole, a true reflection of whole-tumour metabolism. Furthermore, the comparison of the tumour tissue with normal contralateral brain demonstrated significantly elevated glycine, glutamate, and aspartate in keeping with the metabolic profile for high-grade astrocytoma

compared to normal human astrocytes established in Chapter 5 (figure 6-2). However, the astrocytoma cell cultures also had significantly elevated *myo*-inositol and lactate compared to normal astrocytes in the previous study.

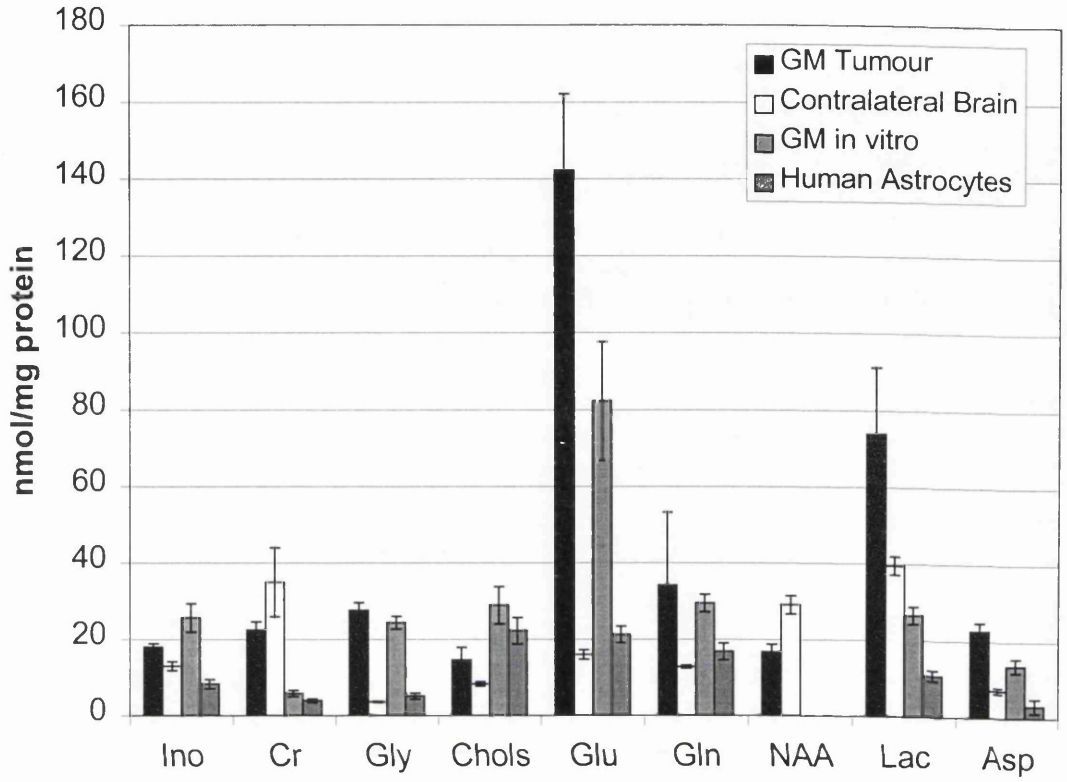


Figure 6-2. **Comparison of the aqueous metabolites from extracted astrocytoma cells grown *in vitro* and *in vivo* and their normal counterparts.** *See table 1 for abbreviations. Metabolites expressed as mean ± SEM. GM tumour = in vivo grade IV astrocytoma.*

6.4.1 Creatine

The comparisons of rat and human astrocytes in Chapter 4 and human astrocytes and astrocytomas in Chapter 5 indicated that low creatine may be a feature of human cells of the astrocytic lineage. The low creatine seen in the cultured astrocytes may be a result of the media composition (low creatine) and a lack of creatine synthesis from precursors (glycine and arginine) available in the media, which indicates a possible reliance by the human cells on creatine uptake. Apart from NAA, the only significant difference between the astrocytomas grown in rat brain and in culture was that creatine was also higher *in vivo*. The high creatine content in the tumours could be due to a greater supply of free creatine for uptake *in vivo*. However as discussed above, elevated

creatine may also reflect contamination by normal rat brain in the excised tumour mass. The fact that the extracted tumour tissue also contained NAA suggests that some normal-tissue contamination occurred. Therefore, it is difficult to draw any conclusions about creatine metabolism in the astrocytomas (i.e. uptake vs. synthesis) from the *in vivo* data. However, the *in vitro* data suggests that uptake may predominate over synthesis.

6.4.2 Inositol

Although the *in vitro* concentration of myo-inositol [Ino] was found to be significantly higher in grade IV astrocytomas compared to normal human astrocytes (Chapter 5), it was not significantly elevated in the implanted astrocytomas compared to contralateral brain in the present study. Therefore, rather than Ino being particularly high in astrocytomas, as was indicated by the comparison to normal astrocytes (Chapter 5), it is more likely that Ino was particularly low in the cultured normal human astrocytes. There are several reasons why this might occur. Firstly, the normal human astrocytes originated from fetal tissue and may not represent a fully mature metabolism in culture. Inositol is known to be lower in neonatal brain and increase with maturation (Bates *et al.*, 1989; Florian *et al.*, 1996). However, if this were the case, one might expect the astrocytomas to have a similarly low Ino content since they represent a de-differentiated, immature metabolism. Secondly, Ino is known to be higher in neonatal plasma compared to adults and to be significantly lower in the CSF of astrocytoma-bearing patients indicating that Ino uptake may be higher in astrocytoma cells compared to immature astrocytes (Pereira *et al.*, 1990). The reason that astrocytoma Ino uptake may not be different to normal brain in the present study may be because the tumours were implanted into adult rat brain which would presumably have a higher Ino uptake than the immature brain.

Finally, mass spectrometry analysis of cell phosphatidylinositol content indicated that the concentration of this membrane phospholipid was significantly lower in the *in vivo* astrocytomas but not significantly different to normal contralateral brain. Thus, if there is elevated uptake of Ino by the astrocytomas and adult brain as postulated above, it does not appear to be for the purpose of increased PtdIno synthesis.

6.4.3 Glycine

Elevated glycine was one of the distinguishing characteristics of the glioblastoma cultures compared to normal human astrocytes in Chapter 5. The abnormal glycine metabolism identified *in vitro* appears to also exist in intact brain tumours from the same cells. In the CNS, glycine is known to function as both a neurotransmitter and neuromodulator (reviewed in Rajendra *et al.*, 1997). Rodent astrocytes have been shown to remove glycine from the extracellular space after release from neurons where it is then converted to serine and released for subsequent neuronal uptake and metabolism (Verleysdonk *et al.*, 1999). This constitutes a glycine-serine cycle similar to the well-established glutamate-glutamine cycle in brain. Rodent astrocyte cultures have also been shown to take up glycine from the medium and metabolise it to serine, lactate, creatine, and glutathione (Verleysdonk *et al.*, 1999; Dringen and Hamprecht, 1998). Although it is still unknown whether the biosynthesis or the uptake of glycine is essential for the maintenance of glycine levels in the human CNS, there is evidence that disturbances in glycine degradation can lead to high intracerebral glycine concentrations in a neurological disease called nonketotic hyperglycinemia (Kure *et al.*, 1997). Thus, in the transformed astrocytes, glycine could accumulate due to either abnormally low glycine cleavage and/or creatine synthesis (e.g., causing the precursor, glycine, to accumulate).

6.4.4 Glutamate—Aspartate

Various tumour types are known to have dramatically enhanced cholesterol synthesis from citrate exported from cell mitochondria resulting in a truncated TCA cycle and high rates of glutaminolysis (Parlo & Coleman, 1984; Kovacevic & Morris, 1972; Kovacevic & McGivan, 1983). Under these conditions, amino acids, which can be oxidised in the remaining "active" half of the TCA cycle (from α-ketoglutarate to oxaloacetate) are a vital source of cellular energy and biosynthetic precursors. Glutamine can provide energy and anabolic precursors in both normal and transformed cells. In fact, a general metabolic feature of tumour cells and many rapidly dividing normal cells is that they are rapid consumers of glutamine. Although most of these glutamine-utilising cells are also known to have very active glutaminase and low glutamine synthetase, the subsequent steps in the oxidation of glutamine can vary with the type of cell, degree of malignancy, or the availability and influence of other exogenous

metabolites (Reitzer *et al.*, 1979; Moreadith & Lehninger, 1984; Matsuno, 1987; Medina *et al.*, 1992; Piva & McEvoy, 1992, 1998). The presence of glutaminase has been correlated with the progression of transformation in hepatomas (Knox *et al.*, 1969)

In standard culture conditions many different transformed cells metabolising labeled glutamine produce labeled glutamate and aspartate, suggesting that the oxidation of glutamine proceeds through aspartate aminotransferase rather than glutamate dehydrogenase (Moreadith & Lehninger, 1984; Hostetler, 1982; Glazer *et al.*, 1974; Piva and McEvoy, 1998). However, when tumour mitochondria were exposed to exogenous glutamate and malate there was a switch from aspartate to alanine efflux (Moreadith & Lehninger, 1984) where alanine aminotransferase replaces aspartate aminotransferase in the truncated TCA cycle. They further showed that it is the pyruvate formed via malic enzyme that causes the inhibition of aspartate aminotransferase. Under normal conditions, however, aspartate is the major product of tumour glutamine oxidation. Piva & McEnvoy (1998) suggested that the reasons for the low alanine aminotransferase activity seen in the intact cell are a restricted flux through malic enzyme and a lack of high cytosolic pyruvate concentrations due to the high affinity of pyruvate dehydrogenase for the substrate. Thus, aspartate aminotransferase is frequently more active and the glutamate formed via glutaminase undergoes transamination to yield aspartate.

Moreadith & Lehninger (1984) also demonstrated that, unlike the transformed cells, mitochondria from normal heart, liver, and kidney cells did not form alanine or citrate from glutamine metabolism but continued to produce aspartate even upon the addition of malate. Further analysis revealed that this was because only the tumour mitochondria contained malic enzyme, which catalyses the oxidation of exogenous malate to pyruvate and CO_2. Because elevated pyruvate can inhibit aspartate aminotransferase activity in favour of alanine aminotransferase, the metabolic fate of glutamine-derived glutamate would most likely depend on the cellular concentration of malate. Furthermore, if the malate-aspartate shuttle is active (i.e. high malate dehydrogenase and aspartate aminotransferase activities) malate would be quickly converted to oxaloacetate, the amino group acceptor for aspartate formation.

In the present study, alanine was not significantly elevated in the astrocytomas, but asparate and glutamate were significantly higher compared to

normal contralateral brain and normal human astrocytes (figure 6-2). If this were due to the partial oxidation of glutamine (present at 4mM in the culture media) then glutaminase and aspartate aminotransferase activity would likely be high in transformed astrocytes. Since this metabolic profile was maintained by the astrocytoma cells grown *in vivo*, this was probably not due to the particular culture media composition used. However, to my knowledge the activity of these enzymes in astrocytomas is not known.

Matsuno & Goto (1992) pointed out that because glutamine oxidation was dominant and glutamine synthetase was low in hepatomas, the existence of an ATP-dissipating futile cycle was negligible in the tumours. Although normal rodent astrocytes have been shown to be the primary site of glutamine synthetase in the CNS, this enzyme would most likely be down-regulated in the astrocytomas. If astrocytomas were utilising exogenous glutamine as an energy source, the *de novo* synthesis of glutamine from glutamate would be a significant futile cycle. In the present study the glutamine concentrations remained low despite very high glutamate concentrations and analysis of the media from astrocytoma cultures (Chapter 8) demonstrated a net loss of glutamine over a 48-hour incubation. Thus, the oxidation rather than the synthesis of glutamine appears to predominate in the astrocytomas.

6.4.5 Choline-containing compounds

There was no significant difference in the total choline-containing compounds between the astrocytoma cells grown *in vitro* and *in vivo* and the individual choline-containing compounds were also very similar. On the other hand, the transformed cells *in vivo* had a relatively enriched phosphocholine concentration compared to normal contralateral brain (figure 6-3).

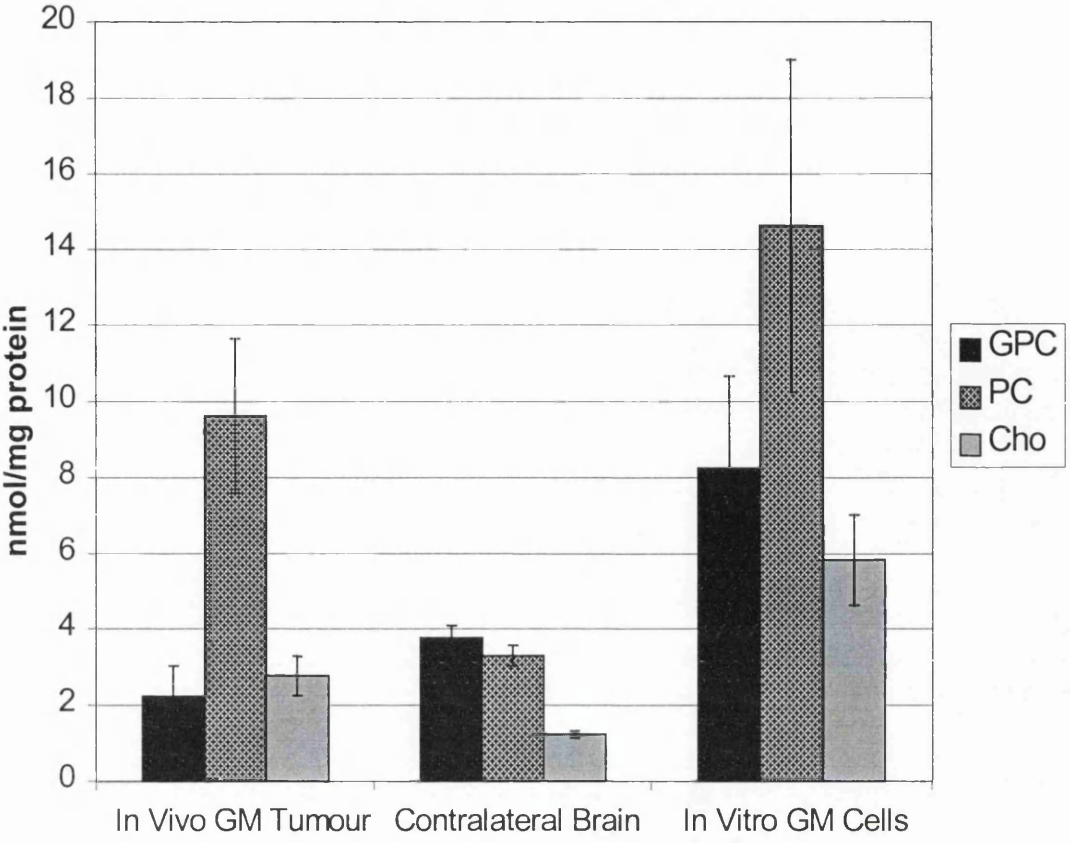


Figure 6-3. **The individual choline-containing compounds from extracts of human astrocytoma grade IV grown *in vitro* and *in vivo* compared to normal contralateral rat brain**. *Metabolite concentrations are expressed as mean ± SEM. Abbreviations are as follows: GPC = glycerophosphocholine; PC = phosphocholine; Cho = choline.*

Florian, *et al.* (1996) have also observed enriched PC in cultured astrocytomas but, as mentioned previously, PC is also enriched in normal human astrocytes (Chapter 5). Furthermore, the membrane phospholipid PtdCho was similarly low in the astrocytomas both *in vivo* and *in vitro* and is therefore not influenced significantly in culture by media concentrations of metabolic precursors. This suggests that the elevated PC in the transformed cells may be due to PtdCho hydrolysis while the elevated PC in normal human astrocytes (Chapter 5) may be due to PC synthesis.

6.4.6 Membrane Phospholipids

The astrocytomas grown *in vivo* had significantly more PtdEth and less PtdIno than those grown in culture. The data from the *in vivo* tumour extracts

were in good agreement with another biopsy tissue extract of a human glioma (Kostic & Buchheit, 1970; table 6-6). As suggested previously, the low PtdEth in the cultured astrocytomas may be due to a lack of ethanolamine precursor in the culture media. Although the cells are capable of synthesising PtdEth from PtdCho and PtdSer, this data suggests that synthesis from free ethanolamine may predominate in the astrocytoma cells.

	HUMAN ASTROCYTOMA *IN VITRO*	HUMAN ASTROCYTOMA *IN VIVO*	NORMAL CONTRALATERAL BRAIN
PtdCho	42 –50	37 – 51	70 – 84
PtdEth	9 - 11	24 – 28	4 – 6
PtdSer	20 -28	11 – 21	7 – 13
PtdIno	19 -21	13 –15	8 – 10
Ref	Present study	present study	present study
	ST HAMSTER GLIOMA *IN VITRO*	ST HAMSTER GLIOMA *IN VIVO*	HUMAN ASTROCYTES
PtdCho	72	68	74 – 81
PtdEth	19	17	3 – 5
PtdSer	4	6	9 – 11
PtdIno	5	9	7
Ref	(1)	(1)	Chapter 4 & 5

	HUMAN GLIOMA CELL LINE	PRIMARY HUMAN ASTROCYTOMA
PtdCho	56	50
PtdEth	14	27
PtdSer	20	17
PtdIno	10	7
Ref	(2)	(3)

Table 6-6. **Comparison of phospholipid yields in the astrocytomas with other gliomas in the literature grown *in vitro* and *in vivo*.** *The phospholipid concentrations from the present study have been calculated as a percentage of the total phospholipid measured for comparison with the literature. The following references have been used: (1) Hauser et al., 1976; (2) Poduslo et al., 1983; (3) Kostic & Buchheit, 1970. ST = spontaneously transformed.*

In the previous *in vitro* study (Chapter 5) cultured astrocytomas had significantly lower PtdCho compared with cultured normal human astrocytes while all the other phospholipids were elevated. In the present *in vivo* study,

although PtdCho was also significantly reduced in the tumour compared to normal contralateral brain, only PtdEth was significantly elevated. This demonstrates that cell culture conditions may affect cell membrane composition more than cell intermediary metabolism, as determined by the relatively small differences observed between the aqueous metabolites. Since the PtdCho decrease *in vivo* was accompanied by concomitant increase in the other PME-contributing metabolite, PtdEth, this would not have been detectable by ^{31}P-NMR because no change in the total PME concentration would be detected. As mentioned previously, the decreased PtdCho concentrations in the lipid fraction and the concomitant elevation in PC in the aqueous fraction of the extracts of astrocytomas grown both *in vivo* and *in vitro* compared to normal astrocytes and normal brain indicates that hydrolysis of the membrane phospholipid may occur in the transformed cells. The elevated PtdIno and PtdSer in the cultured cells may be due to the availability of the inositol and serine precursors in the culture media.

Although there were some differences in the phospholipid concentrations detected between the *in vitro* and *in vivo* samples, there was very little difference in the fatty acid acyl compositions of the phospholipids. High concentrations of exogenous oleic acid has been shown by Gilham & Brindle (1996) to activate membrane phospholipase C and to cause the hydrolysis of membrane PtdCho in myeloma cells. The oleic acid acyl group was similarly high in the astrocytoma cell- and tumour-phospholipids of the present study compared to normal astrocytes in Chapter 5. This increased uptake of media and plasma oleic acid by the astrocytoma cells could have activated phospholipase C and contributed to the decreased PtdCho observed in cell membranes and to the elevated PC seen in the aqueous fraction of the extracts.

Overall there were very few differences between the astrocytoma cells grown in culture and in rat brain although the membrane phospholipids where more affected than the aqueous metabolites. This serves as a strong validation for cell culture studies of astrocytoma metabolism as a model for the *in vivo* metabolism of the tumours.

Chapter 7: **Fatty Acid β-oxidation in Human Astrocytomas**

7.1 Introduction

Fatty acid β-oxidation is known to occur in the brain of neonatal rats and in primary rodent astrocyte cultures from developing brain. In fact, astrocytes are the only CNS lineage which have been demonstrated in rodent cell to be able to β-oxidise endogenously supplied fatty acids. Human tumour cells have been demonstrated to take up endogenously supplied fatty acid from culture media which is accumulated as lipid droplets in the cytosol. Intratumoural lipids are a common feature of high grade astrocytomas *in vivo* (Callies *et al.*, 1993; Remy *et al.*, 1997; Negendank & Sauter, 1996). However, it has not been determined if the brain tumour cells are capable of oxidising the stored lipid. In particular, high-grade tumours of astrocytic lineage may possess this ability known to exist in their normal counterparts from developing rodent brain. This would be consistent with the loss of cell cycle control and reversion to an embryonic phenotype/metabolism, which has been associated with malignancy.

The studies in this chapter explore this hypothesis by determining the rates of fatty acid oxidation by human astrocytoma cultures and the extent to which fatty acid can be used by the cells to maintain cellular ATP during normal substrate deprivation. Additionally, the importance of carnitine for astrocytoma fatty acid utilisation is investigated. Carnitine is not thought to be synthesised to a significant extent by the brain but is taken up from the blood supply (Bremer, 1983; Brooks & McIntosh, 1975). Thus, cultured astrocytomas may be capable of taking up fatty acids and forming cytoplasmic lipid droplets but if the media is missing essential components for its utilisation, like carnitine, the *in vivo* metabolism capable by the cells may not be fully represented by culture conditions.

