Title Expression of ornithine decarboxylase enzyme in central nervous system tissue in experimental allergic encephalomyelitis (EAE) in the Lewis rat

Name E.M. Walemba

EXPRESSION OF ORNITHINE DECARBOXYLASE ENZYME IN CENTRAL NERVOUS SYSTEM TISSUE IN EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS (EAE) IN THE LEWIS RAT

BY

ELVIN M. WALEMBA BSc

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ABSTRACT

Ornithine decarboxylase (ODC) is the primary and rate-limiting enzyme in the natural synthesis of polyamines putrescine, spermidine and spermine, which have been implicated in the breakdown of the blood-brain barrier (BBB), the initial event in the pathogenesis of experimental allergic encephalomyelitis (EAE) and multiple sclerosis (MS). This thesis would like to suggest that there is increased expression of ODC during heightened disease expression in EAE, and therefore that ODC is involved in the development of EAE and MS through the polyamine synthetic pathway.

To assess the levels of ODC enzyme in central nervous system (CNS) tissue, which included cerebellum, cervical spinal cord and medulla, the tissue was first homogenised and the protein concentration of the resulting sample determined. This required the establishment of an appropriate protein estimation method i.e. a suitable protein assay. Much time and experimentation was spent on achieving this because of the effect of interfering substances on the assays used.

Assessment of the levels of ODC in a model of EAE using normal tissue, CFA treated tissue (control), day-13 post inoculation (PI) (height of disease) and day-21 PI (early recovery stage) was carried out by Western blotting. The studies show that there is increased expression of ODC at the height of disease i.e. day-13 PI in cerebellum and medulla pons tissue but not cervical spinal cord tissue.

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ABBREVIATIONS USED

ANOVA – analysis of variance

APC – antigen presenting cell

APS – ammonium persulfate

AZ – antizyme

BBB – blood-brain barrier

BCA – bicinchoninic acid

BSA – bovine serum albumin

CFA – complete Freunds adjuvant

CNS – central nervous system

CSF – cervical spinal fluid

DAO – diamine oxidase

ddH_2O – double distilled water

DFMO - diflouromethyornithine

DNA – deoxyribonucleic acid

EAE – experimental allergic encephalomyelitis

ECL – enhanced chemiluminescence

EDTA – ethylenediaminetetraacetic acid

ELAM – E-selectin

GP-140 – P-selectin

HCl – hydrochloric acid

HEPES - *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulphonic acid, sodium salt

HLA – human leukocyte antigen

HRP – Horse Radish Peroxidase

HSV – herpes simplex virus

ICAM – intercellular adhesion molecule

Ig – immunoglobulin

IL – interleukin

IM – infectious mononucleosis

KDa – kilo Daltons

LAM – L-selectin

LFA – leukocyte functional antigen

MAG – myelin-associated glycoprotein

MBP – myelin basic protein

MHC – major histocompatibility complex

MS – multiple sclerosis

NaCl – sodium chloride

NMDA – N-methy-D-aspartate

NO – nitrogen oxide

NSAID – non-steroidal anti-inflammatory drug

ODC – ornithine decarboxylase

PA – polyamine(s)

PAGE – polyacrylamide gel electrophoresis

PI – post-inoculation

PLP – proteolipid protein

PMSF - Phenylmethylsophonyl fluoride

PP – primary progressive

RNA – ribonucleic acid

RR – relapsing remitting

RTI – respiratory tract infection

SAMD – S-adenosyl methionine decarboxylase

SAO – serum albumin oxidase

SDS – sodium dodecyl sulphate

TEMED – 1,2-Bis-(dimethylamino)ethane

Th – T helper

TIFF – tagged image file format

TNF – tumour necrosis factor

VCAM – vascular adhesion molecule

VLA – vascular leukocyte antigen

WR – Working Reagent

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1.0. INTRODUCTION

1.1. Autoimmune Disease

Autoimmune diseases arise when the body's immune system attacks its own cells and/or tissue having identified the cell(s) and/or tissue as an antigen. Autoimmune diseases include myasthenia gravis in which muscle tissue is attacked, periarteritis nodusa in which arterial walls become inflamed, systemic lupus erythamatisis in which there is superficial inflammation of the skin with scaling patches, rheumatoid arthritis in which cartilage and synovial membrane become enlarged through inflammation, and multiple sclerosis (MS) in which there is inflammation of CNS tissue and demyelination.

The common clinical characteristics of autoimmune diseases include higher incidence in the female gender, a relapsing course, and precipitation of disease activity by viral infections. Autoimmune diseases also have genetic linkage through immune response genes in the major histocompatibility (MHC) complex region of chromosome six. The mechanism by which the autoimmune response is initiated is not clearly understood, however various theories have been put forth and are discussed later on in this thesis.

1.2. Multiple Sclerosis

MS is a chronic disease of autoimmune origin that begins in late adolescence or early adulthood and has both highly variable expression and severity (Lynch, Rose, 1996). MS is best described as an inflammatory disease of the central nervous system (CNS) white matter characterized by focal T cell and macrophage infiltrates leading to demyelination, axonal injury and loss of neurological function (Bar-Or *et al.* 1999). In the earliest stages of MS the myelin sheaths at the patches/plaques, where focal inflammation occurs, break up and are absorbed leaving the nerve fibres bare thus forming the sclerosis or patch. Connective tissue comprised of neuroglia is later formed between the fibres, further hindering proper nerve function.

Symptoms of MS, among others, include; muscle weakness and plasticity; impairment of pain; inability to differentiate temperatures; abnormal touch sensation with pain ranging from moderate to severe; ataxia, tremor, speech and vision disturbances; vertigo;

bladder, bowel, and sexual dysfunction; depression; euphoria, and cognitive abnormalities. In the worst cases MS can render a person unable to write, speak, or walk, and may result in death.

MS may be manifested as a relapsing-remitting (RR) condition, in which case the disease is characterized by a series of attacks that result in varying degrees of disability from which the patient recovers partly or completely, usually followed by a remission period of variable duration before another attack. Alternatively in the primary-progressive (PP) form of the disease there is lack of acute attacks and instead there is a gradual clinical decline (Bar-Or, *et al.* 1999). The PP form of the disease shows a more rapid rate of deterioration than the RR form of the disease (Cottrell *et al.* 1999). About two thirds of MS patients have the RR course (Poser and Brinar, 2001).

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1.2.1. Incidence and Prevalence of MS

Although MS is found in many geographical regions it is often in clusters. The main areas of occurrence are areas inhabited by peoples of Northern European descent (Oger, Lai, 1994; Hader, Elliot, Ebers, 1988; Poser, Brinar, 2000) such as North America, Europe (especially western Europe), the United Kingdom (Chataway, 1989) and N. Ireland (McDonnell, Hawkins, 1998), and Australia (Hammond, *et al.* 2000) suggesting a genetic basis to the disease and also implicating commonality in lifestyle i.e. similar diet, socio-economic, and maybe even cultural factors. This is consistent with the observation that Caucasians are more than twice as likely as other races to develop MS (Poser and Brinar, 2001; Giovannoni, 1997; Hogancamp, *et al.* 1997). MS is the major cause of neurological disability in the Western Hemisphere (Bar-Or, *et al*, 1999).

MS typically manifests between the ages of 20 and 40 and rarely has onset after the age of 60 years. Onset of MS in juveniles younger than 15 years old is rare and disease progression in juveniles is not dependent on age of onset; nor does it determine severity of neurological involvement, or polysymptomatic or monosymptomatic involvement at presentation (Pinhas-Hamiel *et al*, 1998).

In general, women are affected at almost twice the rate of men (Granieri, *et al.*, 1996; McDonnell, Hawkins, 1998) however, among patients who develop the symptoms of MS at a later age (≥ 40), the gender ratio is more balanced. The high prevalence of MS in women led to the study of MS in association with pregnancy and the hormonal cycle of women. It has been shown that the rate of relapse of MS declines during pregnancy, especially in the third trimester, and increases during the first three months after delivery before returning to the pre-pregnancy rate (Confavreux *et al*, 1998). Endogenous steroids may play a part in the remission of symptoms during pregnancy due to their immunosuppressive effect. This is an observation consistent with the effect of pregnancy on other autoimmune diseases (Abramsky, 1994). Although a protective effect of estrogens on the development and progression of MS has also been suggested by experimental and clinical data, use of oral contraceptives, i.e. synthetic steroids, has not been associated with significant reduced risk of MS (Hernan *et al*, 2000; Abramsky, 1994). As the aetiology of MS is yet unknown, the treatment and prevention of MS is directed at the treatment of the associated symptoms. Synthetic steroids have been used in the treatment of the inflammation reaction in MS probably working in much the same manner as natural immunosuppressive substances (Abramsky, 1994) released by the body during pregnancy such as human placental lactogen, human choroinic gonadotropin, early pregnancy factor, pregnancy-associated plasma protein A, and α-Fetoprotein.

1.2.2. Suggested Causes of MS

Although the specific cause(s) of MS is unknown, epidemiological studies support both genetic and non-genetic factors supporting the theory that non-genetic influences work on pre-existing genetic factors to cause MS (Kurland, 1994; Poser, 1993; Calder *et al*, 1989; Lynch, 1996). The environmental or non-genetic component is supported firstly, by the presence of clusters or small epidemics of MS, secondly geographic variation in prevalence and thirdly by the fact that changes in susceptibility to the development of MS can be altered by migration, i.e. increasing risk with migration from an area of low prevalence to one of high prevalence. That MS has a genetic basis is supported by the

increased risk for development of MS in relatives of MS patients. Further support is provided by the presence of MS-resistant ethnic groups e.g. in some groups Africans especially in sub-Sahara Africa that have low incidence levels (Hogancamp *et al*, 1997).

1.2.2.1 Genetic Causes of MS

Relatives of patients with MS have been shown to have a higher incidence or greater susceptibility to autoimmune diseases and at the same time, studies into autoimmune disease have shown a general diathesis in patients with MS (Broadley *et al*, 2000). For example a sibling of an MS patient is 15 times more likely to develop MS than the rest of the population (Chataway, 1989), and the risk of developing MS among first-degree relatives of patients with MS is increased 15-20 times over the risk of the general population (Chataway, 1989; Tournier-Lasserve and Bach, 1993).

The higher incidence rate for MS in monozygotic when compared with di-zygotic twins also supports genetically influenced susceptibility (Calder *et al*, 1989). In distant relations, the chance of developing MS is higher than chance expectation in the general population, but lower than that expected for closely related individuals. Multiple interacting genes seem to play a role in this increased risk of MS (Dessa *et al*, 2001).

Genetic susceptibility in a person will determine if that person will develop the MS "trait" (Poser, 1993). The MS trait is defined as, "a permanent state of hyperactive or intensified immunocompetent responsiveness or capability, which is triggered in the genetically susceptible individual by exposure to a non-specific antigen that is almost certainly of viral origin, either an acute viral infection or a vaccination." (Poser, 1993). The MS trait thus makes a person a potential victim of MS.

The events that trigger the genes of a susceptible include infectious agents, bacteria or viruses, may possess proteins that induce an autoimmune response due, to possession of proteins that share similar epitopes to host (self) proteins, i.e. they mimic self. This molecular mimicry theory proposes that infectious agents contain proteins with peptide sequences that mimic autoantigen peptides, which upon presentation in the periphery

inadvertently activate autoreactive T-cells (Bar-Or, *et al*, 1999) that may find their way into the CNS.

Alternatively, association between MS and thyroid disorders, in particular hypothyroidism, also lends support to the autoimmune pathogenesis of MS. The principle function of the thyroid is the control of growth and the rate of metabolism achieved though production and secretion of thyroxin hormone, which controls the metabolism of all cells and tissues. Thyroid disorders are at least three times as common in women with MS than in female controls (Karni and Abramsky, 1999). The high rate in of hypothyroidism disease in women supports the proposition of a genetic component to MS susceptibility as genetic diseases seem to be preponderate in women i.e. genetic diseases are more prevalent in the female gender and thyroid disorders are more common in women with MS than controls suggesting a possible genetic predisposition to MS.

Another genetic component to MS may involve the major histocompatibility complex (MHC) also known as the human leukocyte antigen (HLA) region. The MHC/HLA region is a set of closely linked genes situated on the short arm of chromosome 6, that are involved in the presentation of antigens to the immune system in general and T cells in particular. HLA class II genes, i.e. HLA-DR, DQ and DP with separate sub-regions for each of the DR, DQ and DP, are important in the regulation of the immune response against peptide antigens by coding for polymorphic proteins expressed on the surface of most nucleated cells. The HLA class II molecules determine whether the individual will react immunologically to a given antigen thus making HLA class II molecules the most important immune response genes.

Strong associations between HLA and susceptibility to autoimmune disease have been described. Conversely, certain HLA antigens occur together more frequently than would be expected by chance, and may have a protective effect, conferring resistance to a disease. Barcellos *et al* (1997) discuss gene loci on the HLA as a potential factor conferring susceptibility to MS however only the haplotype DR15/DQ6 has a confirmed

role in MS (Hillert, 1994). The observation that MS patients have similar gene sequence in the HLA region in particular the DR15/DQ6 haplotype, supports the proposition that genes are an important factor in the autoimmune aetiology of MS although the mechanism by which the DR15/DQ6 haplotype contributes to the ability to develop MS is not clear.

1.2.2.2 Non-Genetic Causes

Non-genetic factors have are also important in the aetiology of MS (Casetta and Granieri, 2000; Giovanni, 1997). These include geographical region especially as related to latitude, exposure to solar light, and socio-economic status or level of development i.e. the hygiene hypothesis (Rook and Brunet, 2002).

MS is five times more prevalent in temperate climates, such as those found in the northern United States, Canada, and Europe, than in tropical regions. There is also an indication that an individual migrating between areas of variable risk before the age of 15 tends to adopt the risk of the new area (Chataway, 1989; Kurland, 1994; Giovannoni, 1997). In the United States where much work has been done in relation to geographical distribution of MS, a higher incidence of MS has been found in northern areas compared with the south, an observation consistent with the north/south divide disease profile in other countries and regions (Hernan *et al*, 1999). Considering that the large majority of this population in this region is of similar genetic make up i.e. largely Caucasian, the difference in the occurrence of MS is more correctly attributed to non-genetic factors. Over the last few decades, a more balanced occurrence of MS in the southern areas of Europe as compared to the northern areas has led to questioning whether latitude is indeed a factor affecting the development of MS.

The observation that people of the same geographical region share the same diet in general, are exposed to similar conditions such as sunlight intensity (influencing vitamin D production) and agents of infection, rather than just the fact that the people are by and large of the same race with similar genes, is probably a large factor in the aetiology of

MS. That is, the increased incidence associated with a certain area may be due to environmental rather than genetic factors.

Although there is currently insufficient evidence for a single or unique non-genetic cause of MS, various agents have been suggested. Of these viruses have received the most attention (Marrie *et al*, 2000; Giovannoni, 1997; Kurland, 1994; Johnson, 1994). Other suggested factors include allergies arising from exposure to animals and plants, toxins, trauma, and diet (Wynn, *et al* 1989).

One explanation given for the role of viruses in the initiation of MS is that common viruses such as measles share epitopes with MS autoantigens (Giovannoni, 1997). The proposition that viruses may be a cause of MS is supported by the three main findings (Johnson, 1994) as follows. Firstly, epidemiological studies indicate that exposure in childhood to certain viral agents precedes exacerbation. Secondly, studies in animal models and patients of autoimmune diseases have shown that viruses can cause diseases with long incubation periods, remitting and relapsing courses and myelin destruction mediated by variety of mechanism. Thirdly, patients with MS have been shown to have abnormal immune responses to virus infection such as measles, influenza C and Herpes simplex viruses (HSV).

No specific virus to which the onset of MS can be attributed has been definitively shown (Cermelli and Jacobson, 2000). Some of the viruses that have been studied include the HSV types 1 and 2 for which the age at which HSV-2 is first detected and the age at peak incidence are similar to those for MS (Martin, 1981); *Ixodes* genus tick viruses to which high rates of MS have been linked, especially with the distribution of certain island human populations relate to the distribution of *Ixodes* genus tick viruses (Brown, 1996). *Ixodes* ticks and their associated viruses are globally distributed by polar-migrating seabirds, which are important food sources for some island and coastal communities that also have a high rate of MS. However, this increased incidence may instead be due to the European ancestry of the island population.

An association between a history of infectious mononucleosis (IM) and MS has was shown by case-control study in which increased frequency of respiratory tract infections

(RTI) was associated with a significantly increased risk of MS (Marie *et al*, 2000), thus supporting the proposition that a history of IM may precipitate disease onset.
In contrast, adults with a history of childhood optic neuritis seem to have a lower risk of recurrence and progression of MS compared to adults who do not have a history of optic neuritis (Lucchinetti *et al*, 1997) possibly suggesting a suppressive or preventative effect on the presentation of MS.

Environmental conditions, however adverse they may be, cannot on their own be considered triggering factors for the initiation of MS. It is likely that a multiplicity of environmental conditions working together on a genetic framework, i.e. "MS trait", lead to the initiation of MS disease. The genetic factors that make an individual susceptible to MS are varied and the mechanisms involved in disease aetiology have not been uncovered. In addition, the importance of ethnic homogeneity of patients and control subjects cannot be overemphasized in prevalence and risk factor considerations. Both nature, genetic, and nurture, non-genetic factors play important interdependent roles in the aetiology of MS.

1.2.3. Immunology of MS

Whatever the cause or initiation of the inflammatory response in MS, activation and proliferation of leukocytes will occur. In the case of MS, the inflammation lasts over a long period of time i.e. leads to the development of state of chronic inflammation.
Activated macrophages and T lymphocytes are the predominant cell types in the CNS of MS patients (Hafler and Weiner, 1989; Calder *et al*, 1989; Fretland, 1992; Paul *et al*, 2000). Macrophages are of central importance in MS pathology because they are antigen-presenting cells (APC), produce both myelinotoxic and neurotoxic factors, and are also capable of phagocytosis and remyelination (Giovannoni, 1997). They also produce cytokines, chemokines and tumour necrosis factor alpha (TNF-α), which drive the inflammatory response.

In particular MS is suggested to be a T cell mediated disease of the CD4+ T cell subtype (Prat and Martin, 2002; Hafler and Weiner, 1989; Raine and Scheinberg, 1988). CD4+ T helper cells, subdivided according to the cytokines they produce i.e. T helper 1 (Th1) cells secrete proinflammatory cytokines such as interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α) and lymphotoxin which enhance antigen presenting cell activation and help to clear intracellular pathogens and T helper 2 (Th2) cells on the other hand secrete cytokines such as interluekin (IL) 4, IL-5, and IL-13 that activate antibody class-switching and eliminating blood-borne infectious agents (Bar-Or *et al*, 1999), are the principal cell types involved. TNF-α is found in plaques of MS patients being produced by astrocytes and macrophages, but not in non-MS sufferers (Bolton, 1997; Bar-Or *et al*, 1999). White matter damage is associated with CD4+ cells which enter the CNS, migrate to white matter and interact with local antigen presenting cells such as microglial cells i.e. the endogenous macrophages (Raine and Scheinberg, 1988). CD8+ cells later on downregulate the CD4+ mediated response (Raine and Scheinberg, 1988), however CD8+ cells themselves may be responsible for disease initiation through the complexion of myelin peptides, myelin basic protein (MBP) and proteolipid protein (PLP) and myelin-associated glycoprotein (MAG), with the HLA-A2-restricted CD8+ cell response (Giovannoni, 1997) through an MHC-I cell response unlike the CD4+ cell mediated response, which is a MHC-II cell response (Hafler and Weiner, 1989; Calder *et al*, 1989; Oksenberg *et al*, 2001). In MS patients' cerebrospinal fluid, fewer circulating CD8+ cells are found than in controls (Giovannoni, 1997) suggesting absence of the down-regulating effect.

In general the immune response associated with MS involves infiltration of activated CD4+ Th cells across the BBB, activation of these cells by APC to proliferate and produce cytokines. The antigen-T cell receptor-MHC complex with the help of co-stimulatory pathways and specific cytokines initiate cell-mediated inflammatory reaction, which activates macrophages, microglia, astrocytes and possibly endothelial cells, further enhancing production of cytokines and recruitment of inflammatory cells. Inflammatory cells are recruited by chemoattractants such as eicosanoids, NO, and leukotrienes and

upregulation of adhesion molecule expression on endothelial cells. Recruitment of lymphocytes into the CNS across the BBB is achieved by the action of adhesion molecules some of which include intercellular adhesion molecule-1 (ICAM-1), intercellular adhesion molecule-2 (ICAM-2), vascular adhesion molecule-1 found on cerebral endothelial cells (VCAM-1), E-selectin (ELAM-1) and P-selectin (GP-140) and leukocyte functional antigen-1 (LFA-1), vascular leukocyte antigen-4 (VLA-4) and L-selectin (LAM-1) found on various different leukocytes. The interaction/recognition between the ligands presented on the BBB cells and leukocyte facilitate the migration of the cells across the BBB (Vries *et al*, 1997; Giovannoni, 1997).

Cyclophosphamide, a drug that can penetrate the CNS, is one of the most effective immunosuppressants in MS patients (Calder *et al*, 1989; Hafler and Weiner, 1989) and has been used for a long time. However, MS therapy is predominantly through the use immunosuppressive substances including steroids, (Hafler and Weiner, 1989; Bolton, 1997; Fretland, 1992; Jacobs *et al*, 1999) such as dexamethasone, cyclosporin-A and methotrexate, and non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen and aspirin (Fretland, 1992). Glucocorticoids have a very powerful anti-inflammatory effect but use in MS therapy fails to inhibit disease progression (Jacobs *et al*, 1999). Interferon (IFN) which down-regulates macrophage production of cytokines and increased expression of glucocorticoid receptors has also been used in the treatment of MS (De Vries *et al*, 1997; Jacobs *et al*, 1999). MS patients treated with IFN-α exhibit reduced number of clinical exacerbations and significantly lower accumulation of disability characterisitics (Jacobs, 1996; Jacobs *et al*, 2000). Vitamins B_{12} and D when included in regulated amounts in the diet improve prognosis in MS patients (Marniemi *et al*, 1998; Trojano and Paolicelli, 2001). Activated vitamin D i.e. 1,25-dihydroxyvitamin D(3) (1,25-(OH)(2)D(3)) has immunomodulatory effects on MS/EAE as shown by various studies (Muller *et al* 1995; Lemire *et al* 1995; Muller *et al* 1996; Jirapongsananuruk 2000; Boonstra *et al* 2001) as an immunosuppressant (Fukoka *et al* 1997; Issa, *et al.*, 1998; Oksenberg *et al*, 2001). Vitamin D synthesis has therefore been proposed as a non-

genetic aetiological factor in MS pathology (Oksenberg *et al*, 2001) a proposition supported by another proposition in which it is suggested that MS has a higher prevalence rate in latitudes where insufficient vitamin D is synthesized from solar sources. Vitamin D compounds (in particular 1,25-dihydroxyvitamin D_3) are shown to be selective immunosuppressants and either prevent or significantly suppress disease in animal models of autoimmune disease.

Zinc deficiency in the diet may also contribute to MS pathology (Harbige, 1996) due to the increased levels of the superoxide CuZnSuperoxide dismustase (CuZnSOD) (Cunane *et al*, 1994). It is thus necessary to ensure that adequate levels of zinc are supplied (Johnson, 2000).

