VICTORIA UNIVERSITY OF WELLINGTON

AVAILABILITY OF THESIS

AUTHORS NAME Ian J. Bloodworth

TITLE OF THESIS A Search for circulating protein markers in neurological disease using 2-dimensional SDS PAGE....

DEGREE PhD

SUBJECT Biochemistry

YEAR 1995

I hereby consent to the above thesis being consulted, borrowed, copied or reproduced in accordance with the provisions of the Library Regulations from time to time made by the academic Board.

(Signature) [redacted] ..... AUTHOR

17-10-95 ..... DATE

Victoria University of Wellington

Biochemistry and Genetics Section
School of Biological Sciences

A Search for Circulating Protein Markers in Neurological Disease Using 2-Dimensional SDS PAGE and Quantitative Densitometry.

by

Ian John Bloodworth

A Thesis

submitted to the Victoria University of Wellington
in fulfilment of the
requirements for the degree of
Doctor of Philosophy
in Biochemistry

July,
1995

Abstract.

Alzheimer's disease and multiple sclerosis are two prevalent neurological diseases that have no laboratory based tests to confirm their clinical diagnosis. In this study, protein abnormalities were searched for in the plasma and platelets of patients with Alzheimer's disease, and in the plasma and CSF of patients with multiple sclerosis. The aim of the search was to detect protein abnormalities that had the potential to be used as marker proteins for these diseases. Such marker proteins could be used as the basis of laboratory tests.

The procedures used to perform the search were 2-dimensional SDS PAGE and quantitative densitometry. Before performing the search, a study was made to find the optimum method for obtaining and analysing protein abundance data. It was found that quantitating only discrete protein regions, and converting the abundance data into compositions yielded the greatest reproducibility. Logratio transforms of the compositional data were analysed statistically to detect quantitative differences between control and affected individuals..

In the analysis of plasma proteins, many proteins were seen to alter in relative abundance in multiple sclerosis and Alzheimer's disease. Almost all of the protein changes observed involved acute phase reactants, and so were of little diagnostic utility. In Alzheimer's disease, orosomucoid, haptoglobin α, haptoglobin β, apo D, RBP, and a group of unidentified proteins were significantly different ($P < 0.01$) between the Alzheimer's patients and a group of age and sex matched controls. In multiple sclerosis, plasma levels of orosomucoid, haptoglobin α, haptoglobin β, apo A1 and apo D, Ig G, Ig J, and a group of unidentified proteins were found to be significantly different ($P < 0.01$) between a group of multiple sclerosis patients and their age and sex matched controls. Differing plasma levels of various acute phase reactants were seen for both diseases. It is possible that these differences are great enough to support clinical diagnosis. Changes in the acute phase reactant profile over time may also be useful in prognostic assessment. A larger study would be required before this could be assessed. A qualitative protein abnormality was also detected in a

group of females affected with multiple sclerosis. This protein was isolated and a partial amino acid sequence was obtained for it. Based on sequence homology, the protein was identified as haptoglobin α. However, as the haptoglobins are a class of acute phase reactants, this protein would be of limited diagnostic utility.

Several quantitative differences were also detected between the CSF proteins of multiple sclerosis patients and their controls. As in the plasma studies, all of the protein changes detected were acute phase reactants, and so were of limited diagnostic utility. The proteins significantly different ($P < 0.05$) between the controls and multiple sclerosis patients were Apo A1, haptoglobin β, fibrinogen β, α1 AT, and actin. A study was also made to see if any disease related pattern in the distribution of proteins in the plasma and CSF could be detected. Such a pattern could suggest differences in protein processing in the disease state, or may indicate a breach in the blood-brain barrier or choriod plexus. Two separate studies were done. In the first a search was made for proteins in the plasma and CSF that appeared to correlate in terms of relative abundance. For this study, correlation analysis was performed on the $\log_{10}$ ratio values of all of the plasma and CSF proteins. Those protein combinations found to correlate significantly ($P<0.05$) were subjected to further analysis, where compositional data for plasma and CSF proteins for every individual were expressed as a series of CSF:Plasma ratios. These values were analysed statistically to detect any disease related changes. There were correlations between nineteen potential multiple sclerosis related proteins, although almost all involved acute phase reactants. Most of the correlations were positive; that is, increasing CSF concentration was associated with increasing plasma concentration. Based on a knowledge of the biochemistry of the proteins studied, no obvious pattern to the correlations was seen. The complexity of the correlations may reflect the complexity of the acute phase response to chronic inflammation. In the second study, proteins that could be identified in both the plasma and CSF were examined. Eleven proteins were studied. The proteins were orosomucoid, ceruloplasmin, α-1 antichymotrypsin, α2 HS glycoprotein, haptoglobin β (2), haptoglobin α (3), Apo D, and Apo A1. No pattern emerged between the relative plasma and CSF concentrations of these proteins. The observations did suggest a complex pattern of protein synthesis in response to multiple sclerosis. The pattern may have emerged as the result of protein

movement between the CSF and plasma and/or co-regulated hepatic and CNS protein synthesis.

A quantitative study of platelet proteins in Alzheimer's disease was also carried out, where platelets prepared in the presence of a protease inhibitor cocktail (1 mM phenylmethyl sulphonylfluoride, 100 μM N-ethylmaleimide, 10 mM EDTA, 100 μM iodoacetamide, 1 μM pepstatin, and 150 Kallikrein inhibitor units/ml of aprotinin) were compared to platelets prepared in the absence of the inhibitor cocktail. By comparing platelets prepared in the presence of protease inhibitors for controls and Alzheimer's patients, an assessment was made of changes in protein expression and/or abundance in Alzheimer's disease. An assessment of the effect of proteolysis in disease was made by comparing platelet proteins prepared in the absence of protease inhibitors from Alzheimer's patients and controls, and also by comparing platelets prepared in the absence of protease inhibitors to those prepared in the presence of protease inhibitors for both controls and patients. Proteins that were seen to be proteolytically processed differently, but which were not observed to alter in abundance between the platelets of patients and controls prepared in the presence of protease inhibitors, were considered more likely to represent disease related proteolytic events, as altered rates of expression could be ruled out. The major assumption made was that proteolysis was inhibited by the protease inhibitor cocktail used. When platelet proteins prepared in the absence of protease inhibitors were examined, several significant differences were observed. The proteins significantly different ($P < 0.05$) between Alzheimer's patients and controls were ER-60 protease inhibitor, serotransferrin, and three unidentified proteins. When platelet proteins prepared in the presence of protease inhibitors were examined, several differences were also seen. Those proteins found to differ significantly ($P < 0.05$) between controls and Alzheimer's patients were ER-60 protease inhibitor and five unidentified proteins. It is difficult to assess the significance of the other platelet protein changes observed in this study due to the limited number of platelet proteins that are currently identified in the Swiss-Prot database. The results obtained however, do add further support to the suggestion of Inestros *et al.* (1993), that platelets may provide a systemic marker for Alzheimer's disease.

Extension of the results obtained in this project could yield useful information regarding the aetiology and pathogenesis of both disorders. Further study may also yield information that may be used in the detection and diagnosis of the diseases.

Acknowledgements.

Foremost, I would like to thank my supervisor, Dr. Bill Jordan (Biochemistry and Genetics Section, School of Biological Sciences, Victoria University), and co-supervisor Dr. Sandy Ford (Department of Pathology, Wellington Clinical School of Medicine) for their invaluable advice, patience, and encouragement throughout the duration of this work. I would also like to thank Mr P. Wakeham (Haematology Section, Clinical Services Department, Wellington Hospital) for performing blood counts, and supplying blood collection tubes. Additionally, I would like to thank Drs. C. Shaw and D. Abernethy (Neurology Department, Wellington Hospital) for supplying clinical samples and patient summaries. I would especially like to thank those patients and other volunteers who generously volunteered to supply blood and CSF for this study.

From Victoria University, I would like to thank the staff of the Electron Microscope Facility for preparing samples for TEM. I would also like to thank Mr Brett Robertson from the Photographic Facility for all of the photographic work in this thesis. Many thanks are also owed to Dr. Ross. Renner (Institute of Statistics and Operations Research, Victoria University) for his assistance with the statistical component of this thesis.

I would also like to thank Fei Lu, Pisana Rawson, Kim Wheeler, Hua Feng, Jane Maxwell, Tim Penny, Matthew Adlam, Tania Clough, Sarah Thompson, Simon Glanville, Tim Markwell, Mary Murray, and Mary Morgan-Richards for supplying advice, technical assistance, blood, many laughs, and for generally making my life at Vic. a lot more pleasurable than it could have otherwise been.

Finally, I would like to give special thanks my parents Ann and Ian, and my brother Keith. Without their support, I doubt whether I would have finished this project.

Table of contents.

List of Figures.

List of Tables.

List of Abbreviations.

ACD	Acid citrate dextrose
ACT	α-1 Antichymotrypsin
APP	Amyloid precursor protein
APR	Acute phase reactant
AT	α1-Antitrypsin
BSA	Bovine serum albumin
CNS	Central nervous system
Crp	Ceruloplasmin
CSF	Cerebrospinal fluid
DEAE	Diethylaminoethyl
EDTA	Ethylenediaminetetra-acetic acid
ER	Endoplasmic reticulum
Hb	Haemoglobin
HDL	High density lipoprotein
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HLA	Human lymphocyte antigen
Hp	Haptoglobin
IDS	Identified protein series
IEF	Isoelectric focusing
Ig	Immunoglobulin
IL	Interleukin
KIU	Kallikrein inhibitor units
LDL	Low density lipoprotein
MHC	Major histocompatibility complex

MS	Multiple sclerosis
MSN	Master spot number
mw	Molecular weight
NEM	N-ethylmaleimide
NEPHGE	Non-equilibrium pH gradient gel electrophoresis
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
pI	Isoelectric point
PLS	Unidentified protein series
pMao-B	Platelet monoamine oxidase B
PMSF	Phenylmethyl sulphonylfluoride
POP	Protein population name
PPO	2,5-Diphenyloxazole
PRP	Platelet rich plasma
RBP	Retinol binding protein
SP	Swiss-Prot database
SAA	Serum amyloid A
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
VI	Volume integration
VLDL	Very low density lipoprotein

Aim.

The aim of this project was to search for protein abnormalities in the blood and CSF of patients with MS, and in the blood and platelets of patients with Alzheimer's disease. If any protein abnormalities were found, they were to be assessed for suitability as protein markers. A suitable protein marker could form the basis of a laboratory marker for the disease. Currently, no such protein markers exist for either disease.

Chapter 1. General Introduction.

The aim of this project was to use the technique of 2-dimensional PAGE to search for protein variation in Alzheimer's disease and MS. In both Alzheimer's disease and MS, the primary site of pathology lies within the CNS. Consequently, it is reasonable to assume that any potentially diagnostic proteins that may exist in the plasma will be either APRs, or will be proteins originating in the CNS that can cross the blood brain barrier. In normal circumstances, transport of molecules across the blood brain barrier is strictly regulated (for reviews, see Rowland *et al.*, 1991 and Goldstein, 1986). However, for both Alzheimer's disease and MS, blood brain barrier abnormalities have been reported (McLean, (1993); Harik *et al.*, 1991; Kalaria, 1992). Considering these observations, it is possible that proteins can cross the blood brain barrier in both diseases, implying that proteins associated with the pathology of the diseases may be present in the plasma. A brief summary of Alzheimer's disease and MS follows.

1.1) Alzheimer's disease.

Dementia is the term used to describe a group of symptoms including a global decline in intellectual ability and cognitive functioning, the decline being sufficient to impair an individual's ability to perform routine daily tasks, and interact socially. The mental processes affected can include mathematical ability, vocabulary, abstract thinking, judgement, speaking, and coordination. Approximately 5% of the population over the age of 65 years suffer from severe dementia, and a further 10% suffer from mild dementia (Goldstein *et al.*, 1991).

Of those individuals autopsied, approximately 50% of all cases of dementia are caused by Alzheimer's disease, 20% are caused by multiple infarcts, and a further 20% are caused by a mixture of the two (Terry, 1978; Roth, 1980). In the United States, Alzheimer's disease is the fourth most prevalent cause of death (Powell *et al.*, 1986), and takes more than 100,000 lives annually (Shapira, 1994). Alzheimer's disease has a huge economic cost. In the United States,

Alzheimer's disease accounts for $90 billion in health care costs annually (Roth, 1993). Between 1984 and 1991, the minimum estimate of the long-term dollar losses to the United State's economy caused by Alzheimer's disease was $1.75 trillion (Ernst *et al.*, 1994).

Alzheimer's disease shows a strong correlation to age. Between the ages of 60 - 65 years, 0.1% of the population is affected; between the ages of 65 - 80 years, 4% of the population is affected; between the ages of 80 -85 years, 20% of the population is affected, while in the age group of 86 years and above, 47% of the population is affected (Brody, 1982, Katzman *et al.*, 1991). The life expectancy of an Alzheimer's disease patient is reduced by approximately one third when compared to an age and sex matched control (Powell *et al.*, 1986). An increasing trend in the rate for Alzheimer's disease has been recorded in England (Martyn *et al.*, 1988), Australia (Jorm *et al.*, 1989), Norway (Flaten, 1989), and the United States and Canada (Newman *et al.*, 1987). This trend could be due to an increase in the prevalence of Alzheimer's disease, as could be caused by demographic factors (such as in increase in the population size in the over 65 years age group). The trend could also be due to physicians diagnosing and listing Alzheimer's disease as the cause of death more frequently.

Although the pathology of Alzheimer's disease is now well characterized, no peripheral biochemical marker for the disease has yet been found. Therefore, a definitive diagnosis of Alzheimer's disease can only be made after the characteristic pathology is observed in a histological examination of the cerebral cortex, which in most cases, is only done at the time of autopsy. Brain biopsies are not routinely done, because even if a positive diagnosis is made, no treatment is available. The cause of Alzheimer's disease is unknown.

1.11) Clinical Features.

Symptoms vary from patient to patient, and often from day to day in the same patient. The course of Alzheimer's disease usually involves a gradual and progressive deterioration in neurological functioning. Several authors have proposed various stages of the disease (Riesberg,

1982; Powell *et al.*,1986). The first symptom is a mild indiscriminate loss of memory. As the disease progresses, the memory deficit becomes more pronounced, and other cognitive functions become affected; the patients' verbal and mathematical abilities begin to decline. Up to this stage of the disease, the affected individuals are relatively alert, and aware of their condition. Depression and anxiety are common symptoms of affected individuals at this stage of the disease, and displays of aggression and hostility are common (Lopez *et al.*, 1990). As the disease progresses further, the patients lose orientation with respect to place and time, and often require assistance with daily activities and self-care. Eventually, severe dementia manifests, and the affected individuals lose all verbal and self-care abilities; the victims are now physically and intellectually impaired, and require full time care or institutionalisation. The progression of the disease to this stage can take anywhere from three to fifteen years after onset. Death usually results from complications arising from long term confinement to bed. These are usually pneumonia (which results from impaired clearing of the lungs) or a pulmonary embolism (the clot usually forming in a major vessel of either leg) (Dr.C.Shaw, personal communication).

1.12) Pathology.

There are several brain changes that accompany Alzheimer's disease. They are:

Cell loss and dendritic degeneration.

Individuals with Alzheimer's disease have brains that show marked atrophy when compared to age-matched controls (Katzman *et al.*, 1991). Up to 50% of the neurons in the neocortex may be lost (Pearson *et al.*, 1985). This loss is mainly in the frontal and temporal cortical regions of the brain. Autopsy has shown dendritic degeneration (mainly of horizontal dendrites) in many neurons within the neocortex (Rogers *et al.*, 1985). These dendrites may be involved in memory.

Neuritic plaques.

The mature form of the neuritic plaque consists of a central core of a fibrous, extracellular protein, amyloid ß/A4 (Yankner *et al.*, 1991). Apo E is closely associated with the amyloid β/A4 (Kida *et al.*, 1994). The central core also contains IL-1, ACT, and aluminium (Clauberg *et al.*, 1993). Surrounding this core are degenerating axonal and dendritic nerve endings, which contain abnormal mitochondria and many lysosomes. Kawai *et al.*, (1992) have proposed that the lysosomal system of the neurons present in the plaques is responsible for the accumulation of amyloid precursor protein (which forms amyloid ß/A4). Such a neuronal origin for the amyloid precursor protein has been supported by Mann *et al.*, (1992). Clauberg *et al.*, (1993) however, suggested that aluminium may accelerate the proteolytic processing of the amyloid precursor protein by enhancing the activity of ACT by almost 100-fold. The density of the neuronal plaques has been shown to correlate to the mental status scores of Alzheimer's disease patients (Sparks *et al.*, 1990). The role of amyloid ß/A4 in the pathogenesis of Alzheimer's disease has been extensively reviewed (Katzman *et al.*, 1991; Rumble *et al.*, 1991; Ishiura *et al.*, 1991; and Selkoe, 1990).

Neurofibrillary tangles.

Neurofibrillary tangles are found within the neuronal cell bodies. They are composed of paired helical filaments, which consist of two twisted filaments (14 - 18 nm in diameter each) coiling counter clockwise around each other. The principal protein component of the paired helical filament is tau, a cytoskeletal protein (Katzman *et al.*, 1991). Lee *et al.*, (1991) showed that when tau is phosphorylated in vitro, it forms paired helical filaments that are indistinguishable from those found in the brains of Alzheimer's disease patients. This suggests that an abnormal phosphorylation event may be involved in the pathogenesis of Alzheimer's disease. Neurofibrillary tangle formation has recently been reviewed (Masters *et al.*, 1990; Mattson, 1992). It has recently been suggested that neurofibrillary degeneration may be more important than the deposition of amyloid β/A4 in

the pathogenesis of Alzheimer's disease (Bandareff *et al.*, 1993).

Neurotransmitter abnormalities.

Many neurotransmitter abnormalities have been detected in the brains of Alzheimer's disease patients. Such abnormalities include abnormally low levels of acetylcholine, noradrenaline, serotonin, and somatostatin (Price, 1986). Of these, the biggest reduction is found in acetylcholine (Mesulam *et al.*, 1991, Nemeroff *et al.*, 1991). The reduced level of acetylcholine is thought to result from reduced levels of choline acetyltransferase and degeneration of cholinergic axons. Mental impairment in Alzheimer's disease is thought to result from low levels of acetylcholine. Parnetti *et al.*, (1994) recently showed that increased levels of pMAO-B correlate significantly with late onset Alzheimer's disease. Although an increased level of pMAO-B could not account for decreased levels of acetylcholine, it may influence the brain levels of catecholamine neurotransmitters such as adrenaline and dopamine. Neurotransmitter abnormalities in Alzheimer's disease have been reviewed (Decker *et al.*, 1991; Chan-Palay, 1990; Fibiger, 1991).

Recently, the role of the excitatory amino acid neurotransmitters, aspartate and glutamate, have been implicated in neurodegenerative disease. It is thought that high levels of these amino acids in the brain result in neurodegeneration (Foster, 1990; Olney, 1990). Tohgi *et al.* (1992) showed reduced levels of these amino acids in the CSF of patients with Alzheimer's disease. The changes were not specific to Alzheimer's disease however.

1.13) Diagnosis.

There are two phases in the diagnosis of Alzheimer's disease (Amaducci *et al.*, 1990). The first step is to verify the presence of dementia. There are various tests available to make this assessment (Siu, 1991). All of the tests require a progressive decline in mental functioning before dementia is diagnosed. The second step is to exclude all other possible sources of dementia. Such

sources can include infection, trauma, nutritional deficiencies, neoplasms, vascular disease, metabolic disorders, and heavy metal poisoning (Pearce, 1983). Recently, clinical tests have been developed which can lend support to a diagnosis of probable Alzheimer's disease. Such tests include topographical mapping of the brain's electrical activity (Martin-Loeches *et al.*, 1991), detection of endogenous ammonia production in the brain (Hoyer *et al.*, 1990), the use of NMR imaging to detect cortical atrophy (Goldstein *et al.*, 1991), and the use of positron emission tomography to detect abnormal cerebral blood flow (Powers *et al.*, 1992) and abnormalities in brain glucose metabolism (Hoyer, 1991; Guze *et al.*, 1992). More recently, Barinaga (1994) described how the pupillary response time to Tropicamide eye drops may be used to support a clinical diagnosis of Alzheimer's disease, although this finding is still controversial. Definite diagnosis of Alzheimer's disease can only be made at the time of autopsy, when the characteristic pathology is observed. In this study, diagnosis was based upon the National Institute of Neurological and Communicative Diseases and Stroke - Alzheimer's Disease and Related Disorders Association diagnostic criteria (McKhann *et al.*, 1984) and the mental status score devised by Kokmen *et al.*, (1987). There is currently no laboratory test available to confirm clinical diagnosis.

1.14) Treatment.

There are two approaches in the treatment of Alzheimer's disease (Davies, 1991). The first involves attempting to alleviate the symptoms, via replacement therapy (Bowen, 1990; Francis *et al.*, 1985). This either involves oral administration of cholinergic compounds, which either inhibit brain cholinesterase (eg. Tacrine), or promote acetylcholine production in the brain (Fisher *et al.*, 1990; Fitten *et al.*, 1990; Eagger *et al.*, 1991), or involves transplantation of cholinergic grafts into the brain (Dunnett *et al.*, 1991; Lindvall, 1991). The other approach to treating Alzheimer's disease is to try to slow the rate of progression of the disease. Such attempts can involve the use of anti-inflammatory agents (McGeer, 1992), nerve growth factor (used to slow the rate of degeneration (Olsen, 1993)), or more commonly, treatments based upon knowledge of the molecular pathology of the disease. For a review, see Gandy *et al.* (1992). Up to the present time, the lack of an

adequate animal model has slowed treatment development (Duff *et al.*, 1995). Recently however, Games *et al.* (1995) claim to have developed a transgenic mouse model for Alzheimer's disease.

1.15) Aetiology.

The cause of Alzheimer's disease is unknown. Several factors have been suggested to contribute to the risk of developing Alzheimer's disease however. As discussed previously, age is the major risk factor. A family history of Alzheimer's disease is also a major risk factor. Up to 40% of individuals diagnosed as having probable Alzheimer's disease have a family history of the disease, suggesting a genetic component to the risk (Price, 1986). Additionally, Payami *et al.* (1994) have shown that a negative family history of Alzheimer's disease with long lived relatives has a protective association against Alzheimer's disease when compared to random controls. Metals such as aluminium, iron, and tin have also been implicated in the disease, although they have not been demonstrated to play a principal role in pathogenesis (Forbes *et al.*, 1992; Walton, 1992; Carrigan *et al.*, 1992). Recently however, iron has been shown to have a potential physiological role in controlling the processing of the amyloid precursor protein (Bodovitz *et al.*, 1995). Head trauma may also contribute to the disease (Gentleman *et al.*, 1992).

A transmissible agent has also been postulated as a potential cause of the disease. Recent reviews suggest that indirect evidence indicates that such an agent may play a role in pathogenesis (Manuelidis *et al.*, 1991; Rohwer, 1992; Baker *et al.*, 1993). Metabolic disorders are also thought to play a major role in disease pathogenesis. Abnormal phosphorylation of tau, and the abnormal proteolytic processing of the amyloid precursor protein (as reviewed by Hardy *et al.*, 1992- A, B; Ishiura, 1991; Gajdusek *et al.*, 1991) are thought to be major contributors to the disease. Other factors that may be involved in the disease are microtubule processing (Matsuyama *et al.*, 1989); membrane abnormalities of red blood cells and platelets (Schoenberg, 1988); cellular calcium homeostasis (Katzman, 1986); antioxidant stress (Pappolla *et al.*, 1992); abnormal DNA repair and vitamin B_{12} metabolism (Gorelick *et al.*, 1991); and oestrogen deficiency in aging women (Pagnini-

Hill *et al.*, 1994).

Two factors have recently been suggested as having protective associations against Alzheimer's disease. Lee (1994) has shown that smoking has a protective role against Alzheimer's disease. Education was shown by Stern *et al.*, (1994) to reduce the risk of incidence of Alzheimer's disease, either by decreasing the ease of clinical detection, or by imparting an intellectual reserve that delays the onset of Alzheimer's disease.

1.16) Genetics.

A genetic component in the aetiology of Alzheimer's disease is supported by the observation that familial clustering is seen in up to 40% of the individuals with Alzheimer's disease. There is evidence to suggest that early onset Alzheimer's disease may be inherited in an autosomal dominant fashion , although it is a genetically heterogenous disorder (Pericak-Vance *et al.*, 1991). However, Farrer *et al.*, (1991) and Tzourio *et al.*, (1991) have suggested that other non-genetic factors must also occur.

Further evidence for a genetic component to the disease has come from the observation that most individuals with trisomy 21 (Down's syndrome) over the age of 50 years become demented, and show the same pathological features as individuals affected with Alzheimer's disease (Stewart *et al.*, 1989; Franceschi *et al.*, 1989; Potter, 1991). There is also an increased risk of Alzheimer's disease in mothers of adults with Down's syndrome, supporting the hypothesis of a shared genetic abnormality between the two disorders (Schupf *et al.*, 1994). This link is also supported by the fact that the gene encoding the amyloid precursor protein is found on chromosome 21 (Muller-Hill, 1989). Recently, attention has been focused on mutations found in this gene, at codon 717. The mutations either cause substitution of valine with isoleucine (Lantos *et al.*, 1992) or with glycine (Mann *et al.*, 1992). It has been proposed that these mutations alter the transmembrane spanning domain of the amyloid precursor protein (Zubenko *et al.*, 1992). Several other mutations in this

gene have also been demonstrated to segregate with Alzheimer's disease. Mullan *et al.*, (1993) for example showed how mutations at codons 670/671 co-segregated with Alzheimer's disease in two Swedish pedigrees.

Several non-mutated alleles also appear to be associated with an increased risk of developing Alzheimer's disease. They include the transferrin C2 subtype (VanRensburg *et al.*, 1993) and the Apo E ϵ4 allele (Yu *et al.*, 1994). The association between Apo E ϵ4 and Alzheimer's disease has received substantial coverage in the scientific literature over the past two years.

1.2) Multiple sclerosis.

MS is a common neurological disease involving relapsing and remitting demyelination of CNS white matter. In New Zealand, the prevalence of MS is approximately 5 per 10,000 (Skegg *et al.*, 1987). The cause of MS is unknown, but it is commonly thought to be an autoimmune disease triggered by an environmental factor, possibly a virus (Mehta, 1991).

1.21) Clinical features.

Onset age for MS is commonly between 20 to 50 years. Females are affected more commonly than males by a ratio of approximately 2:1 (McLoed, 1982). The initial onset symptoms include motor weakness, optic neuritis, and diminished cutaneous sensitivity. In approximately 90% of cases, MS follows a relapsing and remitting course (McLeod, 1982). Clinical symptoms become more severe with each relapse, and neurological dysfunction more widespread. Usually, the disease eventually enters a chronic stage, and the patient becomes bed ridden. The rate of progression for the disease varies widely, it taking between five and thirty years after disease onset for the disease to enter the chronic stage. Death usually occurs due to opportunistic infection. For some individuals however, the disease follows a benign course (Swingler *et al.*, 1992).

1.22) Pathology.

The major pathological feature of MS is the presence of areas of demyelination (plaques) in the white matter of the CNS. The plaques, which have diameters ranging in size from 1 to 5 mm, initially develop within the vicinity of small blood vessels. As demyelination progresses, the plaques become infiltrated with macrophages, neutrophils, lymphocytes, and microglia. Macrophages are responsible for the demyelination (Deber *et al.*, 1991). The exposed axon within the plaque remains intact however (Waxman, 1982). The cause of demyelination in MS is unknown, although it is thought to be immunologically mediated. The immunological aspects of demyelination in MS have been reviewed (Martin *et al.*, 1992; Fretland, 1992).

1.23) Diagnosis.

Currently, the diagnosis of MS is based upon clinical evaluation. Diagnosis requires there to be at least two separate CNS lesions, and is usually categorised as possible, probable, or definite, based upon the criteria established by McAlpine *et al.*(1972). Magnetic resonance imaging is playing an increasingly important role in the diagnosis of MS (as reviewed by Wallace *et al.*, 1992). Currently, there is no definitive laboratory test that can be used to diagnose MS. However, changes in the composition of CSF can support the diagnosis of MS and may be of value in prognostic assessment. The diagnostic utility of CSF in MS has been reviewed by Mehta (1991).

1.24) Treatment.

There is no cure for MS. Although immunological involvement has been demonstrated in MS, immunosuppressive agents have not conclusively been shown to provide any long term relief (Goodin, 1991). The current immunosuppressive agents available for administration pose health risks for the patients. Specific immunosuppressive agents are currently under development that may be of some benefit however (Hintzen *et al.*, 1991). One such agent is mitoxantrone, which was shown by Mauch *et al.* (1993) to slow disease progression in most of patients with rapidly

progressive MS. Mitoxantrone has not yet been approved as a general treatment for MS.

Conventional treatment for MS involves relieving the symptoms of the particular CNS lesions present. Established therapeutic strategies for this purpose have been reviewed (Noseworthy, 1991). One new treatment for treating MS symptomology is based on administration of aminopyridines. These agents are potassium ion channel blockers. Bever (1994) demonstrated the efficacy of two aminopyridines in relieving the symptoms of MS. To date, the most effective conventional way for temporarily slowing the rate of progression of MS is the administration of large doses of steroids (Hughes, 1991). This is thought to interfere with macrophage activity. However, the administration of large doses of steroids poses health risks for the patients, and can not be continued over long periods.

During 1994, two new drugs were introduced for MS treatment. They were a form of recombinant interferon β, and methylprednisolone. These treatments are reviewed by Jacob *et al.* (1994). Currently, therapies based on oral tolerance are undergoing clinical trials for MS (Weiner *et al.*, 1993). Oral tolerance therapy has been reviewed by Friedman *et al.* (1994) and Weiner *et al.* (1994). The variability seen in MS and the lack of specific diagnostic tests for the disease is hindering the development of curative agents.

1.25) Aetiology.

The cause of MS is presently unknown. Epidemiological studies however, have shown that latitude correlates to MS distribution (Skegg, 1991; Mcleod *et al.*, 1994), that exposure to an infectious agent before the age of fifteen years in high risk endemic areas appears to occur (Kurtzke, 1991), and that immunological dysfunction may be related to the development of MS (Harbige *et al.*, 1990). Many conflicting hypotheses have been proposed to explain the epidemiological findings. For example, Swingler *et al.* (1986) proposed that population genetics could be used to explain the correlation between latitude and MS prevalence, by showing that the frequency of the HLA-DR2 antigens in Britain correlated to MS prevalence. However, Miller

et al. (1990) showed that the HLA-DR2 antigen frequencies in New Zealand and Australia did not correlate to MS prevalence. Viruses have also been targeted as a potential cause of MS. No single virus has yet been demonstrated to be causal. However, Lindberg *et al.* (1991) demonstrated that Epstein-Barr infection in childhood, with subsequent development of infectious mononucleosis correlated to MS prevalence, and was consistent with epidemiological findings. This observation is currently being investigated further. Initial findings published by Martyn *et al.* (1993) suggest that the pathogenesis of MS may involve an age-dependent host response to Epstein-Barr virus infection. The role of viruses and infection in the aetiology of MS has been extensively reviewed (Sarchielli *et al.*, 1993; Kurtzke, 1993). Currently, most scientists regard MS as caused by an environmental element (probably one or more infectious agents) triggering an autoimmune response in individuals with a genetic predisposition to MS, leading to myelin destruction (Mehta, 1991; Sadovnic *et al.*, 1993; Kahana *et al.*, 1994).

1.26) Genetics.

Epidemiological evidence suggests that genetics may play a role in the pathogenesis of MS. Such evidence includes: the fact that different ethnic groups show different susceptibilities for MS development; first degree relatives of MS patients show an increased susceptibility to MS (Ransochof, 1992; Binzer *et al.*, 1994); twin studies have shown that monozygotic twin pairs have a concordance rate of 26 to 30% for MS development, while for dizygotic twin pairs, the concordance rate is 2 to 5% (Ebers *et al.*, 1986; Kinnunen *et al.*, 1988); and certain HLA antigens are associated with MS (McDonald, 1986). Recent studies however, have shown no consistent pattern of HLA antigen frequencies in various MS populations (Moller *et al.*, 1990; Spurkland *et al.*, 1991; Grasso *et al.*, 1992). The fact that no MS specific mutations have been detected in the MHC genes (or any other genes), despite the epidemiological evidence pointing to a genetic component, has been taken to suggest that the genetic component of MS risk may be uncommon combinations of common alleles of polymorphic genes (Cowan *et al.*, 1991). Ebers (1994) has shown that oligogenic hypotheses encompassing epistatic interactions best fit the current genetic data for MS. The immunogenetics of MS has recently been reviewed by Tournier-Lasserve *et al.* (1993).

1.27) Protein abnormalities in MS.

Many protein abnormalities have been observed in plasma, serum, and CSF samples from individuals affected with MS. These abnormalities have been reviewed elsewhere (Ford, 1985; Mehta, 1991). None of the abnormalities were shown to be specific to MS however. Since the time of the review of Mehta (1991), several other protein abnormalities have been reported for MS. Maura *et al.* (1992) observed oligoclonal IgD bands in CSF; Sharief *et al.* (1991) observed oligoclonal IgM bands in both serum and CSF; Back *et al.* (1992) showed differential isoform profiles for α2-macroglobin; Harrington *et al.* (1993) found a brain specific protein abnormality; and Pirttila *et al.* (1991) observed further oligoclonal bands in serum and CSF that have yet to be characterised. However, none of these abnormalities were shown to be strictly specific to MS.

1.3) Protein Markers in Disease.

In any disease, there are different biochemical and physiological levels that an associated protein abnormality may occur at. The protein may be found at the level of cellular organelles or it may be a component of a biochemical pathway. The protein may also be found at the level of individual tissues, at the level of individual organs or organ systems, or the protein may be systemic. The protein abnormality may cause the disease, or may arise directly or indirectly because of the disease process. The abnormal protein may be genetic or environmental in character.

Certain criteria must be fulfilled for a protein abnormality to be regarded as a useful and specific marker for a particular disease. These include (Wheeler, 1988):

i The protein must be disease specific and allow a distinction to be made between the particular disease and symptomatically similar diseases.

ii The protein should be present in the vast majority of affected individuals.

iii The protein needs to be readily obtainable from an organ or tissue from affected individuals, and for a reasonable amount of time during the disease.

iv The protein has to be at a concentration sufficient for its detection, and has to be stable enough to withstand the detection method.

It is possible that a protein may not be recognised as a marker for a particular disease in its own right until its relative abundance in the disease state is compared to that in the non-disease state, and possibly correlated to the relative abundances of other proteins. Possible protein markers that could fall into this category are certain acute phase reactants.

As well as providing insights into the aetiology and pathogenesis of a disease, protein markers may form the basis of routine laboratory tests for the presence of a particular disease. The major aim of this project was to search for circulating protein markers in Alzheimer's disease and MS that could form the basis of a diagnostic test for each disease. Currently, no definitive laboratory test exists that can confirm a diagnosis of Alzheimer's disease or MS.

Many different body fluids and tissues can be used in the search for protein markers. For example, Wheeler *et al.* (1988a) searched plasma for protein markers associated with depressive disease; Tracey *et al.* (1984) showed the existence of specific urinary proteins associated with renal tubular and glomerular disease; Maura *et al.* (1992) showed oligoclonal IgD bands in CSF that may be associated with MS; and Comings (1979) showed the presence of a brain specific protein polymorphism in brain tissue from depressed subjects. Table 1.31 lists a selection of other searches for disease related protein markers.

Two-dimensional PAGE is a useful technique for looking for protein variation in disease. The 2-dimensional separations can be analysed to detect differences between affected individuals and controls, and thus identify potential protein markers. In both Alzheimer's disease and MS, such a protein marker could reflect the pathological changes occurring in the CNS; it may be part of a generalised systemic response to the disease (it may be an acute phase reactant for example); or it could possibly be pathogenic in origin. To be an adequate marker, it must meet the criteria discussed above, and preferably, the amounts of the protein detected would reflect disease severity.

There are limitations to using 2-dimensional PAGE in searching for protein markers. The

Table 1.31. Searches for protein markers in various diseases.

Disease/disorder	Body fluid/tissue investigated	Reference
Down's syndrome	Fibroblasts	Weil *et al.* 1979
Down's syndrome		Brown *et al.* 1981
Bernard-Soulier disease	Platelets	Clematson *et al.* 1982
Grey platelet syndrome	Platelets	Nurden *et al.* 1982
Huntington's disease	Brain tissue	Rosenberg *et al.* 1981
Huntington's disease	Lymphocytes	Goldman *et al.* 1982
Idiopathic sclerosis	Muscle tissue	Whalen *et al.* 1982
Dermatomyositis	Muscle tissue	Danon *et al.* 1981
Duchenne's muscular dystrophy	Fibroblasts	Rosenmann *et al.* 1982
Monoclonal gammopathy	Serum	Tracy *et al.* 1982a
Neurological diseases	CSF	Endler *et al.* 1987
Leukemia	Lymphocytes	Anderson *et al.* 1983
Colon cancer	Colonic mucosa	Tracy *et al.* 1982b
Arthritis	Urine	Clark *et al.* 1980
Pancreatic cancer	Pancreatic juice	Scheele *et al.* 1981
Organ rejection	Urine	Tracey *et al.* 1982c
Psychiatric disorders	CSF	Wildenauer *et al.* 1991
Connective tissue disorders	Saliva	Beeley *et al.* 1991
Lung cancer	Lung tumor tissue	Franzén *et al.* 1991
Prostrate cancer	Urine	Edwards *et al.* 1982
Brain tumors	Tumor cell lines	Müller *et al.* 1991
Psoriasis	Dermal fibroblasts	Easty *et al.* 1991
Rheumatoid arthritis	Plasma	Hansen 1994
Pulmonary tuberculosis	Serum	Ameglio *et al.* 1994
Homozygous sickle cell disease	Serum	Hedo *et al.* 1993
Hepatocellular carcinoma	Serum	Hwanghee *et al.* 1992

technique only resolves a subset of proteins within any given complex mixture of proteins. In blood plasma for example, several thousand proteins can be resolved, depending upon the methodology used. However, between 10,000 and 20,000 proteins can be expressed at any time in any given cell (Williams *et al.*, 1975). Therefore, there may be many potential marker proteins that will not be detected using 2-dimensional PAGE. Also, it is possible that proteins could be mutated in disease without their size, charge or rates of synthesis being altered, rendering them undetectable using this technique.

In this study, 2-dimensional PAGE was used to compare plasma proteins in individuals affected with either Alzheimer's disease or MS to those of their controls; to compare CSF proteins in individuals with MS to those of their controls; and to compare platelet proteins in individuals with Alzheimer's disease to the platelet proteins of their controls.

1.4) Biomedical applications of 2-dimensional PAGE.

Recent advances in protein chemistry have made 2-dimensional PAGE a valuable tool in biomedical research. Using this technique, it is possible to isolate unidentified and uncharacterised proteins in a single process, and to obtain adequate quantities of sufficient purity to allow a partial amino acid sequence to be determined. When this technique is used with PCR, it is possible to clone genes for previously unknown proteins. Many papers have been published in which 2-dimensional PAGE has been used for biomedical research. For example, Lapin *et al.* (1991) used 2-dimensional PAGE to study urinary proteins during human disease; Clarke *et al.* (1991) used the technique to study tumour induced protein synthesis; and Mattila *et al.* (1994) used 2-dimensional PAGE to study brain proteins in Alzheimer's disease. Table 1.41 lists some additional biomedical studies that had 2-dimensional PAGE as the central technique.

Table 1.41. Biomedical applications of 2-dimensional PAGE.

Disease/disorder	Body fluid/tissue investigated	Reference
Alzheimer's/Down's syndrome	Brain	Savage *et al.* 1994
Inner ear disease	Perilymph	Thalmann *et al.* 1994
Cancer	Keratinocytes	Rasnussen *et al.* 1994
Urological disease	Urine	Grover *et al.* 1993
Alzheimer's/schizophrenia	CSF	Johnson *et al.* 1992
Heart disease	Serum	Burgess-Cassler *et al.* 1992
Graft vs. host disease	Platelets	Prodouz *et al.* 1990
Aging	Serum	Ai *et al.* 1989
Duchenne muscular dystophy	Plasma	John *et al.* 1989
Rheumatoid arthritis	Plasma	Powlowski *et al.* 1989
Dementia	Plasma/CSF	Alafuzoff *et al.* 1986
Neurological diseases	CSF	Wiederkehr *et al.* 1985

The protein abnormalities detected using 2-dimensional PAGE in biomedical research may be of genetic or environmental origin. There are several categories of protein abnormalities of genetic origin. The first includes those abnormalities that arise because of disease causing mutations either in structural genes or in regulatory genes that alter the rate of protein synthesis. A second category includes abnormalities resulting from protein polymorphism, where one of the polymorphic forms of the protein is associated with the disease. 2-Dimensional PAGE is ideally suited to the detection of disease related protein polymorphisms, due to its ability to resolve proteins differing by as little as one charge unit. The third category of protein abnormalities of genetic origin consists of those protein changes due to aberrant processing. It is thought that aberrant processing of amyloid precursor protein may be the central event in the pathogenic processes underlying Alzheimer's disease (Hardy *et al.*, 1992).

Protein abnormalities observed in the disease state may also be of environmental origin. For

example, infectious diseases may result in the presence of proteins derived from the pathogen. In degenerative diseases, tissue breakdown may release breakdown products, either from the disruption of cellular compartments, or from changes to the metabolism of the affected cells. Also, in many diseases, the plasma level of certain proteins is seen to alter; this is the acute phase response. The majority of disease associated protein changes observed in this project were thought to be involved in the acute phase response, the proteins being known as APRs.

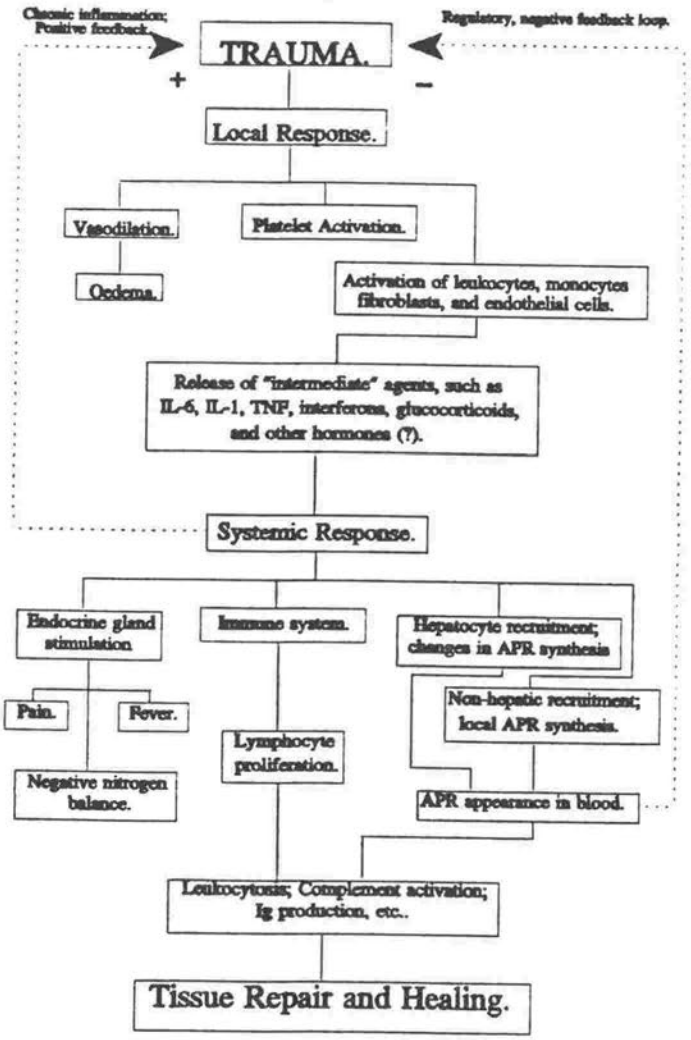
1.5) The acute phase response.

APRs are glycoproteins that experience altered rates of synthesis, from 1.5 to 1000 fold (Steel *et al.*, 1991), because of physical trauma. Trauma capable of inducing the acute phase response include local inflammation, thermal or mechanical injury, surgery, bacterial infection, endotoxin injection, and neoplastic growth. Early work in this area indicated that the increased synthesis of APRs was probably due to increased rates of transcription, as opposed to increased RNA stability. However, some recent studies have shown that for some APRs such as α_2 macroglobin, the increased rate of transcription is not great enough to account for the increased mRNA levels (Gehring *et al.*, 1987). This implies that post transcriptional mechanisms may play a role in the control of APR synthesis. This observation is supported by work done by Lowell *et al.* (1986) which showed that in the murine acute phase response, both increased rates of transcription and increased mRNA stability were responsible for the elevated levels of the APR SAA mRNA.

The acute phase response is part of the systemic response of an animal to injury. Koj (1974) suggested that there are two components of an animal's response to injury/trauma. The first component is the local response, and among other things includes: vasodilation, oedema (due to endothelial cell leakage), platelet aggregation, fibrin formation, leukocyte accumulation with the subsequent release of their hydrolytic enzymes, and the formation of low molecular weight mediators, such as histamine and cytokines. As well as initiating synthesis of APRs by hepatocytes, these mediators also act on many extra-hepatic cells, initiating the systemic response. Koj

suggested that the local response to trauma not only promoted cellular necrosis, but also contributed to and promoted the overall process of response to injury. The second component of the animals' response to injury is the systemic response that includes fever, pain, leukocytosis, synthesis of APRs, and increased activity of the pituitary and adrenal glands. Koj suggested that this component of an animal's response to injury exerted protective and inhibitory effects and promoted the process of healing. Figure 1.51 summarises the events described above.

Figure 1.51. The acute phase response.



The APRs fall into two categories. The first, and largest, category is that of the positive APRs. These are proteins whose synthesis is increased because of trauma. In humans, this category includes Hp, orosomucoid, ACT, ceruloplasmin, C-reactive protein, fibrinogen, HS glycoprotein,

and apo A1 lipoprotein. The second category is that of the negative acute phase reactants, and is made up of proteins whose expression is decreased as a result of trauma. This may include albumin, transferrin, and RBP. However, all APRs in humans have at least two features in common (Koj, 1974). Almost all have relatively high carbohydrate contents, and all may be synthesised in liver parenchymal cells. In mice, extra-hepatic synthesis of APRs has also been demonstrated to occur as part of the acute phase response (Kalmovarin, *et al.*, 1991). Extra-hepatic tissues in which expression occurred included kidney, liver, thymus, heart, brain, lung, testis, and epididymis. Although extra-hepatic synthesis of APRs in humans has not been conclusively shown, papers have been published which indirectly support such synthesis (eg. DeStrooper *et al.*, 1993). The response pattern of the individual APRs to various forms of trauma has been shown to differ considerably (Koj, 1974).

It is thought that cytokines mediate the acute phase response. Cytokines are released at the site of injury by macrophages. In humans, IL-6 is thought to be the major regulator of the acute phase response. Gauldie *et al.*, (1987) showed that IL-6 was responsible for the induction of the acute phase response in human hepatocyte cultures. Moshage *et al.*, (1988) showed that while other cytokines could promote synthesis of various APRs, IL-6 stimulated synthesis of all of the human APRs at times of physical trauma. Ray *et al.*, (1988), showed that the human IL-6 gene promoter could be activated by viruses, bacterial products, and other cytokines (usually via the action of some second messenger agonist). This observation implies that IL-6 has a major role in the human acute phase response. Other cytokines involved in the acute phase response include IL-1 (α and β), tumour necrosis factor, interferon τ, and transforming growth factor β. The cytokines are thought to operate in a cooperative manner, producing heterogeneous APR profiles.

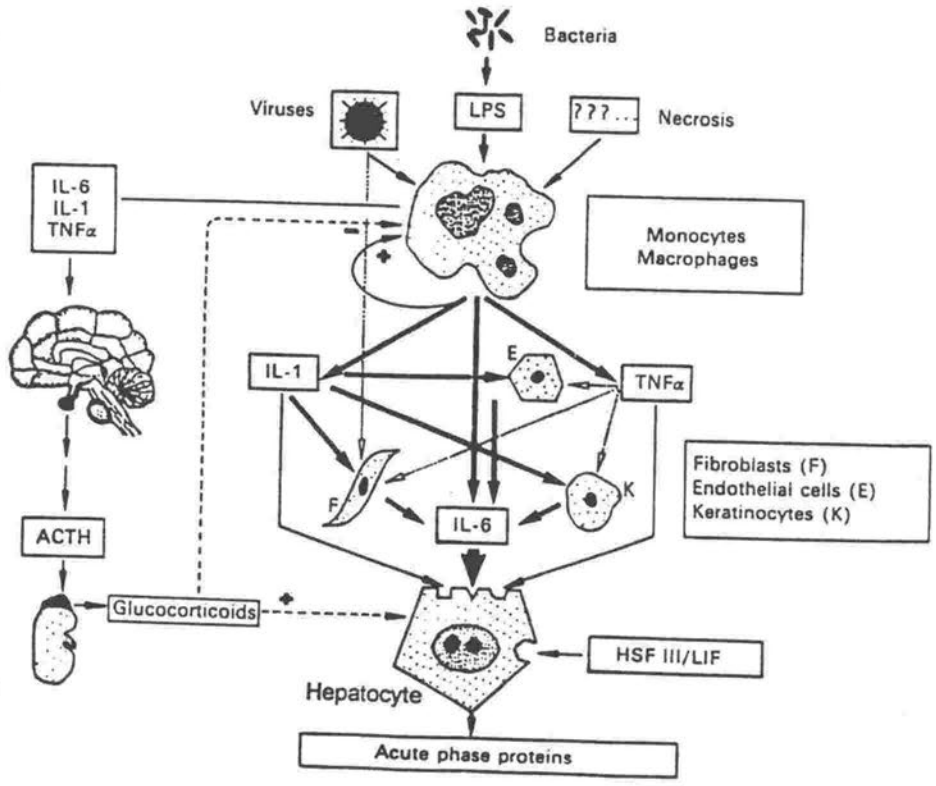
When cultured hepatocytes were studied, the cytokines invoked different responses for each of the APRs (Steel *et al.*, 1991). In Steel's study it was shown that cytokines had different combinatorial effects when added to cultured hepatocytes. Sometimes for a given APR, two cytokines added together appeared to have synergistic effects, while for a different APR, they showed down-regulatory effects, etc. Such effects have also been observed for rats. Baumann *et*

al. (1987) showed that in rat hepatoma cells, various cytokines derived from human squamous carcinoma cells evoked differential APR synthesis. Figure 1.52 illustrates the regulation of hepatic APR synthesis by the various cytokines. Although the various cytokines cause the cellular recruitment of hepatocytes to specific APR production, multiple APRs can be synthesised simultaneously by a given hepatocyte (Kushner, 1988). It is also possible that the receptors for the various cytokines on individual hepatocytes may play a role in determining the different APR profiles observed.

There are other possible mediators of the acute phase response. As trauma stimulates both the pituitary and adrenal glands (and other endocrine glands), it is possible that certain hormones may be involved in initiating or modifying the acute phase response. Purified IL-1 was shown to result in increased APR synthesis when injected intraperitoneally into rats, but was shown not to induce increased APR synthesis in cultured hepatocytes (Kushner, 1988); this may show that hormonal and other metabolic influences may play a role in regulating the acute phase response.

Work has been undertaken to investigate the nature of the IL-6 responsive elements in the various APR genes. Typically, the promoter regions of the APR genes were isolated and ligated to reporter genes, mainly the chloramphenicol acetyl transferase gene. By using deletion mutants in transfection experiments, it was possible to define the *cis*-elements involved in the induction of the increased APR gene transcription via IL-6 stimulation. Oliviero *et al.*, (1987) defined the structure of three *cis*-elements for the human Hp gene promoter region. Arcone *et al.*, (1988) defined the nature of the IL-6 responsive *cis*-element present in the promoter region of the human C-reactive protein. Fung *et al.*, (1987) defined the nature of the *cis*-elements for the rat APRs α_1-protein and kininogen. A common consensus sequence (5'-**CTGGGA**-3') has been found in all of the IL-6 responsive *cis*-elements referred to above, the sequence also being found in the promoter regions of the fibrinogen, α_2 macroglobin, α_1 acid glycoprotein, transthyretin, murine SAA, and AT genes (Heinrich *et al.*, 1990). It appears that the consensus sequence must play an important role in APR gene regulation. A recent observation regarding the induction of APR gene synthesis was made by Yiangou *et al.* (1991), who showed that certain heavy metals could directly induce

Figure 1.52. The regulation of hepatic APR synthesis (from Heinrich et al., 1990).



transcription of certain APR genes. The increased transcription was shown not to be due to a metal mediated inflammatory response, but rather to the activation of some metal responsive elements in the promoter regions of the α_1 acid glycoprotein and C-reactive protein genes. Both proteins are known to have a high affinity for heavy metals.

Both positive and negative APRs can be classified within three groups of proteins (Koj, 1974). These groups are:

i "New" proteins that appear only in pathological states. An example would be Hp related protein (Shurbaji *et al.*, 1991), which only appears in the presence of certain neoplasms.

ii "Foetal" proteins not normally present in adult human plasma, but produced in response to trauma. An example of this would be α-foetoprotein (Adinolfi *et al.*, 1976), which is associated with hepatocellular carcinoma.

iii "Normal" constituents of plasma that are synthesised at a greater/lesser rate in response to trauma. An example would be orosomucoid.

To date, no strictly specific APR has been discovered. In most cases of trauma, the acute phase response is stereotyped; either the plasma concentration of certain APRs rises, or it falls. This makes it difficult to distinguish between different types of disease and inflammation based upon a certain acute phase response profile. Currently, changes in APR plasma concentrations are regarded as a sensitive, but nonspecific test used for diagnostic and prognostic assessment. Elevated levels of positive APRs accompanied by decreased levels of negative APRs are now regarded as being an important indicator of acute and chronic inflammation (Maes *et al.*, 1992).

Currently, an issue receiving much attention is the relationship between the observed "level" of the acute phase response, and the severity of the disease/trauma. It appears that there is a relationship between trauma severity and the level of change observed in the APRs. Maes *et al.* (1992), observed that the severity of depression was significantly linked to increments in positive APRs and decrements in negative APRs. In rheumatoid arthritis, increased levels of APRs have been observed, these levels dropping in response to corticosteroid therapy, and a decrease in symptom severity (Uddhammar *et al.*, 1992). In addition, Apo A1 levels were shown to correlate to recovery in myocardial infarction patients (Slunga *et al.*, 1992). The Apo A1 level also correlated to the level of LDL and serum cholesterol, indicating that there may be a link between Apo A1 levels and the risk of myocardial infarction.

Chapter 2. Sources of Materials.

The reagents used in this project were sourced from several suppliers. The major supplier for each reagent is indicated in the text. When possible, individual reagents were obtained from the same supplier over time. The suppliers were as follows:

Amersham International, Bucks., UK:

^{14}C-labelled formaldehyde.

Amicon Corp., Danvers, MA, USA:

Centricon centrifugal microconcentrators.

Becton Dickson Vacutainer Systems, Rutherford, NJ., USA:

Blood collection tubes.

BDH Ltd., Poole, Dorset, England:

Glacial acetic acid, scintillation fluid.

BioRad Laboratories, Richmond, CA, USA:

Two-dimensional PAGE apparatus and required reagents.

Eastman Kodak Co., Rochester, NY., USA:

X-ray film, developer and fixer.

Molecular Dynamics, Sunnyvale, CA., USA:

Laser densitometer (model 300 A), Image Quant™ (version 3.3) software.

Sigma Chemical Co., St. Louis, MO., USA:

Protease inhibitors, theophylline, adenosine, buffer reagents.

Chapter 2. Optimization of Techniques.

2.10) *Introduction.*

Two-dimensional PAGE was used in this project to search for circulating protein markers in Alzheimer's disease and MS, and to investigate changes in relative protein abundance in neurological disease. What follows is a description of the development of the technique, and factors that affect reproducibility. As reproducibility of the techniques was a major factor in determining whether any observed protein changes were significant, much of this section focused on the issue of reproducibility.

There are many published reports of protein variation in neurological disease. These studies usually had two goals. The first was to detect proteins whose appearance could be used to diagnose the presence or severity of individual diseases. The second was to provide information about changes that may help to understand the aetiology of the disease. Many of these studies used the 2-dimensional PAGE technique developed by O'Farrell (1975) to search for protein variation.

The term 2-dimensional electrophoresis has been used to describe a variety of methods employing separation of molecules in two different electrophoretic systems. The term high resolution two-dimensional PAGE is now specifically used to describe the method of protein separation that separates proteins according to their isoelectric points in the first dimension (in the presence of carrier ampholytes, and after the reduction of disulphide bonds), followed by separation in the second dimension, using SDS PAGE, according to differences in molecular weight (Dunbar, 1990).

High resolution 2-dimensional PAGE can resolve individual proteins contained within complex protein mixtures. Using this technique, it is possible to separate more than one thousand proteins from a complex mixture, the actual number depending upon the sample in question and

the methodology employed. Although the resolution of the technique is high it only separates a subset of the total cellular proteins. The usefulness of the technique however, is reflected in the large number of citations to O'Farrell (1975). O'Farrell's method of 2-dimensional electrophoresis is the most widely used.

A recent development of this technique has come about with the development of computer hardware and associated software that allows gels to be digitised and then manipulated electronically. The technique is computer aided quantitative 2-dimensional gel electrophoresis, as has been described by Burgess-Cassler *et al.* (1989). This technique is considered more accurate and convenient than its qualitative forerunner, as the analysis of 2-dimensional gels can be presented numerically, as opposed to photographically, and often, in a statistically meaningful way. The present project used computer aided quantitative 2-dimensional PAGE for analysis of protein variation in Alzheimer's disease and MS.

2.11) Development of 2-dimensional PAGE.

Raymond was one of the first investigators to use the 2-dimensional PAGE technique (Raymond, 1964). Many other researchers used modified forms of this technique to separate a diverse range of proteins, from plasma proteins to ribosomal proteins (Kaltschmidt *et al.*, 1970). However, there were major limitations to all of these techniques. There was a relatively low level of resolution and most of the proteins separated tended to cluster around a diagonal line from the origin. The reason for this was that the factors influencing the mobility of proteins in each dimension were not totally independent. Commonly, protein size was influencing mobility in both dimensions, with the resultant diagonal clustering.

These limitations were partially overcome by using isoelectric focusing in gels for the first dimension, and electrophoresis in the presence of SDS in the second dimension. Barrett *et al.* (1973) used this technique in the study of the tissue specificity of non-histone proteins associated with chromatin. The resolution of the technique was greatly enhanced by O'Farrell (O'Farrell,

1975). O'Farrell solubilised the sample proteins with urea and Triton X-100, and then treated them with 2-mercaptoethanol to reduce disulphide bonds and further denature the protein. The proteins were then separated according to their isoelectric points in the first dimension by isoelectric focusing in a polyacrylamide tube gel. A 3.5% polyacrylamide gel was used to avoid molecular sieving effects on all but the largest of proteins, and to ensure that the molecular weights of the proteins had little influence on their mobilities. In the second dimension, proteins were separated according to their molecular weight. This was achieved by attaching the tube gel to the top of a polyacrylamide slab gel, and then separating the proteins via discontinuous SDS electrophoresis, as first described by Laemmli (1970). The technique of O'Farrell separated proteins according to two completely independent properties, the result being a more uniform distribution of protein spots over the 2-dimensional field. Also, the protein spots were much smaller and more intense due to the high resolution of isoelectric focusing. Using this procedure, O'Farrell was able to resolve over one thousand proteins from *E. coli*, and he calculated a theoretical resolving power of five thousand proteins, including proteins that differed by as little as one charge unit. However, in practice, this level of resolution is difficult to achieve.

The 2-dimensional PAGE procedure of O'Farrell, as described above, has been modified by O'Farrell and other investigators to enable proteins of various kinds, and from greatly differing sources, to be better resolved. For example, better resolution of basic proteins can be obtained by using NEPHGE for the separation in the first dimension (O'Farrell *et al.*, 1977). In this technique, the sample is loaded onto the anodal (ie. acidic) end of the isoelectric focusing gel, the separation being stopped before the most basic proteins have reached the cathodic end of the gel. The resolution of basic proteins has also been improved by incorporating basic amino acids into the focusing gel, and by using concentrated cathodic solutions. Both methods have been used to extend and stabilise the cathodic region of the pH gradient in the focusing gel using O'Farrell's original equilibrium technique (Breithaupt *et al.*, 1978; Nguyen *et al.*, 1977; Tracey *et al.*, 1982). Also, the conditions of sample preparation have been modified from O'Farrell's original procedure, in which proteins were solubilised using urea and Triton X-100. SDS is now usually added to further increase the solubility of proteins in the sample. SDS was used for this purpose in this project. It

has been demonstrated that as long as the ratio of Triton X-100: SDS is 8:1, or greater, the SDS will not interfere with the isoelectric focusing step (Ames *et al.*, 1976). 2-Mercaptoethanol was shown by Righetti *et al.*, (1982) to cause flattening of the pH gradient at the cathodic end, and thus contributes to poor resolution of basic proteins. This problem can be overcome by substituting dithiothreitol for 2-mercaptoethanol (Gelsema *et al.*, 1989), although cost then becomes an issue. More recently, immobilised pH gradients have been used to overcome the problem of pH gradient instability over time. This instability can result in "cathodic drift" and the "plateau phenomenon", both of which result from electroendosmotic effects (Righetti, 1982). The technique of IPG IEF (immobilised pH gradient isoelectric focusing) involves covalently binding carrier ampholytes to the polyacrylamide support matrix. As well as greatly improving the resolution of basic proteins, this technique allows focusing over a narrow pH gradient.

Resolution of the 2-dimensional technique has been increased by scaling up the dimensions of the two dimensional field, using so called "giant" gels. The "giant" gel format was originally developed by Young *et al.* (1984). Using this format, they showed the existence of steroid inducible protein synthesis. Since then, many other researchers have used large format gel systems to detect new or modified gene products (eg. Levenson *et al.*, 1989; Maytin *et al.*, 1985). More recently, the utility of the large format gel system in comparison to the more common intermediate format gel system was investigated by Levenson. He concluded that while the large format gel system was both more expensive and labour intensive, it yielded the greatest resolution (Levenson *et al.*, 1990).

2.12) Reproducibility of 2-dimensional PAGE.

One limitation of 2-dimensional PAGE is reproducibility. Gel to gel variability is nearly always seen (McConkey, 1979). Both the position and the intensity of the spots vary slightly from gel to gel.