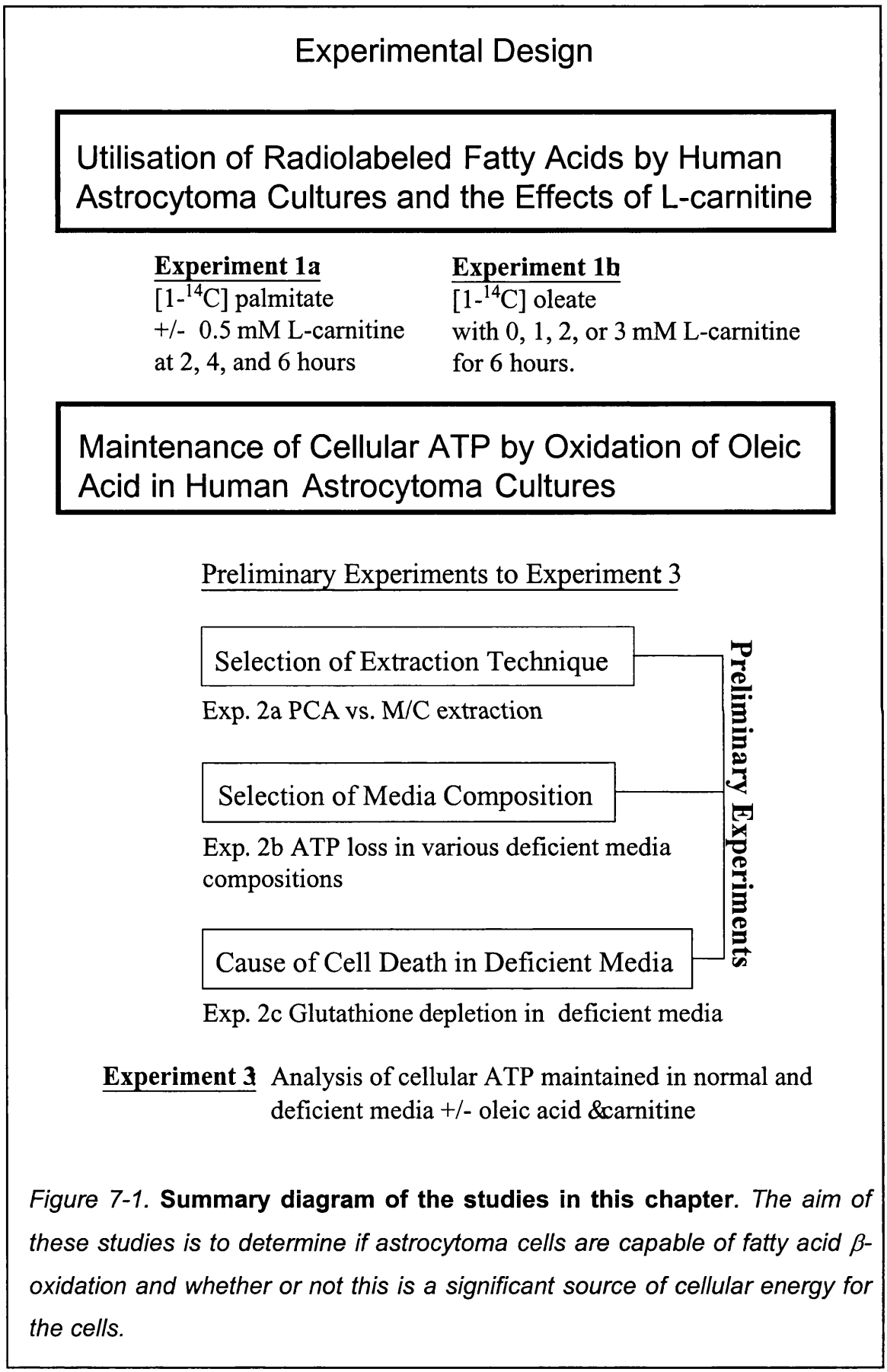


Figure 7-1. **Summary diagram of the studies in this chapter.** *The aim of these studies is to determine if astrocytoma cells are capable of fatty acid β-oxidation and whether or not this is a significant source of cellular energy for the cells.*

7.2 Methods

Through the following studies I hope to establish a possible use for tumour cell lipid-loading in the presence of high media (or plasma) fatty acid concentrations. Two main studies encompassing 6 separate experiments and employing a variety of methodologies were used to determine: (1) whether human astrocytoma cells in culture are able to β-oxidise endogenously supplied fatty acid and (2) the extent to which this metabolic pathway may contribute to the generation of cellular ATP. The experimental design for the studies in this chapter is summarised in figure 7-1.

7.2.1 Study 1: Utilisation of Radiolabeled Fatty Acids by Human Astrocytoma Cultures and the Effects of L-carnitine

400 μM [1-^{14}C] oleic or palmitic acid were incubated with cultured astrocytoma cells at a range of L-carnitine concentrations (0-3mM) to determine (1) whether astrocytomas are capable of fatty acid β-oxidation, (2) the rates of oxidation, and (3) the effect of increasing concentrations of the fatty acid transporter, L-carnitine, on oxidation rates.

Experiment 1a:

Astrocytoma monolayer cultures were incubated with 400 μM [1-^{14}C]palmitic acid in a deficient media that contained no glucose, amino acids, or FCS (Chapter 2, 2.1.1.2) with or without 0.5 mM carnitine supplementation for 2, 4, and 6 hours.

Group 1: 400 μM ^{14}C-palmitic acid + 0.5 mM carnitine---2 hour incubation

Group 2: 400 μM ^{14}C-palmitic acid (no carnitine)---2 hour incubation

Group 3: 400 μM ^{14}C-palmitic acid + 0.5 mM carnitine---4 hour incubation

Group 4: 400 μM ^{14}C-palmitic acid (no carnitine)---4 hour incubation

Group 5: 400 μM ^{14}C-palmitic acid + 0.5 mM carnitine---6 hour incubation

Group 6: 400 μM ^{14}C-palmitic acid (no carnitine)---6 hour incubation

Experiment 1b:

Astrocytoma monolayer cultures were incubated with 400 μM [1-^{14}C]oleic acid in a deficient media that contained no glucose, amino acids, or FCS

(Chapter 2, 2.1.1.2) with or without 1, 2, or 3mM carnitine supplementation for 6 hours.

Group 1: 400 μM ^{14}C-oleic acid (no carnitine)---6 hour incubation

Group 2: 400 μM ^{14}C-oleic acid + 1 mM carnitine---6 hour incubation

Group 3: 400 μM ^{14}C-oleic acid + 2 mM carnitine---6 hour incubation

Group 4: 400 μM ^{14}C-oleic acid + 3 mM carnitine---6 hour incubation

Cells were grown in 6-well plates under normal culture conditions. When confluent, the cells were incubated in deficient DMEM supplemented with a 400 μM [1-^{14}C] fatty acid-30 μM BSA complex with a specific activity of 0.4 μCi/μmol palmitate or 5.0 μCi/μmol oleate. $^{14}CO_2$ was trapped in filter paper which was secured above each well and soaked with 250 μl of 3.5 M NaOH. The incubation was quenched at the time points indicated with 100 ml of 20% PCA. The samples were then prepared for scintillation counting and then placed in a scintillation (radioactivity) counter (Chapter 2, 2.13). Cell protein content was determined in identical parallel cultures grown at the same time and under the same conditions as the experimental cells. It was not possible to determine the cellular protein on the experimental cells because the extracted cell pellet contained not only cellular protein but also the PCA-precipitated albumin-protein which was bound to the fatty acid during incubation. The amount of albumin-protein in the pellet would vary depending on the amount of fatty acid used by the cells. Therefore, parallel cultures were used for cellular protein calculations.

7.2.2 **Preliminary Experiments to Experiment 3**

A series of preliminary experiments were performed in order to determine: (1) the most consistent extraction technique for the analysis of cellular ATP content, (2) the optimal deficient media composition to produce a substantial loss of cellular ATP over 24 and 48 hour incubations in order to later test (in Experiment 3) the extent to which oleic acid oxidation can be used to maintain cellular ATP in the deficient media, and (3) the extent to which oxidative stress may contribute to cell death in the deficient cell cultures by measuring glutathione depletion.

Preliminary Experiment 2a:

The first preliminary study undertaken was to determine which extraction technique, PCA or M/C, was best for ATP extraction. Half of the samples were

spiked with a known concentration of ATP prior to extraction and the amount of recovery calculated for each technique. Astrocytoma cultures were grown in 4 six-well plates and divided into the following treatment groups (N = 6/group):

Group 1: PCA extract

Group 2: PCA extract spiked with 10 μmol ATP

Group 3: M/C extract

Group 4: M/C extract spiked with 10 μmol ATP

Immediately after rinsing with PBS, the extraction solvent (PCA or methanol) was added directly to the plate-wells and 10 μmoles ATP was added to the spiked samples at this point. The cells in the wells were then collected by manual scraping and the cell/solvent mixture transferred to microcentrifuge tubes to complete the extraction process as described in Chapter 2, section 2.5. ATP content was determined by HPLC analysis (2.11.2) and the recovery efficiency for each extraction technique compared.

Preliminary Experiment 2b:

The second preliminary study was designed to determine the optimum composition of culture media to cause a significant ATP loss over 48 hours. This information was necessary in order to choose a media composition which would cause cell death through energy failure if the astrocytoma cells were unable to utilise oleic acid supplemented as an alternative energy substrate. Astrocytoma cultures were grown in six-well plates for this study to determine which media composition sufficiently depletes ATP in the cells and whether or not a sufficient ATP loss is possible with fetal calf serum (FCS) still present in the media. It was desirable to use FCS even in the deficient medium so that the cells were not deprived of important growth factors which could compromise the cells' ability to metabolise normally. Therefore for the first set of experiments cellular ATP, ADP, and AMP content was examined in astrocytomas in the following media groups for 24 & 48 hours: (N = 6 wells/group/incubation time):

Group 1 (D): Normal DMEM + 10% FCS + 4 mM gln

Group 2 (DDF): glucose- and amino acid-free DMEM + 10% FCS

Group 3 (DD): glucose- and amino acid-free DMEM

Group 4 (DD2DF): glucose- and amino acid-free DMEM + 10% FCS
+ 6mM 2-deoxyglucose

Group 5 (DD2D): glucose- and amino acid-free DMEM
+ 6mM 2-deoxyglucose

Group 6 (DD2DFOA): glucose- and amino acid-free DMEM + 10% FCS
+ 6mM 2-deoxyglucose + 400 μM Oleic Acid
+ 3 mM carnitine

PCA extractions were used to prepare HPLC samples for the analysis of cellular adenine nucleotide content according to the methods in Chapter 2, 2.11.2.

Preliminary Experiment 2c:

The third preliminary study was designed to confirm that the primary cause of the cell death in deficient media was due to energy failure and not oxidative stress as a result of lipid peroxidation. Glutathione (GSH) is an important endogenous antioxidant in the cascade of reactions involved in quenching lipid free radicals potentially generated through the peroxidation of the cell membrane or triglyceride stores. The deficient media used in these studies was deficient in the precursor cysteine for the synthesis of endogenous glutathione. Therefore, the loss of cellular glutathione and the inability to synthesise new glutathione could potentially have caused or significantly contributed to cell death rather than energy failure due to the lack of energy substrate in culture. Thus, in order to unambiguously attribute ATP loss to substrate deprivation, intracellular glutathione content was analysed by HPLC in the cultures to determine if the amount of oxidative stress being generated was sufficient to deplete the endogenous antioxidant, thus indicating a high level oxidative stress in these conditions.

Astrocytomas were grown in 6-well plates, divided into the following treatment groups and incubated in the specified media for 48 hours prior to extraction: (6 wells/group):

Group 1: DMEM + 10% FCS + 4mM gln + 400 μM oleic acid
+ 3 mM carnitine

Group 2: glucose- and amino acid-free DMEM + 10% FCS +
400 μM oleic acid + 3 mM carnitine

Group 3: glucose- and amino acid-free DMEM + 10% FCS +
400 μM oleic acid + 3 mM carnitine + 10 μmol GSH

Group 4: glucose- and amino acid-free DMEM + 400 μM oleic acid +
3 mM carnitine + 10 μmol cystine

Astrocytoma cultures were extracted by adding the mobile phase (methanol/phosphate buffer) for HPLC onto the plate-wells following rinsing with PBS. The cells were then mechanically scraped and the cell/mobile phase mixture was put into microcentrifuge tubes and processed according to the methods in Chapter 2, 2.11.1. In addition to glutathione, the amino acids glutamate, glycine, and glutamine were also simultaneously measured in the same samples.

7.2.3 Study 2: **Maintenance of Cellular ATP by the Oxidation of Oleic Acid in Human Astrocytoma Cultures**

Experiment 3:

Once the appropriate extraction technique (PCA) was determined and a sufficiently deficient media to cause cellular energy failure was identified (glucose- and amino acid-free DMEM + 10% FCS + 6 mM 2-deoxyglucose), astrocytoma cultures were divided into six groups for the following 48-hour incubations: (6 wells/group)

Group 1 (**D**): Normal DMEM + 10% FCS + 4 mM gln

Group 2 (**DOA**): Normal DMEM + 10% FCS + 4 mM gln
+ 400 μM Oleic acid

Group 3 (**DOAC**): Normal DMEM + 10% FCS + 4mM gln
+ 400 μM Oleic acid + 3mM carnitine

Group 4 (**DD**): glucose- and amino acid-free DMEM + 10% FCS
+ 6mM 2-deoxyglucose

Group 5 (**DDOA**): glucose- and amino acid-free DMEM
+ 6mM 2-deoxyglucose + 400 μM oleic acid

Group 6 (**DDOAC**): glucose- and amino acid-free DMEM + 10% FCS
+ 6mM 2-deoxyglucose + 400 μM Oleic Acid + 3 mM carnitine

Following incubation, the cells were extracted and analysed by HPLC for ATP, ADP, and AMP concentrations calculated from the known concentration of adenine nucleotide standards (Sigma, UK) run concurrently. Both the quantitative concentrations of the adenine nucleotides and the ratio of ATP/ADP for each group and was calculated and compared to determine the extent to which the astrocytoma cultures were able to use oleic acid to maintain cellular energy stores when it is the main substrate available over a 48-hour period.

The studies in Chapter 8 will further explore the use of fatty acids by astrocytomas by looking at intermediary metabolism and membrane incorporation of exogenous fatty acids.

7.3 Results

7.3.1 Study 1: Utilisation of Radiolabeled Fatty Acids by Human Astrocytoma Cultures and the Effects of L-carnitine

Experiment 1a: β-oxidation flux of [1-^{14}C] palmitate was measured in astrocytoma cultures with or without L-carnitine for 2, 4, and 6 hour incubation periods. The results indicate that the astrocytoma cultures were capable of fatty acid β-oxidation and that L-carnitine significantly increased the rate of oxidation by up to 51% ($p = 0.007$; table 7-1).

[1-^{14}C]palmitic acid (pmol/min/mg protein)			
	+ Carnitine	- Carnitine	%Difference
Avg 2h	30.9 ± 4.7	15.8 ± 1.3	51
Avg 4h	14.7 ± 6.0	5.7 ± 1.8	39
Avg 6h	14.4 ± 2.4	5.7 ± 1.6	39

Table 7-1. Palmitic acid flux in astrocytoma cultures. *The rates are given as a sum of $^{14}CO_2$ and ^{14}C-products according to the methods of Veerkamp, et al. (1986) and expressed as mean ± SEM. The addition of 0.5 mM carnitine resulted in a 39-50% increase in palmitate oxidation.*

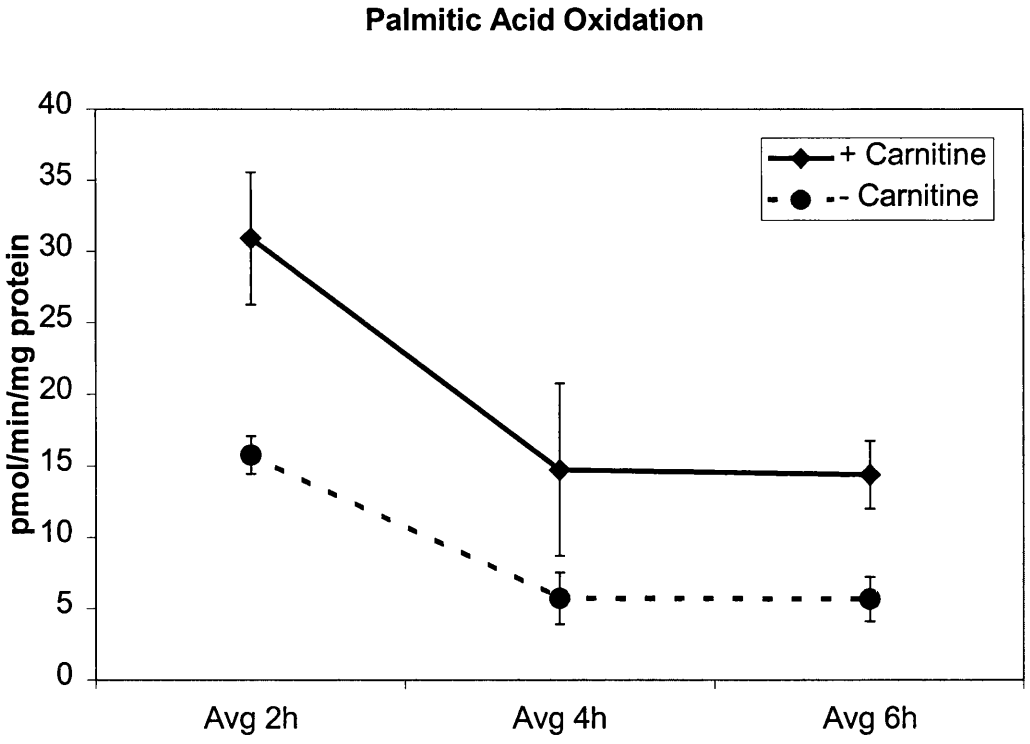


Figure 7-2. [1-^{14}C]palmitic acid oxidation over 6 hours. *The rate of palmitate oxidation in the first two hours of incubation was approximately 2-fold higher than at 4 hours. The oxidation rate remained quite stable from 4 to 6 hours of incubation both with and without carnitine.*

Experiment 1b:

β-oxidation flux of [1-^{14}C] oleate was measured in astrocytoma cultures with increasing carnitine concentrations (0-3 mM) for a 6 hour incubation period. This experiment demonstrated that the astrocytoma cultures were capable of β-oxidation of oleic acid (in addition to palmitic acid, exp. 1a) and that L-carnitine significantly ($p = 0.03$) increased the rate of oxidation by up to 37% (table 7-2).

[1-^{14}C]oleic acid (pmol/min/mg protein)	
Carnitine	**OA oxidised**
0 mM	59.3 ± 2.5
1 mM	76.6 ± 3.0
2 mM	77.7 ± 3.1
3 mM	93.9 ± 3.3

Table 7-2. **Oleic acid flux in astrocytoma cultures**. *The rates of oleic acid oxidation are given as a sum of $^{14}CO_2$ and ^{14}C-products according to the methods of Veerkamp, et al. (1986). The addition of carnitine resulted in a 23 - 37% increase in oleate oxidation. There was a significant difference (p= 0.03) between 0 and 3 mM carnitine only.*

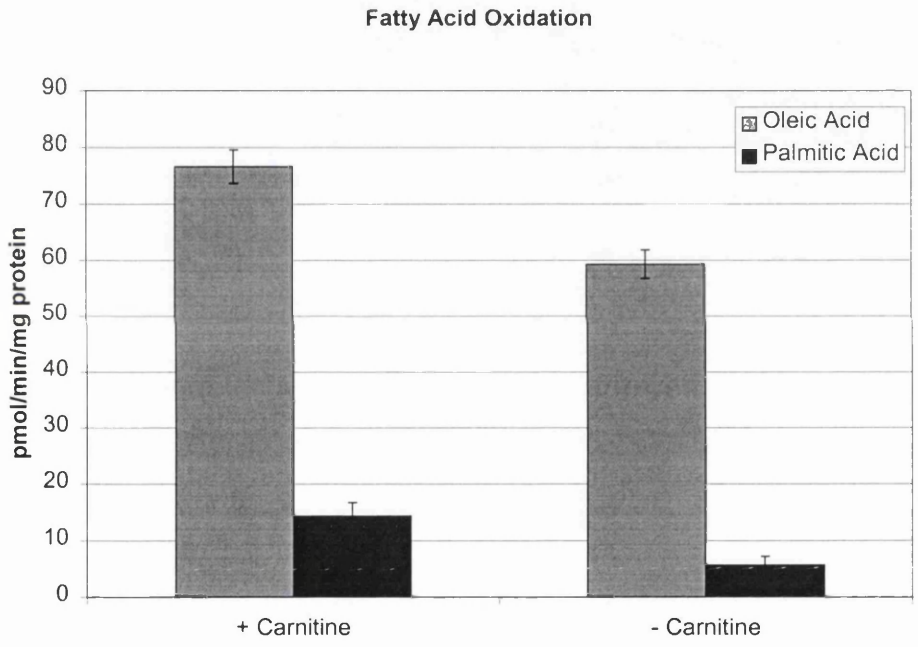


Figure 7-3. ***Comparison of fatty acid oxidation rates from* Experiment 1a and 1b**. *There was a significantly higher rate of oleic acid utilisation by the astrocytoma cultures both with and without carnitine, demonstrating an apparent substrate preference for oleic acid (p=0.0001).*

7.3.2 **Preliminary Experiments to Experiment 3**

Experiment 2a: Preliminary Experiment 2a was undertaken to determine the optimum extraction technique, PCA or M/C, for evaluating cellular ATP concentrations. The PCA extraction was found to be slightly less variable and had a slightly higher recovery of spiked ATP although there was no significant

difference in ATP yield between extract techniques (p = 0.66 natural abundance; p = 0.63 spiked; table 7-3).

ATP Extractions	nmol/mg protein	Percent recovered
M/C	40.3 ± 1.1	
M/C + 10 μmoles ATP	48.4 ± 3.1	81
PCA	40.9 ± 0.7	
PCA + 10 μmoles ATP	50.0 ± 0.6	91

Table 7-3. **ATP extractions: M/C vs. PCA**. *The percent recovered was calculated as: ((spiked sample concentration – natural abundance concentation)/10 μmoles (actual spiked concentration))*100.*

Experiment 2b: The depletion of cellular ATP in astrocytoma cultures incubated with various deficient media compositions was evaluated (table 7-4). There was a loss of cellular ATP in all deficient media, even when FCS was present in the media. The largest loss of ATP resulted from incubation in the glucose- and amino acid-free DMEM containing 6 mM 2-deoxyglucose without FCS (0.2% of control ATP remaining after 48 hours; DD2D). However, even with FCS present in the media there was a 72% drop in ATP over 24-hours and a 94% drop in ATP over 48-hours (DD2DF). When oleic acid and carnitine were added to this media composition, the cells were able to maintain ATP concentrations 37-47% of control (an increase of 31% over the deficient media levels; DD2DFOA). See table 7-4.