A study on the effect of fat consumption in the diet of MS patients showed that consumption of 20.1 g of fat and less as compared with consumption of amounts greater than 20 g was beneficial with only 31% dying and only slight deterioration while for those whose consumption was high, there was serious disability (79%) and deaths (81%) (Swank and Grimsgaard, 1989).

It has long been suggested that dietary therapy may improve fatty acid metabolism (Horrobin, 1979) and also that dysfunction in fatty acid metabolism may lead to biochemical changes in myelin and blood cells (Neu, 1985). Fatty acid metabolism is important because fatty acids are precursors to the prostaglandins, mediators of immune reaction of inflammation and play an important role in the maintenance of the CNS and the myelin sheath. They also maintain cell membranes and transport fats around the body. More recent work has suggested that proper nutritional intake of fatty acids may alleviate disease and improve health (Harbige, 1996; Timmerman and Stuifbergen, 1999). In cell culture and animal feeding studies it has been shown that polyunsaturated fatty acids can influence lymphocyte proliferaton, the production of cytokines by lymphocytes and natural killer cell activity (Calder, 1993; Calder *et al*, 2002). The type and amount of fatty acid in the diet greatly affects the content of serum and spleen lymphocyte fatty acid content (Peterson *et al*, 1999; Jeffery *et al*, 1996; Jeffery *et al*, 1997). EAE mice orally fed on plant lipid rich in the Ω-6 fatty acid λ-linolenic acid from

Borago officinalis showed inhibited clinical incidence and histological manifestations of acute EAE, and the clinical relapse phase of chronic relapsing EAE (CREAE) was also markedly inhibited (Harbige *et al*, 2000). Harbige and Fisher (2001) also showed that feeding fish oil rich in long-chain n-3 fatty acids decreased both T-helper (Th) 1- and Th2-like responses. In contrast, borage (*Borago officinalis*) oil rich in n-6 poly-unsaturated fatty acids increased Th1-like responses but decreased Th2-like responses, and possibly enhanced suppressor cell or Th3-like activity.

Modulation of the immune system in MS therapy has only small clinical benefit and therefore it has been proposed that immunosuppressants used in therapy need to be facilitated access to the site of ongoing immune response (Calder *et al*, 1989).

Many new and different treatments and therapies for MS are being tried, however, due to the fact that the cause of MS is to date unknown, effective therapy and cure of MS will evade us until we can better understand the underlying causes of MS.

1.3. Experimental Allergic Encephalomyelitis

Encephalitis is the inflammation of the brain. EAE is an autoimmune disease model is an important experimental tool because of its similarities to the human ailment, MS. The use of such animal models allows the study of the progression of the disease from inception especially well, given that the life span of the animals used (i.e. rats) is relatively short and the progression and development of the disease can be tracked in animals more readily than MS in humans, which has a longer period of manifestation. It is important however to note that although there are many similarities between EAE and MS, important immunological differences do exist (Juhler, 1988), such as the fact that where as EAE is induced MS is not.

Although highly reproducible, EAE is genetically restricted (Bolton, 1997; Paterson, 1976) requiring different agents to elicit the initiation of pathology in different species e.g. the strain-13 as compared with the Hartley guinea pig, Lewis rat compared with the Sprague Dawley and there are also differences among animals e.g. mice, rats, guinea pigs and primates (Paterson, 1976; Fretland, 1992; Tournier-Lasserve, 1993). Once EAE has been

initiated in an animal species, EAE may be transferred from one animal to another of the same species using T-cells, but not antibodies from the infected animal showing that EAE is a cell-mediated disease. EAE cannot be transferred from one species to another using T-cells probably because of the difference in the HLA/MHC genes of each animal species.

1.3.1. Induction of Experimental Allergic Encephalomyelitis

The most effective and rapid way to induce acute EAE in animals is by a single intracutaneous or footpad injection of central nervous system (CNS) tissue, usually incorporated into an immunologic adjuvant, which is commonly Freund's complete adjuvant (CFA) (Juhler, 1988). Freund's adjuvant is comprised of killed mycobacterium suspended in paraffin oil and emulsifying agent to ensure creation of a stable water-in-oil emulsion (Paterson, 1976; Bolton, 1990) and increases the efficacy of the toxin and in this case, accelerates the development of disseminated EAE.

CNS tissue homogenates and extracts, used in the induction of EAE, show extraordinary stability. The encephalitogenic activity (ability to produce the encephalitis reaction) of whole CNS tissue is retained even after: boiling; autoclaving; autolysis; treatment with formalin, or acid or alkali; ultrasonic vibration, and repeated cycles of freeze thawing (Paterson (1976). That autologous, homologous, or heterologous brain or spinal cord can be used to induce EAE suggests that the encephalitogenic property of CNS tissue is attributed to organ specificity rather than species specificity. That is, the antigens involved are unique to the brain and spinal cord across different species. Thus although it is possible to induce EAE in an animal with the use of CNS tissue from another animal of a different species, it is not possible to use the T-cells of the EAE diseased animal to transfer the disease from one species to another.

1.3.2. Course and Features of Experimental Allergic Encephalomyelitis

The disease EAE occurs over a period of 3 weeks (21 days), with the height of disease at 10-12 days post-induction and death following shortly within 5 – 7 days after this.

However, in the Lewis rat model, natural recovery without any pharmacological intervention may occur at around 21 days after inoculation (Paterson, 1976). The disease is "forward" progressing with signs exhibiting from the posterior end of the body towards the anterior i.e. the tail shows flaccidity then there is hind limb paralysis, fore limb paralysis, and finally cognitive/mental symptoms which may be followed by death.
EAE is histologically characterized by perivenular foci of mononuclear cellular inflammation, primarily in the spinal cord and brainstem, and demyelination (Juhler, 1988). Acute EAE shows demyelination in limited areas only and never in areas covered by cellular inflammation. The common clinical manifestations of EAE induced by the immune reaction are ataxic gait, paresis or paralysis of hind limbs, faecal impaction and urinary retention due to autonomic nervous system dysfunction from spinal cord injury, which appear within 10-21 days of induction with EAE (Paterson, 1976).

1.3.4. The BBB in Experimental Allergic Encephalomyelitis

Onset and progression of EAE involves both the cellular and humoral components. The interaction of the two components is important in EAE as evidenced by the fact that T cell subsets have regulatory influence on B cell activity, and humoral responses require action of both T cells and macrophages. T cells are the main agents in EAE and also in MS however, the suppression of B-cell function leaving the T cell function intact prevents the development of EAE but does not stop cellular infiltration (Juhler, 1988). The activation of the T cell requires an antigen to be presented with the MHC by antigen presenting cells such as astrocytes, monocytes and endothelial cells.
The BBB effectively keeps out humoral substances and immunoreactive cells. By providing an avenue through which cellular infiltrates that would have otherwise been excluded from the CNS by the BBB are allowed into the CNS, BBB breakdown plays an important role in disease initiation.
That BBB breakdown is an initial feature of EAE is shown by the development of EAE lesions in the areas that "leak". Also, in Lewis rats it has been found that even before the infiltration of cells into the CNS appear during EAE, the blood protein fibrinogen exhibits

perivascular accumulation and persists along with and mixes with infiltrating inflammatory cells (Paterson, 1976), a sign of increased permeability of the BBB suggesting that there is a period between the breakdown of BBB function and the infiltration of inflammatory cells. The naturally permeable regions of the CNS, which circumvent the BBB, may contribute to the degeneration of tolerance and play an important role in the pathogenesis of EAE.

It has been suggested that vasoactive amines such as histamine, are possible mediators in the breakdown of the BBB. Vasoactive compounds stimulate the retraction of endothelial cells thereby increasing capillary permeability and allowing the infiltration of large sized serum proteins. Histamine is found in excessive amounts during early neurological disease, being released from mast cells it has an up-regulatory effect on pinocytic vesicle activity in neurovascular isolates, an action that enhances transport across the BBB up and above the norm *in vivo*.

Prostaglandins cause dilation of blood vessels and are mediators in the process of inflammation suggesting that they may also play a part in the enhanced permeability of the BBB during disease. Prostaglandins of the E and F series have also been found in differentially increased amounts in CNS tissue from EAE animals and CSF of MS patients (Fretland, 1992). Nitric oxide (NO) which is vasodilatory and can be generated by the N-methyl-D-aspartate (NMDA) receptor (Bolton, 1997) is also found in increased amounts.

Cytokines such as such as interferon (IFN) and tumour necrosis factor (TNF), are mediators of the immune response that interact with specific cell-surface receptors. Cytokines produced by leukocytes are interleukin, and those produced by lymphocytes, lymthokine. Cell derived cytokines Interleukin – 1 (IL–1) and TNF– α are found in increased levels in the CNS fluid of MS patients as compared to non-MS patients (Fretland, 1992) with levels of TNF– α found to correlate closely with gravity of neurovascular disturbances in MS patients with active disease. IL-1 and TNF have been shown to cause demyelination and lassitude (Fretland, 1992).

1.3.4. Immunotherapy of Experimental Allergic Encephalomyelitis

The prevention and treatment of EAE is important because it gives insight into the mechanism and pathogenesis of MS. Two approaches to the treatment and prevention of EAE were suggested historically (Paterson, 1976). In the first, CNS tissue homogenate was injected into newborn animals that were later on assessed on their capacity to develop EAE with a CNS-adjuvant mixture. In the second approach mature animals are given a series of injections of CNS tissue homogenate or MBP, either incorporated in incomplete adjuvant or without carrier, prior to being challenged with CNS-adjuvant. In the first instance Wistar outbreed rats, inbred DA rats and Lewis-BN or Lewis-DA hybrids showed a decrease in their capacity to develop EAE when challenged with CNS tissue-adjuvant at 8 – 10 weeks of age. In the second instance, there was dramatic inhibition of EAE in adult rabbits given CNS tissue homogenates prior to spinal cord-adjuvant sensitisation possibly through acquired immunological tolerance (Paterson, 1976). The MBP rather than whole CNS tissue in guinea pigs, rats or monkeys, shows significant inhibition in EAE development, suggesting that MBP confers a broad base protective cover against development of disease. This may arise due to desensitisation of the animal's immune system to the circulating effector lymphoid cells (Paterson, 1976). The use of the two approaches given above suggests therapy and treatment in/for two groups of subjects. The first approach seems suitable for a population that may be exposed to predisposing factors to the development of MS i.e. an immunization of the young people in a population. The second approach seems suitable for an adult sub-population that is present in a larger population group having subjects with MS.

Therapeutic control of EAE makes use of a number of drugs of which the immunosuppressive drugs are the most common. These include nitrogen mustard used in a disease model of guinea pigs, that shows decreased mortality and paralysis, and 6-mercaptopurine which, when administered to rabbits, suppresses development of EAE, although on cessation of use, EAE symptoms recurred. In a rat model, a combination of 6-mercaptopurine with duazomycin-A inhibited EAE development much better than 6-mercaptopurine administered alone, and the inhibitive effect persisted even after stopping

drug administration. Cyclophosphamide injections gave an extended inhibition of EAE in guinea pigs (Peterson, 1987) showing that drug intervention is a viable control measure in the therapy of EAE. Also, injections of antilymphocyte serum have shown inhibition of EAE in rabbits and guinea pigs probably arising from the diminished production of lymphokines (Paterson, 1976).

Corticosteriod treatment a few days after induction of EAE in particular delays development of disease and may even suppress it completely. The hormonal steroid oestrogen has a suppressive effect on development of EAE while progesterone actually appears to aggravate the symptoms, enhancing clinical neurological signs of the disease. The importance of EAE in the study of MS is evidenced by the similarities in both diseases. The initial occurrence in both diseases is dysfunction of the BBB leading to infiltration of T-cells and macrophages actions that precipitate the development of lesions and cause neurological dysfunction. Substances that can cause disruption of the BBB integrity of through enhanced permeability or by causing physical breakdown such as is displayed by vasodilation, are important in the study of BBB pathology.

1.4. L-Ornithine decarboxylase (ODC)

1.4.1. Physical properties of L-Ornithine Decarboxylase

ODC is a key enzyme in polyamine (PA) biosynthesis (Murakami, 1994; Morris, 1991; Kumar, Butler, 1999) as the rate-limiting enzyme in the biosynthetic pathway of PA by catalysing the conversion of ornithine to putrescine (Baskaya, 1996). ODC is a 53 kDa dimer that is extremely labile, and occurs in relatively low levels in cells i.e. it has been estimated that there may be 100-200 molecules of enzyme in a quiescent cell, however levels are higher in active cells and in the blood, being in the range of 85 micromole per litre. ODC has a short biological half-life (Morris, 1991; Bernstein, Muller, 1999) estimated at 5-60minutes (Paschen, 1992), although the half-life of ODC mRNA is considerably longer (Morris, 1991) suggesting that as ODC diminishes in concentration, new ODC may be produced. Ornithine is derived from diet supplement, metabolism in urea cycle or as a constituent of blood plasma while ODC is produced in response to various growth factors.

ODC has been shown in highest concentration in the smallest, most rapidly proliferating cells (Anehus, 1984) implicating it in cell development and growth. Immunocytochemical studies of various rat tissues showed high cytoplasmic immunostaining of ODC in epithelial cells of kidney, prostate, and adrenal medulla of testosterone-treated rats, in glandular epithelium of small intestine, and in pancreas of neonatal and adult rats (Schipper *et al*, 1999). Other studies localisation studies of ODC in rat tissues in different cell lines show that that strongest staining for ODC was found in the nucleoplasm of mitotic cells, whereas confluent cells showed moderate perinuclear staining (Schipper, *et al.*, 1999). The main cellular locus of ODC in both the immature and adult central nervous system however is the neuron (Bernstein and Muller, 1999).

1.4.2 Regulation of ODC under normal physiological conditions

In normal conditions a careful balance between synthesis, degradation, and uptake maintains the optimum levels of ODC. There are various means of regulating ODC/PA, which include regulation of both the synthetic degradative arms of the PA biosynthetic cycle i.e. the regulation of ODC and the regulation of S-adenosylmethionine decarboxylase (SAMD). Furthermore, cells have an efficient transport system for utilization of exogenously derived PA. SAMD catalyses the formation of decarboxylated S-adenosylmethionine an enzyme required for the metabolism of spermidine synthase that is necessary for the production of spermidine from putrescine (Bernstein, Muller, 1999). The present study concentrates on the synthetic arm of ODC.

The amount of ODC present in the cell is regulated by growth factors (which normally increase expression), and by polyamine interactions at the transcriptional and translational stage of the cell cycle and also by enzyme degradation (Suzuki, *et al.*, 1994) where PA may bind with DNA and RNA. Growth factors, hormones, regenerative stimuli, tumour promoters, immunoadjuvants and various drugs affect normal ODC activity. In adverse conditions associated with pathological insults, ODC activity is induced by

seizures, excitotoxic conditions, cerebral ischemia and instances of blood-brain barrier breakdown (Mustafa, *et al* 1996).

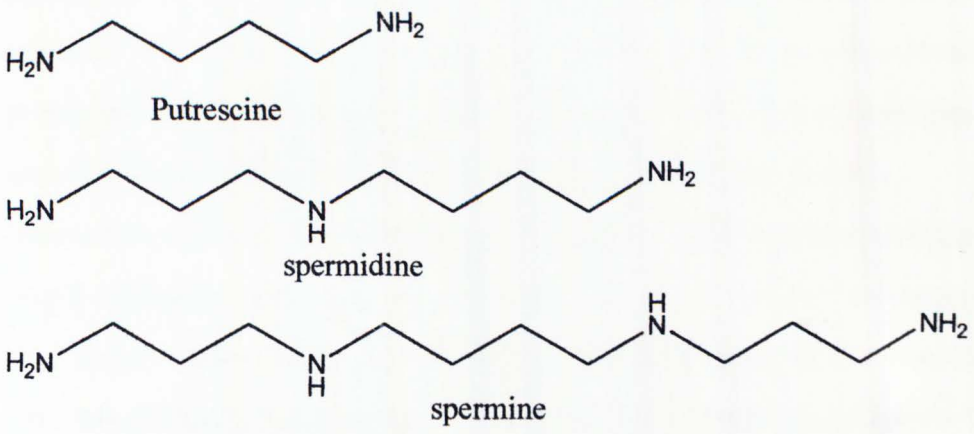
ODC expression is mainly regulated post-transcriptionally (Rohn, *et al.*, 2001) although a transcriptional factor (Sp1) may be involved in neoplastic progression due to altered regulation (Kumar, Butler, 1999). The binding of transcription factors Sp1 and Sp3 may, dependant on relative amount and binding activities, has an important role in ODC regulation during epidermal tumour development (Kumar, Butler, 1999).

Negative feedback regulation by the PA normally regulates ODC activity and degradation of ODC is accelerated by antizyme, an ODC-inhibitory protein induced by the build up of PA. ODC and antizyme forms an ODC-antizyme complex (Paschen, 1992; Murakami, *et al.*, 1994; Seiler, Atanassov, 1994; Suzuki, *et al.*, 1994) thus inactivating ODC. Antizyme also degrades ODC by a proteosome i.e. 26S proteosome and may function as a negative feedback regulator of polyamine transport (Suzuki, *et al.*, 1994) in addition to inactivating the enzyme by complexing.

ODC expression and activity is responsible for the synthesis of PA, which have been shown to mediate in the breakdown and dysfunction of the BBB the initial action in the pathogenesis of MS and EAE. The obvious relationship between the enzyme ODC and synthesis of polyamines themselves suggest that regulation of polyamines through regulation of their rate-limiting enzyme may provide an avenue for prevention or therapy of MS. It should be noted however, that normal physiological processes are dependant on the synthesis and normal function of PA. Indeed any action to regulate or inhibit ODC and thus affecting polyamine synthesis and function would have to be well executed to produce the desired effect while yet not being of fatal consequence.

1.5. Polyamines

Figure 1.1. showing the structure of the polyamines putrescine, spermidine and spermine.



PA spermine, spermidine, and putrescine are linear aliphatic molecules of small molecular mass (figure 1.1). All PA have positively charged amino groups at physiological pH that have steric and cationic properties and are thus water-soluble organic bases. One or more of the PA occur in every living cell and are closely regulated according to the stage and state of growth. PA have a wide, but uneven, distribution in mammalian and non-mammalian tissues and cells. All have been found in eukaryotes while spermidine rarely occurs in prokaryotes. PA are ornithine-derived molecules with various roles whose precise function(s) *in vivo* have yet to be determined (Cochet and Chambaz, 1983; Koenig *et al*, 1983; Oetken *et al*, 1992; Mehta *et al*, 2002).

The best-known physiological function of PA is their interactions with anions such as RNA and DNA thus affecting cell growth, division and differentiation (Hoet and Nemery, 2000). At physiological concentration PA can condense DNA, chromatin and promote transitions from B to Z DNA (Balasundaram and Tyagi, 1991). PA can modulate functions of RNA, DNA, nucleotide triphosphates, proteins, and other acidic substances by modulating phosphorylation. PA stimulate synthesis of special proteins and stimulate the assembly of 30 S ribosomal subunits and regulate ion channels (Igarashi and Kashiwagi, 2000) and reduce the activity of cellular protein tyrosine phosphates towards endogenous substrates

(Oetken *et al*, 1992). A minimal level of PA is required for a cell to move from G1 through S phase and PA are directly involved in DNA synthesis (Balasundaram and Tyagi, 1991). One of the earliest events occurring during cell growth, replication and differentiation is the increase in PA (Koenig *et al*, 1983). Cell proliferation is associated with increased turnover of PA (Oetken *et al*, 1992), which seem to have a nutritional function in certain growing tissues and a role in tissue repair after injury (Bernstein, 1999). PA are also found in high quantities during cellular multiplication, differentiation and even regeneration. A change in the hydrogen bonding capacity of PA leads to a decrease in protein synthesis and growth rate i.e. cells in a polyamine deficient environment have a lower protein synthesis and growth rate (Balasundaram and Tyagi, 1991). In a rat model, oral feeding of spermine to pups (day 10-14) postpartum at elevated levels from that found in natural/physiological levels showed enhanced/advanced development of the small intestine (Buts *et al*, 1993). Progesterone modulates sperm function by stimulating biosynthesis of polyamine (putrescine and spermidine) (Calogero *et al*, 2000).

In addition PA stabilise membranes and act as messengers of cell signals (Bernstein and Muller, 1999). PA modulate receptor action and signalling pathways such as in the effect of spermine on neuronal nicotinic acetylcholine receptors, which is a voltage dependant action and occurs when there is an increase in extracellular spermine (Haghighi and Cooper, 1998). PA interact with acidic amino acid residues lining and flanking potassium ion channel pores thus modulating their action (Lin *et al*, 1997).

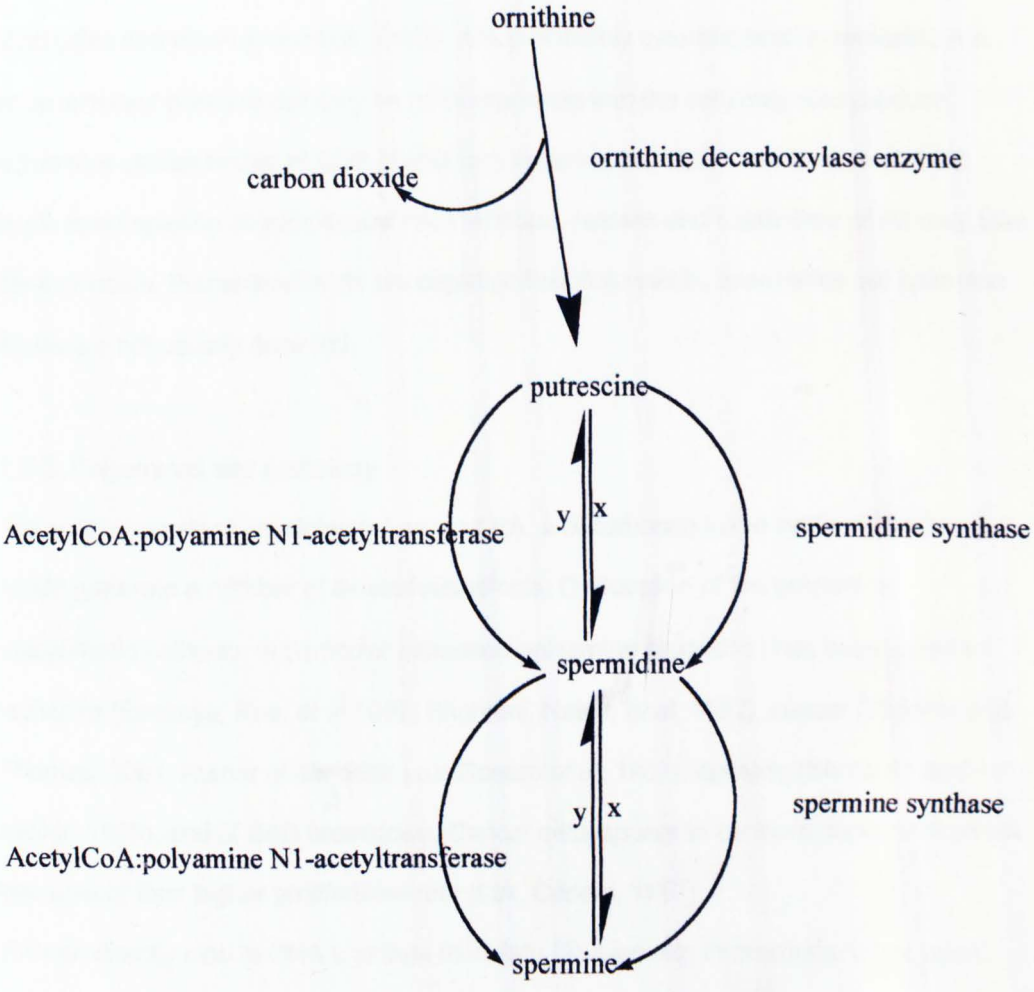
By binding to anionic sites, PA stabilize mitochondria (Toninello *et al*, 1985) possibly contributing to the homeostatic balance of Ca^{2+} for which mitochondria operate as low affinity high capacity buffer systems. Koening *et al* (1983) from findings in their work suggest that polyamine synthesis is necessary for stimulation of membrane transport functions and PA serve as messengers to generate a Ca^{2+} signal by increasing Ca^{2+} influx and mobilizing intracellular calcium via a cation-exchange reaction. ODC is Ca^{2+} dependant enzyme i.e. the production of ODC is upregulated by Ca^{2+}, and this is a suggested mechanism in the polyamine mediated BBB breakdown (Bolton, 1997).