Variation in spot position occurs due to minor variations in the mobility of individual

proteins within the sample. Many factors could cause this variation. Such factors include differences in the pH gradient (as could be caused by any variation in electrolytes used in the first dimension), temperature effects (which will influence the rate of migration for instance), and slight differences in electrophoretic conditions in either dimension (for example, if the running buffer concentration for the second dimension is altered slightly, it can influence the temperature, duration, and amount of current during the SDS-PAGE separation). Other factors include stretching of the first dimension gel, inhomogeneities in polyacrylamide concentration, and minor variations in sample preparation, which may affect the degree to which individual proteins in the sample are solubilised, or may modify some of the proteins (eg. by carbamylation). This list covers only the major sources of variation in spot size and is not comprehensive.

There are several factors that influence the intensity of protein spots on the second dimension gel. This includes the isoelectric focusing conditions. Conditions that promote tight banding patterns will result in more discrete and intense protein spots in the second dimension slab gels. For example, any variation in isoelectric focusing conditions will influence spot intensity. Variation in the amount of protein loaded onto the isoelectric focusing gels will also result in spot intensity variability. The transfer of the protein from the first dimension gel to the second dimension gel will also result in variability. The physical manipulation of the first dimension gel as it extruded from the focusing tube and as it is put onto the second dimension gel also has substantial effects. Stretching or contraction of the gel can affect spot shape and to a certain extent spot size. Other factors can produce a difference between the amount of protein loaded onto the first dimension gel and the amount of protein transferred to the second dimension gel. There are several sources of such protein loss in the 2-dimensional PAGE system before the loading of the IEF gel onto the slab gel. The first is sample preparation as discussed above. The second is protein adsorption to activated surfaces on pipette tips, Eppendorf tubes, loading tips etc. A third source of protein loss is the process of equilibrating the IEF tube gels. This process is associated with loss of low molecular weight proteins, and also, diffusion of the protein bands, which can result in a less well defined border for the protein spots.

A second type of variability is that which is seen to occur between individuals electrophoresing the same type of sample. This mainly occurs because different laboratories use different procedures, often resulting in different peptide patterns being obtained for the same sample type (Gorg, 1991).

2.13) Quantitative 2-dimensional PAGE.

Developments in computer hardware and software (which allows gels to be digitised and manipulated electronically) has produced computer aided quantitative 2-dimensional PAGE. Many studies are now being done, using quantitative 2-dimensional PAGE. For example, Higginbotham *et al.* (1991) used the technique for studying inbred lines of maize; Humphrey-Smith *et al.* (1992) used the technique for studying tissue specific mitochondrial polypeptides; and Taylor *et al.* (1992) used the technique for detection of mutations.

Digitisation of 2-dimensional gels can be performed using either a high resolution video camera or a scanning densitometer (as used in this project). After digitisation, gels can be qualitatively compared (via the superimposition of scanned images) and quantitatively compared, via comparison of relative protein abundance data (Garrels, 1983). The initial image processing step in quantitative analysis is common to all studies, and involves the selection of proteins for study, optimisation of the image, subtraction of background, and the calculation of relative protein abundance data. The data is then processed to allow comparisons to be made between gels (Zeineh *et al.*, 1987). This processing is necessary to remove effects that cause all of the spots on a particular gel to be darker or lighter than on a typical gel. Various methods are currently used for this processing. Kuick *et al.* (1987) compared the commonest methods.

An important factor in quantitative 2-dimensional PAGE is the method of protein visualisation. Methods vary between researchers. In this project, Coomassie blue staining, and fluorography of ^{14}C-labelled proteins, were the methods used for protein visualisation.

2.14) Protein visualization by Coomassie blue staining.

Except for the naturally coloured proteins such as myoglobin, ferritin, and cytochrome c, the visualisation of proteins on gels requires the use of dyes and stains, or, the incorporation of radioactive isotopes into the proteins, and subsequent detection of the radioactively labelled proteins.

The triphenylmethane stain Coomassie blue, is the most sensitive of a number of organic protein stains, including Fast Green and Amido Black (Dunbar, 1987). Originally developed as an acid wool stain, Coomassie blue was first used by St. Groth in 1963 to stain proteins separated by electrophoresis (St. Goth, 1963). The dye used in this instance was Coomassie blue R -250 (the "R" means that the dye has a reddish hue, while the "250" is an ICI dye strength indicator (Merril, 1990)). Other Coomassie stains have been developed since Coomassie blue R-250 was first used. For example, Coomassie blue G-250 was developed for use in post electrophoresis staining. Coomassie blue G-250 exhibited a marked decrease in solubility in 12% trichloroacetic acid, which allowed it to be used as a colloidal suspension. This prevented the dye from penetrating the gel, and so allowed more rapid staining of proteins without undesirable background staining (Diezel, *et al.*, 1972). More recently, Neuhoff (1988) showed that Coomassie blue R-250 could be made to exhibit better colloidal properties when ammonium sulphate and additional methanol were added to the staining solution. This resulted in similar results to those obtained using Coomassie blue G-250. Another Coomassie stain, Violet R-150, has been developed for use with polyacrylamide gels. This stain has the desirable characteristics of not staining carrier ampholytes (a problem with the Coomassie blue stains) and requiring limited destaining. This stain is not as sensitive as the Coomassie blue stains however (Radda, 1980).

For Coomassie blue staining, an acidic medium is required (Merril, 1990). Protein staining is thought to occur via ionic interactions between the dye molecules and the basic amino acid residues of the protein, forming a dye-protein complex (Righetti, *et al.*, 1978). This complex is further stabilized via secondary interactions. These interactions are due to hydrogen bonding, van

der Waals forces, and hydrophobic bonding between the dye molecules and the protein, and also between the free dye molecules and the protein bound molecules (Dunbar, 1987). This ionic interaction was shown to be reversible (St. Groth, 1963). All of the above interactions can be influenced by factors such as pH, ionic strength, and solvents used. It is quite likely therefore that proteins will bind different amounts of dye under different conditions (Wilson, 1983). The number of lysine, histidine, and arginine residues in the protein that are accessible to the dye has been shown to correlate reasonably well to the intensity of staining (Tal *et al.*, 1985). This provides further evidence for the binding of the Coomassie blue dye molecules to the basic amino acid residues in the proteins. Usually 0.2 to 0.5 μg of protein are required for Coomassie blue staining (Hames, 1981). While the Coomassie stains do appear to give linear binding responses for protein ranges up to 20 μg per spot, the relationship between stain density and protein concentration varies from protein to protein (Meyer *et al.*, 1965). Staining variations can also occur if SDS is bound to the proteins (Matheka *et al.*, 1977).

Coomassie stains do not possess ideal qualities for the quantitative staining of proteins on gels for the reasons outlined above. The variation in Coomassie blue staining between different proteins (Tal *et al.*, 1985) has important implications when quantitating Coomassie blue stained gels. If a standard curve is to be used to quantify absolute amounts of various proteins, care will be needed when selecting a "standard" protein for the calibration curve. If the standard protein has a higher or lower proportion of stain reactive groups than a typical protein, then the curve will either underestimate or overestimate the abundance of proteins with normal amounts of such reactive groups.

The following experiments were carried out to find the conditions that yielded the greatest separation and reproducibility. This was necessary so that proteins from affected individuals could be compared with protein samples obtained from appropriate age and sex matched controls.

2.2) *Isoelectric focusing and pH gradient formation.*

2.21) Materials and Methods

Isoelectric focusing, in the presence of carrier ampholytes, is the first dimension protein separation technique used in 2-dimensional PAGE. The following experiments were carried out to determine the conditions that yielded the most linear and reproducible pH gradient. The first dimension IEF gels were 105 mm long, with an external diameter of 2.5 mm. The glass tubes in which the gels were cast were approximately 140 mm long, with an internal diameter of 1.5 mm, and a wall thickness of 1 mm. Before the gels were cast, the glass tubes were washed in a dichromate cleaning solution (6.3 % (w/v) $K_2Cr_2O_7$; 62 % (v/v) H_2SO_4), and silanized by immersion in 10% (v/v) hexamethyldisilazane (in hexane) for ten minutes. The tubes were then left to dry at room temperature for a minimum of four hours. A Parafilm seal was then placed at one end of the tube, and a mark was made on the tube 105 mm from the sealed end. The IEF gels consisted of (16 ml total volume) 4% (v/v) glycerol ; 7.8 M urea (BioRad); 3.4% (w/v) acrylamide: N,N'-methylene-bis-acrylamide in a ratio of 36.5:1 (w/v) (BioRad); 1.7% (w/v) Triton X-100 ; 7.15 mM arginine monohydrochloride (Sigma); 7.2 mM lysine monohydrochloride (Sigma); 7.2 mM aspartic acid; 1.7% (w/v) pH 3-10 Pharmalytes (Sigma); 0.85% (w/v) pH 4-6.5 Pharmalytes (Sigma); and 0.85% (w/v) pH 5-8 Pharmalytes (Sigma). The gel mixture was filtered through Whatman #1 filter paper and degassed for approximately 10 minutes. 30 µl of 10% (w/v) ammonium persulphate (BioRad) and 20 µl of TEMED (BioRad) were added, and the gel mixture was cast into the glass tubes using a BioRad loading needle and a disposable syringe. The gel mixture was overlaid with 20 µl 8 M urea, and left to polymerise for 90 minutes. The 8 M urea was removed, and replaced with diluted IEF buffer, which contained 8 M urea; 1.7% (w/v) Triton X-100; 1.7% (w/v) pH 3-10 Pharmalytes; 0.83% (w/v) pH 4-6.5 Pharmalytes; 0.83% (w/v) pH 5-8 Pharmalytes; and 4.2% v/v 2-mercaptoethanol (Sigma). After one hour, this solution was removed, and replaced with 20 µl of undiluted IEF buffer, containing 9.6 M urea; 2% (w/v) pH 3-10 Pharmalytes; 1% (w/v) pH 4-6.5 Pharmalytes; 1% (w/v) pH 5-8 Pharmalytes; and 5% v/v

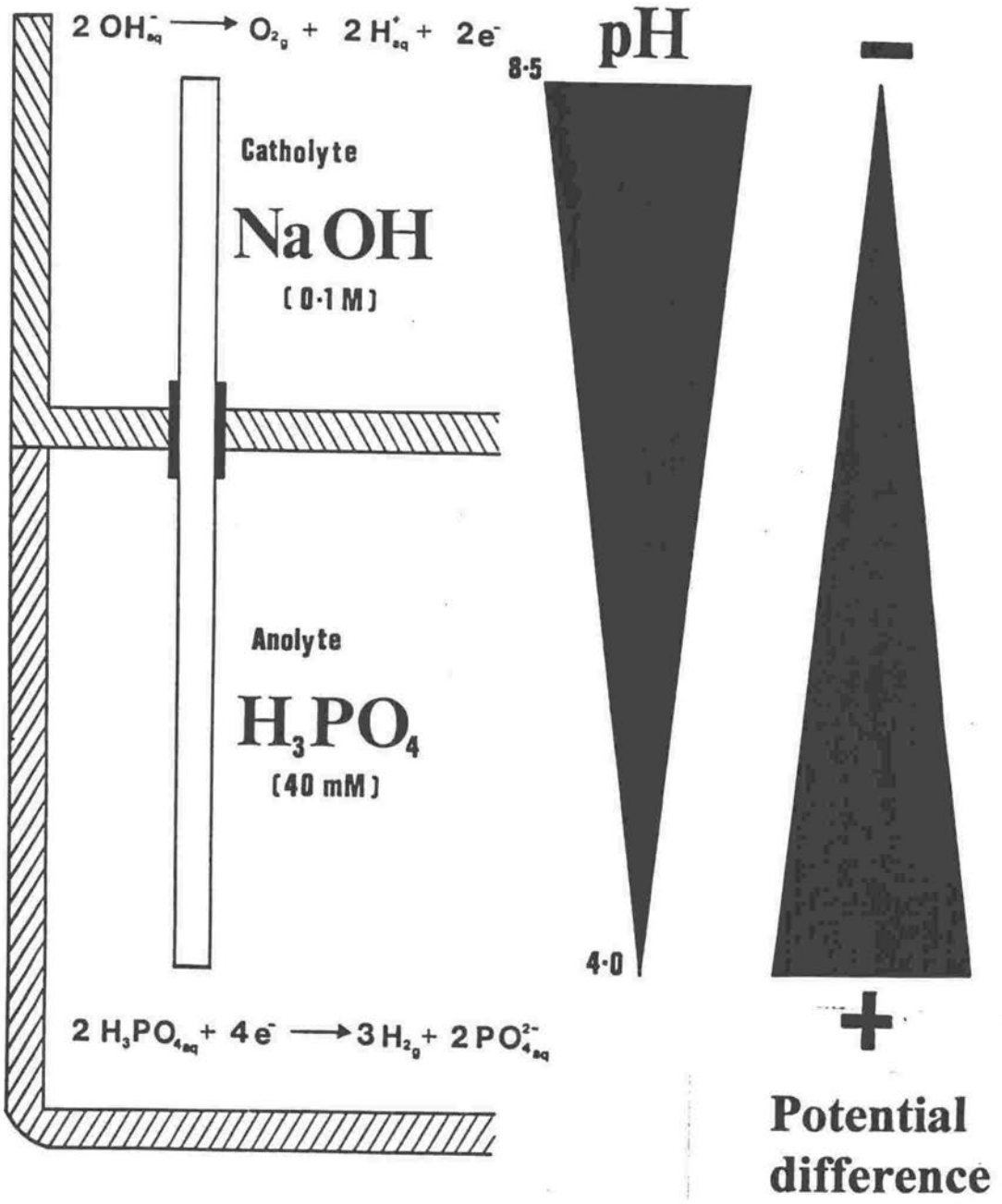
2-mercaptoethanol. The gels were placed in the IEF chamber (BioRad model 175 Tube Cell) so that the top surface of the gels was 1.5 cm below the surface of the catholyte. The catholyte was 0.1 M NaOH (which was boiled and degassed before use), and the anolyte was 0.04 M H_3PO_4. Figure 2.20 illustrates the IEF conditions employed. The Tube Cell was then placed on a magnetic stirrer (the anolyte was continuously stirred throughout focusing) and attached to a cooling water supply. The gels were focused at 200 V for one hour, using a BioRad model 1000/500 power supply.

The sample for separation was prepared by adding one volume of sample to one volume of undiluted IEF buffer and one volume of solid urea (for this purpose, 1 mg of solid urea was taken as equivalent to 1 µl). After vortexing to dissolve the urea, the sample preparation was centrifuged at 10,000 rpm for ten minutes (room temperature) using a Heraeus Biofuge A centrifuge, and kept at 37 °C using a Techne Dri-Block©, before loading.

The IEF tubes were removed from the Tube Cell, and the tops of the gels were rinsed several times with water to remove NaOH. The prepared sample was loaded onto the top (ie. cathodal) surface of the IEF gels, the volume loaded depending upon the sample type, and whether Coomassie blue was to be used to visualise the proteins. The sample was overlaid with 20 µl of solution containing 9 M urea; 1% (w/v) pH 3-10 Pharmalytes; 0.5% (w/v) pH 4-6.5 Pharmalytes; and 0.5% (w/v) pH 5-8 Pharmalytes. The tubes were placed back into the Tube Cell, fresh catholyte was added, and the gels were focused for twenty hours at 400 V, and one hour at 800V.

After focusing, the tube gels were extruded from the glass tubes by applying pressure to the tops of the gels via water applied with a syringe. The syringe was attached to the top of the glass tubes with a short piece of flexible hose. The gels were then cut into 5 mm segments, each segment being placed into a small test-tube containing 1 ml of boiled, degassed, 0.01 M KCl. The tubes were capped, and left for two hours with occasional shaking. After two hours, the pH of the eluate was measured at 20 °C using a pH meter with a single electrode.

Figure 2.20. The isoelectric focusing conditions used in this study.



Several parameters were investigated to determine which would yield the most favourable and reproducible pH gradient. First, the effect of tube height in the focusing chamber was investigated, to decide whether a larger head of pressure for the catholyte would contribute to the collapse of the basic portion of the pH gradient. For this experiment, six tube gels (set #1) were placed in the tube cell, so that the tops of the IEF gels were 3 cm below the top surface of the tube cell floor, and six tube gels (set #2) were placed so that the top surfaces of the IEF gels were 6.5 cm above the top surface of the chamber floor. This resulted in a head of pressure of 1.5 cm NaOH (approximately 14.7 Pa) for set # 2, and a head of pressure of 11.0 cm NaOH (approximately 107.9 Pa) for set # 1. Both sets of gels were formed and focused as described previously.

Secondly, different Pharmalyte combinations were used to decide which combination would yield the most linear and reproducible pH gradient. Three separate sets, each of 4 IEF gels each were used. The first set had a Pharmalyte combination as previously described (ie. 1.7% (w/v) pH 3-10 Pharmalytes; 0.85 % (w/v) pH 4-6.5 Pharmalytes; and 0.85 % (w/v) pH 5-8 Pharmalytes). The second set of gels contained 1.7% (w/v) pH 3-10 Pharmalytes; 0.85% (w/v) pH 3.3-8.5 Pharmalytes; and 0.85% (w/v) pH 5-8 Pharmalytes. The third set of gels contained 1.7% (w/v) pH 3-10 Pharmalytes; and 1.7% (w/v) pH 6.5-10.5 Pharmalytes. All three sets of gels were focused together, as described previously. However, for sets # 2 and # 3, the IEF buffers used were modified to be compatible to the IEF Pharmalyte combinations that were being used. All other components of the buffer were the same as described previously. For set # 2, the Pharmalytes used in the IEF buffer were 2% (w/v) pH 3-10 Pharmalytes; 1% (w/v) pH 3.3-8.5 Pharmalytes; and 1% (w/v) pH 5-8 Pharmalytes. The IEF buffer of set # 3 contained 2% (w/v) pH 3-10 Pharmalytes; and 2% (w/v) pH 6.5-10.5 Pharmalytes.

Thirdly, the effect of various amino acid combinations on the pH gradient was investigated. Four sets of tube gels were used in this experiment, with three gels in each set. In the first set, the normal amino acid combination was used, ie. 7.15 mM arginine monohydrochloride; 7.20 mM lysine monohydrochloride; and 7.20 mM aspartic acid. In the second set, the amino acid combination was 14.3 mM arginine monohydrochloride, 14.4 mM lysine monohydrochloride; and 14.4 mM aspartic acid. In the third set, the amino acid combination was 21.5 mM arginine

monohydrochloride; 21.6 mM lysine monohydrochloride; and 21.6 mM aspartic acid. In the fourth set, no amino acids were added to the gel mixture, the volume being made up with double distilled water. All four sets of gels were focused together as previously described.

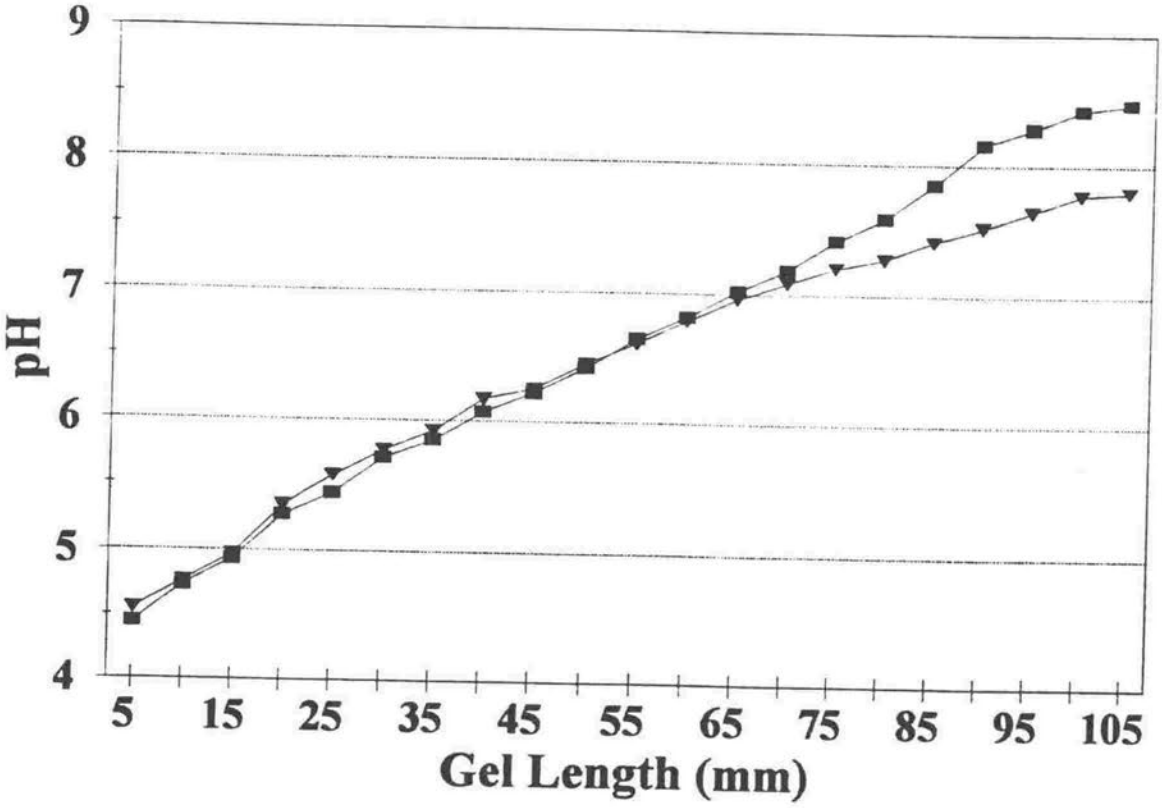
The fourth parameter investigated was the effect of various catholyte and anolyte concentrations on the pH gradient. For this experiment, four sets of IEF gels, with twelve gels in each set, were compared, the tube gel composition being that which was described originally. For the first set, standard catholyte (0.1 M NaOH) and anolyte (0.04 M H_3PO_4) concentrations were used. For the second set, the catholyte concentration was 0.05 M, while the anolyte concentration was 0.04 M. For the third set, the catholyte and anolyte concentrations were 0.02 M and 0.1 M respectively. For the final set, the concentration of the catholyte was 0.04 M, and the concentration of the anolyte was 0.1 M. Constant electrolyte volumes were used for each set, the focusing being carried out as described previously.

The final parameter investigated was the effect of protein load on the pH gradient. For this experiment, three sets of IEF gels (with four gels in each set) were used. The composition of the gels was that which was described initially, all three sets of gels being focused together. For the protein preparation, one volume of undiluted human plasma was mixed with one volume of undiluted IEF buffer, and one "volume" of urea (for this purpose, 1 mg of urea was considered to be equivalent to 1 µl). For set # 1, no plasma proteins were loaded onto the gels. For set # 2, 100 µg of protein was loaded onto each gel, and the gels in set # 3 each received a 750 µg protein load.

2.22) Results.

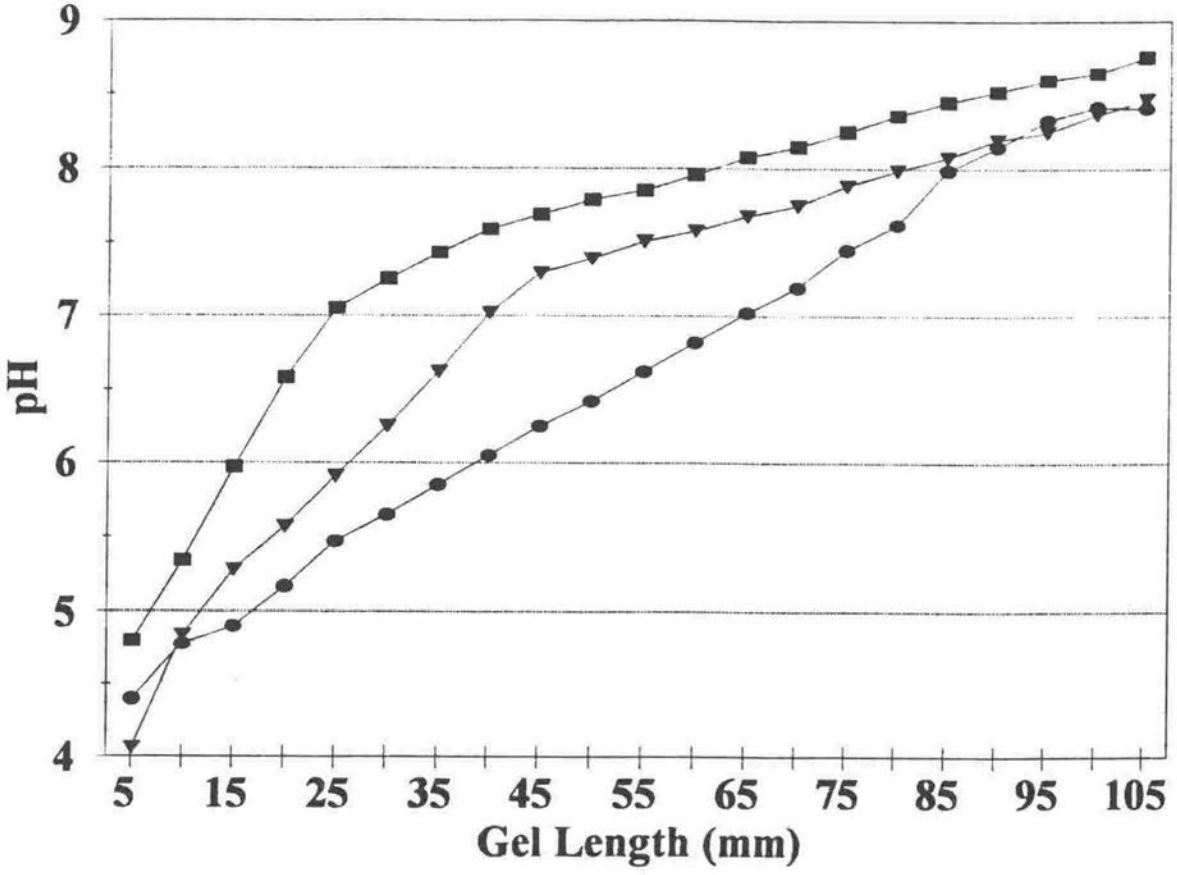
The results of the investigations outlined above are shown Figures 2.21 to 2.25. To aid clarity error bars are not shown. In addition, Figure 2.26 shows the standard deviation associated with each pH measurement for the various regions of a set of tube gels, made using the method found to give optimum results. Figure 2.27 shows the results that were obtained comparing the pH gradients of three sets, each of 12 IEF gels, focused on three separate occasions, approximately one week apart. Each data point, for all of the figures, represents the average pH value for all members of the set.

Figure 2.21. Effect of tube height in tube cell on the pH gradient of tube gels.



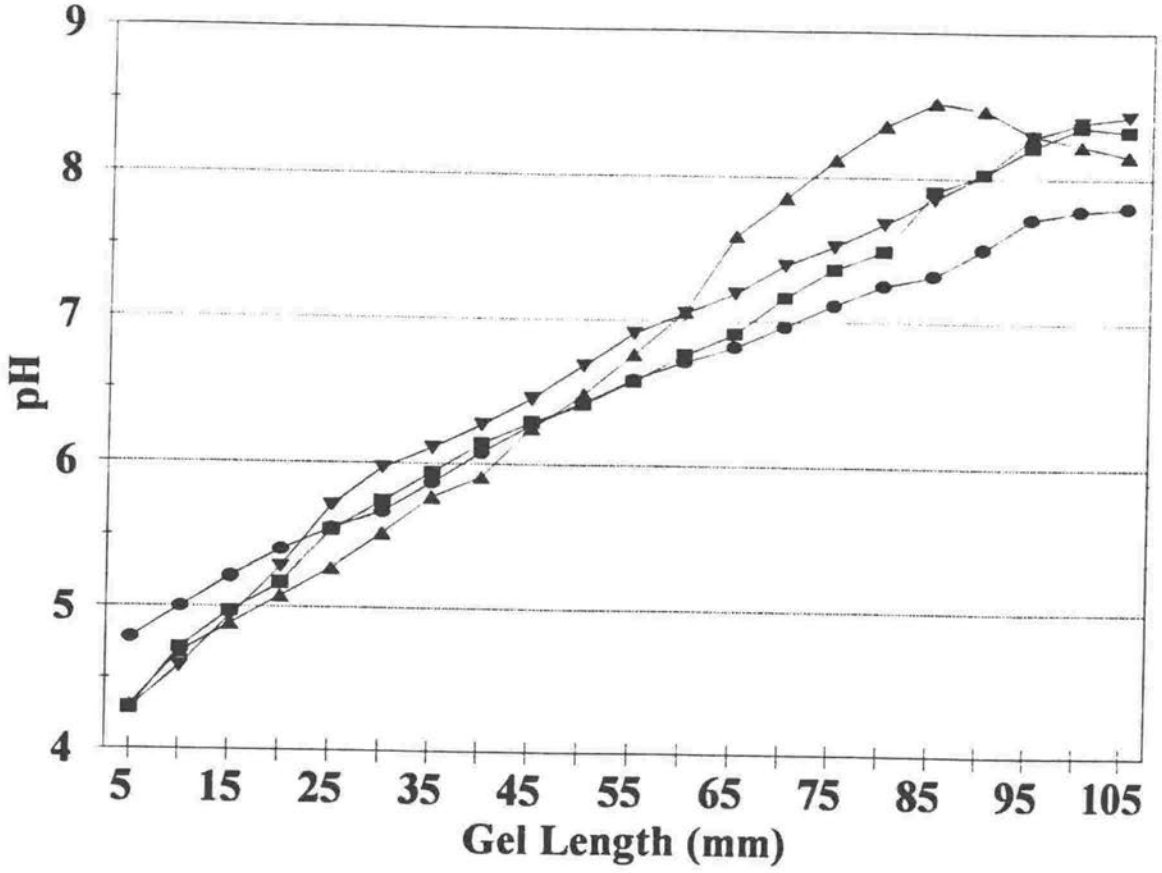
pH gradients in IEF gels placed at different heights in the tube cell were measured after IEF. (▼) set # 1. Tubes placed so that the top of the IEF gels had a head of approximately 11.0 cm NaOH. (■) set # 2. Tubes placed so that the tops of the IEF gels had a head of approximately 1.5 cm NaOH. The data points for each set represent the average of six gels. For set # 1, the average standard deviation between pH 4.0 and 7.0 was ± 0.06 pH units, while between pH 7.01 to 9.0, the average standard deviation was ± 0.15 pH units. For set # 2, the average standard deviations between these pH values were ± 0.05 and ± 0.15 pH units respectively.

Figure 2.22. ***The effect of Pharmalyte combinations on the pH gradient.***



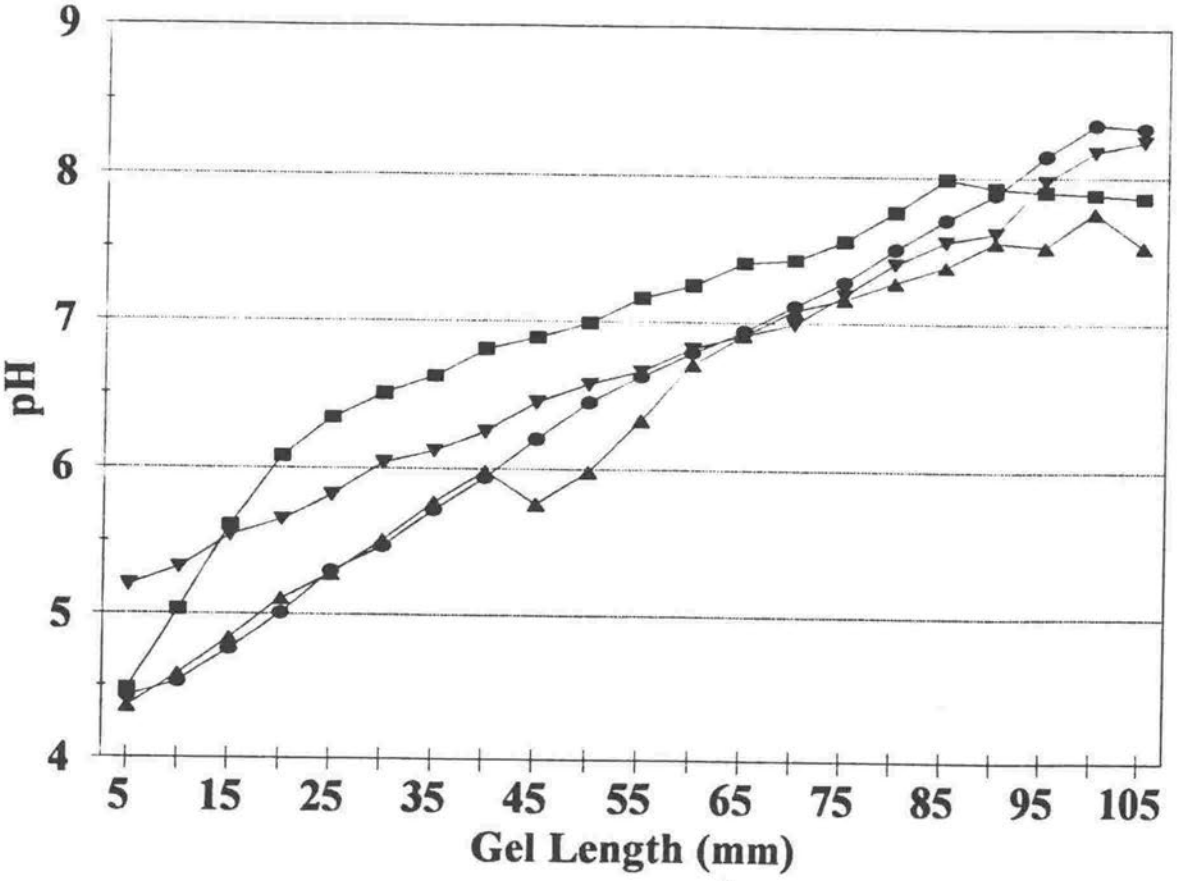
pH gradients in IEF gels with various Pharmalyte combinations were measured after IEF. (●) set # 1. 3.4% (w/v) Pharmalytes (pH 3-10; pH 4-6.5; pH 5-8; 2:1:1; v/v/v). (▼) set # 2. 3.4% (w/v) Pharmalytes (pH 3-10; pH 3.8-8.5; pH 5-8; 2:1:1; v/v/v). (■) set # 3. 3.4% (w/v) Pharmalytes (pH 3-10; pH 6.5-10.5; 1:1; v/v). The average standard deviations between pH 4.0 to 7.0 for sets 1 to 3 were ± 0.04, ± 0.04, and ± 0.05 pH units respectively. The average standard deviations for sets 1 to 3 between pH 7.01 to 9.0 were ± 0.14, ± 0.15, and ± 0.14 pH units respectively. Each data point for each set represents the average of four gels.

Figure 2.23. *Effect of amino acid composition on the pH gradient.*



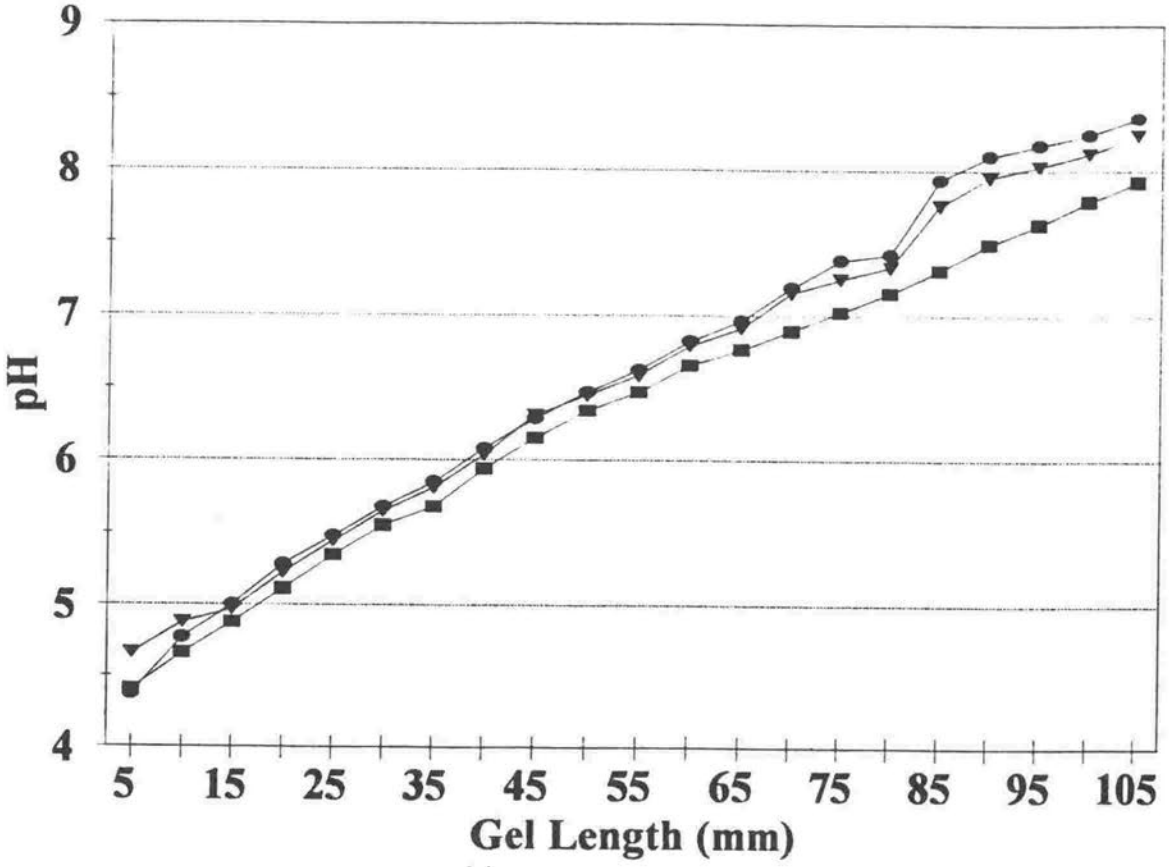
pH gradients in IEF gels with different amino acid combinations were measured after IEF. (■) set # 1. 7.15 mM arginine monohydrochloride; 7.2 mM lysine monohydrochloride and aspartic acid. (▲) set # 2. 14.30 mM arginine monohydrochloride; 14.4 mM lysine monohydrochloride and aspartic acid. (▼) set # 3. 21.45 mM arginine monohydrochloride; 21.6 mM lysine monohydrochloride and aspartic acid. (●) set # 4. No amino acids were added. Average standard deviations between pH 4.0 and 7.0 for sets 1 to 4 were ± 0.05, ± 0.05, ± 0.03, and ± 0.04 pH units respectively. The average standard deviations between pH 7.01 and 9.0 for sets 1 to 4 were ± 0.10, ± 0.17, ± 0.16, and ± 0.12 pH units respectively. Each data point represents the average of three gels.

Figure 2.24. ***The effect of electrolyte composition on the pH gradient of gels.***



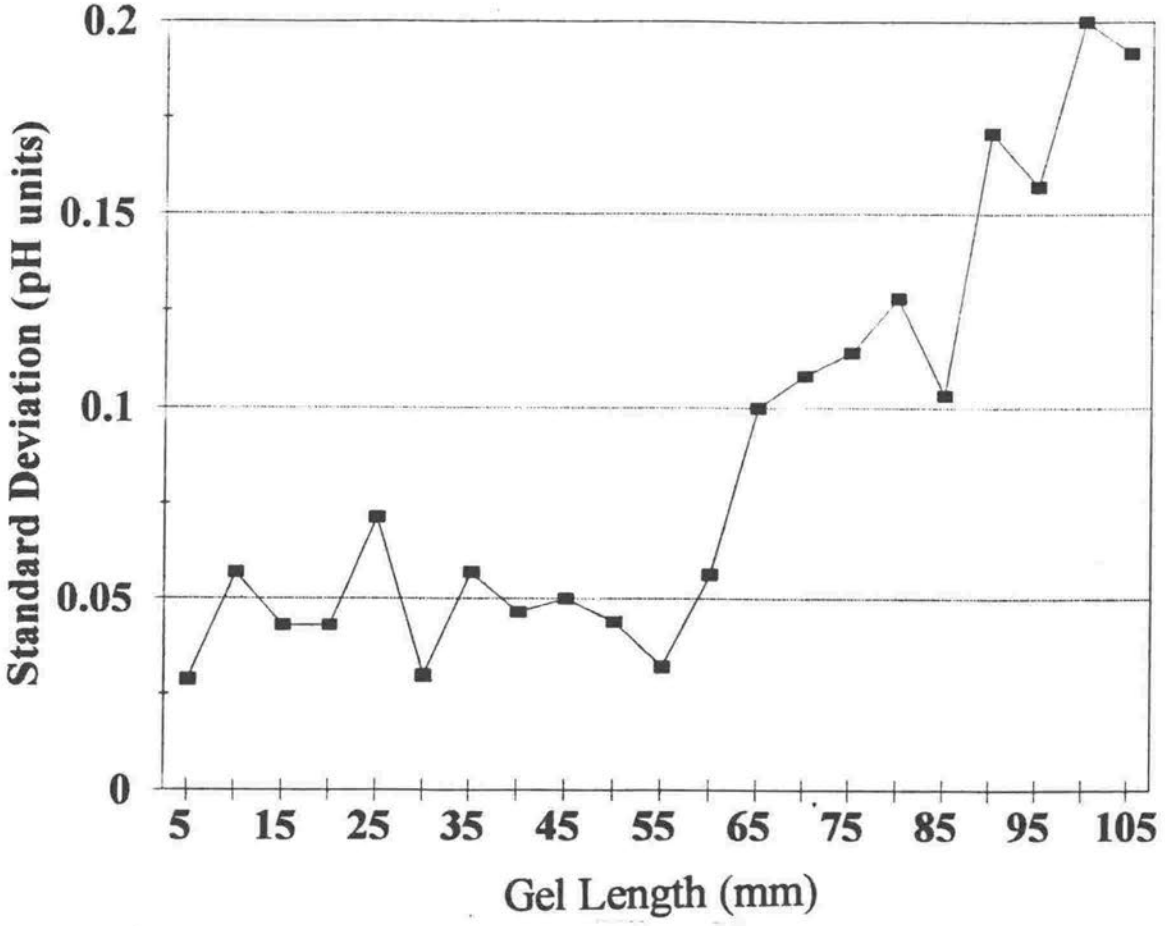
pH gradients were measured in IEF gels that were focused using different electrolyte combinations. (●) set # 1. Catholyte: 0.1 M NaOH; anolyte: 0.04 M H_3PO_4. (▼) set # 2. Catholyte: 0.05 M NaOH; anolyte 0.04 M H_3PO_4. (▲) set # 3. Catholyte: 0.1 M NaOH; anolyte: 0.1 M H_3PO_4. (■) set # 4. Catholyte: 0.2 M NaOH; anolyte 0.04 M H_3PO_4. The average standard deviations between pH 4.0 to 7.0 for sets 1 to 4 were ± 0.03, ± 0.06, ± 0.04, and ± 0.04 pH units respectively. The average standard deviations between pH 7.01 to 9.0 for sets 1 to 4 were ± 0.12, ± 0.17, ± 0.09, and ± 0.18 pH units respectively. Each data point for each set represents the average of twelve gels.

Figure 2.25. *The effect of protein load on the pH gradient.*



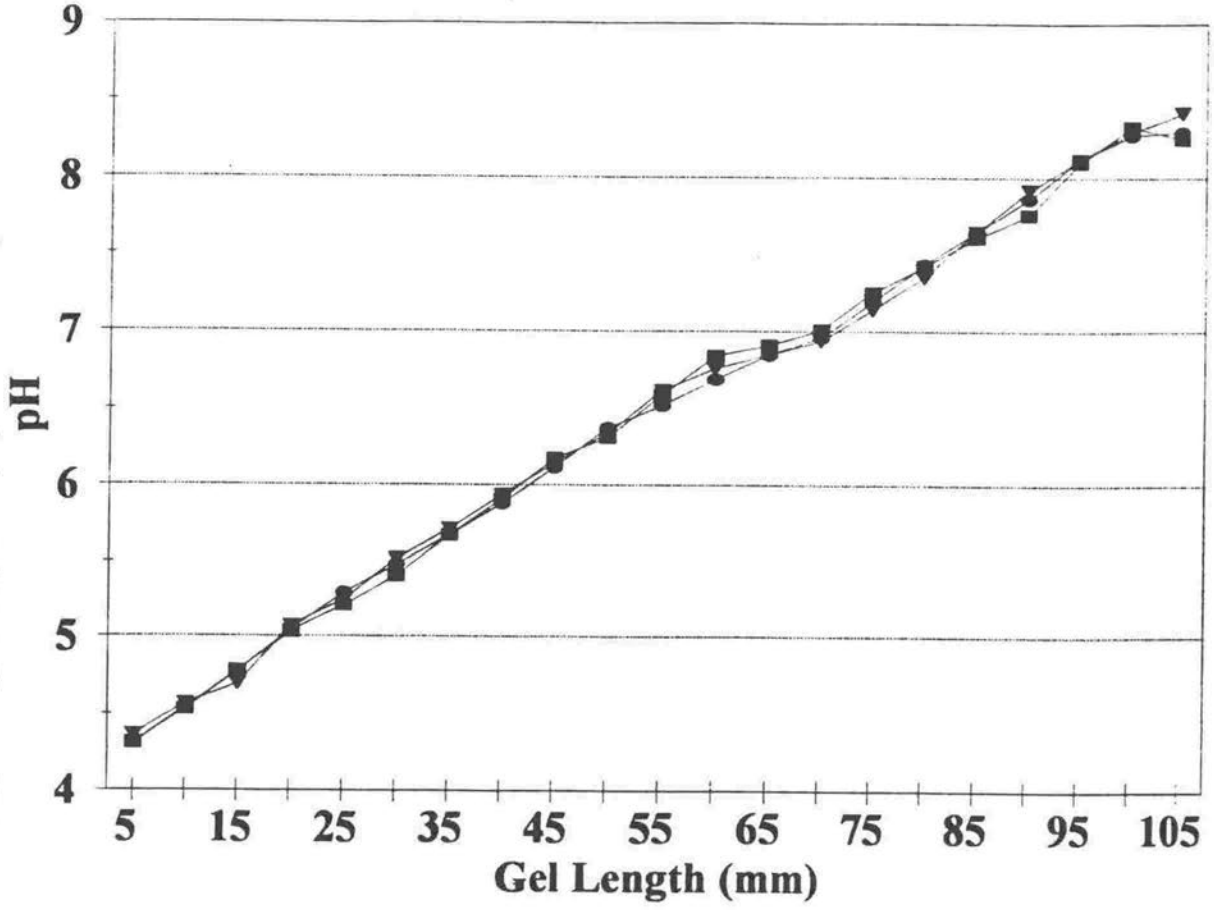
pH gradients were measured in IEF gels that had different loads of human plasma proteins. (●) set # 1. No plasma proteins loaded. (▼) set # 2. 100 µg of plasma proteins loaded. (■) set # 3. 750 µg of plasma proteins loaded. The average standard deviations between pH 4.0 to 7.0 for sets 1 to 3 were ± 0.05, ± 0.05, and ± 0.04 pH units respectively. The average standard deviations between pH 7.01 to 9.0 for sets 1 to 3 were ± 0.13, ± 0.12, and ± 0.14 pH units respectively. Each data point for each set represents the average of four gels.

Figure 2.26. Reproducibility of the pH gradient formed in gels focused at the same time.



pH gradients were measured in IEF gels of the standard composition after focusing. Each point represents the standard deviation of measurements from twelve gels.

Figure 2.27. Reproducibility of the pH gradient formed in gels focused at different times.



pH gradients were measured in IEF gels focused at three different times, using the same methods and reagents. The range of standard deviations between pH 4.0 to 7.0 for the 3 sets was 0.04 to 0.05 pH units. The range of standard deviations between pH 7.01 and 9.0 was 0.14 to 0.17 pH units. Each data point for each set represents the average of twelve gels.

2.23) Discussion.

Figure 2.21 shows the effect of tube height in the isoelectric focusing chamber on the pH profile of the IEF gels. Tube position affected the pH gradient. A possible explanation for this observation is that the greater head of NaOH pressure that existed for the gels of set # 1, caused greater penetration of the NaOH into the gel matrix after the gel overlay had entered the gel. This could overcome the buffering capacity of the ampholytes and amino acids in this region and cause the local pH gradient to collapse.

Figure 2.22 shows that different Pharmalyte combinations affected the shape and linearity of the pH gradient in IEF gels. The optimum Pharmalyte combination was that of set # 1; 1.7 % (w/v) pH 3-10 Pharmalytes; 0.85 % (w/v) pH 4-6.5 Pharmalytes; 0.85 % (w/v) pH 5-8 Pharmalytes. Sets # 1 and # 2 were different except for the most basic 25 mm of the gel. Sets # 1 and # 3 were different over their entire length. The gels from sets # 2 and # 3 had extended basic regions, presumably due to their greater content of ampholytes with basic pI values. Although the pH gradient of the gels from set # 2 and set # 3 covered a larger pH range than the pH gradient of the gels of set # 1, their gradients were not linear, and had much smaller acidic regions. For set #2, the acidic region averaged 40 mm. For set #3, the acidic region averaged 20 mm. However, for set #1, the acidic region averaged 65 mm. I decided that the Pharmalyte combination of set # 1 would give the best separation of plasma proteins, as it resolved more neutral to acidic proteins; there are 429 proteins with neutral to basic pI values in the database of Anderson *et al.* (1991) compared to 298 proteins with basic pI values. The reason that the gels of set # 1 had a more linear pH gradient than the other sets is probably the greater proportion of Pharmalytes in the pH 4-8 region. This region requires a greater concentration of Pharmalytes due to the dilution effect of water. Water molecules will be concentrated in the pH 7 region of the gradient due to the repulsion of H^+ ions from the anode and OH^- ions from the cathode. This will dilute the concentration of Pharmalytes in this region of the gradient.

The amino acid composition of the gel mixture was found to affect the pH profile of the

IEF gels. The addition of aspartic acid (pI 2.77), to buffer the pH gradient at the acidic end, and the addition of lysine (pI 9.74) and arginine (pI 10.76), to buffer the basic end of the pH gradient, resulted in an increased range of the pH gradient by up to 1.17 pH units (shown by Figure 2.23). This observation is consistent with that made by Breithaupt *et al.* (1978). Figure 2.23 showed that for the most acidic 55 mm of all gels that have amino acids in their composition, the pH profile did not vary. This showed that the buffering capacity of the lowest aspartic acid concentration (ie. 7.2 mM) was adequate for this region of the gel. The pH profiles for all sets over remaining gel length however, are different except the most basic 25 mm of sets # 2 and # 3.

Figure 2.24 shows that different electrolyte concentrations altered the pH profiles of the IEF gels. The most linear gradient was formed when the anolyte was 0.04 M H_3PO_4, and the catholyte was 0.1 M NaOH. When a higher catholyte concentration was used (set #4; 0.2 M NaOH), a less linear pH gradient was produced, with cathodic drift being evident. When a lower catholyte concentration was used (set #2; 0.05 M NaOH), a less linear pH gradient was obtained, which covered a reduced pH range. And when higher anolyte concentrations were used (set #3; 0.1 M H_3PO_4), a less linear gradient was obtained, which also covered a reduced pH range. These observations are consistent with those of Nguyen *et al.* (1977).

Figure 2.25 shows that protein load had an effect on the pH gradient in IEF gels. An increased protein load resulted in a flattening of the cathodic region of the pH gradient. This observation is consistent with those published by Cantrell *et al.* (1981). However, as the protein preparation used also included SDS, urea, 2-mercaptoethanol, and Triton X-100, it is possible that one of these agents may have been responsible for the flattening of the cathodic region of the pH gradient. 2-Mercaptoethanol in amounts within 30% of those used in the protein preparations in this study, was shown by Righetti *et al.* (1982) to cause flattening of the pH gradient in the cathodic region. 2-Mercaptoethanol ionizes at the cathodic end of the gel and migrates towards the anode, sweeping away the already focused Pharmalytes, until it reaches its isoelectric point. The buffering capacity of Pharmalytes in the pH 8-10 region of the IEF gels used in this study, was approximately 3 μequiv. pH^{-1} ml^{-1}. The buffering capacity for 2-mercaptoethanol used in the

protein preparation was approximately 0.3 μequivalents pH^{-1} ml^{-1}. When any species has a buffering capacity of at least 10% of that of the surrounding Pharmalytes, the pH gradient in IEF gels will be affected. This problem can be overcome by substituting dithiothreitol for 2-mercaptoethanol when loading large amounts of protein onto IEF gels. In this project, 2-mercaptoethanol was used throughout, but in an attempt to overcome the problems associated with its use, protein loads were kept as small as possible.

From the results outlined above, I decided upon a set of standard conditions for isoelectric focusing. The IEF gel mixture consisted of 4% (v/v) glycerol; 7.8 M urea; 3.4% (w/v) acrylamide: N, N'-methylene-bis-acrylamide, in a ratio of 36.5:1 (w/w); 1.7% (w/v) Triton X-100; 7.15 mM arginine monohydrochloride; 7.2 mM lysine monohydrochloride; 7.2 mM aspartic acid; 1.7% (w/v) pH 3-10 Pharmalytes; 0.85% (w/v) pH 4-6.5 Pharmalytes; 0.85% (w/v) pH 5-8 Pharmalytes; 0.02% (w/v) ammonium persulphate; and 0.001% TEMED (v/v). IEF tube gels were placed in the tube cell so that the top surface of the gel was 1.5 cm below the surface of the catholyte, the catholyte being 0.1 M NaOH, and the anolyte being 0.04 M H_3PO_4. When loading plasma proteins, the maximum protein load was set at 350 μg.

Figure 2.26 shows that the reproducibility of the pH gradient of a typical IEF gel produced using the method outlined above, was less for the more basic regions of the gel. This was due largely to cathodic drift. Figure 2.27 shows the pH gradients from three sets of gels, electrophoresed at different times (the gels were electrophoresed at approximately one week intervals, using the same stock reagents). No obvious difference existed for any of the data points.

2.3) *The relationship between protein abundance and measured intensities of Coomassie blue staining.*

2.31) Introduction.

An aim of this project was to investigate changes in amounts of individual proteins in neurological disease. This required quantitative analysis of proteins separated by 2-dimensional PAGE, and visualised using Coomassie blue staining. Others have shown that Coomassie blue does not bind to all proteins consistently (St. Groth, 1963). Also, the relationship between protein load and staining intensity is non linear for certain proteins (Wilson, 1983). I therefore investigated the relationship between protein load and VI values for human plasma proteins.

2.32) Materials and Methods.

The relationship between protein load and quantitative response was investigated for a purified protein (BSA) separated by 1-dimensional electrophoresis and for human plasma proteins separated by 2-dimensional PAGE.

2.321) The relationship between protein load and the quantitative response for BSA.

Differing amounts (5 - 30 μg) of BSA were separated on a 10% 1-dimensional polyacrylamide gel using discontinuous SDS electrophoresis (Laemmli,1970). Samples were prepared by adding equal volumes of BSA stock solution (1 mg ml^{-1}) and sample buffer (0.08 M SDS, 0.06 M Tris, 10% (v/v) glycerol, 5% (v/v) mercaptoethanol, pH 6.8). The solution was heated in a boiling water bath for four minutes. After cooling, 5 μl of 0.1% (w/v) bromophenol blue was added to the solution, and the volume was adjusted to 70 μl with distilled water. The samples were then vortexed to mix, and centrifuged at 10,000 x *g* for five minutes at room

temperature to remove any insoluble proteins.

The gels were prepared and electrophoresed using BioRad Protean II vertical slab cell equipment. Slab gels were 1 mm thick. The resolving gel measured 13.5 cm x 20 cm, and contained 10% (w/v) acrylamide: N, N'-methylene-bis-acrylamide, in a ratio of 36.5:1 (w/v); 0.38 M Tris (pH 8.8); 0.1% (w/v) SDS; 0.05% (w/v) ammonium persulphate; and 3 mM TEMED. The stacking gel consisted of 3.9% (w/v) acrylamide: N, N'-methylene-bis-acrylamide; 0.1 M Tris (pH 6.8); 0.1% (w/v) SDS; 0.05% (w/v) ammonium persulphate; and 9.8 mM TEMED. A comb was inserted into the stacking gel before polymerization, to form wells for sample application. The apparatus was assembled, and attached to a cooling water supply. Samples were then loaded into the wells and the gels were electrophoresed at 30 mA per gel, using an electrophoresis buffer that contained 24 mM Tris; 192 mM glycine; and 4 mM SDS. Electrophoresis was continued until the tracking dye, 0.1% (w/v) Bromophenol blue (BioRad), had reached the bottom of the gels (approximately four hours).

After the slab gels had been electrophoresed, the proteins were fixed by soaking in a solution containing 50% (v/v) methanol and 10% (v/v) glacial acetic acid for four hours. The slab gels were then soaked for a minimum of eight hours in a staining solution containing 0.1% (w/v) Coomassie Brilliant Blue R-250 ; 10% (v/v) methanol; and 10% (v/v) glacial acetic acid. Staining was carried out at room temperature on a rocking table.

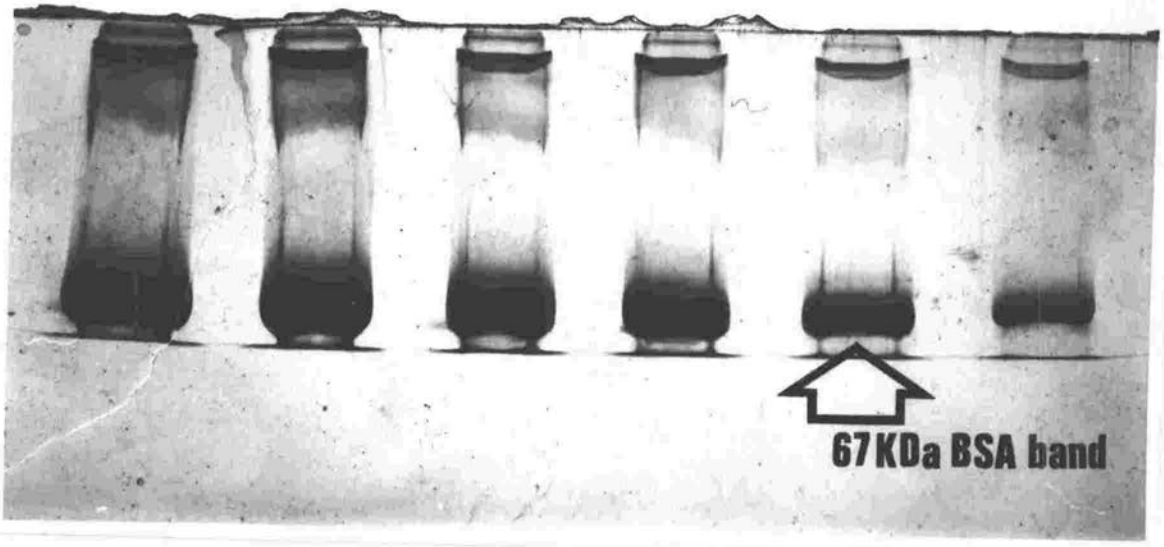
After staining, the gels were destained in a solution containing 10% (v/v) glacial acetic acid and 5% methanol. Destaining was carried out at room temperature on a rocking table. Gels were destained until a clear background was obtained (approximately fourteen hours). Figure 2.321 shows the 1-dimensional separation. Coomassie blue stained gels were scanned and digitised using a model 300 A Molecular Dynamics Computing Densitometer. Subsequent analysis of the scanned image was done using Molecular Dynamic's Image Quant™ version 3.3 software, the data generated here being analysed further using the software package Excel. The major function of the densitometer, and its allied software, was to compute and compare extinction values for various regions of a gel containing separated proteins.

The class of quantitative analysis performed by the Image Quant software was volume integration (VI) analysis. For this analysis the software calculated the total extinction at all of the pixels within a predefined area, subtracted the pixel density of a predefined background area, and expressed the result as a VI value. This represented the extinction of the particular protein being studied. An important step in this type of analysis was the assigning of a representative background. As background staining for Coomassie Blue stained gels was found to vary, separate background values were calculated for each protein spot of interest. The first step in background determination was to draw a line through the image of the protein spot of interest, and obtain an extinction profile along the line. A record was made of the minimum extinction value. This was used as the initial background value. Two regions were then drawn around the protein spot using the mouse. One region was drawn around the perimeter of the protein, taking care not to let the line touch the protein spot itself, but came very close to the boundary of the protein. This was necessary to ensure that the protein did not contribute to the background determination and therefore reduce the VI value. The second region was drawn around the first region, leaving as much clear gel as possible between the two regions. Using the initial background value found from the extinction profile for the protein spot, VI values were obtained for each of the two regions. When the VI value for the outermost region was greater than the VI value for the inner region, it was taken to imply that the background was being included in the VI determinations, and the background value was increased. When the VI value of the innermost region exceeded the VI value for the outer region, it was taken to imply that the background value was set too high, and it was decreased. This process was repeated until the VI values for both regions were approximately equal. The background level used to achieve this was recorded and used as the background value when obtaining a VI value for that particular protein.

Occasionally, a background determination was required for a protein spot that was not totally resolved from the surrounding proteins. In cases such as this, an object of approximately the same shape and size of the protein in question was drawn in the closest region of protein free gel, and used to calculate the background value. A region was then drawn around the perimeter of the protein spot and VI values were calculated.

Figure 2.321. 1-Dimensional PAGE separation of BSA using a 10% gel.

The loadings in each lane (from left to right) were 30 µg, 25 µg, 20 µg, 15 µg, 10 µg, and 5 µg of BSA.



The major 67 KDa band indicated in Figure 2.321 was taken to be albumin and the amount of BSA in the sample was calculated by quantitative densitometry as shown in Table 2.321.