Mean ATP (nmol/mg protein)				
24-hours	**ATP**	**ADP**	**AMP**	**[ATP/ADP]**
D	40.9 ± 2.6	3.6 ± 0.2	0.9 ± 0.1	11.4
DDF	32.5 ± 4.6	5.6 ± 0.6	0.9 ± 0.1	5.8
DD	16.1 ± 4.3	4.9 ± 1.3	1.3 ± 0.4	3.3
DD2DF	11.4 ± 3.5	3.9 ± 1.3	0.4 ± 0.2	2.9
DD2D	3.5 ± 1.4	1.7 ± 0.9	0.4 ± 0.2	2.0
DD2DFOA	19.3 ± 5.3	3.4 ± 0.2	0.9 ± 0.2	5.6
48-hours	**ATP**	**ADP**	**AMP**	**[ATP/ADP]**
D	39.4 ± 0.8	3.8 ± 0.3	0.4 ± 0.2	10.5
DDF	13.1 ± 1.6	5.4 ± 0.8	0.4 ± 0.2	2.4
DD	7.8 ± 1.1	3.7 ± 0.9	1.0 ± 0.2	2.1
DD2DF	2.4 ± 1.8	1.6 ± 1.2	0.2 ± 0.2	1.5
DD2D	0.3 ± 0.3	0.3 ± 0.2	0.00	1.3
DD2DFOA	14.8 ± 0.8	3.4 ± 0.5	0.3 ± 0.2	4.4

Table 7-4. **Adenine nucleotide concentrations after incubation for 24 or 48 hours in various media compositions**. *ATP content is expressed as mean ± SEM. Media abbreviations as follows:*

***D** = Normal DMEM + 10% FCS + 4 mM gln;*

***DDF** = glucose- and amino acid-free DMEM + 10% FCS;*

***DD** = glucose- and amino acid-free DMEM;*

***DD2DF** = glucose- and amino acid-free DMEM + 10% FCS + 6mM 2-deoxyglucose;*

***DD2D** = glucose- and amino acid-free DMEM + 6mM 2-deoxyglucose;*

***DD2DFOA** = glucose- and amino acid-free DMEM + 10% FCS + 6mM 2-deoxyglucose + 400 μM Oleic Acid + 3 mM carnitine.*

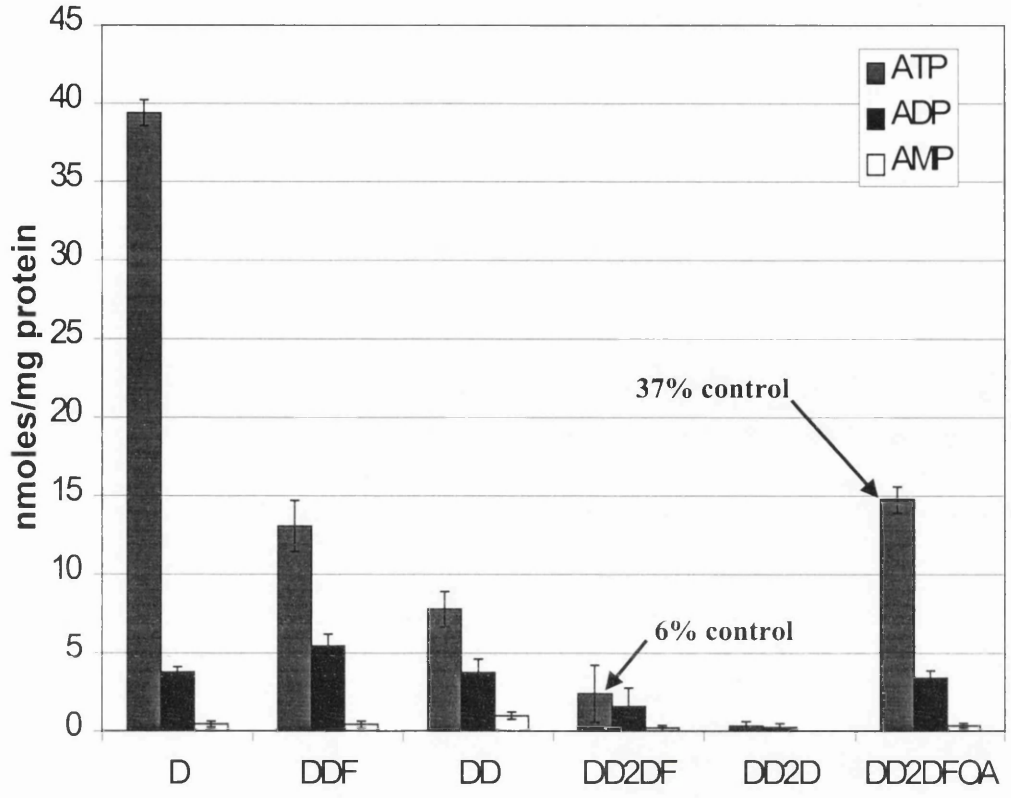


Figure 7-4. **Differences in cellular adenine nucleotide content from 48-hour incubation in different media compositions**. *See table 7-4 for media group abbreviations. Nucleotides are expressed as mean ± SEM. Although the DD2D media resulted in the most dramatic loss of ATP, the cells in DD2DF media retained only 6% of control ATP despite the presence of fetal calf serum.*

Experiment 2c: The loss of cellular glutathione was evaluated in astrocytoma cultures incubated with various deficient media. There was a 48% drop in cellular glutathione content in cells in the deficient media lacking the GSH precursor cysteine compared to cells in normal media ($p = 0.003$; figure 7-5, DDOA). Control levels of GSH were not restored by the supplementation of 2 mM cystine in the deficient media (cysteine auto-oxidises in culture so the precursor of cysteine, cystine, was used; DDOACys). However, in cells cultured in deficient media supplemented with 2 mM GSH there was a significant elevation (57%) of intracellular GSH content ($p = 0.003$; DDOAGSH). Oxidised glutathione (GSSG) was below the limits of detection in all of the samples. The glutamine and glycine concentrations were not significantly different between groups but glutamate was significantly elevated in the GSH-supplemented cultures ($p = 0.001$;DDOAGSH).

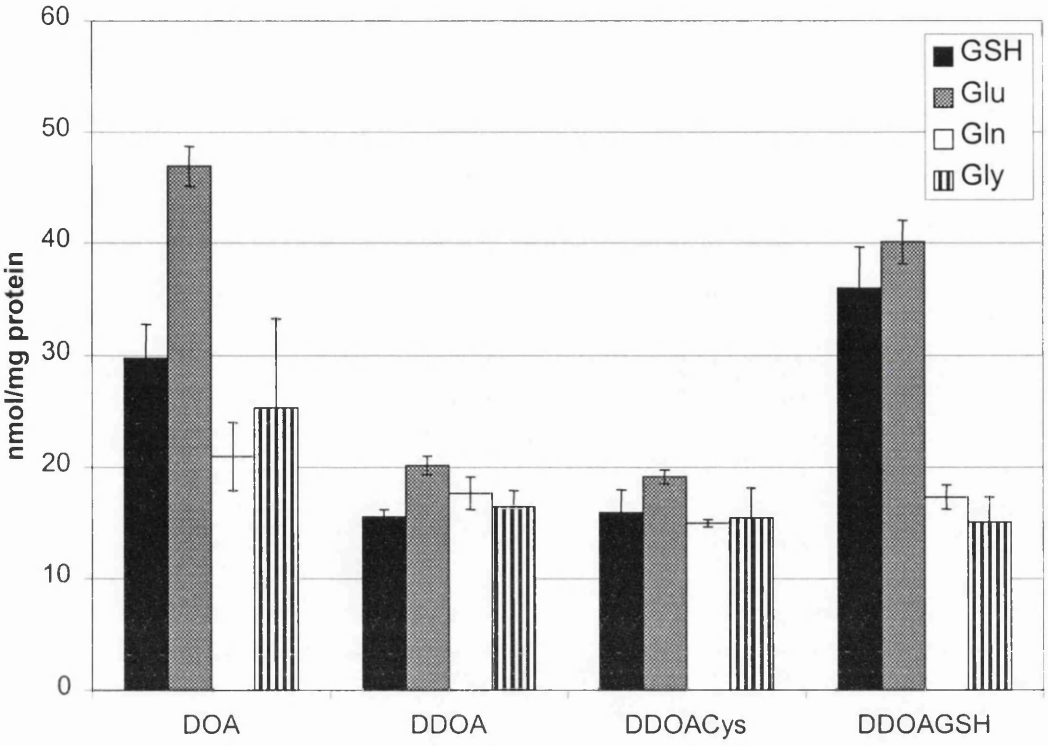


Figure 7-5: **The effects of deficient media on cell glutathione content**. *The deficient media (DDOA) resulted in a 48% decrease in GSH content compared to control (DOA) which could be restored by supplementation of 2mM GSH (DDOAGSH) but not the GSH precursor cysteine (DDOACys). Glutamate was elevated by 50% in the GSH-supplemented deficient media (DDOAGSH). Abbreviations are as follows: Glu = glutamate, Gln = glutamine, Gly = glycine, GSH = glutathione (reduced form),*

DOA *= Normal DMEM + 10% FCS + 4 mM gln + 400 μM oleic acid + 3mM carnitine,*

DDOA *= glucose- and amino acid-free DMEM + 10% FCS + 4 mM gln + 400 μM oleic acid + 3mM carnitine,*

DDOACys *= DDOA + 2 mM cystine,*

DDOAGSH *= DDOA + 2 mM GSH*

7.3.3 Study 2: Maintenance of Cellular ATP by the Oxidation of Oleic Acid in Human Astrocytoma Cultures

Experiment 3: The ability of astrocytoma cultures to use oleic acid β-oxidation to supply cellular ATP was evaluated (table 7-5). The addition of oleic acid alone

(DOA) did not have an effect on the ATP content of cells in normal media (D). However, when oleic acid and carnitine were added to normal media (DOAC), the cells produced a 57% increase in ATP. Likewise, the addition of oleic acid on its own in deficient media (DDOA) did not increase cellular ATP content but when combined with carnitine (DDOAC) produced ATP concentrations up to 32% of control cultures (D). Without oleic acid and carnitine, cellular ATP dropped to approximately 9% of control levels in deficient-media cultures (DD). See table 7-5.

	ATP	ADP	AMP	[ATP/ADP]
D	41.8 + 0.6	6.1 + 1.7	1.1 + 0.1	6.9
DOA	41.6 + 1.8	3.3 + 0.3	0.3 + 0.04	12.5
DOAC	65.7 + 3.6	6.0 + 2.2	0.9 + 0.6	11.0
DD	3.7 + 0.2	0.6 + 0.1	0.4 + 0.1	5.8
DDOA	4.2 + 0.3	0.8 + 0.4	1.1 + 0.2	5.3
DDOAC	13.2 + 1.1	2.0 + 1.2	1.0 + 0.3	6.5

Table 7-5. **Adenine nucleotide concentrations from astrocytoma cultures in normal and deficient media supplemented with oleic acid +/- carnitine**. *Concentrations are expressed as nmol/mg protein and as mean ± SEM. The supplementation of oleic acid and carnitine in both normal media and deficient media resulted in an increased ATP content (32-57%) over cultures in equivalent unsupplemented-media. The supplementation of oleic acid and carnitine in the deficient media restored the ATP/ADP ratio (6.5) to very close to the ratio obtained in control media (6.9). Abbreviations as follows:*

***D** = DMEM + 10% FCS + 4 mM Gln*

***DOA** = D + 400 μM oleic acid*

***DOAC** = DOA + 3 mM carnitine*

***DD** = glucose- and amino acid-free DMEM + 10% FCS + 6 mM 2-deoxyglucose*

***DDOA** = DD + 400 μM oleic acid*

***DDOAC** = DDOA + 3 mM carnitine*

7.4 Discussion

7.4.1 Study 1: Utilisation of Radiolabeled Fatty Acids by Human Astrocytoma Cultures and the Effects of L-carnitine

The Experiments (1a) and (1b) demonstrated that cultured astrocytoma cells are capable of fatty acid oxidation and that oleic acid is a preferred substrate over palmitic acid. Furthermore, carnitine was shown to be an import co-factor in incubation media for the oxidation of fatty acids. The preliminary experiments used to set up Experiment 3 further demonstrated that the oxidation of oleic acid can contribute up to 32% of cellular energy in substrate-deprivation conditions and that it can be used to boost ATP synthesis by 57% in normal, carbohydrate-containing media.

Experiments 1a & 1b:

The astrocytoma cultures were demonstrated to be capable of β-oxidising both palmitic and oleic acid presented at high concentration (400 μM) in incubation media both with and without carnitine. Study 1a with palmitic acid demonstrated that the rate of fatty acid utilisation by the cells was highest within the first two hours of incubation and then remained stable between 4 and 6 hours. It is unknown if this rate remains stable beyond 6 hours if sufficient fatty acid substrate is available. Longer incubation experiments were attempted but resulted in the condensation of water droplets on the filter paper covering the culture wells and NaOH dripping into the cultures and killing the cells. Study 1b demonstrated that increasing the concentration of carnitine incubated with fatty acid-supplemented media resulted in an increase in the rate of oxidation. However, this increase was not linear and a significant increase was only reached with 3 mM carnitine. It should be noted that the fatty acids in Experiments 1a & 1b were supplemented into a glucose-, fatty acid-, and FCS-deficient medium. Thus, the overall rates of fatty acid oxidation may be even higher for astrocytoma cells in normal media. Experiment 3 supports this speculation since the supplementation of fatty acid and carnintine in normal media resulted in a 57% increase in ATP compared to a 32% increase in ATP in deficient media. Even without carnitine, the rate of oleic acid oxidation was 10-fold higher than palmitic acid and suggests a preference for the monounsaturated fatty acid. The reasons for this preference are unknown.

Even though the astrocytoma cultures were capable of fatty acid β-oxidation and only rodent astrocytes from developing brain are known to share this ability, this does not fully support the hypothesis that lipid-loading is an adaptive response of astrocytoma tumours to the increased energy demands of high rates of proliferation by ultilising the developmentally primitive pathway of fatty acid oxidation. Unless the rates of fatty acid flux observed in Experiment 1a & 1b are similar or higher than the rates seen in normal human astrocytes, the accumulation of lipid in astrocytomas could still be due to a mitochondrial dysfunction in which the rate of fatty acid oxidation is actually lower in tumour cells. Table 7-6 summarises the rates of substrate oxidation measured in normal CNS cells in the literature.

Rate of fatty acid oxidation (pmol/min/mg protein)							
	Astrocytes	Neurons	Oligoden-drocytes	whole brain	Ref.	Carnitine	Fatty Acid
Fatty Acids	40-51			470-670	1	0.5mM	200μM palmitate
	19-49				2	2mM	20μM palmitate
				220-390	3	none	27μM oleate (U)
	260-290				4	none	500μM octanoate
	114-121				5	0.5mM	200μM palmitate
	12-36*					0.5mM	400μM palmitate
	57-97*					0-3mM	400μM oleate
Glucose	700-1400	900	80		6		
	190-211	260-270	520-560		4		
Ketone Bodies				90-130	3		
	630	430	20		7		
	183-216	500	400-467		4, 8		

Table 7-6. **Rates of substrate oxidation in the literature.** *The rates listed above are compiled from the following references: (1) Murphy, et al., 1992; (2) Esfandiari, et al., 1997; (3) Bossi, et al., 1982; (4) Edmond, 1987; (5) Auested, et al., 1991; (6) Yu and Hertz, 1983; (7) Lopez-Cardozo, et al., 1986; (8) Edmond, et al., 1985. *The rates in bold are those reported for the current study.*

The rates of fatty acid oxidation by astrocytoma cultures in the present study were comparable with the rates from normal rodent astrocytes and whole brain homogenates in the literature (table 7-6). Whole brain refers to homogenates where the cell mitochondria are directly exposed to the substrate during incubation and therefore utilisation was not subject to the restrictions of intracellular uptake. Additionally, octanoate is not a long-chain fatty acid and can freely diffuse across the mitochondrial membrane and is not subject to the restrictions of carnitine transport across the membrane. These factors may account for the substantially higher rates of fatty acid oxidation in these studies.

However, there is a large range of rates in the literature most probably due to methodological differences such as: monolayers vs. suspensions vs. homogenates, different concentrations of fatty acids and carnitine, and differently labeled fatty acids. Needless to say, there are many reasons why a direct comparison of rates can not be made but another important factor may be that the normal rates of metabolism reported in the literature are all measured in non-human cells. The only way to be certain whether the rate of fatty acid utilisation by the human astrocytomas in the present study was significant or not would be to compare them to human astrocytes under identical conditions (monolayer cultures, 400 μM [1-^{14}C]fatty acids + 0-3 mM carnitine in deficient media). However, the difficulty in obtaining normal human astrocytes and the prohibitive expense of obtaining them commercially did not make this a tenable option. Thus, the significance of this fatty acid utilisation was assessed *indirectly* by monitoring the extent to which the cells can use fatty acid for generating ATP in deficient and normal cell media in Experiment 3.

7.4.2 **Preliminary Experiments to Experiment 3**

Experiment 2a:

The comparison of PCA and M/C extraction of cellular ATP revealed no significant differences between the techniques. However, the PCA extraction was slightly less variable and had a slightly higher recovery rate for ATP spiked into the samples. Therefore, for Experiment 3 the PCA technique was used to extract cell cultures for HPLC analysis of the adenine nucleotides.

Experiment 2b:

Although the most significant loss of cellular ATP content occurred in the glucose- and amino acid-free DMEM + 2-deoxyglucose media, the addition of 10% FCS still resulted in a large drop (94%) in cell ATP content compared to controls. Thus, glucose- and amino acid-free DMEM + 10% FCS + 2-deoxyglucose was chosen as the basic deficient media for Experiment 3 where the ability of oleic acid and carnitine supplements into this media composition was used to test the cells ability to use the fatty acid to produce ATP. Although FCS contains some fatty acid, carbohydrate, amino acids and ketone bodies that the cells could potentially have utilised, this preliminary study has shown that FCS alone was not sufficient to maintain viable levels of ATP in the astrocytoma cultures. Yager, *et al.* (1994) demonstrated that cell death occurs when cellular ATP content drops below 10% control values in rat astrocyte cultures. The ATP levels in normal rat astrocytes in hypoglycemic conditions are known to have a gradual reduction in ATP when substrate is withdrawn from control values of 50 nmol/mg protein to 7 nmol/mg protein over 30-36 hours (Hertz *et al.*, 1995). Under these conditions cell death has been directly correlated with ATP depletion. Furthermore, the 24-hour media incubations in the present study indicated that cells maintained ATP levels above the viability threshold of 10% control values even in the basic deficient media (table 7-4). Therefore, 48 hours incubations, which produced a drop in ATP levels below 10% control values in the deficient media, were used in Experiment 3.

Experiment 2C:

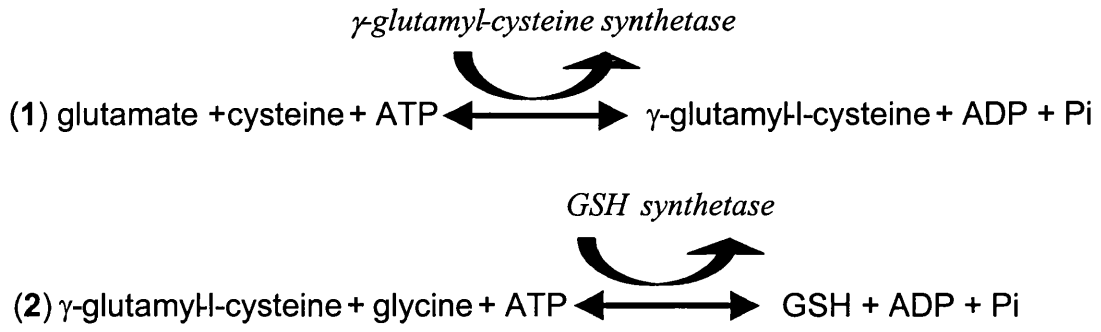
Mature astrocytes can survive the absence of media energy substrates for more than 6 hours in the absence of oxygen and up to 36 hours in the presence of oxygen. Immature astrocytes can survive approximately twice as long under those conditions (Hertz *et al.*, 1995; Yager *et al.*, 1994; Juurlink, 1992). It is believed that astrocytes have a superior ability to survive long periods of substrate deprivation compared to other CNS cells because of their high intracellular stores of compounds that can be used as alternative energy substrates such as glutathione (20 nmol/mg protein yielding up to 40 nmol/mg protein ATP) and glycogen (60 nmol/mg protein yielding up to 180 nmol/mg ATP) (Juurlink *et al.*, 1996, 1997). The ability of immature astrocytes to survive even

longer may be due to the ability in developing brain to β-oxidise fatty acids, providing an additional substrate store for generating ATP.