PA play a role in remodelling the extracellular matrix (Stabellini *et al*, 2002) by their interaction with proteins and may also facilitate cell death through a complex cascade of events (Thomas, 2001). PA are involved in neoplastic disorders. This is shown by the therapeutic effect observed by inhibition of polyamine uptake in such disorders (Seiler *et al*, 1996).

1.5.1. Polyamine Regulation

Irregular spatial and temporal PA levels are detrimental to proper physiological body function. Adequate intracellular levels of PA are necessary for optimal growth and replication of all cell types. PA are normally sequestered suggesting that only free PA are physiologically active (Bernstein, *et al.*, 1999). Multiple pathways regulate cellular PA levels. PA can be synthesised from amino acid precursors, obtained through cellular uptake mechanisms that salvage PA, from the diet and intestinal microorganisms, and from stepwise degradation (Bernstein, Muller, 1999). The decarboxylation of ornithine by the enzyme ODC to form putrescine, which in turn is converted to spermidine and spermine, is the rate-limiting step in the biosynthesis of PA. 90% of the enzyme activity involved in PA synthesis is located in the cytoplasm (Baskaya, *et al*, 1996).

Figure 1.2. The synthetic pathway of polyamine production.



x = AcetylCoA:spermidine N8-transferase, y = N^8-acetylspermidine deacetylase

Ornithine loses a molecule of carbon dioxide through the action of ODC. The action of AcetylCoA:polyamine N^1-acetyltransferase and polyamine oxidase (PAO) are responsible for interconversion of spermine to spermidine and spermidine to putrescine in the cytosol and AcetylCoA:spermidine N^8-transferase (nuclear) and (7) N^8-acetylspermidine deacetylase catalyse the interconversion in the nucleus (Seiler and Antanassov, 1994).

Other enzymes not shown include serum albumin oxidase (SAO) that participates in the oxidative deamination of PA, the products of which are hydrogen peroxide, ammonia, and aldehydes and diamine oxidase (DAO), which is mainly cytosolic and re-sealeable (i.e. once released from the cell may be re-incorporated into the cell) may also produce substrates similar to that of SAO (Seiler and Antanassov, 1994). Inactivation of ODC leads to a depletion of intracellular PA. Depletion, release and catabolism of PA may take several hours. Putrescine levels are depleted first, followed by spermidine but spermine levels are not usually depleted.

1.5.2. Polyamines and pathology

Due to the important physiological roles of PA, a disturbance in the synthetic pathway would generate a number of deleterious effects. Dysfunction of the polyamine biosynthetic pathway, in particular increased polyamine production has been linked to ischemia (Baskaya, Rao, *et al* 1997; Shohami, Nates, *et al*, 1992), cancer (Thomas and Thomas, 2001; Kumar *et al*, 1999; Luk, Casero *et al*, 1987), epilepsy (Bernstein and Muller, 1999), and of BBB breakdown. Cancer cells appear to be more dependant on PA because of their higher proliferative rate (Luk, Casero, 1987).

PA can directly bind to DNA and thus modulate DNA-protein interactions. It has been suggested (Brooks, 1995) that at the start of S phase during the cell cycle, expression of PA leads to disruption of transcription and splicing, giving priority to DNA and histone synthesis in non-viable cells and may be a cause of autoimmunity (Brooks, 1995). Binding of PA to cell constituents has been proposed as a disruptive event to the regulatory feedback inhibition mechanism of PA synthesis (Davis 1990) leading to an accumulation of PA in the cell which can be toxic to the cell. Alterations in cellular polyamine levels may lead to apoptosis as a result of production of hydrogen peroxide during PA catabolism (Thomas and Thomas, 2001).

An important aspect of dysfunctional PA activity is the effect on the BBB. Localized BBB breakdown, vasogenic brain oedema including abnormalities in neuronal function, and a

reduction in local cerebral glucose utilization caused by focal cold injury of rat cerebrum are associated with a rapid biphasic increase in the concentrations of PA and their rate limiting synthetic enzyme ODC (Koenig *et al*, 1989). In separate studies pharmacological antagonism of NMDA receptor has been shown to reduce polyamine levels, prevent BBB breakdown, and reduce EAE neurological symptoms (Bolton, 1997).

Polyamines have been shown to mediate in the breakdown of the BBB, the initial event in the development of MS. They have also been shown to interact with DNA and also to facilitate DNA-protein interactions that may cause alteration in proteins to and initiate an autoimmune response by the formation of an antigenic protein or by causing breakdown in self-tolerance.

1.6. Blood-brain Barrier

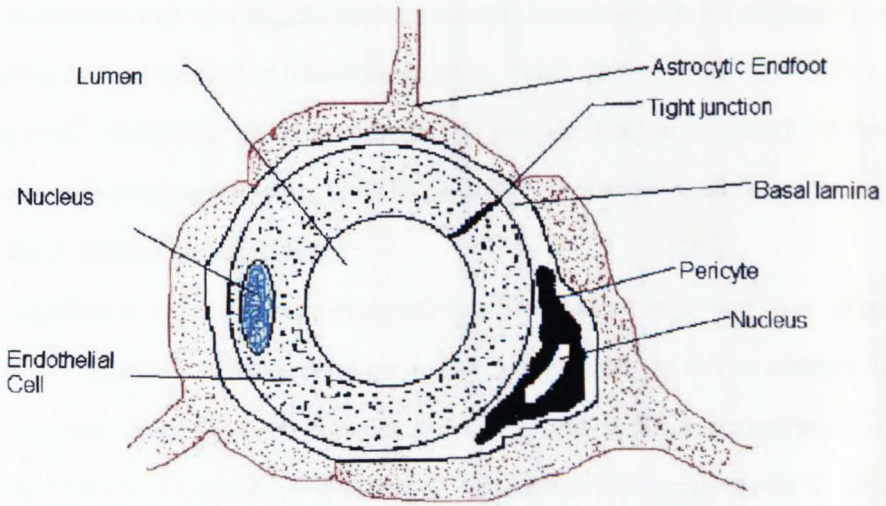
1.6.1. Blood-brain barrier morphology and physiology

The BBB is a complex structure the function of which is to impede the random movement of substances into the brain. It is comprised of endothelial cells of the capillaries, pericytes, microglia and astrocytes (figure 1.3). The endothelial capillary cells are completely surrounded by a basal lamina, in turn covered by astrocytic endfeet. Astrocytes, a distinct type of glial cell, support the neurons and are essential for the induction and maintenance of the endothelial cells.

The BBB is highly dependent for proper function on the tight junctions of neighbouring endothelial (Bradbury, 1985; Bolton, 1997). The reduced number of pinocytic vesicles in the cells of the BBB also effectively restrict movement of substances across the BBB (Schlosshauer, 1993). Polymorphic and multi-branched pericytes control endothelial cell proliferation and are responsible for the regulation of vessel contractility and the synthesis and secretion of vasoactive compounds. The metabolic needs of the brain are met by specific transport systems of the BBB defined by the plasma membrane of the endothelium, which is similar in cytochemistry to an actively transporting epithelium (Bradbury, 1985). The passage of substances through the BBB is achieved through

passive diffusion, facilitated diffusion, active transport or transcytosis in coated vesicles. Passive diffusion accounts for the passage of lipophilic substances although P-glycoprotein actively exports specific lipid soluble molecules into the capillary lumen. Glucose, amino acids and ions are transported by facilitated diffusion by the action of the glucose transporter, other similar carrier systems, and ion pumps respectively (Schlosshauer, 1993). Still other proteins are transported across the BBB by transcytosis through the action of different endothelial cells e.g. iron is carried by transferrin (Schlosshauer, 1993), and small molecule nutrients (Pardridge, 1999).

Figure 1.3. The structure of the BBB.



Schematic diagram of the functional BBB showing the tight apposition of astrocytic end-feet, and the tight junction of the endothelial cells. (Modified from Schlosshauer, 1993.)

Pathology involving the BBB results from disruption of the physical and physiological mechanisms responsible for the BBB's unique properties.

Inflammation can cause BBB dysfunction. During normal function, mononuclear phagocytes and non-activated T-cells may be found in the CNS (Perry et. al., 1997). In human chronic inflammatory disease such as MS, lesions are dominated by large numbers of activated T-cells and macrophages (Perry *et al.*, 1997; Paul *et al.*, 2000; Rabchevsky, *et al.*, 1999) contributing to myelin and axon damage (Perry *et al.*, 1997). Similar pleocytosis occurs in EAE, however there is little evidence of significant demyelination or neuron damage (Rabchevsky *et al.*, 1999).

MS is characterized by episodic malfunction of the BBB that allows oedema formation and inflammatory cell invasion of CNS tissue (Bolton, 1997), while in EAE neurovascular dysfunction is a prominent pathological feature. The mechanisms leading to the breakdown of the BBB in both MS and EAE are similar (Hawkins *et al.*, 1991) and the earliest lesions occur around the BBB (Pardridge, 1999).

The mechanisms that lead to BBB dysfunction may be unknown but the dysfunction itself can be tracked and quantified (Rabchevsky *et al.*, 1999; Guerin *et al.*, 2001;Bolton, 1997; Koenig *et al.*, 1992) along with the inflammation process (Paul *et al.*, 2000). PA have been shown to mediate the dysfunction of the BBB probably by increased production of PA (Koenig, Goldstone, Lu, 1989).

The PA synthesis rate-limiting enzyme ODC thus presents an important point for the study of a mechanism that may be of use in the understanding of MS. In addition, MS therapy or treatment may be possible through the regulation of polyamines and the associated breakdown of the BBB, through the regulation of the enzyme ODC.

1.7 Aims and Objectives of this study

The course of EAE, from induction to recovery, lasts for a period of approximately 21 days, with the height of symptom presentation at 10-14 days PI. Accordingly, increased levels of ODC should be seen prior to, or at the height of, disease. Similarly the days pre and post day 13 PI EAE should exhibit lower levels of ODC. To test this proposition, a profile of ODC levels in the CNS tissue during the course of the disease must be carried out.

This study compares the levels of ODC in EAE disease tissue at the height of disease, i.e. Day-13, with the levels of ODC in normal and recovering tissue for cerebellum, medulla and cervical spinal cord from Lewis rat CNS. Secondly, to establish a profile of ODC expression and location during EAE disease in the tissues exhibiting altered enzyme levels and ascertain if there is differential expression of ODC between cerebellum, medulla, and cervical spinal cord. In so doing, the present study will be able to determine the relative amount of ODC in these three tissues during EAE in the Lewis rat. The techniques developed and used included an appropriate protein assay, SDS-PAGE and Western blotting. During the course of this project, problems were met with in the homogenisation of tissue and the protein assay therefore a significant part of this study was dedicated to this. There were also problems with sectioning and staining tissue for immunohistochemical and this was not carried out to completion.

2.0. MATERIALS AND METHODS

At the start of this project it was necessary to first establish the techniques that would later be used to achieve the aims and objectives of the study. In order to establish and verify this methodology, pig brain tissue samples were used. The pig brain tissue samples were available in large quantity, readily obtainable, and easy to handle and process for use in experimentation. As the establishment of the necessary technique was a process that required numerous experimentation trials, large amounts of sample tissue were required. It was not possible to use rat brain tissue from the animal model of EAE that was being assessed i.e. Lewis rat model, because this tissue was available in limited

quantity only and was not easily obtainable because of scarcity of both the tissue itself and the funds to acquire the tissue. It was not possible to use human tissue either, as human tissue is quite hard to obtain and extremely valuable. Also, the use of human tissue would have required getting permission from various health and ethics committees and would have also necessitated the use of more stringent handling and usage control measures. However, once the techniques required for assessment of the tissue had been established, sufficient amounts of Lewis rat EAE tissue samples were available for experimentation.

It was necessary to evaluate two techniques of homogenisation of the rat tissue to be assessed because in the first technique the yield of total sample was low. For a tissue sample of 200 mg a sample volume of about 2-3 ml was obtained. This was an equivalent to approximately 65-100 mg/ml of which only 10% was protein. Such low yield in total sample was inefficient for the quantity of rat brain tissue samples at hand and if this homogenisation method had been used the tissue samples available would not have been sufficient for experimentation. The second homogenisation method (Min, editor, 1999) assessed produced greater volumes of protein from each sample (4-6 ml as compared to 2-3 ml for method one) for subsequent assaying.

Once an appropriate homogenisation procedure had been established, the next phase in the project consisted of establishing an appropriate assay for estimation of protein quantity within the homogenised sample i.e. tissue supernatant. Three different assay methods i.e. BioRad™, Lowry and Bicinchoninic Acid (BCA) methods were used because during the experimentation it was discovered that some of the assays were incompatible with reagents used in the preparation of the homogenisation medium. The BCA method was finally chosen as the method for use in the analysis of the rat brain tissue supernatant because it proved the most efficient in terms of accuracy and consistency in reproducing similar results.

In chapter three, the problems and solutions to these problems, leading to the decision of which assay should be used, will be discussed in detail.

2.1 Animal tissue

The for induction of EAE in animals and subsequent preparation of tissue was carried out at the John Harvey Research Institute, UK, by other research staff at the university using the method of Bolton and Flower, 1987; Bolton and Paul, 1997; Paul *et al*, 2000. Tissue collected consisted of normal Lewis rats and CFA treated rats at day-13, EAE rats at day-13 post inoculation (PI) and day-21 PI, the latter two representing the height of clinical manifestation of disease and early recovery of the rats respectively. Three animals each of normal, CFA-treated, day-13 and day-21 PI EAE were used. The tissues were stored at –70° C. The weights of the rat brain tissues are given in appendix I.

2.2 Homogenisation of Protein

Tissue was solubilised in preparation of electrophoresis and Western blotting. Homogenisation releases and solubilises the proteins for easier analysis. During the study it was found that the first homogenisation method used produced a low yield of total sample volume (2-3 ml) and the efficiency of solubilisation was circumspect. As the brain samples available for analysis were in limited quantity, this method had to be replaced with a more efficient method for higher yield.

A second homogenisation method produced a higher yield of total sample, in the range of 4.5-6 ml i.e. effectively 2-3 times more total sample. The second homogenisation method used was an enhanced method for solubilisation of membrane proteins. Due to financial constraints, brain tissue available was shared with a colleague doing work on a membrane protein. However, the fact that the homogenisation method was particularly good for solubilisation of membrane proteins did not make it any less viable for the homogenisation of the protein that this study was looking to analyse i.e. ODC.

2.2.1. Homogenisation Method One

Preparation of reagents.

All reagents were obtained from Sigma-Aldrich unless otherwise stated and all equipment was properly sterilised including autoclaving where appropriate. Protease inhibitors leupeptin and pepstatin (Sigma, UK) were available in 100 mM concentration prepared by colleague. 100 mM ethylenediaminetetraacetic acid (EDTA) was prepared by dissolving 37.22 g of EDTA powder in 1.0 ml of double distilled water (ddH_2O) in an Eppendorf(s). EDTA was then aliquoted into 100 µl volumes in Eppendorfs and kept on ice for immediate use or stored at 20°C. 100.0 mM *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulphonic acid, sodium salt (HEPES) (pH 7.5) was made by dissolving 23.83 mg of HEPES powder in 1.0 ml of ddH2O then aliquoted into 100 µl volumes and kept on ice for immediate use or stored at 20°C. Phenylmethylsophonyl fluoride (PMSF) was purchased at 200 µM concentration and stored at 20°C until required for use. 100 mM NaCl was prepared by dissolving 0.2922 g of NaCl crystals in ddH2O in a sterile 20 ml universal.

Procedure

The procedure used was that of Bolton *et al* (1990). Tissue sample was removed from storage at -70°C and put into a 20 ml universal while still frozen for easier handling and thawed on ice. Individual tissue was cut up in 0.5 ml of homogenisation medium (containing 10 mM HEPES, 5 mM EDTA, 200 µM PMSF, and 100 mM NaCl) per 100 g of protein to be homogenised using dissection scissors.

A homogenate was prepared by equal and repeated aspiration using sterile 1 ml syringe fitted with 21 and 19 gauge needle. This was followed by three cycles of freez-thawing, aliquoted in 1.2 ml volumes and centrifuged at 4°C in a HAWK Sanyo refrigerated centrifuge at 200 x 100 *g* for 30 minutes. The supernatant was then pipetted off and aliquoted into sterile Eppendorfs in 150 µl volumes. The supernatant was then placed on ice ready for immediate use or stored at –80°C for use when required. The pellet was stored in aliquots at –20°C.

2.2.2.Homogenisation Method Two

The preparation of reagents and homogenisation of proteins was carried out on ice as for method one.

Preparation of reagents.

Protein Inhibitors leupeptin and pepstatin were available in ready to use concentrations (prepared by colleagues) of 200 mM and 100 mM respectively. 50mM NaCl was prepared in a 20 ml universal. The EDTA and PMSF were prepared as described in method I (p 54). One molar (1M) homogenisation medium II was prepared using Tris-HCl powder in ddH2O and the pH of the resulting adjusted to 9.0 by titration with concentration hydrochloric acid (HCl) (Anachem). A 10% w/v SDS solution was prepared in ddH2O using 5.768 g of SDS powder was weighed out in a fume cupboard using Metler Toledo scale into a 20 ml universal. A 20% v/v concentration of Triton X-100 (Sigma) was made by dissolving 100 µl of Triton X-100 in 400 µl ddH2O, the dilution making it easier for dissolving in sample.

Procedure

Tissue was cut up in 0.5 ml homogenising medium per 100 g tissue in 20 ml universal on ice. PMSF, EDTA, and protease inhibitors leupeptin and pepstatin were added to give final concentration of 0.5 mM, 2.5 mM, 1 µg/µl, and 1 µg/µl w/v respectively. SDS was added to a final concentration of 2% of total volume. Finally, NaCl was added to a final concentration of 200 mM.

The homogenised sample was then placed in boiling water for 5 minutes, centrifuged at 4°C in a HAWK Sanyo refrigerated centrifuge at 200 x 100 *g* for two hours, the supernatant pipetted off the pellet into Eppendorfs in 1.5 ml aliquots and to this was added 20 % v/v Triton X-100 to make up 2% of the total volume. The sample was then mixed on vortex and stored.

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2.3. Protein Estimation

To ensure that equal total protein is loaded onto gels and thus carry out accurate SDS-PAGE and Western blotting, it is important to know the protein concentration of the sample to be assessed. This is achieved using a protein assay. Initially, the BioRad™ assay based on the Bradford method was used. This assay however gave inconsistent results over varying dilutions of sample (table 3.2 page 71) and numerous replications and so a second assay method, the Lowry assay method was used.

The timing required in the Lowry assay for both the addition of reagents and mixing of samples, is intricate and may contribute to inconsistencies in this assay (Smith, *et al*, 1985). The Lowry assay method was also inconclusive in the assessment of protein content because it also gave inconsistent results over varying sample dilutions and numerous replicates (tables 3.5 and 3.6 page 76). For this reason a third protein assay, the BCA method, was assessed. The BCA method copes with higher levels of interfering agents and detergents than either the BioRad™ or Lowry assay methods (Smith, *et al*, 1985).

2.3.1. BioRad™ Protein Assay

The assay method used with the BioRad™ protein assay kit is based on the method described in Bradford *et al.*, (1976). In this method, the maximum absorbance for an acidic solution of Coomassie Brialliant Blue G-250 dye shifts from 465 nm to 595 nm when bound to a protein. The BioRad™ is more compatible with a wider range of detergents than the original Bradford method.

A stock solution of concentration 1 mg/ml of bovine serum albumin (BSA) was prepared in 25 ml universal. The BioRadTM reagent (BioRad™) was prepared by diluting 1:4 in water. Volumes 0.0 µl (zero blank), 1.0 – 5.0 µl, were pipetted into a 96 well microtitre plate in duplicate and made up to 5.0 µl each using ddH2O. 200 µl of BioRad™ reagent was then added to each well and the microtitre plate was shaken by hand and left standing for 5 minutes. The absorbance was read using a Rosys Anthos II spectrophotometer at 595 nm, corrected for the zero blank, and the results plotted with

absorbance/volume of sample used. The protein concentration was calculated from the standard curve.

2.3.2. Folin Phenol Method (Lowry Assay).

The Lowry assay is based on the biuret reaction in which a protein placed in an alkaline system containing Cu^{2+} ion forms a coloured complex with the peptide bonds. The Lowry assay works in a two-step nature, a reduction of Cu^{2+} to Cu^{+} at the complexation site on the protein followed by reaction of the Cu^{+1} with Folin-Ciolcalteau reagent to form the final intense colour (Lowry, 1955; Smith, *et al.*, 1985).

Preparation of reagents

The reagents 2% (w/v) hydrated copper sulphate ($5H_2O$), 4% (w/v) sodium potassium tartrate, 3% (w/v) sodium carbonate in 0.2 M sodium hydroxide were prepared fresh. Folin and Ciocalteu's phenol reagent was bought in ready-to-use concentration Absorbance at 640 nm was read on a visible light spectrophotometer (CeCil CE 2021). All reagents were supplied by Sigma.

Procedure

A solution of 1 mg/ml of the BSA standard was prepared. Triplicate volumes of sample 5-50 µl were pipetted into Eppendorfs and the volume of each aliquot made up to 200 µl with buffer. The same was done for BSA. At the time of assay, 1 ml of copper sulphate solution was mixed with 1 ml of tartrate solution in a 100 ml beaker to which 48 ml of freshly prepared 3% (w/v) sodium carbonate in 0.2 M sodium hydroxide was added. 1 ml of the final solution was added to sample and BSA. The mixture was shaken and left to stand for 10 min at room temperature then 50 µl of phenol reagent added to each. The mixture was again shaken and left to stand for 25 minutes at room temperature. After the 25-minute incubation the solution was mixed again and 5 min later the absorbance of each sample and standard was read using CeCil CE 2021 spectrophotometer, at 640 nm using the water blank to zero the spectrophotometer. The mean for each sample's

absorbance was calculated, plotted with absorbance/volume and concentration of the sample calculated from the plot using a linear regression from the BSA standard curve.

2.3.3. Bicinchoninic Acid (BCA) Assay Method

BCA is a stable water-soluble sodium salt capable of forming an intense purple complex with cuprous ion (Cu^{1+}) in alkaline environment. This colour increases in a proportional fashion over a broad range of increasing protein concentration The BCA method shows greater tolerance of the bicinchoninate reagent toward interfering substances such as non-ionic detergents and simple buffer salts (Smith, *et al*, 1985; BCA Assay kit, Pierce Endogen).

Procedure for BCA Protein Assay using the Microtitre Plate method.