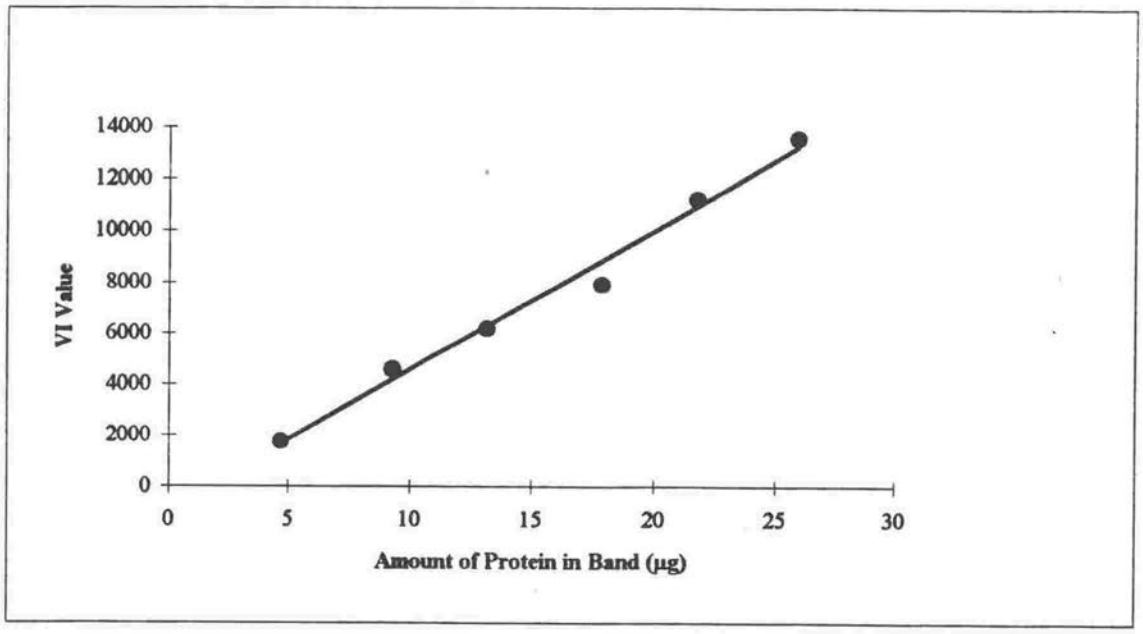
Table 2.321. The amount of BSA in the sample loaded onto the gel shown in Figure 2.321.

Lane	Amount of protein loaded	% of total protein in BSA band [1]	Amount of protein in BSA band
1	5 µg	94.28	4.71 µg
2	10 µg	92.93	9.29 µg
3	15 µg	87.81	13.17 µg
4	20 µg	89.39	17.88 µg
5	25 µg	87.14	21.78 µg
6	30 µg	86.47	25.95 µg

1 The VI value for BSA as a percentage of the total VI value for all protein bands in the lane.

Figure 2.322 shows the relationship between quantitative response and protein load for BSA. The relationship was nearly linear up to VI values of approximately 14,000, which corresponded to approximately 26 μg of protein.

Figure 2.322. Relationship between protein load and VI values for BSA.



The R square value for the plot was 0.99.

2.322) The relationship between protein load and quantitative response for plasma proteins.

Differing amounts of human plasma proteins (120 - 1900 µg) were separated using five 2-dimensional PAGE gels. For 2-dimensional PAGE, the method of O'Farrell (1975) was modified as previously described. The first dimension tube gels were prepared and focused as described previously. After focusing, the IEF gels were extruded from the glass tubes, and equilibrated for ten minutes in sample buffer (0.08 M SDS; 0.06 M Tris; 10% (v/v) glycerol; 5% (v/v) mercaptoethanol; pH 6.8). After equilibration, the gels were immediately subjected to SDS electrophoresis (the second-dimension separation technique).

The second dimension separation technique was discontinuous SDS electrophoresis, as was described previously. The IEF gels were attached to the tops of the stacking gels of the SDS slab gels using a molten agarose solution that contained 1% (w/v) agarose; 40 mM SDS; 30 mM tris; 5% (v/v) glycerol; and 2.5% (v/v) mercaptoethanol. After the agarose had set, the apparatus was assembled, and attached to a cooling water supply. The gels were electrophoresed, and spot quantitation was performed as previously described. Figure 2.323 shows the proteins selected for study.

Figure 2.323. The plasma proteins studied in this section. The proteins were separated by 2-dimensional PAGE, on a 10% gel.

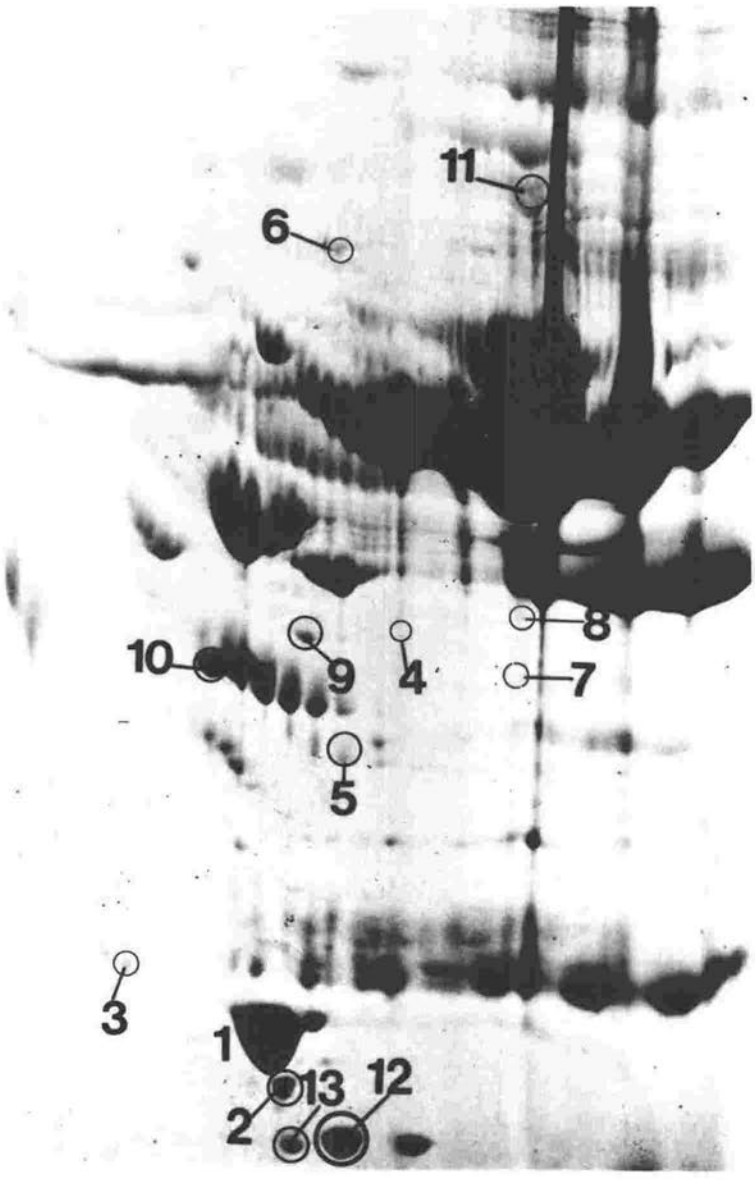
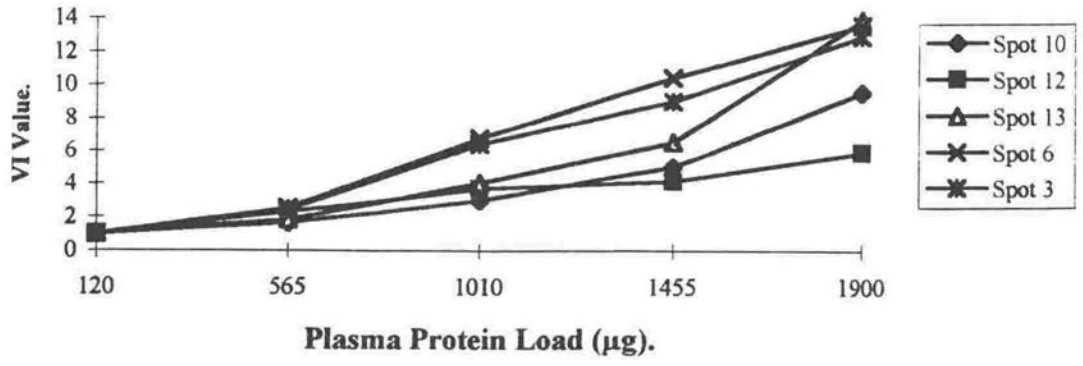


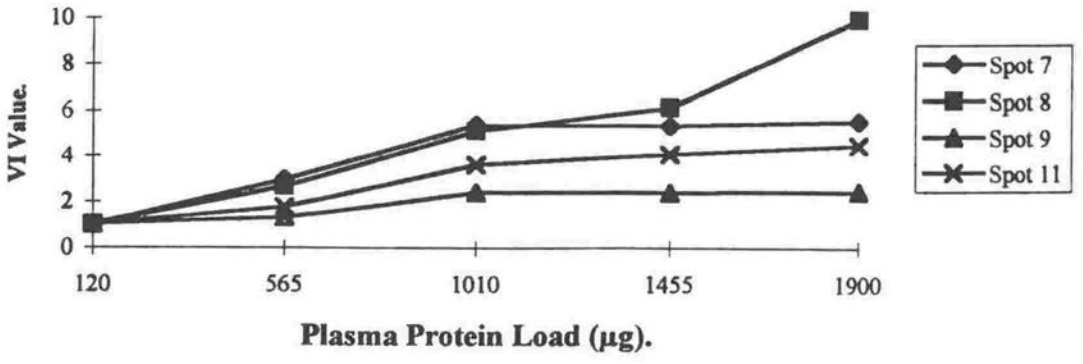
Figure 2.324 shows plots for the thirteen protein spots after the data had been normalised for each protein by dividing the VI values by the VI value obtained for that protein at the lowest protein loading. This allowed all of the data to be displayed on axes with the same scale. For a linear response between VI value and protein load, the theoretical normalised VI values for each protein loading (starting at 120μg) would be 1, 4.7, 8.4, 12.1, and 15.8. None of the proteins studied gave such a response. Proteins with responses similar to this were spots 2, 4, 5, 10, and 13. These proteins covered a wide range of molecular weights, pI's, and abundances. For protein spots 7, 9, 11, and 12 , a plateau was observed when protein load was plotted against VI values. Again, these proteins covered a wide range of molecular weights, pI's, and abundances. These observations illustrate the value of determining the nature of this relationship for any protein found to differ in abundance in the plasma in the disease state. There are several possible explanations for the apparent lack of linearity between VI values and protein load. First, there could be several classes of binding site for Coomassie blue for any protein. A second possible explanation is that saturation of the dye binding sites may occur for some proteins at relatively low protein concentrations (Burgess-Cassler *et al.*, 1989). A third possible explanation is that the mode of sample preparation may have contributed to the non-linear response. When the plasma was diluted (as necessary for the smaller protein loads), the ratio between urea and plasma proteins on a weight to weight basis was altered in the sample preparation. This is shown in Table 2.322.The significance of the urea: plasma protein ratio is that urea is used to solubilize proteins during sample preparation.

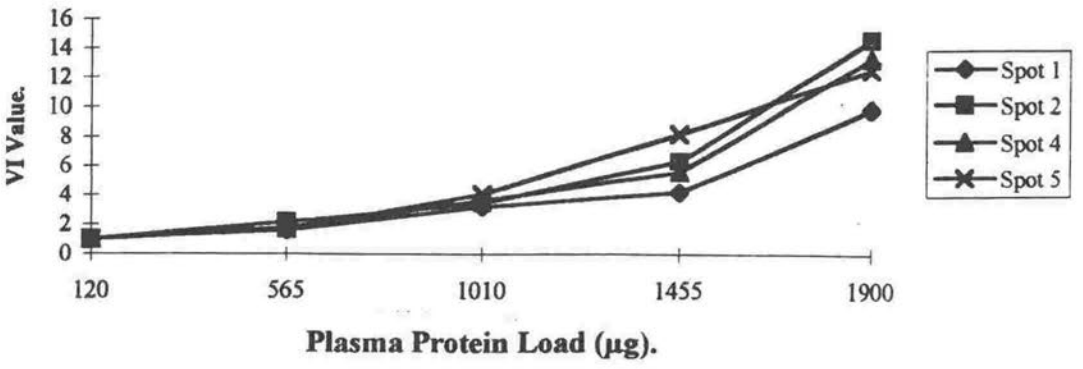
Table 2.322 The urea: plasma protein ratio, on a weight to weight basis.

Gel #	Urea: Protein Ratio (w/w)
1	224:1
2	112:1
3	45:1
4	22:1
5	22:1

Figure 2.324. ***Plots for normalised data showing the relationship between protein load and VI values.***







To decide if differences in the urea: plasma protein ratio were influencing the relationship between protein load and quantitative response, the following experiment was done. A series of six 2-dimensional gels were prepared and electrophoresed. Each gel was loaded with 930 µg of protein, but for each gel, the protein preparation had a different urea: plasma protein ratio. Table 2.323 shows the ratios for each sample, and sample preparation details. The urea: plasma protein ratio was calculated on a weight to weight basis. The calculation was based on a knowledge of the concentration of urea in IEF buffer (9.6 M), the volume of IEF buffer used, the amount of protein contained in the volume of plasma used (based on a total protein concentration for plasma of 70 µg μl^{-1}; Ganong (1985)), and the volume of the sample preparation (measured using a micropipette). The molarity of urea in the sample was calculated from the molarity of urea in IEF buffer and the volume of the sample preparation.

Table 2.323. The urea: plasma protein ratio (w/w)for each sample.

Gel Number	Urea: Protein ratio	Volume of plasma	Mass of Urea	Volume of IEF buffer	[Urea] M
1	10:1	13.3 µl	5.3 mg	5.3 µl	6.4
2	22:1	13.3 µl	11.7 mg	11.7 µl	9.3
3	45:1	13.3 µl	24.0 mg	24.0 µl	11.4
4	112:1	13.3 µl	59.7 mg	59.7 µl	13.1
5	224:1	13.3 µl	119.3 mg	119.3 µl	13.8
6	500:1	13.3 µl	266.4 mg	266.4 µl	14.2

During preparation, the samples were kept at approximately 30°C as a precaution against the urea crystallising out of solution. From a visual inspection of all of the samples however, it did not appear that the urea had reached its saturation point. The solubility of urea in water is 1 kg l^{-1} at 25°C (Windholz, 1976). For the sample with the highest urea concentration, the urea was present at a concentration of 0.828 kg l^{-1}.

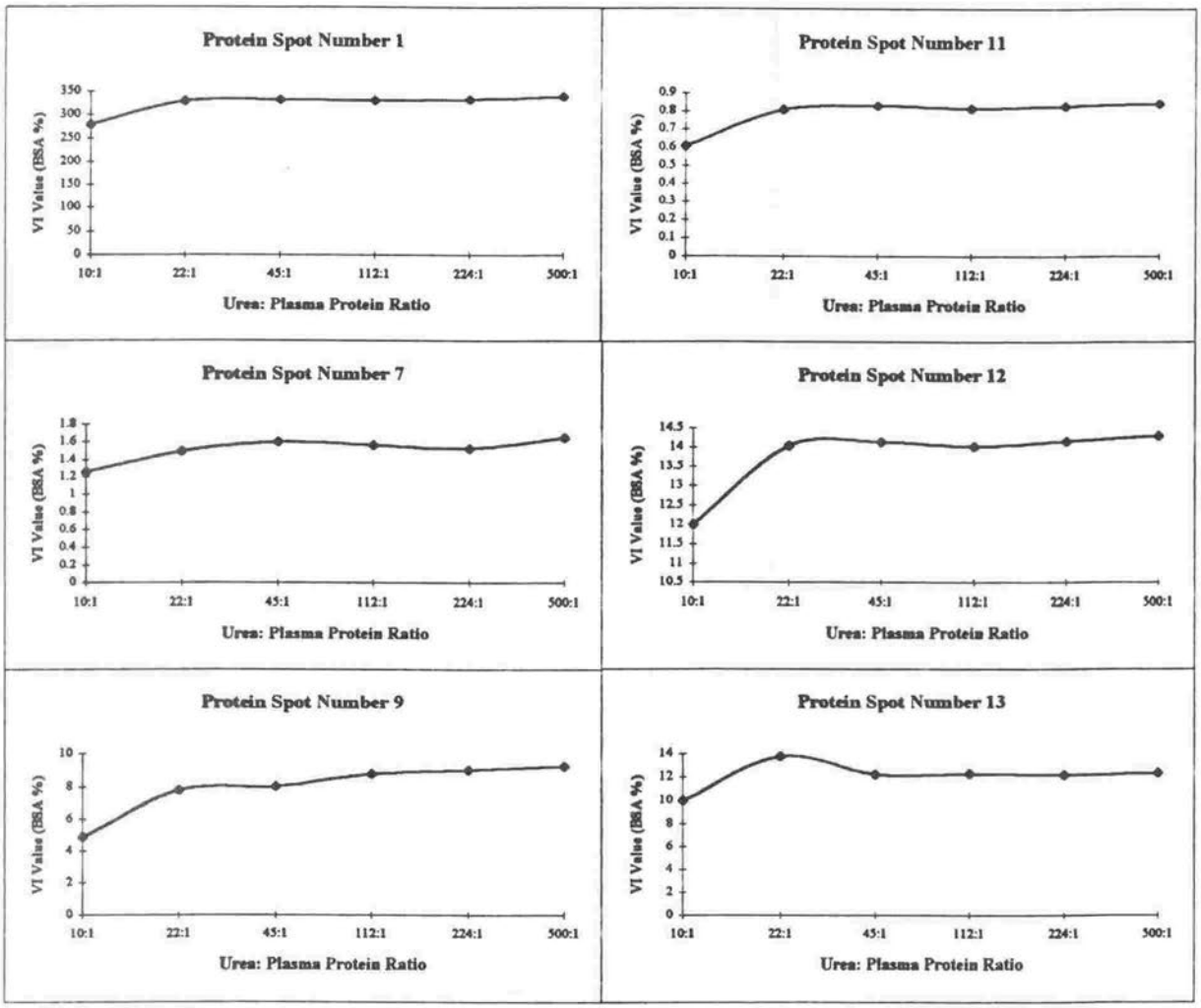
Each gel had 30 μg of BSA electrophoresed in a well beside the IEF gel. This was used as a calibration standard against which the abundances of other proteins were compared.. After staining and destaining, the VI values were measured for six of the protein spots which had previously been used to examine the relationship between protein load and quantitative response. Figure 2.325 shows plots of the VI values expressed as a percentage of the VI value for BSA on the same gel. Four of the proteins selected for Figure 2.325 were those which showed a plateau when VI was plotted against protein load. These proteins were spots # 7, # 9, # 11, and # 12 (Figure 2.324). Protein spot # 1 was also included. This protein had a staining response that increased with protein load, although the response was not linear. Protein spot # 13 was the final protein selected. This protein had a relationship between protein amount and VI value that most closely resembled the normalised theoretical values. If the urea: plasma protein ratio had no influence on the VI values, the plots shown in Figure 2.325 would be parallel to the X axis.

One observation that can be made from Figure 2.325 is that the measured abundance of each protein when the urea: plasma protein ratio was 10:1 was substantially lower than the values obtained for the same proteins at higher urea: plasma protein ratios. This observation can probably be attributed to solubility effects as the concentration of urea was only 6.4 M. Urea is usually effective for solubilizing proteins at concentrations exceeding 8 M (Dunbar, 1985). Although the 10:1 urea: protein ratio was lower than that used in the previous experiment (Table 3.222), it illustrates that the ratio can influence the relationship between protein load and VI value. In the absence of solubility effects, the VI values should not be affected by the urea: protein ratio. This was seen for all of the protein spots. However, at higher urea: protein ratios some of the proteins showed an increase in VI values, suggesting that some of the proteins remained slightly insoluble at all but the highest urea: protein ratio. A problem with using high urea: protein ratios however, is that the urea crystallises out of solution. There is also an increased risk of carbamylation (Dunbar, 1987).

These observations suggest that when using standard protein preparation techniques for 2-dimensional PAGE, solubility effects do not substantially influence the relationship between protein load and VI value. For the range of urea: protein ratios shown in Table 3.222, the

observed VI values did not differ greatly from the expected values; the differences genarally falling within the limits of experimental error. A ratio of 22:1 for the urea: protein ratio was subsequently used in all sample preparations for IEF.

Figure 2.325. The relationship between urea: plasma protein ratio and VI values for six plasma proteins.



As previously shown, the relationship between protein load and quantitative response differed among the proteins studied. One way to overcome this problem would be to produce a standard curve for each protein studied. An alternative method, based on log transformation of measured abundances was developed as described in the next section.

2.4) *Quantitative Reproducibility of 2-Dimensional PAGE.*

2.41) Introduction.

The following experiments were performed to study the reproducibility of quantitation of proteins separated on replicate gels. This was done to assess the amount of variation generated by the gel separation and the protein quantitation techniques.

2.42) Methods and Results.

For 2-dimensional PAGE, the method of O'Farrell (1975) was modified as described in section 2.322. Spot quantitation was performed as described in section 2.321.

The following experiments were carried out for three reasons. First, to determine the degree of reproducibility of the process of quantitation. Second, to assess the reproducibility of quantitations made on gels electrophoresed simultaneously and at different times. And thirdly, to compare different methods for trying to improve reproducibility.

2.421) *Reproducibility of the densiometric procedure.*

The reproducibility of the densiometric procedure was investigated. For this analysis, a single 10% 2-dimensional gel was scanned on six separate occasions over six days, the densitometer being calibrated before every scan. Initially, four discrete protein spots (with acidic to neutral pI values) were selected for quantitation. Discrete protein spots were defined as those spots with a minimum of 1 mm of clear gel surrounding their boundary. For each spot, the image was magnified by a factor of four, and the pixel density range was maximised to enhance the image. A boundary was then drawn around the perimeter of the protein spot for the background determination calculation. A printout was obtained of the magnified protein spot and its background boundary, and of the maximum and minimum values for the density range. VI was then carried out. This process was repeated for the remaining three protein spots. After completion of this process, a further five VI values were obtained for each spot, the boundary being redrawn each time using the initial image printout as a guide. The VI determinations were done alternately for each protein spot, ie. the second VI value was calculated for the entire group of spots before the third VI value was calculated, etc. Mean and standard deviations for the six measurements on each of the four protein spots were then calculated (Table 2.421).

Table 2.421. Reproducibility of protein spot VI values for human plasma proteins separated by 2-dimensional PAGE. Results of six separate quantitations of four discrete protein spots on a single gel.

Reproducibility of VI values as measured by computerised densitometery for discrete[1] protein spots from a single gel.				
VI Value	**Protein Spot 1**	**Protein Spot 2**	**Protein Spot 3**	**Protein Spot 4**
1	45.9	112.7	53.0	12.9
2	44.9	120.0	55.4	14.1
3	45.3	115.9	54.0	12.7
4	41.2	122.6	56.2	13.2
5	44.9	120.2	54.6	14.4
6	44.0	114.4	58.3	13.6
Mean Value	44.4	117.7	55.3	13.5
Standard Deviation[2]	3.5%	3.0%	3.1%	4.5%

1. Discrete protein spots had a minimum of 1mm of clear gel surrounding their boundary.
2. One standard deviation expressed a percentage of the mean.

The process outlined above was repeated for three non-discrete protein spots chosen at random. Non-discrete protein spots were defined as spots whose boundaries blurred into background or overlapped with other spots. A lower degree of reproducibility was expected for non-discrete protein spots, due to the difficulty of assigning consistent boundaries. The results of these calculations are shown in Table 2.422.

Table 2.422. Reproducibility of protein spot VI values for human plasma proteins separated by 2-dimensional PAGE. Results of six separate quantitations of three non-discrete protein spots on a single gel.

Reproducibility of VI values as measured by computerised densitometery for non-discrete[1] protein spots from a single gel.			
VI Value	**Protein Spot no. 5**	**Protein Spot no. 6**	**Protein Spot no. 7**
1	77.0	111.6	133.0
2	87.4	103.2	132.3
3	91.2	121.4	135.4
4	78.5	107.0	124.2
5	94.0	127.6	116.0
6	95.0	121.6	115.5
Mean Value	87.2	115.4	126.1
Standard Deviation[2]	8.1%	7.6%	6.4%

1. Non-discrete protein spots did not have clearly defined borders.
2. One standard deviation expressed as a percentage of the mean.

2.422) *Quantitative reproducibility of proteins isoelectrofocused at the same time.*

The second category of quantitative reproducibility investigated was analysis of protein samples that had been focused and electrophoresed together in batches of eight gels. For this experiment a sample of human plasma was separated on 24 2-dimensional gels. Each gel was loaded with 350 µg of protein and isoelectric focusing was performed using eight tube gels on each of three occasions, subsequently called series 1 to 3. A separate well containing 30 µg of BSA was included on each of the 24 2-dimensional gels. Eight 2-dimensional gels were electrophoresed on three separate occasions. Thirteen spots were selected (Figure 2.323), and after scanning, VI values for each of the thirteen spots on each gel were obtained. Means and standard deviations

were calculated for each of the thirteen proteins analysed on the eight gels (Table 2.423).

Table 2.423. Reproducibility of protein quantitation from 2-dimensional gels. Protein densities for thirteen proteins and a BSA standard on three sets of eight gels.

VI values and standard deviations[1] of human plasma proteins measured by computerized densitometry.			
Protein Spot Number[2]	**Series 1[3]**	**Series 2**	**Series 3**
1	3460.0 ±6.2%	4500.0 ±8.9%	3380 ±7.1%
2	364.0 ±7.3%	471.3 ±7.6%	353.5±6.4%
3	9.7 ±6.8%	12.8 ±9.5%	9.6 ±7.4%
4	47.5±8.1%	62.7 ±8.4%	45.9 ±9.3%
5	8.6 ±9.3%	11.4 ±6.1	8.4±6.1
6	20.5 ±4.8%	27.8 ±7.9%	19.9 ±7.1%
7	12.5 ±3.7%	16.0 ±8.7%	12.0 ±4.2%
8	257.2 ±6.2%	319.3 ±9.3%	236.4±6.3%
9	212.5 ±11.4%	279.8 ±13.1%	208.8±12.7%
10	250.0 ±12.7%	333.4 ±11.8%	248.0 ±13.6%
11	10.9 ±9.3%	14.9±6.8%	11.0 ±6.4%
12	146.2 ±6.8%	191.9 ±7.1%	138.8 ±8.5%
13	53.9 ±8.2%	72.8 ±8.1%	53.6±9.8%
BSA[4]	1161.0 ±4.6%	1367.0 ±5.1%	1117.0 ±4.2%
Range[5]	3.7 - 12.7	5.1 - 13.1	4.2 - 13.6

1 One standard deviation, expressed as a percentage of the mean.
2 Refer to Figure 2.323
3 Each series represents eight two-dimensional polyacrylamide gel separations performed simultaneously.
4 BSA, electrophoresed in a well beside the two-dimensional separation.
5 The range of standard deviations for each set, expressed as percentages of the means.

In an attempt to correct for any discrepancies that may have been caused by differences in staining with Coomassie blue, three calibration methods were examined. In the first, the VI values for each of the thirteen spots were expressed as a percentage of the VI value for the BSA standard electrophoresed on that gel. The average percentage value for each of the thirteen spots was then calculated for each set with means and standard deviations. These values are shown in Table 2.424.

Table 2.424. Reproducibility of protein quantitation from 2-dimensional gels. Protein densities for thirteen protein spots on three series of eight gels each. The amount of each protein is expressed as a percentage of a BSA standard included on each gel.

VI values of human plasma proteins measured by computerised densitometery, and expressed as a percentage of the BSA values shown in Table 2.423.			
Protein Spot [3].	Series 1 [4].	Series 2.	Series 3.
1	298.0 ±2.9 % [1]	329.2±5.3	302.6 ±3.5%
2	31.4 ±4.1	34.5 ±3.6%	31.7 ±4.7%
3	0.8 ±3.4%	0.9 ±5.1%	0.9 ±3.4%
4	4.1 ±3.7%	4.6 ±5.1%	4.1 ±4.9%
5	0.7 ±3.7%	0.8 ±4.4%	0.8 ±4.1%
6	1.8 ±3.3%	2.0 ±5.3%	1.7 ±3.4%
7	1.1 ±3.5%	1.2 ±3.9%	1.1 ±3.9%
8	22.2 ±4.0%	23.4 ±4.4%	21.2 ±4.8%
9	18.3 ±13.2%	20.5 ±14.3%	18.7 ±13.9%
10	21.6 ±15.7%	24.3 ±14.6%	22.2 ±14.7%
11	0.9 ±14.8%	1.1 ±11.9%	0.9 ±10.1%
12	12.6 ±4.14%	14.1 ±3.9%	12.4 ±4.1%
13	4.6 ±4.5%	5.3 ±3.5%	4.8 ±4.9%
Range.[2]	2.9 - 15.7%	3.5 - 14.6%	3.4 - 14.7%

1. Standard deviation, expressed as a percentage of the mean.
2. The range of standard deviations expressed as percentages of the means.
3. Refer to Figure 2.323.
4. Each series represents eight two-dimensional polyacrylamide gel separations performed at the same time.

For the second calibration method, a protein spot was selected (protein spot number 8) to act as an internal standard. All VI values for a gel were then expressed as a percentage of the VI value obtained for protein spot 8 on that gel. These values are given in Table 2.425.

Table 2.425. Reproducibility of protein quantitation from 2-dimensional gels. Protein densities for thirteen protein spots on three series of eight gels each. The amount of each protein is expressed as a percentage of protein spot # 8 on each gel.

VI values of human plasma proteins measured by computerised densitometery, and expressed as a percentage of the VI value of protein spot # 8.			
Protein Spot number[3].	Series 1[4].	Series 2.	Series 3.
1	1337.7 ± 3.6%[1]	1379.6 ± 4.9%	1422.4 ± 3.9%
2	140.6 ± 3.2%	141.7 ± 3.5%	145.3 ± 4.1%
3	3.7 ± 3.6%	4.0 ± 4.1%	4.0 ± 2.9%
4	18.3 ± 3.5%	19.5 ± 5.2%	19.1 ± 4.2%
5	3.3 ± 3.1%	3.5 ± 4.1%	3.5 ± 1.9%
6	8.1 ± 3.1%	8.5 ± 3.3%	8.2 ± 2.6%
7	4.8 ± 2.9%	4.9 ± 4.1%	4.9 ± 3.0%
8	100.0	100.0	100.0
9	80.9 ± 7.9%	86.6 ± 6.9%	86.9 ± 4.2%
10	96.7 ± 11.0%	101.8 ± 10.4%	102.1 ± 8.2%
11	4.2 ± 9.3%	4.6 ± 9.8%	4.6 ± 8.3%
12	55.9 ± 4.2%	59.6 ± 4.6%	57.7 ± 3.7%
13	20.5 ± 3.6%	22.3 ± 4.0%	21.5 ± 2.9%
Range[2]	2.9 - 11.0	3.3 - 10.4	1.9 - 8.3

1. Standard deviation, expressed as a percentage of the mean.
2. The range of standard deviations expressed as percentages of the means.
3. Refer to Figure 2.323
4. Each series represents eight two-dimensional polyacrylamide gel separations performed simultaneously.

The third calibration method involved expressing each protein as a percentage of the total VI value for the thirteen spots quantitated on that gel (this type of data is subsequently called compositional data. Appendix 1). Table 2.426 shows the compositional values for the thirteen protein spots discussed above.

Table 2.426. Reproducibility of protein quantitation from 2-dimensional gels. Protein densities for thirteen protein spots on three series of eight gels each. The amount of protein is expressed as compositional values for that gel.

Protein spot number [1].	Series 1.	Series 2.	Series 3.
1	71.3 ± 2.5%[2]	71.3 ± 2.0%	71.5 ± 2.1%
2	7.5 ± 2.9%	7.5 ± 2.4%	7.5 ± 1.9%
3	0.2 ± 2.6%	0.2 ± 6.0%	0.2 ± 3.5%
4	1.0 ± 2.6%	0.9 ± 3.8%	0.9 ± 5.2%
5	0.2 ± 5.0%	0.2 ± 2.9%	0.2 ± 6.6%
6	0.4 ± 3.2%	0.4 ± 4.2%	0.4 ± 5.5%
7	0.3 ± 2.6%	0.3 ± 5.0%	0.3 ± 4.4%
8	5.3 ± 2.7%	5.1 ± 3.6%	5.0 ± 1.9%
9	4.4 ± 7.4%	4.4 ± 7.9%	4.4 ± 6.9%
10	5.2 ± 9.6%	5.3 ± 8.8%	5.3 ± 7.0%
11	0.2 ± 4.5%	0.2 ± 6.4%	0.2 ± 7.2%
12	3.0 ± 2.7%	3.0 ± 3.7%	2.9 ± 4.3%
13	1.1 ± 4.5%	1.2 ± 4.9%	1.1 ± 3.1%
Range	2.5 - 9.6%	2.0 - 8.8%	1.9 - 7.2%

1. Refer to Figure 2.323
2. Standard deviation, expressed as percentages of the means.

Table 2.427 summarises the results given in Tables2.423 to 2.426, and allows an assessment to be made of the three calibration techniques.

Table 2.427. Reproducibility of protein quantitions for proteins isoelectrofocused at the same time and electrophoresed on replicate gels. Values shown are the three series range of standard deviations expressed as a percentages of the means.

Protein spot. [1]	Uncalibrated	BSA Calibration	Internal Calibration	Compositional Calibration
1	6.2% - 8.9%	2.9% - 5.3%	3.6% - 4.9%	2.0% - 2.5%
2	6.4% - 7.6%	4.1% - 4.7%	3.2% - 4.1%	1.9% - 2.9%
3	6.8% - 9.5%	3.4% - 5.1%	2.9% - 4.1%	2.6% - 6.0%
4	8.1% - 9.3%	3.7% - 5.1%	3.5% - 5.2%	2.6% - 5.2%
5	6.1% - 9.3%	3.7% - 4.4%	1.9% - 4.1%	2.9% - 6.0%
6	4.8% - 7.9%	3.3% - 5.3%	2.6% - 3.3%	3.2% - 5.5%
7	3.7% - 8.7%	3.5% - 3.9%	2.9% - 4.0%	2.6% - 5.0%
8	6.2% - 9.3%	4.0% - 4.8%	N.A.	1.9% - 3.6%
9	11.4% - 13.1%	13.2% - 14.3%	4.2% - 7.9%	6.9% - 7.9%
10	11.8% - 13.6%	14.6% - 15.7%	8.2% - 11.0%	7.0% - 9.6%
11	6.4% - 9.3%	10.1% - 14.8%	8.3% - 9.8%	4.5% - 7.2%
12	6.8% - 8.5%	3.9% - 4.1%	3.7% - 4.6%	2.7% - 4.3%
13	8.1% - 9.8%	3.5% - 4.9%	2.9% - 4.0%	3.1% - 4.9%

1. Refer to Figure 2.323

2.423) *Quantitative reproducibility of proteins isoelectrofocused at different times.*

The third category of quantitative reproducibility that was investigated was designed to be similar to the conditions which were subsequently used in the analysis of disease related protein variation. This required analysis of samples that had been electrophoresed at different times. For this analysis the data for the thirteen protein spots which had been quantitated on the 24 gels in section 2.422 were analysed as a total set of 24 measurements for each protein. This was done to give a measure of the variation in protein abundance which occurred for data from gels which had been electrophoresed and stained on different occasions.

The four methods of spot quantitation summarised in Table 2.4247 were used. First, an assessment was made of the reproducibility of quantitations made on proteins separated at different times, without considering discrepancies caused by differences in staining with Coomassie blue (these values are subsequently called uncalibrated values). To do this, the mean VI values for each of the measured proteins on the 24 gels were calculated. Standard deviations were also calculated and expressed as a percentage of the mean VI values for each protein spot (subsequently called SD/Mean % values). Second, an assessment was made of the reproducibility after VI values for each gel had been calibrated against the BSA standard electrophoresed on each gel. These results were also expressed as SD/Mean % values. Third, reproducibility of VI values was assessed after the VI values from each gel were calibrated against an internal protein standard (protein spot 8). Values were expressed as SD/Mean %. Finally, reproducibility was assessed after the VI values for each gel had been converted into compositional values; the results were expressed as SD/Mean % values. The results of the above measurements are summarised in Table 2.428.

Table 2.428. Reproducibility of proteins quantitated on different gels electrophoresed at different times. Values shown are standard deviations expressed as percentages of the means.

Protein spot.[1]	Uncalibrated	BSA Calibration	Internal Calibration	Compositional Calibration
1	14.4%	5.6%	4.3%	1.8%
2	14.7%	5.5%	3.3%	2.0%
3	15.3%	6.3%	4.6%	5.0%
4	16.2%	6.6%	4.4%	4.0%
5	15.9%	7.1%	4.1%	5.6%
6	15.6%	7.6%	3.5%	4.7%
7	14.3%	5.4%	3.1%	4.0%
8	14.5%	5.4%	N.A.	3.5%
9	18.1%	11.3%	6.2%	6.1%
10	18.9%	11.6%	8.5%	7.0%
11	18.0%	8.9%	8.3%	4.4%
12	15.9%	6.5%	4.3%	3.4%
13	16.5%	6.9%	4.4%	4.4%
Range	14.3% - 18.9%	5.4% - 11.6%	3.1% - 8.5%	1.8% - 7.0%

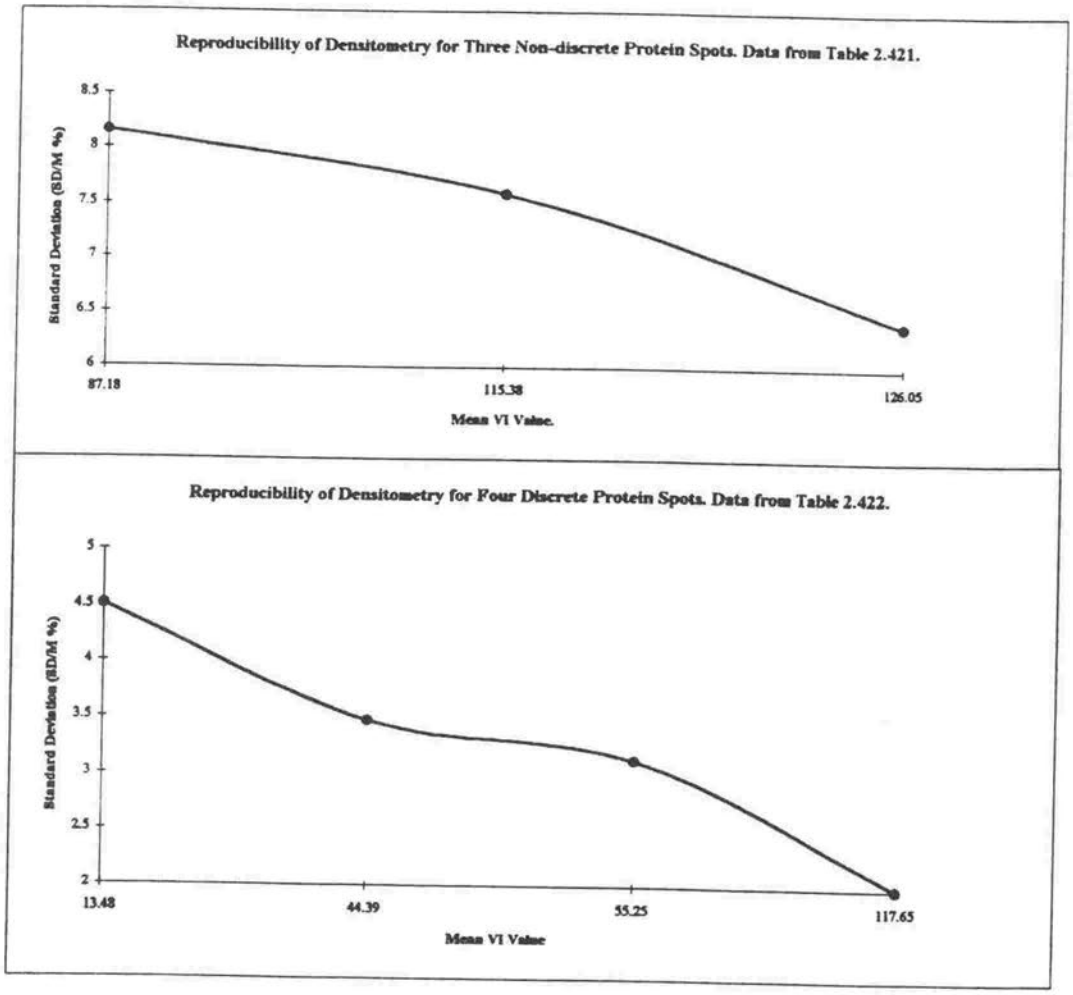
1. Refer to Figure 2.323

2.44) Discussion.

The results in Tables 2.421 and 2.422 show that greatest reproducibility was obtained when quantitating discrete protein spots (range 2.05 - 4.5% for standard deviations as percentages of the means) compared to non-discrete protein spots (range 6.4% - 8.1%). In addition, the quantitation of more abundant spots was more reproducible than the quantitation of less abundant spots (Figure 2.41). These differences probably reflect the difficulties in distinguishing the borders of low abundance and non-discrete spots during the quantitative procedure.

Table 2.423 allows an assessment to be made of the reproducibility of quantitations made on the same proteins separated on different gels (made using the same reagents, electrophoresed together, and stained/destained simultaneously). For these quantitations, when the three series of gel were considered, the range of standard deviations (expressed as percentages of the means) was from 3.7 to 13.6%, the mean standard deviation being 8.8%. When the VI values were calibrated against a BSA standard electrophoresed with each gel, the range of standard deviations was from 2.9 to 15.7% of the mean, the average standard deviation representing 5.7% of the mean (Table 2.424). When an internal standard was used to calibrate the VI values, the range of standard deviations (expressed as percentages of the means) was from 1.9 to 11.0%, and the mean standard deviation was 4.8% (Table 2.425). When VI values were calibrated after conversion into compositional values, the range of standard deviations was from 1.9 to 9.6% of the mean, the average standard deviation being 4.5% (Table 2.426). Figure 2.42 shows the mean standard deviations for each protein spot for uncalibrated VI values, and for VI values calibrated as described above. The results indicate that calibration usually increased the reproducibility of quantitations made on different gels that had been electrophoresed together.

Figure 2.41. ***Reproducibility of Densitometry for discrete and non-discrete protein spots.***



The data have been plotted to show the relationship between spot abundance (mean VI value) and the magnitude of the standard deviation (expressed as a percentage of the mean) for each protein spot.

Figure 2.42. Quantitative reproducibility of proteins isoelectrofocused together, and electrophoresed on replicate gels.

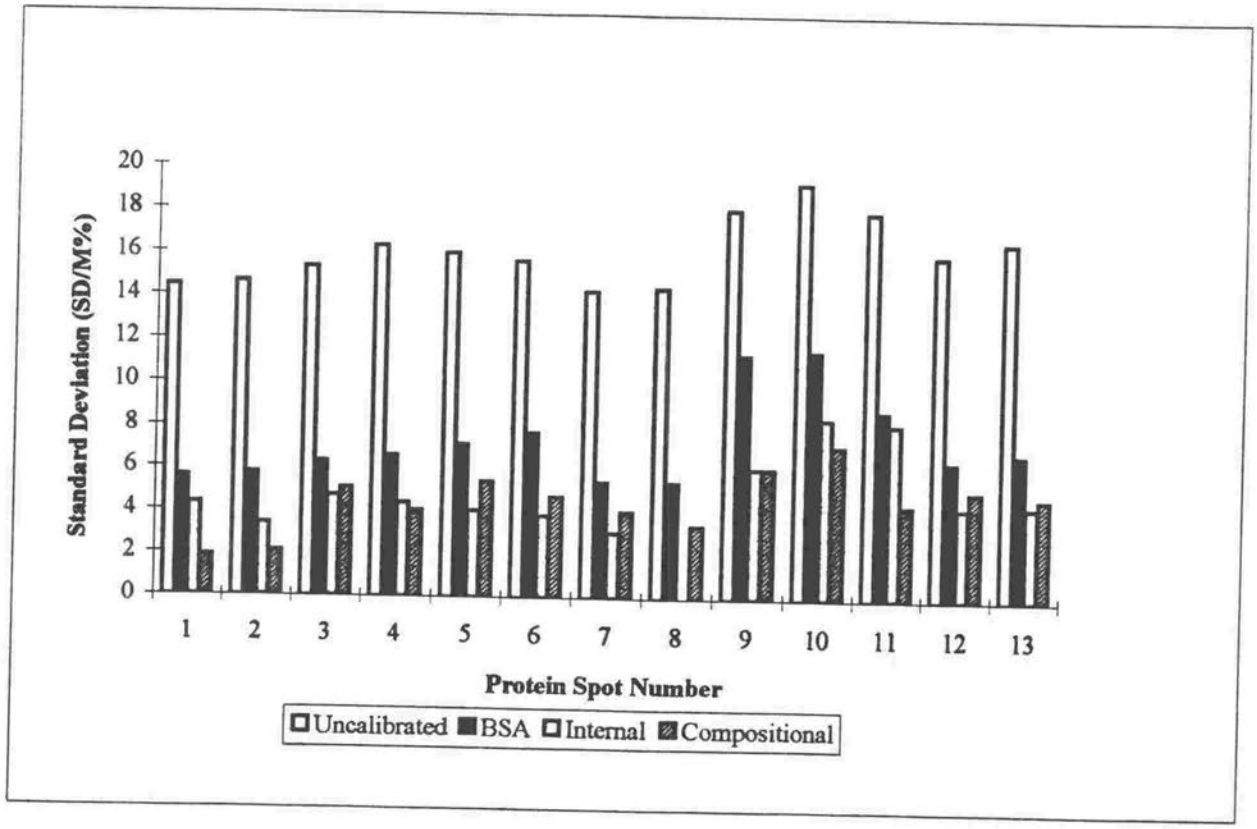


Table 2.428 allows an assessment to be made of the reproducibility of quantitations made on gels electrophoresed at different times, without trying to account for differences in protein staining, and after accounting for staining differences using three calibration techniques. For uncalibrated VI values, the range of standard deviations (SD/Mean%) was from 14.3 to 18.9%, the average value being 16.0%. When the same VI values were calibrated against a BSA standard electrophoresed with each gel, the range of standard deviations was from 5.4 to 11.6%, the mean value being 7.8%. When the VI values were calibrated using protein spot number 8 as an internal standard, the range of standard deviation values was from 3.1 to 8.5%, with an average value of 4.9%. When compositional calibration was used, the range of standard deviation values was from 1.8 to 7.1%, the mean value being 4.3%. These observations show that VI values obtained for proteins separated on different 2-dimensional gels electrophoresed at different times are most reproducible after the values obtained for each gel have been calibrated by conversion into compositional data (Figure 2.43).

From the observations made above, it is clear that when comparing protein samples electrophoresed both simultaneously, and at different times, it is necessary to calibrate the VI values from each gel before comparison. Of the calibration methods investigated here, conversion of VI values into compositional data gave the most reproducible results. For all subsequent protein quantitations done in this project, VI values were converted into compositional data prior to statistical analysis of differences between samples.

Two other issues were investigated. The first concerned the shape of the distribution curve obtained when VI values obtained from gels electrophoresed at different times were compared. It was important to examine if the distributions were non-Gaussian or non-symmetrical, as under these circumstances the arithmetic values for the mean, median, and mode, may not have been numerically equal. This would mean that multivariate statistical tests, such as the *t*-test, could not be validly applied. Figure 2.44 shows the proportion of VI values falling within the standard deviation values shown, for uncalibrated VI values, for compositionally calibrated values, and also for the theoretical values that would be expected for true Gaussian distributions. This data was

obtained from Tables 2.423 and 2.426. For the uncalibrated values, the distribution was both non-Gaussian, and non-symmetrical. The negative skew to the distribution may have arisen due to one series of gels being more intensely stained than the other series, as could occur when a new staining solution was used. The compositional values however, had near Gaussian proportions, and were distributed symmetrically, indicating that the data could be analysed using the student *t*-test.

The second issue concerned whether a relationship existed between the amount of variance (as indicated by standard deviation values) and the relative protein abundance (as measured by VI values). Knowledge of such a relationship was important when quantitating proteins thought to change in relative abundance in the disease state. In such cases it was important to know whether to assume equal variance for comparisons of high and low abundance protein spots. The normal student *t*-test assumes equal variances. Figure 2.45 is a scatter diagram (derived from the data contained in Table 2.426) of protein abundance versus variance. A positive relationship existed between the two variables. When linear regression analysis was done on the data, an R^2 value of 0.88 was obtained. For a sample size of 39 pairs (13 spots from three sets of data), this value is significant at the 99% confidence level. This implied that there was a significant positive correlation between variance and relative protein abundance. That is, as relative protein abundance increased, so did variance. Based on this observation, variance was assessed for each group of proteins studied.

Figure 2.43. ***Quantitative reproducibility of proteins isoelectrofocused and electrophoresed at different times.***

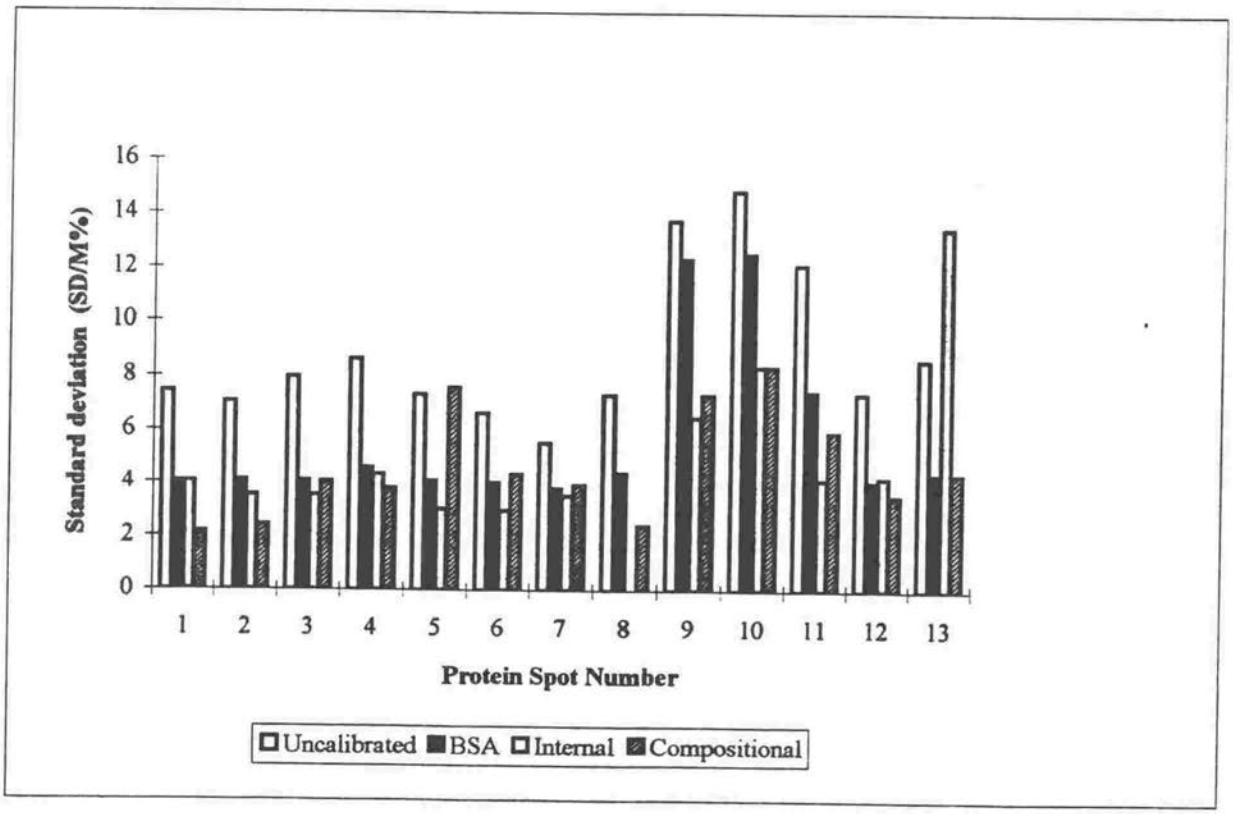


Figure 2.44. Normalised deviations from the mean for uncalibrated and compositional data.

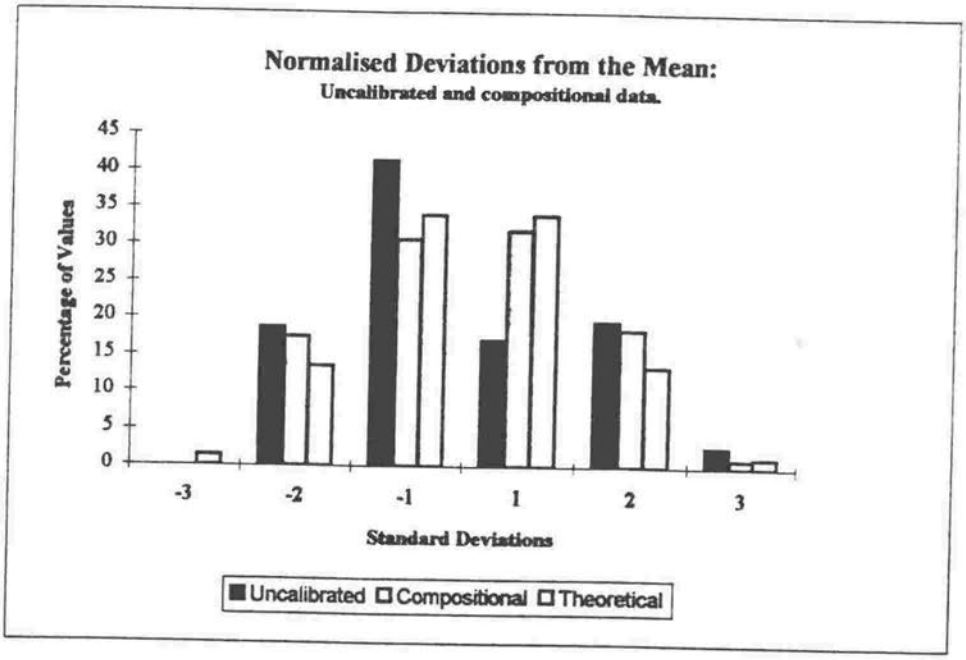
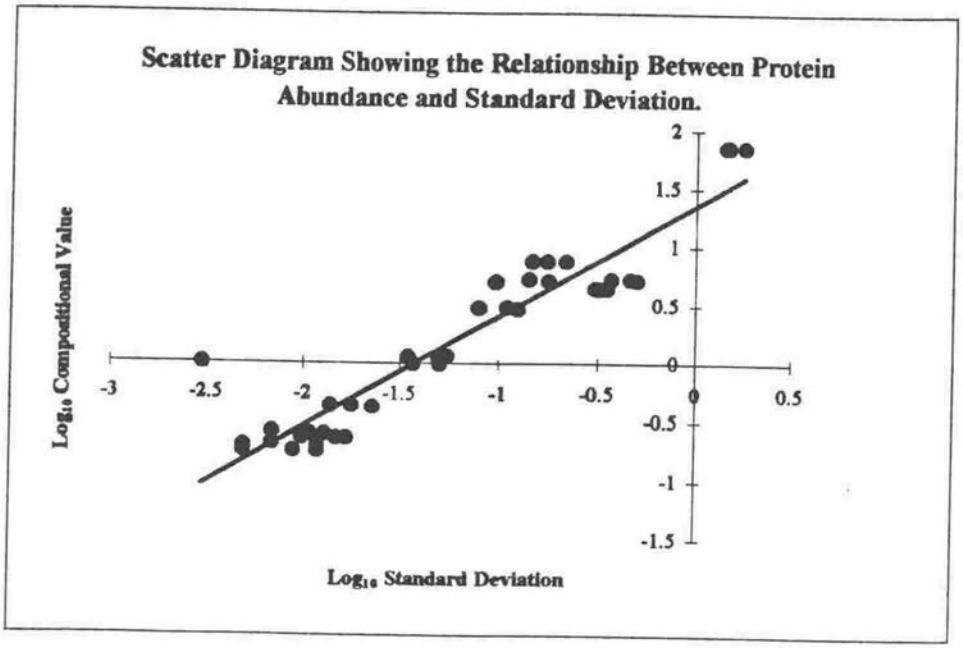


Figure 2.45. Scatter diagram of protein abundance against variance.



Chapter 3. Control Map for Human Plasma Proteins.

3.10) *Plasma proteins Selected for Study.*

This section gives a brief description of the proteins that were included in this study. This information is given to explain why the individual proteins were chosen and to aid in explaining the observations made for the groups of patients studied during this project. Unless otherwise stated, the information contained in this section was derived from current biochemistry and physiology texts.

Apo A-1

Synthesis of apo A-1 occurs mainly in the liver, and in the epithelial cells of the intestinal mucosa to a lesser extent. The half-life of apo A-1 is approximately four days. Apo A-1 is involved in the metabolism of VLDL and chylomicra, and in the metabolism of cholesterol. Apo A-1 also activates lecithin: cholesterol acyltransferase, a plasma enzyme responsible for the formation of most of the plasma cholesterol ester in humans.

A large range of values occur for apo A-1 concentrations in human plasma. Some factors that may affect this concentration include:

1) Age. The concentration of the apoproteins in plasma increases with age.

2) Sex. Women have higher apo A-1 levels than men. Differences in timing of the menstrual cycle also cause differences to be observed between women. The observed differences in apo A-1 levels between men and women can be explained by the effect of the sex hormones on lipoprotein metabolism. Oestrogens increase the concentration of apoproteins, while

androgens cause the level of apoproteins to decrease.

3) Diet. The diet and nutritional state of an individual can affect the concentration and composition of apoproteins, depending upon the types of foods consumed and the time that has elapsed since the last meal. For example, after eating a meal containing fat, hyperlipidaemia will result, usually peaking after two to four hours, but taking between six to eight hours to clear. As the chylomicra plasma level rises with the hyperlipidaemia, apoprotein levels increase in response. Also, the level of carbohydrate intake will influence the level of hepatic lipogenesis, which may influence apo A-1 production.

4) There are various other factors that may influence plasma levels of the apoproteins. These include genetic factors, the emotional state, the level of physical activity before sampling, and the general health status of the individual.

This protein was included in this study, despite the large number of variables that can influence its plasma level, because several recent papers have implicated abnormal lipid metabolism as having a role in the etiology of MS (eg. Bates, 1990), and because changes in lipid metabolism may have some relevance to Alzheimer's disease (Sun *et al.*, 1990; Corrigan *et al.*, 1991). Another reason for studying apo A-1 is that SAA, the APR seen to increase in plasma most dramatically in humans, preferentially binds to HDL (apo A-1 is a constituent of HDL). As SAA plasma concentrations can increase by up to 1000-fold in disease (Benditt *et al.*, 1988) it was decided to investigate whether apo A-1 was altered in MS or Alzheimer's disease.

Apo D and apo E

Apo D is an apoprotein found in HDL. The major function of apo D is to transfer esterified cholesterol from HDL to LDL, chylomicra, or VLDL. This allows the transport of esterified cholesterol to the liver, where it is hydrolysed and metabolised. A common name for apo D is cholesterol ester transfer protein.

Apo E has three isoforms, termed ϵ2, ϵ3, and ϵ4. Apo E is found in VLDL, HDL, IDL, chylomicra, and chylomicra remnants. Apo E acts to mediate the uptake of chylomicra by the liver. It achieves this by binding to specific receptors for apo ϵ3 and ϵ4. Apo D and E are essential for the metabolism of cholesterol ester. The reasons for studying these proteins are essentially the same as those outlined for apo A-1, and because of the association of the ϵ4 isoform with Alzheimer's disease (Strittmatter *et al.*, 1993).

Immunoglobulin Light Chains

There are two isotypic forms of Ig light chains, the lambda (λ) chains and the kappa (κ) chains. A given Ig molecule will have either two λ chains, or two κ chains. Neither the λ nor κ chains contain carbohydrate. They are distinguished from each other by differences in their constant regions (ie. C_L regions). The C_L regions of the light chains form the basis of the isotypic "typing" and "subgrouping" of all Igs. No allotypes have yet been found for the λ chains, but for the κ chains, three allotypes have been observed. Allotypes are a form of variance that depends upon the existence of allelic forms at a single locus. Usually, allotypic differences at a given locus only involve a few amino acid changes in the peptide.

In humans, Igs containing κ chains occur more frequently than those with λ chains, so the proteins observed on the 2-dimensional gels used in this project, will mainly be κ chains. However, due to the small difference in mw between the two chains (the λ chains are larger) the resolution of the type of gels used in this project would not separate the two chains. There are two sources for the variability in mw and isoelectric point for the Ig light chains. They are allotypic differences and idiotypic differences. Allotypic differences are differences in the C_L region. Idiotypic differences are differences in the variable (C_V) regions, mainly the hypervariable regions. Unlike allotypic differences, idiotypic differences are a type of variance that depends upon the existence of many genes at different loci.

There were two reasons for including the light chains in this study. First, the oligoclonal

banding seen in many individuals with MS occurs in the light chains of the Igs (Ford, 1985), although these changes are only usually observed in CSF. Secondly, other investigators working in this laboratory had observed some differences in the Ig light chain region of individuals with Alzheimer's disease when compared with controls.

Immunoglobulin J chains

The IgJ chains consist of low mw, cysteine-rich, polypeptides that are necessary for the polymerisation of the polypeptide subunits of IgA and IgM. IgA is a dimer consisting of two tetrameric polypeptide subunits, while IgM is a pentamer, consisting of five tetrameric polypeptide subunits. The polymerisation of both IgA and IgM occurs intracellularly. It is thought that IgJ chains act to stabilise the Fc sulphydryl groups during Ig synthesis so that they remain available for cross-linking to other subunits. The Fc regions are the heavy chain constant regions, which are obtained when Ig molecules are digested with papain.

IgJ chains were chosen for inclusion in this study because of their association with IgA and IgM. Both IgA and IgM synthesis is seen to increase in many individuals with MS (Martin *et al.*, 1992), although this is intrathecal synthesis, and only occurs after increased lymphocyte trafficking into the brain (Rowland *et al.*, 1991). The IgJ protein in plasma was studied to see if any difference between control and affected individuals could be observed. Such a difference could result from either an increase in IgA/IgM synthesis, or from the passage of IgJ from the brain into the plasma. I have not been able to find any reference to the passage of molecules such as IgJ from the brain to the plasma. But, as asymmetric transport systems do exist in the blood brain barrier (Rowland, 1991), and the blood-brain barrier can be compromised in neurological disease (Harik *et al.*, 1991), it is possible that such movement of IgJ could occur.

Retinol Binding Protein.

RBP is an α-globulin synthesised in the liver and involved in the transport of retinol (vitamin A). RBP is bound to transthyretin in equimolar amounts. Dietary retinol is esterified to long chain fatty acids in the intestine. The retinyl esters are then transported via the lymphatics to the liver where they are either transported to storage vesicles, or are bound to RBP for transport in the plasma. The RBP-retinol complex binds specifically to receptors on the choroidal surface of the pigment epithelial cells of the retina.

There are many clinical symptoms of retinol deficiency. These include lesions in the central nervous system (White *et al.*, 1979) of individuals who are thought to have been deficient in retinol during childhood.