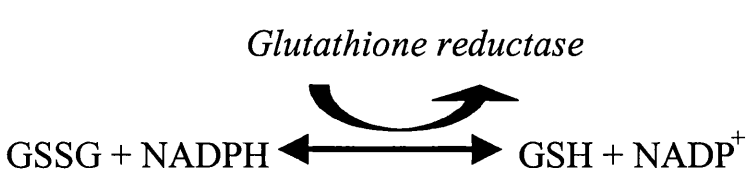
Juurlink, *et al.* (1993 & 1997) believe it is unlikely that oxidative stress plays a major role in causing astrocyte cell death in hypoglycemia because mitochondrial membrane potentials collapse just prior to cell death when ATP levels are severely depleted. However, the role of free radicals under these conditions is not completely ruled out. Furthermore, in experimental conditions where high concentrations of free fatty acids were available in the media, the astrocytoma cells would have an additional risk factor for oxidative stress (e. g., the formation of lipid peroxides) from the triglyceride stores produced by cell lipid-loading. Furthermore, the deficient media lacks the precursor cysteine for the synthesis of an important endogenous antioxidant, glutathione. Glutathione (GSH) is vital for preventing free radical damage to cells by: (1) scavenging reactive oxygen species directly, (2) scavenging peroxides (e.g., lipid peroxides and H_2O_2) via glutathione peroxidase, and (3) reducing the last step in the chain of scavenging reactions that prevent the propagation of lipid peroxidation (GSH reduces the ascorbic acid radical produced from reducing the vitamin E radical which is in turn produced from reducing lipid peroxides). Thus, in the present study a reduction in cellular GSH may indicate the occurrence of oxidative stress generated in deficient media containing large amounts of fatty acid. Although lipid peroxides are propagated from PUFA (North *et al.*, 1994) and the astrocytoma cultures have been given oleic acid (a MUFA), high concentrations of oleic acid is known to activate phospholipase C (Gillham & Brindle, 1996), which can release PUFA from the breakdown of membrane phospholipid. Murphy *et al.*, (1995) showed that when membrane choline- and ethanolamine-glycerophospholipids were broken down from combined oxygen and glucose deprivation, it resulted in the subsequent release of primarily PUFA.

In Experiment 2c the level of GSH was reduced by 52% in deficient, fatty acid-supplemented media when compared to control. However, there was not a concomitant increase in the oxidised form of glutathione to detectable levels. In most cells, including CNS cells, 98% of intracellular glutathione exists primarily in the reduced form (GSH) rather than the oxidised disulfide form (GSSG) (Slivka *et al.*, 1987). The control concentrations of GSH in the present experiment are in good agreement with the literature which ranges from 8 – 50 nmol/mg protein for normal astrocytes (Papadopoulos *et al.*, 1997; Yudkoff *et al.*, 1990; Barker *et al.*,

1996; Juurlink *et al.*, 1996; Makar *et al.*, 1994; Dringen *et al.*, 1998; Sagara *et al.*, 1993). Astrocyte cultures under prolonged ischemia have been shown to release GSH into the media which is subsequently catabolised to its constituent amino acids (Juurlink *et al.*, 1996). Since there was no oxygen in the stimulated ischemia in that study the loss of GSH from the media couldn't have been due to the oxidation of GSH. Additionally, GSH catabolism didn't begin until ATP had fallen by 67% from control (pre-ischemia) values. The ATP decrease favours the transition from GSH synthesis to catabolism and the amino acids released from the catabolism of GSH may also be used as further energy substrates. Thus, the loss of GSH but lack of GSSG generation from the astrocytomas in Experiment 2c may also just be due to GSH catabolism. The ATP levels maintained by the astrocytoma cells (32-37% control) are similar to the levels at which the astrocyte cultures in the Juurlink study began catabolising GSH for ATP. Furthermore, when cystine was supplemented into the deficient media, [GSH] did not increase. Sagara, *et al.* (1993b) showed that primary cultures of fetal rat brain cells lost GSH upon culturing unless the media contained cystine. Thus, the fact that the cells in Experiment 2c were supplemented with cystine but didn't synthesise GSH may indicate that this was not a priority for the cells under these conditions. The cells in deficient media might not be able to use the supplemented cystine to synthesise GSH because this requires ATP in the following reactions, which are reversible:



However, if a lack of cellular energy was responsible for the lack of GSH synthesis by the astrocytoma cells in deficient media supplemented with cystine, then GSSG should have been substantially elevated if oxidative stress were significant because the reduction of GSSG to GSH requires NADPH in the following reaction:



In fact, Ben-Yoseph, et al (1994) have even suggested that pentose phosphate pathway (PPP) activity can be used as an indirect measure of oxidative stress because glutathione peroxidase activity is coupled (via glutathione reductase) to the PPP enzyme glucose-6-phosphate dehydrogenase by generating NADPH. The deficient media in Experiment 2c contained 2-deoxyglucose to block the phosphorylation of glucose and this would have subsequently stopped the reduction of any GSSG generated by oxidative stress. Thus, if oxidative stress were significant then the GSSG concentration should have been high but this was not the case.

Finally, when cells in deficient media had GSH supplemented in the media the intracellular GSH content was significantly increased along with cellular glutamate. This indicates that the glutathione taken up by the cells may be primarily catabolised rather than oxidised with glutamate released from the glutathione molecule. However, glutamine and glycine were not similarly elevated in these cell extracts. Glutathione is degraded outside of the cells (transported out via the enzyme γ-glutamyltranspeptidase) where it is degraded to glycine + cysteine + γ-glutamyl-amino acid. Cystine, in particular, is a good amino acid-acceptor forming γ-glutamylcysteine. The γ-glutamyl-amino acid is then transported back into the cell where γ-glutamyltransferase catalyses its reduction to: amino acid + 5-oxoproline which is further reduced to glutamate via 5-oxoprolinase. Thus, a possible reason why intracellular glutamate increased in the GSH-supplemented cultures may be because GSH was broken down to its constituent amino acids for metabolism rather than being used as an antioxidant. Although glycine is also a GSH precursor amino acid, it did not increase in the GSH-supplemented cultures but this may be because GSH catabolism occurs outside of the cell as described above. If the γ-glutamyl-amino acid transported back into the cell was not glycine then glutamate but not glycine would be elevated in cell extracts. Furthermore, the astrocytomas do not appear to be converting the glutamate to glutamine, which is thought to be an important pathway in normal astrocytes.

7.4.3 Study 2: Maintenance of Cellular ATP by the Oxidation of Oleic Acid in Human Astrocytoma Cultures

Experiment 3:

Through the preliminary Experiments 2a & b the extraction technique (PCA) and deficient media composition (glucose- and amino acid-free DMEM +10% FCS + 6mM 2-doxyglucose) were selected. Furthermore, the loss of ATP and cell death in deficient media was not correlated with oxidative stress in the preliminary Experiment 2c. Experiment 3 was designed to establish the extent to which astrocytoma cells can use fatty acid oxidation to generate ATP in order to interpret whether or not the rates of β-oxidation flux measured in Experiment 1a & b are indicative of an active or dysfunctional metabolic pathway.

Several studies have shown that astrocyte cell death, measured by the release of the cytosolic enzyme lactate dehydrogenase, occurs when cellular ATP drops below 5-10% control (Juurlink *et al.*, 1993; Hertz *et al.*, 1995; Yager *et al.*, 1994; Sochocka *et al.*, 1994). The deficient media from Experiment 2b produced a drop in ATP to 1-10% control levels which indicates a sufficient energy failure to cause cell death. In Experiment 2b and Experiment 3 when this deficient media was supplemented with 400 μM oleic acid + 3 mM carnitine, the cells were able to maintain 29 – 38% of control ATP. So, although fatty acid oxidation was not able to generate the same amount of ATP as in normal media, it was able to maintain ATP well above the critical level at which cell death occurs. Furthermore, in Experiment 3 the oleic acid + carnitine supplementation was able to restore the ratio of ATP:ADP to near control values. This is significant because the ATP: ADP ratio represents the metabolic energy status of tissues and cells and therefore the functioning of oxidative phosphorylation (for review see Tager *et al.*, 1983; Brown, 1992). The lower [ATP] but normal [ATP:ADP] measured in the astrocytomas from deficient media + oleic acid + carnitine could indicate that ATP synthesis and utilisation have become uncoupled. Fatty acids are known to displace nucleotides from mitochondrial uncoupling proteins and interfere with their action (i.e., increasing proton leak). Additionally, they can bind directly to the mitochondrial adenine nucleotide carrier and block ATP from leaving the mitochondria (see Brown, 1992 for review). However, the ATP and ADP measured in Experiment 3 are extracted from both the cytosolic and the mitochondrial compartments and therefore this total cell [ATP:ADP] ratio is not representative of those types of metabolic

regulation. Thus, the maintenance of a normal ratio of total cell [ATP:ADP] with low total ATP in the astrocytoma cells in oleic acid-supplemented deficient media could indicate that the overall cell metabolic rate is lower than control but the balance of respiration and ATP synthesis is fully maintained through the utilisation of oleic acid. A similar scenario is seen in quiescent cells that lower their overall metabolic rate while maintaining normal respiration. It has been shown in quiescent/hibernating human fibroblasts and rat cardiomyocytes that ATP consumption/production is down-regulated without measurable changes in energy status (James *et al.*, 1999; Stumpe & Schrader, 1997).

When 400 μM oleic acid + 3 mM carnitine was supplemented into normal media even more cellular ATP was generated than when supplemented into the deprivation conditions. This may be due to: (1) the fact that cell metabolism is normal in these cultures and not slowed as in the deprivation cultures, (2) the fact that glucose-derived oxaloacetate is required for fatty acid acetyl-CoA to enter the TCA cycle ('fat burns in the flame of carbohydrate'), (3) fat metabolism can not completely replace glucose because it can not use the pentose phosphate pathway where NADPH and ribose units are primarily produced, or (4) the higher efficiency of fat-fuels over glucose. Although the rates of fatty acid oxidation by astrocytes in the literature are 12-16 times lower than that of glucose (table. 7-6), the ATP yield per mol of fatty acid is approximately 4 times greater than glucose if CO_2 is the end-product of glucose oxidation and 73 times greater if lactate is the end-product of glucose oxidation. Therefore, less fatty acid would need to be oxidised in order to produce the same amount of ATP generated from other, less efficient substrates. The ADP content of the astrocytomas remained relatively constant in the non-deprivation conditions even though ATP changed considerably. Even in the deprivation media, ADP didn't change as dramatically as ATP. This is most likely due to the PCA extraction liberating a large amount of previously bound ADP which would not have taken part in enzyme catalysed reactions of metabolism. Brindle, *et al.* (1989) have shown that the majority of intracellular ADP is not free. Therefore, the changes in the free cytosolic pool of ADP due to ATP breakdown or creatine kinase activity would be masked by the extract-release of bound ADP. However, the loss of total adenine nucleotides observed in deficient media indicates high adenlyate kinase activity with further AMP breakdown via IMP to inosine. Creatine kinase should prevent the net loss of adenine nucleotides by rephosphorylating ADP to

ATP from PCr stores (reviewed in Wallimann *et al.*, 1992). However, the ^{1}H-NMR of extracts from the astrocytomas in Chapter 5 showed that, similar to human astrocytes (Chapter 4), the transformed cells contained very low [PCr + Cr]. It has been shown in human lung cancer cells, neuroblastomas, and gliomas that low [PCr + Cr] corresponds to low creatine kinase activity (Kristiansen *et al.*, 1991; Lowry *et al.*, 1977).

Even though Experiment 1a & b showed that the astrocytoma cultures were capable of fatty acid β-oxidation without carnitine supplementation, there was very little contribution to cellular ATP from oleic acid oxidation in the absence of carnitine in Experiment 3. Thus, high extracellular fatty acid concentrations require concomitantly high carnitine concentrations to be utilised by astrocytoma cells. Many of the studies in the literature on human tumour cells which have concluded that fatty acids are not significantly utilised as an energy substrate have not used carnitine supplements (Spydevold & Gautvik, 1989; Furth *et al.*, 1992; Rofe *et al.*, 1986). In rat hepatoma cells, Prip-Buus, *et al.* (1992) have shown that the capacity for long-chain fatty acid oxidation is low because CPT-I sensitivity to malonyl-CoA is enhanced and is therefore more easily inhibited. However, octanoate, a short-chain fatty acid that does not require carnitine-transport, was actively oxidised by the hepatoma cells in the study and isolated mitochondria from the hepatoma cells had high rates of oleic acid oxidation. Thus, the tumour cells *were* capable of fatty acid β-oxidation even though this metabolic pathway may be restricted in culture by the availability of carnitine. Furthermore, the highest concentration of carnitine used in the experiments of the present study was 3 mM and there is still the possibility that astrocytomas are capable of even higher rates of fatty acid metabolism at higher carnitine concentrations since CPT is thought to be the rate-limiting factor in fatty acid oxidation (Guzman and Geelen, 1993; Zammit, 1994; McGarry and Brown, 1997). Although it is known that plasma fatty acid concentrations are elevated in tumour-bearing patients (in particular oleic acid concentrations) it is not known if carnitine concentrations are similarly elevated in these patients. Seelaender *et al.* (1996) showed that rat lymphocytes cultured with the serum from carcinosarcoma tumour-bearing rats had a 178-fold higher rate of oxidation of intracellular pools of [1-^{14}C]plamitate compared to lymphocytes in normal serum. They suggest that the enhanced capacity for β-oxidation by the

lymphocytes was due to 'factors' circulating in the serum of the tumour-bearing rats which could include elevated carnitine.

Thus, Experiment 3 has, at least in part, answered the question as to whether or not the rates of fatty acid oxidation in astrocytomas represent an active or dysfunctional metabolism by showing that it is sufficient to maintain viable levels of cellular ATP in the absence of all other substrates and to almost double the amount of ATP produced by cells in normal media. In the absence of normal human astrocytes for a direct comparison, this data is in strong support of the hypothesis that astrocytomas are able to utilise fatty acids in a similar way to normal astrocytes from the developing brain.

Chapter 8: **Fatty Acid Metabolism in Human Astrocytomas: Intermediary Metabolism and Membrane Incorporation**

8.1 Introduction

Little is known about fatty acid metabolism by tumour cells and even less is known about this metabolism in CNS tumours. From the studies in Chapter 7, it has been established that human astrocytoma cells in culture are capable of fatty acid oxidation and that this metabolism can provide up to 37% of cellular ATP during substrate deprivation and up to 50% more ATP when other substrates are present. Although these experiments using ^{14}C-labeled fatty acids were able to demonstrate considerable metabolic flux in the tumour cells, the precise products of fatty acid metabolism in the aqueous fraction of the cell extracts was not determined. The purpose of the present study was to describe the distribution of ^{13}C label from [1-^{13}C] oleic acid within the aqueous and membrane lipid pools of cultured astrocytoma extracts and from that distribution to evaluate the metabolic pathways involved in fatty acid metabolism by human astrocytomas. The effect of oleic acid on astrocytoma intermediary metabolism in the presence and absence of other energy substrates was also evaluated. Although the astrocytoma cells were shown to oxidise fatty acid in the presence and absence of other substrates (Chapter7), the effect of fatty acid utilisation on other aspects of cell metabolism is not known. For example, if fatty acid is able to supply sufficient cellular energy this may lead to reduced rates of glycolysis and/or glutaminolysis. In normal cells the oxidation of fatty acid results in decreased glycolysis and consequently decreased lactate production due to the glucose-fatty acid cycle (Randle cycle) but it is not known how the astrocytoma cells respond to these conditions. This can be indirectly assessed by the changes in ^{1}H-NMR-observed metabolites as well as the incorporation of ^{13}C label into aqueous metabolites. The quantitative incorporation of labeled fatty acid into the individual membrane phospholipids can also be assessed by mass spectrometry as well as any elongation/desaturation/recycling of fatty acid side-chains.

Two CO_2 molecules are produced from each acetyl-CoA molecule that enters the TCA cycle. If one of the carbons of the acetyl-CoA is labeled from [1-^{13}C]oleic acid not all of the label will end up in CO_2 as a result of the symmetry of citrate molecule and randomised decarboxylation. Thus, label can be

incorporated into any TCA cycle-derived metabolite or intermediate. For example, label can be lost from α-ketoglutarate to glutamate and glutamine or from oxaolacetate to aspartate. If the fatty acid-derived acetyl-CoA doesn't enter the TCA cycle, the label could also be found in the ketone bodies. See figure 8-1 for the possible fates of labeled carbon.

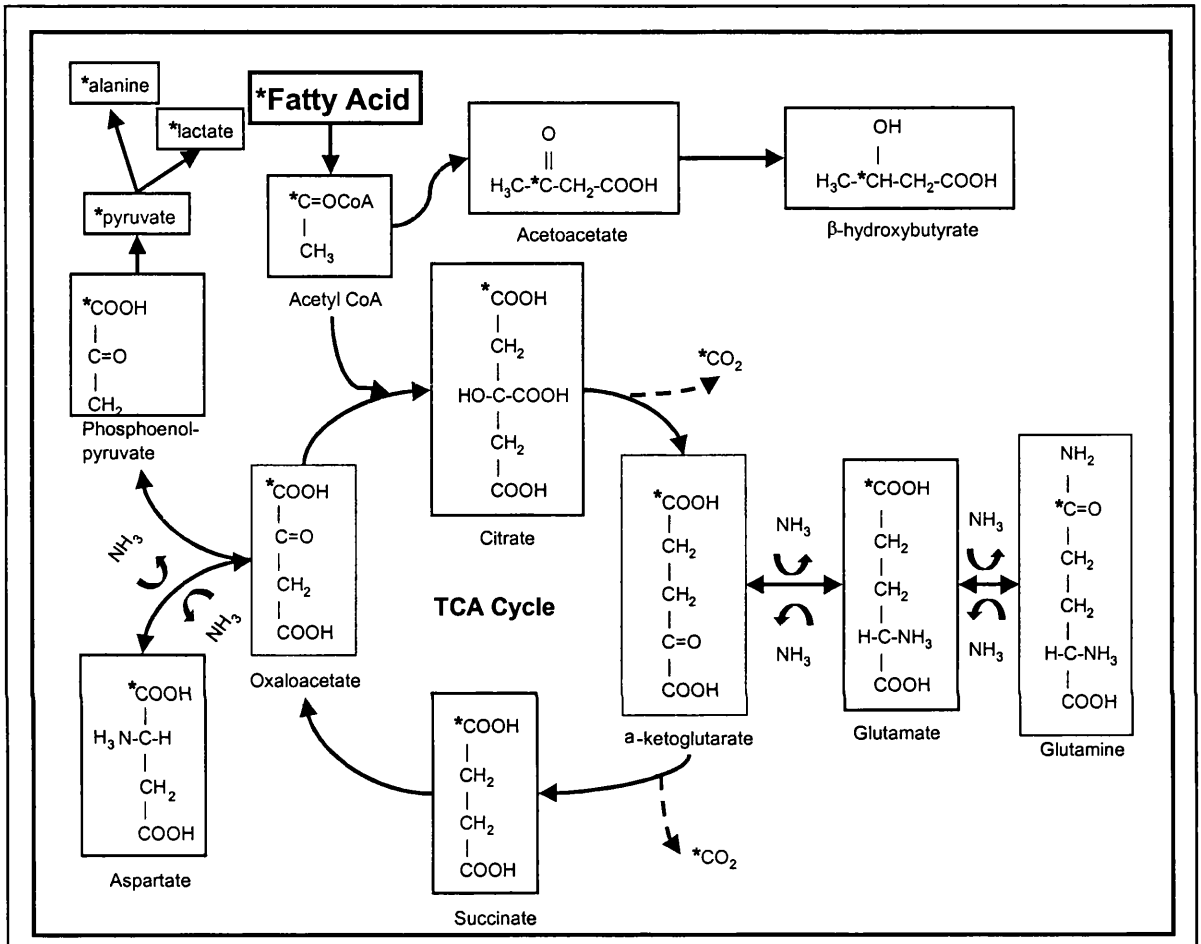


Figure 8-1. **Summary of the potential pathways involved in metabolism of fatty acid-derived acetyl-CoA.** ** indicates the possible fates of the [1-^{13}C]oleic acid carbon after incubation with astrocytoma cell cultures.*

8.2 Methods

Human astrocytoma cultures were divided into the following 3 groups for a 48-hour incubation in different media:

Group 1 (**D**): Normal media (DMEM + 10% FCS + 4mM glutamine)

Group 2 (**DOA**): Normal media + 400 μM [1-^{13}C]Oleic acid + 3mM carnitine

Group 3 (**DDOA**): Glucose- and amino-acid-free DMEM + 6 mM 2-deoxyglucose +

400 μM [1-^{13}C]Oleic acid + 3mM carnitine

After the 48-hour incubation, the media was collected and the cells were harvested. Fresh media samples of the same volume which had not been exposed to the cells were also collected. Both the cells and the media samples were extracted with the M/C technique. The aqueous fraction was prepared for ^{1}H & ^{13}C-NMR analysis and the lipid fraction of the cell extracts was prepared for mass spectrometry analysis. The ^{1}H-NMR analysis of the cell extracts was used to evaluate the effect of the different media compositions on cell intermediary metabolism. From the studies in Chapter 7 it is known that the oleic acid supplemented into normal and deficient astrocytoma culture media was oxidised by the cells but it was not demonstrated how the utilisation of the fatty acid may have affected the overall metabolism of the cells. Therefore, the proton spectra of the aqueous cell extracts were compared for metabolite yields. ^{13}C-NMR was also performed on the same samples to detect any ^{13}C-label enrichment in the aqueous metabolites of the cells.

The ^{1}H-NMR of the aqueous fraction of the culture media extracts was used to calculate natural abundance concentrations of the metabolites so that when the metabolite concentrations were calculated using ^{13}C-NMR spectra from

$$\frac{\int \text{glu} \,|\, \#\text{C TSP} \,|\, [\text{TSP}]}{\int \text{TSP} \,|\, \#\text{C glu} \,|} = [\text{glu}] = \frac{\int \text{glu} \,|\, \#\text{H TSP} \,|\, [\text{TSP}]}{\int \text{TSP} \,|\, \#\text{H glu} \,|}$$

the same samples the differences would reflect ^{13}C-label enrichment. Example of media enrichment calculations for glutamate (glu):

Although the integral (∫) of natural abundance glutamate in the ^{13}C spectrum would be only 1.1% of the glutamate integral in the ^{1}H spectrum, the metabolite concentration is calculated from comparison to the same internal standard (TSP) within the same sample. Therefore, unless the glu was enriched with ^{13}C, the

calculated concentration of glu should be the same from ^{1}H & ^{13}C spectra and any increase in concentrations in the ^{13}C spectrum would represent the enriched fraction. The ^{13}C-enrichment was also assessed in the control group even though this media did not contain any ^{13}C-enriched substrate in order to establish the experimental error/variability in the ^{13}C measurement. Therefore, ^{13}C-enrichment in the DOA and DDOA groups were only considered "real" or significant if they are significantly greater than the natural abundance control group. Additionally, the aqueous metabolite concentrations from the cell culture media were compared between fresh media and the media exposed to the astrocytoma cells for 48 hours to assess metabolite uptake or release.