BSA standards with differing concentration 0.0 – 2.0 μg/μl were prepared in water from 2 μg/μl stock provided with the kit following the instruction of the BCA Reagent Kit. Concentration of 10 - 100% of homogenised tissue was made in water. 25 μl of each in triplicate of the BSA standards and samples was pipetted into wells of a 96 well microtitre plate. BCA working reagent (WR) was made up with 1:50 solutions A:B of which 200 μl were added to each well, the plate shaken, covered and left at room temperature for 2 hrs. The absorbance was read at 570 nm on spectrophotometer (Rosys Anthos HIII) and analysed using STINGRAY™ application software. Absorbance values were corrected for blank and plotted against standard curve and a calculation of protein concentration made using a linear regression equation (figure 3.8 page 80).

2.4. Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE) and Western blotting.

The Western blotting technique is a semi-quantitative technique for assessment of specific protein contained within a sample. Proteins were separated by gel electrophoresis and were transferred to a nitrocellulose membrane then incubated in antibody (primary) raised against the antigen, followed by a second incubation an antibody (secondary) raised against the primary antibody and which is also conjugated

with horseradish peroxidase (HRP) enzyme. The HRP is allowed to react with a chemiluminescent substrate that luminesces blue light at the sites at which the primary antibody is bound. The light is detected by film sensitive to blue light and developed. This film is assessed by densitometry of the bands of the specific protein.

Samples were removed from storage at -20°C and thawed on ice. Samples containing 15 μg of protein were prepared. A four times concentration of Laemli sample buffer was added to the tissue sample to make up one third of the final volume, i.e. 10 μl, and the solution made up to a final volume of 30 μl with water (Laemmli, 1970). The samples were mixed on vortex and placed in boiling water for 5 min then loaded onto the polyacrylamide gels for electrophoresis.

Preparation of reagents.for 12% Resolving Gel.

The gel-casting module was set up as per kit instructions (BioRad™) on a flat surface. 10% Ammonium persulfate (APS) (Sigma) was prepared i.e. 0.26 g of APS crystals in 2.34 g of ddH_2O. 10% SDS was available ready-to-use. 25 ml of 12% resolving gel was prepared i.e. 3.55 ml ddH_2O, 2.5 ml 40% Acrylamide (Sigma), 3.75 ml 1 M Tris-Base pH 8.8, 100μl of 10% SDS, and 100μl of 10% APS. 10μl of TEMED (Sigma) was added, the mixture shaken and poured into the gel-casting module to a level approximately 2 cm from the top of the plates. A layer of H_2O saturated isobutanol was applied to the top of the resolving gel to prevent extended polymerisation allowing the gel to set with a straight surface. The gel set for 20 min after which the H_2O saturated butanol layer was blotted off using Whatman absorbent paper.

5% Stacking Gel

15 ml 5% stacking gel was prepared by mixing 3.64 ml of distilled water, 630 μl of 40% acrylamide, 630 μl of 1 M Tris-Base pH 6.8, 50 μl of 10% w/v APS, and 50 μl of 10% w/v APS. 10 μl of TEMED (Sigma) was added the mixture swirled and poured into the gel-

casting module. The gel combs were inserted into the gel immediately on pouring in the stacking gel and the gel was left to set for 30 minutes.

Laemmli Sample Buffer

20 ml of Laemli sample buffer was made up by mixing 7 ml of ddH_2O, 4 ml of 10% SDS, 4 ml of glycerol (Amersham, UK) ultra grade, 5 ml of Tris-Base pH 6.8, and 40 mg of bromophenol blue. The solution was mixed thoroughly with a vortex machine.

ODC solution

The preparation of ODC solution was carried out on ice. The L-ODC (Sigma) was removed from storage at -20°C and 0.762×10^3 g of powder was weighed out into Eppendorfs. 500 µl of water was then added, and then the solution was mixed on a "whirly" mixer and then aliquoted into 15 x 30 µl volumes, which were stored at -20°C for later use. The concentration is 1.538 µg/µl.

The prepared gels are mounted in the Novex® gel electrophoresis module. A one times strength gel running buffer was poured between the two gels mounted in their glass plates to overflow at the tops of the plates. Using gel-loading tips, the boiled samples were loaded onto the wells as follows, 8 µg ODC solution, and 15 µg each of normal, CFA treated, Day-13 EAE and Day-21 EAE cerebellum, medulla pons, or cervical spinal cord and molecular weight marker. An example of the gel-loading pattern for an SDS-PAGE analysis is given in figure 2.1a. and 2.1b. pages 46 and 47 respectively.

Figure 2.1a Gel loading pattern for SDS-PAGE of rat brain sample

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10
Positive control	Normal CNS tissue	CFA treated CNS	Day-13 EAE tissue	Day-21 EAE tissue	Normal CNS tissue	CFA treated CNS	Day-13 EAE tissue	Day-21 EAE tissue	Molecular weight marker

The figure above shows the layout of tissue samples for an SDS-PAGE analysis of rat brain sample used in the western blot.

In later experiments the molecular weight markers was loaded in well separating the two CNS tissue types i.e. tissues were loaded as, positive control, normal, CFA treated, Day-13 EAE, Day-21 EAE, molecular weight marker, normal, CFA treated, Day-13 EAE, and Day-21 EAE (figure 2.1b pg 56). The molecular weight marker was in a position in which easier comparison could be made with respect to each tissue type in the different wells.

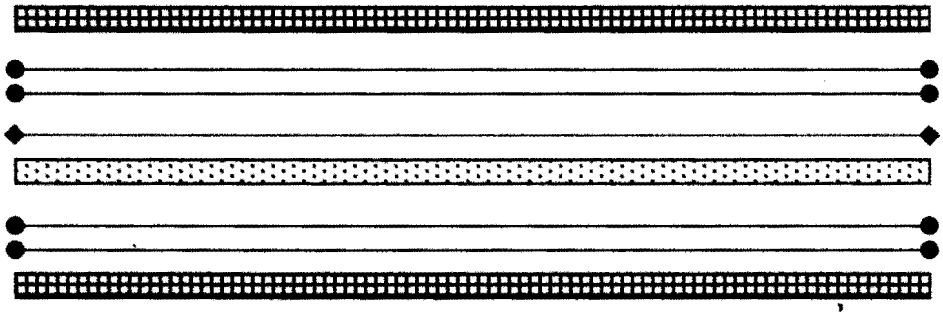
Figure 2.1b Gel loading pattern for SDS-PAGE of rat brain sample.

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10
Positive control	Normal CNS tissue	CFA treated CNS	Day-13 EAE tissue	Day-21 EAE tissue	Molecular weight marker	Normal CNS tissue	CFA treated CNS	Day-13 EAE tissue	Day-21 EAE tissue

The figure shows loading of samples onto gel with molecular weight marker separating CNS sections.

Electrophoresis was carried out at 80mAmps in a Novex® X Cell II Blot module connected to an Anachem ScotLab electrophoresis power supply for 75-90 minutes after which transfer onto the nitrocellulose was accomplished using the wet transfer method as follows. The glass plates were seperated leaving the gel flat on one plate and the stacking gel cut off. Hybond-C nitrocellulose (Amersham, UK) and two pieces of gel-sized blotting paper pre-wetted in transfer buffer were placed on top of the gel with the nitrocellulose in contact with the gel. Two pieces of pre-wetted blotting paper were placed on the other side of the gel.

Figure 2.2 Protein transfer sandwich for transfer of protein from gel onto nitrocellulose showing arrangement of components.



Key:

= Electrophoresis pad = Nitrocellulose membrane

= Blotting paper = Polyacrylamide gel

The transfer cassette was placed in the Novex® X Cell II Blot module and filled with transfer buffer inside and tap water outside then placed in an ice bath. Transfer was carried out at 200 mAmps for 80 minutes using an Anachem ScotLab electrophoresis power supply. The transfer efficiency was tested using Ponceau S (Sigma) bought as ready-to-use solution, for visualisation of protein bands then washed off in three 5-minute washes of 20 ml of (0.1% v/v) PBS Tween-20. The nitrocellulose was then bathed in 20ml of blocking buffer, prepared by dissolving 5% w/v Marvel (Premier Brands, UK Ltd) non-fat dried milk powder in PBS Tween (polyoxyethylene sorbitan monolaurate), on a plate rocker for 1 hour at room temperature to block non-specific binding of the primary antibody then placed in primary antibody (monoclonal mouse α-ODC, Sigma; 1:400), prepared in (0.1% v/v) PBS Tween-20and incubated at 4°C overnight. The nitrocellulose was removed from the primary antibody solution and washed three times for 5-minutes each time in 20 ml volumes of PBS Tween then placed in secondary antibody (goat anti-mouse, IgG Peroxidase conjugate, Sigma; 1:4000) at room temperature for 1 hour on a plate rocker.

2.5. Enhanced Chemilumeniscence

The membrane was then washed in PBS Tween solution and the excess solution blotted off. The nitrocellulose was then placed on a strip of cling film place on a flat workbench with no air bubbles trapped underneath. The surface of the nitrocellulose with the transferred protein was flooded with ECL reagent (Santa Cruz) prepared by mixing equal volumes of reagents A and B provided as part of ECL kit. The excess ECL reagent was drained off and the nitrocellulose was wrapped in cling film, taking care not to allow air bubbles to form, and was placed in a film cassette (Kodak) and exposed to light sensitive Kodak GBX film for 5 minutes. The film was then developed in a darkroom by bathing the film in Kodak GBX developer solution as directed by kit (Kodak GBX, UK) i.e. 5 minutes in developer then 8 minutes in fixer then film was then removed and allowed to air dry.

2.6. Analysis of Gels

The film was scanned with an Epson Twain scanner with Adobe Photoshop 5 software and densitometry of bands analysed using Gel-Pro Analyzer software. Densitometry values were used to plot graphs in Microsoft Excel®, with relative absorbance against tissue types as shown in results section.

2.7. Statistical Analysis of Results

The densitometry analysis gave a relative comparison of the optical density of the protein bands. The values obtained were analysed for significance using a one-way analysis of variance (ANOVA) with the variation arising due to point in disease course i.e. normal (day 0), CFA treated (control), day-13 EAE PI, and day-21 EAE PI.

3.0. STANDARDISATION OF PROTEIN ASSAY

3.1. Protein Assay

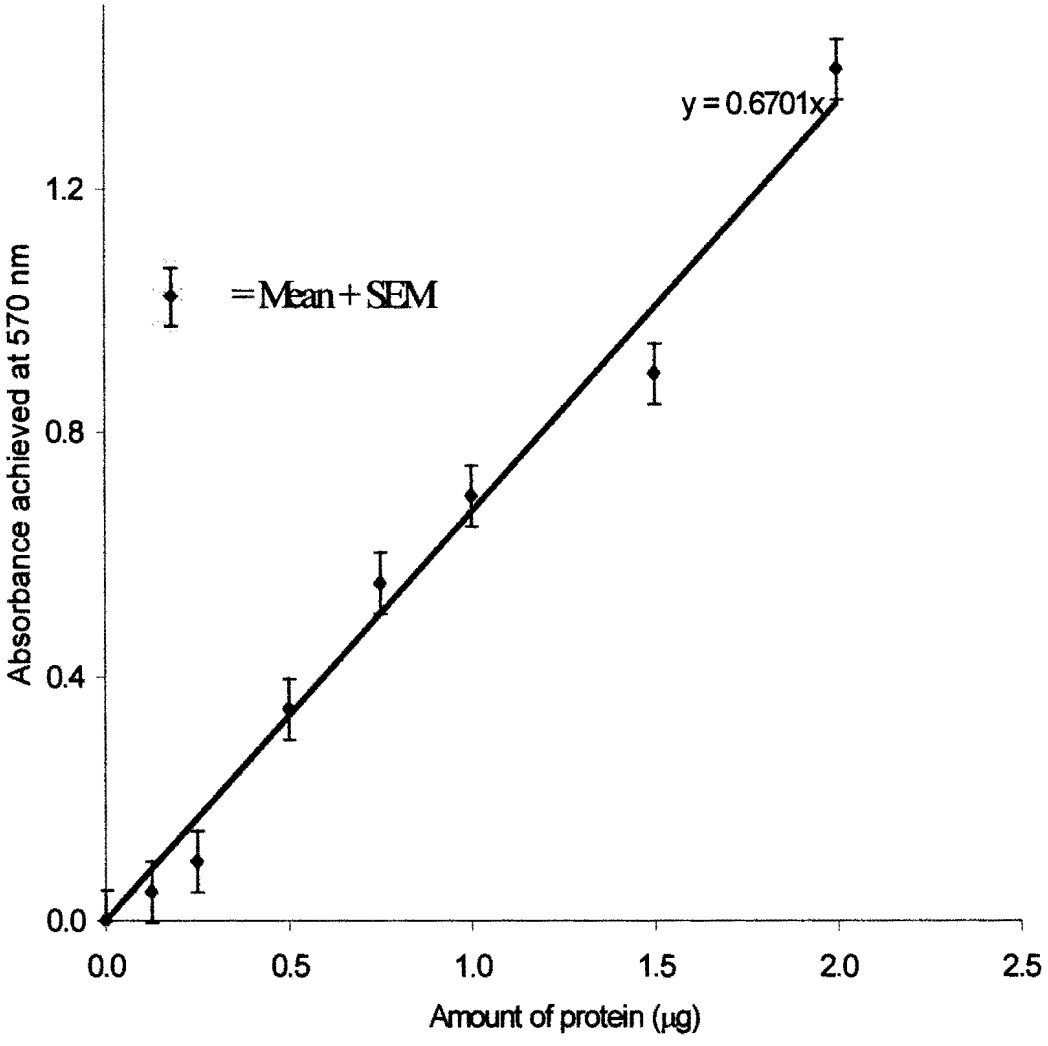
The protein assay is used to determine concentration of a solution. The choice of protein assay depends on various factors, an important one being presence of substances that interfere and thus distort the result. Initial stages of this study established an appropriate protein assay because the Western blotting technique relies on the proper determination of protein concentration i.e. the results obtained by Western blotting are based on the assumption that the total protein content of each sample loaded the same. However establishing an accurate and reliable protein assay proved more difficult than expected, due to problems detailed in the following sections.

BSA that was readily available and was easy to prepare and store was chosen as protein standard. A 1.0 μg/μl BSA was made as described previously and used to plot a standard curve. The protein assay methods assessed were the Bradford assay method (BioRad™), the Lowry assay method (Folin Phenol method) and the Bicinchoninic Acid (BCA) method.

3.1.1. Bradford Assay

In assessing the Bradford assay, BioRad™ kit and protocol were used. This assay method is easy to use and the reagents and equipment required for its use were readily available. Using BSA (1.0 μg/μl) for concentration 0.0 – 2.0 μg /μl a standard curve was plotted. This is shown in the standard curve achieved in figure 3.1 below.

Figure 3.1 Standard curve for absorbance achieved per unit protein measured using bovine serum albumin (1 μg/μl) as protein standard using the BioRad™ (Bradford) assay



Standard curve of BSA (1.0 μg/μl). Plots are mean ± SEM for n = 3. (Appendix IV shows data). The figure shows a good relationship between protein concentration and absorbance with little deviation. The plot provides a linear regression equation from which the protein concentration of samples may be calculated.

Assay of prepared ODC solution

As ODC was the protein to be assessed in the rat brain tissue, an assay was carried out to determine the effects of the assay on ODC. ODC was processed using homogenising

method II. Volumes of the prepared solution (concentration 1.538 µg/µl) ranging from 0-15 µl were assayed using the BioRad™ method and the absorbance achieved measured. (Table 3.1 below shows result and data is given in appendix V).

Table 3.1. The protein concentration calculated for ODC sample on a BSA standard curve measured using BioRad™ assay.

Linear regression equation for protein calculation, y = 0.0799x.

Volume of sample (µl)	Absorbance at 570 nm	Protein concentration (µg/µl)	Concentration corrected for sample volume (µg/ul)
0	0.000	0.000	0.000
3	0.829	10.375	3.458
5	1.049	13.129	2.626
6	1.191	14.906	2.484
7	1.253	15.682	2.240
8	1.315	16.458	2.057
10	1.337	16.733	1.673
15	1.415	17.710	1.181

As sample volume increased protein concentration determined increased. The table shows an inconsistency in the protein estimation over the range of volumes of sample taken with most volumes of sample showing a higher protein concentration than the known concentration of 1.538 µg/µl.

The differences between the known protein concentration of 1.538 µg/µl and the calculated protein concentration are too large to be attributed to experimental error, suggesting that there is interference with the assay probably arising from substances within the homogenising medium. Further experimentation, using ODC sample of volumes ranging from 5-15 µl gave results that failed to tally with the known protein concentration of 1.538 µg/µl. A table showing the results of such experiments using the BioRad™ method is given below (Data shown in appendix VI).

Table 3.2 Protein estimation for ODC solution assays

ODC solution Volume assayed (µl)	Expected concentration (µg/µl)	Replicate One Estimated protein (µg/µl)	Replicate Two Estimated protein (µg/µl)
5	1.583	2.626	4.008
10	1.583	1.673	2.339
15	1.583	1.181	1.607

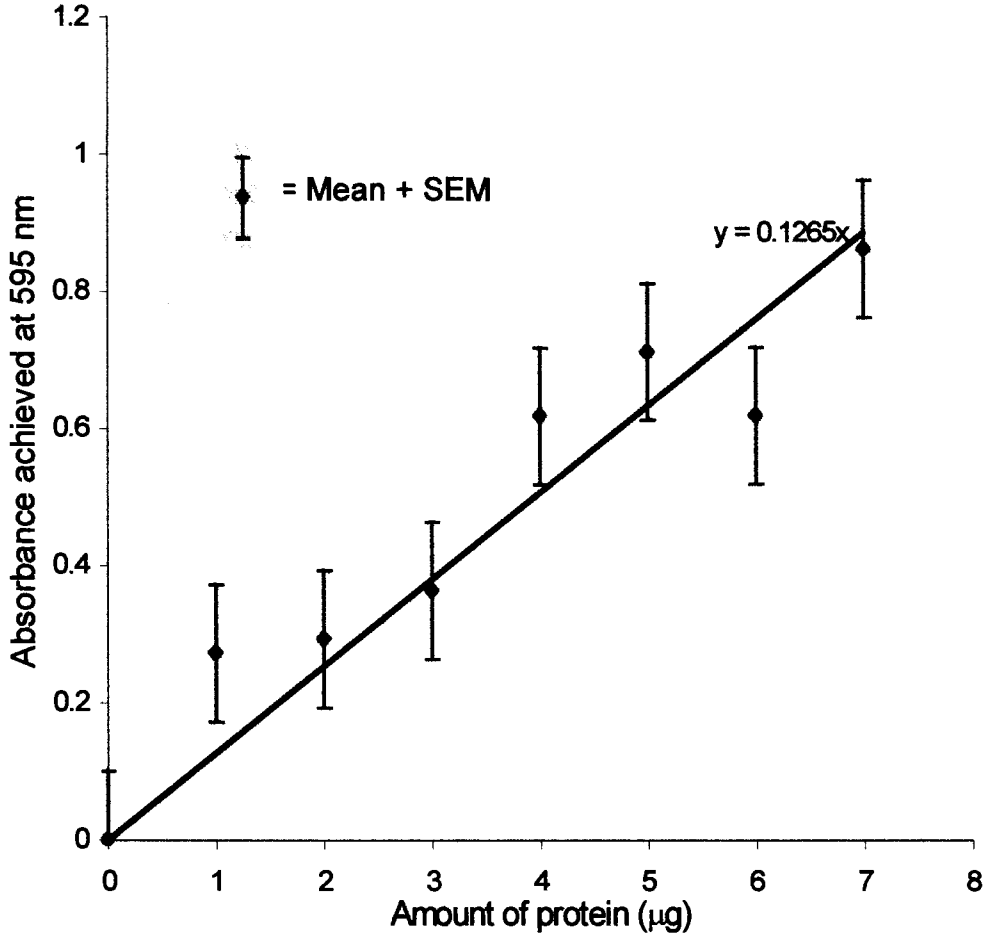
Duplicate assays of ODC solution of known concentration showed inaccurate protein concentration determination again suggesting interference. (Data shown in Appendix VI).

Use of Pig Brain in protein assay standardisation.

The standardisation of the techniques required the use of a large amount of sample meaning that large volumes of rat brain sample would be required. Rat brain sample was in limited quantity and not readily available therefore pig brain, which was readily available, was used in early stages of this study. Initial experiments performed on undiluted pig brain sample (data not shown) showed that the homogenised undiluted sample was too concentration and the absorbance being out of the range of the standard curve. Subsequent assays were carried out at lower concentration as shown below (figures 3.3 and 3.4).

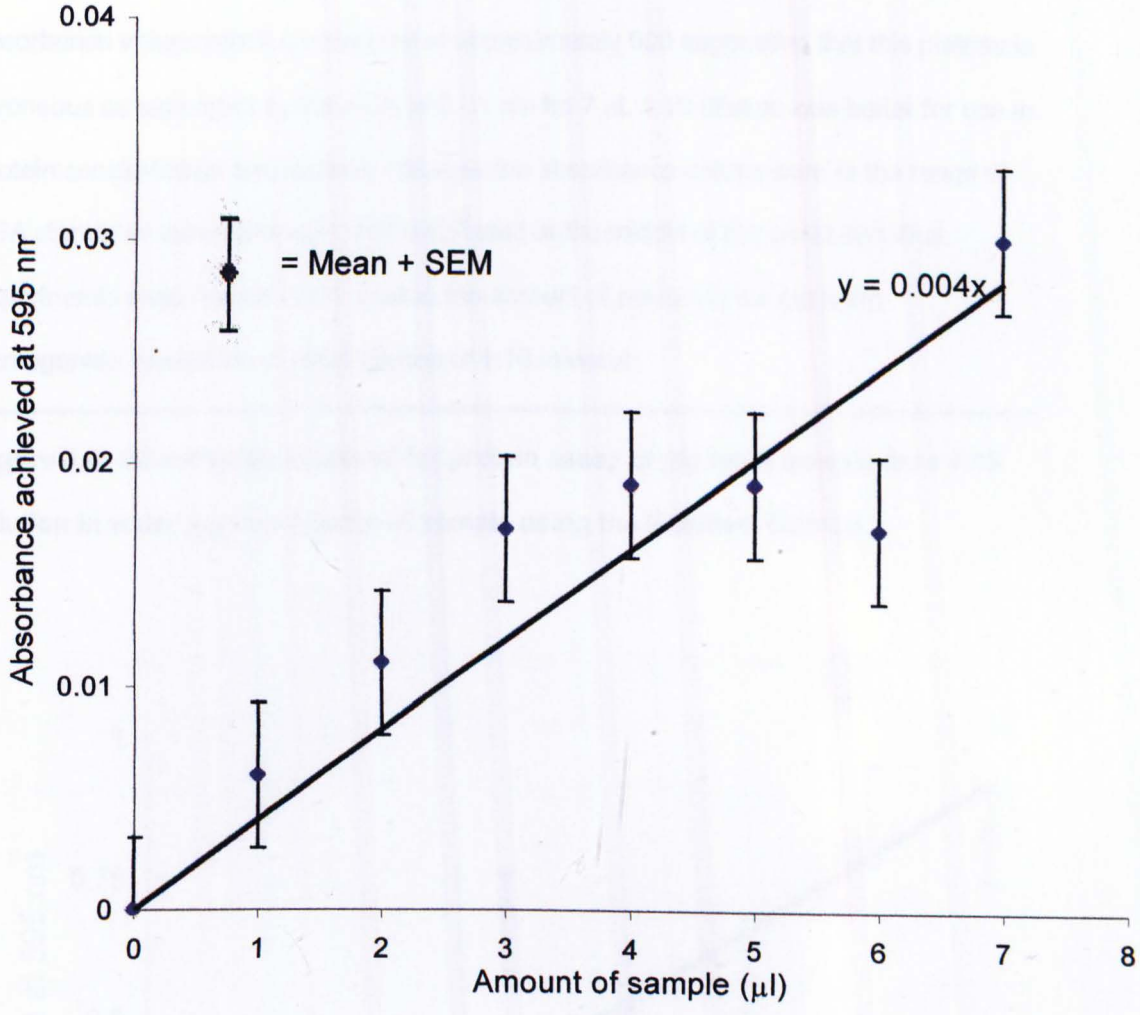
A 1:10 dilution of pig brain sample in homogenising medium was assayed using the BioRad™ method and this was compared with an assay of 1:1000 of pig brain sample in homogenising medium also using the BioRad™ method.