RBP was included in this study because of the association with retinol, and the previous finding that retinol deficiency in childhood often results in central nervous system lesions. It is possible that lower levels of RBP could produce symptoms of retinol deficiency due to retinol accumulation in the liver. Also, elevated plasma levels of RBP have been shown in depressed patients, where the increased RBP was thought to have arisen because of inflammation (Maes *et al.*, 1992).

Haptoglobins.

The Hps are members of the α-2 globulin family. The Hps have two major functions. They are the primary determinants of the renal threshold for Hb. Hp achieves this by binding to free Hb in the blood, which prevents the renal uptake of Hb as the Hp-Hb complex is too large to pass through the glomerular membrane. This process has a role in the recycling of haem iron (Morimatsu *et al.*, 1991) and reduces the amount of damage done to the kidneys by Hb, as the degradation product haem, is cytotoxic to renal tubular epithelium (Hashizume *et al.*, 1988). The second major function of Hp is in the acute phase response. Plasma Hp levels increase several fold

during inflammatory reactions.

Hps are tetrameric polypeptides, containing two identical heavy ß chains that are responsible for Hb binding (Lustbader *et al.*, 1983), and two identical light α chains. The structure of the α and ß chains is governed by the Hpα and Hpß loci. Both chains are generated by a posttranslational cleavage from a single polypeptide encoded by the Hp gene (Maeda, 1991). Very little variation has been observed at the Hpß locus and the Hp ß chains from mammals have a high homology with one another (Morimatsu *et al.*, 1991). A possible reason for the conservation of the ß chain is that it is responsible for Hb binding. At least three alleles are commonly observed at the human Hpα locus ($Hp\alpha^{1s}$, $Hp\alpha^{1f}$, and $Hp\alpha^{2}$). The $Hp\alpha^{1s}$ and $Hp\alpha^{1f}$ polypeptides have mws of approximately 9100, while the $Hp\alpha^{2}$ polypeptide has a mw of 16,000.

Hp molecules have varying degrees of glycosylation, and varying contents of sialic acid. The sialic acid content may be important, as disialylation is thought to be involved in the catabolism of glycoproteins. The major site of Hp synthesis is the liver, although the spleen and lymphoid tissue are also thought to produce small amounts of Hp. Hb in the circulatory system is almost immediately complexed with Hp. This complex is then removed by hepatocytes, and by the spleen and bone marrow to a lesser extent. However, only approximately 50% of the plasma Hp is removed in this manner. The remainder is catabolised independently and has a half-life of approximately 96 hours. There is a negative correlation between the level of haemolysis and the plasma Hp level (Giblet, 1974).

In a healthy individual, the plasma Hp level remains constant, but varies by as much as one order of magnitude between apparently healthy individuals and tends to be lower in children and young adults (Giblet, 1974). Inflammation, stress, and neoplasia can cause Hp levels to increase. Plasma Hp levels can increase 10-fold during inflammatory processes. Usually such increases are accompanied by increasing plasma levels of orosomucoid, and fibrinogen (Maes *et al.*, 1992). Hereditary factors also influence plasma Hp levels.

The concept of Hp phenotype is complicated. Not only does it consider the type of Hp present in the plasma, but also the relative amount. The three commonest phenotypes are Hp1, Hp2, and Hp2-1 (Putnam, 1975). Individuals who have the Hp2 phenotype tend to have lower plasma Hp levels than Hp1 type individuals. The frequency of the three genotypes producing these phenotypes differs between different ethnic groups. For example, in caucasians and negroes, the commonest genotype is Hp 2-1, but this is followed by Hp 2-2 in caucasians and Hp 1-1 in negroes. Also, it appears that the presence of certain haemoglobinopathies within a population can participate in a selection mechanism that alters the Hp gene frequencies within that population over time (Moreira *et al.*, 1990).

There are thought to be at least six multimers of Hp2-1, and there is a wide spectrum of Hp2-1 phenotypes. The variants that have received the most attention are the Hp2-1 (mod) variants. Hypohaptoglobinemia (phenotypic designation Hp0) is often found in individuals suffering from hepatocellular damage or greatly increased haemolysis. Genetic factors can also result in this phenotype. Individuals homozygous for the $Hp\alpha^2$ genes tend to be of the Hp0 phenotype. Individuals with the genotype $Hp\alpha^1$: $Hp\alpha^2$ can also be phenotypically Hp0, especially if one or both of their parents were of the Hp2-1 (mod) phenotype. Currently, it is not known whether individuals with the Hp0 phenotype produce no Hp, or whether they produce levels of Hp that are too low to be detected by the techniques in common practice. Two important points need to be raised. First, in all of the populations studied, Hp polymorphism has been observed. Second, because the normal Hp plasma levels vary so widely, it is difficult to interpret the clinical significance of the laboratory value for Hp obtained from an isolated specimen of blood (Koj, 1974).

Despite these limitations there were two major reasons for analysing Hp variation. First, differences were detected in the Hp levels in the blood samples taken from control patients and those taken from affected patients. Most differences between control individuals and affected individuals in this project were differences in Hp expression. Second, several blood samples were obtained for three Alzheimer's patients over a period of nine months so that it was possible to study whether the plasma level of Hp and the other APRs varied with time.

Transthyretin.

Transthyretin, formerly known as pre-albumin, is a glycoprotein. Usually, glycoproteins are designated α-1 or α-2 depending upon their electrophoretic mobility. However, the electrophoretic mobility of transthyretin is between those of α-1 and α-2 globulins, so it is said to be an inter-α-glycoprotein. Transthyretin binds and transports both thyroxine and RBP. As thyroxine binding proteins influence the activity of thyroxine and triiodothyronine, they can have a large influence on general metabolism, development, and tissue differentiation. The rate of hepatic synthesis of thyroxine binding proteins is increased by increasing oestrogen levels, and decreased by increasing androgen levels, liver disease, and glucocorticoid therapy.

Transthyretin was analysed in this study because of the involvement of thyroid hormones in brain function and development. Some regions of the brain of Alzheimer's patients have much reduced levels of oxygen consumption when compared to controls (Heiss *et al.*, 1991; Wurtman, 1987). Since the general function of thyroid hormones is to increase oxygen consumption, it seemed reasonable to study transthyretin, given the link with the activity of thyroid hormones. It is also known that reduced levels of thyroid hormones during human development appear to cause abnormalities in the central nervous system (Granner, 1988). However, the hypothyroidism that would be necessary to cause such abnormalities would probably be readily diagnosed. The major assumption made in studying transthyretin levels in this project is that increased levels of transthyretin will reduce the total amount of free triiodothyronine and thyroxine available for biological activity.

Orosomucoid.

Orosmucoid, also known as α-1 acid glycoprotein, is an α globulin with at least three genetic variants (phenotypically designated I, II, and III). The carbohydrate content of this protein is approximately 42% and the half life of orosomucoid is approximately 125 hours.

Orosomucoid is an APR, its concentration in plasma increases during trauma, inflammation, rheumatoid arthritis, and in the presence of some malignancies. Surgery can double the plasma concentration of orosomucoid (Koj, 1974).

Orosomucoid is involved in regulating progesterone levels in pregnancy and in normal and pathological states. It does this by binding to progesterone. Orosomucoid also competitively inhibits the transformation of prothrombin to thrombin and may inhibit increased blood clotting in certain inflammatory states. Orosomucoid is also thought to have immunosuppressive functions (Arnaud *et al.*, 1988). This protein was included in this study because of its known role in the acute phase response.

α-1 Antichymotrypsin.

ACT is a glycoprotein that is a serine protease inhibitor that specifically inhibits chymotrypsin. ACT is synthesized in the liver, and has a carbohydrate content of approximately 25%. ACT is regarded as an APR. The plasma concentration increases during certain types of infection and in the presence of some malignancies. The physiological function of ACT has not yet been adequately defined (Matsubara *et al.*, 1990). It may afford protection against enzymes released into the blood and may have roles in blood coagulation. It may also be involved in the complement system, and may be involved in removing intravascular blood clots.

ACT was included in this study because it is a component of the acute phase response. Also several researchers have shown that ACT is a component of senile plaques, and that plasma levels of ACT are elevated in the plasma of individuals affected with Alzheimer's disease (Shoji *et al.*, 1991; Carmela *et al.*, 1989).

Actin.

Actin is found in muscle and non-muscle cells, and in plasma. In nerve cells, actin is involved in the exocytotic release of neurotransmitters from pre-synaptic membranes, into the synaptic cleft. As this process is relevant to neurological functioning it was decided to include actin in this study, although plasma actin is probably derived from a range of cellular sources.

Gamma Fibrinogen.

Gamma fibrinogen is a soluble plasma glycoprotein with a carbohydrate content of approximately 4%. Fibrinogen is synthesised in the liver and is a dimer of two identical trimeric polypeptides containing α(A), ß(B), and γ chains. The genes encoding the three polypeptide chains are genetically linked, and are expressed in a coordinated fashion. The half life of fibrinogen varies between 84 and 96 hours. The major function of fibrinogen is in the blood coagulation cascade where it is the precursor of fibrin. γ Fibrinogen is involved in both the intrinsic and extrinsic blood coagulation pathways. Fibrinogen was chosen for inclusion in this study due to its role in the acute phase response (Fuller *et al.*, 1988).

Ceruloplasmin.

Crp is an α-2 globulin, with a carbohydrate content of approximately 8%. Crp is synthesized in the liver, and has a half-life of approximately 102 hours. There are four alleles that produce ten phenotypes.

Crp is an APR whose plasma concentration rises during infection. However, the Crp plasma concentration often decreases with increasing age. Other factors known to affect Crp plasma levels include genetic influences, hormones (oestrogens increase Crp synthesis, while androgens act to reduce Crp concentrations in some situations), and the amount of copper in plasma. It is thought that heavy cigarette smoking can also result in elevated plasma Crp levels (Duthie *et al.*, 1991).

Crp is involved in copper transport, and possibly also regulates the copper levels in the liver and other tissues. Crp has eight copper binding sites, and can bind Cu (I) and Cu (II). Crp also has a catalytic activity of unknown physiological significance. It exhibits both ferroxidase and polyamine oxidase activities. The copper serves as the sole prosthetic group in this oxidative function of Crp. Another major function of Crp is to deliver copper to cytochrome reductase in the mitochondrial respiratory chain.

Apart from its role in the acute phase response, Crp was included in this study because characteristic changes that accompany the low Crp plasma levels in Wilson's disease include copper accumulation in the brain, and lesions in the CNS. Also imbalances in copper metabolism in lambs result in demyelination (White *et al.*, 1978).

Other proteins.

For the remainder of the plasma proteins studied in this project, inclusion was not based upon prior knowledge of their biochemical/physiological activity. Such proteins were included because a difference was seen between control patients and affected patients or because the protein was selected at random, to increase the reference panel of proteins that should remain invariant during quantitative analysis.

3.20) *Control Map for Human Plasma Proteins.*

3.21) Introduction.

To make a map of human plasma proteins that included all of the proteins studied in this project, it was necessary to create a composite control map. This had as its source, several different gels. Before including proteins on the control map, a check was made to ensure that each protein occurred on replicate gels, and gels taken from similar individuals. When possible the proteins were then identified from published maps.

3.22) Materials and Methods.

Patient Summary.

Blood and CSF were collected by Drs. C. Shaw and D. Abernethy of the Neurology Department of Wellington Hospital. Drs. Shaw and Abernethy were responsible for patient diagnosis, and for selection of patients and their age and sex matched controls for this study. Alzheimer's diagnosis was based upon the National Institute of Neurological and Communicative Diseases and Stroke - Alzheimer's Disease and Related Disorders Association diagnostic criteria (McKhann *et al.*, 1984) and the mental status score devised by Kokmen *et al.* (1987). Controls for almost all of the Alzheimer's patients were spouses judged to be healthy by Dr. D. Abernethy. MS diagnosis was based upon clinical evaluation and the application of the criteria established by McAlpine *et al.* (1972). Controls for the MS study were Neurology Department inpatients not suffering from neurodegenerative disorders. Appendix 2 summarises the MS patients and controls.

This study was approved by the Ethics Committee of the Wellington Area Health Board, and informed consent was obtained from each subject. Clinical information is not provided for individual patients, but is held by Wellington Hospital. Table 3.221 gives a summary of the

patients and their matched controls that were used in this study.

Table 3.221. Summary of patients and their matched controls used in this study.

	MALES			FEMALES		
	n[1]	Age (y)	Duration[2] (y)	n	Age (y)	Duration (y)
Alzheimer's Patients	5	67-84	5-8	5	54-84	4-9
Matched Controls	5	64-82	...	5	54-88	...
MS Patients	4	27-45	Unknown	6	36-42	Unknown
Matched Controls	4	26-41	...	6	16-54	...

1. Number of individuals in sample group.
2. Years since onset of disease was first diagnosed.

Control Map for Human Plasma Proteins.

The proteins identified below are those used in the quantitative analysis of plasma variation in patients with Alzheimer's disease or MS, and their matched controls. To make the control map, four 10% two-dimensional gels were selected from three of the groups studied in this project; normal healthy individuals, individuals affected with MS, and individuals diagnosed as having Alzheimer's disease. When selecting these gels, a visual inspection was made to ensure that they represented the group from which they came. For the MS group, the gel chosen was from a replicate set of pooled control females. This gel was chosen as it shared all of the proteins that were consistently shown on gels from control and affected male and female individuals. It also contained a group of proteins that were only found in several affected female individuals. For the

Alzheimer's group, the gel chosen was from a total pool of affected individuals (both males and females), as no consistent differences had been detected between affected individuals and controls, or between sexes. For the third group, the two gels chosen were from the healthy adult population. In summary, the details of the chosen gels are:

Gel Number	*Sample Used*	*Protein Load (μg)*
1	*Individual A (healthy)*	*175*
2	*Individual B (healthy)*	*175*
3	*Pooled MS females*	*175*
4	*Pooled Alzheimer's disease patients*	*175*

The four gels were scanned and digitised using a Molecular Dynamics scanning densitometer. The magnification was then increased by a factor of four. One of the regions of interest was then scrolled into position, the colour/density range was maximised, and a contour map of the region was printed. This process was then repeated for the remaining gels, a contour map for each of the nine regions of interest being obtained for each gel. For each of the nine regions of interest, the appropriate contour maps from each gel were compared. If any contour map showed a protein difference not present on the maps of the other gels, a check was made to see if the difference also occurred on replicate gels, and gels from within the group from which the gel was taken (eg. affected Alzheimer's patients). If the change was consistent, the contour maps were overlaid and a new contour map was drawn.

In making the control map there were several potential sources of error. Error may have arisen during the overlaying process. As different colour/density ranges were used for each contour map, slight differences occurred in the contour maps. This meant that judgement was required during the overlaying process when different contour maps were being aligned. Contour regions deemed to be due to either background or ampholyte staining were considered artifacts and were

not included in the control map.

The plasma proteins studied in this project were identified from several published maps (Harrison *et al.*, 1992; Anderson *et al.*, 1977; and Anderson *et al.*, 1991), although identification tended to focus on the database contained in Anderson *et al.* (1991). The proteins identified were given the appropriate identification number from the 1991 database of Anderson *et al.*, (1991). The mw cited with each of the identified proteins is that given by Anderson *et al.*, (1991). Internal mw standards were used to make a mw standard curve using the following plasma proteins: α2 macroglobin (mw 181,250), albumin (mw 66,458), AT (mw 54,000), apo A1 (mw 28,076), and transthyretin (mw 13,745). Unless the mw determined differed from that cited by Anderson by more than 20%, the value of Anderson was quoted. Where a difference of more than 20% was obtained, it was assumed that the protein had been incorrectly identified on the control gels. A second attempt was then made to locate the protein on the database. If the protein could still not be accurately identified, its MSN was termed unknown.

3.23) Results.

Figures 3.231 and 3.232 show the control map at the size of the original scanned image. Figure 3.232 identifies the nine individual regions that were analysed at 4X magnification and are shown in the regional maps. In the data which accompanies each of the regional maps, MSN refers to the master spot number quoted in the database of Anderson *et al.* (1991). POP Name refers to the name of the protein population which the individual protein is thought to belong to. POP names are referred to as either IDS, when the protein series has been identified, or PLS, when the protein series has not yet been identified. Figures 3.233 to 3.241 show the nine map regions indicated in Figure 3.232. Table 3.240 summarises the proteins studied, and gives the location of each protein.

Figure 3.231. 10% 2-Dimensional PAGE composite plasma protein map.

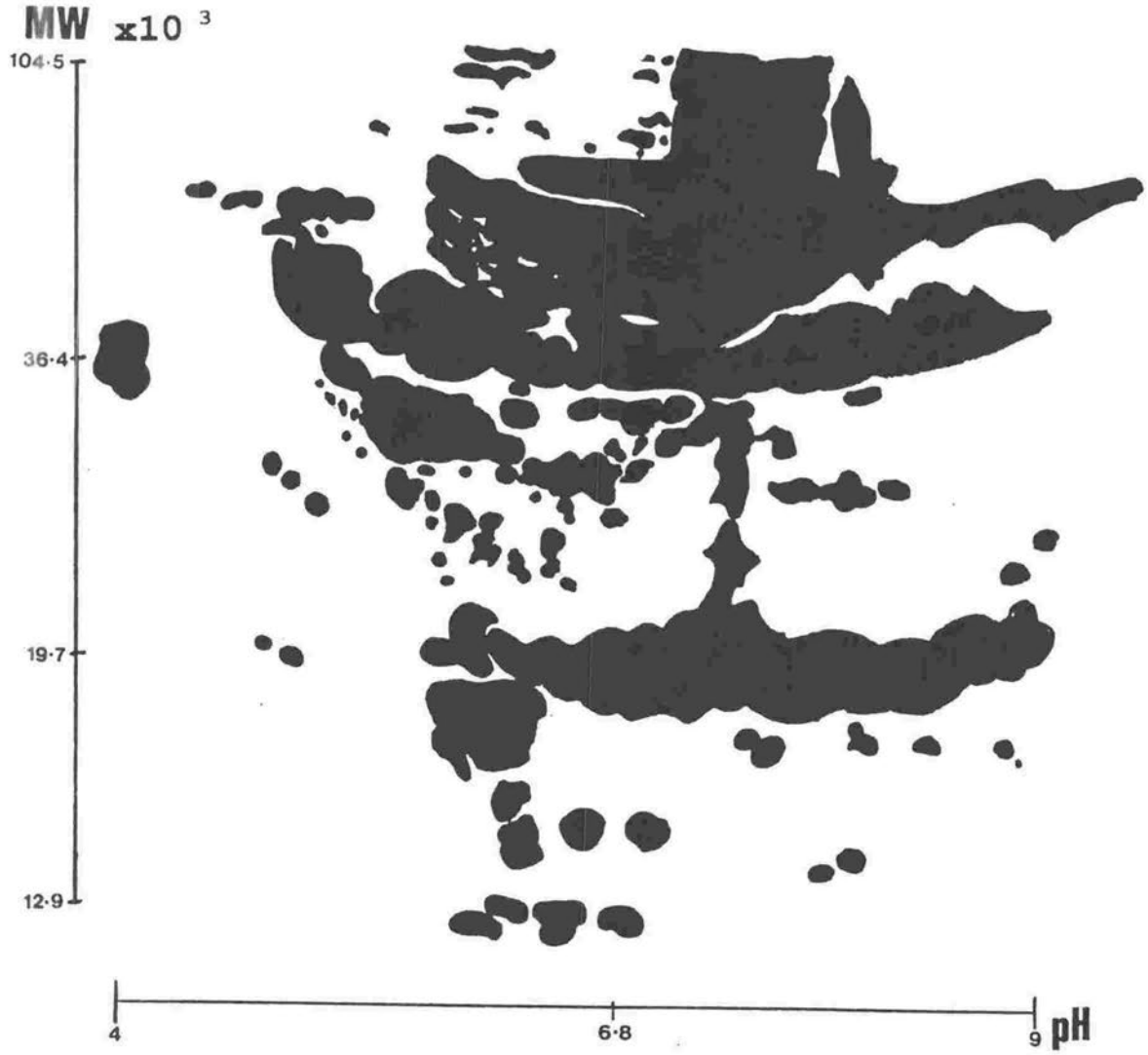


Figure 3.232. Composite plasma protein map, showing the location of the nine regions shown in Figures 3.233 to 3.2311 .

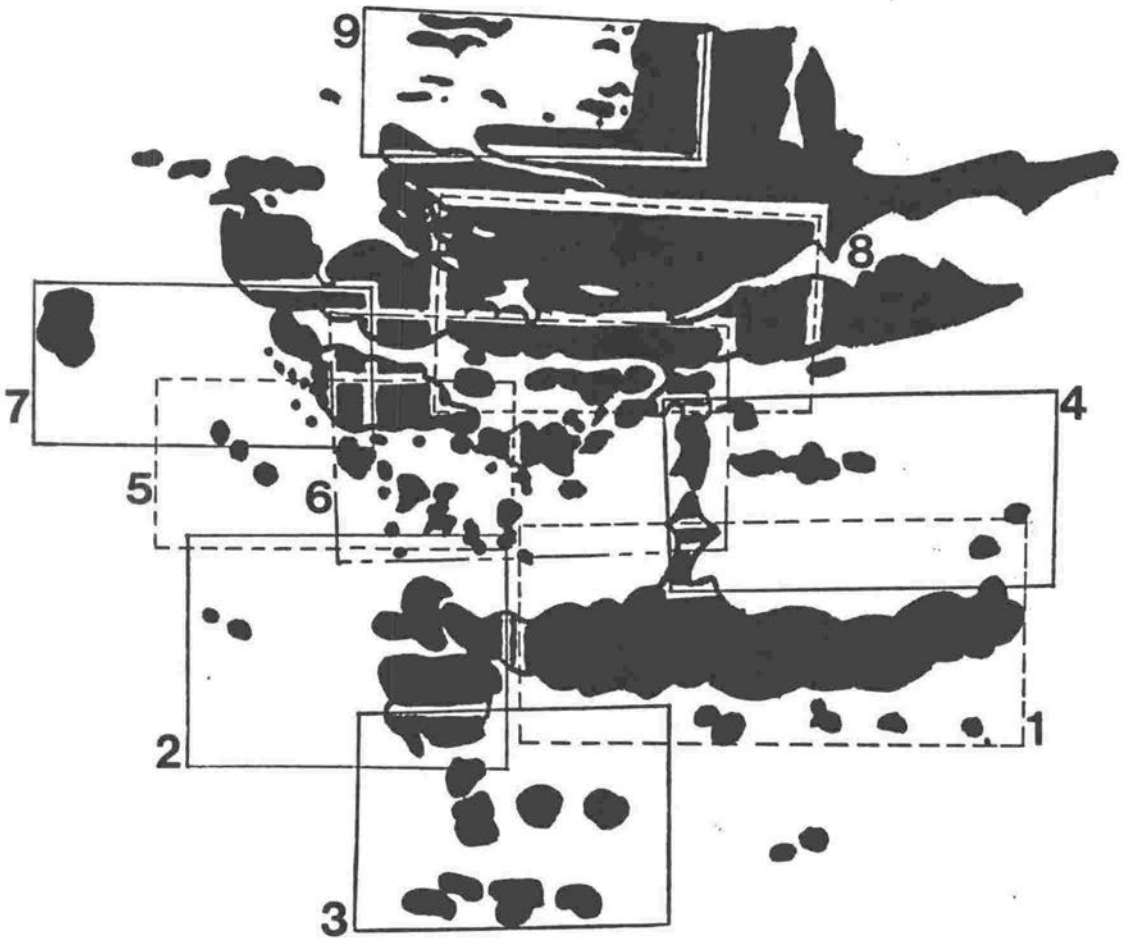


Table 3.231. The proteins shown in Figure 3.233.

Spot I.D.	POP Name	MSN	Common Name	mw
18	Unidentified	173	Unidentified	27,900
30	IDS: IG_LIGHT_ CHAIN	173	Ig light chains (λ and κ).	21,500
31	IDS: IG_LIGHT_ CHAIN	115*	Ig light chains	21,400
32	IDS: IG_LIGHT_ CHAIN	124	Ig light chains	21,200
33	IDS: IG_LIGHT_ CHAIN	100	Ig light chains	21,400
34	IDS: IG_LIGHT_ CHAIN	323	Ig light chains	21,400
35	IDS:PRO_APO_A1	153	Pro-form of Apo A1	20,500
36	IDS:PRO_APO_A1	511	Pro-form of Apo A1	20,300
A	IDS: HP:_Alpha2	140	Hp α-2 chain (allelic with α1F and α1s chains)	15,600

* This protein may also have incorporated MSN's 159 and 324. Due to the lack of resolution in this portion of the gel, it is difficult to be certain.

The Ig light chain region (spot numbers 30 to 34) show both the λ and κ chains. The λ chains have a higher mw, so will have migrated to a point above the κ chains. However, with the separation techniques employed in this study, the resolution is not great enough in this region of the gel to resolve the two chains.

Figure 3.233. ***Plasma map region number 1. Ig light chain region.***

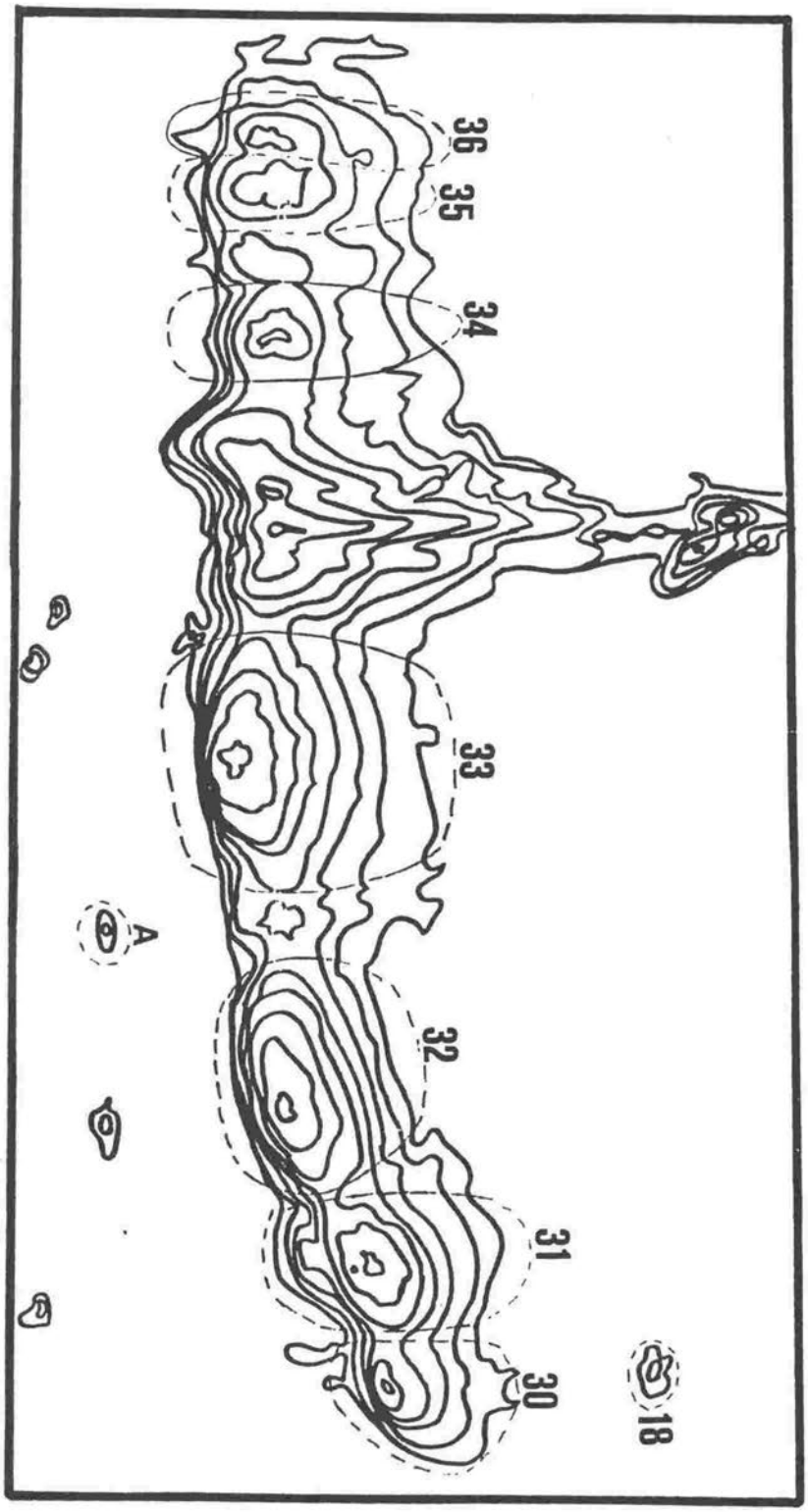


Table 3.232. The proteins shown in Figure 3.234.

Spot I.D.	MSN	POP Name	Common Name	mw
1	57,65, 133,436	IDS: APO_A1	Apo A1, mature circulating form.	20,200 to 20,300
2	203	IDS: IG_J_ CHIAN	IgJ chain	19,700
3	235	IDS: IG_J_ CHAIN	IgJ chain	19,800

Table 3.233. The proteins shown in Figure 3.235.

Spot I.D.	MSN	POP Name	Common Name	mw
1	37,65, 133,463	IDS: APO_A1	Apo A1. Mature circulating form.	20,200 to 20,300
14	140	IDS: HP_ALPHA_2	Hp α-2 chain	15,600
15	95	IDS: HP_ALPHA_2	Hp α-2 chain	15,600
16	86	IDS: HP_ALPHA_2	Hp α-2 chain	15,800
17	81	IDS: SRBP	Serum RBP.	18,300
A	263	IDS: TRANSTHYRETIN	Transthyretin; formerly pre-albumin	12,900
B	89	IDS: TRANSTHYRETIN	Transthyretin.	13,000

Figure 3.234. ***Plasma map region number 2. Low mw acidic proteins.***

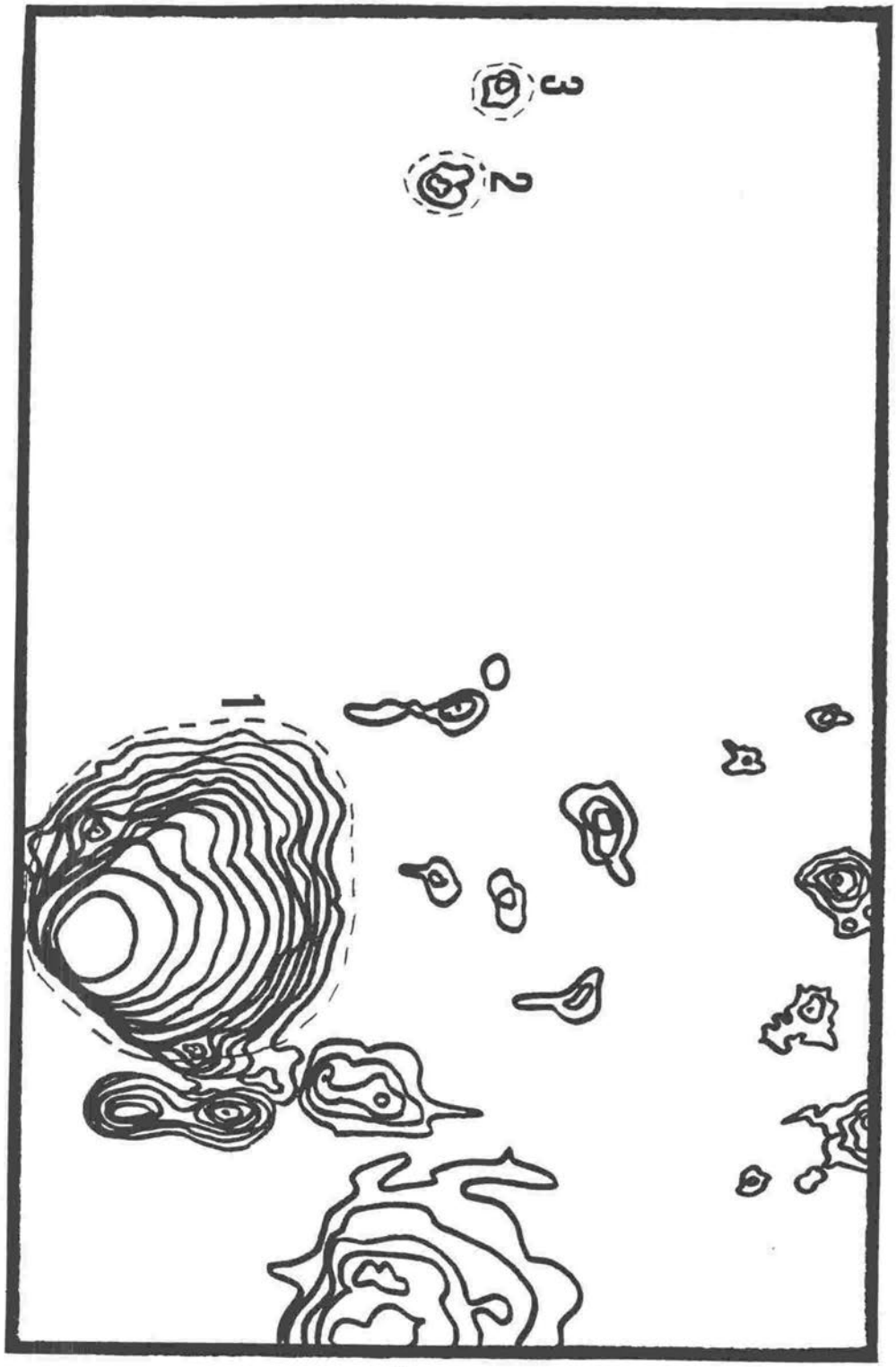


Figure 3.235. ***Plasma map region number 3. Low mw acidic/neutral proteins.***

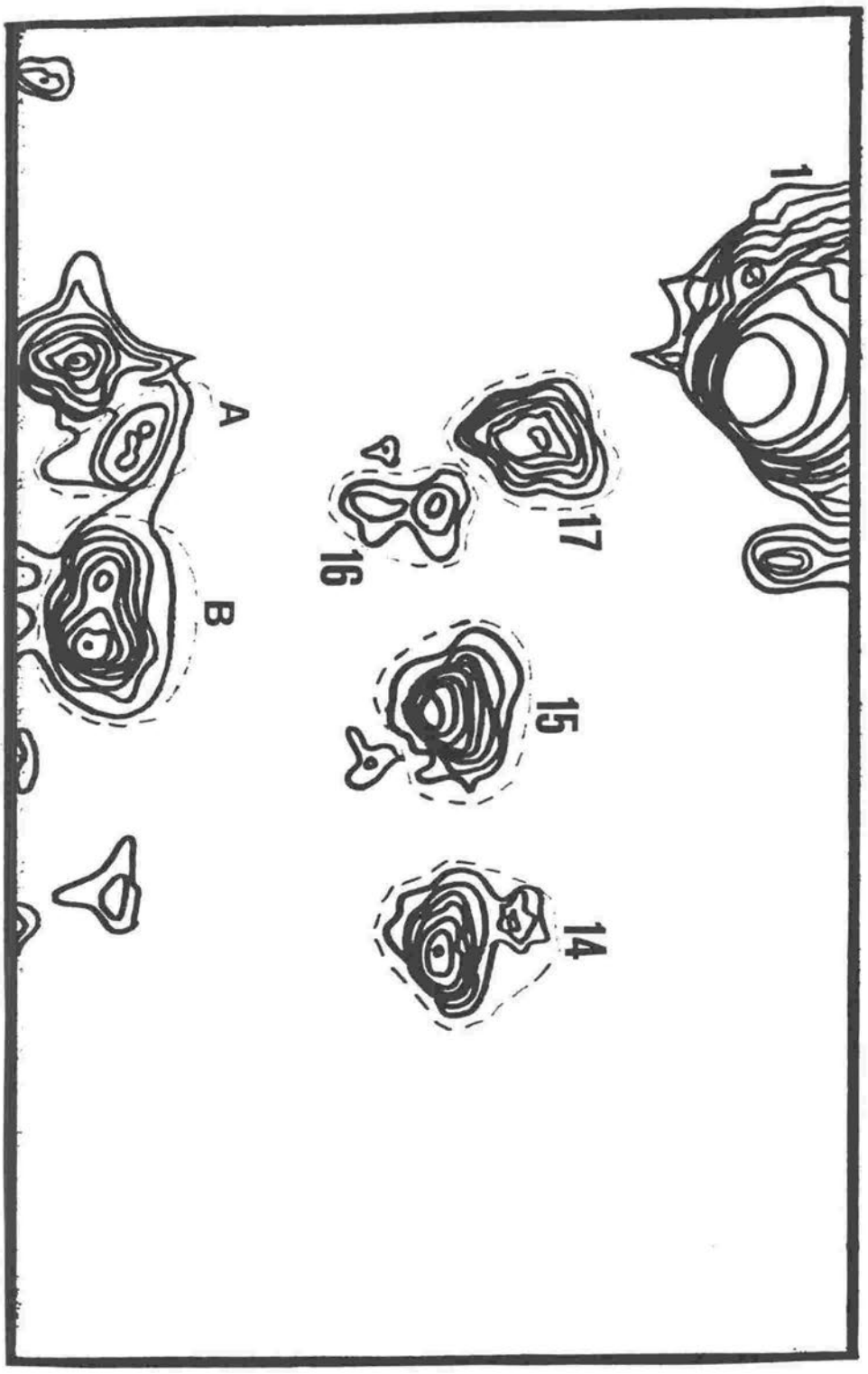


Table 3.234. The proteins shown in Figure 3.236.

Spot I.D.	MSN	POP Name	Common Name	mw
18	613	Unidentified	Unidentified	27,900
19	601	PLS: 37	Unidentified	34,000
20	597	PLS: 37	Unidentified	34,100
21	599, 600	Unidentified	Unidentified	33,700 to 34,500

Table 3.235. The proteins shown in Figure 3.237.

Spot I.D.	MSN	POP Name	Common Name	mw
5	535	Unidentified	Unidentified	25,700
7	523, 551	Unidentified	Unidentified	28,900 to 29,700
9	487, 529	Unidentified	Unidentified	43,300 to 43,800
10*	80,92, 217	IDS: HP_BETA	Hp ß chain	33,800 to 39,000
25	617	IDS: APO_D	Apo D	32,500
26	702	IDS: APO_D	Apo D	34,900
A	530	IDS: APO_D	Apo D	33,600
B	215, 238	IDS: APO_E	Apo E	27,500 to 28,100
C	532	Unidentified	Unidentified	24,600

* See Table 3.237 for more detail.

Figure 3.236. ***Plasma map region number 4. Intermediate mw basic proteins.***

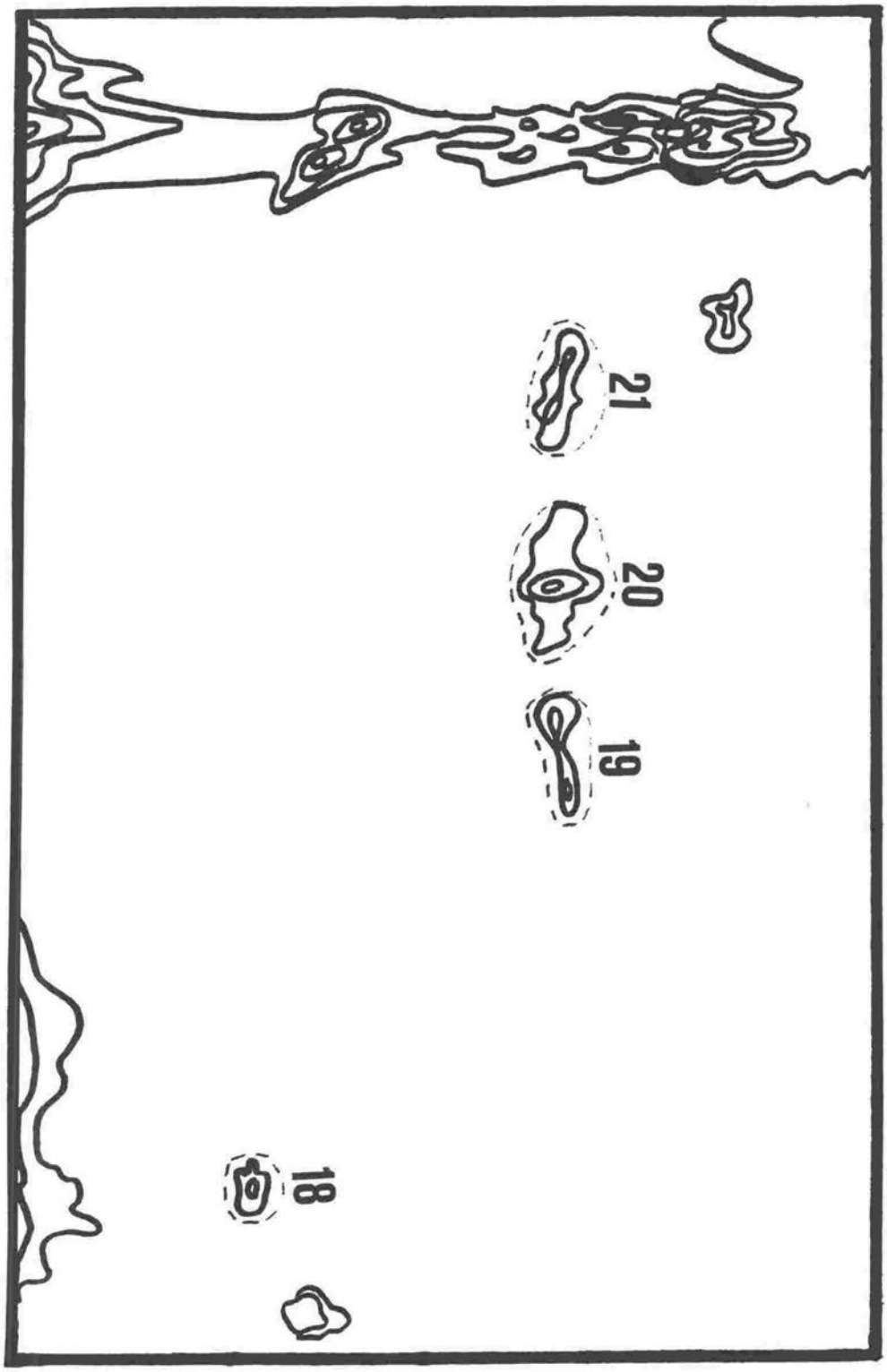


Figure 3.237. ***Plasma map region number 5. Intermediate mw acidic proteins.***

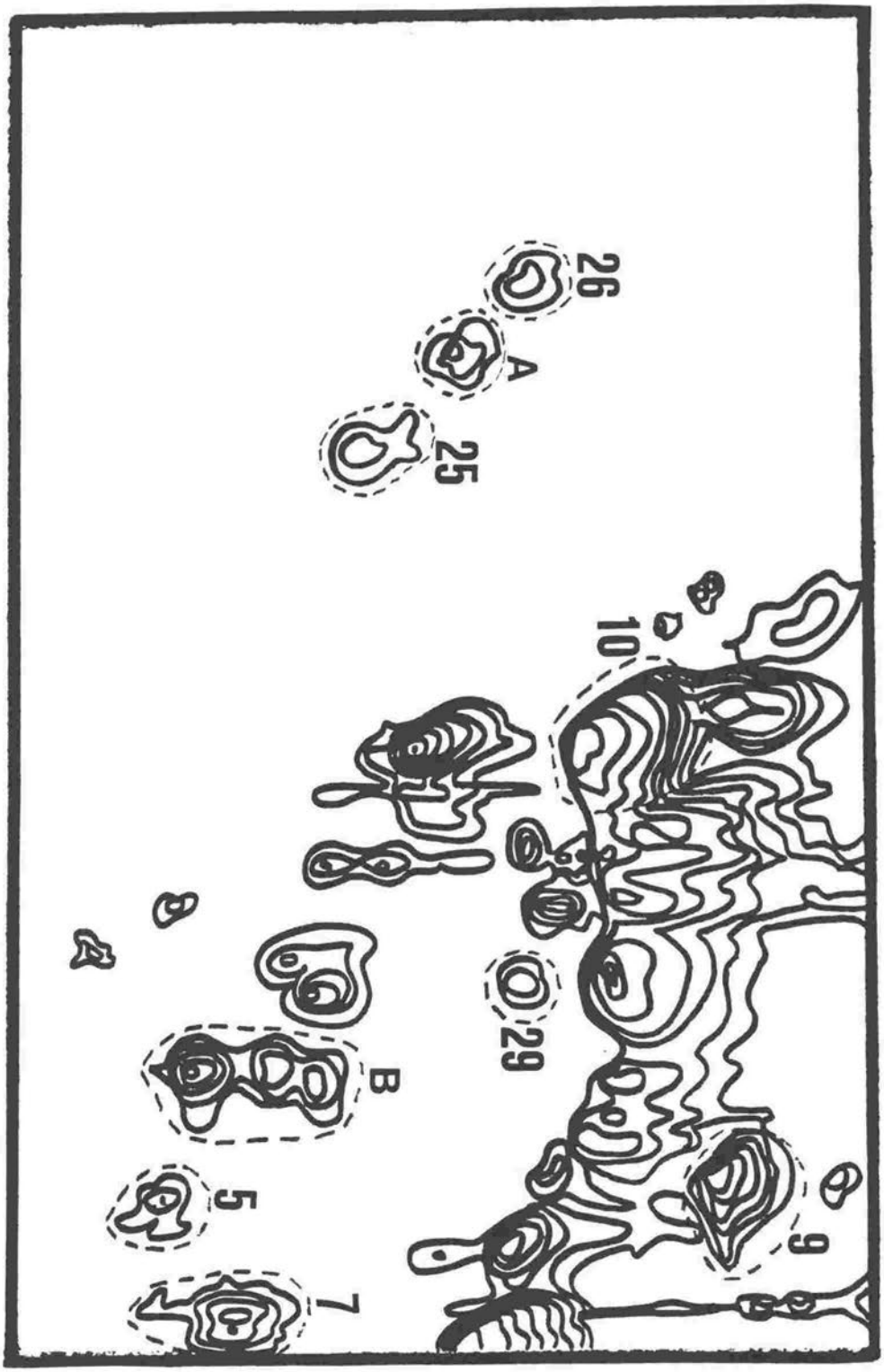


Table 3.236. The proteins shown in Figure 3.238.

Spot I.D.	MSN	POP Name	Common Name	mw
4	501	Unidentified	Unidentified	43,600
5	535	Unidentified	Unidentified	25,700
7	523, 551	Unidentified	Unidentified	28,900 to 29,700
9	487, 529	Unidentified	Unidentified	43,300 to 43,800
10*	80,92, 217	IDS: HP_BETA	Hp ß chains	33,800 to 37,200
22	479	Unidentified	Unidentified	32,100
23	332, 193	IDS: HP_BETA _CLEAVED	Hp ß chains	31,700 to 31,80
24	207,363, 524	IDS: HP_BETA _CLEAVED	Hp ß chains	32,200 to 32,800
27	458,502	Unidentified	Unidentified	43,600 to 44,900
28	412,499	Unidentified	Unidentified	41,700 to 43,500
29	384	IDS: APO_E	Apo E	29,000
37	Unknown	Unidentified	Unidentified	
38	Unknown	Unidentified	Unidentified	
39	357	Unidentified	Unidentified	36,000
40	555,550	Unidentified	Unidentified	38,900 to 39,300
A	554	Unidentified	Unidentified	31,300

* See Table 3.237 for more detail.

Figure 3.238. ***Plasma map region number 6. Intermediate mw acidic/neutral proteins.***

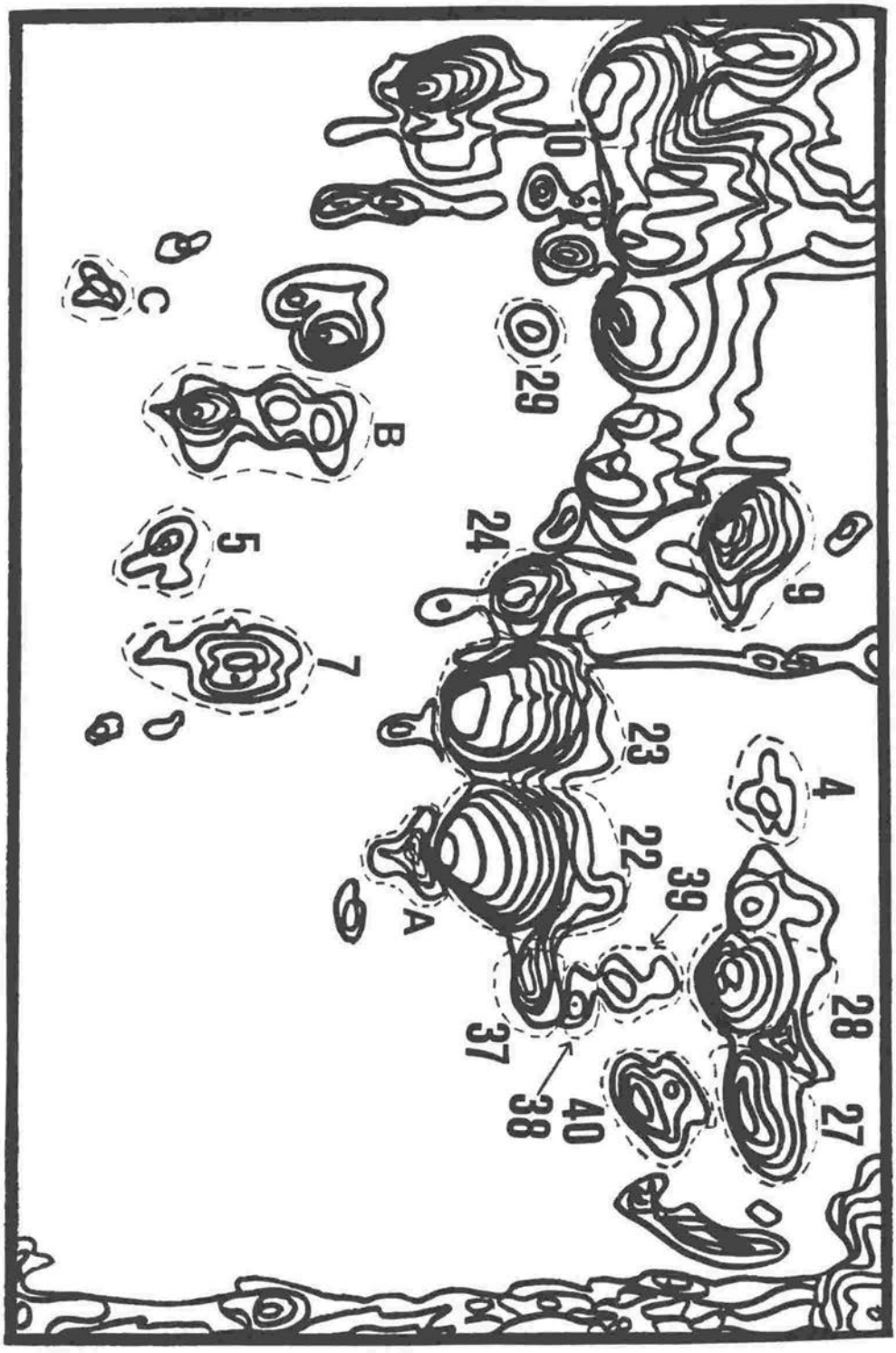


Table 3.237. The proteins shown in Figure 3.239.

Spot I.D.	MSN	POP Name	Common Name	mw
10*	80,92, 217 725	IDS: HP_BETA IDS: HP_BETA_CLEAVED IDS: ACTIN GAMMA	Hp chain Cleaved Hp ß chain gamma actin	33,800 to 37,200 33,800 33,800
13	686,690 691,694 695,696 722	IDS: OROSOMUCOID	Orosomucoid; α-1 acid glycoprotein	33,900 to 38,800
26	702	IDS: APO_D	Apo D	24,900
41	55,54	IDS: HP_BETA	Hp ß chain	36,600 to 37,500
42	64,66	IDS: HP_BETA	Hp ß chain	37,400 to 38,500
43	43,104	IDS: HP_BETA	Hp ß chain	38,700 to 39,900
44	87	IDS: HP_BETA	Hp ß chain	39,900
45	268,402	Unidentified	Unidentified	39,400 to 40,700
A	34,37,385 0,62,63 82,127	IDS: A1-ANTICHY	ACT	54,900 to 61,800
B	12,32,454 6,85, 116,258	IDS: ALPHA2-HS	α-2 HS glycoprotein	48,100 to 51,600
C	608,9	PLS: 30	NA2 (HDL associated protein).	30,400 to 30,700
D	216,237	PLS: 29,30	NA1,2 (HDL associated protein).	3,300 to 33,400
E	8,9,13, 16,23,31 69,110, 134	IDS: A1-AT	AT, mature circulating form	49,500 to 53,200

* This was a non-discrete protein spot. Due to the similar characteristics of these proteins they were not resolved with the method used in this project.

Figure 3.239. Plasma map region number 7. Intermediate mw acidic proteins.

Table 3.238. The proteins shown in Figure 3.240.

Spot I.D.	MSN	POP Name	Common Name	mw
4	501	Unidentified	Unidentified	43,600
8	482	Unidentified	Unidentified	48,400
9	487,529	Unidentified	Unidentified	43,300 to 43,800
27	458,502	Unidentified	Unidentified	43,600 to 44,900
28	412,499	Unidentified	Unidentified	41,700 to 43,500
A	36,59,70 79,90,136 170,284	IDS: FIB_GAMMA	Fibrinogen γ chain	45,900 to 74,600

Table 3.239. The proteins shown in Figure 3.241.

Spot I.D.	MSN	POP Name	Common Name	mw
6	374	Unidentified	Unidentified	82,500
11	351	PLS: 9	Unidentified	85,300
12	445,457 459,486 496	PLS: 10	Unidentified	90,900 to 92,100
A	1,4,83,88 101,160, 184,242 257,312	IDS: CERULOPLAS	Crp	163,300 to 106,400

Figure 3.240. ***Plasma map region number 8. High mw acidic proteins.***

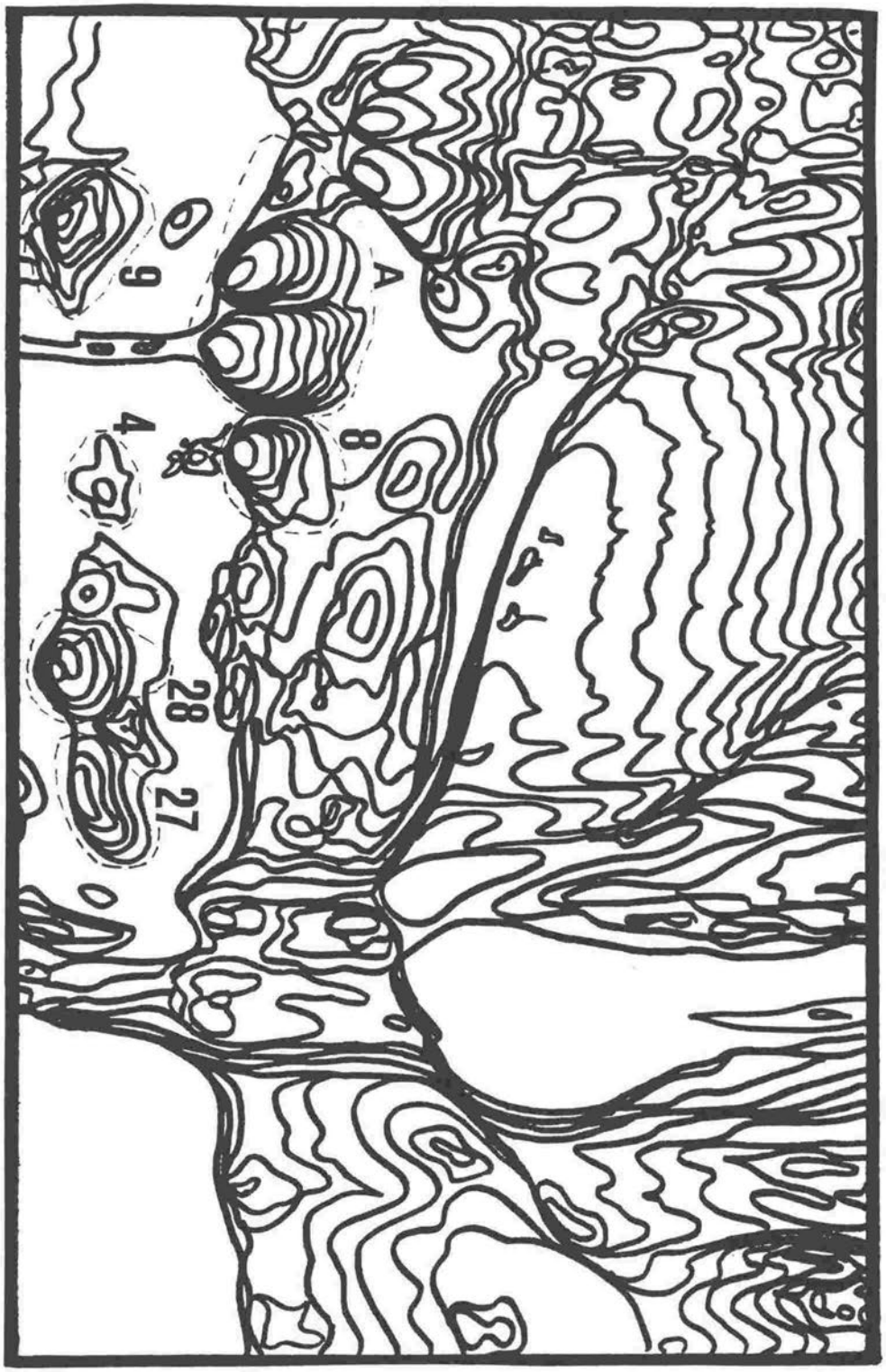


Figure 3.241. ***Plasma map region number 9. High mw acidic proteins.***

Table 3.240. The location of the plasma proteins studied in this project.

Spot I.D.	Map #	Protein Name	Spot I.D.	Map #	Protein Name
1	2	Apo A1 Circulating form	30	1	Ig G light chains
2	2	Ig J chain	31	1	Ig G light chains
3	2	Ig J chain	32	1	Ig G light chains
4	6	Unidentified	33	1	Ig G light chains
5	5	Unidentified	34	1	Ig G light chains
6	9	Unidentified	35	1	Pro form of Apo A1
7	5	Unidentified	36	1	Pro form of Apo A1
8	8	Unidentified	37	6	Unidentified
9	5	Unidentified	38	6	Unidentified
10	5	Hp β chain	39	6	Unidentified
11	9	Unidentified	40	6	Unidentified
12	9	Unidentified	41	7	Hp β chain
13	7	Orosomucoid	42	7	Hp β chain
14	3	Hp α2	43	7	Hp β chain
15	3	Hp α2	44	7	Hp β chain
16	3	Hp α2	45	7	Unidentified
17	3	Serum RBP	..	3	Transthyretin
18	1	Unidentified	..	3	Transthyretin
19	4	Unidentified	..	5	Apo D
20	4	Unidentified	..	5	Apo E
21	4	Unidentified	..	7	ACT
22	6	Unidentified	..	7	α2 HS glycoprotein
23	6	Hp β chain	..	7	NA2 (HDL associated protein)
24	6	Hp β chain	..	7	NA1, 2(HDL associated protein)
25	5	Apo D	..	7	AT
26	5	Apo D	..	8	Fibrinogen gamma chain
27	6	Unidentified	..	9	Crp
28	6	Unidentified	..	1	Hp α2
29	6	Apo E			

Chapter 4. Plasma Protein Abnormalities in Patients with Alzheimer's disease or MS.

4.10) *Introduction.*

2-Dimensional SDS PAGE was used to separate the plasma proteins of individuals with MS or Alzheimer's disease and their matched controls. The 2-dimensional gels were scanned and quantitative data were obtained for 45 proteins (as described in Chapter 3). The proteins were selected either because of their known biological function (as discussed previously) or were selected at random to increase the reference panel of proteins for quantitative analysis. The VI values were converted into compositional data, and were analysed statistically to determine if significant differences could be detected between the affected and control states. Ten patients with MS (four males, six females) and ten patients diagnosed as having Alzheimer's disease (equal numbers of males and females) and their age and sex matched controls were used in this study. A patient summary is given in Section 3.22.

4.11) *Materials and Methods.*

Venous blood was collected in Vacutainer blood collection tubes containing sodium heparin as anticoagulant. Plasma was collected from the supernatant after centrifugation of whole blood at 1500 x g for ten minutes (20°C). Aliquots of plasma were either used immediately or stored frozen (-20 or -80°C) until required.

Two-dimensional gels were made and electrophoresed as described previously, to separate the plasma proteins in the samples. For the statistical analysis, the VI values were converted into compositional data. For the analyses, quantitative data were obtained for control and affected individuals. The data for a given group (eg. female affected individuals) were then combined into a pool. The pooled data for each group was then compared and analysed statistically.

For the statistical analysis of the compositional data, the methods of Aitchison (1986) were followed (Appendix 1). Compositional values for each protein on each gel were converted into log ratios using the formula $\mathbf{Log_{10}\ [X/(100\text{-}X)]}$, where X was the particular compositional value (as a percent). The SAS statistical software package was then used to analyse variation in protein abundance. The significance of variation between experimental groups was assessed using a two-tailed student *t*-test, and variance was tested using the F-test. A double tailed *t*-test was used as it required no assumptions to be made regarding the type of quantitative change that may occur. It would detect proteins that experience either increased or decreased synthesis during disease, as well as proteins that were synthesised either only during disease, or, only in the healthy state. As no assumptions are required to be made when using a double tailed *t*-test, it is considered a rigorous test. A more detailed description of compositional data, and Aitchison's method of analysis is given in Appendix 1. The statistical test used was based upon a double tailed *t*-test.

In addition to the *t*-test, an F-test was also performed to determine the degree of variance for the data sets. The *t*-test assumed equal variances, and although a probability *t* value could be obtained for data sets of differing degrees of variance, it is not as accurate as a probability *t* value calculated for data sets exhibiting equal variance.

After a preliminary analysis of the statistical data, I decided to use a 99% confidence level for the *t*-test. This was done to reduce the number of false positives, and as an attempt to consider the small sample sizes. It is possible however that a 99% confidence level may have resulted in some potentially significant differences being overlooked.