The lipid fraction of the cell extracts was analysed by gas ionisation tandem mass spectrometry for membrane phospholipid concentration, composition, and ^{13}C-enrichment. The lipid fraction of the media extract was analysed by ^{13}C-NMR to determine the concentration of oleic acid remaining after the 48-hour incubation. [1-^{13}C]oleic acid was not used for calculating metabolic flux rates in this experiment. The 48-hour incubations allowed observation of steady-state label incorporation into particular aqueous and lipid metabolites which in turn may provide information for the involvement of specific metabolic pathways in fatty acid utilisation by astrocytomas. It was hoped that the specific enrichments of the intracellular metabolites labeled with ^{13}C could be determined for both the aqueous and lipid fraction of the cell extracts. Unfortunately, in the aqueous fraction the enrichment of the ^{13}C label was below detectable levels for NMR. Therefore, the 6 samples per group were pooled into one sample, freeze-dried, and reconstituted in a small volume (40 μl) for ^{1}H-^{13}C 2D NMR analysis using a micro-NMR probe and the heteronuclear 2D HMBC sequence to observe long-range couplings of ^{13}C label in the carboxyl carbons of aqueous metabolites.

The experiments in this study are summarised for clarity in figure 8-2.

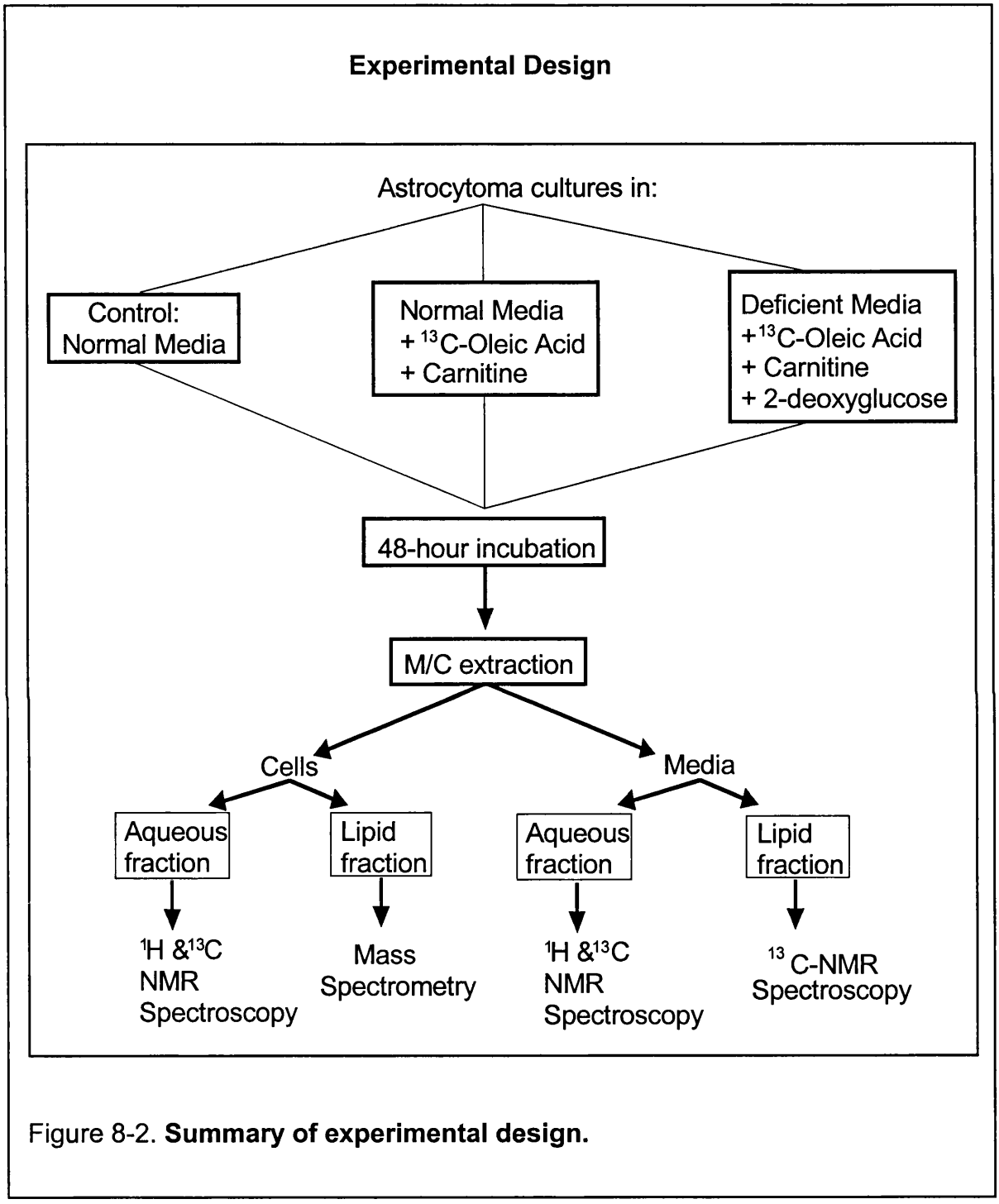
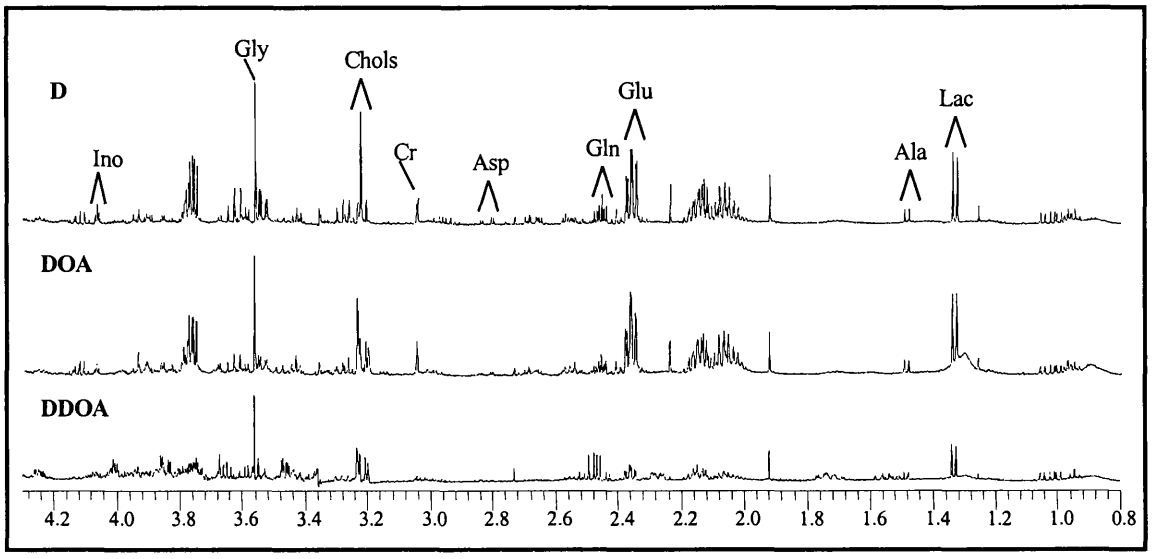


Figure 8-2. **Summary of experimental design.**



Figrue 8-3. **Representative ^{1}H-NMR spectra from M/C extracts of astrocytoma cells incubated in D, DOA, and DDOA culture media for 48 hours**. *Abbreviations are as follows: D = control medium (DMEM + 4 mM glutamine + 10% FCS); DOA = control medium (D) supplemented with 400 μM oleic acid + 3 mM carnitine; DDOA = glucose- and amino acid-deficient DMEM supplemented with 400 μM oleic acid + 3 mM carnitine; Lac = lactate; Ala = alanine; Glu = glutamate; Gln = glutamine; Cr = total creatine (phosphocreatine + creatine); Chols = choline-containing compounds (glycerophosphocholine + phosphocholine + choline); Gly = glycine; Asp = aspartate; Ino = inositol. Fully relaxed spectra were collected (tr = 22 sec) and referenced to TSP as an internal standard.*

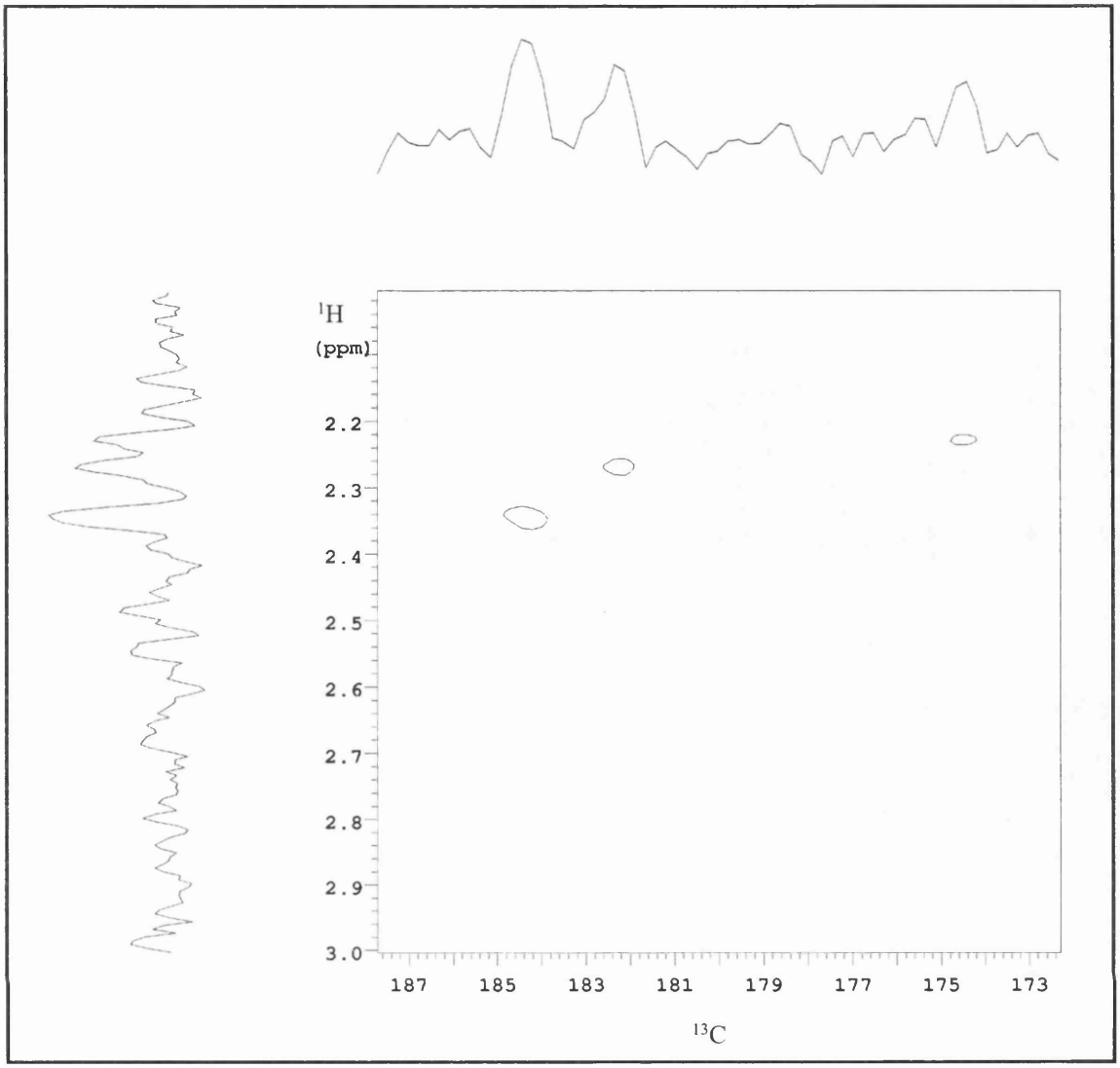


Figure 8-4. **2D ^{1}H-^{13}C HMBC spectrum of pooled cell extracts from the astrocytomas incubated with the DOA media.** *No ^{13}C-label signal was detected in the extracts from the astrocytomas incubated in control media (natural abundance) or in the group incubated in the DDOA media. The ^{13}C label was only detected in the long-range couplings (i.e., the carboxyl carbons) of glutamate. Amino acid standards were run at different pH levels to confirm the glutamate assignment at low pH. See figure 8-3 for abbreviations.*

8.3.1 Cell extracts

8.3.1.1 ^{1}H-NMR Spectroscopy

Extracts of the astrocytoma cells incubated for 48 hours in either control media (D), control media supplemented with oleic acid and carnitine (DOA), or a deficient media supplemented with oleic acid and carnitine (DDOA) demonstrated a significant overall difference between the groups for total creatine (p=0.0002; α=0.0063 (0.05/8)) and glutamate (p=0.0060; table 8-1). Post-hoc tests revealed a significantly increased total creatine in the DOA group compared to both the control group (D) and the DDOA group (p=0.0017 & 0.0008, respectively) and increased GPC (p=0.0059) compared to the DDOA group. The DDOA group had significantly reduced glutamate (p=0.0028) and lactate (p=0.0001) compared to the control group. See table 8-1 for summary.

	D	DOA	DDOA	P-VALUE
Creatine	5.46 ± 0.75	**10.48 ± 0.96**	5.88 ± 0.58	**0.0002**
GPC	8.57 ± 1.85	**18.14 ± 1.45**	8.35 ± 1.35	0.0094
PC	16.76 ± 3.96	4.40 ± 2.09	5.03 ± 1.69	0.0076
Choline	7.80 ± 0.83	7.81 ± 1.91	4.99 ± 1.31	0.2557
Aspartate	14.36 ± 1.56	14.69 ± 2.37	7.92 ± 1.44	0.131
Glutamine	30.13 ± 1.88	22.95 ± 3.60	29.00 ± 5.90	0.2855
Glutamate	79.16 ± 12.31	68.97 ± 16.95	**19.90 ± 3.63**	**0.0060**
Lactate	26.06 ± 1.82	27.67 ± 5.11	**12.72 ± 1.69**	0.0172

Table 8-1. **Comparison of the intracellular metabolites extracted from astrocytoma cultures after a 48-hour incubation in different media compositions.** *Metabolite concentrations (nmol/mg protein) are expressed as mean ± SEM. Abbreviations are as follows:* ***D*** *= control media consisting of DMEM + 4mM glutamine + 10% FCS;* ***DOA*** *= control media + 400 μM oleic acid + 3 mM carnitine;* ***DDOA*** *= glucose- and amino acid-free DMEM + 6 mM 2-deoxyglucose + 400 μM oleic acid + 3 mM carnitine; creatine = total creatine (phosphocreatine + creatine); GPC = glycerophosphocholine; PC = phosphocholine. P-values in bold indicate an overall significant difference between the groups (α=0.05/8) and post-hoc significances are indicated in bold in the individual metabolite concentrations.*

8.3.1.2 ^{13}C-NMR Spectroscopy

Neither direct-detected or indirect-detected ^{13}C spectroscopy revealed any natural abundance or enriched ^{13}C signal from the astrocytoma cell extracts indicating that the samples were too dilute for NMR sensitivity. Therefore, all the samples from each group (n=6/group) which had previously been analysed by ^{1}H-NMR (section 8.3.1.1 above) were then pooled and reconstituted in 40 μl for analysis in a micro-NMR probe. Even with the micro-NMR probe no directly detected or one-bond carbon (^{13}C-^{1}H) couplings could be detected in 2D heteronuclear NMR spectra from any of the three samples representing the D, DOA and DDOA groups. However, long-range couplings in glutamate (^{13}C-O-O-^{1}H) were detected in the DOA group (figure 8-4). The shimming and sample pH were very difficult to adjust in such a small volume of sample. Therefore, the assignment of the peaks in the HMBC spectrum were confirmed by running standards of several different amino acids (aspartate, glutamate, glutamine, alanine, and lactate) at several different pH levels using the same HMBC parameters as used on the cell extract samples. Only glutamate had the appropriate cross peaks at very low pH (<1.0). Thus, a small amount of ^{13}C label from [1-^{13}C]oleic acid was recovered in intracellular glutamate from the astrocytomas incubated in normal media supplemented with the labeled fatty acid. A quantitative concentration was not ascertained due to the dilute nature of the samples.

8.3.1.3 Mass spectrometry

Mass spectrometry analysis of the membrane phospholipids demonstrated that there was a significant overall difference between the groups in PtdCho, PtdEth, PtdIno, and total phospholipid concentrations (table 8-2). Post-hoc analysis revealed a large significant increase in the PtdCho and PtdEth concentrations in the DOA group compared to the other groups (p_{PtdCho}=0.003 D, 0.007 DDOA; p_{PtdEth}=5.9x10^{-7} D, 5.2x10^{-5} DDOA; α=0.013). The DDOA group had significantly elevated PtdEth (p=0.001) and lower PtdIno (p=0.003) compared to controls. For total phospholipids the DOA group had significantly higher concentrations of phospholipids compared to the other groups (p=0.001 D, 0.002 DDOA). There was no significant difference between the total phospholipids in the control (D) and DDOA groups (p=0.405).

	D	DOA	DDOA	P-VALUES
PtdCho	50.50 ± 5.15	**159.77 ± 23.45**	66.84 ± 6.94	**0.00008**
PtdEth	10.43 ± 0.82	**25.50 ± 0.44**	**15.75 ± 0.92**	**1.5×10^{-8}**
PtdSer	25.86 ± 3.89	20.08 ± 2.52	19.26 ± 3.64	0.384
PtdIno	21.26 ± 1.40	19.84 ± 1.72	**14.74 ± 0.74**	**0.008**
Total	108.05 ± 11.26	**225.19 ± 28.13**	116.59 ± 12.24	**1.3×10^{-5}**

Table 8-2. **Comparison of membrane phospholipid concentrations between astrocytoma cells incubated in different media compositions.** *See table 8-1 for media compositions of groups D, DOA, & DDOA. Abbreviations are as follows: PtdCho = phosphatidylcholine; PtdEth = phosphatidylethanolamine; PtdSer = phosphatidylserine; PtdIno = phosphatidylinositol. Concentrations (nmol/mg protein) are expressed as mean ± SEM.*

Further analysis of the fatty acid acyl groups of the individual phospholipids has shown that the majority of exogenously supplied ^{13}C-labeled oleic acid was incorporated intact in several phospholipids (table 8-3). In the DOA group ^{13}C label was found in PtdCho, PtdEth, and PtdIno with the percentage of total phospholipid incorporation ranging from 29 to 79% and was greatest in PtdCho followed by PtdEth and PtdIno. The DDOA samples also contained ^{13}C label in PtdCho and PtdEth with the majority of label in PtdCho (table 8-3).

	DOA				DDOA			
	PtdCho	PtdEth	PtdSer	PtdIno	PtdCho	PtdEth	PtdSer	PtdIno
16:0	--	--	--	--	**8.7 +/- 2.3**	--	--	--
18:0	--	--	--	--	--	--	--	--
18:1	**79.1 +/- 9.3**	**41.3 +/- 10.4**	--	**29.0 +/- 5.9**	**63.4 +/- 8.8**	**38.2 +/- 8.9**	--	--
18:2	--	--	--	--	--	--	--	--
20:4	--	--	--	--	--	--	--	--
22:6	--	--	--	--	--	--	--	--
% of total labeled phospholipids	**73**	**22**	**0**	**5**	**91**	**9**	**0**	**0**

Table 8-3. **% ^{13}C-enrichment in the individual and total phospholipids.** *See table 8-2 for abbreviations. The ^{13}C-enrichment is represented as a percentage of the total label in the acyl groups for each individual phospholipid. The [% of total labeled phospholipids] represents the ^{13}C in all acyl groups from the individual phospholipid as a percentage of the ^{13}C in all of the phospholipids combined.*

8.3.2 Media Extracts

There were surprisingly few significant differences between metabolite concentrations in the different media composition groups. Only the DDOA group had a significant net loss of lactate from the media while the other groups had a net increase in media lactate ($p=0.002$; $\alpha=0.0063$; table 8-4). Although not significantly different from the other groups, the DDOA group had a small increase while the other groups had a small decrease in media β-hydroxybutyrate. Neither glutamate or aspartate was detected in any of the media groups. The ^{13}C enrichment was not significantly different to the control (natural abundance) group for any of the metabolites from the DOA and DDOA groups except for carnitine in the DDOA group which was just outside significance ($p = 0.009$; $\alpha=0.05/6 = 0.008$). Although there was a considerably higher average oleic acid uptake from the media in the DOA group than in the DDOA group, there was a large variability in the uptake in both groups and consequently no significant difference was detected ($p=0.317$; table 8-4).