Figure 3.2. Absorbance achieved for protein assay of pig brain sample for a 1:10 dilution plotted against volume of sample using the Bradford method.



The protein concentration was calculated from a 1:10 dilution using the BioRad™ method and a linear regression equation of an assay of BSA standards (1 μg/μl concentration). The BSA standards were assayed for volume ranging from 0.0-7.0 μl (Data shown in appendix VIII).

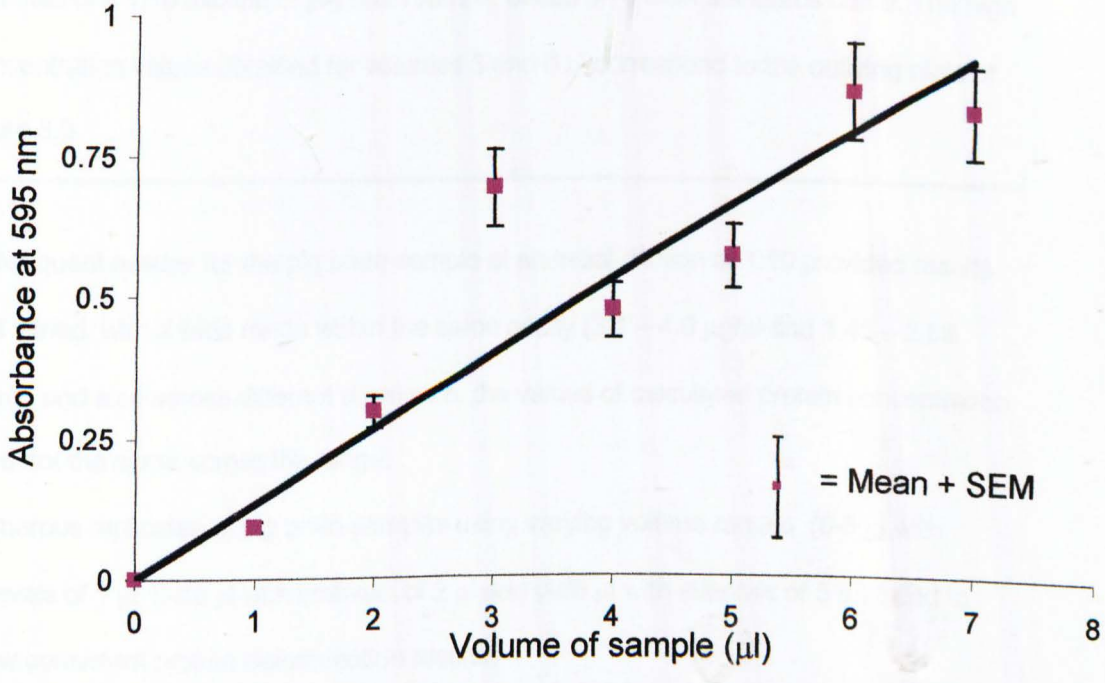
Figure 3.3. Absorbance achieved for protein assay of pig brain sample for a 1:1000 original dilution in water against volume of sample using the Bradford method.



The protein concentration was calculated from a 1:1000 dilution using the BioRad™ method and a linear regression equation of an assay of BSA standards (1 µg/µl conc). The absorbance values seen are less than those for 1:10 dilution in figure 3.2. The decrease is however not proportional to dilution. The graph seems to level of about 0.02 nm, which may denote a saturation of sample however, in the 1:10 dilution it is seen that even above this concentration a linear plot can be achieved. (Data shown in Appendix VIII).

From figures 3.2. and 3.3. it can be seen that the lower concentration, i.e. higher dilution, is more affected by interference. Also, the absorbance values obtained from the lower concentration in figure 3.3. plateau at approximately 0.02 nm while in figure 3.3, the absorbance values reach a maximum of approximately 900 suggesting that this plateau is erroneous as supported by the point at 0.03 nm for 7 μl. 1:10 dilution was better for use in protein concentration calculations, because the absorbance values were in the range of BSA standards curve and were well distributed at the middle of the curve and thus, experiments were carried out to assess the amount of protein in the pig brain homogenate sample at an initial dilution of 1:10 in water.

Figure 3.4. Absorbance achieved for protein assay of pig brain sample for a 1:10 dilution in water against volume of sample using the Bradford Method.



Pig brain sample diluted 1:10 in homogenising medium shows that there is a direct proportional relationship between the concentration of the sample and the absorbance achieved. The result of this experiment also shows that the protein assay is working

within a small range and fits well within the working range of the BSA standard curve. The calculated protein content is shown in table 3.3, page 84.

Table 3.3 Protein estimation for a pig brain sample diluted 1:10 in homogenising medium using the BioRad™ assay.

Linear regression equation, Y = 0.422X

Volume of BSA (μl)	Absorbance at 570 nm	Protein concentration (taking into account dilution) (μg/μl)	Protein concentration corrected for sample volume (μg/μl)
1	0.091	2.156	2.156
2	0.214	5.071	2.536
3	0.689	16.327	5.442
4	0.472	11.185	2.796
5	0.568	13.460	2.692
6	0.865	20.498	3.416
7	0.829	19.645	2.806

Table 3.3 shows the protein estimation for BioRad™ assay performed on different volumes of a 1:10 dilution of pig brain sample based on a BSA standards curve. The high concentration values obtained for volumes 3 and 6 μl correspond to the outlying plots of figure 3.3.

Subsequent assays for the pig brain sample at an initial dilution of 1:10 provided results that varied, with a wide range within the same assay (3.1 – 4.0 μg/μl and 1.46 – 2.59 μg/μl) and also across different assays i.e. the values of calculated protein concentration were not the same across the range.

Numerous replicates of pig brain samples using varying volume ranges (0-5 μl with intervals of 1 μl; 0-20 μl with intervals of 2 μl and 0-35 μl with intervals of 5 μl) failed to show consistent protein determination results.

From the Bradford method, it was seen that higher protein concentration corresponded with smaller volumes. Another observation was that the protein estimations within the assays were not consistent, i.e. the different volumes of sample taken for the carrying out of an assay provided different protein concentration Replicates of experiments using pig

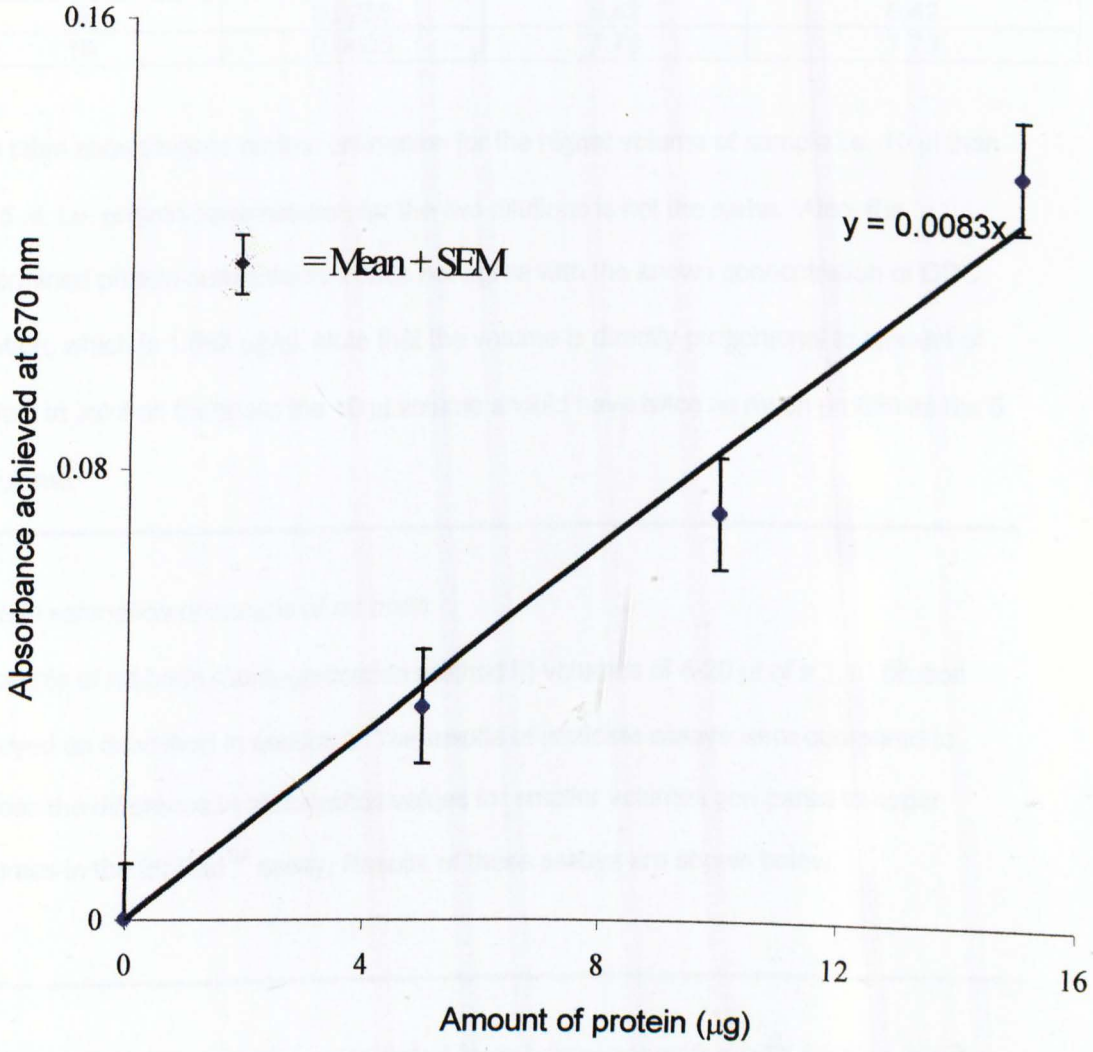
brain sample and ODC solution could not efficiently estimate the protein content within the samples.

In addition the response of the assay to dilutions of the samples was not proportional to the amount of protein in sample, i.e. the absorbance achieved was not proportional to dilutions of sample assayed, except for figure 3.4. Using ODC solution of known concentration it was determined that the Bradford assay could not effectively determine the concentration of prepared samples. It was thus necessary to establish another method for the assessment of protein concentration of the prepared samples, in this case the Folin Phenol method as discussed below. The result of the determination of protein concentration using Folin Phenol assay method could then be compared with the results obtained using the BioRad™. If these results were similar then the irregularities in the assessment of the protein concentration would not be dependant on the method but other factors. However, a difference in the two results would establish that the BioRad™ assay, as had been suggested, had not accurately assessed the protein concentration

3.1.2. Folin Phenol Method (Lowry Assay)

First BSA standard curve was produced. Secondly, an assay was carried out on ODC of known concentration and the determined concentration compared to the known concentration Lastly, protein determination for pig brain was carried out.

Figure 3.5. Standard curve of BSA standards of 1 μg/μl concentration showing absorbance achieved against amount of protein in solution using the Lowry method.



The figure shows a good linear relationship between amount of protein, i.e. concentration, and absorbance achieved. This figure also shows little interference and very small experimental error. A line was used to assess the graph instead of a smooth curve through the points because the points have a good linear relationship.

Prepared ODC solution of concentration 1.538 μg/μl was assayed using the Lowry assay and a calculation of the protein concentration as assessed by the assay method was made (results shown in table 3.4. page 60). A comparison of the calculated protein concentration and the known concentration was then made.

Table 3.4. Protein estimation for prepared ODC solution assessed by Lowry assay method.

Volume of ODC solution (μl)	Absorbance at 640 nm	Amount of protein (μg/μl)	Dilution and volume correction (μg/μl)
5	0.0285	5.42	5.42
10	0.0405	7.79	7.79

The table shows higher protein estimation for the higher volume of sample i.e. 10 μl than the 5 μl. i.e. protein determination for the two dilutions is not the same. Also, the determined protein concentration does not agree with the known concentration of ODC solution, which is 1.582 μg/μl. Note that the volume is directly proportional to amount of protein in solution therefore the 10 μl volume should have twice as much protein as the 5 μl volume.

Protein estimation of sample of rat brain.

A sample of rat brain (homogenisation method II) volumes of 4-20 μl of a 1:10 dilution assayed as described in section 2. The results of replicate assays were compared to assess the difference in absorbance values for smaller volumes compared to larger volumes in the BioRad™ assay. Results of these assays are shown below.

Table 3.5 Protein content as calculated from Lowry assay for 1:10 dilution of rat brain sample for volumes 4, 8, 10, and 20 μl.

	Normal (μg/μl)	CFA treated (μg/μl)	EAE day-13 PI (μg/μl)	EAE day-21 PI (μg/μl)
4 μl	15.45	17.90	19.60	14.70
8 μ	10.06	11.89	13.60	10.67
10 μl	9.62	10.0	9.62	9.04
20 μl	11.92	14.04	14.23	12.5

In table 3.5 a comparison of the amount of protein calculated for rat brain samples using four different volumes of sample of a 1:10 dilution is shown. The calculated protein

concentration for each sample is not the same. The smallest volume shows the highest calculated protein concentration (Data shown in appendix X).

The protein estimation using a range of sample volumes shows two things. Firstly, the protein concentration from the absorbance values achieved is not the same for the four volumes. Secondly, the difference seen in the calculated protein concentration for the four different volumes is too large to be attributed to experimental error. The experiments carried out on the samples using the Lowry assay failed to give accurate results of the protein content, i.e. the assay did not give the expected protein concentration of prepared ODC solution. Also, the assay failed to show a direct relationship between the volume of sample taken, which is directly proportional to the amount of protein, and the absorbance achieved.

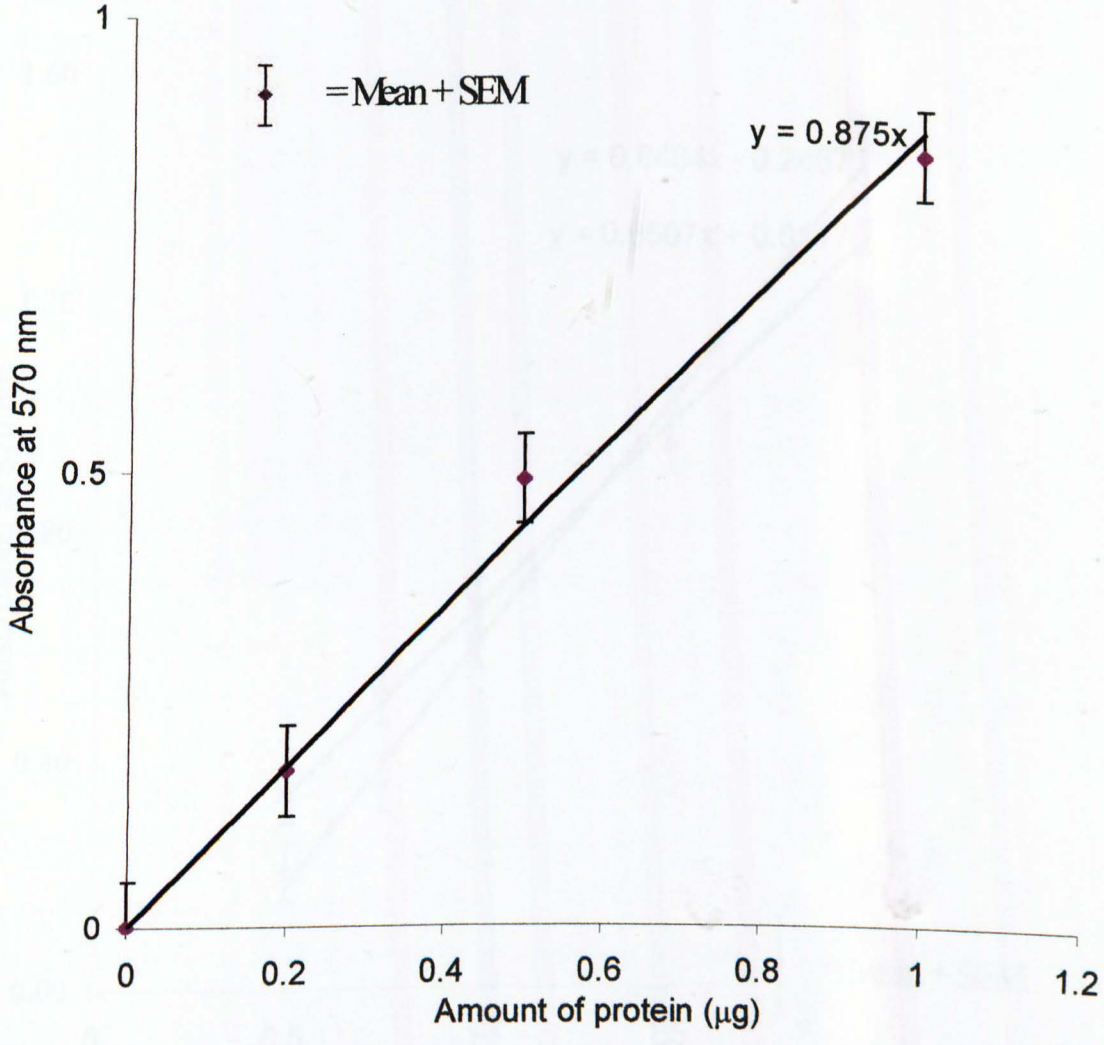
The Lowry and BioRad™ assay showed different protein concentration values (tables 3.3. and 3.5.) for a 1:10 dilution of pig brain with the Lowry assay showing estimated concentration five to six times higher than BioRad™ assay. Thus it was again necessary to establish another assay method. The protein assay method chosen was the BCA method.

3.1.3. Bicinchoninic Acid (BCA) Method

In order to establish the BCA assay in the estimation of protein concentration for the CNS samples, and to determine the accuracy of the method, two aspects of the BCA protein assay were tested. Firstly, BSA standards were prepared using homogenisation medium and water as diluents as per kit instructions. Comparison of these two standard curves obtained would show the effect of any interference due to components of the homogenising medium in which the samples were prepared. Dilutions of the pure sample, 1:1 and 1:4, were prepared in both homogenising medium and water, and a range 0.0 – 2.0 μl assayed in triplicate. The absorbance values were taken and the protein concentration of each sample volume calculated and corrected for volume and dilution factors.

Secondly, a sample of prepared ODC solution of known concentration was assayed and a comparison of the measured protein concentration with the known concentration made i.e. if determined protein concentration equals known concentration of the prepared ODC, then the BCA assay method is reasonably accurate. The first part of the assessment of the BCA assay method was to produce a BSA standard curve as before (figure 3.6).

Figure 3.6. Standard curve of protein concentration against absorbance achieved for BSA standards (1μg/μl) prepared in water using the Bicinchoninic Acid Assay method.



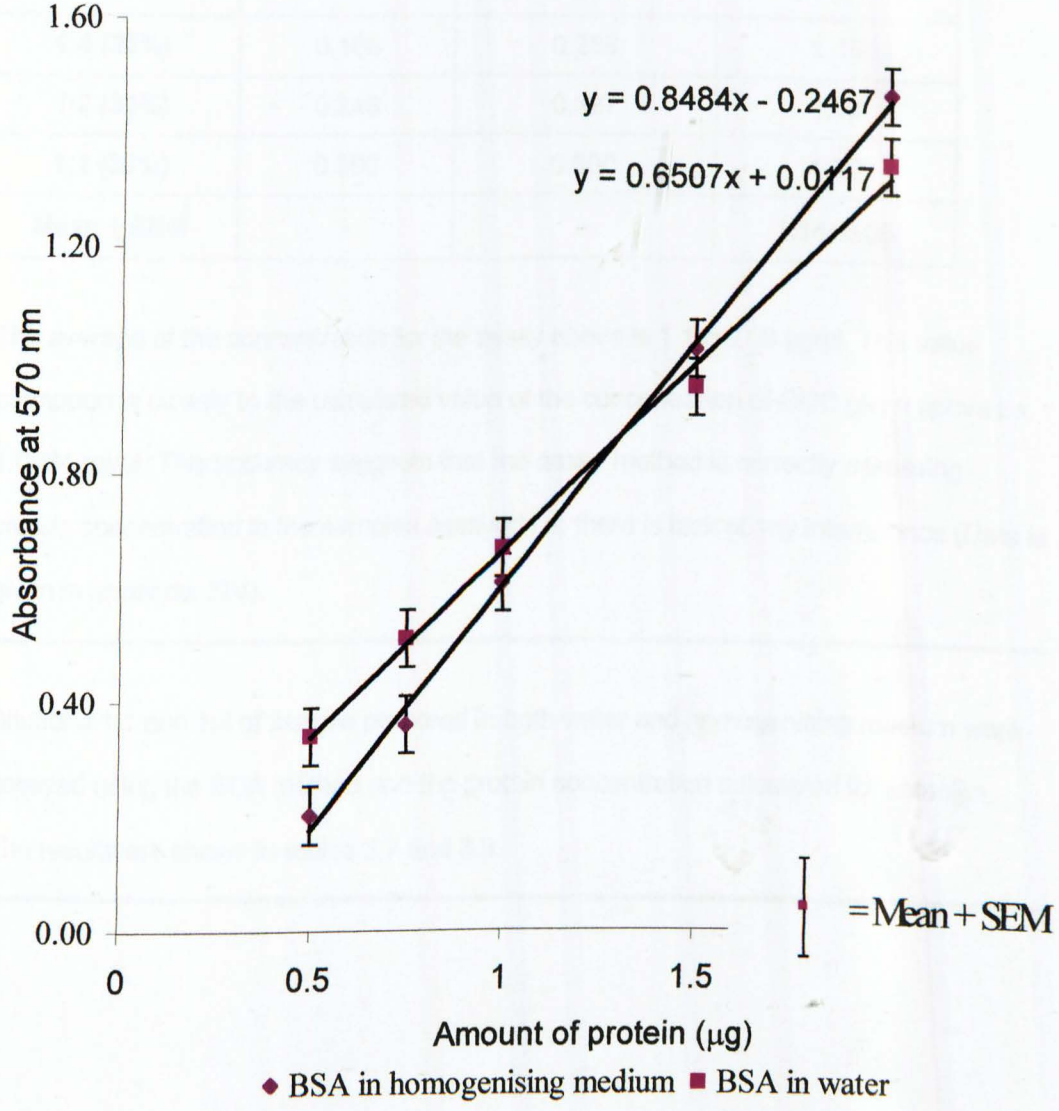
BCA assay of BSA to produce an equation calculated for a straight line by linear regression analysis is shown. The equation may be used for calculations of protein

concentration Absorbance is seen to be linear and proportional to concentration with little deviation from the straight line i.e. little experimental error. (Data shown in Appendix XII)

Assessment of BCA Assay for protein concentration estimation

Standard curves of BSA samples prepared in water and homogenising medium were plotted and compared (figure 3.7) following BCA assay kit protocol. Figure 3.8 shows the result.