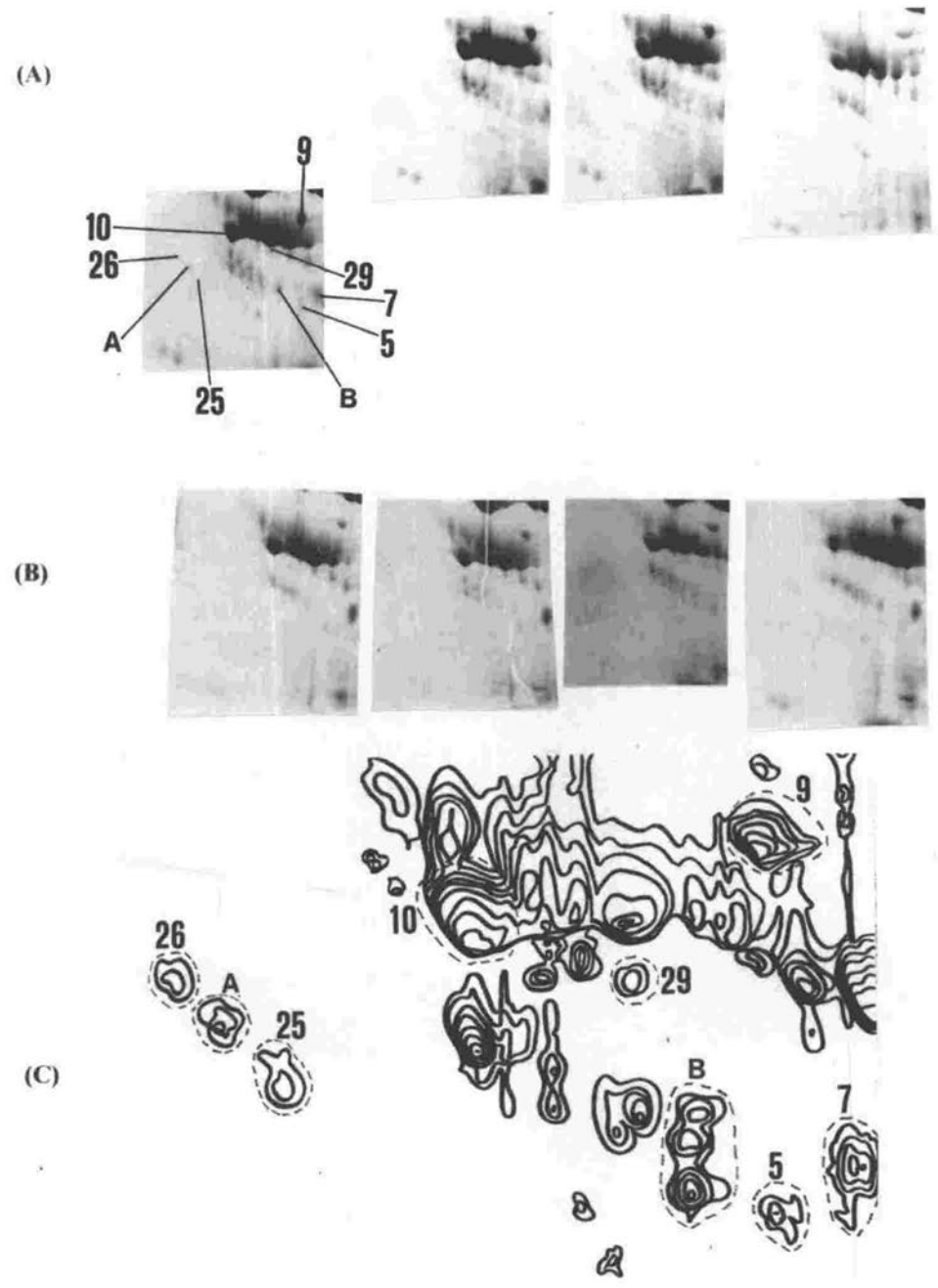
For each analysis, the 45 proteins listed in the plasma maps of Figures 3.233 to 3.241 were quantitated. The number given with each protein name in all of the tables in this section refers to the map number (Figure 3.232) where the protein can be found. Initially, the quantitative data for both males and females for a given group (either affected or control) were analysed together. Then, each population was divided according to sex, and the analyses were repeated.

Proteins for sequencing were separated by 2-dimensional PAGE and were located after brief staining with Coomassie blue. The regions of gel containing the proteins of interest were cut from the gel. Individual gel regions were digested with trypsin, and the resulting peptides were separated by reverse-phase HPLC and were sequenced using an Applied Biosystems (Foster City, CA) gas phase sequencer as described by Hieber *et al.* (1993). Protein microsequencing was carried out by D. Christie and C. Knight of the Auckland University Biochemistry Department. Sequence homology searches were carried out using the NCBI BLAST *e*-mail server.

Section 4.2 gives the results for the MS studies, and section 4.3 gives the results for the Alzheimer's disease studies. Figure 4.10 shows one region of a 2-dimensional PAGE gel (corresponding to plasma protein map region 5, Figure 3.237) for four MS patients (two male and two female) and four controls (two male and two female). This figure also contains a table showing the compositional values that were obtained for these proteins in the MS study group. Figure 4.10 illustrates the variation in spot position and intensity that was observed throughout this study. The figure also contains a histogram showing mean compositional VI values and standard deviations for protein spots 9 and 10. Spot 9 was significantly different ($P = 0.004$) between MS patients and controls. Spot 10 was not significantly different between the two groups. This is clearly shown in the histogram and the photographs.

Figure 4.11 illustrates the magnitude of variation in protein abundance that was required to give statistically significant differences. The data compared were logratio transformants of compositional data for six proteins in female MS patients and female MS controls. The data used was chosen to illustrate the range of variation encountered in this study. Figure 4.11 shows the mean compositional values for the patients and controls with standard deviations. A table is also given which cites *t*-test and F-test values in order of increasing significance. In general, when variances were assumed equal, significant differences ($0.01<P<0.05$) occurred between pairs of proteins whose abundances differed by approximately 1.2 to 1.4 fold (eg. proteins 9 and 23). More significant differences ($0.001<P<0.01$) required an approximately 1.6 fold difference in abundance (eg. protein 1), and highly significant differences ($0.0001<P<0.001$) required at least a 1.8 fold difference in abundance (eg. protein 26).

Figure 4.10. ***Variation in spot position and intensity in one region of gel (plasma protein map region 5) for four MS patients and their controls.***



(A) MS controls. Two male and two female (left to right).
(B) MS patients. Two male and two female (left to right).
(C) Contour map of the gel region (Magnified X4).

Figure 4.10 (contd.). Variation in spot position and intensity in one region of gel (plasma protein map region 5) for four MS patients and their controls.

The following tables give the identity of those proteins labelled in the contour map (C) on the previous page, and give the compositional values obtained after spot quantitation for the MS study group.

Spot I.D.	Name	Spot I.D.	Name
5	Unidentified	26	Apo D
7	Unidentified	29	Apo E
9	Unidentified	A	Apo D
10	Haptoglobin ß chain	B	Apo E
25	Apo D		

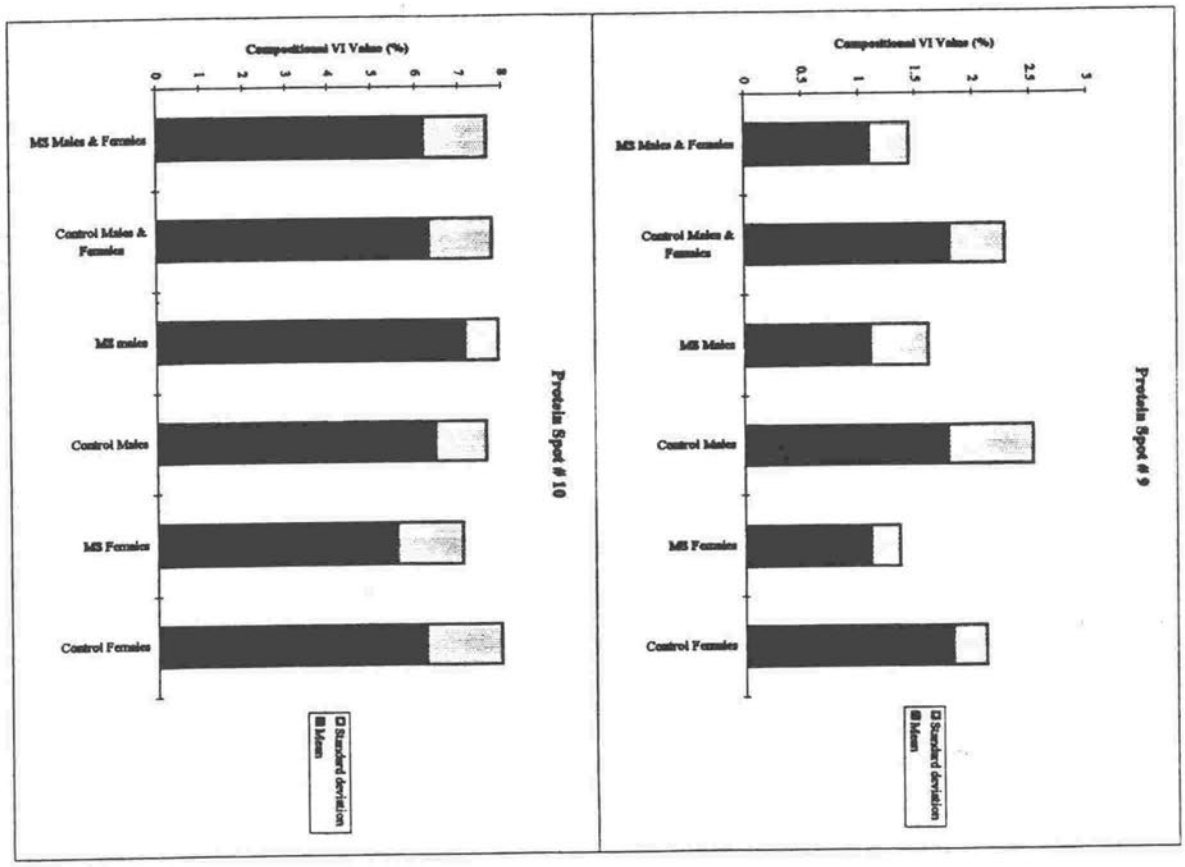
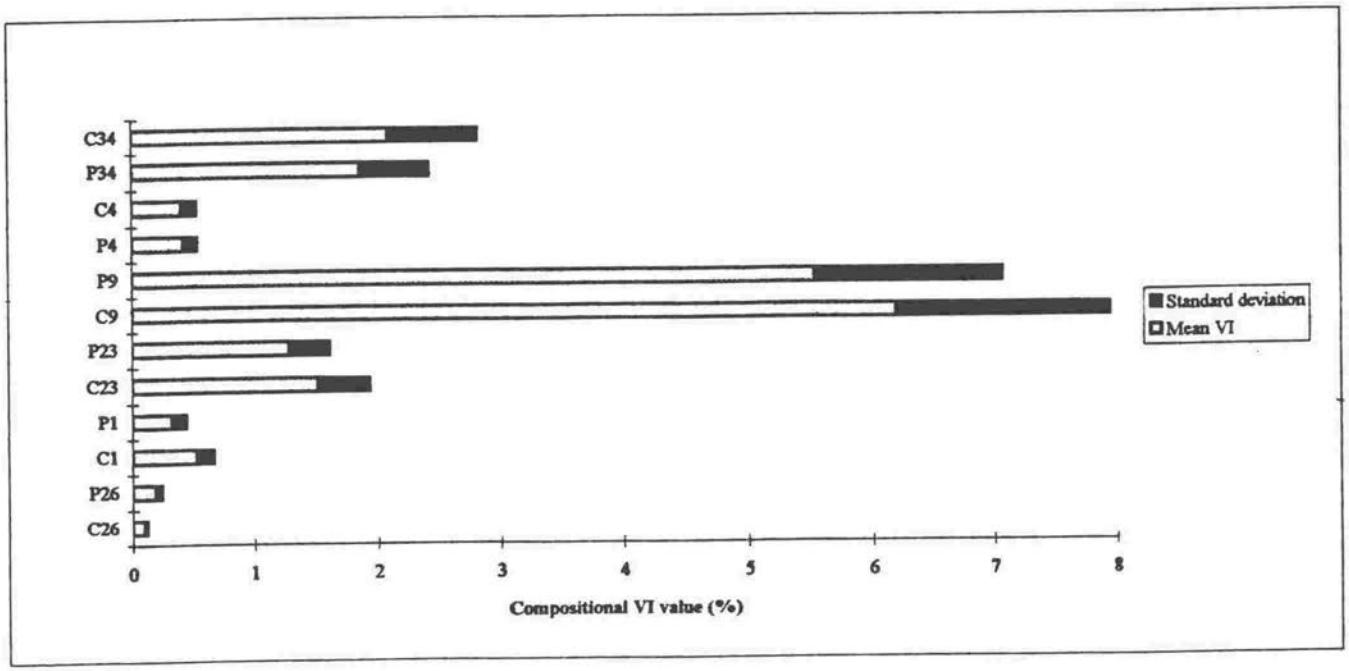


Figure 4.11. ***Logratio analysis of compositional data showing the differences in protein abundance required for statistical significance.***

Spot I.D.	Mean VI Controls	Standard Deviation	Mean VI Patients	Standard Deviation	Percentage Difference	Prob. *t* Value	Prob. F Value
34	2.10	0.73	1.86	0.56	12.42	0.13	0.92
4	0.41	0.12	0.43	0.11	3.32	0.05	0.88
9	6.19	1.76	5.54	1.54	111.87	0.028	0.79
23	1.52	0.42	1.29	0.32	117.83	0.02	0.99
1	0.53	0.15	0.33	0.12	160.06	0.002	0.5
26	0.11	0.02	0.20	0.05	185.71	0.0007	0.47

In the following figure, "P" refers to patients and "C" refers to controls.



4.2) *Multiple Sclerosis.*

Initially, ten patients with MS (four males and six females) were compared to their controls. The null hypothesis adopted for this analysis was *that the affected individuals (both sexes) and the control individuals (both sexes) were members of the same population.* On the basis of the results obtained from the *t*-test, the null hypothesis was rejected and an alternative hypothesis was proposed. The alternative hypothesis was that the control and affected individuals were sufficiently different to justify being considered as coming from different populations. Table 4.21 lists those proteins that were significantly different at the 99% confidence level between the two populations.

Table 4.21. Proteins found to differ significantly between a sample of ten individuals (both sexes) with MS and their controls. The map position of each protein is given with the protein name.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.[1]	APR[4]
3	0.57	0.004	Yes	IgJ (2)[2]	No
9	0.58	0.002	Yes	unidentified (6)	..
12	0.86	0.007	Yes	unidentified (9)	..
13	0.04	0.002	No	Orosomucoid (7)	Yes
14	0.17	<0.000	Yes	Hpα (3)	Yes
15	0.01	<0.000	No	Hpα (3)	Yes
16	0.10	<0.000	Yes	Hpα (3)	Yes
17	0.02	0.002	No	RBP (3)	?
26	0.46	0.006	Yes	Apo D (5)	?
27	0.99	0.001	Yes	unidentified (6)	..
28	0.84	0.001	Yes	unidentified (6)	..
31	0.58	0.004	Yes	IgG light chain (1)	No
36	0.75	0.049	Yes	Apo A1 Pro form (1)	?
38[3]	...	0.009	..	unknown (6) [1]	..
39	<0.00	0.015	No	unidentified (6)	..
40	<0.00	<0.000	No	unidentified (6)	..
41	<0.00	0.001	No	Hpß (7)	Yes
42	<0.00	0.002	No	Hpß (7)	Yes
43	<0.00	<0.000	No	Hpß (7)	Yes
44	<0.00	<0.000	No	Hpß (7)	Yes
45	<0.00	<0.000	No	unidentified (7)	..

1. Proteins were named according to the database of Anderson *et al.*, (1991). Unidentified refers to proteins that were on the database, but have not yet been identified. Unknown refers to proteins that could not be found on the database.
2. This number refers to the map region number in Figure 3.232 where the protein is shown.
3. This protein was only observed in a subset of the affected females, so variance values could not be calculated.
4. " ?" refers to possible APRs.

For the subsequent analyses, the individuals were grouped according to sex (ie. male affected, female affected, male control, and female control) and analysis was performed by comparing data obtained for each group. The format of all of the following tables is the same as that for Table 4.21.

1) *Null hypothesis: That the male controls and the female controls were members of the same population.* After testing, no significant differences were detected between the two groups at the 99% confidence level, and the null hypothesis was upheld.

2) *Null hypothesis: That the MS females and the MS males were members of the same population.* After testing, the hypothesis was rejected based on finding four significant differences between the two groups at the 99% confidence level. Table 4.22 lists those proteins found to differ significantly between the two groups at the 99% confidence level.

Table 4.22. Proteins found to differ significantly between MS males and MS females. The map position of each protein is given with the protein name.

Spot I.D.	Prob. F Value.	Prob. t Value.	Equal Variance.	Protein Name.	APR
38*		0.006	..	unknown (6)	..
39	0.72	0.001	Yes	unidentified (6)	..
40	0.36	<0.000	Yes	unidentified (6)	..
41	0.17	0.001	Yes	Hpß (7)	Yes

* This protein was only observed in a subset of the affected female data set, so no F probability values could be calculated.

3) *Null hypothesis: That the MS males and the control males were members of the same population.* After testing, the hypothesis was rejected upon the basis of finding two significant differences at the 99% confidence level between the two groups, as shown by Table 4.23.

Table 4.23. Proteins found to differ significantly between MS males and control males. The map position of each protein is given with the protein name.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.	APR
15	0.29	0.009	Yes	Hpα (3)	Yes
40	0.25	0.004	Yes	unidentified (6)	..

4) *Null hypothesis: That the MS females and the control males were members of the same population.* The hypothesis was rejected on the basis of finding six significant differences between the two groups at the 99% confidence level. Table 4.24 lists these differences.

Table 4.24. Proteins found to differ significantly between MS females and control males. The map position of each protein is given with the protein name.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.	APR
3	0.69	0.001	Yes	IgJ (2)	No
15	0.23	0.004	Yes	Hpα (3)	Yes
27	0.39	0.005	Yes	unidentified (6)	..
35	0.20	0.005	Yes	Apo A1 pro-form (1)	?
38		0.006	..	unknown (6)	..
40	0.71	<0.000	Yes	unidentified (6)	..

5) *Null hypothesis: That the MS males and the control females were members of the same population.* The hypothesis was rejected based on finding ten differences at the 99% confidence level between the two groups. Table 4.25 gives these differences.

Table 4.25. Proteins found to differ significantly between MS males and control females. The map position of each protein is given with the protein name.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.	APR
14	0.30	<0.000	Yes	Hpα (3)	Yes
15	0.10	0.006	Yes	Hpα (3)	Yes
16	0.13	0.005	Yes	Hpα (3)	Yes
17	0.81	0.009	Yes	RBP (3)	?
28	0.41	<0.000	Yes	unidentified (6)	..
39	0.63	<0.008	Yes	unidentified (6)	..
41	<0.00	0.009	No	Hpß (7)	Yes
43	0.01	0.001	No	Hpß (7)	Yes
44	0.03	0.001	No	Hpß (7)	Yes
45	<0.00	0.001	No	unidentified (7)	..

6) *Null hypothesis: That the affected female MS individuals and the control female individuals were members of the same population.* After testing, the hypothesis was rejected by finding fourteen differences between the two groups at the 99% confidence level. Table 4.26 lists these differences.

Table 4.26. Proteins found to differ significantly between MS females and control females. The map location of each protein is given with the protein name.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.	APR
9	0.47	0.002	Yes	unidentified (6)	..
14	0.24	<0.000	Yes	Hpα (3)	Yes
15	0.04	0.006	No	Hpα (3)	Yes
16	0.32	<0.000	Yes	Hpα (3)	Yes
17	0.20	<0.000	Yes	RBP (3)	?
27	0.47	0.002	Yes	unidentified (6)	..
28	0.26	0.001	Yes	unidentified (6)	..
29	0.33	0.005	Yes	Apo E (6)	?
38		0.001	..	unknown (6)	..
39	0.87	<0.000	Yes	unidentified (6)	..
40	0.44	<0.000	Yes	unidentified (7)	..
43	0.01	0.001	No	Hpß (7)	Yes
44	<0.00	0.001	No	Hpß (7)	Yes
45	0.01	0.001	No	unidentified (7)	..

4.21) Discussion.

Population study.

For the MS group, when both sexes were included in the groups for comparison, twenty one significant differences were detected between the control population and the affected population (Table 4.21). Of these proteins, thirteen could be identified on the database of Anderson *et al,* (1991). Of this group of proteins, eleven are either thought to be, or are, acute phase reactants (the two proteins that were not APRs were IgJ and immunoglobin light chains). All of

the APRs were seen to increase in relative plasma concentration in the disease state. Table 4.27 gives the average percentage change for those proteins that were found to differ significantly between the control population and the affected population. Proteins that were found in higher relative plasma concentrations in the diseased state are represented by positive percentage values, while those proteins that decreased in relative plasma concentration are represented by negative percentage values.

Table 4.27. Average percentage change for those proteins found to differ between ten individuals with MS and their controls.

Spot I.D.	Protein Name	Change (%)	Spot I.D.	Protein Name	Change (%)
3	IgJ	-32.18	31	IgG Light chain	-43.64
9	Unidentified	-39.23	36	Apo A1 (pro form)	-32.51
12	Unidentified	-37.91	38	Unknown	**
13	Orosomucoid	+64.90	39	Unidentified	+216.67
14	Hpα	+111.90	40*	Unidentified	+752.70
15	Hpα	+101.51	41	Hpβ	+659.70
16	Hpα	+153.28	42	Hpβ	+454.55
17	RBP	+64.22	43*	Hpβ	+1328.57
26	Apo D	-31.41	44*	Hpβ	+1750.00
27	Unidentified	+104.12	45*	Unidentified	+1733.33
28	Unidentified	+91.01			

* These proteins all had very low F probability values, the individual values varying widely both between the populations and within the populations. The values associated with these proteins should only be considered approximate estimates for this reason.

** This protein was only present in some MS females.

Of the plasma proteins selected for inclusion in this study because of their known

biochemical/physiological functions (Section 3.10), five were found to differ significantly between the MS and control state. These proteins are discussed below.

1) IgJ.

This protein (spot numbers 2 and 3) was seen to decrease in relative plasma concentration. When the sex linked studies were considered, the difference was seen only when affected females were compared to control males. This possibly suggests that the difference was artifactual, being due to either the small sample size, or possibly control selection. For the control subjects used in the MS study, diagnosis had not been determined for all of the individuals at the time of sample collection - the individuals were all inpatients, thought to be suffering from neurological disorders (non-neurodegenerative). This type of control was used to allow an assessment to be made regarding specificity of any observed changes to MS. However, as several different disorders are involved there will be an increased number of variables affecting the total plasma protein composition. Figure 4.211 shows the range of values obtained for this protein.

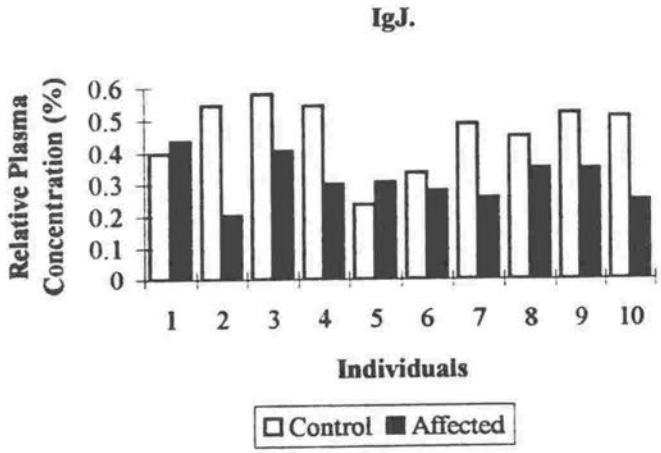
2) Retinol Binding protein.

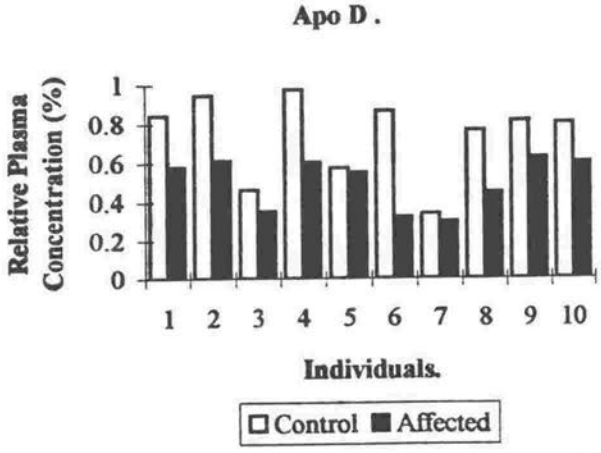
This protein (spot number 17) was seen to increase in relative plasma concentration in MS. There are some reports that suggest that RBP is a negative acute phase reactant Maes *et al.*, (1992). I have been unable to find any other studies reporting altered RBP plasma levels in MS.

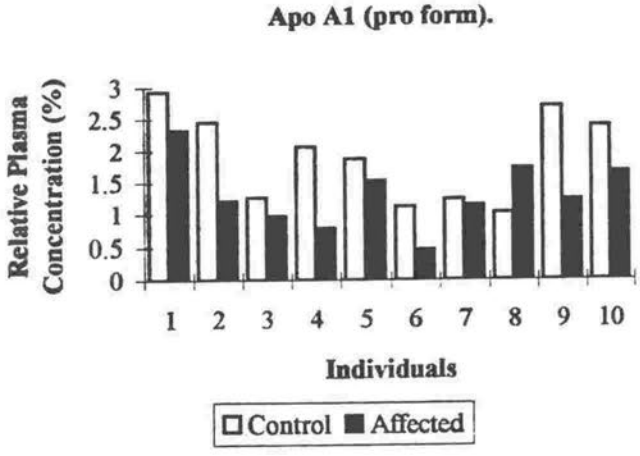
3) Apo D.

This protein (spot numbers 25 and 26) was seen to decrease in relative plasma concentration. I have found no reference to altered Apo D plasma levels in MS. When the sex based studies were examined however, no significant differences could be detected. Figure 4.211 shows the range of values obtained for this protein. In this Figure, relative concentration refers to the compositional values obtained for the proteins.

Figure 4.211. Histograms showing the relative plasma concentration (compositional value) of IgJ, Apo D, and Apo A1 in affected MS individuals and their controls. Individuals 1-4 were males and 5-10 were females.







4) IgG light chains.

This protein was seen to decrease in relative plasma concentration. Again, this change was only significant in the population study. Figure 4.212 shows 6 IgG regions, taken from six individuals, and illustrates how varied these proteins were.

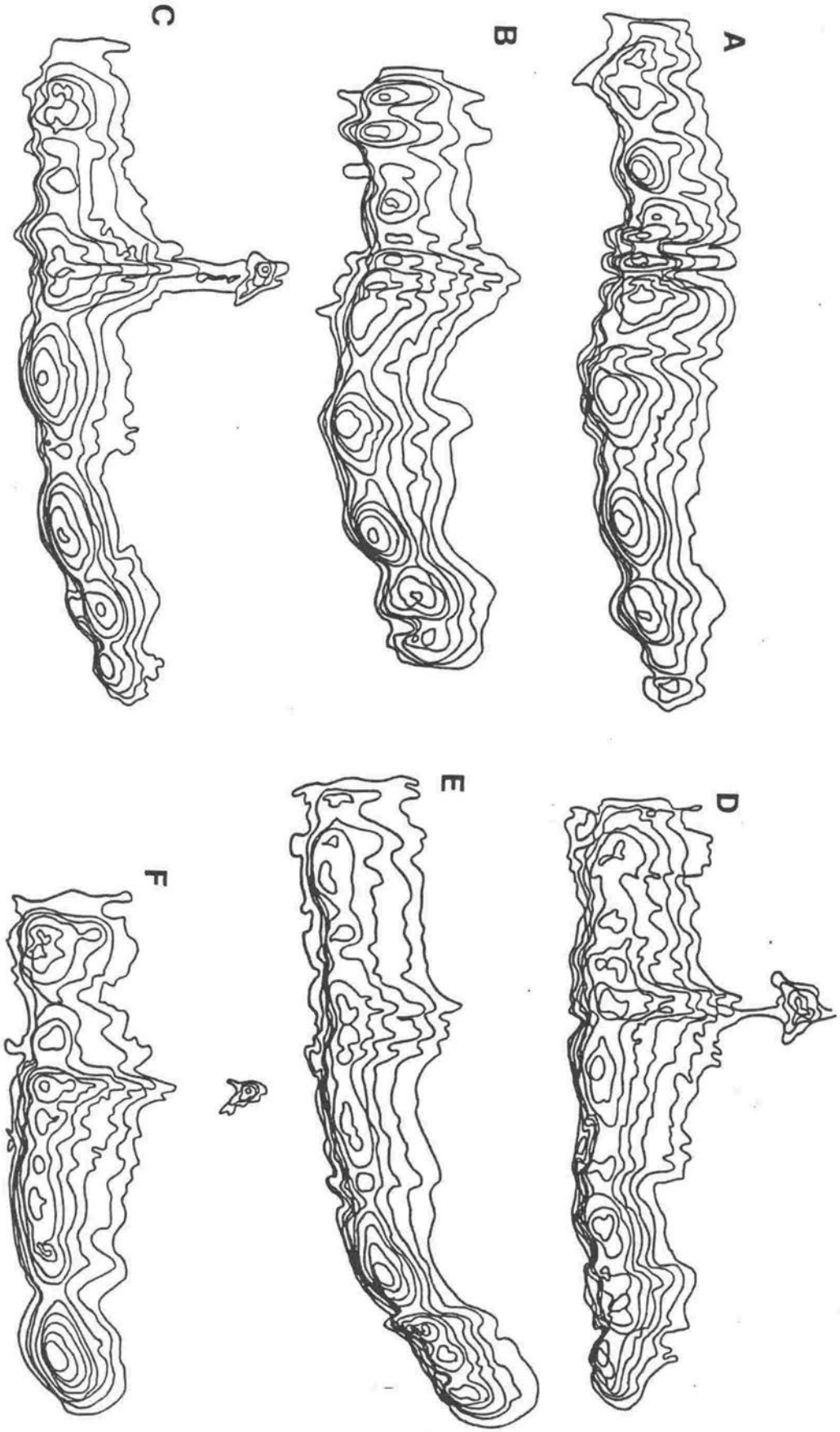
5) Apo A1(pro form).

This protein (spot numbers 35 and 36) was seen to decrease in relative plasma concentration. In the sex based studies, this difference was only seen to occur between MS females and control males. This observation may be an artifact of sample size and control composition. Others have reported that Apo A1 plasma levels are no different between MS patients and controls (Gelman, 1988). The relative plasma concentration values for this protein for control and affected individuals are shown in Figure 4.211.

Sex-linked protein changes.

When differences between male and females were compared, no significant differences were observed when control males were compared to control females. This implied that any significant sex-linked differences between MS individuals could be expected to be disease related, and not simply sex related. However, when MS males were compared to MS females, several significant differences were observed. Because of the above observation, these differences can be assumed to be disease related. One of the proteins was Hpß. The other three proteins were spot numbers 38, 39, and 40. Spot numbers 39 and 40 are in the database of Anderson *et al.* (1991), although they have yet to be identified. Protein number 38 could not be found on the database. A disease related sex difference for MS should not be ruled out as the disease is more prevalent in women than in men (McLeod, 1982).

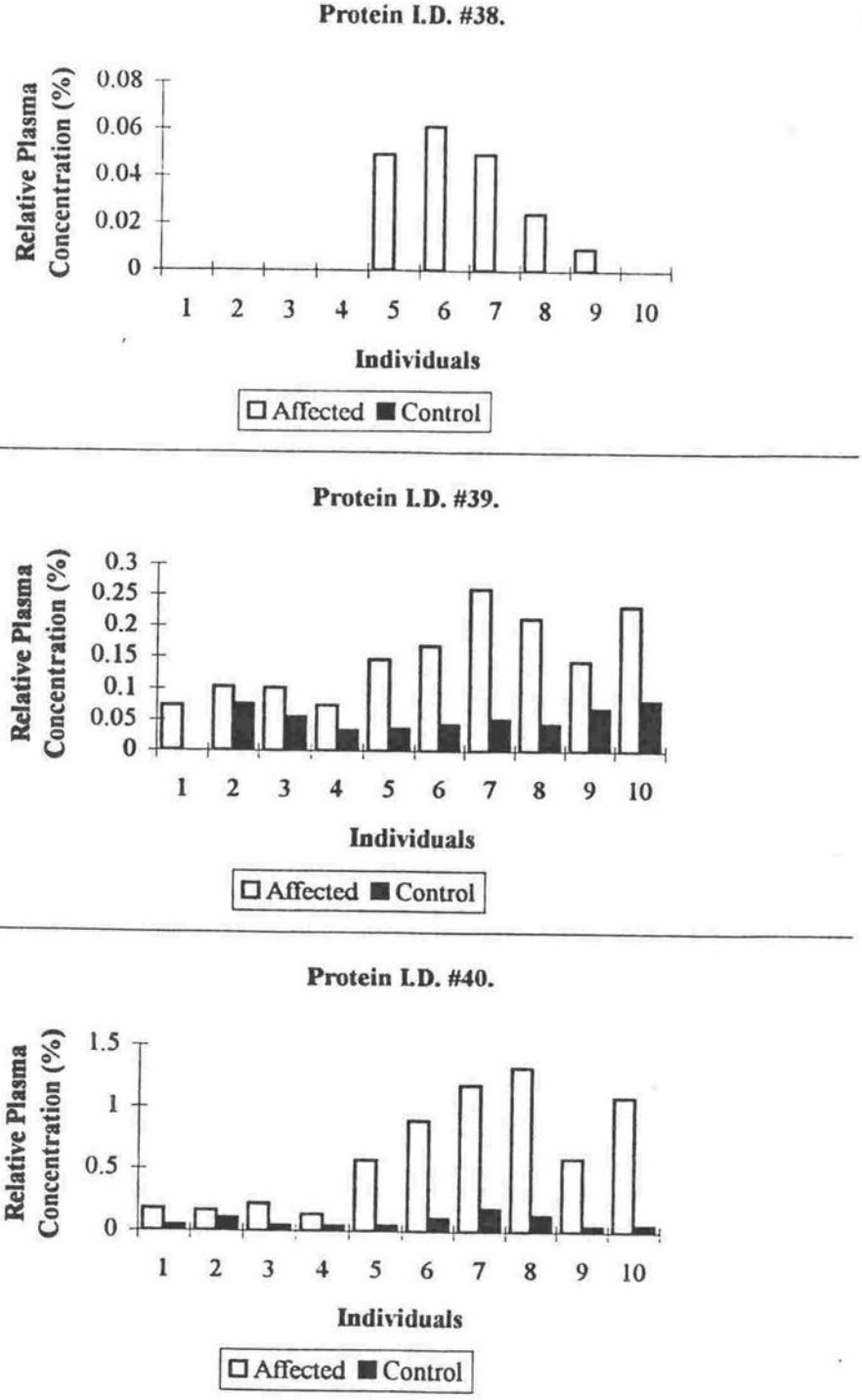
Figure 4.212. *Contour maps of IgG light chains from three MS affected individuals (A to C) and three controls (D to F).*



Because the male and female control groups were not significantly different, it was possible to assess the statistical inferences made. This was done by making several predictions based on the observation that significant sex-linked differences between MS individuals appeared to be disease related and not simply sex related. The first prediction was that any significant differences that were found between MS males and control males would be the same as those that would be observed between the MS males and the control females. Second, it would be expected that any significant differences found between the MS females and the control females would be the same as those found between the MS females and the control males. However, when the results obtained by comparing MS males and control males were compared to those obtained by comparing MS males with control females, different significant changes were seen in the two groups. The same observation was also made when comparing the results obtained by comparing MS females with both male and female controls. These observations were expected. This is probably the result of a small sample size and possibly control selection. These observations make it difficult to assess whether a sex specific, disease related, significant difference exists. Figure 4.213 shows the relative plasma concentrations of proteins 38, 39, and 40, for MS males and females. As can be seen, there may be a sex specific disease related protein change; both MS groups had higher relative plasma concentrations of proteins 39 and 40 than did controls, and MS females had higher relative plasma concentrations than MS males. This apparent sex specific protein change was not observed in the Alzheimer's study (for Alzheimer's disease, prevalence rates for men and women are considered the same, Goldstein *et al.* (1991)). However, a much larger sample size would be needed to assess the validity of this observation, although due to the apparent lack of disease specificity, the diagnostic utility of such an observation is questionable.

One observation made in the MS study was that for four of the six MS females, a unique, apparently disease associated, protein was observed (protein number 22). This difference had a *t* probability value of 0.04. This may suggest that use of the 99% confidence level may have excluded some protein changes. However, at the 95% confidence level approximately 85% of the proteins studied were considered to differ significantly between MS patients and controls.

Figure 4.213. Histograms showing the relative plasma concentrations of proteins 38, 39, and 40 in MS individuals and their controls.



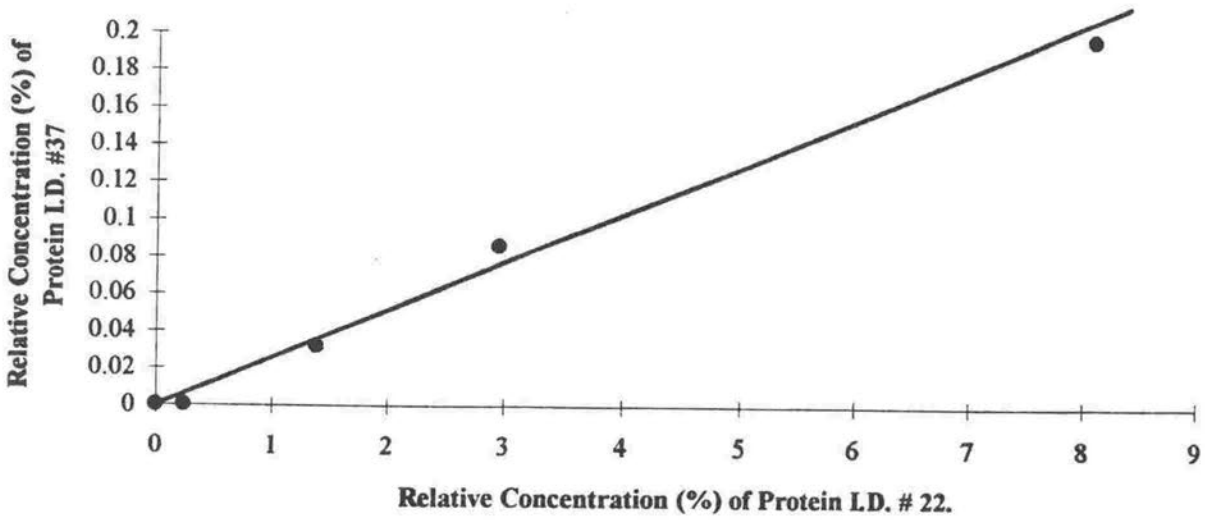
Individuals 1 to 4 were males, and individuals 5 to 10 were females.

A large range of relative plasma concentrations were obtained for this protein. There was a 32 fold difference in amount among the plasmas of the four patients. Figures 4.215 to 4.218 show this protein in the four patients who possessed it and Figure 4.219 shows a pooled sample of control individuals for the MS group. It may be of value to examine a large number of MS patients for the presence of this protein.

A partial sequence for protein number 22 was obtained as described in Section 4.11. Peptide sequencing of a trypsin fragment from this protein spot gave the sequence **RVGYVSG-[ ]GR** (where [ ] represents an unidentified amino acid). The observation of a blank at one position during the sequence determination of a small peptide, while all other positions are unambiguously determined, is good evidence for the location of a tryptophan residue (Allen, 1986). I searched for sequence homology using the NCBI BLAST *e*-mail server at "blast@ncbi.nlm.nih.gov", as described by Altschul *et al.* (1990). A 90% sequence homology was found with residues 116 to 125 of human Hp α (**RVGYVSGWGR**). This is the highest homology that could be obtained, due to the presence of an unidentified amino acid at position eight in the partial sequence. The presence of a tryptophan residue at residue 123 is consistent with the sequence data however. This identification is consistent with the location of protein number 22 on the 2-dimensional gel. It is also consistent with the variation in relative abundance seen in those individuals possessing this protein. Because the protein was identified as a Hp, it seems likely that the protein was present in all of the patients in this study, but was at a concentration too low to be reliably detected by Coomassie blue.

Another observation regarding this protein was that its relative plasma concentration was related to the relative plasma concentration of protein spot 37, as is shown in Figure 4.214. It is possible that this protein is specific to MS.

Figure 4.214. The relationship between proteins 22 and 37.



For the proteins in the MS study that were found to differ significantly between patients and controls, of those identified, the majority were either known APRs, or were thought to be APRs. As such, these proteins are of little utility as disease specific protein markers that could form the basis of a diagnostic test for MS.

Figure 4.215. ***10% 2-dimensional polyacrylamide gel for MS female 6. The arrow indicates the location of protein I.D. no. 22.***

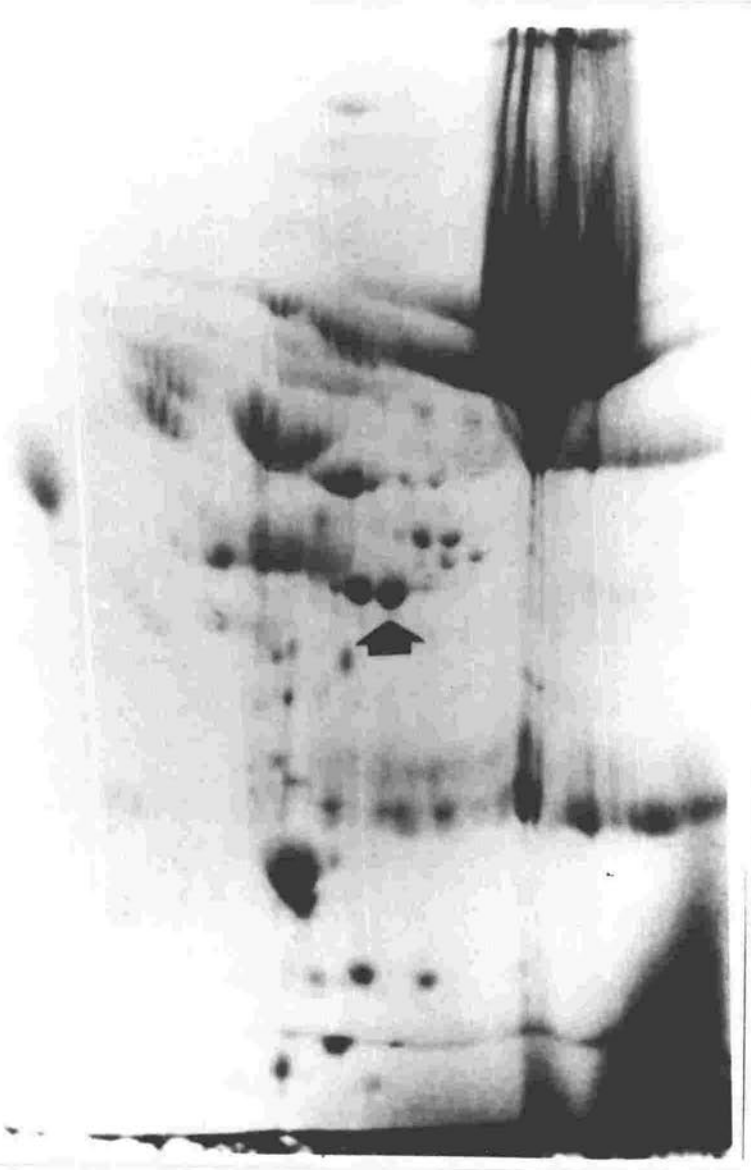


Figure 4.216. 10% 2-dimensional polyacrylamide gel for MS female 8. The arrow indicates the location of protein I.D. no. 22.

Figure 4.217. 10% 2-dimensional polyacrylamide gel for MS female 9. The arrow indicates the location of protein I.D. no. 22.

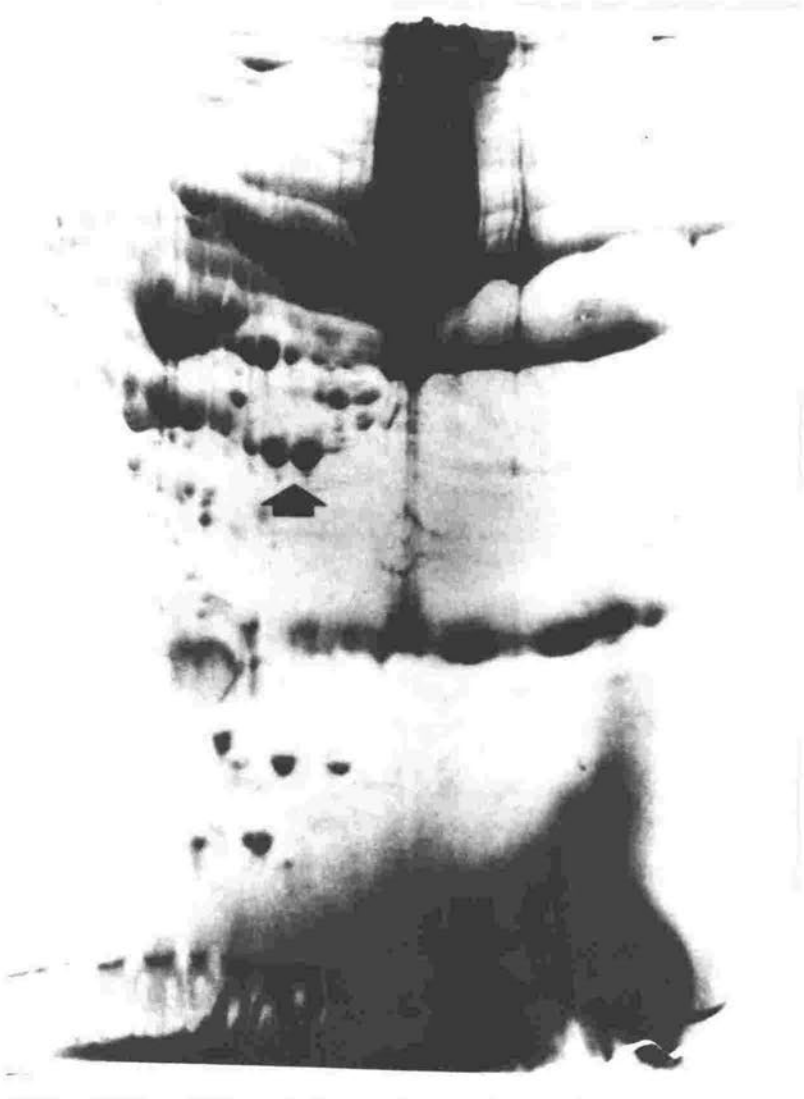
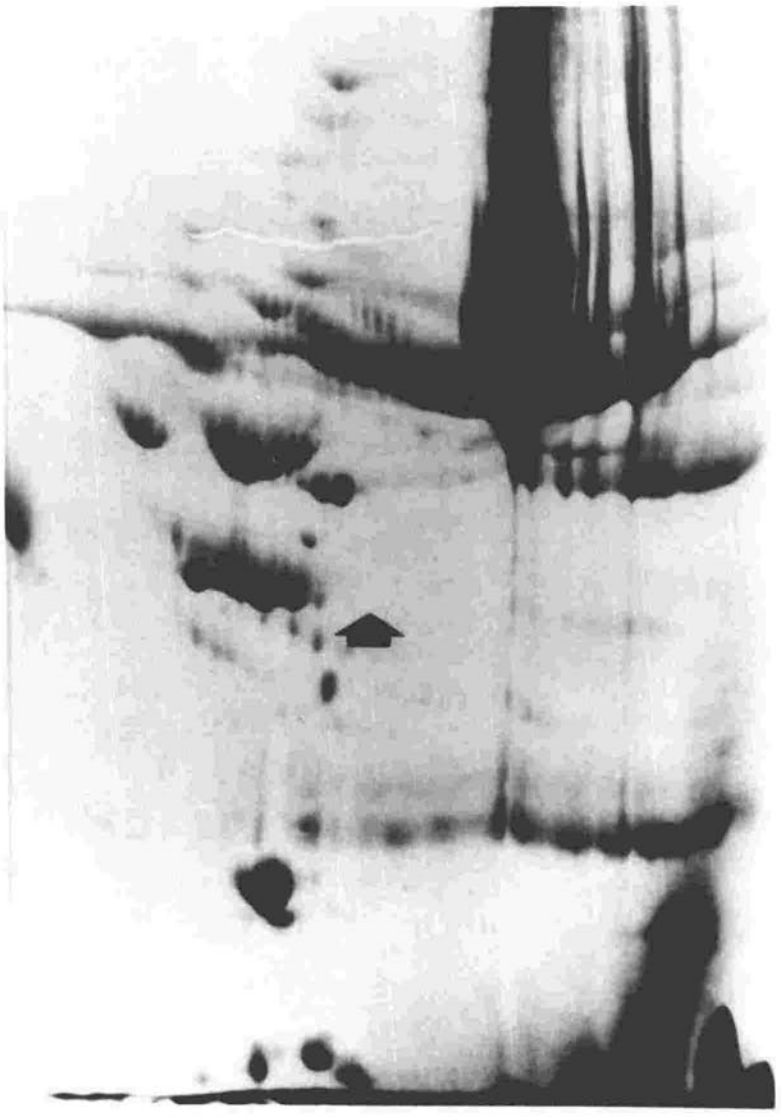


Figure 4.218. 10% 2-dimensional polyacrylamide gel for MS female 10. The arrow indicates the location of protein I.D. no. 22.

Figure 4.219. *10% 2-dimensional polyacrylamide gel for pooled MS controls. The arrow shows the location of protein I.D. no. 22 on the previous Figures.*



4.3) ***Alzheimer's Disease.***

Initially, ten patients with Alzheimer's disease (five males and five females) were compared to their controls (a patient summary is given in Section 3.22). The null hypothesis used for this analysis was *that the affected individuals of both sexes were derived from the same population as the control individuals.* On the basis of the results obtained from the *t*-test, this hypothesis was rejected and an alternative was proposed, that the two data sets appeared sufficiently different to be considered as coming from different populations. Table 4.31 lists those proteins that were significantly different between the control and Alzheimer's disease affected populations at the 99% confidence level.

For the following analyses, the individuals were grouped according to sex, and the analysis was performed by comparing the data obtained for each group.

1) *Null hypothesis: That the female controls and the male controls were members of the same population.* After testing, the hypothesis was upheld by finding no significant difference at the 99% confidence level between the two groups.

2) *Null hypothesis: that the Alzheimer's males and the Alzheimer's females were members of the same population.* After testing, the hypothesis was upheld on the basis of finding no significant differences between the 2 groups at the 99% confidence level.

Table 4.31. Proteins found to differ significantly between ten individuals with Alzheimer's disease and their controls.

Spot I.D.	Prob. F Value.	Prob.t Value.	Equal Variance	Protein Name.	APR
13	0.75	0.003	Yes	Orosomucoid (7)	Yes
14	0.14	0.005	Yes	Hpα (3)	Yes
15	0.44	0.004	Yes	Hpα (3)	Yes
16	0.11	0.001	Yes	Hpα (3)	Yes
17	0.32	0.001	Yes	RBP (3)	?
23	0.93	0.017	Yes	Hpß (6)	Yes
25	0.55	0.013	Yes	Apo D (5)	?
27	0.38	0.012	Yes	Unidentified (6)	..
28	0.97	0.004	Yes	Unidentified (6)	..
39	0.51	<0.000	Yes	Unidentified (6)	..
40	0.91	0.003	Yes	Unidentified (6)	..
41	<0.00	0.002	No	Hpß (7)	Yes
42	<0.00	0.001	No	Hpß (7)	Yes
43	<0.00	<0.00	No	Hpß (7)	Yes
44	<0.00	<0.00	No	Hpß (7)	Yes
45	<0.00	<0.00	No	Unidentified (7)	Yes

3) *Null hypothesis: That the control males and the Alzheimer's males were members of the same population.* After testing, the hypothesis was rejected because of finding three significant differences between the two groups at the 99% confidence level. Table 4.32 lists those differences.

Table 4.32. Proteins found to differ significantly between Alzheimer's males and control males. The map position of each protein is given with the protein name.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.	APR
43	<0.00	0.006	No	Hpß (7)	Yes
44	<0.00	0.003	No	Hpß (7)	Yes
45	<0.00	0.008	No	unidentified (7)	..

4) *Null hypothesis: That the Alzheimer's females and the control males were members of the same population.* After testing, the hypothesis was rejected by finding five significant differences between the two groups at the 99% confidence level. Table 4.33 lists these differences.

Table 4.33. Proteins found to differ significantly between Alzheimer's females and control males. The map position of each protein is given with the protein name.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.	APR
16	0.85	0.007	Yes	Hpα (3)	Yes
17	0.15	0.008	Yes	RBP (3)	?
43	<0.00	0.006	No	Hpß (7)	Yes
44	<0.00	0.002	No	Hpß (7)	Yes
45	<0.00	0.008	No	unidentified (7)	..

5) *Null hypothesis: That the Alzheimer's males and the control females were members of the same population.* After testing, this hypothesis was rejected because of finding four significant

differences between the two groups at the 99% confidence level. Table 4.34 lists these differences.

Table 4.34. Proteins found to differ significantly between Alzheimer's males and control females. The map position of each protein is given with the protein name.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.	APR
39	0.52	0.009	Yes	unidentified (6)	..
43	<0.00	0.003	Yes	Hpß (7)	Yes
44	<0.00	0.002	Yes	Hpß (7)	Yes
45	<0.00	0.003	Yes	unidentified (7)	..

6) *Null hypothesis: That the Alzheimer's females and the control females were members of the same population.* After testing, the hypothesis was rejected on the basis of finding six significant differences at the 99% confidence level between the two groups. Table 4.35 lists these differences.

Table 4.35. Proteins found to differ significantly between Alzheimer's females and control females.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.	APR
16	0.79	0.008	Yes	Hpα (3)	Yes
39	0.45	0.001	Yes	unidentified (6)	..
40	0.69	0.008	Yes	unidentified (6)	..
43	<0.00	0.003	No	Hpß (7)	Yes
44	<0.00	0.002	No	Hpß (7)	Yes
45	<0.00	0.003	No	unidentified (7)	..

4.31) Discussion.

For the Alzheimer's study, when male and females were analysed together, sixteen significant protein differences were detected between the Alzheimer's and control populations. All of the proteins involved were found on the database of Anderson *et al.*, (1991), eleven of the proteins being known by name. All of the known proteins are either thought to be, or are, APRs. Table 4.36 lists the percentage change observed for those proteins that were found to differ significantly at the 99% confidence level between the Alzheimer's and control groups.

Table 4.36. Average percentage change for those proteins found to differ significantly between ten patients with Alzheimer's disease and ten controls.

Spot I.D.	Protein Name	Change (%)	Spot I.D.	Protein Name	Change (%)
13	Orosmucoid (7)	+33.80	28	Unidentified	+28.42
14	Hpα (3)	+33.41	39	Unidentified	+36.96
15	Hpα (3)	+53.63	40*	Unidentified	+35.23
16	Hpα (3)	+47.22	41	Hpβ (7)	+269.23
17	RBP (3)	+45.64	42*	Hpβ (7)	+297.67
23**	Hpβ (6)	-15.43	43*	Hpβ (7)	+762.50
25**	Apo D	-17.78	44*	Hpβ (7)	+1116.67
27	Unidentified	+30.21	45*	Unidentified	+700.00

* These proteins had very low F probability values and very large variances. This indicates that caution is needed when interpreting the results for these proteins.

**Proteins 23 and 25 were only found to differ significantly in Alzheimer's disease.

When the groups were analysed according to sex, no significant differences were detected between the male and female controls. This implies that any differences detected between the Alzheimer's males and females would be expected to be disease related. When Alzheimer's

females were compared to Alzheimer's males, no significant differences were detected at the 99% confidence level. In view of these two observations, it would be reasonable to assume that any significant changes observed between the Alzheimer's males and control males would also be the same as those observed between Alzheimer's females and control females. However, significant differences were detected among the various groups. This may have resulted from the small sample size, or it may be an indication of a certain degree of disease heterogeneity within the Alzheimer's patients. This may have occurred because of including early onset Alzheimer's patients and late onset Alzheimer's patients in the same sample group (the sample size was too small to allow such a separation to be made). The observation may also have been due to the error associated with some diagnoses. However, from the initial results, it would appear that no sex specific disease associated protein change occured in the plasma proteins of Alzheimer's patients. The results do however, suggest the need for larger sample sizes to make an accurate assessment of the situation.

Another observation that can be made is that for almost every F-test performed, Hpß showed unequal variance between the groups; the F probability values were usually less than 0.00, indicating that the groups being compared exhibited highly significant levels of variance (ie. the distribution of plasma abundances of Hpß varied greatly between the two groups being compared). This also indicated that the data relating to Hpß should be treated with caution. This observation also supports the view of Koj, (1974), which is that it is difficult to interpret the clinical significance of laboratory values obtained for Hp obtained from isolated blood samples. However, I am uncertain why the other Hp proteins (such as Hpα chains) did not show the same degree of variance; in almost all instances, the F probability values for Hpα were significantly greater than 0.1, indicating equal variance between the sample groups being compared. Although the average relative plasma concentration values were significantly different between the groups being compared, the standard deviation values associated with each distribution were similar. One possible explanation is that there was more experimental error associated with the Hpα measurements. This could be because Hpα has several allelic forms, while HPß is highly conserved. There is a greater chance for error when quantitating proteins with multiple allelic forms when compared to quantitating proteins with few/no allelic forms. This may have masked

the actual variance of the protein between individuals with the various forms of Hpα. A second possible explanation is that the different allelic forms of Hpα may have different binding capacities for Coomassie Blue. This would alter the quantitative relationship between staining intensity and relative plasma concentration between individuals with the different forms of Hpα. This may have masked the actual variance between individual members of each group. The logratio transformation would minimise these differences however. For Hpß, the different proteins seen are not allelic variants, but rather, are cleaved and uncleaved forms of the same protein, and the same protein with different sialic acid contents. These two factors would alter the pI and the mw, but would not alter Coomassie blue binding capacities. This means that between individuals the quantitative relationship between staining intensity and relative plasma concentration could be regarded as the same.

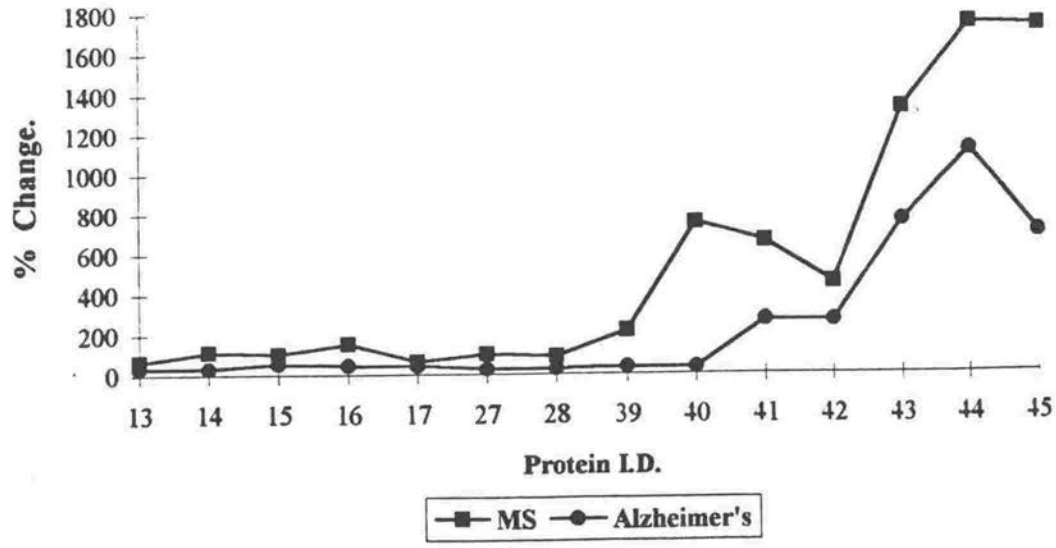
When the data for both Alzheimer's disease and MS were compared, fourteen significant protein changes between the affected and control populations were common to both diseases. Table 4.37 lists those proteins that were found to differ significantly between the affected and control states for both Alzheimer's disease and MS.

Table 4.37. Proteins that were found to differ significantly between pooled samples of affected individuals and pooled samples of their controls for both Alzheimer's disease and MS.

Spot I.D.	Protein Name.	APR
13	Orosomucoid (7)	Yes
14	Hpα (3)	Yes
15	Hpα (3)	Yes
16	Hpα (3)	Yes
17	RBP (3)	?
27	unidentified (6)	..
28	unidentified (6)	..
39	unidentified (6)	..
40	unidentified (6)	..
41	Hpß (7)	Yes
42	Hpß (7)	Yes
43	Hpß (7)	Yes
44	Hpß (7)	Yes
45	unidentified (7)	..

Figure 4.311 shows the average percentage change in relative plasma concentration between the affected and control state for both Alzheimer's disease and MS. To aid clarity, error bars are not shown. The change in relative plasma concentration was always lower for the Alzheimer's group; this may be a reflection of the different pathologies associated with each disease, although this is probably an oversimplification.

Figure 4.311. ***Average percentage change in relative plasma concentration for the proteins which differed significantly between affected individuals and their controls, for both Alzheimer's disease and MS.***



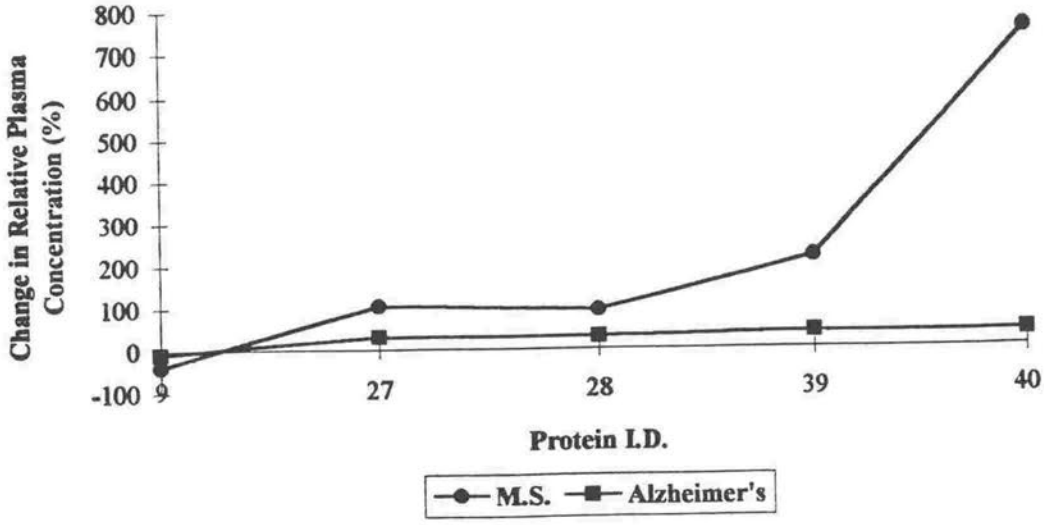
Also, all of the protein changes that the two diseases had in common involved an increase in relative plasma concentration in the affected state. This is surprising, as in chronic inflammation (as expected to occur in Alzheimer's disease and MS; unless the MS was in a period of remission) both positive and negative APRs would be expected (Maes *et al.*, 1992). However, it is possible that the two diseases do not have any negative APRs in common, and that some proteins seen to decrease in relative plasma concentration for each disease may have been negative APRs.