	D	% ^{13}C	DOA	% ^{13}C	DDOA	% ^{13}C
β-HB	- 1.5 ± 0.3	1.5 ± 0.7	- 1.6 ± 0.5	0.9 ± 0.4	+ 2.2 ± 1.3	2.9 ± 1.1
Lactate	+ 83.9 ± 26.3	2.8 ± 1.0	+197.9 ± 53.6	4.9 ± 2.9	**- 20.4 ± 5.8**	2.2 ± 1.1
Glycine	- 1.2 ± 0.3	1.4 ± 0.5	- 1.0 ± 0.2	2.0 ± 1.1	- 0.4 ± 0.2	0.9 ± 0.6
Alanine	- 0.8 ± 0.4	0.9 ± 0.4	+ 5.1 ± 1.4	1.0 ± 0.4	+ 2.1 ± 0.8	1.1 ± 0.8
Glutamine	- 99.7 ± 29.7	1.6 ± 0.9	- 136.0 ± 19.4	3.5 ± 0.8	- 69.2 ± 10.3	2.7 ± 1.2
Glutamate	ND	--	ND	--	ND	--
Aspartate	ND	--	ND	--	ND	--
Choline	- 4.5 ± 1.2	NM-^{13}C	- 6.6 ± 2.9	NM-^{13}C	- 3.7 ± 1.4	NM-^{13}C
Carnitine	- 27.4 ± 5.2	0.8 ± 0.3	- 208.0 ± 46.4	1.2 ± 0.9	-242.6 ± 41.8	**7.6 ± 1.1**
Glucose	-267.1 ± 64.2	NM-^{1}H	- 256.6 ± 47.6	NM-^{1}H	- 192.2 ± 41.4	NM-^{1}H
Oleic Acid	ND	--	- 482.7 ± 197.4	NM-^{1}H	-256.4 +88.7	NM-^{1}H

Table 8-4. **Astrocytoma uptake (-) or release (+) of metabolites from/in different media compositions.** *After incubation with the astrocytoma cultures used media was collected, extracted, and analysed along with samples of the same media which had not been exposed to the cells. These data represent the differences in metabolite concentrations between the media exposed to the cells and the fresh media of the same composition. Metabolite uptake is represented by a negative value and elevated metabolite concentrations are positive. Metabolite concentrations were established from the ^{1}H-NMR spectra of the media extracts for all metabolites except for glucose which was measured using the ^{13}C-spectra. The ^{13}C-spectra were also used to calculate the concentrations of the same metabolites in the same samples previously analysed by ^{1}H-NMR to determine any ^{13}C-enrichment (see methods for calculation). The ^{13}C "enrichment" has also been calculated for the control group (D) even though there would be no ^{13}C enrichment in this sample above natural abundance (1.1%) so that the variability in this measurement could be determined and the significance of ^{13}C-enrichment in the other samples assessed. ^{13}C-NMR was also used to assess the uptake of labeled oleic acid from the lipid fraction of the DOA and DDOA media extracts. Metabolite concentrations (nmoles/mg protein) are expressed as mean ± SEM. Abbreviations are as follows: β-HB = β-hydroxybutyrate; NM = not measured; ND = not detected.*

8.4 Discussion

8.4.1 Glutamate – Aspartate

One of the major differences between the normal and transformed astrocytes *in vitro* (Chapter 5) and between the astroctyoma tumours and normal contralateral brain (Chapter 6) was elevated glutamate and aspartate in the transformed cells. I have speculated in those chapters that the consistent increase in these specific amino acids may be linked to increased glutaminolysis in the astrocytomas. Glutaminolysis has been shown to be elevated and to be a vital source of cellular ATP generation in hepatic tumours, HeLa cells, and other transformed cells (Moreadith & Lehninger, 1984; Parlo & Coleman, 1984, 1986; Street *et al.*, 1993; Piva & McEvoy-Bowe, 1998; Board *et al.*, 1990) but has not, to my knowledge, been investigated in astrocytomas or other CNS tumours. The ^{1}H-NMR data from the cell extracts in the present study supports this suggestion that the elevated glutamate and aspartate in the astrocytomas reflects increased glutaminolysis because the DDOA group in which the cells were incubated in a media deficient in glutamine (and glucose and other amino acids) had significantly decreased glutamate and aspartate. The substrate-deficient composition of the DDOA group (apart from the presence of oleic acid) might have resulted in a general reduction in TCA cycle activity with glutamate and aspartate decreasing because of this general metabolic downturn rather than being specifically related to the lack of glutamine substrate. However, other TCA cycle-derived metabolites such as glutamine and glycine were not significantly decreased, indicating TCA cycle activity may not have been severely affected by the media composition. The oleic acid was shown in Chapter 7 to be able to supply a third of normal ATP even in this deficient media which most likely involved a significant contribution from the TCA cycle metabolism of oleic acid-derived acetylCoA to the total cell ATP.

Furthermore, heteronuclear 2D NMR (HMBC) analysis of the cell extracts in the present study demonstrated that some label from the [1-^{13}C]oleic acid can be found in intracellular glutamate in the DOA group (figure 8-4). It is possible that other metabolites were labeled (e.g., aspartate) but the concentrations were too low for detection in the samples. The fatty acid-derived glutamate can be further metabolised via the same pathway of glutaminolysis (which normally involves the conversion of glutamine to glutamate before entering the TCA cycle at α-ketoglutarate). This is in good agreement with several other studies of fatty

acid metabolism by rat brain and lymphocytes which have shown that glutamate, glutamine, and aspartate are the major aqueous products of fatty acid metabolism (Miller *et al.*, 1987; Newsholme, *et al.*, 1989). The study by Miller *et al.* (1987) showed not only that glutamate and glutamine in rat brain extracts contained the majority of aqueous label (65-75%) from fatty acid oxidation but that this occurred only at the early time points (0-4 hours) after intravenous fatty acid injection. At later time points post-injection (20-44 hours) the majority of label was found in aspartate and acetyl-L-carnitine. Thus, this demonstrated that the glutaminolysis pathway as well as the recycling of metabolised fatty acids was active in the developing brain. Unlike the Miller study, the astrocytoma cells in the present study were exposed to a constant media supply of labeled oleic acid which may explain why label was found in glutamate even after 48 hours of incubation. Although ^{13}C-labeled aspartate was not also detected in the present study, this may be due to the dilute nature of the samples and the relatively lower intracellular concentration of aspartate (14 nmol/mg protein) compared to glutamate (79 nmol/mg protein), it is also possible that when fatty acids are the source of intracellular glutamate, metabolism via the TCA cycle to aspartate does not occur until the fatty acid substrate source is exhausted (i.e., when more energy needs to be extracted from amino acid stores/sources). Otherwise, it seems that the time-frame for the metabolism of labeled glutamate to aspartate in the Miller study is unusually long (20-44 hours post-injection) since several studies on cultured astrocytes using labeled glutamine and glutamate in the culture media observed their metabolism to aspartate and lactate within 2-3 hours (McKenna *et al.*, 1996; Bakken *et al.*, 1998; Piva & McEvoy-Bowe, 1998; Moreadith & Lehninger, 1984).

Although astrocytes are thought to metabolise glutamate extensively to glutamine which is then released for uptake by other cells, McKenna *et al.* (1996) have demonstrated that the extracellular concentration of glutamate can determine the fate of glutamate metabolism by normal rodent astrocytes. At low concentrations ($\leq$0.1 mM) glutamate was primarily used to synthesise glutamine, whereas at higher concentrations (0.2 – 0.5 mM) labeled carbon was mainly incorporated into intracellular aspartate and lactate released into the media. If high intracellular glutamate concentrations have a similar effect on cells of astrocytic origin, then the high glutamate demonstrated in the astrocytoma

extracts in the present study may have contributed to the resultant elevation in aspartate as opposed to glutamine synthesis and release.

8.4.2 Lactate

Although lactate production and release into the media was quite high in the DOA group (significantly higher than the DDOA group), the uptake of glucose from the media was not considerably different to the other groups (table 8-4). This suggests that some of the media lactate may be generated by the cells from a substrate other than glucose. Sonnewald *et al.* (1993) have demonstrated lactate formation from glutamate in normal rodent astrocytes and McKenna *et al.* (1996) also showed that the metabolism of [U-^{13}C]glutamate resulted in recovery of labeled lactate in the media. Therefore, it may be the elevated intracellular glutamate pool which contributed to the lactate released by the cells in this group. The glutamate and therefore the lactate could be produced from the metabolism of fatty acid-derived acetylCoA via the TCA cycle. However, only intracellular glutamate was found to contain a detectable level of ^{13}C-enrichment and the lactate in the media did not have a significantly increased ^{13}C-enrichment over natural abundance samples (group D). A possible explanation why ^{13}C label might not be detected in the fatty acid-derived lactate is that only one in every 9 acetylCoAs produced from [1-^{13}C]oleic acid would contain label and further dilutions of the label can occur via exchange reactions with the TCA cycle such that lactate produced via oxaloacetate and phosphoenolpyruvate may have less label than earlier TCA cycle products like glutamate (figure 8-1).

In the McKenna *et al.* (1996) study, the amount of lactate produced from glutamate was only a small fraction of the total lactate produced by the astrocytes. However, in the present study it may represent a substantial amount of the lactate produced because (1) more lactate is released in the media by the DOA group despite the amount of glucose uptake from the media being similar to the other groups and (2) the DDOA group contained considerably high intracellular lactate given the fact that a glucose-free media was used and any fetal calf serum-provided glucose was blocked from glycolysis by the competitive uptake of high 2-deoxyglucose concentrations (intracellular lactate was still significantly lower in the DDOA group compared to the other groups). This may indicate that the "glutaminolysis" pathway from α-ketoglutarate to oxaloacetate may be even more active in the transformed astrocytes and (in addition to

aspartate) lactate can be formed from oxaloacetate via phosphoenolpyruvate carboxylase (Hamprecht et al, 1994; figure 8-1). However, lactate can also be produced from malate-derived pyruvate via malic enzyme. Therefore, a conclusive understanding of the pathways utilised by the astrocytomas would require the measurement of these enzyme activities. However, I would speculate that malic enzyme activity was low in the astrocytomas. Otherwise, it would have been alanine rather than aspartate that was elevated due to the metabolism of the truncated TCA cycle. Alanine aminotransferase is active when sufficient pyruvate is available to saturate pyruvate dehydrogenase and inhibit aspartate aminotransferase (Moreadith and Lehninger, 1984; Piva and McEvoy, 1998). Some tumour mitochondria have been shown to have increased alanine efflux from glutamate metabolism but only in the presence of artificially high pyruvate concentrations which confirmed that these mitochondria must have a low malic enzyme activity (Kovacevic *et al.*, 1991; Piva & McEvoy, 1998). Similarly, in the present study, the astrocytomas did not have any significant increases in intracellular or media alanine under any of the experimental conditions or compared to the alanine concentrations seen in the normal astrocytes from Chapter 5. Therefore, any non-glucose lactate production would most likely have occurred via phosphoenolpyruvate carboxylase.

The conversion of glutamate to lactate may represent a mechanism for the complete oxidation of the glutamate carbon skeleton for energy. Sonnewald *et al.* (1993) have shown that recycling of TCA constituents through pyruvate occurs in normal astrocytes and this may also occur in the transformed astrocytes. The release of the lactate from the astrocytomas in the present study in the substrate-abundant media of the DOA group where cellular ATP can be up to 50% higher than in normal media (Chapter 7) may be a metabolic mechanism which allows the cells in more vascularised regions of a tumour to release 'excess' lactate for uptake and metabolism by other more deprived regions of the tumour in a similar way to which lactate has been suggested to serve as a source for transferable energy between astrocytes and neurons (Pellerin & Magistretti, 1994). Although a high rate of glycolysis is a well recognised phenomenon in tumour cells and other rapidly dividing cells, the lactate produced has generally been considered to be a by-product of this abnormal metabolic pathway rather than as a potential energy source for other cells in the tumour. However, if lactate is also significantly produced via other metabolic

pathways such as glutaminolysis, then perhaps the transformed metabolism of lactate and its use should be more closely investigated in tumour cells and intact tumours.

Lactate formation through the TCA cycle (i.e., from glutamate) has been observed in rat brain slices (Bachelard, 1994) and rat astrocyte cultures (Sonnewald *et al.*, 1993) but not in cultured rat neurons (Westergaard *et al.*, 1995). Therefore, this metabolic pathway may be unique to the astrocytic lineage and their capacity for clearing potentially toxic extracellular glutamate from the brain. In which case, lactate formation from fatty acid-derived glutamate in astrocytomas may be related to the metabolism of their cell type of origin.

In normal cells the metabolism of abundant extracellular fatty acid should inhibit glycolysis due to the glucose-fatty acid cycle (or Randle cycle). However, in the cells from both groups containing supplemented fatty acid, glucose uptake was not significantly different to controls (D) and lactate production was even increased in the DOA group (table 8-4). Therefore, either the glucose-fatty acid cycle is reversed in the transformed cells with fatty acids *stimulating* glycolysis, or at least some lactate may be produced from another substrate other than glucose.

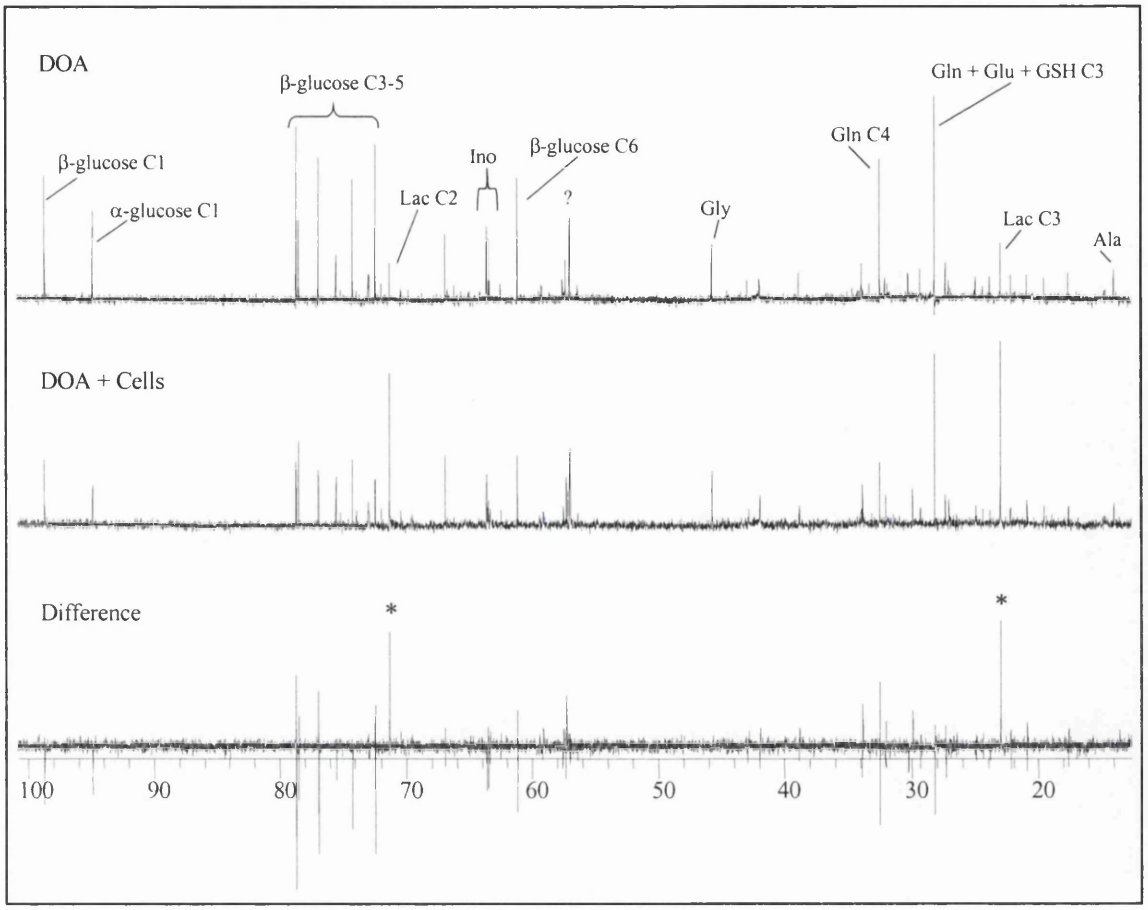


Figure 8-5. **Representative ^{13}C-NMR spectra of media extracts from fresh DOA media and the DOA media collected after 48 hours incubation with the astrocytoma cultures.** *The difference spectrum demonstrates the uptake of many different compounds from the media and * represents the most abundant metabolite, lactate, released into the media. Although this is only natural abundance ^{13}C signal detect and is not significantly enriched compared to control spectra.*

8.4.3 β-hydroxybutyrate

β-hydroxybutyrate (BHB) is a potential carbon source (along with amino acids and fatty acid) for the astrocytomas when glucose is not available to fuel the TCA cycle. BHB was increased in the media extracts from the DDOA group but was decreased in the media from the other groups. The reason may be reduced TCA cycle activity in the DDOA cells since fatty acid-derived acetyl-CoA requires sufficient carbohydrate-derived oxaloacetate to enter the TCA cycle and with no other significant energy substrate available from the DDOA media the fatty acid-acetylCoA would be diverted towards ketone body synthesis. If, as

suggested in section 8.4.2, the lactate produced by the cells from fatty acid oxidation is due largely to phosphoenolpyruvate conversion of oxaloacetate to pyruvate then this would add to the truncated TCA cycle frequently observed in tumour cells by removing more oxaloacetate. In which case, oxaloacetate concentrations may be too depleted for condensation with new acetylCoA entering the cycle. In this case, the fatty acid-derived acetylCoA may be used instead to produce ketone bodies that may be released into the media for consumption when the partial oxidation of fatty acid is exhausted as an energy source. Even normal rodent astrocytes are known to produce and release ketone bodies and 2 out of 3 produced are also oxidised by the cells via the TCA cycle for ATP production (Bixel *et al.*, 1995).

No ^{13}C-enriched NMR signal was detected in the ketone bodies in cell extracts from any group. However, as mentioned previously, this may be due to the limits of detecting the small concentrations in a dilute sample. In the media extracts the ^{13}C enrichment of the β-hydroxybutyrate was higher than the other groups but was not significantly different to the natural abundance sample. However, the DDOA group was the only group which displayed a small increase in BHB concentration in the media while the other groups had a net loss of the ketone body. As discussed above, the synthesis of ketone bodies from exogenously supplied fatty acids would be most likely to occur in the DDOA group because without replenishment of mitochondrial oxaloacetate, fatty acid-derived acetylCoA can not efficiently enter the TCA cycle and would then be available for ketone body synthesis.

8.4.4 Choline-containing compounds

Although borderline in significance, the cell extracts revealed that GPC and PtdCho were increased and PC was decreased when oleic acid was supplemented into the normal media (DOA; table 8-1). This is the opposite to what was expected since Gillham & Brindle (1996) have shown that the concentration of oleic acid used in the present study (400 μM) stimulates a PtdCho-specific phospholipase C in myeloma and hepatic cells. It is possible that PtdCho hydrolysis did occur but that PtdCho synthesis far outweighed the oleic acid-induced breakdown. This could explain why both GPC, a product of PtdCho catabolism, was increased while the PtdCho concentration itself was also increased. PtdEth as well as the total phospholipid concentration were also

significantly elevated by the DOA medium. This suggests that membrane synthesis and turnover was stimulated by the presence of the high oleic acid concentration and this is in keeping with the known growth-stimulatory effect of oleic acid on other tumours (Vignikin *et al.*, 1989; Buckman & Erickson, 1991; Hubbard & Erickson, 1991; De Caterina *et al.*, 1999). However, the measurement of phospholipase C and choline synthase activities would be required to evaluate conclusively whether or not simultaneous membrane synthesis and breakdown have contributed to the metabolic products (GPC and PtdCho) detected in the fatty acid-supplemented astrocytoma cultures.

In the deficient media supplemented with oleic acid (DDOA) the cells did not have the same dramatic increase in PtdCho and although PtdEth was significantly increased compared to controls, it was not as enriched as in the DOA group. There was, surprisingly, no significant difference between the D and DDOA groups for any of the other phospholipids. This is surprising mainly because of the known effects of oleic acid on cell phospholipases. It is possible, though unlikely, that astrocytoma cells do not have the same PtdCho-specific phospholipase C as the other cells in which oleic acid has been shown to cause PtdCho hydrolysis. Further studies would be required in order to rule out this possibility. It is also possible that the effects of energy deprivation are not well reflected in the cell membrane composition because the oleic acid supplement is well suited to act as an energy substrate as well as a cell membrane constituent. Therefore, detectable levels of normal membrane recycling may still be occurring even though only 32-37% of normal ATP is being generated under the DDOA conditions (Chapter 7).

The very large increase in PtdCho and total phospholipids in the DOA group could also result from the storage of lipids within the cells as lipid droplets which are known to be surrounded by a surface monolayer of phospholipids and proteins (Murphy & Vance, 1999). These lipid droplet phospholipids may have contributed to the very large increase in phospholipid concentrations detected in the DOA group extracts. Although electron microscopy was not performed to confirm the presence of lipid droplets, the high concentration of oleic acid used in the medium is known to cause the formation of lipid droplets in other cells (Callies *et al.*, 1993) and it also seems unlikely that such a large increase in phospholipid content (>100%) could be accommodated by incorporation into the normal cell membranes alone. Analysis of the oleic acid uptaken by the cells and

the amount of oleic acid potentially used for oxidation and membrane incorporation is discussed in the next section but indicates that an excess of approximately 14% was taken up from the media, which may be stored intracellularly.

The mass spectrometry analysis of the membrane phospholipids further revealed that the exogenously-supplied labeled oleic acid was incorporated overwhelmingly intact as the 18:1 phospholipid acyl groups predominated and was incorporated mainly into PtdCho compared to the other phospholipids.

8.4.5 Oleic acid

Although there was a greater uptake of oleic acid from the media of the DOA group compared to the DDOA group, the variability within the groups prevented a significant difference between the groups being detected. Furthermore, uptake into the cells does not necessarily reflect fatty acid metabolism by the cells. There are three possible pathways for the exogenous oleic acid to take once entering the cells: (1) fatty acid oxidation, (2) incorporation into membrane lipids, and (3) storage as lipid droplets. Although lipid droplets were not directly measured in these cells, it may be possible to make a rough estimate of the use of the media-supplied fatty acid. This is achieved by calculating the amount of fatty acid incorporated into the membrane phospholipids and the amount of fatty acid that would be oxidised at the rates calculated in Chapter 7 as a percentage of the total fatty acid concentration taken up from the media. Any 'excess' fatty acid taken up by the cells, e. g., not accounted for by oxidation and membrane incorporation, may constitute the amount of fatty acid stored in the cells. For example, the DOA group had an average of 483 nmol oleic acid/mg protein taken up from the media. The rate of oleic acid ^{14}C flux recorded in the experiments from Chapter 7 was approximately 94 pmol/min/mgprotein which over the 48-hour incubation would equal 270 nmol/mg protein. The total amount of ^{13}C-enrichment in the 4 membrane phospholipids measured for this group equals 143 nmol/mg protein (PC= 79% of 160 + PE= 41% of 26 + PI= 29% of 20 from tables 8-2 & 8-3). Therefore, the difference between the fatty acid taken up by this group and the amount potentially used for membrane synthesis or oxidation is 70 nmol/mg protein. This may represent the amount of fatty acid stored as lipid droplets. See figure 6 for the percentage of total fatty acid uptake each activity may represent.