Figure 3.7. Absorbance achieved for BSA standards of 1μg/μl concentration prepared in homogenising medium and water as diluents.



The standard curves of BSA prepared in both homogenising medium and water produce equations of linear regression that are similar suggesting that the use of either diluent has little or no effect on the results. Determination of protein concentration in cerebellum sample prepared in water and homogenising medium (table 3.7 and 3.8) was calculated. (Data in appendices XIII, XV and XVI).

ODC solution of known concentration (1.0984 μg/μl) was assayed using the BCA method at dilutions 1:1, 1:2, and 1:4 with water as diluent. Table 3.6 shows the results.

Table 3.6 Protein concentration measured by BCA assay estimated for prepared ODC solution at different dilutions in water.

Dilutions (concentration)	Absorbance at 570 nm	Amount of protein (μg/μl)	Corrected for dilution
1:4 (20%)	0.168	0.288	1.15
1:2 (33%)	0.246	0.327	1.08
1:1 (50%)	0.398	0.600	1.20
Mean ± SEM	-	-	1.14±0.06

The average of the concentration for the assay above is 1.14±0.06 μg/μl. This value corresponds closely to the calculated value of the concentration of ODC given above i.e. 1.0984 μg/μl. This accuracy suggests that the assay method is correctly assessing protein concentration in the samples assayed i.e. there is lack of any interference (Data is given in appendix XIV).

Dilutions 1:1 and 1:4 of sample prepared in both water and homogenising medium were assayed using the BCA method and the protein concentration calculated for samples. The results are shown in tables 3.7 and 3.8.

Table 3.7. Protein concentration of normal cerebellum prepared in homogenising medium assayed by BCA method at dilutions of 1:1 and 1:4.

Dilution (Concentration)	Absorbance at 570 nm	Amount of protein (μg/μl	Corrected for dilution (μg/μl)
1:1 (50%)	0.302	0.4614	0.9228
1:4 (20%)	0.157	0.2398	1.1990
Mean ± SEM	-	-	1.06±0.27

The protein estimation for the two dilutions is similar i.e. (average ± SEM) 1.06±0.27. Data is given in appendix XIV.

Table 3.8. Protein concentration of normal cerebellum prepared in water assayed by BCA method at 1:1 and 1:4 dilutions.

Dilution (Concentration)	Absorbance at 570 nm	Amount of protein (μg/μl	Corrected for dilution (μg/μl)
1:1 (50%)	0.379	0.5791	1.1582
1:4 (20%)	0.149	0.2277	1.1385
Mean ± SEM	-	-	1.582±0.0098

The determined protein concentration for the two dilutions is similar i.e. (average ± SEM) 1.1582±0.0098 but slightly higher than that for sample prepared in homogenising medium. Data is given in Appendix XV.

From the results (tables 3.6, 3.7, and 3.8), it was seen that that BCA assay gave consistent and accurate results for different assays of same samples as shown by the close agreement between the measured and known concentration ODC solution. Although it was determined that the difference in the determination of protein concentration using samples made up in either water or homogenising medium is minimal (figure 3.7.), water was used as diluent in all subsequent experiments.

4.0. RESULTS

For the western blot analysis, it was important to know the concentration of protein in the samples to be assessed. The rat samples were categorised into three experimental groups for purposes of replicates. Each group comprised of cerebellum, medulla, and cervical spinal cord tissue. Each tissue type was further divided into normal, CFA-treated, day-13 PI EAE and day-21 PI EAE tissue. Having standardised the protein assay and determined the concentration of samples, it was possible to carry out the estimation of protein of the rat samples in preparation for SDS-PAGE and subsequent Western blotting.

4.1. Protein estimation in rat tissue samples for rat brain samples.

All rat brain homogenate samples were assayed using the BCA assay as described in the methods section for SDS-PAGE and following the instructions of the BCA assay kit. Table 4.1 shows a summary of the protein concentration estimations for analysis set one, two and three with dilution factor taken into consideration.

Table 4.1 Protein concentration for tissue samples (All values in the table are µg/µl.).

	Cerebellum				Medulla				Cervical Spinal Cord			
	Normal	CFA-treated	Day-13 PI EAE	Day-21 PI EAE	Normal	CFA-treated	Day-13 PI EAE	Day-21 PI EAE	Normal	CFA-treated	Day-13 PI EAE	Day-21 PI EAE
Three	3.204	3.204	2.964	3.204	3.737	3.577	3.817	3.283	2.763	3.003	2.843	3.043
Two	3.324	3.123	3.123	3.363	2.883	1.683	2.562	2.523	3.065	2.795	3.065	3.025
One	3.570	3.325	3.310	3.625	3.325	2.845	3.180	3.000	3.420	3.505	3.110	3.025

The protein concentration for each rat tissue sample is shown in the table above. The protein estimation shown was used for the calculation of the amount of protein in the samples prepared for loading.

The protein concentration of the samples all fall in the same range, a good indication of the accuracy of the assay as the weights of the rat tissue (Appendix I) were also in the same range.

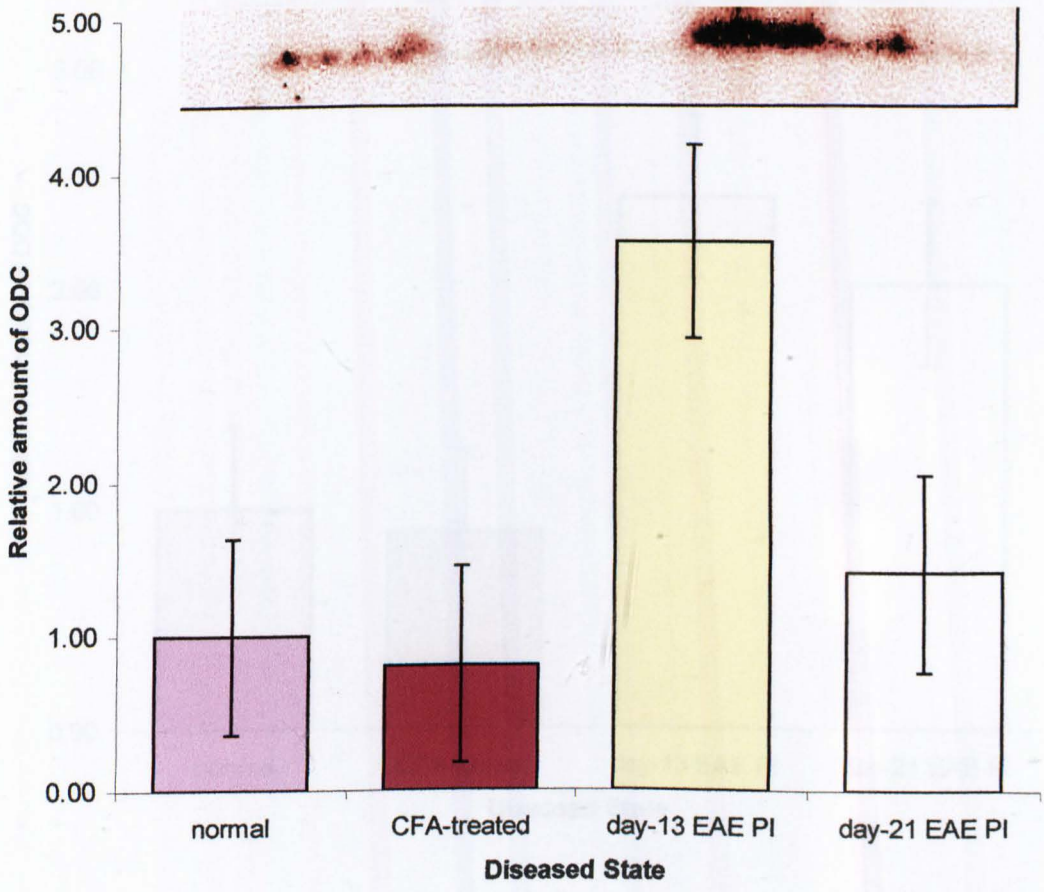
The protein of interest in this Western blotting analysis was ODC. It was therefore important to find out if the processes of homogenisation, SDS-PAGE and protein transfer (from gel to nitrocellulose) would adversely affect ODC. Therefore initial Western blotting was carried out with ODC (not shown). ODC solution was prepared for SDS-PAGE following the method as the tissue samples. This result was compared with the result of a western blot of ODC solution that had not been prepared using the same method as the tissue samples and this showed that homogenising medium does not have an effect on the ODC solution (figure 3.7 page 65).

4.2. Analysis of rat CNS tissue samples for levels of ornithine decarboxylase using the Western blotting technique.

Determination of protein concentration in the homogenised rat tissue samples allowed accurate loading of samples onto gels for the western blot. Samples volumes containing 15 μg of protein from experimental groups one, two, and three were prepared for SDS-PAGE, loaded onto the gels for each sample of rat tissue i.e., normal, CFA-treated, day-13 PI EAE and day-21 PI EAE and assessed for levels of ODC as described previously (section 2.3 page 56).

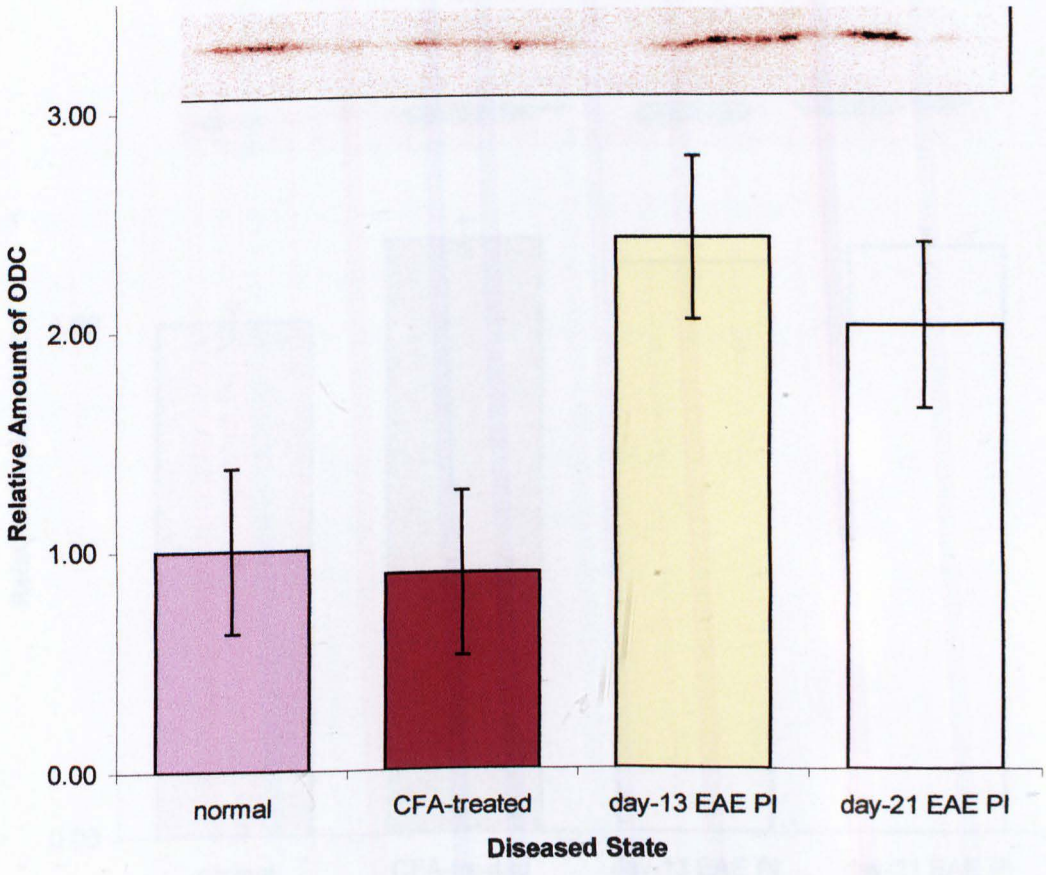
Gel loading was carried out in such a manner as to allow a comparison of the expression of ODC in the tissues. Therefore gels were loaded cerebellum-medulla, cerebellum-cervical spinal cord and medulla-cervical spinal cord. By running two CNS sections on one gel it was possible to compare the expression of ODC levels of one tissue relative to another. Replicate analyses were carried out and the results are shown I figures 4.1., 4.2. and 4.3. Significance of the results was tested using a one-way analysis of variance (ANOVA).The photograph of the gel given in the graph is representative of the tissue type assessed and not the only raw data from which the graph is plotted. The graphs for cerebellum and medulla are for n = 6 (mean ± SEM) while the graph for cervical spinal cord is for n = 9 (mean ± SEM). The data is shown in appendix XVII.

Figure 4.1 Densitometry result for cerebellum tissue showing the levels of ODC for sample loaded onto 12% polyacrylamide gels.



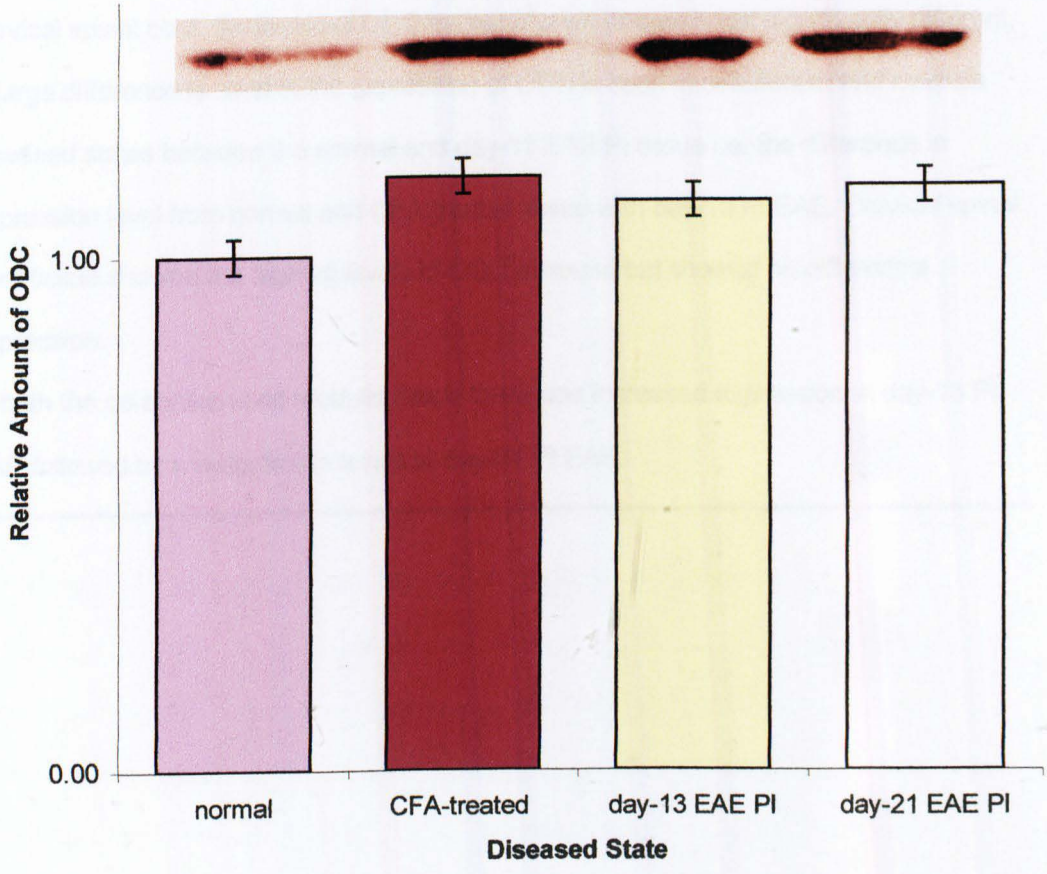
Statistical analysis of difference in ODC expression was performed using one-way ANOVA. The values shown are a relative amount of ODC as determined by absorbance levels observed. The amounts represented are relative to the amount in normal tissue. There is a significant increase in day-13 EAE PI, $P < 0.05$ and $P < 0.01$; $n = 24$.

Figure 4.2. Densitometry result for medulla tissue showing level of ODC for sample loaded onto a 12% polyacrylamide gel.



Statistical analysis of difference in ODC expression was performed using one-way ANOVA. The values shown are a relative amount of ODC as determined by absorbance levels observed. The amounts represented are relative to the amount in normal tissue. There is a significant increase in day-13 EAE PI, $P < 0.05$ and $P < 0.01$; $n = 24$.

Figure 4.3. Densitometry result for cervical spinal cord tissue showing levels of ODC for sample loaded onto 12% SDS-PAGE gels.



Statistical analysis of difference in ODC expression was performed using one-way ANOVA. There is no significant increase in day-13 EAE PI, $P < 0.05$; $n = 36$.

Figures 4.1, 4.2, 4.3, show the analysis of cerebellum, medulla and cervical spinal cord samples for ODC levels of expression in normal, CFA treated, day-13 EAE PI and day-21 EAE PI. The results show that expression of ODC in cerebellum and medulla samples was higher at day-13 PI EAE than in any other disease state (figure 4.1). However in cervical spinal cord, the levels of ODC in the different tissues is not significantly different. A large difference is seen in the expression of ODC is seen in cerebellum and medulla diseased states between the normal and day-13 EAE PI tissue i.e. the difference in expression level from normal and CFA-treated tissue with day-13 PI EAE. Cervical spinal cord tissue showed the highest levels of ODC all round but showed no differential expression.

In both the cerebellum and medulla tissue there was increased expression at day-13 PI EAE followed by a reduction in levels at day-21 PI EAE.

5.0. DISCUSSION

5.1. Homogenisation method

The initial homogenisation method used on the rat brain was replaced by a second method because the initial homogenisation method allowed the collection of only a small volume of total sample volume for the tissue samples homogenised. The standardisation of the protein assay and the Western blotting analysis to follow would both require substantial volumes of homogenised sample and therefore this initial method was inappropriate for use in this study. It was also necessary to optimise use of the rat brain tissue available because the tissue was in limited quantity. The second homogenisation method used allowed the collection of more total sample volume.

5.2. Standardisation of protein assay

Although it was not the aim of this study to evaluate different protein assays, it became evident that establishing an assay that could accurately determine the protein concentration of the homogenised rat brain samples would be an important part of this study. Much time and emphasis was spent on establishing a method of protein concentration determination that would be both accurate and for which replicate assays would give consistent results. With the initial assays used, i.e. BioRad™ and Lowry assays, the results obtained were not an accurate determination of the protein concentration, as evidenced by inaccurate determination of protein concentration of the ODC solution for which the concentration was known. Replicates of experiments carried out using the same samples and same protocols also failed to reproduce consistent results i.e. repeated protein assays did not give the same results for same samples. Initially, to try to rectify the problem of inaccurate determination of protein concentration, dilutions of the original samples made in water were assayed in order to minimise the effect of potential interfering substances that may be present in the homogenising medium used to prepare the samples because diluting with homogenising medium would not achieve the desire effect of minimising the concentration of potential interfering substances such as dithiothreitol (DTT), SDS, EDTA and Triton X-100. Also diluting the

sample would bring the concentration into the working range for the standard procedure of the BioRad™ assay i.e. 0.2-1.4 μg/μl (Bio-Rad™ protocol, US/EG bulletin 1069; BioRad™, 1976). Dilution of the rat brain samples would lessen the noise caused by any interfering substances within the sample thus decreasing the value of the SEM.

When the Lowry assay was used similar problems as those observed with the BioRad™ assay were encountered and the method was also cumbersome because of the time duration and many reagents involved. The range of the Lowry assay for linear results with BSA is 1-1,500 μg/ml (Pierce protocols; Lowry, *et al*, 1951). The Lowry assay also failed to accurately determine the protein concentration of the samples as seen from results of assay of ODC solution. The Lowry assay may also be affected by interfering substances that prevent the accurate determination of protein concentration in a sample. These substances include reducing agents, chelating agents, and strong acids and bases. In particular interfering substances that should be minimized are EDTA to 1 mM, Tris to 250 mM, SDS to 1.0%, Triton X-100 to 0.031%, leupeptin to 10 mg/l, and PMSF to 1 mM (Pierce instruction books No 0389).

For both the BioRad™ and Lowry methods, samples assayed at high dilutions, in particular 20% concentration of the original sample, gave the best results i.e. the results were in the same range for replicates of the same sample (Figure 3.5 for BioRad™ assay; table 3.5 for Lowry assay). The lower the concentration of the sample the closer to expected result and the greater the similarity in protein estimation values across different experiments. When the results from the BioRad™ and Lowry methods were compared, there was a large difference in the calculated protein concentration for similar samples. These differences were too large to be attributed to experimental error alone and were probably brought about by interfering substances in the homogenising medium.

The determination of protein concentration of the samples was finally achieved using the BCA assay method for which accurate and reproducible values were obtained for both the rat brain samples and ODC solution (table 3.6). Experimentation with rat brain sample using water and homogenising medium as diluents also showed results within a similar range and these results were replicable at different dilutions, 10% and 20% (table 3.8 and

3.9) i.e. the BCA assay method was more tolerant to potentially interfering substances present in samples than the two previously used methods. Also, the working range at which the BCA assay works properly is 20-2000 μg/μl. This is a wider range than that of both the BioRad™ and Lowry assays methods. Of the substances that may interfere with the proper working of the BCA assay, the rat brain samples contained NaCl 1 M, EDTA 10 mM, Triton X-100 5%, SDS 1%, Tris 250 mM, and HCl 100 mM (Smith, *et al*, 1985; BCA assay kit 23225-23227, Pierce). The BCA assay was also much easier to use and required less time thus making it possible to carry out multiple analyses quite quickly.
In retrospect, the study would have progressed more efficiently i.e. saving time and resources, had a more extensive literature search been carried out on the various protein assay methods and their limitations and the equipment and reagents for the various assay methods been available such that the assay methods could be assessed simultaneously. Also, the standardisation of assays has shown that all initial assays of samples of non-pure solutions should be done at dilutions ideally dilutions 1:10, 1:100 and 1:1000, in water to establish the concentration of the sample, which fits in with the range of the protein standard to be used.

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5.3. ODC Expression

The work discussed in this thesis was carried out to determine the changes, if any, in ODC expression in CNS tissue during EAE using the Lewis rat model as described by Paul *et al* (2000) and Bolton *et al* (1990). From the evidence available that PA mediate BBB disruption and dysfunction it would follow that expression of ODC, the primary and rate-limiting enzyme, would also be affected and in this case that ODC expression would be increased during the course of the disease.
It was shown in this study that there are significantly increased levels of expression of ODC at day-13 PI EAE for cerebellum and medulla samples with the levels of expression in cerebellum at day-13 EAE PI were at least four times as high as the levels observed in normal tissue (figures 4.1). In samples of cervical spinal cord, there was no observable difference in the expression of ODC between normal and day-13 EAE PI (figures 4.3).

Western blotting results of cerebellum sample run with medulla sample and cerebellum sample with cervical spinal cord sample on the same gel the distinction between the expression of ODC in the four groups (normal, CFA-treated, day-13 PI EAE, day-21 PI EAE) was most evident in the cerebellum samples as a comparison of figures 4.1, 4.2, and 4.3 shows. When cervical spinal cord and cerebellum and cervical spinal cord and medulla run on the same gel it was observed that the density of ODC bands in cervical spinal cord tissue was much higher suggesting that the greatest expression of ODC is found in the cervical spinal cord.