Protein number 38 was also common to both diseases. However, the observations are difficult to explain. Although in the MS study, the protein was only observed in the affected females, the protein was also observed in one affected female in the Alzheimer's study. A possible explanation for this observation could be that the synthesis of this protein is influenced by both sex and the disease state. However, due to its low abundance the protein is not seen in either of the

male control groups, and in only one affected female in the Alzheimer's study. The difference between the affected females in the Alzheimer's and MS studies could be explained by the fact that many APRs are seen to differ in rates of expression in different diseases. However, to assess the significance of this protein difference, it would be necessary to have access to a much larger sample group, and a more sensitive method of protein detection.

The majority of protein differences that were found to differ significantly between the affected and control states in both the MS and the Alzheimer's patients were APRs. However, for some proteins, such as proteins 9, 27, 28, 38, 39, and 40, the differences between the MS and Alzheimer's groups were such that the proteins may be of use in either supporting a clinical diagnosis of MS, or in making prognostic assessment. Figure 4.312 illustrates these differences. However, a more detailed investigation using a much larger sample group would be required before such an assessment could be accurately made.

Figure 4.312. ***Average percentage change in relative plasma concentration of proteins 9, 27, 28, 39, and 40 in MS and Alzheimer's Disease.***



4.4) *Changes in relative plasma APR concentration over time.*

A preliminary study was made of changes in the relative plasma concentration of specific APRs over time. It is possible that such changes could be used as prognostic indicators.

4.41) Materials and Methods.

Two-dimensional SDS PAGE was used to separate the plasma proteins of three late onset Alzheimer's patients who had supplied three blood samples over a period of nine months. The plasma proteins of three controls who had also supplied three blood samples over a similar period were also investigated. Two-dimensional PAGE and quantitative densitometry were performed as described previously. Due to the small sample size, no statistical analysis was done. Table 4.11 lists the proteins studied in this investigation.

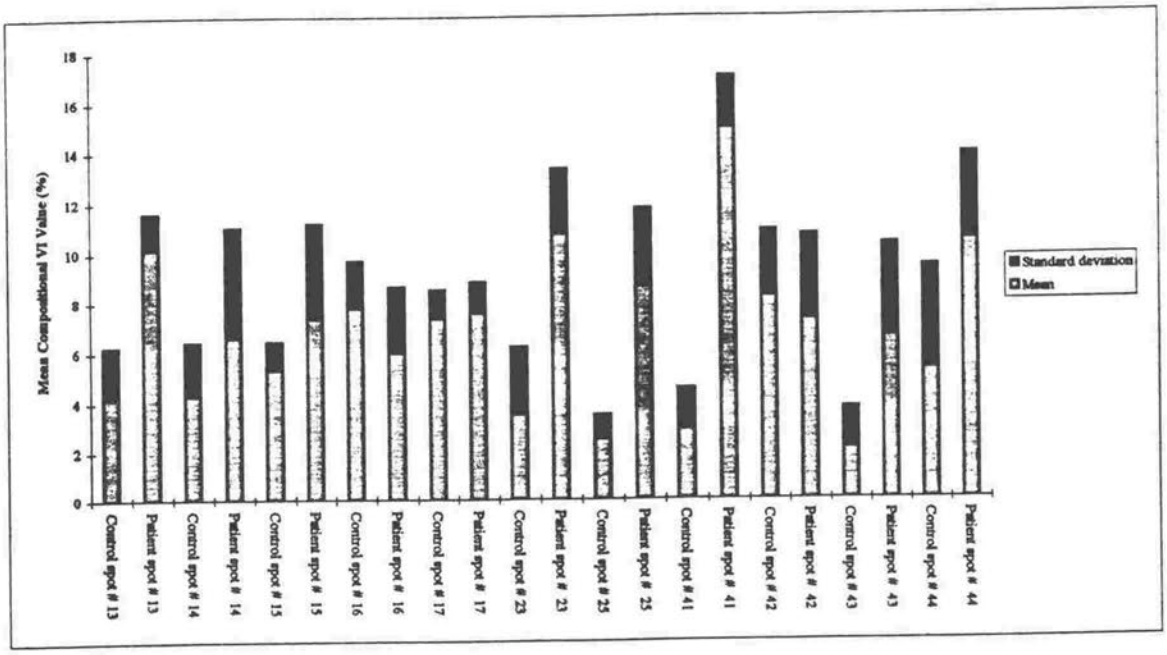
Table 4.41. APRs included in the study of variation in relative plasma concentration over time. The map position of each protein is given in brackets beside the protein name.

Spot I.D.	Protein Name.	Spot I.D.	Protein Name.
13	orosomucoid (7)	25	Apo D (5)
14	Hpα (3)	41	Hpß (7)
15	Hpα (3)	42	Hpß (7)
16	Hpα (3)	43	Hpß (7)
17	RBP (3)	44	Hpß (7)
23	Hpß (6)		

4.42) Results and Discussion.

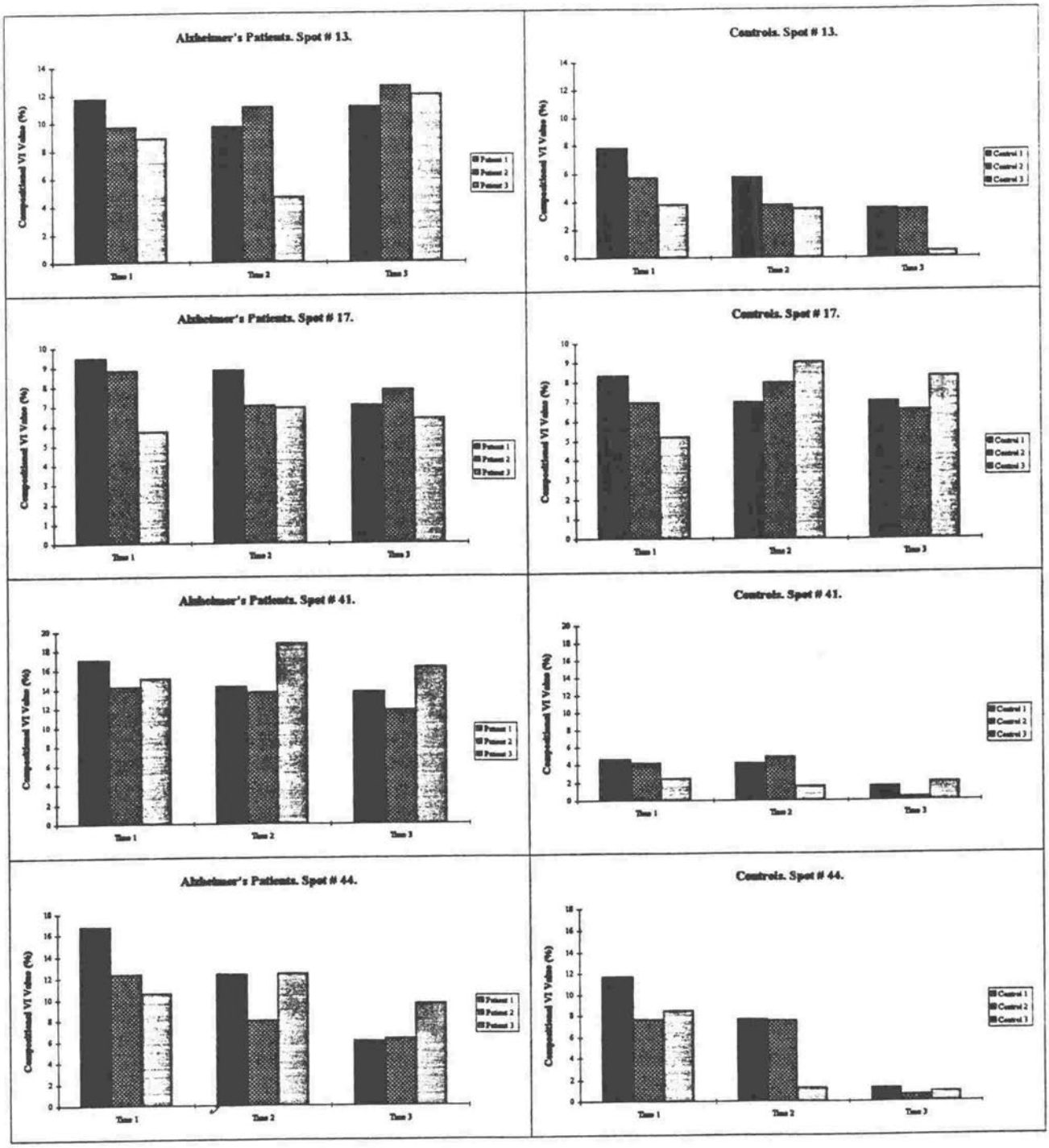
Figure 4.421 shows the mean compositional VI values for each protein with associated standard deviations. The controls were included in the study to determine the amount of change that would be expected to be observed in relative plasma values due to experimental error, natural variation, etc.. Almost all of the relative plasma concentrations for the controls were lower than the corresponding values for patients. As shown in the figure, for most of the proteins in the control and patient groups, standard deviations were large. This made it difficult to interpret the results in terms of disease related protein changes. For some proteins in the patient group (such as spots 13, 17, 41, and 45), standard deviations were smaller. When these proteins were examined in individual patients and controls however (Figure 4.422), the plasma APR levels did not appear to change consistently over time. This indicates that APR levels are of little prognostic value in Alzheimer's disease.

Figure 4.421. Mean relative plasma concentration of APRs in Alzheimer's patients and controls.



Values are means and standard deviations for the abundances of each protein over the three time intervals.

Figure 4.422. The change in relative plasma concentration of four APRs at three-monthly intervals, in Alzheimer's patients and controls.



Chapter 5. Protein Abnormalities in the CSF of MS Patients.

5.10) Introduction.

In adult humans, approximately 135 ml of CSF fills the subarachnoid space and the core of the spinal cord and brain. It is found in the small central canal which runs the length of the spinal cord, and in the four ventricles of the brain (Ganong, 1985). CSF is continually produced by the choroid plexus, which consists of networks of specialised capillaries which protrude from the pia mata into the ventricles. As it is produced, the CSF circulates inferiorly through the ventricles. From the fourth ventricle, the CSF passes into the subarachnoid space. From here, some CSF moves down into the central canal, the rest moving upwards over the brain. Eventually, the CSF is absorbed from the subarachnoid space via structures called arachnoid granulations, which protrude from the arachnoid layer into large blood sinuses. These blood sinuses run through the dura mata and drain into the large jugular veins of the neck. The total CSF volume is turned over once every six hours (Guyton, 1986). The function of the CSF is to protect the brain from mechanical injury, and to transport substances filtered from the blood. In this function it acts as the medium of exchange for nutrients and waste products between the blood and the brain. The CSF also conveys regulatory information to and from the rest of the body.

The composition of CSF is regulated by the selective permeability of both the blood brain barrier and the choroid plexus. Its composition is a composite of the absorption and filtration equilibriums across the blood brain barrier and the choroid plexus.

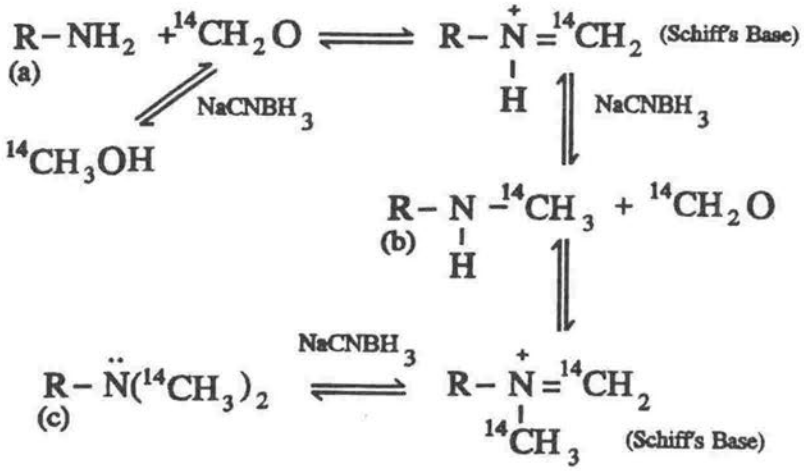
In this investigation, ^{14}C-labelled CSF proteins from individuals diagnosed as having MS and their matched controls were separated by 2-dimensional PAGE. After fluorography, fluorograms for each of the gels were scanned, and quantitative data were obtained for a number of proteins. The quantitative data were then analysed statistically to determine if any significant difference could be detected between MS and controls.

5.11) ^{14}C protein labelling.

Reductive methylation was the method used to radiolabel proteins in this project. The method was based upon that described by Means *et al.* (1968), and incorporated some of the modifications suggested by Jentoft *et al.* (1979).

Reductive methylation of proteins using formaldehyde and a reducing agent (such as $NaCNBH_3$) is a lysine specific protein modification procedure. The free amino groups of proteins are converted to their methyl and N,N-dimethyl derivatives. This involves the formation of a Schiff's base, which is reduced to form the corresponding methyl-derivatives, as shown in Figure 5.111. The primary amine (a) is reductively methylated to form the monomethyl (secondary) amine (b). This derivative can also be reductively methylated to form the dimethyl (tertiary) amine (c). This is the most common product of reductive methylation using $NaCNBH_3$ as the reducing agent.

Figure 5.111. Reductive methylation using $NaCNBH_3$ as reducing agent.



As shown in the figure, the formaldehyde can be reduced directly by $NaCNBH_3$ to form methanol. This reduces the efficiency of the overall reaction. One possible undesirable feature of using formaldehyde to supply the radiolabelled methyl groups is that it has the potential to form methylene bridges. This enables protein molecules to link together. Jentoft *et al.* (1979) however suggested that the efficient reduction of the Schiff's base by $NaCNBH_3$ should reduce the occurrence of methylene bridges to a minimum.

Reductive methylation was chosen for protein labelling in this project because it produces minimal changes in the physicochemical properties of most proteins (Geoghegan *et al.*, 1981). Reductive methylation specifically labels only the ϵ-amino groups of lysyl residues and the α-NH_2 terminus. The procedure only alters the pK values of the amino groups by 0.5 pH units (Means *et al.*, 1968). The total charge of the protein and the spatial distribution of charges is also maintained. The dimethyl derivative is also able to be charged due to the lone pair of electrons on the nitrogen centre. This is important in terms of maintaining the same electrophoretic mobility of the protein.

5.2) Materials and Methods.

Ten ml of CSF was collected via lumbar puncture from the MS patients and controls summarised in Section 3.22. 0.5 ml aliquots of CSF were concentrated by centrifugation at 7500 x g for 90 minutes (10°C) using a centrifugal microconcentrator (Centricon-10, Amicon). Concentrated CSF samples were either radiolabelled immediately or stored at -70°C until required.

Prior to protein labelling, ^{14}C-formaldehyde (Amersham) was passed through an anion exchanger to remove contaminating anions including ^{14}C-formate. The column material was Dowex 1 X-8 (BDH) with acetate as the counter ion. To prepare the Dowex, 30 g of Dowex was soaked in 500 ml 10 mM acetic acid for one hour. The acid was removed and the Dowex was rinsed in water, and then soaked in water for five minutes. The Dowex was washed in water in this manner approximately six times until the pH became alkaline. A 1 ml pipette tip was packed up to a

200 µl mark with Dowex and placed above a clean vial. 500 µCi of ^{14}C-formaldehyde (18.6 mCi/mmol) was made up to a volume of 0.2 ml with water and passed through the anion exchanger. After the elution of formaldehyde the column was washed twice with 0.2 ml of water, the eluates being combined (total volume 0.6 ml) and stored at -20 °C until required for labelling. Liquid scintillation counting was used to determine the amount of radioactivity before and after anion exchange. Usually, 95% of counts applied to the column were recovered in the eluate.

Liquid scintillation counting was performed using a Beckman model 3000 liquid scintillation counter. The scintillation fluid contained 0.3% (w/v) PPO (BDH); 0.01% (w/v) POPOP (Sigma); and 33% (v/v) Triton X-100, in toluene (BDH). Counting efficiency was measured using an internal standard.

Protein labelling was carried out in Eppendorf tubes. The reaction mixture consisted of 10 µl Hepes buffer, pH 7.5; 10 µl 1 M $NaCNBH_3$; 50 µl sample; 20 µl ^{14}C-formaldehyde (approximately 16 µCi). The reaction was allowed to proceed for one hour with occasional mixing. The sample was washed twice by adding 2.0 ml sodium phosphate buffer pH 8.0 and was concentrated by centrifugation at 7500 x g for 90 minutes (10 °C) using a centrifugal microconcentrator (Centricon-10, Amicon). The labelled sample was either stored frozen (-20 °C) or used immediately.

Two-dimensional gels were prepared and electrophoresed as described previously, to separate ^{14}C-labelled CSF proteins from ten individuals with MS, and their matched controls. The gels were processed for fluorography using a method modified from Skinner *et al.* (1983). After gels had been stained and destained, they were soaked in acetic acid for 40 minutes, and then in a solution of 20% (w/v) PPO (BDH) in glacial acetic acid. After being removed from the PPO, the gels were soaked in water for 30 minutes. During this time, the water was changed three times. The gels were then dried on 0.25 mm thick paper using a BioRad model 583 Gel Drier, attached to a Buchi B-169 water pump. The dried gels were then brought into contact with X-ray film (Kodak XAR-5 diagnostic film) and placed in X-ray cassettes (Sigma). The film was left for

approximately two weeks at -70 °C. The films were developed (four minutes) in liquid X-ray developer type 2 (Kodak), and fixed (four minutes) in liquid X-ray fixer (Kodak).

Thirty six protein spots were selected for analysis. The spots were selected to cover a range of molecular weights and isoelectric points, and also to correspond to varying plasma proteins, and to CSF proteins shown to vary in previous studies. Quantitation was performed as previously described. Figure 5.21 shows a typical separation of CSF proteins and identifies five regions which are shown in more detail in Figures 5.22 to 5.26. Proteins that were selected for quantitation are numbered on these figures and their identities are shown in Table 5.21. The identities of the proteins were determined from the 2-dimensional CSF maps of Goldman *et al.* (1980), Weiderkehr *et al.* (1989), and Yun *et al.* (1992).

Figure 5.21. Fluorogram of a 10% SDS PAGE separation of human CSF proteins. The figure shows the location of the five map regions contained in the following five figures.

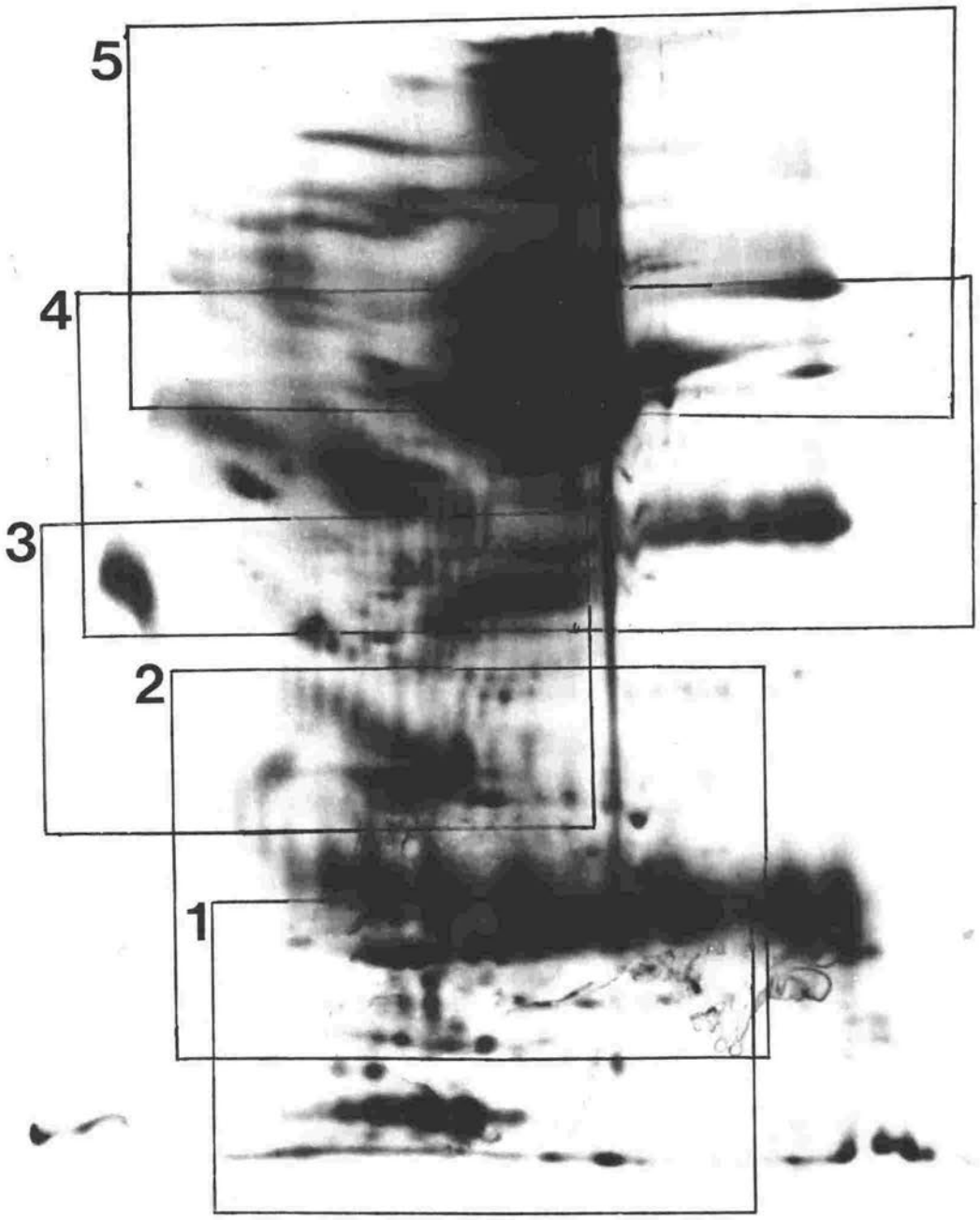


Figure 5.22. ***Map region number 1. Low mw acidic proteins.***

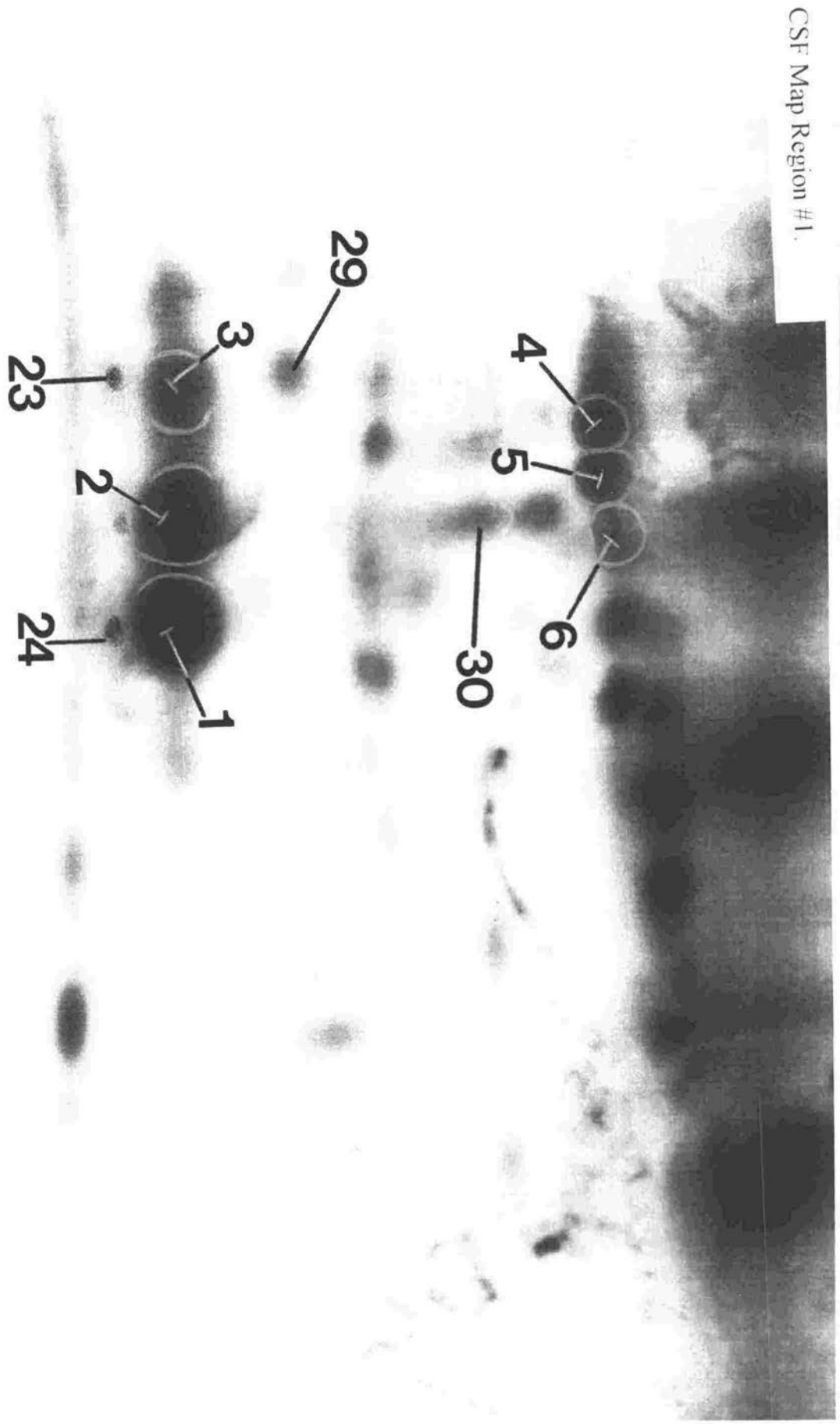


Figure 5.23. ***Map region number 2. Low mw acidic/basic proteins.***

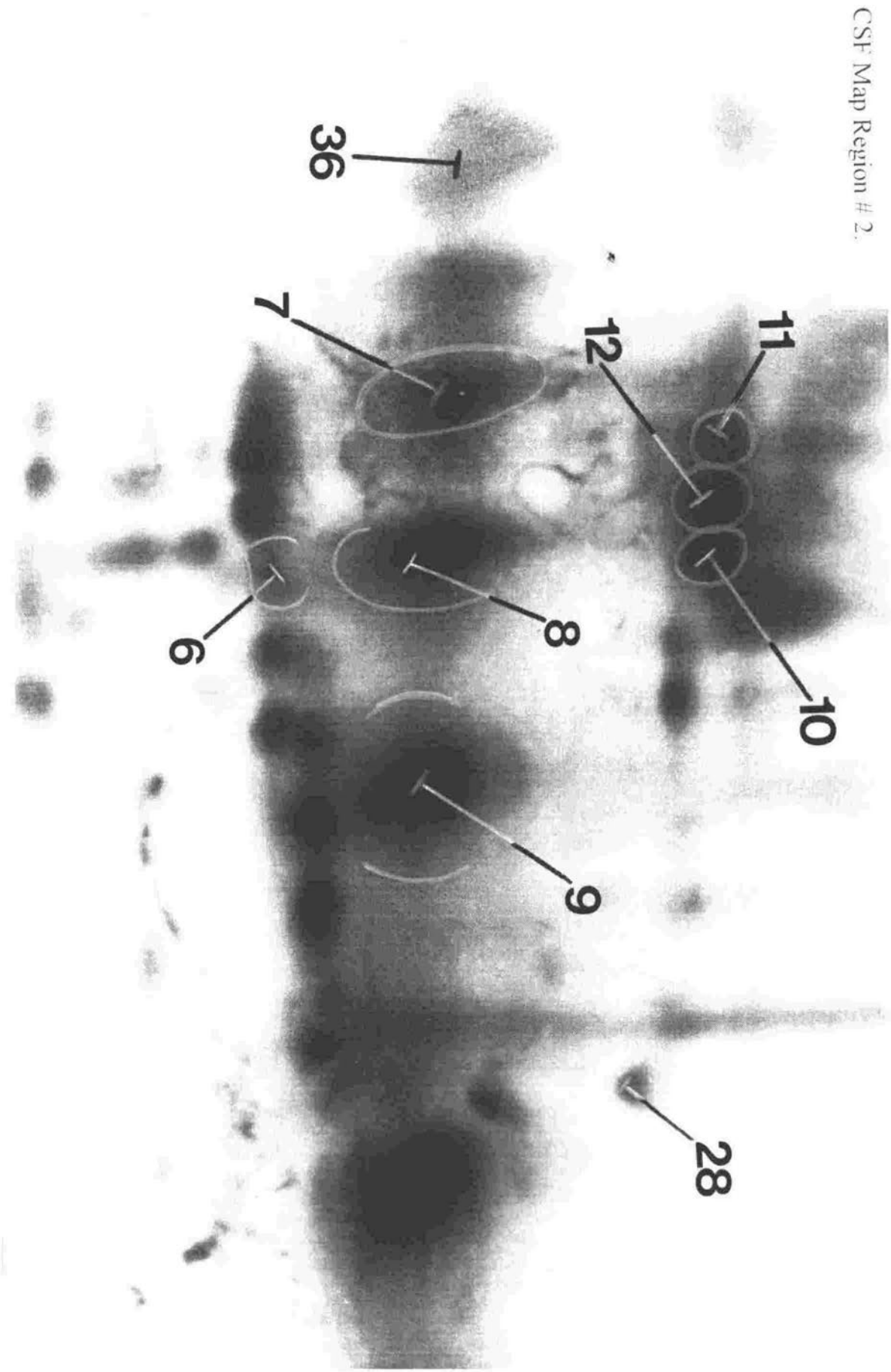


Figure 5.24. ***Map region number 3. Intermediate mw acidic proteins.***

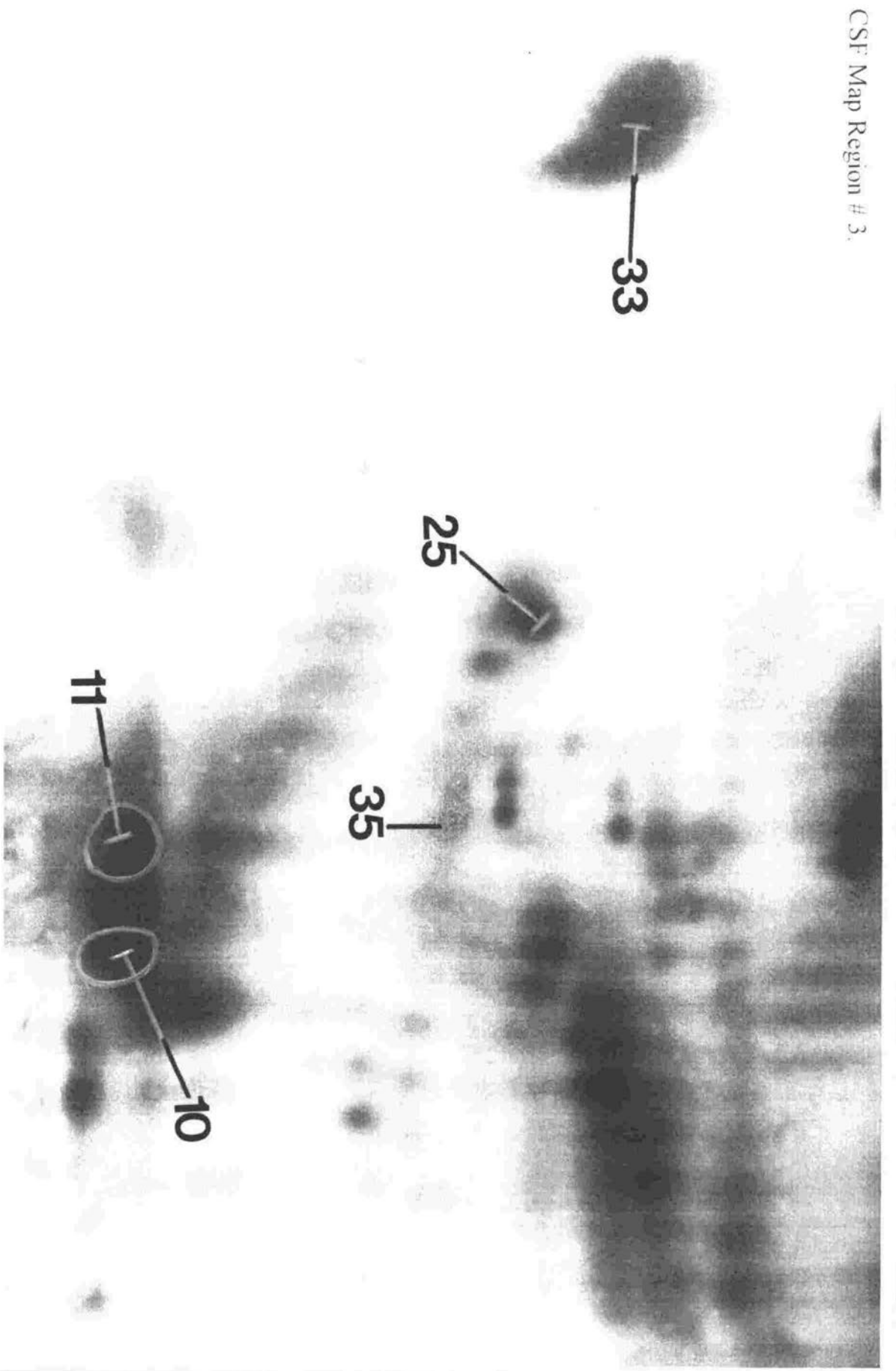


Figure 5.25. *Map region number 4. High mw acidic/neutral proteins.*

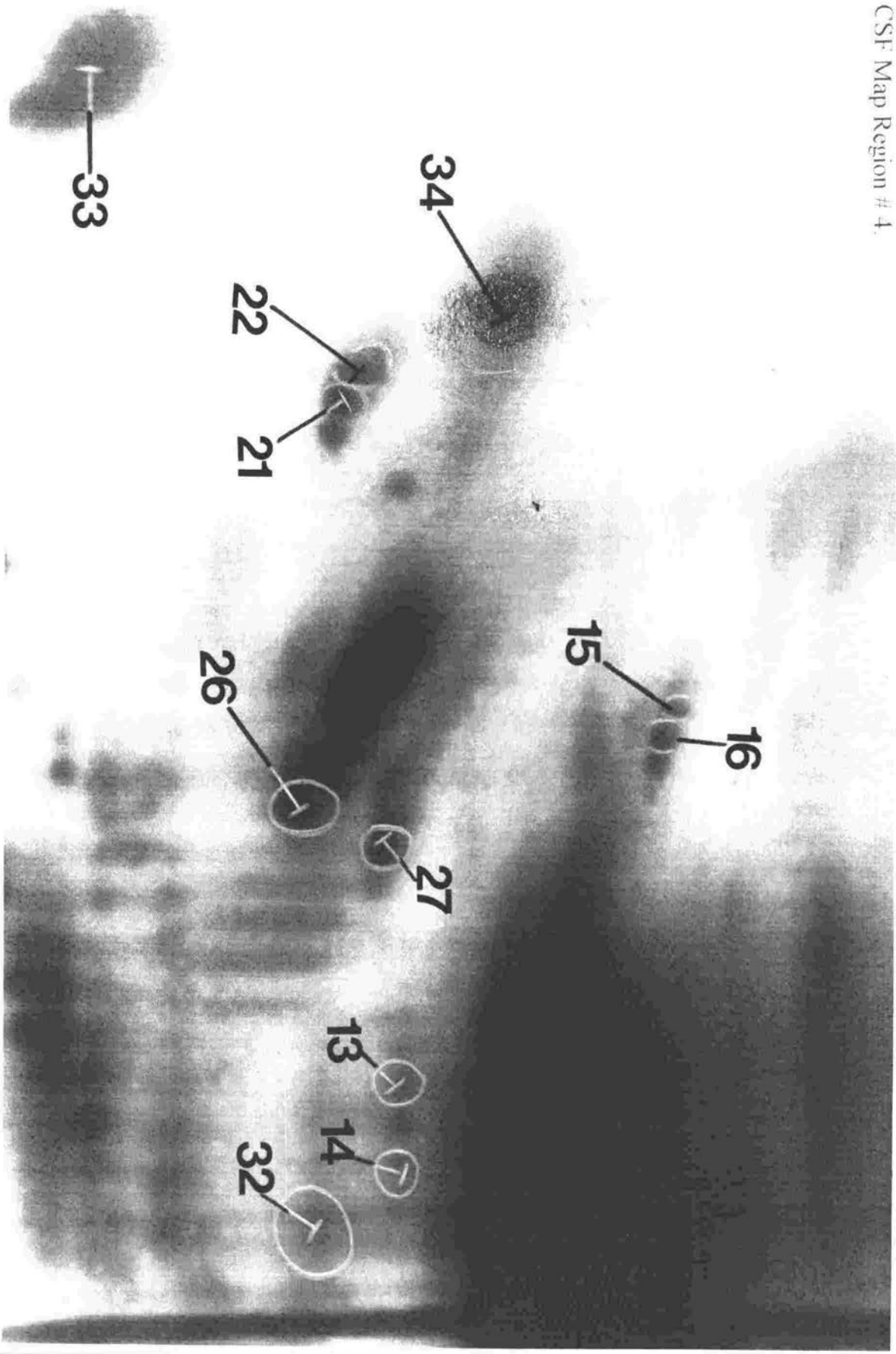


Figure 5.26. ***Map region number 5. High mw acidic/basic proteins.***

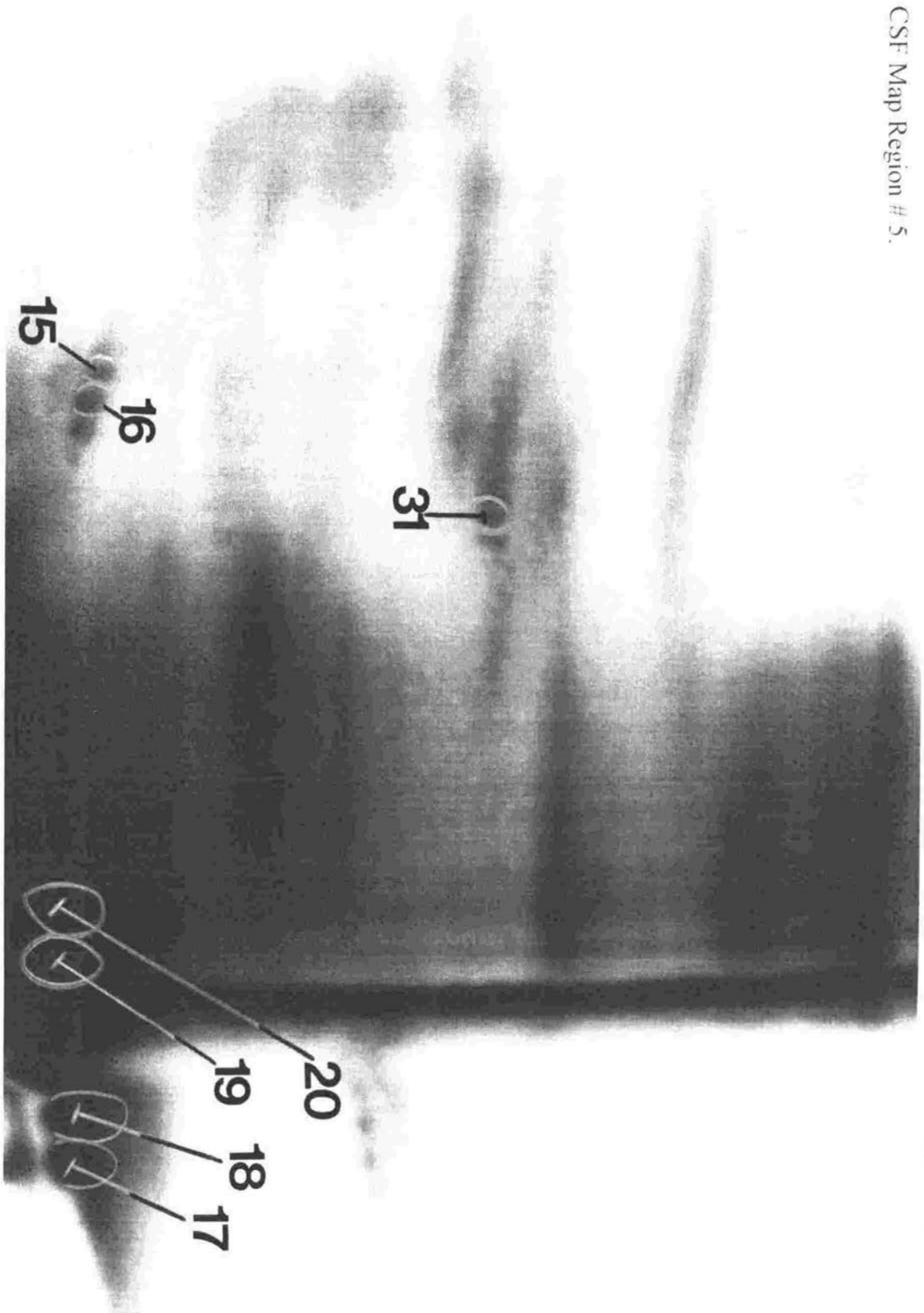


Table 5.21. CSF proteins selected for study in this section.

Spot I.D.	Protein Name	Spot I.D.	Protein Name
1	Hp α2 (1)	19	Transferrin (5)
2	Hp α2 (1)	20	Transferrin (5)
3	Hp α2 (1)	21	α2 HS glycoprotein (4)
4	Apo A1 (1)	22	α2 HS glycoprotein (4)
5	Apo A1 (1)	23	Prealbumin (1)
6	Apo A1 (1)	24	Prealbumin (1)
7	IgG light chain (2)	25	Actin (3)
8	IgG light chains (2)	26	AT (4)
9	IgG light chain (2)	27	AT (4)
10	Hp β (2)	28	C4 Gamma (2)
11	Hp β (2)	29	Unknown (1)
12	Hp β (2)	30	Unknown (1)
13	Fibrinogen (4)	31	Crp (5)
14	Fibrinogen (4)	32	Hp β (4)
15	Hemopexin (4)	33	Orosomucoid (3)
16	Hemopexin (4)	34	ACT (4)
17	Transferrin (5)	35	Hp β (3)
18	Transferrin (5)	36	Apo D (2)

Proteins were identified from the published 2-dimensional CSF maps of Goldman *et al.* (1980), Weiderkehr *et al.* (1989), and Yun *et al.* (1992). The numbers in parantheses represent the map region numbers (Figures 5.22 to 5.26) where the proteins can be found.

5.3) Results.

Figure 5.30 shows one region of a 2-dimensional PAGE gel (corresponding to CSF protein map region 1, Figure 5.22) for four MS patients (two male and two female) and four controls (two male and two female). Figure 5.30 illustrates the variation in spot position and intensity that was observed throughout this study. The histogram contained in the figure shows mean and standard deviation values for proteins 5 (Apo A1) and 23 (prealbumin) for MS males, control males, MS females, and control females. Protein spot 5 was significantly different ($P = 0.046$) between MS patients (both sexes) and their matched controls, while protein spot 23 was not significantly different between MS patients and controls.

Figure 5.30. ***Variation in spot position and intensity in one region of gel (CSF Protein Map Region 1) for four MS patients and their controls.***

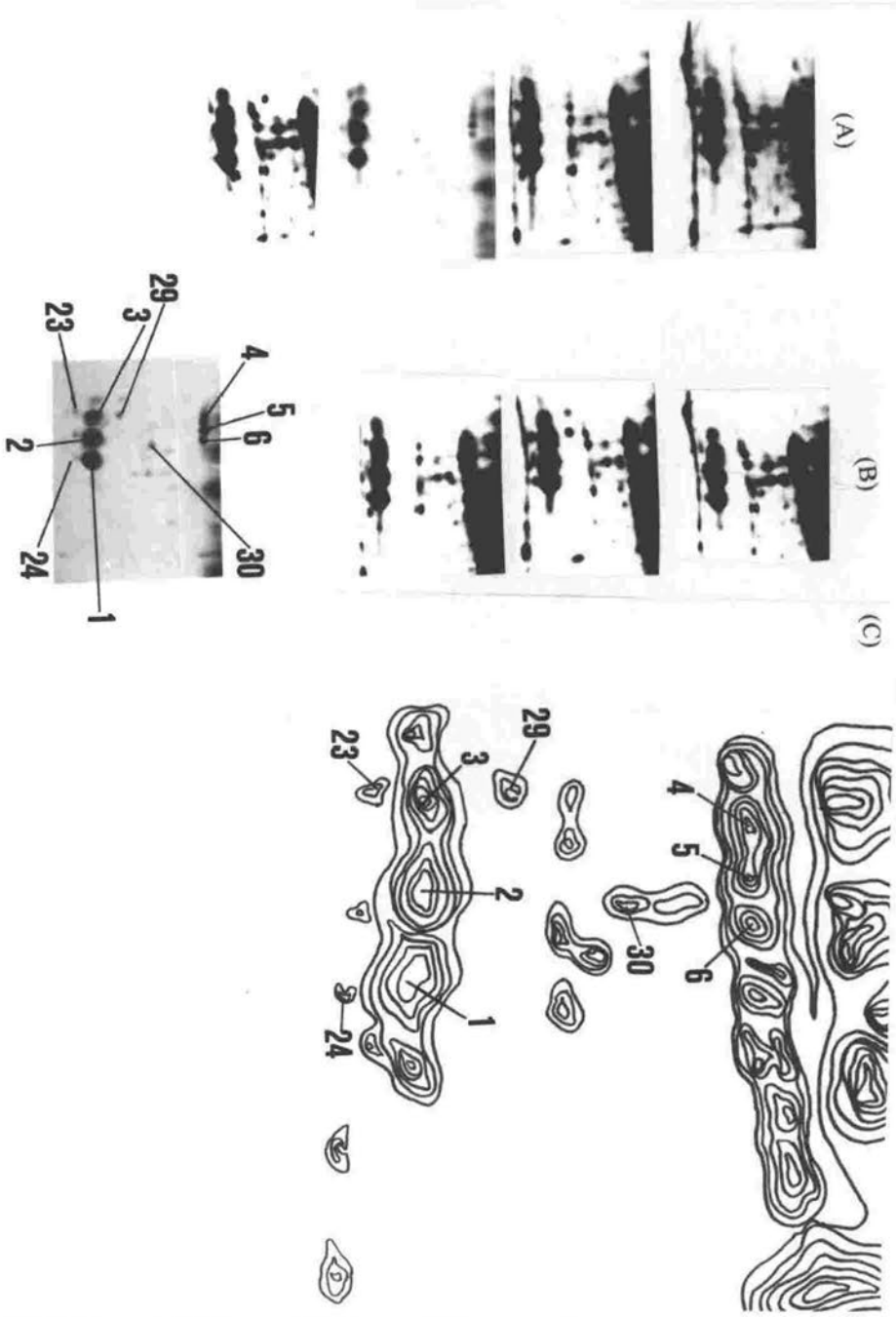
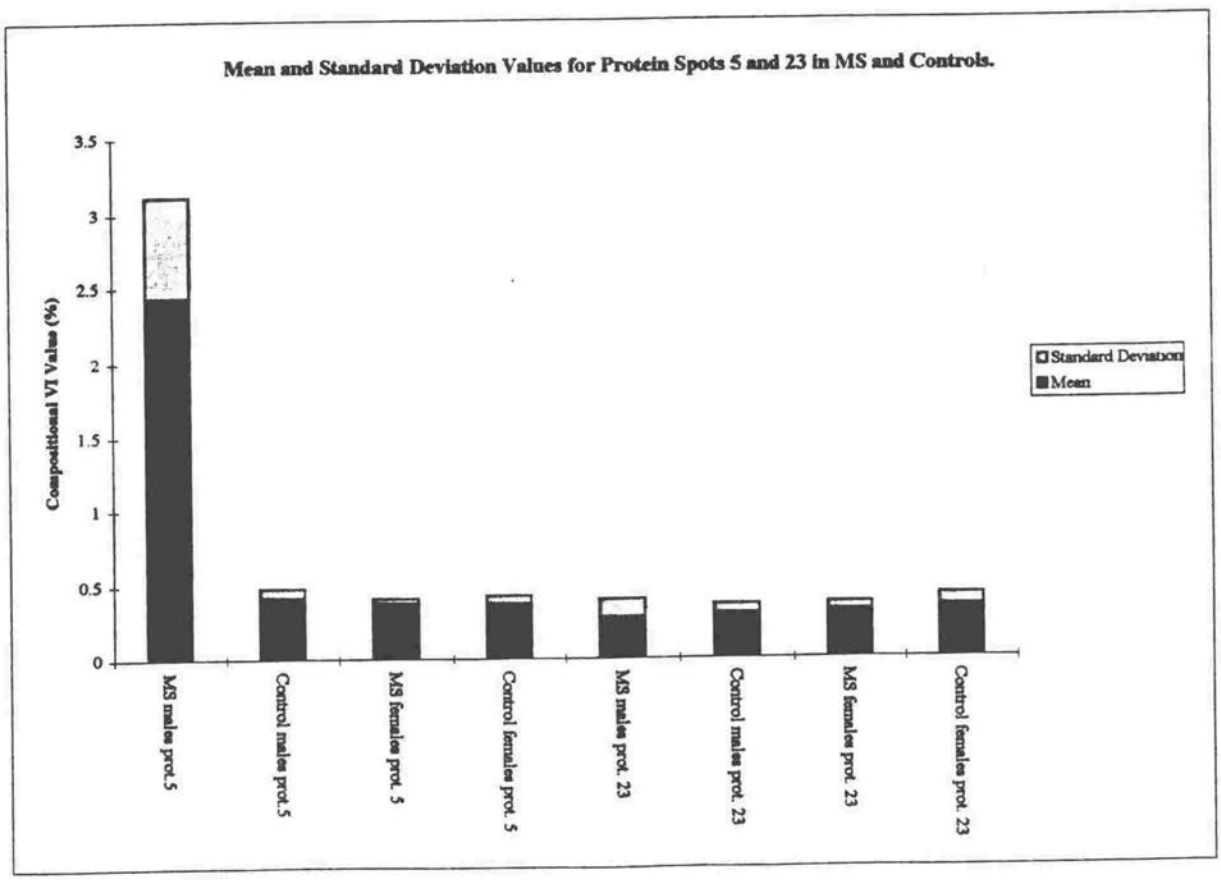


Figure 5.30. (A) MS patients. Two male and two female (top to bottom).
(B) MS controls. Two male and two female (top to bottom).
(C) Contour map of the gel region (Magnified X3).

Figure 5.30 (contd.). Variation in spot position and intensity in one region of gel (CSF Map Region 1) for four MS patients and their controls.

The following tables give the identity of those proteins labelled in the contour map (C) on the previous page.

Spot I.D.	Name	Spot I.D.	Name
1	Hp α2	6	Apo A1
2	Hp α2	23	Prealbumin
3	Hp α2	24	Prealbumin
4	Apo A1	29	Unknown
5	Apo A1	30	Unknown



After a preliminary analysis of the data I decided to use a 95% confidence level for the *t*-test data, as no proteins were found to differ significantly at the 99% confidence level.

Initially, ten patients with MS were compared to their controls. The null hypothesis adopted for this analysis was: *that MS patients of both sexes and controls of both sexes were members of the same population.* After testing, the null hypothesis was rejected on the basis of finding six significant differences between the 2 groups at the 95% confidence level. Table 5.31 lists these proteins.

Table 5.31. Proteins found to differ significantly at the 95% confidence level between MS patients (both sexes) and their matched controls.

Spot I.D.	Prob. F Value	Prob. *t* Value	Equal Variance	Protein name	Plasma APR
5	<.000	.046	No	Apo A1	?
10	.003	.011	No	Hp ß	Yes
12	.952	.001	Yes	Hp ß	Yes
13	.628	.021	Yes	Fibrinogen ß	Yes
14	.878	.005	Yes	Fibrinogen ß	Yes
26	.744	.014	Yes	AT	Yes

For the following analyses, the individuals were grouped according to sex (ie. male affected, male control, female affected, and female control) and analysis was performed by comparing data for each group.

1) *Null hypothesis: that the control males and the control females were members of the same population.* After testing, the hypothesis was upheld on the basis of finding no significant differences between the 2 groups.

2) *Null hypothesis: that the control males and the MS males were members of the same population.* After testing, the hypothesis was rejected on the basis of finding three significant differences at the 95% confidence level between the 2 groups. Table 5.32 lists these proteins.

Table 5.32. Proteins that were found to differ significantly at the 95% confidence level between MS males and control males.

Spot I.D.	Prob. F Value	Prob. *t* Value	Equal Variance	Protein name	Plasma APR
5	.314	<.000	Yes	Apo A1	?
6	.328	.006	Yes	Apo A1	?
25	.925	.008	Yes	Actin	No

3) *Null hypothesis: that the MS females and the control females were members of the same population.* After testing, the hypothesis was rejected on the basis of finding five significant differences between the 2 groups at the 95% confidence level. Table 5.33 lists these differences.

Table 5.33. Proteins found to differ significantly between MS females and female controls.

Spot I.D.	Prob. F Value	Prob. *t* Value	Equal Variance	Protein name	Plasma APR
10	.022	.001	No	Hp ß	Yes
12	.743	.005	Yes	Hp ß	Yes
13	.648	.044	Yes	Fibrinogen ß	Yes
14	.074	.026	Yes	Fibrinogen ß	Yes
26	.651	.048	Yes	α-1 antitrypsin	Yes

4) *Null hypothesis: that the MS males and the MS females were members of the same population.* After testing, the hypothesis was rejected on the basis of finding four significant differences between the 2 groups at the 95% confidence level. Table 5.34 lists these differences.

Table 5.34. Proteins found to differ significantly at the 95% confidence level between MS males and MS females.

Spot I.D.	Prob. F Value	Prob. *t* Value	Equal Variance	Protein name	Plasma APR
5	.030	.001	No	Apo A1	?
6	.269	<.000	Yes	Apo A1	?
20	.139	.050	Yes	Transferrin	No
25	.903	.008	Yes	Actin	No

5) *Null hypothesis: that the MS males and the and the control females were members of the same population.* After testing, the hypothesis was rejected on the basis of finding three significant differences at the 95% confidence level between the 2 groups. Table 5.35 lists these differences.

Table 5.35. Proteins found to differ significantly between MS males and control females.

Spot I.D.	Prob. F Value	Prob. *t* Value	Equal Variance	Protein name	Plasma APR
5	.132	<.000	Yes	Apo A1	?
6	1.000	<.000	Yes	Apo A1	?
18	.757	.026	Yes	Transferrin	No

6) *Null hypothesis: that the MS females and the control males were members of the same population.* After testing, the hypothesis was rejected on the basis of finding five significant differences between the 2 groups at the 95% confidence level. Table 5.36 lists these proteins.

Table 5.36. Proteins found to differ significantly between MS females and control males.

Spot I.D.	Prob. F Value	Prob. *t* Value	Equal Variance	Protein name	Plasma APR
10	.077	<.000	Yes	Hp ß	Yes
11	.623	.042	Yes	Hp ß	Yes
12	.772	.011	Yes	Hp ß	Yes
13	.559	.024	Yes	Fibrinogen ß	Yes
14	.524	.006	Yes	Fibrinogen ß	Yes

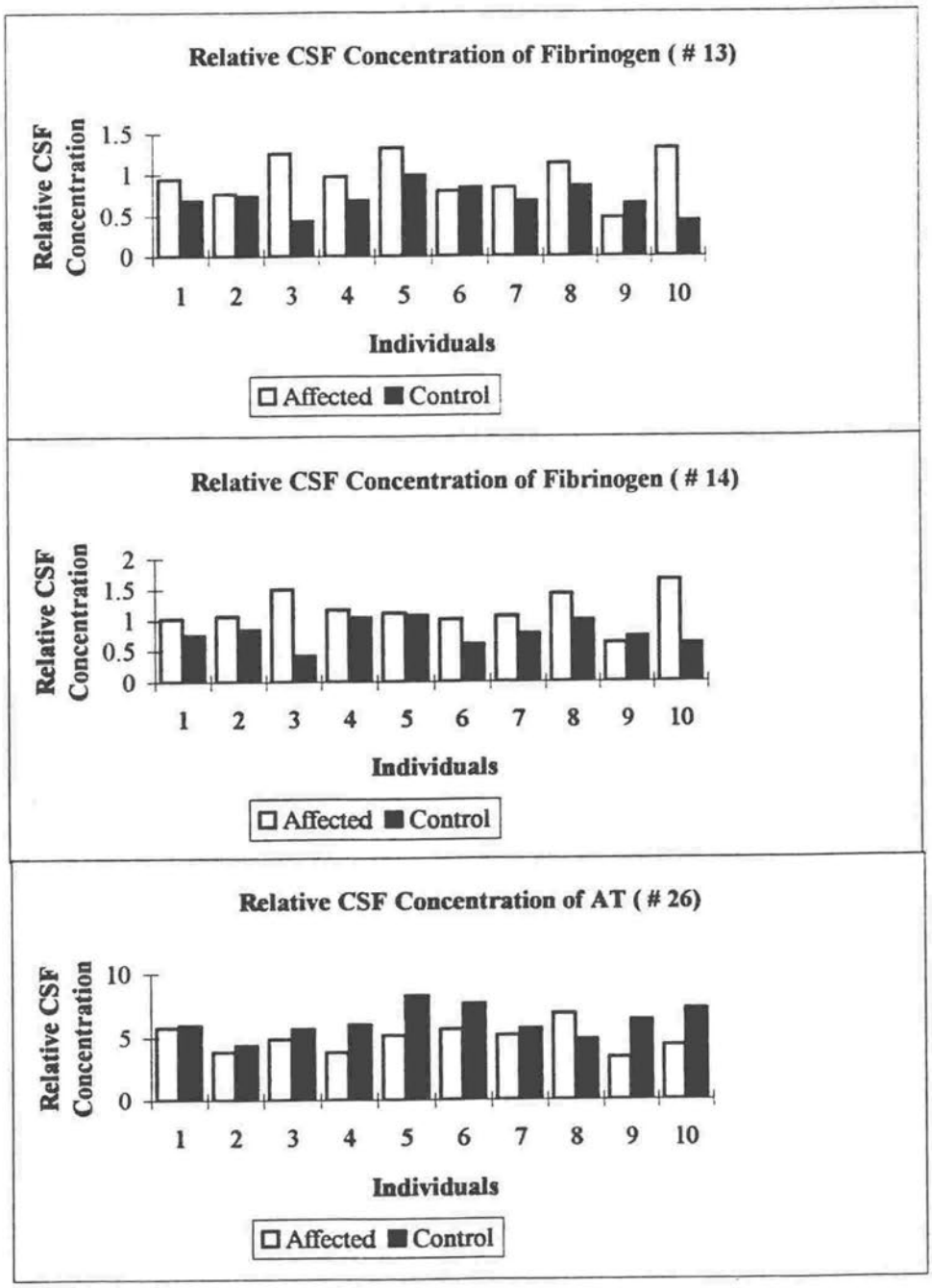
5.4) Discussion.

For the initial study, where the data from both MS males and females were compared to controls, several proteins were found to differ significantly between patients and controls. All of the proteins were either thought to be, or were, APRs. Figure 5.41 shows histograms giving the relative CSF concentrations of each of these proteins in the affected and control individuals. In Figure 5.41 individuals 1 to 4 are males, and individuals 5 to 10 are females. Of those proteins known to be APRs, Hp ß and fibrinogen ß were seen to increase in relative CSF concentration, while AT was seen to decrease in relative CSF concentration. In plasma, patterns of increased and decreased APR concentrations are associated with chronic inflammation (Maes *et al.*, 1992). A possible explanation for the observed increased in plasma APR concentrations in CSF could be abnormalities in the blood brain barrier of the patients with MS. Such abnormalities have been reported in patients with MS (Harik *et al.*, 1991). The increase in CSF of plasma APRs could also be due to local (ie. intrathecal) production.

When male controls were compared to female controls, no significant differences were observed at the 95% confidence level. This implied that any significant differences found between MS males and MS females should be regarded as disease related and not sex related. When this comparison was made, several significant differences were detected, although none of the proteins concerned were APRs.