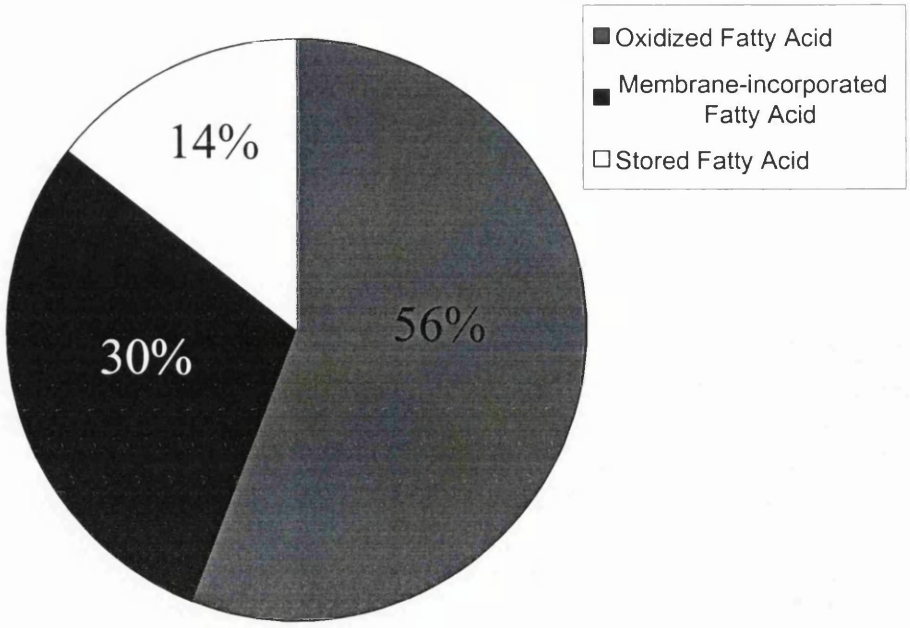


Figure 8-6. **Representation of the theorised use of media supplied oleic acid in the astrocytomas from group DOA**. *The oxidation rate is based on the calculated rate from* Chapter *7 and the membrane incorporation is calculated from the* ^{13}C *enrichment and the phospholipid concentrations calculated from the mass spectrometry analysis in the present* Chapter. *The stored fatty acid is extrapolated from the total fatty acid taken up from the media minus the fatty acids used for oxidation and membrane synthesis. The data is presented as a percentage of the total.*

The same calculation for the DDOA group is not as straightforward because the average oleic acid taken up from the media (256 nmol/mg protein) is considerably less than the amount needed to supply the same oxidation rate (270 nmol/mg protein) and the membrane phospholipid incorporation of 48 nmol/mg protein (62% of 67 + 38% of 16 from tables 2 & 3). However, the data from Chapter 7 also revealed that the amount of ATP generated from fatty acid oxidation in the DDOA group was 13-18 % less than the DOA group so the rate of oxidation used here may be a maximum for the cells. Therefore, if a rate of oleic acid oxidation 18% lower than that in the DOA group is used (77 pmol/min/mg protein = 222 nmol/mg protein over 48 hours) then the total fatty acid supply for oxidation (222 nmol/mg) plus membrane incorporation (48 nmol/mg) is equal to 270 nmol/mg protein and would roughly account for all of

the fatty acid taken up by the cells. Therefore, there would most likely be no fatty acids stored in the cells from the DDOA group. This suggests that the deprivation media of the DDOA group has resulted in fatty acid uptake and use (oxidation and membrane synthesis) that is evenly matched. However, in the substrate-abundant media of the DOA group the cells may have had more energy substrates than required for use in several pathways which could result in the storage of some fatty acid.

It is also worth noting that the uptake of fatty acid in the DOA & DDOA groups did not alter the uptake of glucose from the media compared to the control group. This was unexpected since the glucose-fatty acid cycle should have inhibited glucose uptake in these cells. Therefore, it is possible that the reciprocal inhibition normally caused by these substrates is uncoupled in the transformed cells. However, the uptake of media-glucose by the astrocytoma cells, even in the control group, indicates that the rate of glucose consumption by the transformed cells (260 nmol/mg over 48 hours= 90 pmol/h/mg protein) is considerably lower than what has been reported for normal astrocytes in the literature (190 – 1400 pmol/h/mg protein) (Yu & Hertz, 1983; Edmond, 1987). Thus, glucose metabolism may be more generally abnormal in the astrocytomas.

8.4.6 Carnitine

In the media extracts of the DDOA group there was a small but significant increase in ^{13}C-enrichment in media carnitine (i.e. acetyl-l-carnitine) which indicates that some fatty acid recycling may be occurring in these cells. Indeed, there is some ^{13}C label seen in the 16:0 fatty acid acyl group of membrane PtdCho in the DDOA group cell extracts (table 8-3). With high rates of fatty acid oxidation the cells may need to export acetylcarnitine into the media in order to maintain favorable acetylCoA : CoA balance within the cells. Acetyl-l-carnitine can be taken up from the media again when required as an energy substrate or for fatty acid/phospholipid synthesis.

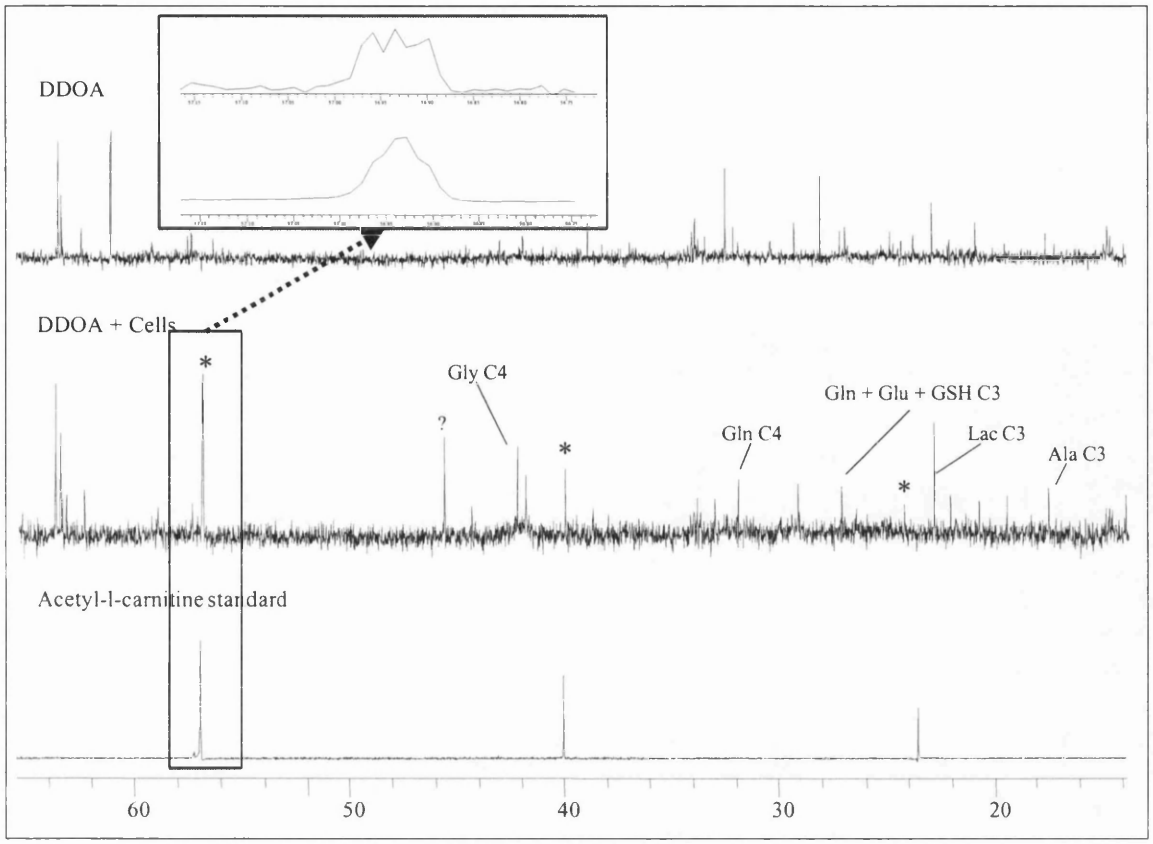


Figure 8-7. **Representative ^{13}C spectra from the media extracts of the DDOA group and an acetyl-L-carnitine standard**. *Abbreviations are as follows: gly = glycine; Glu = glutamate; Gln =glutamine; GSH = glutathione; Lac =lactate; Ala = alanine.*

8.4.7 Creatine

The incubation of the astrocytomas in normal media with supplemented fatty acid (DOA) resulted in a surprising increase in intracellular total creatine (table 8-1). In particular, phosphocreatine was increased in these cells. Therefore, the low total creatine observed in the cultured astrocytomas may reflect a metabolic requirement for creatine synthesis not met by normal culture media. Therefore, it is possible that the high creatine seen in the *in vivo* astrocytoma extracts from Chapter 6 was not due entirely to infiltration of normal brain tissue in the excised tumour sample and elevated plasma oleic acid may have contributed to the elevated total creatine concentrations observed.

In conclusion, the presence of high extracellular concentrations of oleic acid affected several different aspects of astrocytoma metabolism. The aqueous

and lipid metabolites associated with membrane synthesis and breakdown were especially affected. Several aspects of intermediary metabolism observed in the aqueous fraction were also significantly different in the presence of the fatty acid. As demonstrated in Chapter 7, fatty acids can serve as an oxidisable fuel to the astrocytomas which results in a change in intermediary metabolism and an associated change in energy substrate use. Glucose uptake, however, was surprisingly unaffected by the fatty acid metabolism. Therefore, this data has identified some potentially abnormal metabolic pathway activities related to fatty acid utilisation by high-grade astrocytoma cells.

Chapter 9: **General Discussion**

One of the advantages that has come from the development of cell culture preparations of astrocytes and of other cell types in the CNS is the ability to investigate their individual and interactive metabolism through the selective manipulation of culture conditions.

I have examined the metabolic profile of cultured human astrocytes and astrocytomas detected by ^{1}H-NMR spectroscopy and mass spectrometry. Several studies in the literature have identified distinguishing metabolic characteristics in astrocytomas compared to whole brain or other types of tumours. However, in order to identify specific metabolic changes associated with astrocytoma transformation, comparisons must be made between purified human astrocytoma cultures and purified human astrocytes grown in identical culture conditions. Without such specific comparisons, differences due to species, heterogeneous mixtures of cell types, and differences in culture conditions (i.e., serum content) can not be ruled out. I have carried out experiments which have demonstrated that there are several qualitative and quantitative differences in the metabolism of rat and human astrocytes such as an undetectable amount of taurine and hypotaurine and significantly lower creatine and glutamine in the human cells. However, the membrane phospholipids of the cells were not significantly different in concentration or composition. This has demonstrated the importance of using normal cells of the same species for comparisons to abnormal metabolisms.

Thus, for my studies on the metabolic changes associated with the transformation of human astrocytes, cell extracts of human astrocytomas (grade IV; glioblastoma multiforme) were compared with extracts from normal human astrocytes. Dual phase extractions were used, allowing the analysis of both aqueous metabolites (amino acids, etc.) and lipids from each sample. The astrocytomas were found to have significantly elevated *myo*-inositol, glycine, and aspartate. Glutamate was also elevated but with borderline significance. Mass spectrometry analysis of the membrane phospholipids also revealed significantly reduced phosphatidylcholine and a significant elevation in the other phospholipids from the astrocytomas. Furthermore, there was an increase in oleic acid in the phospholipid composition of the astrocytomas. These results

had several implications for understanding the changes in metabolism with transformation of human astrocytes:

(1) Elevated glutamate and aspartate in the astrocytomas may be related to increased rates of glutaminolysis which is the partial oxidation of glutamine through a truncated TCA cycle from α-ketoglutarate through oxaloacetate, producing aspartate. Several studies in the literature have identified high rates of glutaminolysis in other tumour cells such as HeLa cells and hepatomas with the accumulation of either aspartate or alanine as the major products of this pathway. To my knowledge, this particular metabolic pathway has not been investigated in astrocytomas or any other CNS tumour. Thus, the data from this thesis has revealed a metabolic pathway that may be especially active in astrocytomas, which warrants further investigation.

(2) The significant reduction in membrane phosphatidylcholine may be related to the common tumour-related characteristic of elevated phosphocholine in the aqueous fraction of the cell extracts. The dual phase extracts of the normal human astrocytes revealed that the high phosphocholine concentrations in the aqueous fraction was unlikely to be related to membrane phospholipid breakdown because significantly higher phosphatidylcholine concentrations were observed in the normal cells. This may indicate that phospholipid synthesis predominates in the rapidly dividing normal cells while phospholipid breakdown predominates in the rapidly dividing tumour cells. Further investigation of phospholipase and choline synthase activities would be necessary to confirm this hypothesis. This may be helpful in explaining the confounding evidence that elevated phosphocholine is related to both high growth rates in normal and transformed cells as well as with the transformation process itself.

Although the highly controlled environment of the cell culture studies above were vital to unambiguously identifying those metabolic characteristics of the transformed phenotype, the extent to which the metabolic profile for astrocytomas observed in culture occurs *in vivo* remains to be seen. There are many aspects of the *in vivo* environment which may not be accounted for in the culture conditions from which the profiles were developed, especially in the case of heterogeneous brain tumours. Thus, an important step in developing NMR spectroscopic techniques for use in differential clinical diagnosis is to identify

whether metabolic profiles from cultured tumour cells are valid markers of tumour-specific metabolism *in vivo.* Once again, limiting the number of confounding factors influencing the comparison of cells *in vitro* and *in vivo* is of vital importance for the unambiguous identification of changes due to the *in vivo* brain environment. Therefore, I have compared extracts from human astrocytoma cell cultures and tissue extracts of tumours grown *in vivo* in nude rat brain from the same human astrocytoma cells. This comparison should identify any detectable differences as a result of the *in vivo* environment which may not be accounted for in culture. Encouragingly, the only differences between the astrocytoma cells grown in culture and *in vivo* were higher concentrations of creatine and NAA in the *in vivo* tumour. This was most likely due to contaminating normal tissue from the host brain since creatine was shown to be particularly low in normal human astrocytes and astrocytomas *in vitro* while NAA is not present in astrocytes. When the *in vivo* tumours were compared to normal contralateral brain the major transformation-related characteristics identified in culture compared to normal astrocytes were preserved. Specifically, the tumours contained significantly elevated glycine, aspartate, and glutamate. Membrane phosphatidylcholine was also reduced and phosphatidylethanolamine increased similar to the *in vitro* astrocytoma lipid analysis. Overall, the *in vitro* characteristics of the astrocytomas were largely maintained *in vivo* and again implicate glutaminolysis and phosphatidylcholine hydrolysis as a highly active metabolic pathways.

One of the most striking characteristics of high grade astrocytomas observed clinically is the presence of a large NMR signal from mobile lipid which is not observed in cultured tumour cells unless standard culture media is supplemented with high concentrations of fatty acids. This is a potentially important aspect of astrocytoma metabolism that is not normally addressed in culture studies and is poorly understood. There is some debate as to the origin of the lipid signal observed in lipid-loaded cells. There is evidence that the cells contain cytostolic lipid droplets and that the cells undergo changes in cellular membranes which could also contribute to the lipid signal. Since there is evidence that both phenomena occur in lipid-loaded tumour cells, I have investigated the possible purpose of this lipid-loading by following the metabolism of ^{14}C- & ^{13}C-labled fatty acids in cultures of high-grade astrocytomas. By observing the metabolic fate of the labeled fatty acid, the

extent to which it is incorporated into membrane phospholipid or used for other metabolic purposes such as β-oxidation for cellular energy may be ascertained. Since astrocytes from developing brain are the only CNS cell type able to β-oxidise fatty acids I hypothesised that de-differentiated (i.e., fetal-like) transformed cells of the same lineage may be loading lipid for the same purpose.

Therefore, a study using ^{14}C-labeled fatty acids was undertaken and the astrocytoma cells in culture were shown to have a net uptake of the fatty acid from the culture medium. However cells in substrate-deficient media with glycolysis blocked by high levels of 2-deoxyglucose were unable to maintain viable levels of ATP (>10%) even when the cells had taken up fatty acid from the media. This led me to examine whether or not there was something missing from the culture media vital to fatty acid utilisation. Experiments with ^{14}C-palmitate and –oleate and the addition of various concentrations of carnitine, an important metabolite for the transport of fatty acid into cell mitochondria for oxidation, revealed that low levels of fatty acid oxidation were occurring without carnitine but that oxidation rates increased with increasing carnitine concentrations. The experiments also demonstrated that the fatty acids present in the culture media were oxidised by the astrocytoma cells to form metabolic products (e.g., amino acids, Acetyl-CoA, TCA intermediates, ketone bodies, etc.) as well as being completely oxidised to CO_2, at rates comparable to those seen in rodent astrocytes from the literature. A further set of studies demonstrated that astrocytoma cells in substrate-deficient, 2-deoxyglucose-supplemented media supplemented with 400 μM oleic acid were able to maintain viable levels of cellular ATP (i.e., 37% of control) but only in the presence of 3mM carnitine. Therefore, the ability of human astrocytomas to utilise fatty acid for the generation of up to 37% of cellular energy supplies and the formation of metabolic aqueous products has been demonstrated to exist.

I then proceeded to explore precisely which aqueous products were being produced through fatty acid β-oxidation and whether or not the astrocytomas utilised the available fatty acid (in the presence of carnitine) differently when other substrates were present in the culture media compared to when fatty acid was the only available substrate. ^{13}C-NMR spectroscopy was used to detect the label from [1-^{13}C] oleic acid in the aqueous metabolites from dual phase cell and media extractions and mass spectrometry was used to detect the ^{13}C label incorporated into cell phospholipids. Although the ^{13}C label in the cell extracts

was quite weak, intracellular glutamate was found to contain label from the metabolism of the fatty acid in the glucose-containing media. Several membrane phospholipids also contained ^{13}C label from the incorporation of intact oleic acid into predominantly phosphatidylcholine in both the normal and deficient media supplemented groups. There was a small amount of fatty acid recycling in the glucose-deficient media as indicated by the presence of ^{13}C label in 16:0 side chains of phosphatidylcholine.

Overall, these studies of astrocytoma metabolism have revealed that several metabolic pathways that have either not been investigated or that have been considered unimportant to tumour metabolism may be particularly active in astrocytomas. Calculations based on the rates of ^{14}C fatty acid oxidation, the quantitative amounts of fatty acid taken up by the astrocytoma cells from the media, and the amount incorporated into the cell membranes have revealed that fatty acid oxidation may be the dominant use of this substrate by the cells. When other substrates are present such as glucose and amino acids, it was calculated that the cells may take up more fatty acid from the media than is used for oxidation or membrane synthesis and therefore may be stored as lipid droplets. However, in the deprived medium the oxidation and uptake of fatty acid was more evenly matched with no excess for storage. The metabolism of glutamate to asparate and lactate in a truncated TCA cycle may also be a highly active pathway previously undetected in astrocytomas. The glutamate utilised in this pathway may be provided by extracellular (media) glutamine or from the utilisation of fatty acids.

Some anecdotal evidence for the importance of glutamine (and therefore glutaminolysis) to the astrocytoma cells used in this thesis comes from an error made in calculating the concentration of glutamine stock used in making the culture media (DMEM + 10% FCS + 4mM glutamine). At one point during the scaling-up of the astrocytoma cells to obtain the 10^7 - 10^8 cells per sample used in these studies the cell doubling time dropped from 48-72 hours to 2-3 weeks. It was finally discovered that a batch of glutamine stock was made too dilute and instead of adding 4mM glutamine to the DMEM only 0.4 μM was being added. The cells were discarded and new cultures were started from frozen stock and cell doubling times were consistently 48-72 hours henceforth. Although this was not a rigorously tested metabolic requirement for glutamine, the importance of glutamine to cell proliferation was obvious because other energy substrates

(including 6 mM glucose) were still available in the media even when the glutamine was mistakenly low yet they did not support the high rates of proliferation seen with 4 mM glutamine. Although normal astrocytes are known to be the glutamine-producing cells of the CNS due to the localisation of glutamine synthetase in astrocytes, the astrocytoma cells were utilising glutamine as an exogenous energy source. Therefore, glutamine synthetase is most likely absent or inhibited in the transformed cells since the production of glutamine would constitute a considerable futile cycle given the high glutaminase activity that must exist for the oxidation of media glutamine by the cells. Again, this hypothesis would be confirmed by enzymatic analysis of the astrocytomas.