The result seen at day-1 PI EAE is an anticipated result that correlates with evidence showing that PA facilitate development and progression of EAE pathology by mediating in BBB breakdown (Calder, *et al*, 1989; Bolton, 1997; Bolton *et al*, 1990). This increase at day thirteen and subsequent decrease again at day-21 PI EAE correlate with the course of disease. The height of visual clinical symptom development is seen at day-13 EAE PI after which, the animal shows decline in symptoms and recovers, usually, around day-21 EAE PI.

The observation of the different levels of ODC in the different CNS sections also ties in with the progression of EAE symptoms which are ascending i.e. symptoms are seen to occur first at the hind limbs towards the forelimbs and then affecting the cognitive function of the brain typified by flaccidity of the tail followed by hind limb weakness, abdominal dysfunction, fore limb weakness, and finally cognitive/brain dysfunction (Simmons *et al*, 1982). This progression is also explained by the observation that plaques of demyelinated areas are found more commonly in areas of white matter concentration. The progression follows the same trend in the CNS by progressing from the cervical spinal cord to the medulla and finally to the cerebellum i.e. from the posterior or base of the brain to the anterior or fore part of the brain. Ascending progression of symptoms is not seen in MS where the symptoms are so varied that the clinical diagnosis of MS involves both spatial and temporal pathological evidence and many times is left to the "expertise" of clinical neurologists (Poser and Brinar, 2000). It is difficult to correlate pathology of MS with clinical features due to their different sizes, frequency and

distribution. MS symptoms are usually manifested in a relapsing remitting manner and are varied.

If the level of ODC remains high in the days after day-13, this would suggest a longer period of illness because this persistence could be an indication of increased action of PA. This increased expression may also support a role of the enzyme in the propagation of the disease either facilitating development of symptoms or as a result of another arm of the disease process working in a positive feedback process. There seems to be a direct relationship between level of ODC expression and presentation of disease symptoms especially since at day-21 PI EAE there are reduced levels and remission of symptoms.

The initiation of EAE involves the breakdown of the BBB (Hawkins *et al*, 1991; Bolton, 1997; Rabchevsky *et al*, 1999; Huber *et al*, 2001) for which PA have been implicated (Koenig *et al*, 1989; Koenig *et al*, 1992; Paschen, 1992; Shohami *et al*, 1992; Baskaya *et al*, 1997; Bolton, 1997). The accumulation of natural PA is toxic (Urdiales, *et al*, 2001) and may contribute through various ways to pathology as is suggested for MS and EAE (Hawkins *et al*, 1991; Bolton and Paul, 1997; Paul *et al*, 2000).

Significant points along the disease course can be correlated to ODC/polyamine expression and initiation probably correlates with initial increased expression of ODC and concomitant production of PA leading to the breakdown of the BBB. As the level of ODC increases up until day-13 the level of PA also increase and with this increase is an increase in clinical symptoms. At this point, the negative feed back regulatory mechanism of PA on ODC production may come into play thus leading to decreased ODC production and removal of already present ODC through activation of production and action of antizyme later on in the course of disease at day-21.

This progression of events seems be logical and acceptable however, studies have shown that clinical symptoms of EAE in Lewis rats preceded BBB permeability to small molecules by one day in the lumbar spinal cord region, while in other regions permeability to small molecules coincided with onset of clinical disease (Juhler *et al*, 1984). This may

be an explanation for the results obtained in this study in which there is a higher general expression of ODC in cervical spinal cord over cerebellum and medulla. If the levels of ODC are generally higher even in normal tissue of cervical spinal cord as compared to other sections of the brain then this may be a factor determining the early presentation of clinical symptoms of EAE prior to BBB breakdown in this section in comparison to other sections of the brain where BBB breakdown coincides with the onset of clinical disease although other factors may be involved. On the otherhand, results of a study by Juhler *et al* (1984) suggest that presentation of clinical symptoms is not necessarily primarily dependant on a mechanism driven by polyamine mediated BBB breakdown and also that in the spinal cord region, there may be other events that occur that initiate or facilitate EAE before the manifestation of breakdown of the BBB and conversely that there is activation of the immune response within the CNS before cellular infiltrates of T-cells and macrophages not related to the ODC/polyamine synthetic process. In this case, the ODC/PA pathway would serve to further the development of the disease rather than be an initiating factor. In another study with guinea pigs, Hawkins *et al* (1991) showed that lesions that developed during EAE occurred in the areas of maximal BBB breakdown that exhibited diffuse perivascular inflammation. This suggests that although in some instances the ODC/polyamine synthetic pathway may not initiate the presentation of symptoms it does play a role in the initial stages and progression of the course of the disease.

Paul *et al* (2000) in a study in which ^{99m}Tc-RP128 was intravenously administered to animals, showed that in other CNS regions, the breakdown of the BBB was an important event in the initiation of clinical symptoms of EAE. Day-13 PI, at which increased levels of ODC were found in this study (figures 4.1, 4.2, 4.3), corresponds to the time at which increased levels of large size substances have been measured on the CNS side of the BBB (Paul *et al*, 2000) adding support to the proposition the time during the disease when levels of ODC are highest is the time when the BBB is most permeable, thus suggesting that ODC/PA have a role in BBB dysfunction. Results from Paul *et al* (2000)

also showed that large quantities of ^{99m}Tc-RP128 crossed the BBB in CNS tissue of all EAE animals compared to CNS tissue of normal animals, which is an indication of a dysfunctional BBB and that the greatest movement i.e. highest amount of ^{99m}Tc-RP128 was observed in cervical spinal cord. Similarly, in this thesis, it has been shown that the highest levels of ODC detected are in the cervical spinal cord (figure 4.3). Paul *et al* (2000) also studied the accumulation of ^{125}I-RSA within the cerebellum, medulla-pons and cervical spinal cord at 5, 10, 11, 12, 15, 23 and 35-37 days PI. They showed the earliest increase in ^{25}I-RSA in cervical spinal cord and therefore for the same duration of time, the greatest increase should occur in the cervical spinal cord in comparison to the cerebellum and medulla pons. This observation lends support to the finding of greater levels of ODC in the cervical spinal cord especially if the ODC/PA pathway is involved in BBB dysfunction i.e. the level of ODC starts increasing at an earlier stage and possibly at a faster rate and so the accumulation of ODC would be highest in the cervical spinal cord. The accumulation of ^{125}I-RSA in cerebellum is slower and slower yet in the medulla and suggests a later "opening" of the BBB.

Using CFA and incomplete Freund's adjuvant, Rabchevsky *et al* (1999) showed that BBB breakdown could occur without reactive gliosis if cellular damage and T cell infiltration do not occur. This suggests that the mechanism mediating BBB breakdown in itself does not lead to the pathological events seen in EAE and that the opening of the BBB must be accompanied by the infiltration and activation of reactive T cells to cause pathology. Similarly, the opening of the BBB as shown by Paul *et al* (2000) simply suggests a relationship between the development of clinical symptoms with permeability of the BBB which I link with results from this thesis with increased expression of ODC. The following events i.e. cellular infiltration and the inflammatory response are most likely guided by different events.

The vast numbers of studies that have been carried out on the ODC/PA synthetic pathway has concentrated on PA. Not as many studies have been carried out on the difference in levels of expression and localization of ODC in inflammatory disease. PA

synthesis and metabolism are associated with various forms of brain injury (Fage *et al*, 1993) such as ischemia and cold injury. Ischemia is an inadequate flow of blood to a certain area of the body caused by blockage or constriction of blood vessels supplying it. Similar events occur in the pathogenesis of both ischemia and EAE, especially in terms of BBB dysfunction and insight into the mechanisms involved in ischemia may provide a better understanding of the mechanisms involved in the pathology of EAE and therefore, help in the development of therapeutic methods. Cellular changes as a result of ischemia in the brain lead to breakdown of the blood-brain barrier, causing malignant cerebral oedema that can be seen clinically by a rapid neurological deterioration (Croll and Weigand, 2001) and there is a nuclear response with increased expression in the perinuclear cytoplasm and dendrites of neurons (Paschen, 1992). The pathogenesis of ischemia and the subsequent return to normal of blood flow (reperfusion) involves cytokine production, astrogliosis, cytoskeletal protein degradation, inflammatory cell influx and breakdown of the BBB (Chen *et al*, 2001) occurrences that can also be seen in the pathogenesis of EAE and MS. PA and NMDA receptors are both thought to play an important role in secondary neuronal injury after cerebral ischemia (Dogan *et al*, 1997) another feature similar to EAE and MS pathology in which calcium ion dependant NMDA upregulation of ODC leads to PA production (Bolton, 1997) a process generated by activation of the NMDA receptor located at neuronal and cerebrovascular sites (Bolton, 1997; Bolton and Paul, 1997). Dogan *et al* (1997) showed that for an animal model of ischemia, animals given intravenous infusion of ifenprodil showed reduced brain oedema and infarct volume as compared to animals that had saline infusion during ischemia through the inhibition of PA action although the precise mechanism is unknown. These findings are supported by similar studies by Baskaya *et al* (1997) in which it was shown that both ischemic injury size and BBB permeability were smaller in the ifenprodil-treated group, compared with the saline-treated group. Since ifenprodil is a non-competitive inhibitor of PA sites at the NMDA receptor, the use of ifenprodil may, in the case of EAE and MS, provide a mechanism for control of the PA mediated breakdown of the BBB.

An immunohistochemical study of the cerebellum tissue, medulla tissue and cervical spinal cord tissue i.e. CNS sections used in this study, may provide an explanation as to why cervical spinal cord shows the highest band density. There is not much information on the exact cellular or subcellular location of ODC (Anehus *et al*, 1984; Schipper *et al*, 1999) although the highest concentration of ODC in any cell population is always seen in the most rapidly proliferating cells (Anehus *et al*, 1984; Muller *et al*, 1986; Koibuchi *et al*, 1990; McCann *et al*, 1992; Fage *et al*, 1993). Immunohistochemical studies on rat tissues for ODC have shown high staining for ODC in nucleoplasm of mitotic cells, and cytoplasmic immunostaining showed ODC in epithelial cells of kidney, prostate and adrenal medulla of testosterone-treated rats, and in the glandular epithelium of small intestine and pancreas of neonatal and adult rats (Schipper *et al*, 1999) all cells with high activity. This would suggest that it is possible that before the BBB breaks down there is increased cellular activity responsible for the increased expression of ODC and thus increased PA expression leading to BBB disruption, the reason for the increased cellular activity being varied.

Studies in models of ischemia by Muller *et al* (1991) showed immunoreactivity of ODC in neurons, and even higher reactivity in the perinuclear cytoplasm and dendrites of neurons, and in the hippocampus. In the adult human brain ODC reactivity was found in the hippocampus and in the spinal cord (Muller *et al*, 1999). However, the main cellular locus of ODC in both the mature and immature central nervous system is the neuron (Dempsey *et al*, 1988; Bernstein and Muller,1998). An immunohistochemical study at the BBB might show whether the increased ODC expression is internal to the CNS or occurs in the peripheral vascular system or within the cells of the BBB itself i.e. on the luminal or abluminal side of the BBB.

During this present study, it was not possible to carry out an immunohistochemical comparison of ODC immunoreactivity in the CNS for the cerebellum, medulla pons and cervical spinal cord. However, it follows that during PA mediated BBB dysfunction, immunoreactivity for ODC would be highest at the brain vasculature especially at areas of

leakage of the BBB. Regulation of the levels of ODC either through manipulation of the production of antizyme or with the use of ODC inhibitors localized to these sites may prove to be beneficial in the prevention of BBB breakdown and thus retard or stop disease progression.

Between day-13 PI EAE and day-21 PI EAE there is decrease in ODC levels in cerebellum and medulla. Under normal physiological conditions, the levels of ODC are regulated by antizyme (AZ), negative feedback regulation due to build up of PA and the fact that ODC has a short half-life. AZ is a natural inhibitor of ODC and works by negative regulation of PA but may also degrade ODC (Suzuki *et al*, 1994; Mitchell *et al* 2002). The highest levels for AZ genes in the adult rat brain have been detected in the cerebellar cortex, hippocampus, hypothalamic paraventricular supraoptic nuclei, locus coeruleus, olfactory bulb, piriform cortex and pontine nuclei (Kilpelainen *et al*, 2000). AZ production is PA stimulated and seems to function by regulating PA uptake (Coffino, 2001; Scoriconi *et al*, 2001) leading to the accumulation of spermine (Suzuki *et al*, 1994) suggesting that AZ has a higher affinity for the PA transport protein than for ODC enzyme. This implies that although AZ can degrade ODC (Murakami *et al*, 2000), the inhibitory function is mediated through prevention of PA uptake. In the degradation of ODC, AZ dissociates the ODC dimer and forms an inactive heterodimer of ODC: AZ and this heterodimer is degraded by proteosome 26 S (Zhu *et al*, 1999; Murakami *et al*, 2000). Under normal circumstances, ODC levels are closely regulated and a dysfunction in this regulatory mechanism is lethal. Also, since AZ is the only protein that has been shown to interact with ODC, depletion of AZ or dysfunctional synthesis of AZ may lead to the accumulation of ODC and PA levels (Toshikazu *et al*, 1994). The action of AZ provides another pathway that may be studied to determine the action of ODC. The dysfunction in AZ production and function may lead to the increased expression of ODC and thus accumulation of PA. During the disease itself, decrease in ODC levels may be attributed to its regulation by AZ and this may be ascertained by studying the levels of AZ production alongside the ODC expression during the EAE disease course. Manipulation

of AZ levels may thus provide an avenue for therapeutic regulation of ODC and the effects of dysfunctional ODC expression.

As has been mentioned briefly before, an important drug that used to study the effects of ODC inhibition is α-diflouromethylornithine (DFMO). DFMO is accepted as a substrate at the active site of ODC and is then converted into a reactive intermediate that forms a covalent bond with ODC (McCann and Pegg, 1992). DFMO has been shown to slow the growth of tumour cells *in vitro* and *in vivo* in animal models (McCann and Pegg, 1992) and also shows significant reduction in the volume of oedema, necrosis and brain tissue damage, which are indicators of abnormal BBB function (Paschen, 1992). In a time and dose dependant treatment of cells, DFMO inhibits growth of cervical precancerous and cancerous cells *in vitro* by inducing apoptosis (Zou *et al*, 2002); DFMO inhibits tumours in models of skin cancer by inhibiting PA synthesis (Einspahr *et al*, 2002). The use of DFMO or AZ has been shown to have therapeutic effect in various diseases from various studies in both animal models and humans and may provide therapeutic benefit in treatment of MS and EAE through its role in regulating ODC.

5.4. Summary and Conclusions

This study has shown four important observations. Firstly, that there is a significantly increased expression of ODC enzyme in cerebellum and medulla CNS tissue for day-13 EAE PI tissue as compared to normal, CFA treated tissue and day-21 EAE PI tissue. Secondly, that the levels of expression decrease markedly after day-13 EAE PI as the disease progresses to day-21 EAE PI when in the Lewis rat, recovery usually occurs. Thirdly results of this project show that the CNS section with the highest levels of expression of ODC enzyme is the cervical spinal cord. Fourthly, the results show that although the cervical spinal cord tissue did show that highest levels of expression of ODC, the expression of does not follow the expression of the cerebellum and medulla tissue i.e. there is no significant difference when normal, CFA treated, day-13 EAE PI and day-21 EAE PI are compared. These results suggest a role for ODC in EAE pathology. At

the height of symptomatic presentation the highest levels of ODC are observed and when levels of ODC are reduced towards normal levels, there is remission and recovery. This observation supports the suggestion that ODC/PA pathway plays an important role in EAE and MS pathology.

5.5. Recommendations and Future Studies

In future studies it would be beneficial to establish a profile of the expression of ODC in EAE at shorter time intervals such as 24 or 48-hour intervals throughout the duration of the disease. Also, the clinical symptoms and their presentation must be closely monitored during the disease course such that the relationship between ODC expression and clinical symptom presentation can be determined. The point in time at which the level of ODC increases above normal i.e. normal being the levels before disease induction, could be linked to physiological and physical changes that occur at the same time.

By using ODC inhibitors to study the effect of regulation of ODC expression on the development of these changes that occur as a result of increased ODC above the norm, it would also be possible to see the accompanying changes in PA production.

It would also be important to determine the events that bring down ODC levels post day-13. The mechanisms most suspect in this event, i.e. reduction of ODC levels, are negative feedback regulation of the PA and the action of AZ. It would be necessary to determine the profile of the synthesis and metabolism of the PA during the EAE disease course in similar time intervals as that taken for ODC. From this result a plot of level of expression of ODC and PA against time from inoculation could be plotted and a relationship established with reference to presentation of clinical symptoms and recovery from disease while at the same time determining the expression of AZ during the disease course. The expression of AZ would probably be inversely proportional to the expression of ODC during disease expression, however, the point at which antizyme expression would come into play during the disease may also present a picture of when and how the recovery process begins and if indeed AZ regulation of ODC occurs during EAE.

Immunohistochemical localisation of the enzyme would determine the precise location of ODC before and during the disease, again along a time interval of 24 or 48 hours. More specifically, determination of ODC expression at or in close proximity to the BBB from the time of inoculation, through the opening of the BBB to the initiation of disease and through to the point at which recovery occurs could be determined by concentrating on perivascular tissue. It would therefore be insightful to study the changes in PA expression and the physiological and physical changes that occur at 24-hour or 48-hour intervals PI at the BBB and on both sides of the BBB. Since it has already been shown that PA do mediate the opening of the BBB, the accompanying physiological and physical changes at the BBB could be traced along with the levels of ODC and PA. In this way it may be possible to more clearly describe the mechanism by which the PA mediate in the opening of the BBB.

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Sigma product information. Anti-Mouse IgG (Fib SPECIFIC) PEROXIDASE CONJUGATE.

Sigma product information. Monoclonal Anti-Ornithine Decarboxylase (ODC).

APPENDICES

Appendix I. Rat Samples weights of rat brain sections.

Cerebellum

	Rat number	Weight (g)	Diseased State	Date of collection
One	7	0.280	Day 13 EAE	14/11/00
	6	0.270	Day 21 EAE	14/11/00
	8	0.270	Normal	14/11/00
	6	0.270	CFA treated	14/11/00
Two	3	0.2869	Normal	14/11/00
	8	0.2967	Day 21 EAE	08/02/01
	4	0.2361	Day 13 EAE	14/11/00
	4	0.2595	CFA treated	14/11/00
Three	3	0.250	Day 13 EAE	14/11/00
	1	0.300	Day-21 EAE	08/02/01
	7	0.300	Normal	08/02/01
	5	0.260	CFA treated	14/11/00

Medulla

	Rat number	Weight (g)	Diseased State	Date of collection
One	7	0.180	Day 13	14/11/00
	4	0.170	Day 21	08/02/01
	8	0.180	Normal	08/02/01
	6	0.190	CFA treated	14/11/00
Two	4	0.1696	Day 13 EAE	14/11/00
	8	0.1023	Day 21 EAE	08/02/01
	3	0.1516	Normal	14/11/00
	4	0.1613	CFA treated	14/11/00
Three	3	0.190	Day 13 EAE	14/11/00
	1	0.190	Day-21 EAE	14/11/00
	7	0.200	Normal	14/11/00
	5	0.180	CFA treated	14/11/00

Cervical Spinal Cord

	Rat number	Weight (g)	Diseased State	Date of collection
One	7	0.3669	Day 13 EAE	14/11/00
	3	0.2822	Day 21 EAE	14/11/00
	3	0.2423	Normal	14/11/00
	1	0.2609	CFA treated	14/11/00
Two	1	0.327	Day 13 EAE	14/11/00
	2	0.270	Day 21 EAE	08/02/01
	9	0.228	Normal	08/02/01
	7	0.229	CFA treated	14/11/00
Three	4	0.310	Day 13 EAE	14/11/00
	8	0.247	Day 21 EAE	08/02/01
	8	0.325	Normal	08/02/01
	5	0.269	CFA treated	14/11/00

Appendix II

Interfering substances (BCA Assay kit, PIERCE ENDOGEN).

PIERCE
a brand of

Table 2: Compatible Substance Concentrations in the BCA Protein Assay (see text for details)

Substance	Compatible Concentration
Salts/Buffers	
ACES, pH 7.8	25 mM
Ammonium sulfate	1.5 M
Asparagine	1 mM
Bicine, pH 8.4	20 mM
Bis-Tris, pH 6.5	33 mM
Borate (50 mM), pH 8.5 (# 28364)	undiluted
B-PER® Reagent (#78248)	undiluted
Calcium chloride in TBS, pH 7.2	10 mM
Na-Carbonate/Na-Bicarbonate (0.2 M), pH 9.4 (#28382)	undiluted
Cesium bicarbonate	100 mM
CHES, pH 9.0	100 mM
Na-Citrate (0.8 M), Na-Carbonate (0.1 M), pH 9.0 (#28368)	1:8 dilution*
Na-Citrate (0.8 M), MOPS (0.1 M), pH 7.5 (#28386)	1:8 dilution*
Cobalt chloride in TBS, pH 7.2	0.8 mM
EPPS, pH 8.0	100 mM
Ferric chloride in TBS, pH 7.2	10 mM
Glycine	1 mM
Guanidine-HCl	4 M
HEPES, pH 7.5	100 mM
Imidazole, pH 7.0	50 mM
MES, pH 6.1	100 mM
MES (0.1 M), NaCl (0.9%), pH 4.7 (#28390)	undiluted
MOPS, pH 7.2	100 mM
Modified Dulbecco's PBS, pH 7.4 (#28374)	undiluted
Nickel chloride in TBS, pH 7.2	10 mM
PBS; Phosphate (0.1 M), NaCl (0.15 M), pH 7.2 (#28372)	undiluted
PIPES, pH 6.8	100 mM
RIPA lysis buffer; 50 mM Tris, 150 mM NaCl, 0.5% DOC, 1% NP-40, 0.1% SDS, pH 8.0	undiluted
Sodium acetate, pH 4.8	200 mM
Sodium azide	0.2%
Sodium bicarbonate	100 mM
Sodium chloride	1 M
Sodium citrate, pH 4.8 or pH 6.4	200 mM
Sodium phosphate	100 mM
Tricine, pH 8.0	25 mM
Triethanolamine, pH 7.8	25 mM
Tris	250 mM
TBS; Tris (25 mM), NaCl (0.15 M), pH 7.6 (#28376)	undiluted
Tris (25 mM), Glycine (192 mM), pH 8.0 (#28380)	1:3 dilution*
Tris (25 mM), Glycine (192 mM), SDS (0.1%), pH 8.3 (#28378)	undiluted
Zinc chloride in TBS, pH 7.2	10 mM

Substance	Compatible Concentration
Detergents	
Brij®-35	5.0%
Brij®-56, Brij®-58	1.0%
CHAPS, CHAPSO	5.0%
Deoxycholic acid	5.0%
Lubrol® PX	1.0%
Octyl β-glucoside	5.0%
Nonidet P-40 (NP-40)	5.0%
Octyl β-thioglucopyranoside	5.0%
SDS	5.0%
Span® 20	1.0%
Triton® X-100	5.0%
Triton® X-114, X-305, X-405	1.0%
Tween®-20, Tween®-60, Tween®-80	5.0%
Zwittergent® 3-14	1.0%
Chelating agents	
EDTA	10 mM
EGTA	------
Sodium citrate	200 mM
Reducing & Thiol-Containing Agents	
N-acetylglucosamine in PBS, pH 7.2	10 mM
Ascorbic acid	------
Cysteine	------
Dithioerythritol (DTE)	1 mM
Dithiothreitol (DTT)	1 mM
Glucose	10 mM
Melibiose	------
2-Mercaptoethanol	0.01%
Potassium thiocyanate	3.0 M
Thimerosal	0.01%
Misc. Reagents & Solvents	
Acetone	10%
Acetonitrile	10%
Aprotinin	10 mg/L
DMF	10%
DMSO	10%
Ethanol	10%
Glycerol (Fresh)	10%
Hydrazide (Na_2BH_4 or $NaCNBH_3$)	------
Hydrochloric Acid	100 mM
Leupeptin	10 mg/L
Methanol	10%
Phenol Red	------
PMSF	1 mM
Sodium Hydroxide	100 mM
Sucrose	40%
TLCK	0.1 mg/L
TPCK	0.1 mg/L
Urea	3 M
o-Vanadate (sodium salt), in PBS, pH 7.2	1 mM

* Diluted with dH_2O. A blank indicates that the material is incompatible with the assay.