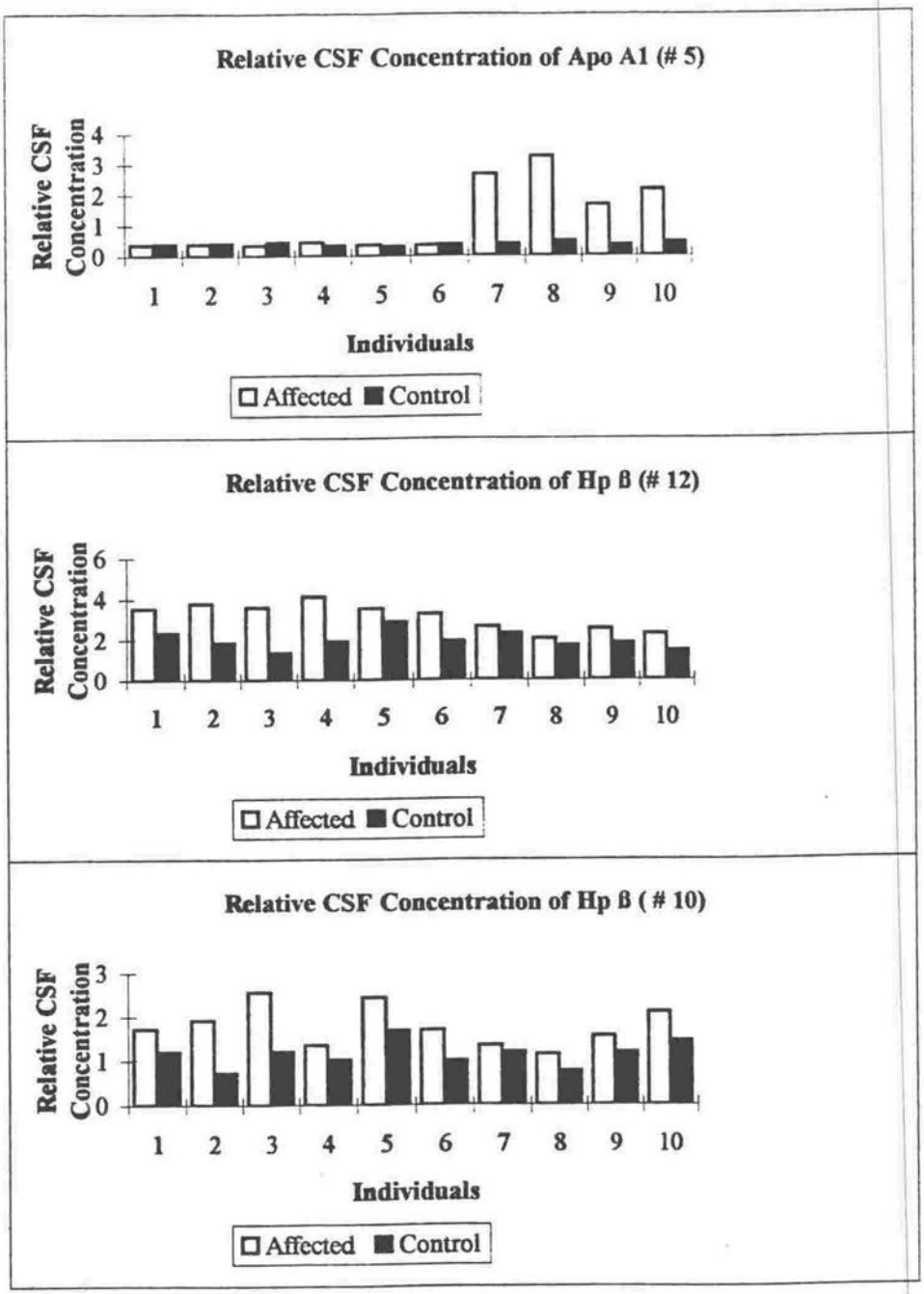
Because male and female controls were not significantly different, it was possible to test the stringency of the observations made, by making two predictions. First, it would be expected that any differences found between MS males and control males would also be found between MS males and control females. When the tests were done, the two comparisons shared two out of the four proteins found to differ significantly at the 95% confidence level. These proteins were Apo A1 (spots 5 and 6). A second prediction that could be made was that any proteins found to differ significantly between MS females and control females would also be found to differ significantly between MS females and control males. When these tests were performed, the two comparisons shared four out of the five proteins found to differ significantly at the 95% confidence level. The proteins were Hp β (spots 10 and 12) and fibrinogen β (spots 13 and 14).

Figure 5.41. Relative CSF concentration (ie. compositional value) of proteins found to differ significantly between the MS patients (both sexes) and their controls.



Individuals 1 to 4 were males and individuals 5 to 10 were females.

Figure 5.41 (contd.). ***Relative CSF concentration (ie. compositional value) of proteins found to differ significantly between the MS patients (both sexes) and their controls.***



Individuals 1 to 4 were males and individuals 5 to 10 were females.

5.5) Comparison of the levels of proteins in the plasma and CSF of MS patients and their controls.

5.51) Introduction.

A study was made to see if any disease related pattern in the distribution of proteins in the plasma and CSF could be detected. Such a pattern could suggest differences in protein processing in the disease state, or may indicate a breach in the blood-brain barrier or choriod plexus.

Two separate studies were done. In the first (described in section 5.52), a search was made for any proteins in the plasma and CSF that appeared to correlate in terms of relative abundance. In the second study (section 5.53), an examination was made of the relationship between the abundances of proteins which could be detected in both the plasma and CSF.

5.52) Correlation between the relative abundance of proteins in the plasma and CSF.

The study group consisted of ten individuals affected with MS and ten control subjects. The plasma proteins quantitated were those described in Chapter 4. The CSF proteins quantitated were those described in the first section of this chapter. Initially, correlation analysis was performed on the $\log_{10}$ ratio values of all of the quantitated plasma and CSF proteins. This was done to examine whether there was any relationship between CSF and plasma protein levels.

Sample correlation was used, as given by the following:

$$\rho_{X,Y} = \frac{cov\ (X,Y)}{\sigma_X \cdot \sigma_Y}$$

Where ρ is the correlation coefficient, *cov* is the covariance, $\sigma_x^2 = \frac{1}{n}\sum (X_i - \mu_x)^2$

and $\sigma_y^2 = \frac{1}{n}\sum (Y_i - \mu_y)^2$.

Theoretical certainty values for the correlation analysis were calculated as follows:

$$Ser = \frac{1-(\rho)^2}{\sqrt{n-1}}$$

Where S er = standard error, and ρ = correlation coefficient.

This equation represents the standard deviation of a hypothetical distribution of ρ among samples of a given size drawn from the same population. The null hypothesis was defined as there being no relationship between the relative abundances of the two proteins. Therefore, the values of ρ would be normally distributed with an error of $\frac{1-(0)^2}{\sqrt{n-1}}$. So, in approximately 95% of samples from such a population, ρ should fall within two standard errors of zero, and in approximately 99% of samples, ρ should fall within two and one-half standard errors of zero. Therefore, to be significant at the 95% confidence level, the value of ρ must be at least 0.67. To be significant at the 99% confidence level, ρ must have a value of at least 0.83.

Each group of values for every CSF protein was correlated against each group of values for every plasma protein. This process was performed for the MS patients and their controls. Those protein combinations that correlated significantly (at least $P < 0.05$ at the 95% level) were subjected to a further analysis. In this analysis, the compositional values for plasma and CSF proteins for every individual were expressed as a series of CSF: Plasma ratios. A *t*-test comparing MS patients to their controls was then done on the values. Table 5.521 shows those protein combinations that correlated significantly, and differed significantly between the disease and non-disease state.

Table 5.521. Protein combinations that showed significant correlation and were significantly different between MS patients and their controls.

Protein Combination[1]	Correlation Coefficient (ρ)[2]	Prob. *t* Value	Protein names
CSF1: P16	0.69(C)	0.004	Hpα2 : Hp α2
CSF1: P19	0.70(C)	0.007	Hpα2: Unidentified
CSF5: P15	-0.77(D)	0.040	Apo A1: Hpα2
CSF5: P38	-0.88(D)	0.039	Apo A1: Unidentified
CSF5: P39	-0.94(D)	0.026	Apo A1: Unidentified
CSF6: P33	0.73(C)	0.046	Apo A1: IgG light chain
CSF6: P38	-0.86(D)	0.029	Apo A1: Unidentified
CSF6: P39	-0.89(D)	0.025	Apo A1: Unidentified
CSF6: P40	0.87(D)	0.002	Apo A1: Unidentified
CSF9: P11	0.71(D)	0.027	IgG Light chain: Unidentified
CSF10: P3	0.68(C)	0.015	Hpβ: IgJ
CSF10: P28	0.79(D)	0.028	Hpβ: Unidentified
CSF12: P22	0.71(D)	0.007	Hpβ: Unidentified
CSF20: P11	0.70(D)	0.0003	Transferrin: Unidentified
CSF20: P39	0.70(D)	0.021	Transferrin: Unidentified
CSF21: P13	-0.85(C)	0.003	α-2 HS glycoprotein: Orosomucoid
CSF22: P24	0.77(D)	0.047	α-2 HS glycoprotein: Hpβ
CSF22: P26	-0.69(D)	0.023	α-2 HS glycoprotein: Apo D
CSF25: P31	0.80(D)	0.028	Actin: IgG light chain

1. The protein combinations are given in the order of CSF protein: plasma protein
2. ρ values followed by a "C" represent correlations found in the control group. ρ values followed by a "D" represent correlations found in the disease group.

Fourteen protein combinations were seen to correlate in MS patients but not in controls. The combinations included eight CSF proteins and ten plasma proteins. The CSF proteins were Apo A1 (spots 5 and 6), IgG light chain (spot 9), Hpβ (spots 10 and 12), α-2 HS acid glycoprotein (spot 22), and actin (spot 25). The plasma proteins included Hpα (spot 15), Hpβ (spot 24), Apo D (spot 26), IgG light chain (spot 31), and six unidentified proteins (spots 11, 22, 28, 38, 39, and 40). Positive and negtive correlations were seen in the protein combinations.

Five protein combinations were seen to correlate in controls but not in MS patients. The combinations included four CSF proteins and five plasma proteins. The CSF proteins were Hpα (spot 1), Apo A1 (spots 5 and 6), Hpβ (spot 10), and α-2 HS acid glycoprotein (spot 21). The plasma proteins included Hpα (spot 16), IgG light chain (spot 33), IgJ (spot 3), orosomucoid (spot 13), and an unknown protein (spot 19). Again, positive and negtive correlations were seen in the protein combinations.

Most of the correlations for the MS patients and the control group involved APRs. This is not surprising as the majority of disease related protein changes in the CSF and plasma of MS patients (detected in this study) involved APRs. Most of the correlations (63%) were positive. That is, increasing relative CSF concentration was associated with increasing relative plasma concentration. Based on knowledge of the biochemistry of the proteins studied, I can find no obvious pattern. The complexity of the correlations may reflect the complexity of the acute phase response to chronic inflammation.

5.53) The relationship between proteins found in both the CSF and plasma.

A study was also made of individual proteins that could be identified in both the CSF and plasma. Table 5.531 gives the proteins studied, and gives the plasma and CSF map numbers. The study group consisted of four MS patients and four controls. For each individual, the proteins given in Table 5.531 were quantitated. Means and standard deviations were calculated for each protein.

Table 5.531. The proteins investigated in this study.

Protein Name	CSF #	Plasma #	Protein Name	CSF #	Plasma #
Orosomucoid	33	13(7)	Apo A1	4.5.6(1)	1(2)
Crp	31	A(9)	Hpβ	32(4)	23(6)
ACT	34	A(7)	Hpα2	3(1)	16(3)
α2 HS glycoprotein	21(4)	B(7)	Hpα2	2(1)	15(3)
Hpβ	35(3)	41(7)	Hpα2	1(1)	14(3)
Apo D	36(2)	25.26(5)			

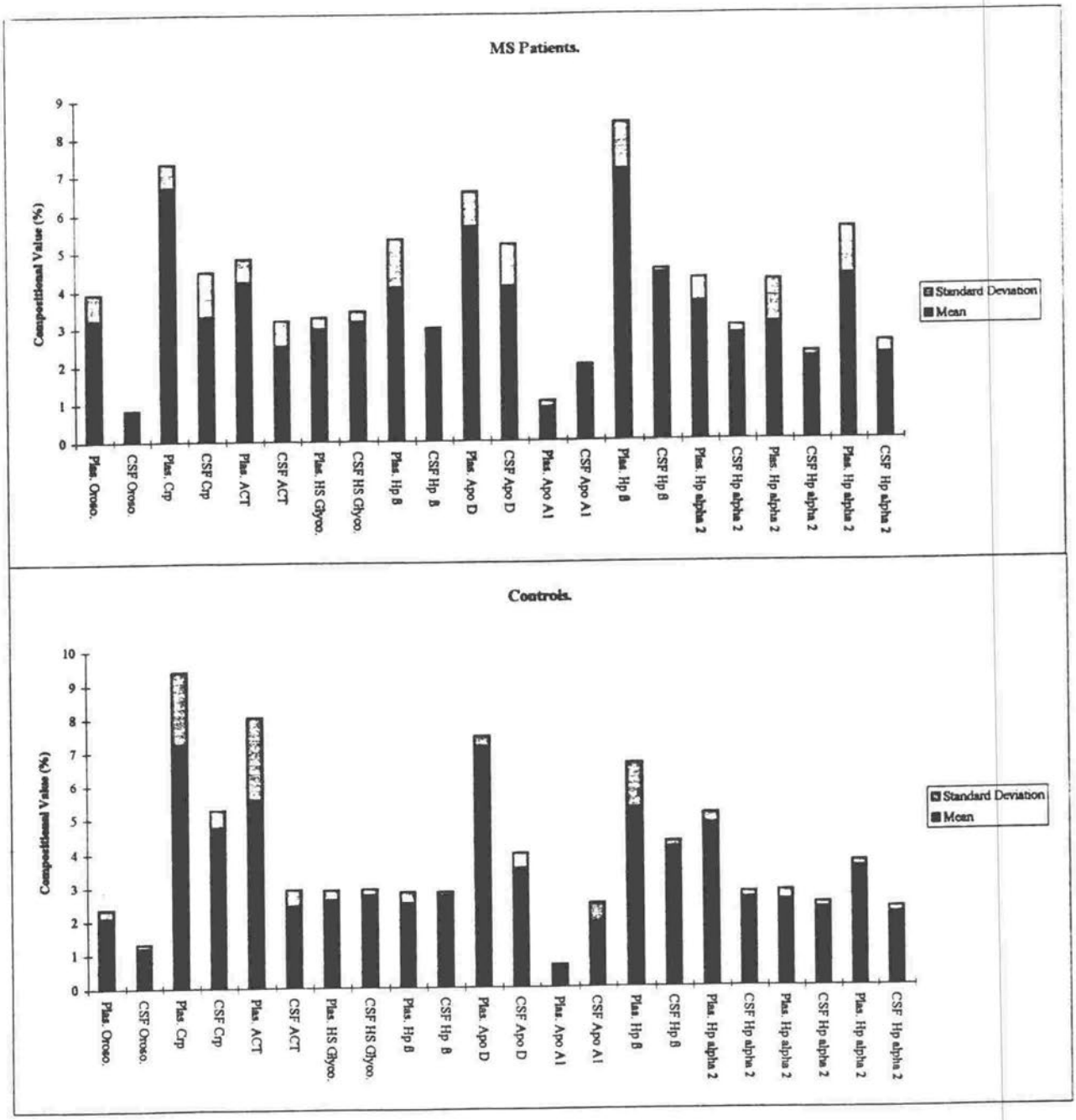
Numbers in parentheses give the relevant map number.

Figure 5.531 shows plots of the mean and standard deviation values for the proteins in CSF and plasma, for MS patients and controls. No clear pattern can be seen. For some proteins in MS, high plasma concentrations were associated with low CSF concentrations (eg.Crp), while the opposite was seen for others (eg. Apo A1). For other proteins (eg. Apo D), low plasma levels in the disease state were associated with low CSF levels. The CSF level of other proteins (eg. orosomucoid) remained fairly constant despite greatly differing plasma levels. These observations produce no clear evidence for a breached blood-brain barrier or choroid plexus. In such a situation, a less complex pattern would be expected. Generally, high plasma levels would be expected to be associated with high CSF levels (if no extra-hepatic synthesis of the proteins concerned occured). This was not consistently observed in this study however.

These observations suggest a complex pattern of protein synthesis in response to disease. Due to recent suggestions that human plasma proteins may be synthesised in the CNS (as discussed in Chapter 1), it is not yet possible to know how the pattern observed was achieved. It may have been the result of protein movement between the CSF and plasma and co-regulated hepatic and CNS protein synthesis. Although the results presented here were obtained from a small sample group the complexity of the results would support further study. If such a study was to be undertaken, a larger sample group and a greater number of proteins would need to be studied. A more rigorous protein identification method would be required; obtaining sequence data for each

protein in the study would be a priority. It would also be useful to obtain a number of CSF and plasma samples for every individual over a period of at least several months.

Figure 5.531. Average abundance values for proteins found in both the CSF and plasma in MS affected individuals and their controls. Values are expressed as SD/Mean% values.



Chapter 6. Platelet Protein Abnormalities in Alzheimer's Disease.

6.10) *Introduction.*

Platelets are small, anucleate, biconvex disk shaped cells, 2-4 μm in diameter. They are formed from fragmentation of megakaryocytes in the bone marrow, and are involved in the haemostatic process. In the peripheral blood, the normal platelet count is approximately 300,000/μl. The life span of a human platelet is about eight days, platelets being removed from the circulation either by the reticulo-endothelial system, or by incorporation into haemostatic plugs (Ganong, 1985; Gordon, 1981).

The reasons for studying platelet proteins in this study were: first, an increase in platelet membrane fluidity (thought to be due to accumulation of SER membranes) has been observed in Alzheimer's disease patients (Zubenko 1990; Hajimohammadreza *et al.*,1990). Second, various metabolic studies on the platelet metabolism of asymptomatic individuals and those affected with neurological diseases have so far been inconclusive (Kanof *et al.*, 1991). Third, studies have shown that platelets exhibit significant proteolytic activity (Scharpe *et al.*,1991); many recent reports suggest that proteases and their inhibitors may play a role in the pathogenesis of Alzheimer's disease (Delamarche *et al.*, 1991). Some of these proteins may be expressed in platelets. Fourth, APP is abundantly expressed in platelets (Li *et al.*, 1994). Fifth, a specific reduction in platelet cytochrome c oxidase activity has been observed in Alzheimer's disease patients (Parker *et al.*,1994). Some of these changes may be observable in platelet protein profiles. APP does not appear in the Swiss-2D PAGE platelet protein database.

In this study, platelets were harvested from the blood of Alzheimer's disease patients and their age and sex matched controls (a patient summary is given in Section 3.22). A study was then made between platelet proteins from Alzheimer's disease patients and their controls, looking for both qualitative and quantitative protein changes.

Initially, several studies were performed to develop techniques that were subsequently used for this section of work. These studies included the method for isolation of platelets from human whole blood; the effect of proteolytic inhibitors; determining the effect of the platelet activation state on the platelet protein profile; and determining if the platelet protein profile was consistent both over time, and between unrelated individuals. For all of these studies, 1-dimensional SDS PAGE was used, as described in previous chapters. The following sections describe these studies.

6.20) *Development of a platelet isolation technique.*

Two methods were investigated to decide which gave better platelet preparations, in terms of yield and purity. The first method was modified from Timmons *et al.*,(1989), and involved isolating human platelets by centrifugation over an albumin layer, followed by gel filtration on Sepharose 2B. The second method involved isolating human platelets by differential centrifugation. (Giometti *et al.*, (1984) and Gerard *et al.*,(1989)).

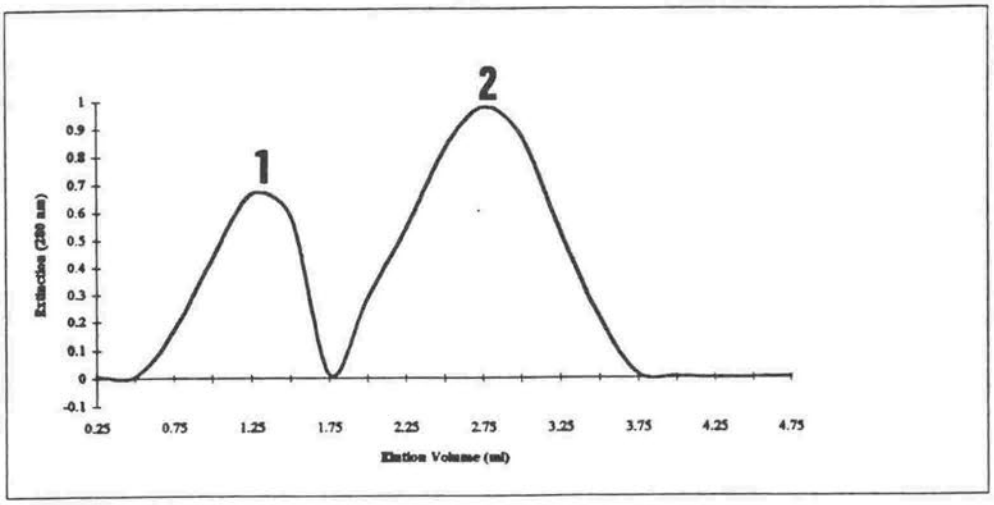
6.21) Platelet isolation by centrifugation over albumin followed by Sepharose 2B Gel filtration.

20 ml of blood was drawn from the cubital vein into Vacutainer blood collection tubes, containing 143 USP units of sodium heparin as anticoagulant. The tubes were centrifuged at 160 *g* for 15 minutes at 7 °C, and the supernatant (PRP) was removed. The PRP was then applied to the top of a 50% albumin (bovine) solution, and centrifuged at 1200 *g* for 15 minutes (7 °C). The plasma was removed, and the platelet fraction was collected from the top of the albumin layer. The platelet fraction was diluted with column elution buffer (137 mM NaCl; 2.7 mM KCl; 1 mM $MgCl_2$; 5.5 mM glucose; 0.35% v/v BSA; 3 mM NaH_2PO_4; 3.5 mM HEPES; pH 7.4) to a total volume of 0.25 ml, and stored at 4 °C.

Sepharose 2B was washed in four volumes of acetone, followed by six volumes of 0.9%

NaCl. The Sepharose was then poured into a plastic 1 ml syringe, containing a Nybolt microfilament mesh (pore size 20 μm) bottom filter. The flow rate through the column was regulated with a small clamp attached to a piece of polyethylene tubing which was attached to the bottom of the syringe. The column was equilibrated with elution buffer at room temperature for one hour, the flow rate being adjusted to 1 ml min^{-1}. The platelet sample was then applied to the top of the column, and 0.25 ml fractions were collected. Each fraction was diluted to 1 ml with elution buffer, and the extinction for each was measured at 280 nm. Figure 6.211 shows the elution profile.

Figure 6.211. Elution Profile for Platelets Separated From Plasma using Sepharose 2B.



Platelets eluted between 0.25 and 1.75 ml (peak 1). The second peak has been shown to contain plasma proteins (Timmons *et al.*, 1989). Fractions 5 and 6 (peak 1) were pooled, and the sample was diluted to 1.5 ml in 0.02 M sodium phosphate buffer, pH 7.4. A 1 ml aliquot was analysed using an automated cell counter (Stak F Coulter Counter; Coulter Inc. CA) at the Haematology Section, Clinical Services Department, Wellington Hospital. The red cell count was 0.39 x 10^9 l^{-1}; the white cell count was 11.49 x 10^9 l^{-1}; and the platelet count was 823 x 10^9 l^{-1}. The remaining 0.5 ml aliquot was sonicated (Heat Systems Ultrasonic Inc. Sonicator, model w-380) with 12, 1 second bursts, at 50% duty cycle with a power setting of 3.5. The protein content of the sample was

assayed at 8.5 mg ml^{-1} using the Bradford method (Bradford, 1976). For this assay, 8 µl of the sonicated aliquot was diluted with 0.15 M NaCl to a total volume of 0.1 ml. One ml of protein reagent (0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, 8.5% (w/v) phosphoric acid) was then added, and the solution was mixed by inversion. After five minutes the extinction was measured at 595 nm. Protein amount was extrapolated from a BSA standard curve.

6.22) Platelet isolation by differential centrifugation.

Twenty ml of blood was drawn into evacuated Vacutainer blood collection tubes that contained sodium heparin as anticoagulant. The tubes were centrifuged at 200 *g* for ten minutes (20°C) to sediment red blood cells. The supernatant was transferred to a plastic centrifuge tube and centrifuged at 100 *g* for ten minutes (20°C). The upper 75% of the supernatant was transferred to a new plastic centrifuge tube and centrifuged at 160 *g* for five minutes (20°C) to sediment the white blood cells. The upper 60% of the supernatant was then transferred to another plastic centrifuge tube, and centrifuged at 1500 *g* for thirty minutes (20°C) to sediment the platelets. The pellet was resuspended in 1 ml of 1.5 M NH_4Cl, 1 mM EDTA, 0.1 M $KHCO_3$, and left on ice for twenty minutes to lyse any remaining red and white blood cells. The suspension was then resedimented by centrifugation at 2000 *g* for 20 minutes (20°). The supernatant was discarded, and the pellet was resuspended in 1.5 ml 0.02 M sodium phosphate buffer, pH 7.4. A 1 ml aliquot was then subjected to automated cell counting. The red cell count was 0.02 x 10^9 l^{-1}; the white cell count was 0.3 x 10^9 l^{-1}; and the platelet count was 447 x 10^9 l^{-1}. The remaining 0.5 ml aliquot was sonicated as described previously, and its protein concentration was found to be 5.3 mg ml^{-1}.

On the basis of the above results I decided to use differential centrifugation to isolate platelets. Although this method resulted in a lower platelet yield, the platelet preparation had a much lower level of contamination by red and white cells.

6.3) *Inhibition of proteolysis.*

One goal of the current study was to investigate whether any aberrant proteolytic event could be detected in the platelet proteins of patients affected with Alzheimer's disease. To do this study, two blood samples were taken from each individual. One sample was drawn into a Vacutainer containing anticoagulant and a cocktail of protease inhibitors. The other sample was drawn into a Vacutainer containing anticoagulant only. By comparing inhibited and uninhibited blood samples, an assessment could be made of whether any observed protein change might be due to aberrant proteolytic processing.

Before such a study could be carried out it was necessary to decide if whether protease inhibitors affected the platelet protein profiles in a consistent manner. It was also necessary to know if the timing of the addition of protease inhibitors to the collected blood affected the protein profiles.

6.31) The consistency of protease inhibition between unrelated individuals.

Two 10 ml blood samples were supplied from each of four healthy volunteers. Each sample was drawn from the cubital vein into Vacutainer blood collection tubes containing 143 USP units of sodium heparin as anticoagulant. One Vacutainer for each individual contained anticoagulant only. The other contained anticoagulant and a protease inhibitor cocktail. The final concentrations of the inhibitors (all obtained from Sigma) in the cocktail were: 1 mM PMSF; 100 μM NEM; 10 mM EDTA; 100 μM iodoacetamide; 1 μM pepstatin; and 150 KIU /ml aprotinin. This cocktail was selected to inhibit proteases of all four catalytic classes (serine, cysteine, aspartic, and metallo proteases). No attempt was made to target specific proteases. Platelets were isolated via differential centrifugation as described in section 6.22. The platelet proteins were separated by 1-dimensional SDS PAGE, as described previously. Figure 6.311 shows 1-dimensional platelet

protein profiles with and without protease inhibitors for two unrelated individuals. The protein profiles for both inhibited and uninhibited platelet preparations were similar, although the samples prepared in the presence of protease inhibitors had additional high mw bands.

6.32) The effect of time of addition of protease inhibitors to venous blood.

The effect of adding protease inhibitors three hours after collection of venous blood was also studied. This was done to establish the conditions which were subsequently used in the study of platelet proteins. For this investigation, four aliquots of blood (each 10 ml) were taken from a group of volunteers. The first three aliquots were collected into Vacutainers containing anticoagulant (lithium heparin) only. The fourth aliquot was collected into a Vacutainer containing anticoagulant and the inhibitor cocktail. The sample with protease inhibitors and one of the samples without protease inhibitors were immediately processed to isolate platelets. The remaining two samples were left at room temperature for three hours, after which time one of the samples had the inhibitor cocktail added to it. After a further ten minutes, both samples were processed to isolate platelets. The platelet proteins were then separated by 1-dimensional SDS PAGE (Figure 6.321). The sample left at room temperature for three hours prior to the addition of the inhibitor cocktail differed from the uninhibited samples and from the samples to which protease inhibitors had been added immediately. It possessed some high mw bands associated with the inhibited protein profile, but also had some lower mw bands associated with the protein profile of the samples prepared in the absence of protease inhibitors. Some of these bands are highlighted by arrows in Figure 6.321. Based on these observations, I decided that the inhibitor cocktail had to be present in the Vacutainers at the time of blood collection.

Figure 6.311. ***10% 1-dimensional PAGE separation of human platelet proteins from two unrelated individuals.***

Figure. 6.311

Platelets prepared in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of protease inhibitors.

6.4) *The effect of platelet activation.*

A study was made to assess whether the activation state of the platelets influenced their protein profile. This assessment was necessary because the majority of platelets *in vivo* are normally in the resting state, but many are activated by the process of blood collection.

For this experiment, blood was drawn into three 10 ml Vacutainers containing lithium heparin as anticoagulant. The first Vacutainer contained anticoagulant only, and yielded a mixture of platelets in the resting and activated states. The second Vacutainer contained 0.01 mmol of theophylline and 0.015 mmol of adenosine. This yielded resting state platelets. The final Vacutainer contained 0.1 µmol of the calcium ionophore A23178. This yielded activated platelets. The protein yield for each preparation (per 1 g of whole blood) was 390 µg for the resting state platelet preparation, 326 µg for the mixed state platelet preparation, and 286 µg for the activated platelet preparation. Figure 6.320 indicates the site and mode of action of theophylline, adenosine, and ionophore A23178. The figure also summarises the intercellular events involved in the release reaction, resting state maintenance, and platelet activation.

The proteins for each preparation were separated by 1-dimensional SDS PAGE (Fig 6.41). No qualitative differences could be detected between the preparations. A quantitative study was also done, where the bands shown in Figure 6.42 were quantitated and converted into compositional values. Again, no differences could be detected (Fig 6.43).

Figure 6.320. ***The events which maintain the resting state in platelets, and those which lead to platelet activation.***

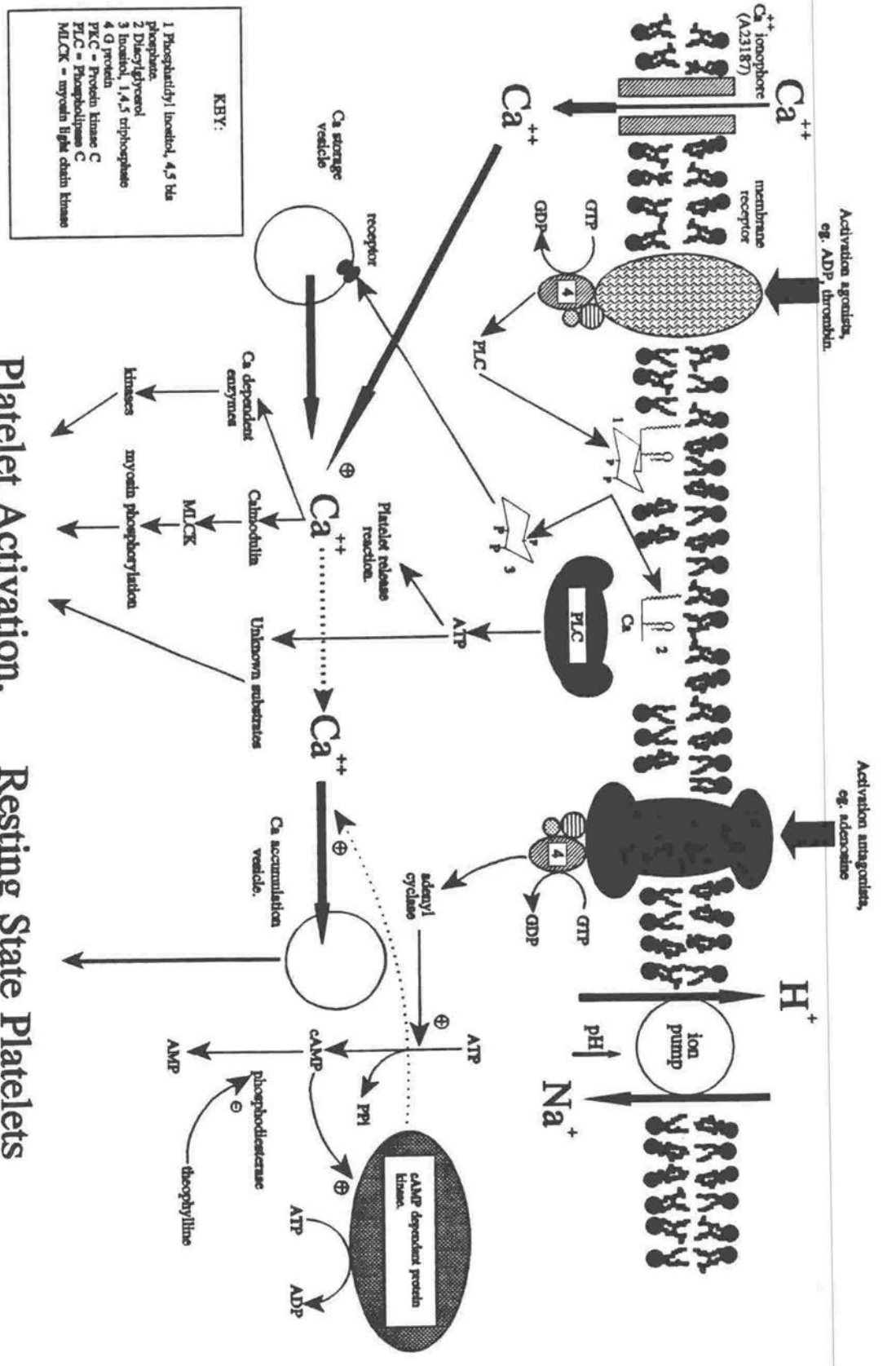


Figure 6.321. ***10% 1-dimensional SDS PAGE separation of human platelet proteins, showing the effect of adding the inhibitor cocktail at different times.***

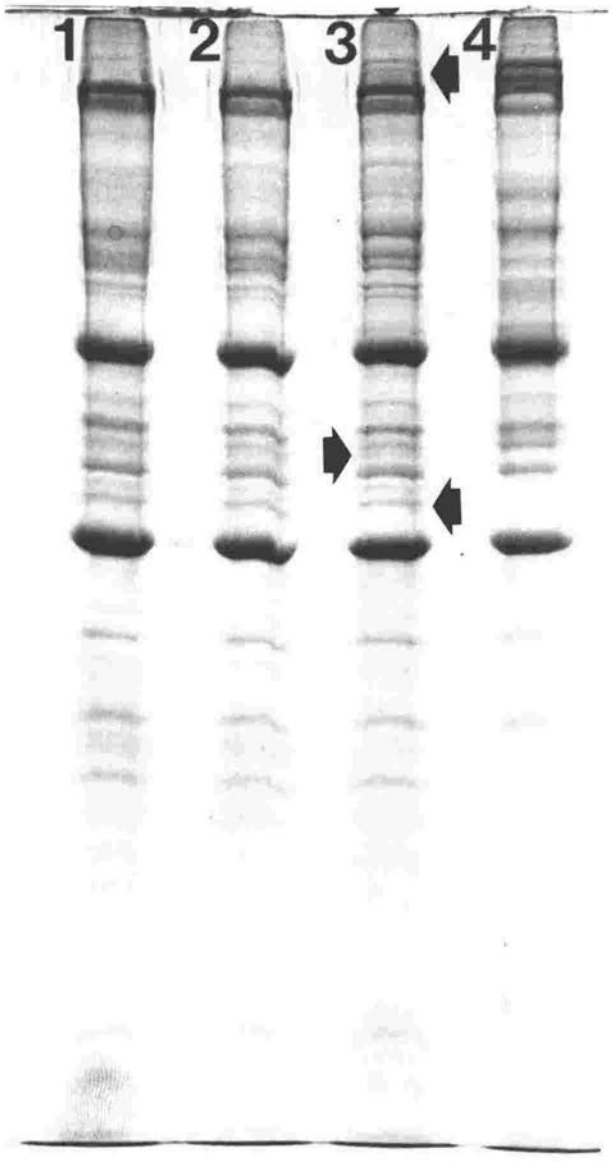


Figure 6.321. Lane 1: 75 µg platelet proteins from a blood sample prepared in the absence of protease inhibitors and left at room temperature for three hours prior to platelet isolation. Lane 2: 75 µg platelet proteins from a blood sample prepared in the absence of protease inhibitors from which platelets were isolated immediately after blood collection. Lane 3: 75 µg platelet proteins from a blood left at room temperature for three hours before having the protease inhibitor cocktail added to it and subsequent platelet isolation. Lane 4: 75 µg platelet proteins from a blood sample collected into a Vacutainer containing the inhibitor cocktail, and from which platelets were isolated immediately after blood collection. Arrows show proteins that varied between the samples.

Figure 6.41. ***10% 1-dimensional SDS PAGE separation of human platelet proteins from activated , resting state, and mixed state platelet preparations.***

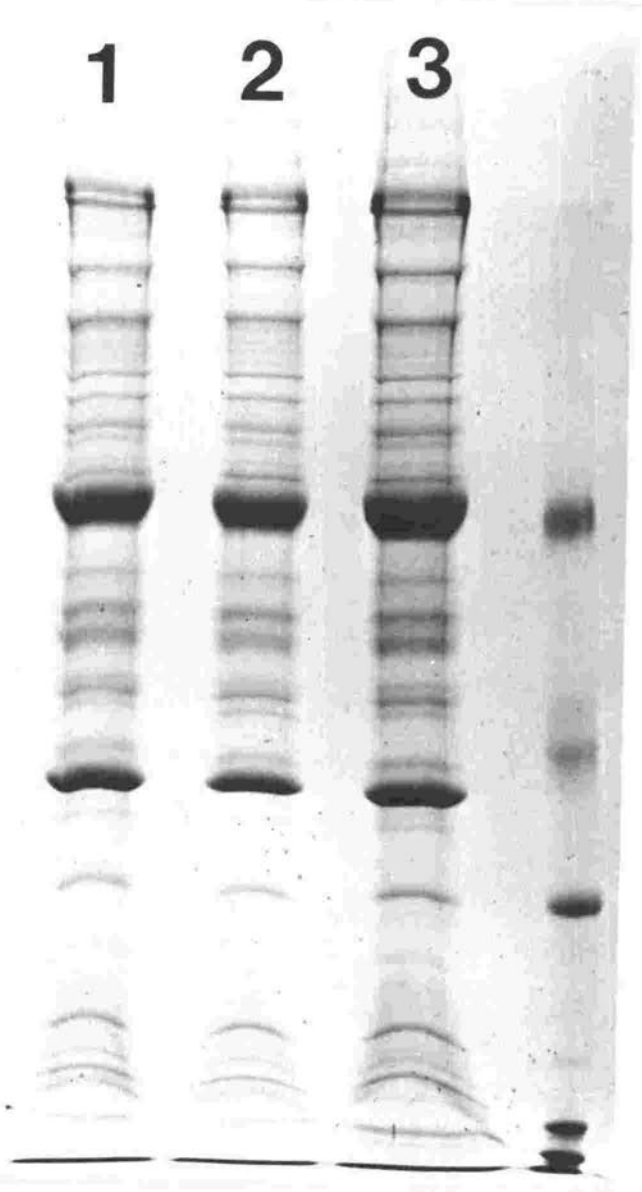


Figure 6.41. Lane 1: 75 µg human platelet proteins from mixed state preparation. Lane 2: 75 µg human platelet proteins from an activated platelet preparation. Lane 3: 75 µg human platelet proteins from resting state platelet preparation.

Figure 6.42. ***The protein bands quantitated to produce Figure 6.43.***

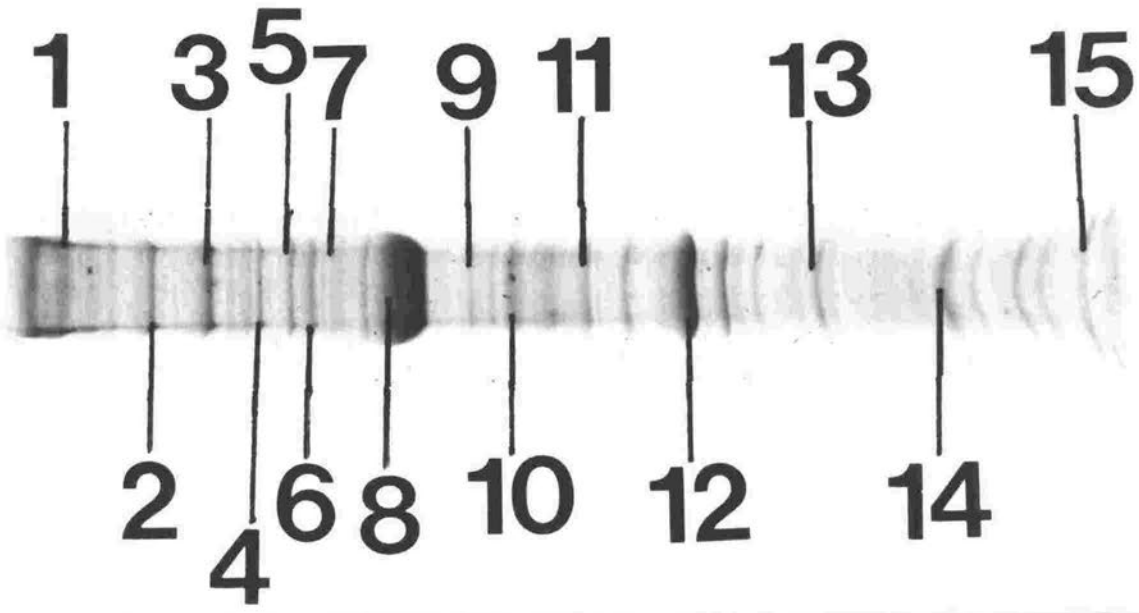
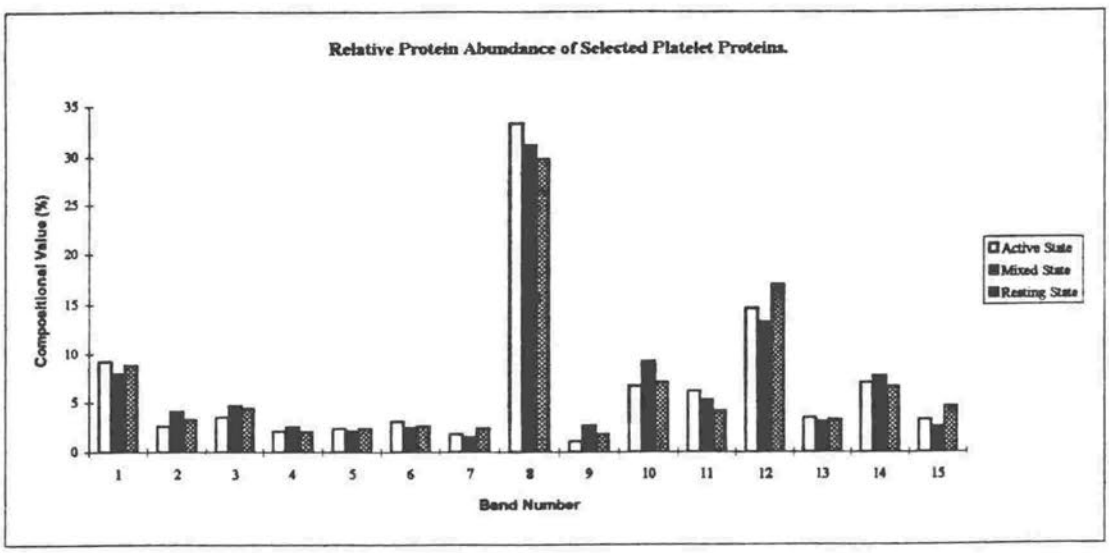


Figure 6.43. ***The effect of the platelet activation state on relative protein abundance.***



The abundance of each of the protein bands was measured by quantitative densitometry and the amounts of each band were expressed as compositional values which reflect the abundance of each band as a percentage of all bands measured in that lane.

To confirm the state of platelets in each type of preparation, unsonicated platelet pellets from resting, activated, and mixed state platelet preparations were obtained. Each pellet was gently resuspended in 2 ml buffered saline that contained 140 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 0.8 mM NaH_2PO_4, 5.5 mM glucose, and 25 mM HEPES. For the resting state preparation, the buffered saline also contained 1 mM theophylline, and 1.5 mM adenosine. The activated platelet preparation contained 10 μM A23187 in place of the theophylline and adenosine. The suspensions were incubated at room temperature for fifteen minutes and then fixed by the addition of 4 ml 1.5% (w/v) glutaraldehyde, 0.05 M sodium cacodylate. After dehydration, and staining with saturated uranyl acetate and 2% lead citrate, the cells were examined using transmission electron microscopy. Predominantly resting state platelets were observed in the resting state preparation, both active and resting state platelets were observed in the mixed state preparation, and mainly activated platelets were observed in the activated platelet preparation. Figures 6.44 and 6.45 show activated and resting platelets.

Based on the observations made in this section I decided to add theophylline and adenosine to maintain platelets in the resting state. Although the platelet activation state did not appear to influence the protein profile, a greater yield of platelets was obtained when the resting state promoters were present. This was probably due to a smaller proportion of resting state platelets sedimenting with the leukocytes.

6.5) The effect of anticoagulants on the platelet protein profile.

A study was made to assess if the anticoagulant commonly used in Vacutainers (sodium heparin) affected the platelet protein profile for inhibited and uninhibited platelet protein preparations. To make this study, three anticoagulants were compared. The first was sodium heparin that was present in the Vacutainers when purchased. The second was ACD which consisted of 93 mM monosodium citrate, 7 mM citric acid, and 140 mM dextrose, pH 6.5. To each washed 10 ml blood collection tube, 1 ml of fresh ACD was added to prevent blood coagulation.

Figure 6.44. ***Transmission electron micrograph of an activated platelet (X38, 000).***

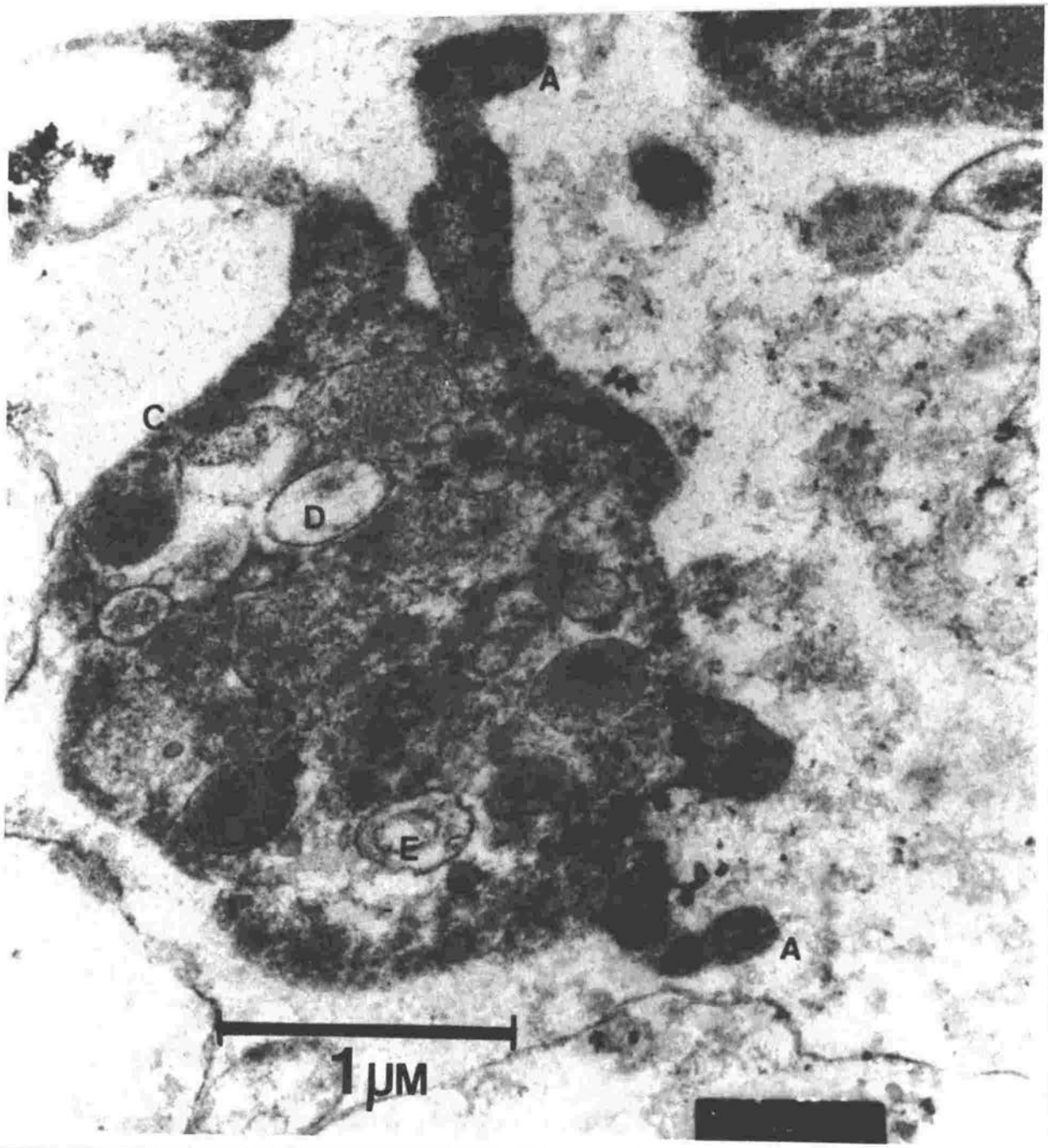
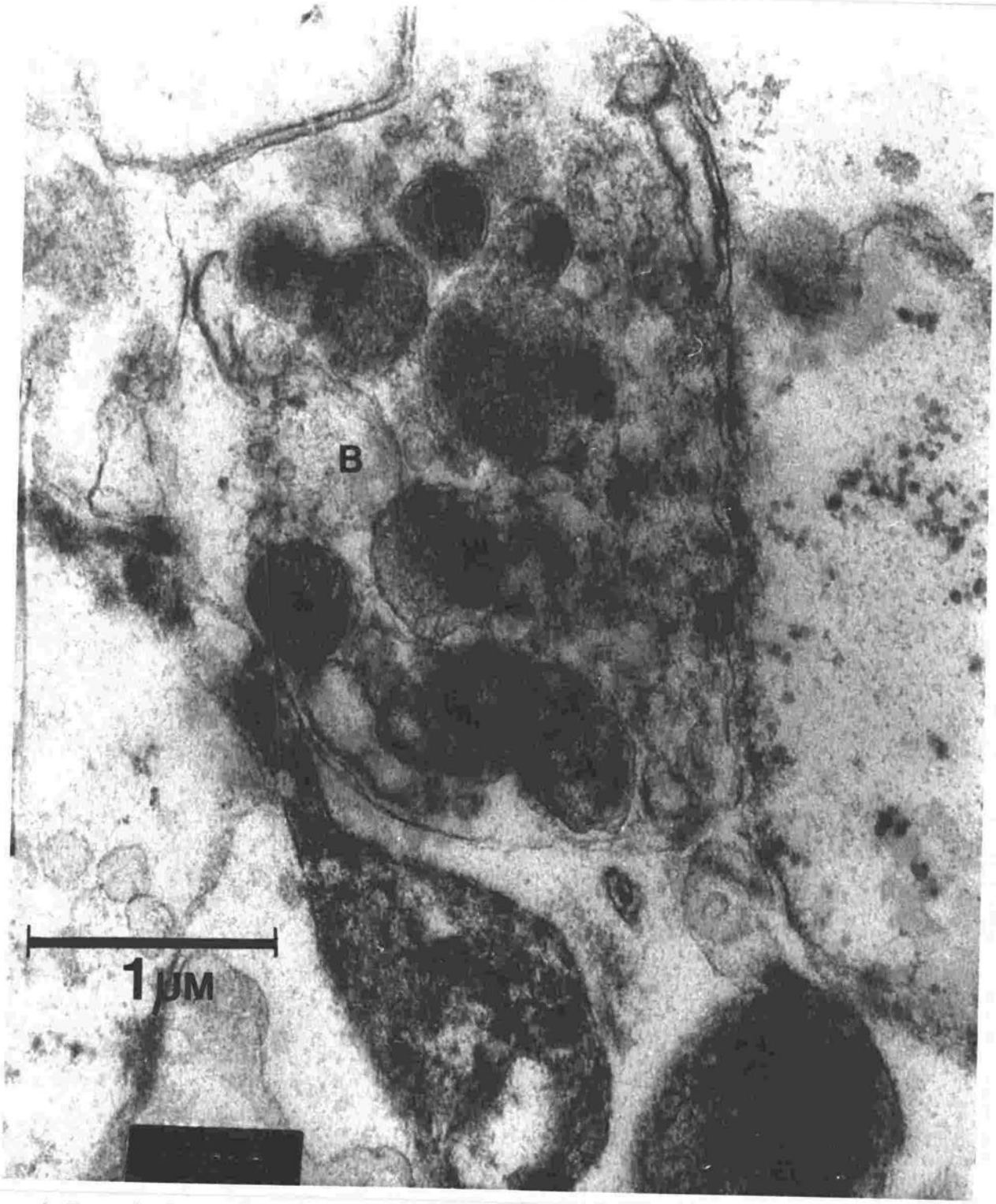


Figure 6.44
A. Pseudopod. B. Dense body. C. Microtubules. D. Granule. E. Canalicular opening.

Figure 6.45. ***Transmission electron micrograph of a resting state platelet (X38, 500).***



A. Dense body. B. Granule.

The third anticoagulant tested was buffered citrate, which consisted of 84 mM trisodium citrate, and 21 mM citric acid. One ml of buffered citrate was used in each washed 10 ml collection tube.

The three anticoagulants act by different mechanisms. ACD chelates divalent cations such as Ca^{++}, which is required for the coagulation cascade. Buffered citrate also chelates Ca^{++}, but additionally interacts with Factor VIII, the antihaemophilic factor (Rock *et al.*, 1988). Heparin acts mainly by enhancing the activity of antithrombin III, which is a potent serine protease inhibitor. There were two reasons why this study was necessary. First, the effect of protease inhibitors on the platelet protein profile was to be investigated. As a control for this experiment, preparations were required that had no protease inhibitors present. Given the mode of action of heparin, it was necessary to find out if any of the proteases present in the samples would be inhibited by the process of collecting blood in heparinised tubes. Second, as ACD and buffered citrate act via chelating Ca^{++}, they will also prevent the platelet release reaction (where platelets release their hydrolytic enzymes to their environment). The significance of this is that the release reaction can occur independently of platelet activation. Therefore, it is possible that when heparin is used as anticoagulant, the release reaction may occur inconsistently, and may influence the platelet protein profile.

For this investigation, six 10 ml blood samples were collected from several healthy individuals. Blood samples were drawn into tubes containing each anticoagulant in the presence or absence of protease inhibitors. The platelet proteins were isolated as previously described, and separated by 1-dimensional SDS PAGE. Figure 6.51 shows one such separation. No qualitative differences could be detected among the three anticoagulants for samples collected in the presence or absence of protease inhibitors. A quantitative study was also done on the uninhibited samples, where the bands shown in Figure 6.52 were quantitated and expressed as compositional values. Again, no differences could be detected among the three anticoagulants (Figure 6.53). However, the platelet yield for the buffered citrate preparations was consistently higher than for the two other anticoagulants. Consequently, buffered citrate was chosen as the anticoagulant for this study.

Figure 6.51. ***1-dimensional SDS PAGE separations of human platelet proteins, harvested from blood collected in the presence of different anticoagulants.***

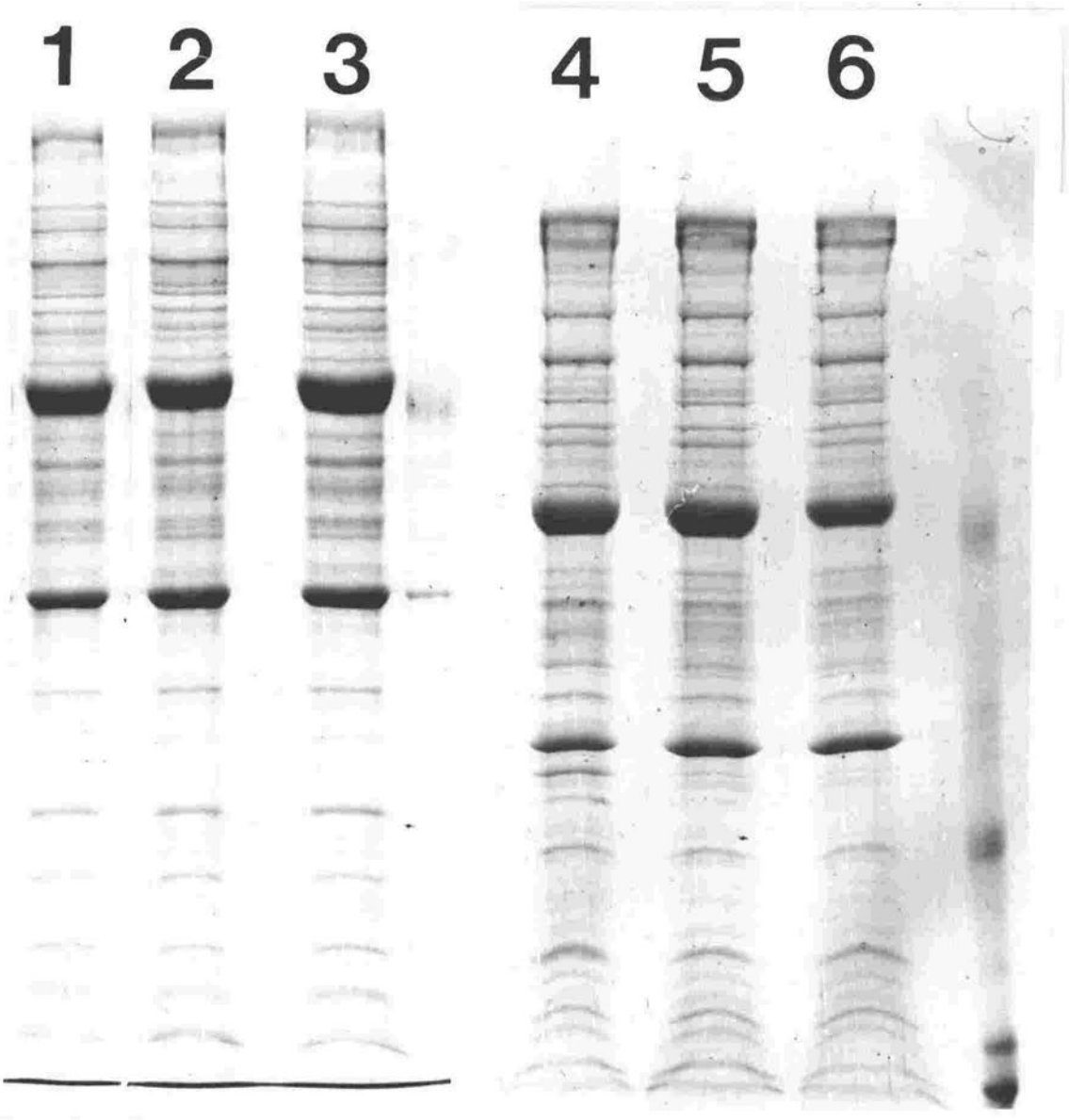


Fig.6.51.Platelets prepared in the presence (lanes 1-3) or absence (lanes 4-6) of protease inhibitors. Lanes 1,4: Sodium heparin anticoagulant. Lanes 2,5: ACD anticoagulant. Lanes 3,6: Buffered citrate anticoagulant. Lanes 1-3 were separated on a 10% gel, and lanes 4-6 were separated on a 12% gel.

Figure 6.52. The protein bands quantitated to produce Figure 6.53.

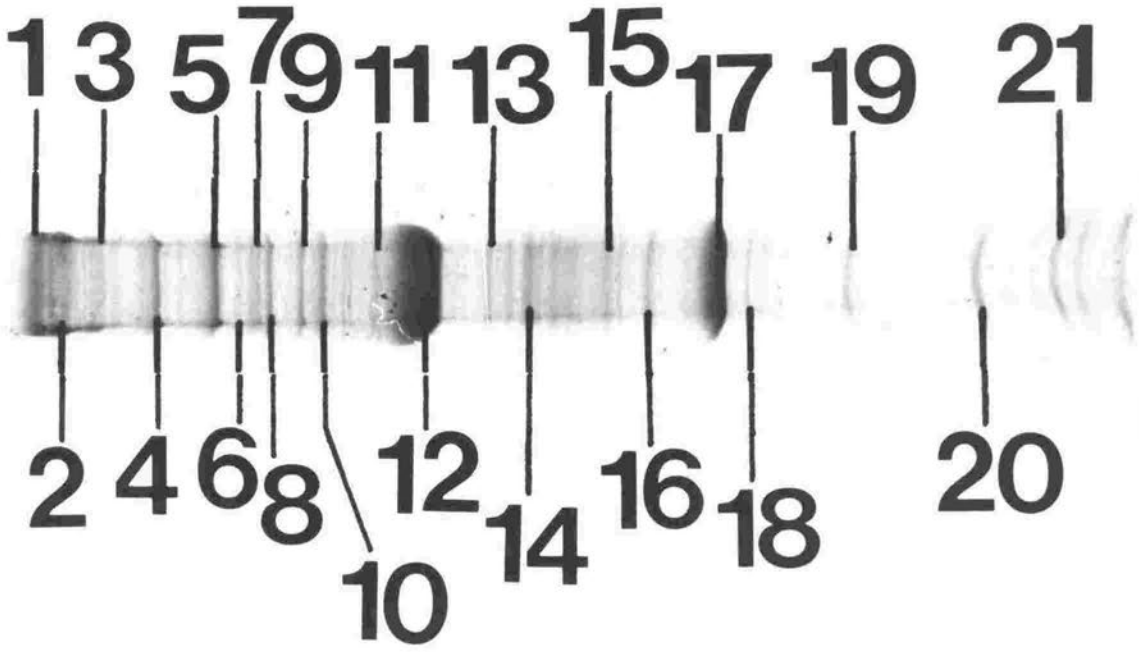
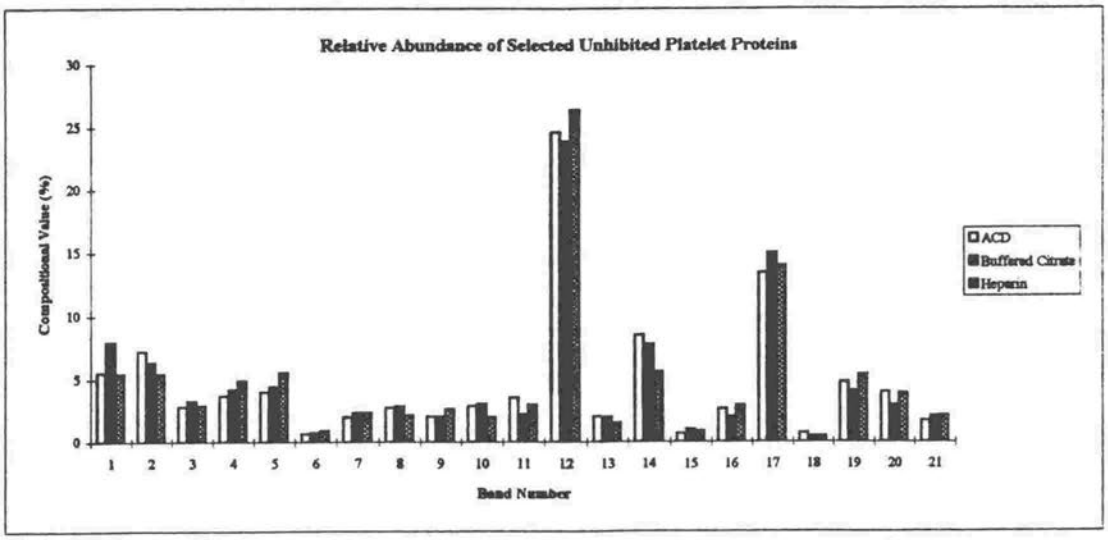


Figure 6.53. The effect of anticoagulants on relative protein abundance.