Thus, the astrocytomas appear to rely heavily on the *partial* use of several metabolic pathways (glycolysis, glutaminolysis, and partial fatty acid oxidation—most of the ^{14}C-radioactivity from ^{14}C-oleic and –palmitic acid oxidation was found in the aqueous products rather than $^{14}CO_2$) to provide the necessary cellular energy for high rates of proliferation. Studies in the literature on other tumour cells and rodent glial cells have shown that glycolysis typically supplies 30% of cellular ATP (Portais *et al.*, 1993). Experiment 3 in Chapter 7 measured the contribution of fatty acid oxidation to astrocytoma ATP and demonstrated that up to 37% of ATP can be provided by fatty acid metabolism. Together with high rates of glutaminolysis by the cells, this could provide all of the cellular energy and metabolic precursors required by the rapidly dividing cells. This may reduce reliance on a particular substrate by the tumour cells for ATP generation and would be most advantageous given the heterogeneous supply of oxygen and vascularisation (i.e. substrate supply) in tumours *in vivo*. Therefore, I propose the following metabolic paradigm in astrocytoma cells (figures 9-1 & 9-2):

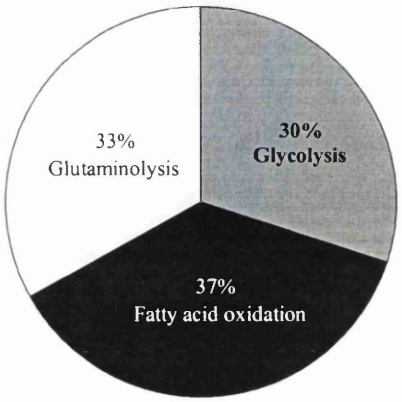


Figure 9-1. **Proposed substrate metabolism by astrocytomas**

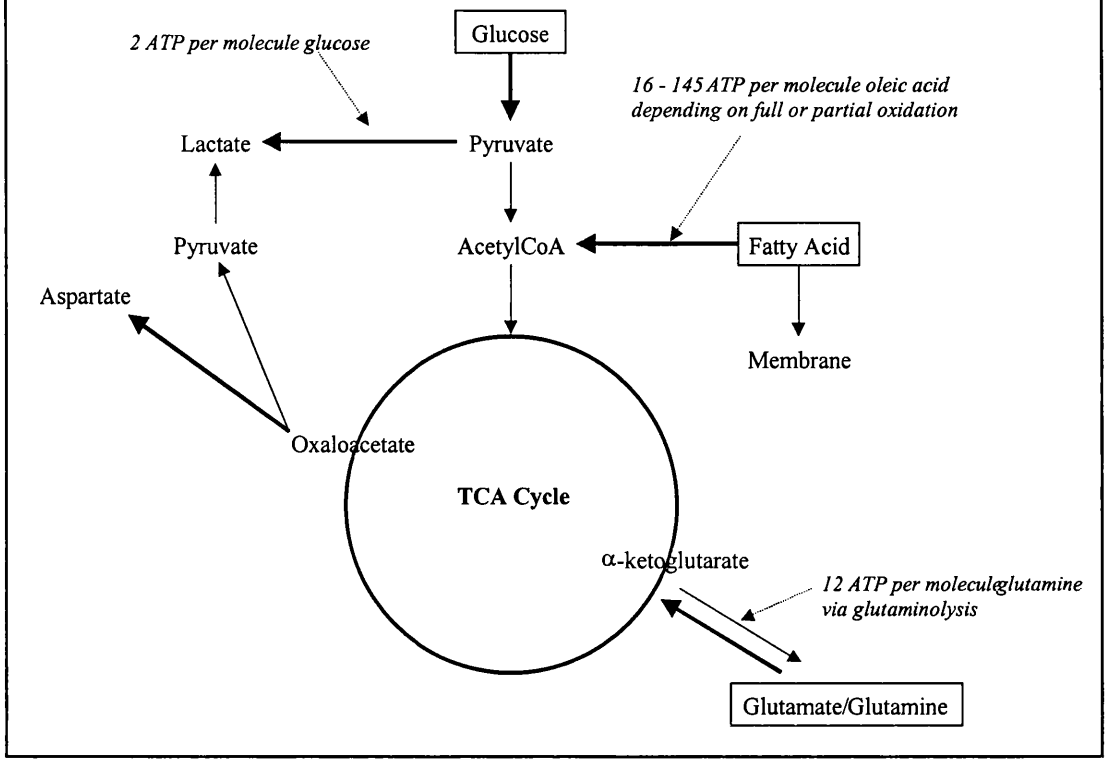
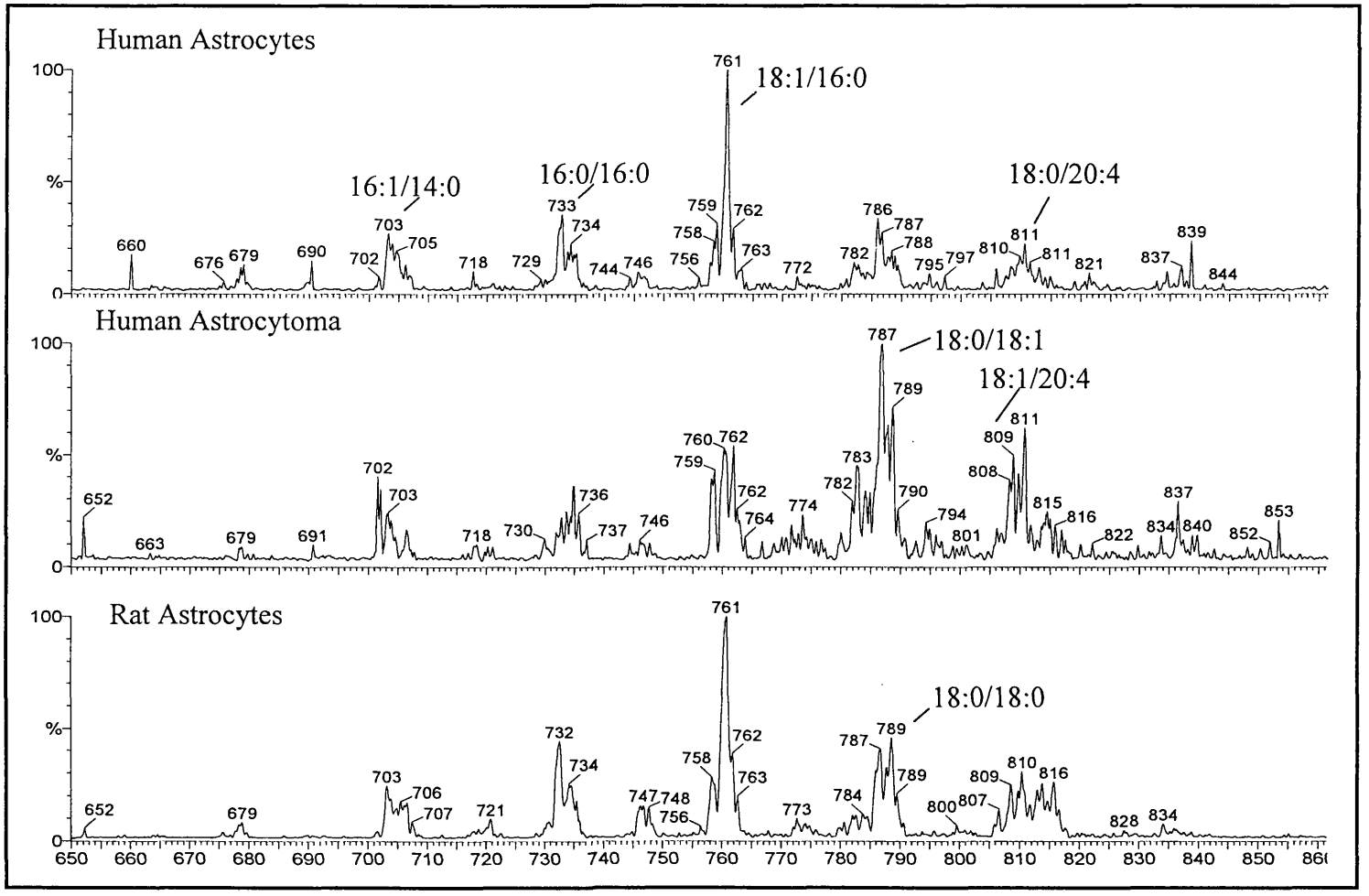


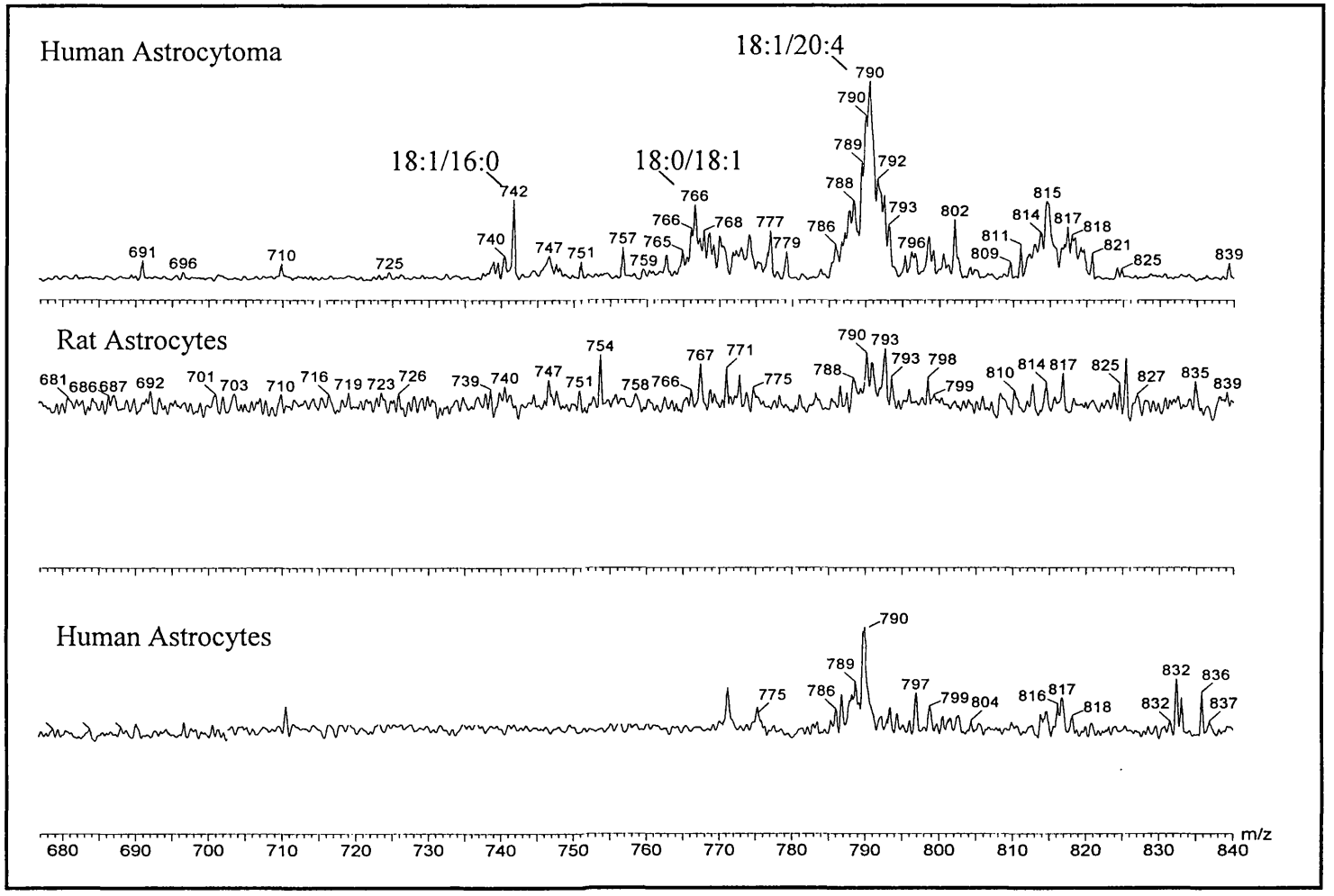
Figure 9-2. **Diagram of proposed metabolic pathways of importance in astrocytoma metabolism.** *The heavier print arrows indicate the more active pathways.*

In order to determine more conclusively if this metabolic arrangement exists in astrocytomas the metabolic enzyme activities in the proposed pathways need to be measured and more labeled precursor studies need to be carried out and compared to normal human astrocytes in identical culture conditions. The difficulty of obtaining normal human CNS cells and/or their prohibitive cost commercially has been a major deterrent to answering these types of questions in the past. However, the development of human neural stem cell cultures in many laboratories may make normal human CNS cells more readily available and the comparison to astrocytoma metabolism more feasible. Thus, although the experiments in this thesis have not determined conclusively the metabolic pathways most important to astrocytoma survival and proliferation, they may serve as the first step in identifying those transformed pathways through the description of the abnormal products and/or intermediates in the metabolic "snapshots" provided by analysis of cellular extracts.

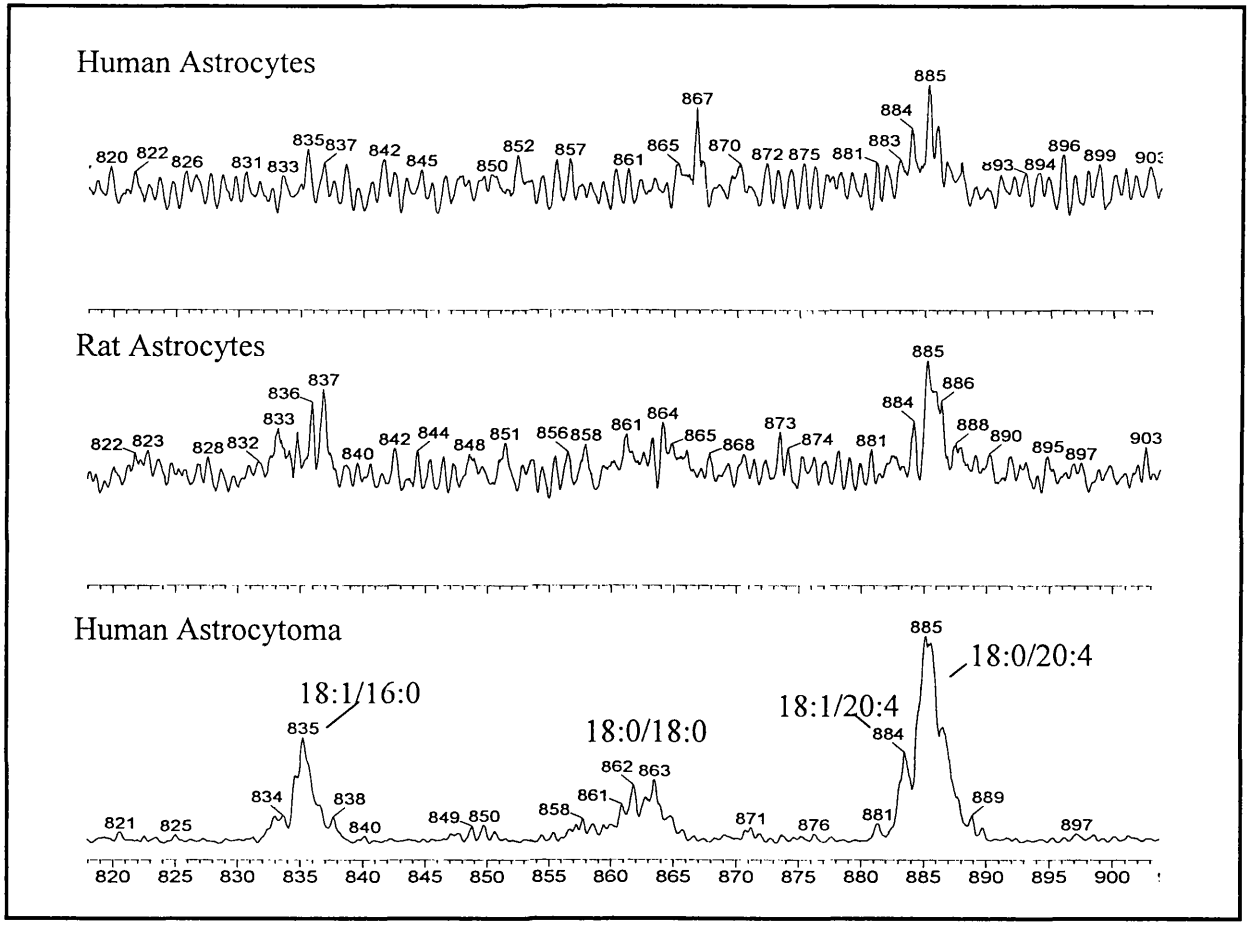
Phosphatidylcholine



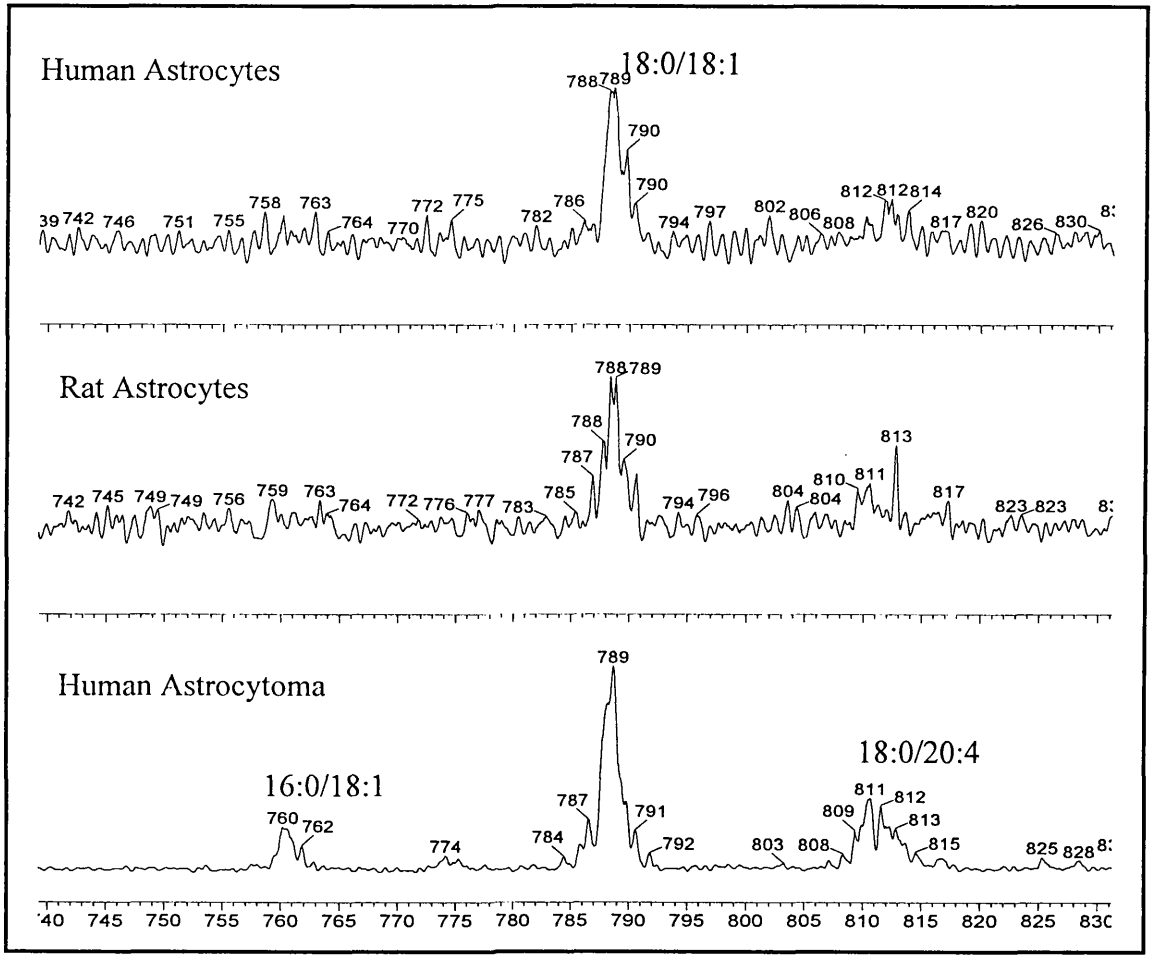
Phosphatidylethanolamine



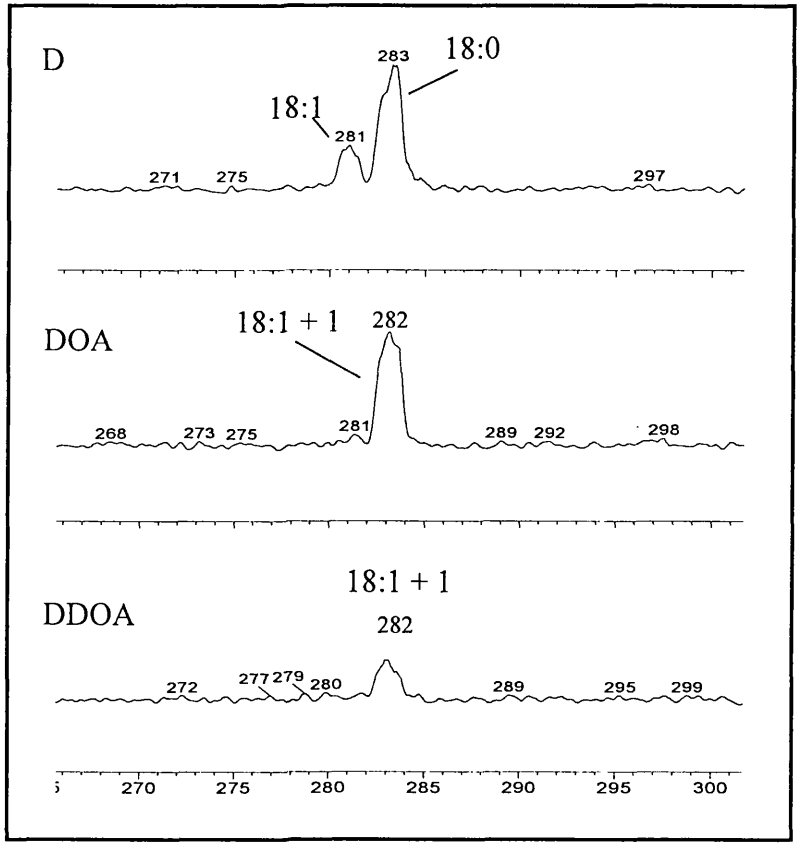
Phosphatidylinositol



Phosphatidylserine



^{13}C-enrichment



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