Interfering substances. (Smith, P. K., *et al*, 1985, page 79.)

TABLE I

EFFECT OF SELECTED POTENTIAL INTERFERING COMPOUNDS

Sample (50 μg BSA) in the following:	BCA assay (μg BSA found)		Lowry assay (μg BSA found)	
	Water blank corrected	Interference blank corrected	Water blank corrected	Interference blank corrected
50 μg BSA in water (reference)	50.00	—	50.00	—
0.1 N HCl	50.70	50.80	44.20	43.80
0.1 N NaOH	49.00	49.40	50.60	50.60
0.2% sodium azide	51.10	50.90	49.20	49.00
0.02% sodium azide	51.10	51.00	49.50	49.60
1.0 M sodium chloride	51.30	51.10	50.20	50.10
100 mM EDTA (4 Na)	No color		138.50	5.10
50 mM EDTA (4 Na)	28.00	29.40	96.70	6.80
10 mM EDTA (4 Na)	48.80	49.10	33.60	12.70
50 mM EDTA (4 Na), pH 11.25	31.50	32.80	72.30	5.00
4.0 M guanidine HCl	48.30	46.90		Precipitated
3.0 M urea	51.30	50.10	53.20	45.00
1.0% Triton X-100	50.20	49.80		Precipitated
1.0% SDS (lauryl)	49.20	48.90		Precipitated
1.0% Brij 35	51.00	50.90		Precipitated
1.0% Lubrol	50.70	50.70		Precipitated
1.0% Chaps	49.90	49.50		Precipitated
1.0% Chapso	51.80	51.00		Precipitated
1.0% octyl glucoside	50.90	50.80		Precipitated
40.0% sucrose	55.40	48.70	4.90	28.90
10.0% sucrose	52.50	50.50	42.90	41.10
1.0% sucrose	51.30	51.20	48.40	48.10
100 mM glucose	245.00	57.10	68.10	61.70
50 mM glucose	144.00	47.70	62.70	58.40
10 mM glucose	70.00	49.10	52.60	51.20
0.2 M sorbitol	42.90	37.80	63.70	31.00
0.2 M sorbitol, pH 11.25	40.70	36.20	68.60	26.60
1.0 M glycine	No color		7.30	7.70
1.0 M glycine, pH 11.	50.70	48.90	32.50	27.90
0.5 M Tris	36.20	32.90	10.20	8.80
0.25 M Tris	46.60	44.00	27.90	28.10
0.1 M Tris	50.80	49.60	38.90	38.90
0.25 M Tris, pH 11.25	52.00	50.30	40.80	40.80
20.0% ammonium sulfate	5.60	1.20		Precipitated
10.0% ammonium sulfate	16.00	12.00		Precipitated
3.0% ammonium sulfate	44.90	42.00	21.20	21.40
10.0% ammonium sulfate, pH 11.	48.10	45.20	32.60	32.80
2.0 M sodium acetate, pH 5.5	35.50	34.50	5.40	3.30
0.2 M sodium acetate, pH 5.5	50.80	50.40	47.50	47.60
1.0 M sodium phosphate	37.10	36.20	7.30	5.30
0.1 M sodium phosphate	50.80	50.40	46.60	46.60
0.1 M cesium bicarbonate	49.50	49.70		Precipitated

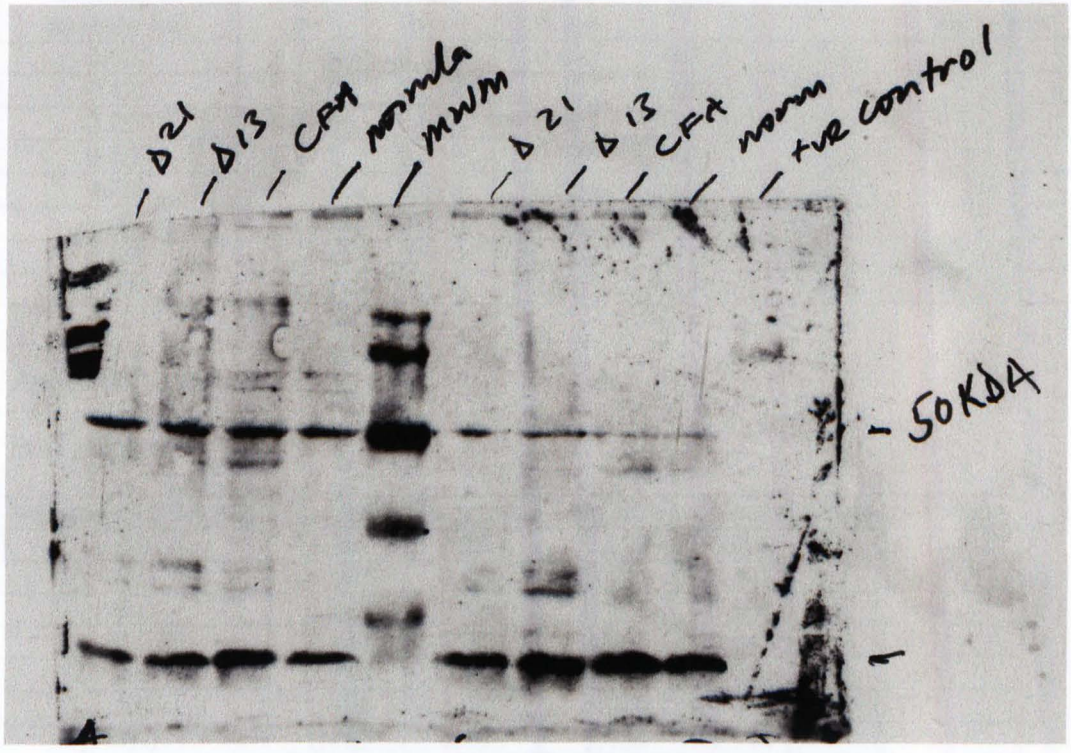
itations of the Lowry procedure. As shown in Fig. 1, bicinchoninic acid can form a 2:1 complex with Cu^{1+}, resulting in a stable, highly colored chromophore with an absorbance maximum at 562 nm (3). We have found that it is possible to make use of this reaction to

The table shows substances that interfere with the proper assessment of protein concentration when using the BioRad™, Lowry and BCA assay methods.

Appendix for III

Whole gel

The gel below shows cervical spinal cord and cerebellum tissue run together. The lanes were loaded (from right to left) using prepared ODC solution for positive control, cerebellum, i.e. normal, CFA-treated, day-13 PI EAE and day-21 PI EAE, molecular weight marker, and cervical spinal cord, i.e. normal, CFA-treated, day-13 PI EAE and day-21 PI EAE.



Appendix IV. Figure 3.1

The range of BSA used was 0.0, 0.125, 0.250, 0.500, 0.750, 1.000, 1.500, and 2.000 µl from a 1µg/µl solution. Tables show the achieved absorbance for samples of BSA using the BioRad™ assay method.

Table for BSA standards with three replicates given as runs one, two and three. (Volume and concentration are equivalent since concentration is 1 µg/µl.)

	Absorbance Achieved at 570 nm					
Volume of sample (µl)	Run one	Run two	Run three	Total	Mean	Corrected for blank
0.000	0.003	0.005	0.002	0.010	0.003	0.000
0.125	0.047	0.052	0.051	0.150	0.050	0.047
0.250	0.097	0.098	0.105	0.300	0.100	0.097
0.500	0.270	0.410	0.370	1.050	0.350	0.347
0.750	0.550	0.570	0.550	1.670	0.557	0.554
1.000	0.680	0.800	0.620	2.100	0.700	0.697
1.500	1.000	0.800	0.900	2.700	0.900	0.897
2.000	1.430	1.470	1.300	4.200	1.400	1.397

Appendix V. Figure 3.2.

BSA standards

Volume of BSA (µl)	Run one	Run two	Run three	Mean	Corrected value
0.0	0.299	0.288	0.290	0.292	0.000
3.0	0.483	0.482	0.481	0.482	0.190
5.0	0.843	0.863	0.897	0.868	0.576
6.0	0.929	0.950	0.948	0.942	0.650
7.0	0.916	0.939	0.912	0.922	0.630
8.0	1.076	1.093	1.075	1.081	0.789
10.0	1.113	1.106	1.124	1.114	0.822
15.0	1.261	1.217	1.261	1.246	0.954

Prepared ODC solution

Volume of ODC (µl)	Run one	Run two	Run three	Mean	Corrected value
0.0	0.290	0.287	0.295	0.291	0.000
3.0	1.005	1.142	1.212	1.120	0.829
5.0	1.340	1.304	1.376	1.340	1.049
6.0	1.446	1.476	1.523	1.482	1.191
7.0	1.504	1.563	1.564	1.544	1.253
8.0	1.587	1.558	1.672	1.606	1.315
10.0	1.540	1.649	1.694	1.628	1.337
15.0	1.648	1.690	1.781	1.706	1.415

Appendix VI. Table 3.2.

Retrial with spot volumes of ODC

BSA standards

	Absorbance achieved at 570 nm				
Volume of BSA (µl)	**Run one**	**Run two**	**Run three**	**Mean**	**Corrected value**
5.0	1.055	0.870	0.360	0.762	0.436
10.0	1.315	1.274	1.304	1.298	0.999
15.0	1.425	1.420	1.669	1.505	1.206
20.0	1.507	1.527	1.559	1.531	1.232
25.0	1.597	1.607	1.636	1.613	1.314

Prepared ODC solution

	Absorbance achieved at 570 nm				
Volume of ODC (µl)	**Run one**	**Run two**	**Run three**	**Mean**	**Corrected value**
5.0	1.507	1.352	1.451	1.437	1.145
10.0	1.721	1.597	1.712	1.677	1.385
15.0	1.723	1.708	1.752	1.728	1.436

Appendix VII 1:10 Dilution of pig brain sample – figure 3.3.

BSA standards

	Absorbance achieved at 570 nm			
Volume of BSA (1µg/µl)	**Run one**	**Run two**	**Mean**	**Corrected value**
0.0	0.340	-		0.000
1.0	0.431	-		0.091
2.0	0.554	-		0.214
3.0	1.029	-		0.689
4.0	0.812	-		0.472
5.0	0.908	-		0.568
6.0	1.205	-		0.865
7.0	1.169	-		0.829

Pig brain sample

	Absorbance achieved at 570 nm				
Sample volume (µl)	**Run one**	**Run two**	**Run three**	**Mean**	**Corrected value**
0.0	0.322	0.322	0.332	0.325	0.000
1.0	0.353	0.385	0.371	0.370	0.045
2.0	0.426	0.343	0.491	0.420	0.095
3.0	0.417	0.474	0.462	0.451	0.126
4.0	0.518	0.508	0.476	0.501	0.176
5.0	0.533	0.576	0.574	0.561	0.236
6.0	0.640	0.569	0.520	0.576	0.251
7.0	0.626	0.629	0.536	0.597	0.272

Appendix VIII 1:1000 Dilution of pig brain sample – figure 3.4.

BSA standards

	Absorbance achieved at 570 nm			
Volume of BSA (1µg/µl)	**Run one**	**Run two**	**Mean**	**Corrected value**
0.0	0.340	-		0.000
1.0	0.431	-		0.091
2.0	0.554	-		0.214
3.0	1.029	-		0.689
4.0	0.812	-		0.472
5.0	0.908	-		0.568
6.0	1.205	-		0.865
7.0	1.169	-		0.829

Pig brain sample

	Absorbance achieved at 570 nm			
Sample volume (µl)	**Run one**	**Run two**	**Mean**	**Corrected value**
0.0	0.328	0.323	0.326	0.000
1.0	0.336	0.326	0.331	0.005
2.0	0.333	0.339	0.336	0.010
3.0	0.342	0.343	0.343	0.017
4.0	0.345	0.344	0.345	0.019
5.0	0.352	0.337	0.345	0.019
6.0	0.343	0.342	0.343	0.017
7.0	0.351	0.360	0.356	0.030

Appendix IX. Figure 3.5

BSA standards

	Absorbance achieved at 570 nm					
Volume of sample (µl)	**Run one**	**Run two**	**Run three**	**Total**	**Mean**	**Corrected for blank**
0	0.322	0.322	0.332	0.976	0.325	0.000
1	0.353	0.385	0.371	1.109	0.370	0.044
2	0.426	0.343	0.491	1.260	0.420	0.095
3	0.417	0.474	0.462	1.353	0.451	0.126
4	0.518	0.508	0.476	1.502	0.501	0.175
5	0.533	0.576	0.574	1.683	0.561	0.236
6	0.640	0.569	0.520	1.792	0.576	0.251
7	0.626	0.629	0.536	1.791	0.597	0.272

Amount of protein BSA (µg)	0	1	2	3	4	5	6	7
Absorbance achieved At 570 nm	0.340 (0.000)	0.451 (0.091)	0.554 (0.214)	1.029 (0.689)	0.812 (0.472)	0.908 (0.568)	1.205 (0.865)	1.169 (0.829)

Values in brackets are absorbance values corrected for blank.

Appendix X. Figure 3.6

	Absorbance achieved at 570 nm				
Volume of sample (μl)	Run one	Run two	Run three	Total	Mean
0	0.000	0.000	0.000	0.000	0.000
5	0.040	0.036	0.035	0.114	0.038
10	0.082	0.063	0.073	0.218	0.072
15	0.102	0.165	0.134	0.401	0.134

Table 3.5 Medulla (4 and 8 μl)

Volume of BSA (μl)	Run one	Run two	Mean	Corrected value
5.0	0.052	0.062	0.057	N/A
10.0	0.080	0.078	0.079	N/A
20.0	0.100	0.101	0.101	N/A
30.0	0.143	1.135	0.139	N/A

Normal

Volume of sample (μl)	Run one	Run two	Mean	Corrected value
4	0.030	0.330	0.315	N/A
8	0.044	0.043	0.044	N/A

CFA-treated

Volume of sample (μl)	Run one	Run two	Mean	Corrected value
4	0.035	0.038	0.037	N/A
8	0.051	0.046	0.049	N/A

Day-13 PI EAE

Volume of sample (μl)	Run one	Run two	Mean	Corrected value
4	0.042	0.038	0.040	N/A
8	0.056	0.055	0.056	N/A

Day-21 PI EAE

Volume of sample (μl)	Run one	Run two	Mean	Corrected value
4	0.029	0.031	0.030	N/A
8	0.043	0.044	0.435	N/A

Figure 3.6 Medulla (10 and 20 μl)

Volume of BSA (μl)	Run one	Run two	Mean	Corrected value
0.0	0.036	0.035	0.036	N/A
5.0	0.054	0.052	0.053	N/A
10.0	0.084	0.087	0.086	N/A
15.0	0.098	-	0.098	N/A
25.0	0.126	0.127	0.127	N/A
35.0	0.160	0.166	0.163	N/A

Normal tissue

Volume of sample (µl)	Run one	Run two	Mean	Corrected value
10	0.052	0.047	0.050	N/A
20	0.063	0.061	0.062	N/A

CFA-treated

Volume of sample (µl)	Run one	Run two	Mean	Corrected value
10	0.051	0.053	0.052	N/A
20	0.070	0.076	0.073	N/A

Day-13 PI EAE

Volume of sample (µl)	Run one	Run two	Mean	Corrected value
10	0.052	0.047	0.050	N/A
20	0.077	0.072	0.075	N/A

Day-21 PI EAE

Volume of sample (µl)	Run one	Run two	Mean	Corrected value
10	0.048	0.046	0.047	N/A
20	0.067	0.062	0.065	N/A

ODC

Volume of sample (µl)	Run one	Run two	Mean	Corrected value
10	0.027	0.030	0.029	N/A
20	0.039	0.042	0.041	N/A

Appendix XII. Figure 3.6

	Absorbance achieved at 590 nm				
Concentration of BSA	Run one	Run two	Run three	Mean	Corrected value
0.000	-0.018	-0.017	-0.037	-0.024	0.0000
0.200	0.223	0.188	0.173	0.195	0.1710
0.500	0.549	0.476	0.524	0.516	0.4896
1.000	0.854	0.852	0.915	0.874	0.8497

Appendix XIII. Figure 3.8

Comparison of absorbance achieved when sample is diluted in water and homogenising medium.

Buffer as diluent

	Absorbance achieved at 570 nm				
Vol. of BSA (µl)	Abs. 1	Abs. 2	Abs. 3	Mean	Corrected value
0.000	-0.021	-0.019	-0.016	-0.019	
0.025	-0.013	-0.012	-0.010	-0.012	
0.125	-0.002	0.022	0.009	0.010	
0.250	0.041	0.055	0.077	0.058	
0.500	0.172	0.205	0.179	0.185	
0.750	0.330	0.342	0.345	0.339	
1.000	0.597	0.581	0.585	0.588	
1.500	1.027	0.991	0.969	0.996	
2.000	1.408	1.469	1.450	1.442	

Water as diluent

	Absorbance achieved at 570nm				
Vol. of BSA (µl)	**Abs. 1**	**Abs. 2**	**Abs. 3**	**Mean**	**Corrected value**
0.000	0.010	0.006	0.006	0.007	
0.025	0.022	0.024	0.024	0.023	
0.125	0.107	0.117	0.119	0.114	
0.250	-	-	-	-	
0.500	0.346	0.347	0.344	0.346	
0.750	0.498	0.535	0.519	0.517	
1.000	0.690	0.662	0.669	0.674	
1.500	0.971	0.937	0.953	0.954	
2.000	1.425	1.298	1.308	1.344	

Appendix XIV

Concentration of prepared ODC solution

0.00404 g of ODC powder was dissolved in 1.160 ml of analogue water.

1 mg of solid has 0.41 units i.e. 0.001 g has 0.00041 units.

10^3 x 0.00404 g has (0.041/0.00404) units. There are 1.66 x 10^3 units in solution. But 1 unit contains 1/1.3 mg of protein. 1/1.3 x 1.66 x 10^3 mg of protein = 1.27 mg of protein in solution.The concentration of the prepared ODC solution is 1.27/1.160 (mg/ml)

The concentration of the prepared ODC solution is 1.098 mg/ml

Figure 3.10

BSA standards

	Absorbance achieved at 570 nm				
Volume of BSA (µl)	**Run one**	**Run two**	**Run three**	**Mean**	**Corrected value**
0.025	0.025	-0.004	0.000	0.007	0.000
0.125	0.231	0.094	0.103	0.099	0.092
0.250	0.174	0.194	0.187	0.185	0.178
0.500	0.332	0.376	0.345	0.351	0.344
0.750	0.488	0.477	0.491	0.485	0.478
1.000	0.657	0.659	0.695	0.670	0.663
1.500	1.012	1.034	0.927	0.991	0.984
2.000	1.333	1.295	1.139	1.256	1.249

Prepared ODC solution

ODC	**Run one**	**Run two**	**Run three**	**Mean**	**Corrected value**
1:3 dilution	0.171	0.178	0.186	0.178	0.168
1:2 dilution	0.251	0.257	0.259	0.256	0.246
1:1 dilution	0.409	0.407	-	0.408	0.398

Appendix XV. Table 3.7.

Normal cerebellum in homogenising medium dilutions 1:1 and 1:4

Dilution	Abs. 1	Abs. 2	Abs. 3	Mean	Corrected value
1:1	0.283	0.246	0.321	0.283	0.302
1:4	0.160	0.133	0.121	0.138	0.157

Appendix XVI. Table 3.8

Normal cerebellum in water dilutions 1:1 and 1:4

Dilution	Abs. 1	Abs. 2	Abs. 3	Mean	Corrected value
1:1	0.412	0.359	-	0.386	0.379
1:4	0.173	0.156	0.138	0.156	0.149

Appendix XVII. ANOVA for the rat tissue samples.

The F value is the ratio of the difference between groups compared to the difference among/within groups. F calculated i.e. mean of squares between groups/mean of squares within groups. $F = MS_{bg}/MS_{wg}$; the degrees of freedom $F_{x,y}$ with x = degrees of freedom for the groups and y = degree of freedom for total number of samples.

$MS_{bg} = SS_{total} - SS_{wg}$; $SS_{wg} = SSx + SSy + SSz + SS\alpha$

$\therefore MS_{bg} = SS_{total} - (SSx + Ssy + SSz + SS\alpha)$

df_{bg} is number of groups – 1; df_{wg} is total number of samples – number of groups

CEREBELLUM

	Normal	CFA-treated	day-13 PI EAE	day-21 PI EAE
Rep. 1	72.58	116.25	80.49	48.45
Rep. 2	100.00	48.47	622.22	57.30
Rep. 3	100.00	32.46	256.18	60.57
Rep. 4	17.60	41.75	106.79	97.25
Rep. 5	39.96	23.77	102.57	47.23
Rep. 6	99.59	86.01	359.81	293.77
Mean	71.62	58.12	254.68	100.76
SS	6314.61	6356.41	221491.28	46370.98
SS total	453230.40		MSwg = 14026.66	
SS wg	280533.28 df = 20		MSbg = 57565.71	
SS bg	172697.12 df = 3		F = 4.104	

MEDULLA

	Normal	CFA-treated	day-13 PI EAE	day-21 PI EAE
Rep. 1	15.24	7.93	42.43	100.00
Rep. 2	48.77	10.83	114.10	91.44
Rep. 3	40.95	46.25	83.65	64.59
Rep. 4	29.98	22.64	207.66	69.84
Rep. 5	45.42	63.50	50.64	100.00
Rep. 6	95.34	94.32	165.98	128.26
Mean	45.95	40.91	110.74	92.36
SS	3670.75	5717.73	21468.37	2684.72
SS total	58458.06		MSwg = 1677.08	
SS wg	33541.57 df = 20		MSbg = 8305.50	
SS bg	24916.49 df = 3		F = 4.95	

CERVICAL SPINAL CORD

	Normal	CFA-treated	day-13 PI EAE	day-21 PI EAE
Rep. 1	230.88	251.08	273.27	282.90
Rep. 2	114.89	114.92	96.68	92.95
Rep. 3	99.96	265.82	237.11	278.71
Rep. 4	96.12	81.35	84.11	86.34
Rep. 5	98.68	21.52	298.86	195.78
Rep. 6	178.72	198.20	64.62	136.07
Rep. 7	84.79	47.18	68.64	71.37
Rep. 8	771.22	971.30	758.63	770.16
Rep. 9	99.96	97.95	82.32	99.97
Mean	197.25	227.70	218.25	223.81
SS	389220.33	681881.345	399126.5149	388084.5886
SS total	1863284.01		MSwg = 58072.27	
SS wg	1858312.78 df = 32		MSbg = 1657.08	
SS bg	4971.23 df = 3		F = 0.03	