The abundance of each of the protein bands was measured by quantitative densitometry and the amounts of each band were expressed as compositional values which reflect the abundance of each band as a percentage of all bands measured in that lane.

6.6) Reproducibility of the platelet protein profile over time.

An assessment was made regarding the consistency of the protein profiles obtained from platelets isolated from the same individual at different times.

For this study, four individuals supplied two 10 ml blood samples (one taken in the presence of protease inhibitors, one taken in the absence of protease inhibitors, both with buffered citrate as anticoagulant) on five separate occasions (with a minimum of a six week interval between blood collection times). Platelet proteins were isolated and separated using 1-dimensional SDS PAGE. Figures 6.61 to 6.63 show one analysis of platelet proteins prepared in the presence or absence of protease inhibitors. Few qualitative differences could be detected between the samples collected in either the absence or the presence of protease inhibitors for the same person over time. A quantitative study was also done on the protein samples collected in the presence of protease inhibitors. The bands shown in Figure 6.62 were quantitated and expressed as compositional data. It appeared that platelet proteins were expressed consistently over time, there being no large differences in VI values for any of the protein bands studied over time. This level of consistency was observed for all of the individuals studied.

6.7) Consistency of the platelet protein profile between unrelated individuals.

A study was performed to assess the consistency of the platelet protein profile between different individuals. Knowledge of the level of consistency between unrelated individuals was necessary before an assessment could be made of the differences between the protein profiles of patients and controls. Blood was drawn from each of five healthy individuals into a 10 ml Vacutainer that had buffered citrate as anticoagulant. The blood samples were collected and platelets were isolated from each of the individuals at the same time. The platelet proteins were

Figure 6.61. ***10% 1-dimensional SDS PAGE separation of human platelet proteins taken from the same individual at different times.***

Fig 6.61. Each lane contained approximately 75µg of platelet proteins isolated from blood samples taken from the same individual. Lanes 1 and 2 were samples prepared in the absence of protease inhibitors, and lanes 3 and 4 were samples prepared in the presence of protease inhibitors. The platelet proteins shown in lanes 1 and 3 were isolated from a blood sample taken in September, 1992. The platelet proteins shown in lanes 2 and 4 were isolated from a blood sample taken in February, 1993.

Figure 6.62. The protein bands quantitated to produce Figure 6.63.

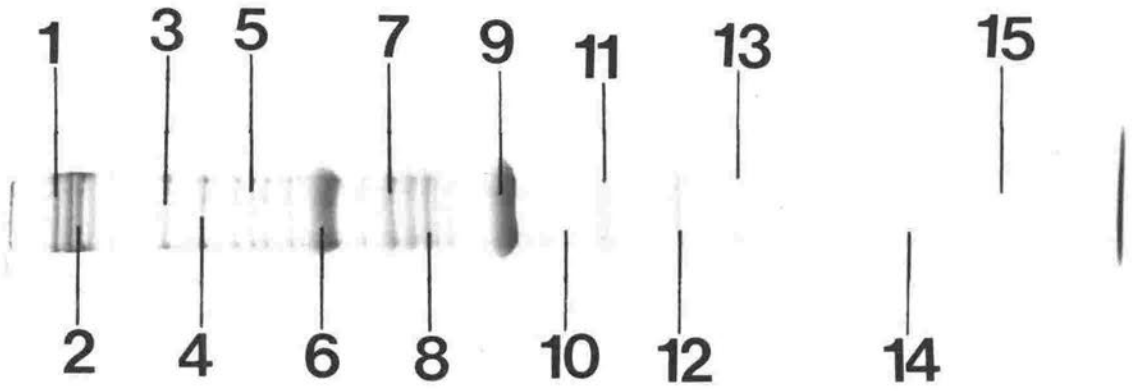
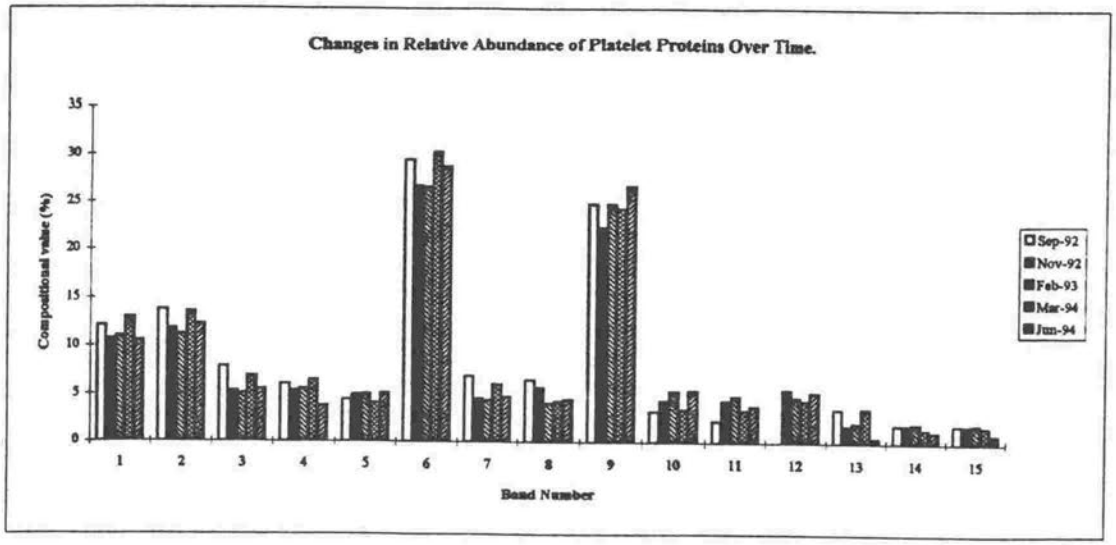


Figure 6.63. Relative protein abundance for the same individual over time.



The abundance of each of the protein bands was measured by quantitative densitometry and the amounts of each band were expressed as compositional values which reflect the abundance of each band as a percentage of all bands measured in that lane.

separated using 1-dimensional SDS PAGE (Figure 6.71). Both the protein profiles and the quantitative data were similar for all individuals. A few differences were seen however. Bands 2 and 9 were fainter for individual 3. This is shown in Figure 6.71 and is also clearly shown in Figure 6.73. These differences may represent analytical differences (differences that arise as a result in slight differences in sample preparation, sample loading, etc.), or may simply represent natural variation.

Based on the qualitative and quantitative observations made in this section, it appeared that the complement of platelet proteins was consistent between unrelated individuals. It also appeared that the relative abundance of various proteins was similar between individuals.

Figure 6.71. ***10% 1-dimensional SDS PAGE separation of human platelet proteins (prepared in the absence of protease inhibitors) from five unrelated individuals.***

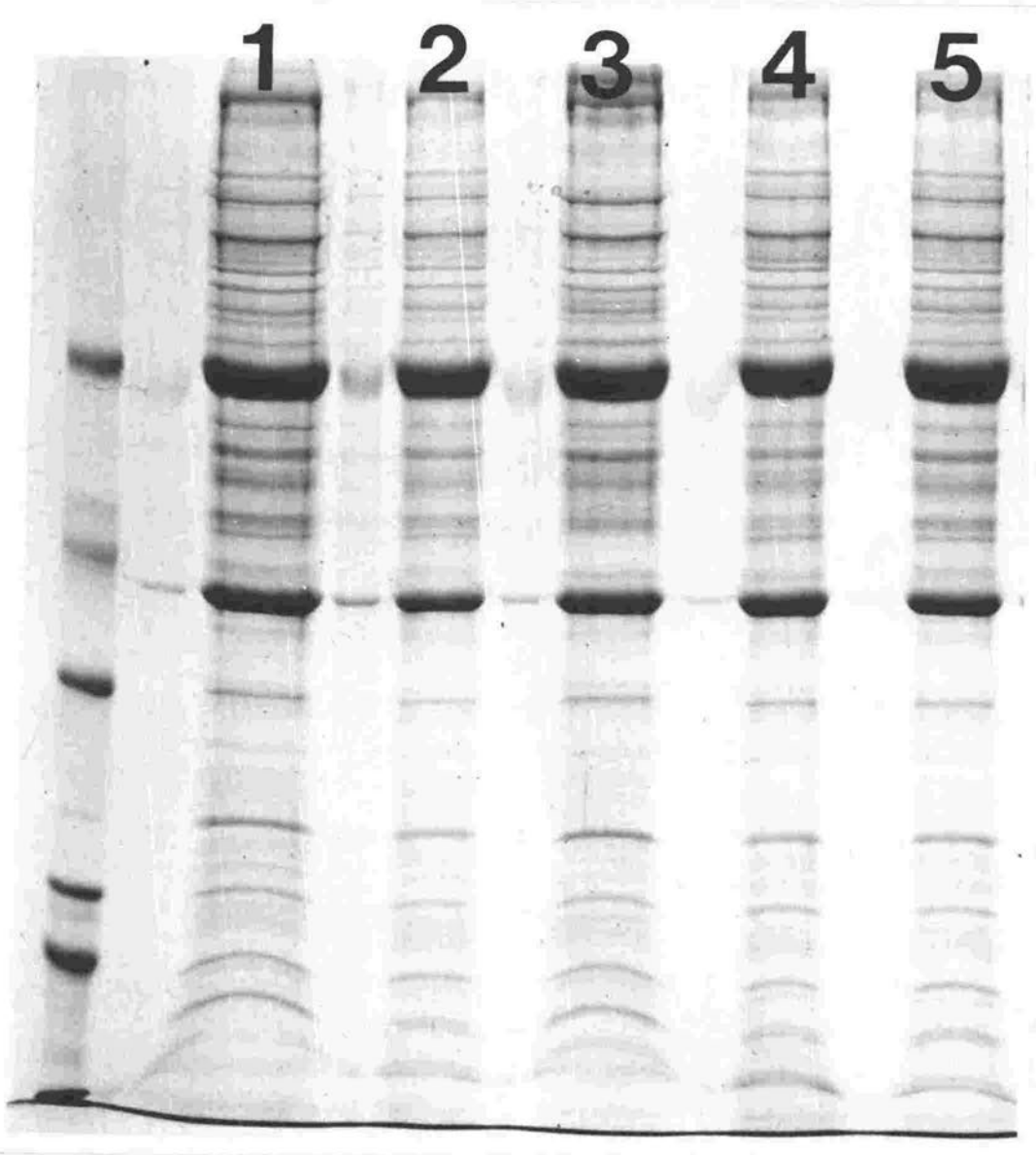


Fig6.71. Each lane was loaded with approximately 75μg of platelet proteins from a different individual.

Figure 6.72. The protein bands quantitated to produce Figure 6.73.

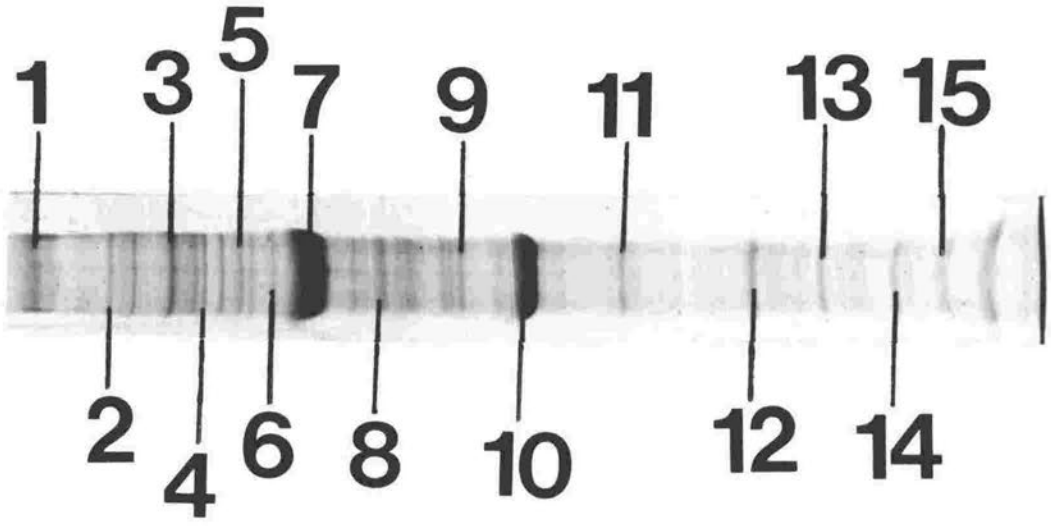
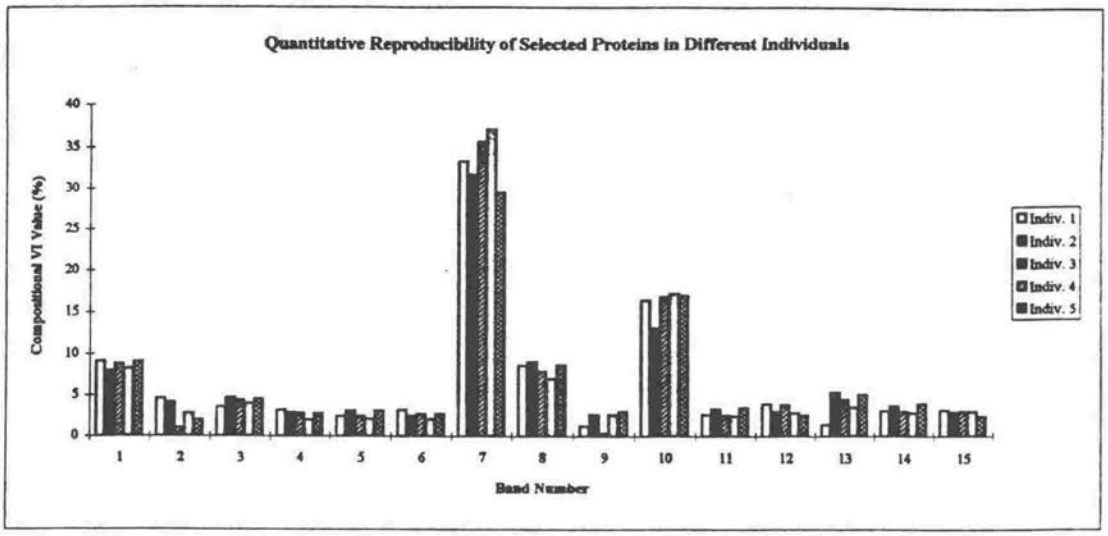


Figure 6.73. Relative protein abundance for five unrelated individuals.



The abundance of each of the protein bands was measured by quantitative densitometry and the amounts of each band were expressed as compositional values which reflect the abundance of each band as a percentage of all bands measured in that lane.

6.8) *Analysis of platelet proteins from individuals with Alzheimer's disease and their matched controls.*

6.81) Introduction.

Two-dimensional PAGE was used to separate ^{14}C-labelled platelet proteins of ten individuals affected with Alzheimer's disease and their matched controls. After fluorography, fluorograms of the gels were scanned and quantitative data were obtained for one hundred proteins. The quantitative data were analysed statistically to determine whether any significant differences could be detected between patients and controls.

6.82) Materials and Methods.

Two 10 ml blood samples were drawn from each individual. Blood was drawn into two 10 ml Vacutainers containing buffered citrate as anticoagulant. One Vacutainer for each individual contained the protease inhibitor cocktail described in section 6.31. Platelets were isolated by differential centrifugation. Approximately 30 μg of platelet proteins from each individual were radioactively labelled by reductive methylation, as described previously.

Initially a study was made to see whether any consistent qualitative differences could be detected between control and affected individuals. To do this, four pooled platelet protein samples (pooled affected individuals with and without protease inhibitors, and pooled controls with and without protease inhibitors) were separated by 2-dimensional SDS PAGE (Figures 6.831 to 6.834). The gels were processed for fluorography and dried as described previously. Fluorograms had an average of twelve days film exposure. A visual search was then made for differences in abundance

of individual proteins among the four pooled samples.

A quantitative study of variation among one hundred platelet proteins was then carried out. In this study protein abundances were measured in forty fluorograms representing the platelet proteins from ten individuals with Alzheimer's disease and their matched controls, prepared in the absence or presence of protease inhibitors. The proteins were selected to include spots that appeared to vary in the pools and to cover a wide mw range, a wide range of relative abundances, and a wide range of pI values. Quantitation was performed as described previously. Figure 6.835 shows the master map that gives the location of the proteins. The map was made from a fluorogram of a pooled sample of Alzheimer's disease patients. The figure also shows the position of each region of the gel in Figures 6.836 to 6.840. Figures 6.836 to 6.840 are laser printed images of regions of the fluorogram made after scanning. They are at a magnification of three times. In the figures the outline of certain proteins has been highlighted to aid in determining their boundaries. This was necessary because to show some low abundance proteins, the sensitivity of the image had to be increased to the point where some protein boundaries merged into the background. In Figure 8.637, the location of protein number 69 was added to the image. This was done as that protein did not show clearly on the scanned image at a sensitivity low enough to keep the images of the other proteins from blurring with the background. Table 6.831 gives the identity of those proteins located in the Swiss-2D PAGE platelet database, located on the World Wide Web ExPASy server at http://expasy.hcuge.ch (Appel *et al.*, 1993).

For the statistical analysis, VI values were converted to compositional data, and both a *t*-test and a F-test were performed on the data. These procedures are discussed in previous sections.

6.83) Results.

6.831) Qualitative analysis.

Figures 6.831 to 6.834 show fluorograms of pooled platelet proteins from the Alzheimer's disease patient group collected in the presence (P AD+) or absence (P AD-) of protease inhibitors, and pooled platelet proteins from the control group also collected in the presence (P C+) or absence (P C-) of protease inhibitors. By comparing platelets prepared in the presence of protease inhibitors for controls and Alzheimer's patients, an assessment can be made of changes in protein expression/abundance in Alzheimer's disease. An assessment of the effects of proteolysis in disease can be made by comparing platelet proteins prepared in the absence of protease inhibitors from Alzheimer's patients and controls, and also by comparing platelets prepared in the absence of protease inhibitors to those prepared in the presence of protease inhibitors for both controls and patients. Proteins that were seen to be proteolytically processed differently, but which were not observed to alter in abundance between the platelets of patients and controls prepared in the presence of protease inhibitors were considered more likely to represent disease related proteolytic events, as altered rates of expression could be ruled out. The major assumption made here is that proteolysis was inhibited by the protease inhibitor cocktail used.

Differences were detected between the platelet proteins collected in the presence of protease inhibitors and those collected in the absence of protease inhibitors for both the control and patient pools. These differences varied when the patient pools were compared to the control pools. The differences observed in the pooled samples were then examined by visual inspection of the 2-dimensional PAGE separations of platelet proteins from the twenty control and affected individuals. Again, no consistent protein differences could be detected, both within the control and patient groups, and also when the two groups were compared. The differences seen may represent analytical differences; that is, differences resulting from sample preparation and the experimental procedure. The differences may also represent natural variation. Figure 6.841 illustrates some of these differences, and shows how an individual protein (solid arrow) and a group of proteins

(unfilled arrow) differed between individual patients and controls. Proteins such as these were not quantitated as they only appeared in subsets of both patient and control groups. Also, as some of the proteins were of high abundance, including them in the compositional analysis for a particular gel in which they were present would have reduced the relative abundance values of the low abundance proteins also included in that analysis. This would have increased the variance associated with the low abundance proteins, and would have made any statistics in which they were included, less rigorous.

Figure 6.831. Fluorogram of a 10 % 2-dimensional SDS PAGE separation of human platelet proteins (collected in the presence of protease inhibitors) from a pooled sample of ten Alzheimer's disease patients.

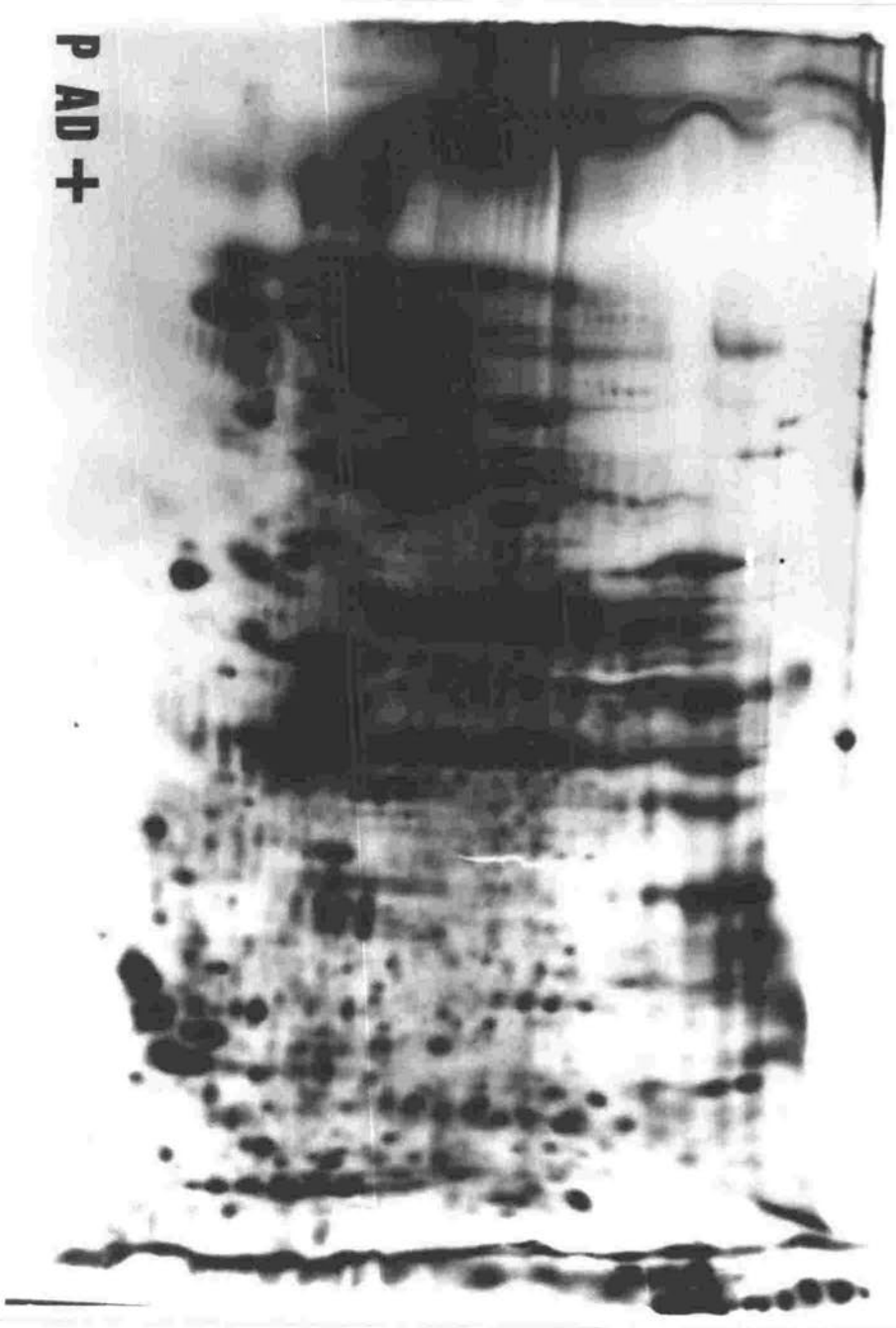


Figure 6.832. Fluorogram of a 10 % 2-dimensional SDS PAGE separation of human platelet proteins (collected in the absence of protease inhibitors) from a pooled sample of ten Alzheimer's disease patients.

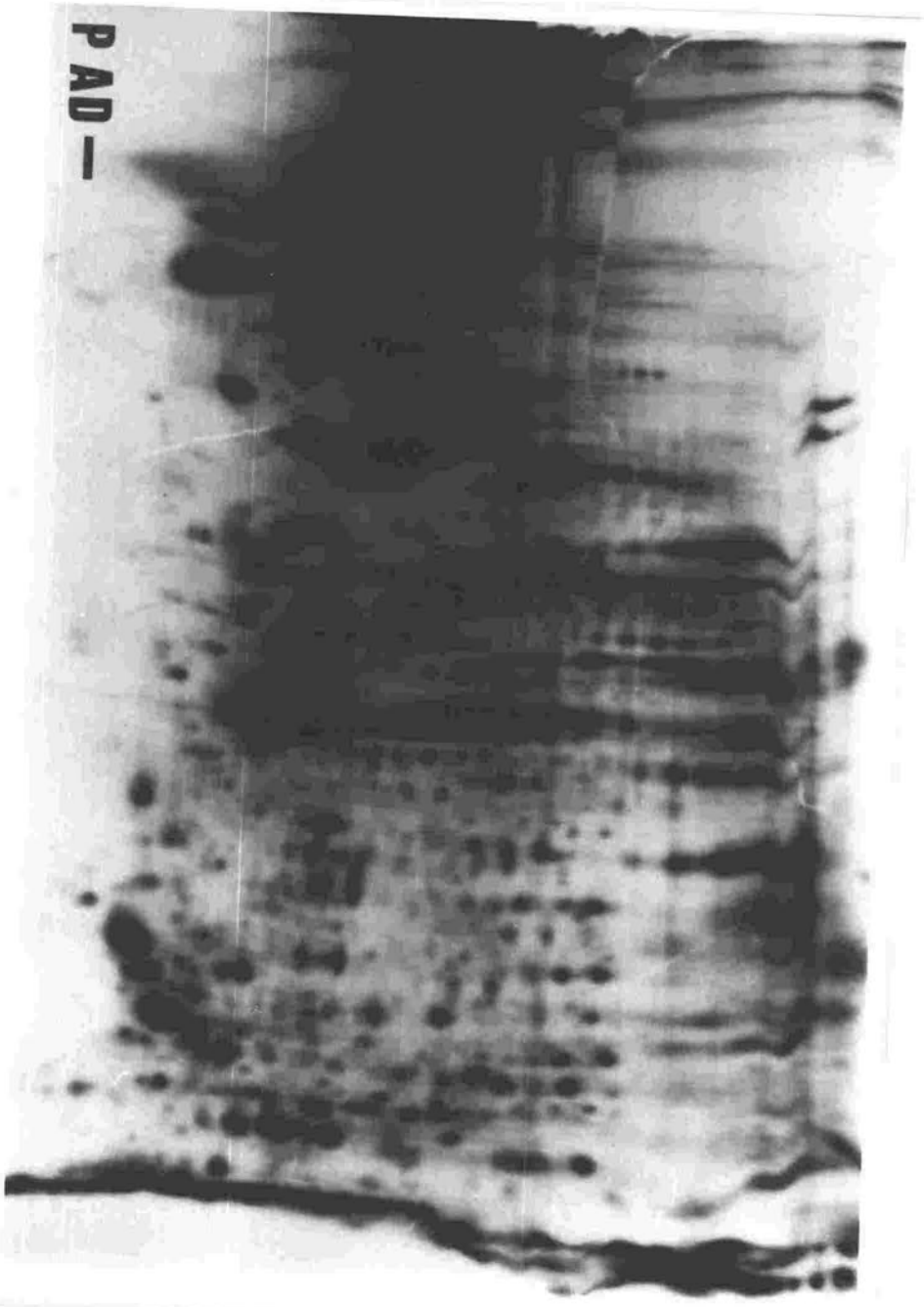


Figure 6.833. Fluorogram of a 10 % 2-dimensional SDS PAGE separation of human platelet proteins (collected in the presence of protease inhibitors) from a pooled sample of ten controls.

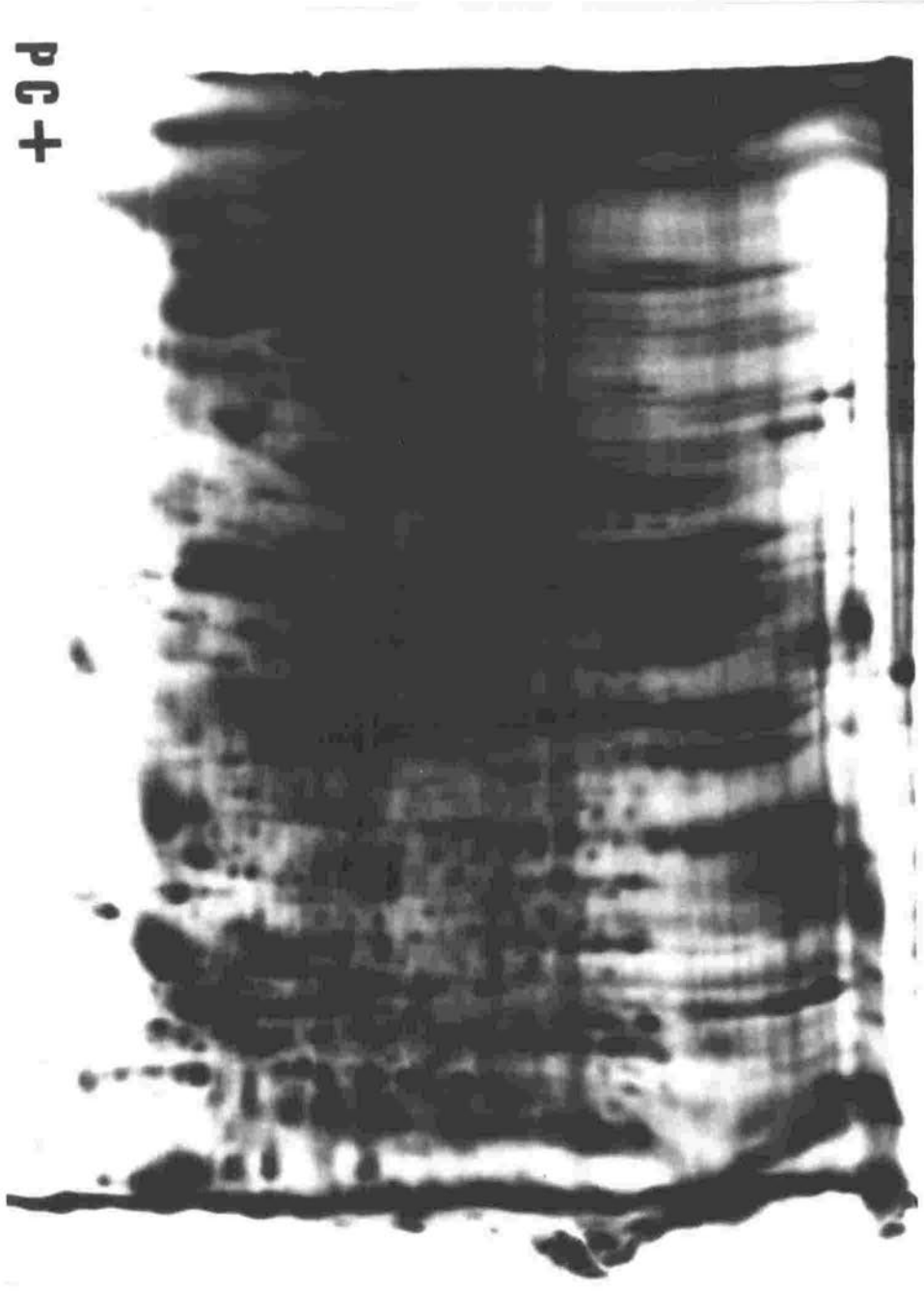
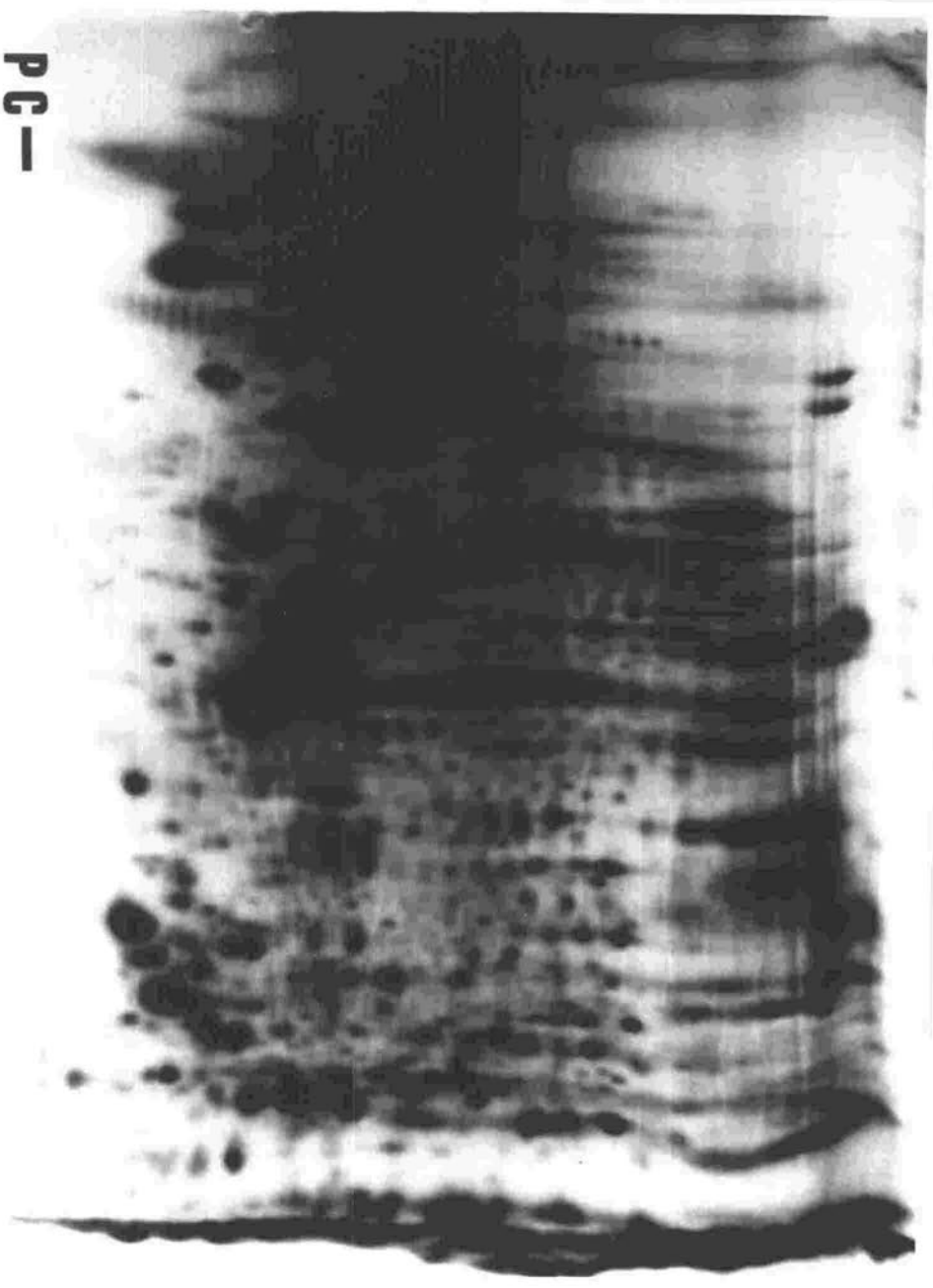


Figure 6.834. ***Fluorogram of a 10 % 2-dimensional SDS PAGE separation of human platelet proteins (collected in the absence of protease inhibitors) from a pooled sample of ten controls.***



6.832) Quantitative analysis.

Figures 6.835 to 6.840 show the gel location of the platelet proteins that were selected for quantitation. Table 8.631 lists the proteins that could be identified.

Figure 6.835. ***Fluorogram of human platelet proteins separated by 2-dimensional PAGE on a 10% gel, showing the location of the five map regions contained in Figures 6.836 to 6.840.***

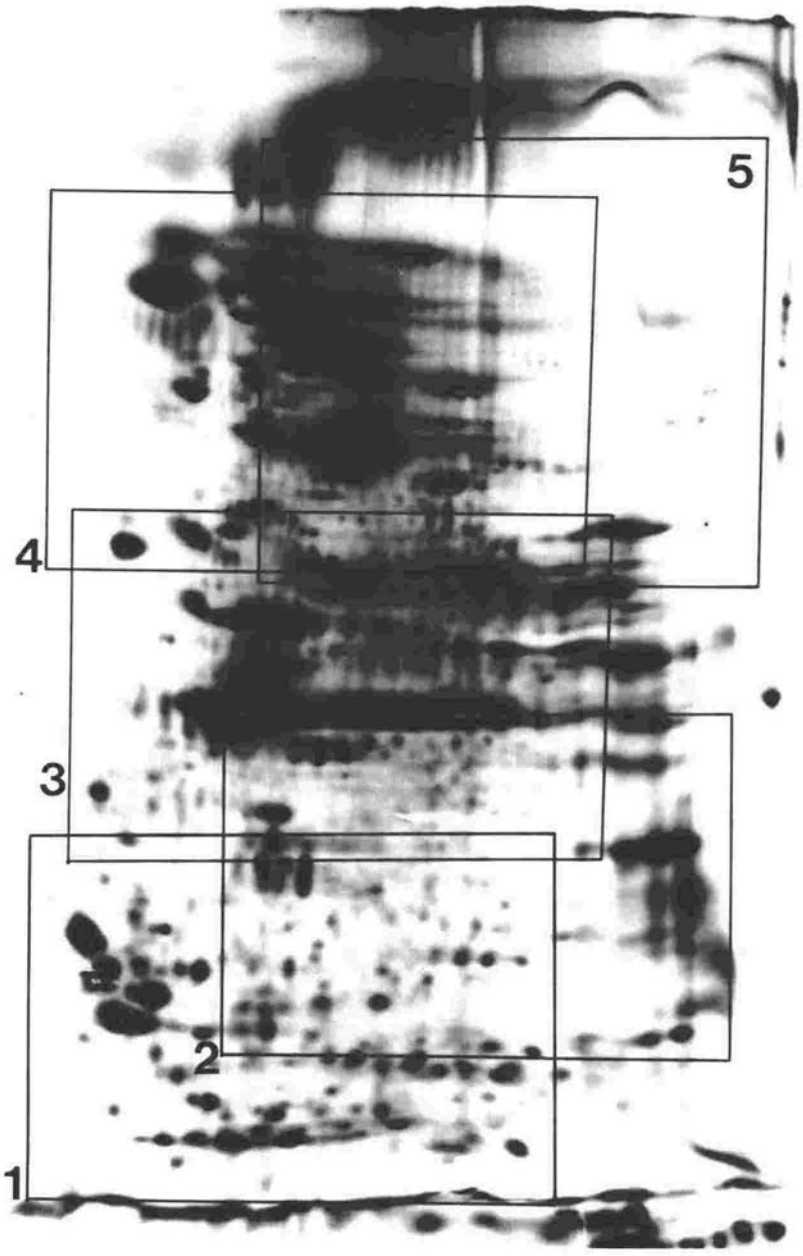


Figure 6.836. Platelet map region number 1. Low mw acid/neutral proteins

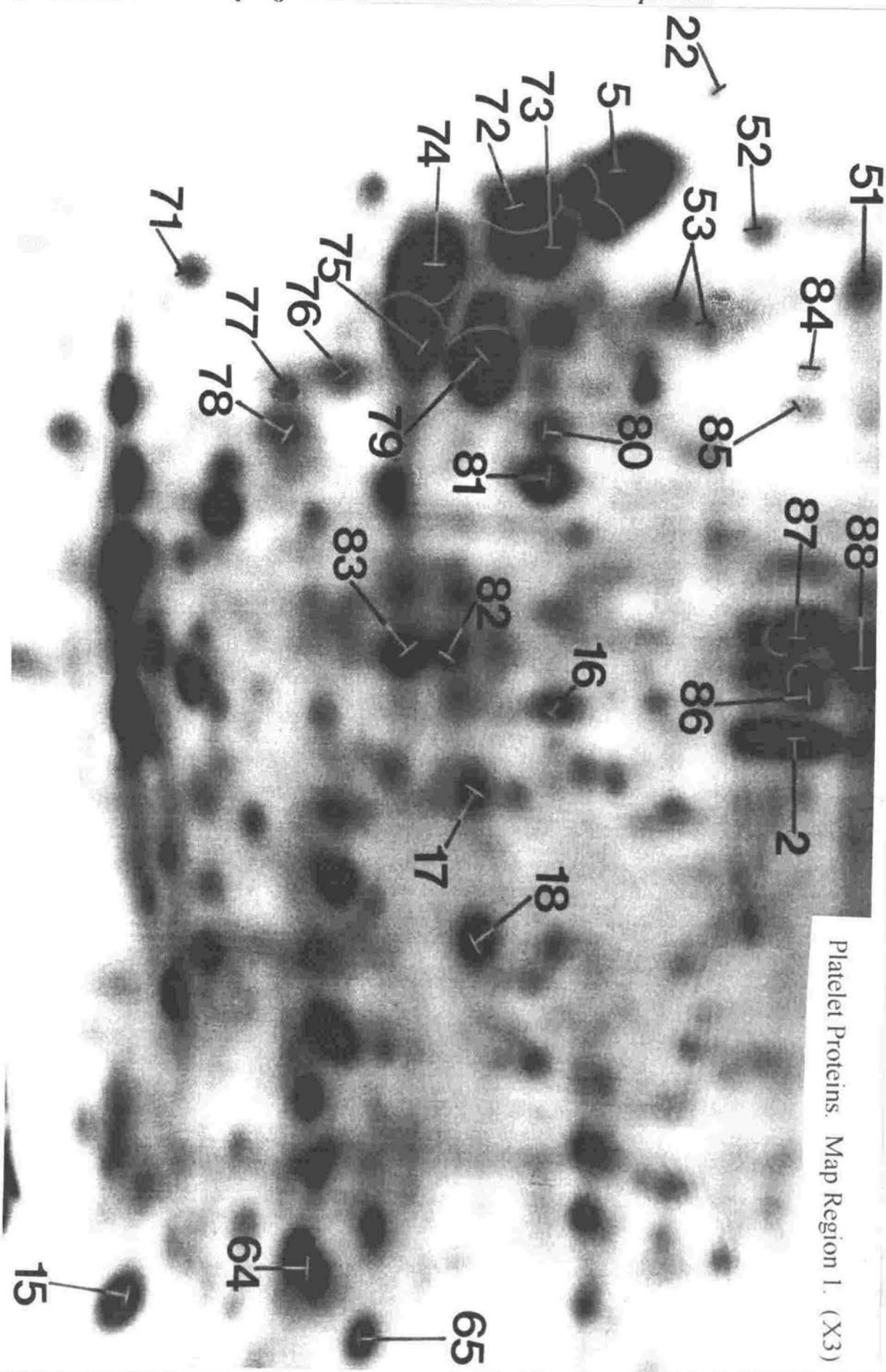
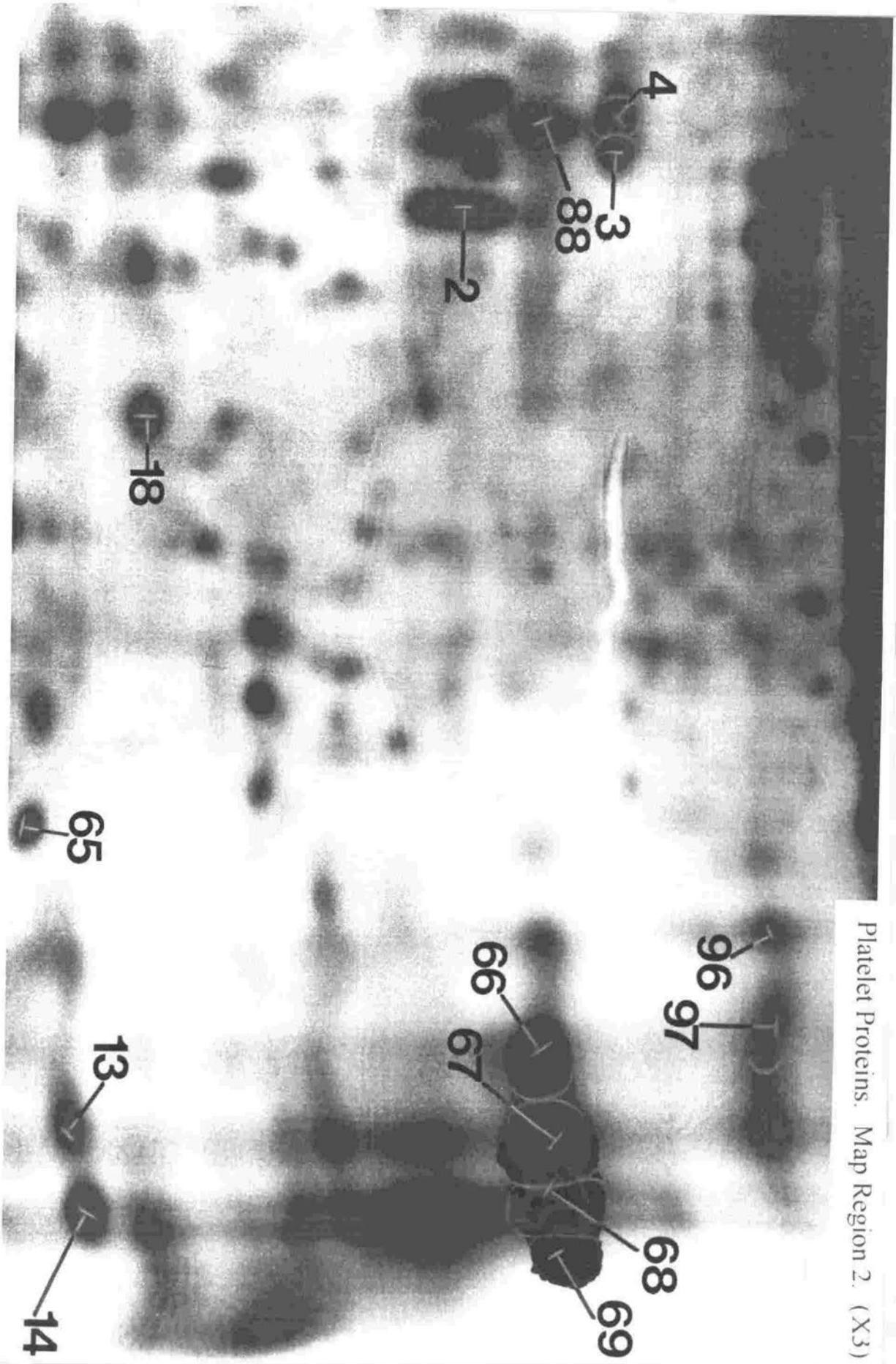


Figure 6.837. ***Platelet map region number 2. Low mw neutral/basic proteins.***



Platelet Proteins. Map Region 2. (X3)

Figure 6.838. Platelet map region number 3. Intermediate mw acidic/basic proteins.

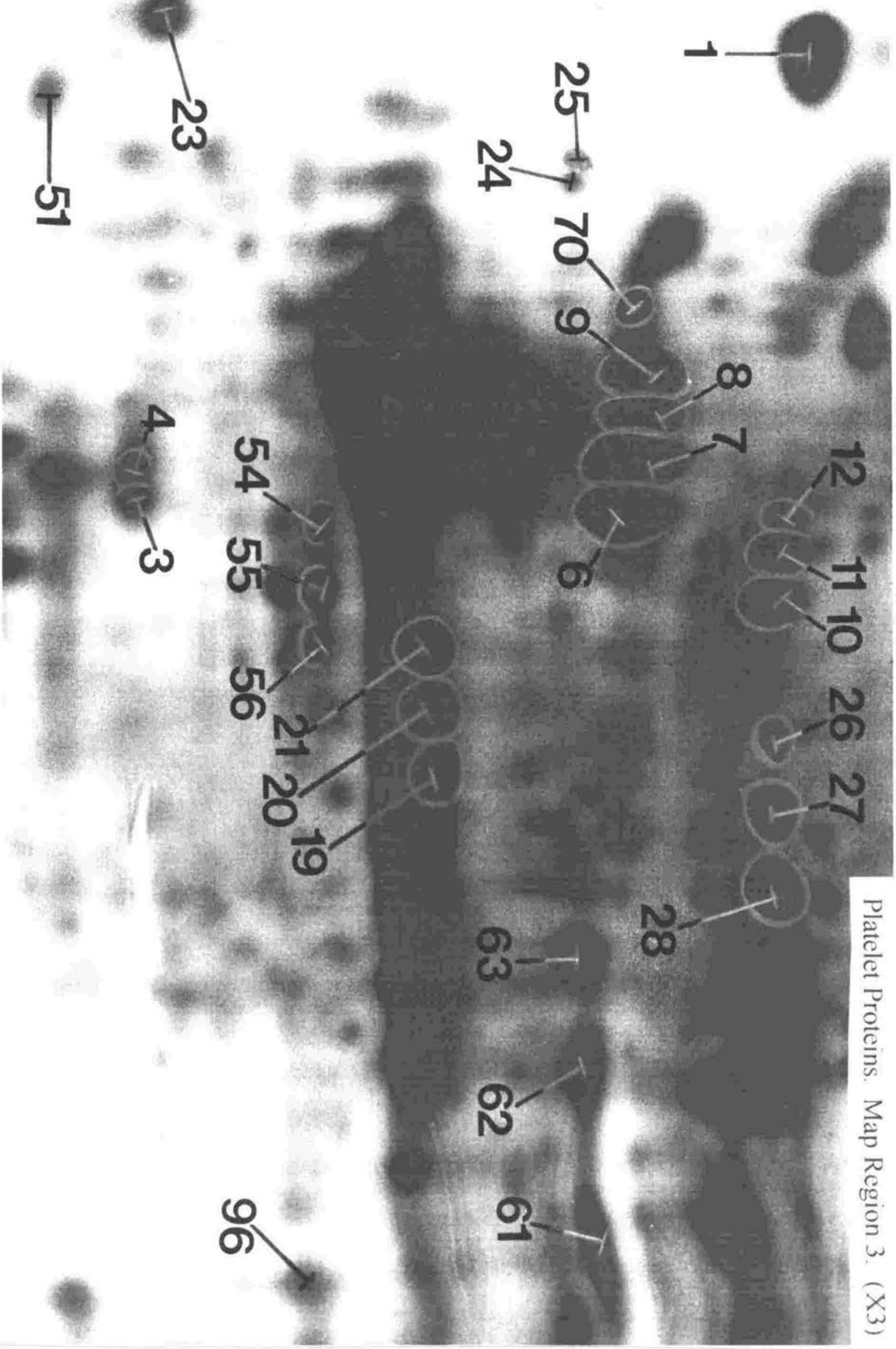
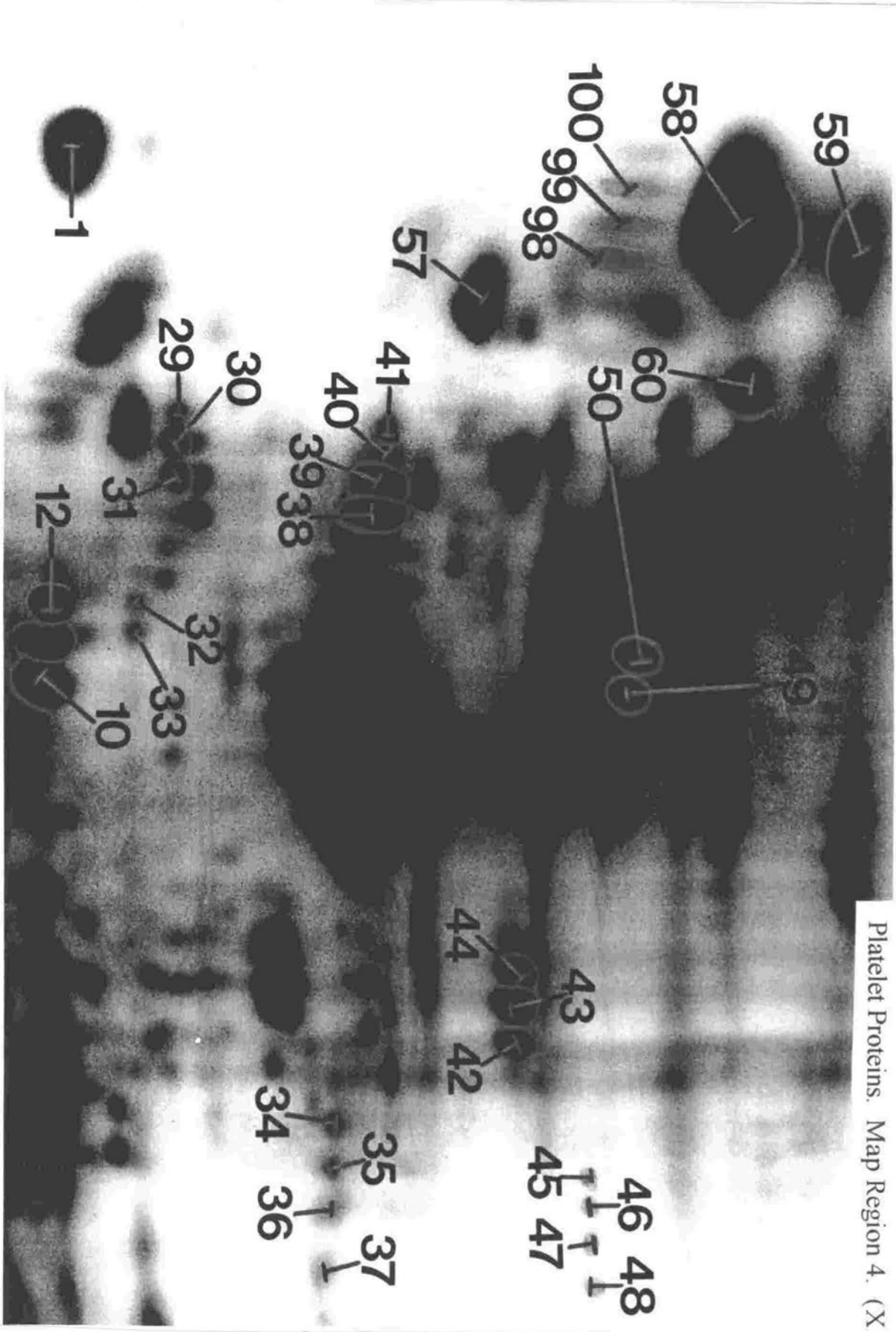


Figure 6.89. ***Platelet map region number 4. High mw acidic/neutral proteins.***



Platelet Proteins. Map Region 4. (X

Figure 6.840. ***Platelet map region number 5. High mw neutral/basic proteins.***

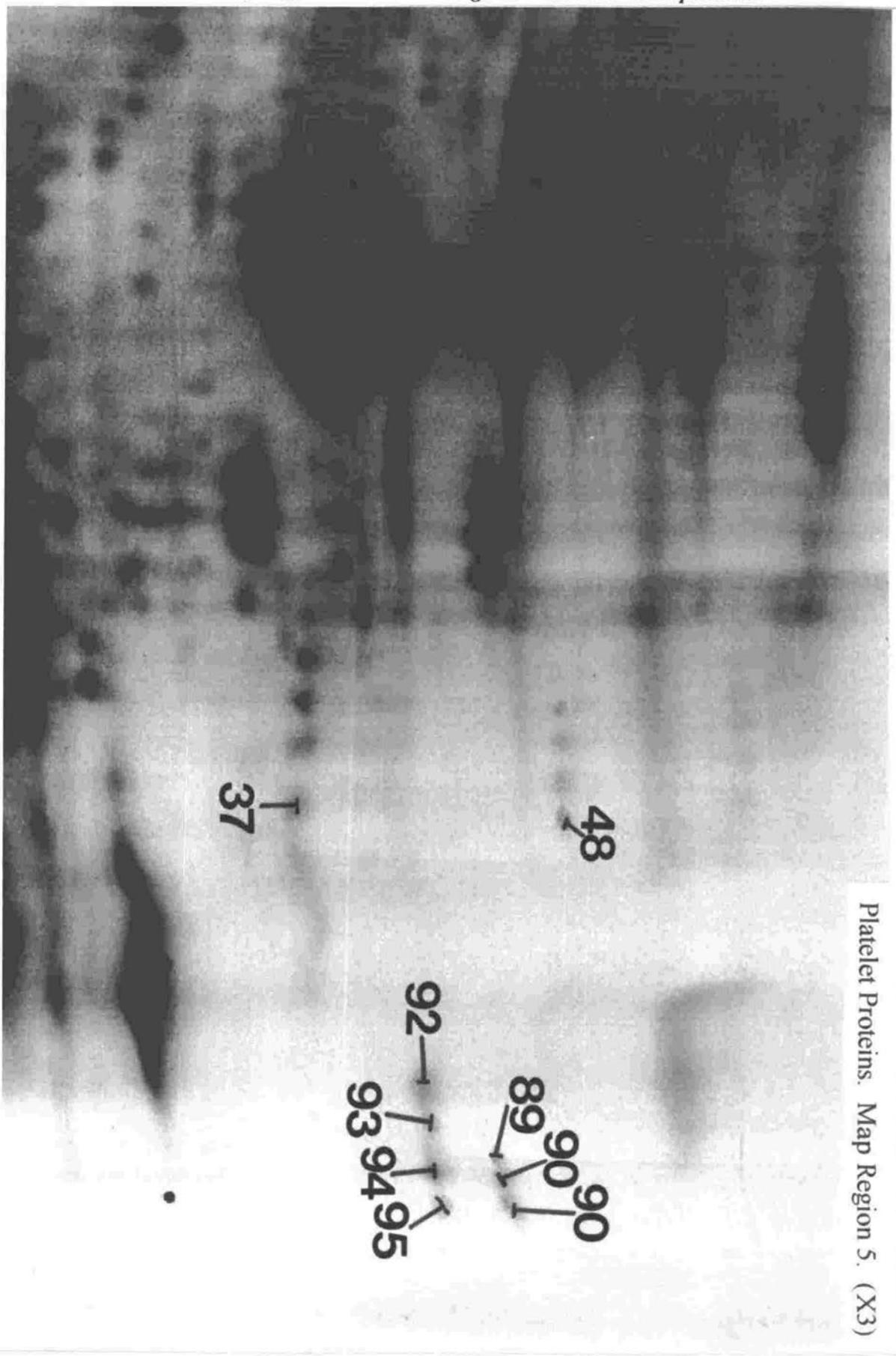


Figure 6.841. Variation in the same region of gel (platelet map region 2) for three Alzheimer's patients and three controls. Platelets prepared in the presence or absence of protease inhibitors are shown.

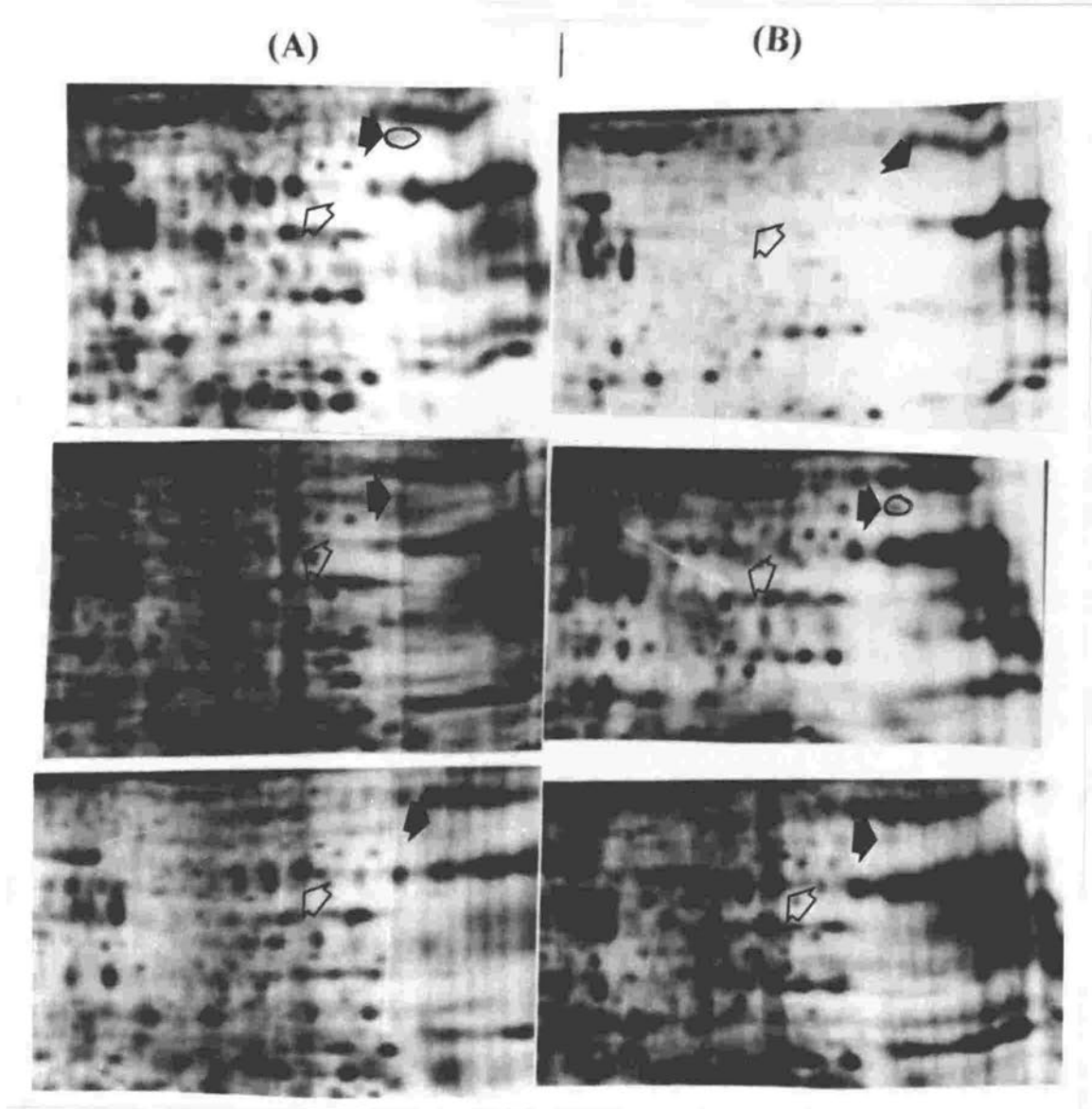


Figure 6.835. (A) Platelets obtained from three Alzheimer's patients, and prepared in the absence of protease inhibitors.
(B) Platelets obtained from three Alzheimer's patients, and prepared in the presence of protease inhibitors.

Figure 6.841. (contd.). Variation in the same region of gel (platelet map region 2) for three Alzheimer's patients and three controls. Platelets prepared in the presence or absence of protease inhibitors are shown.

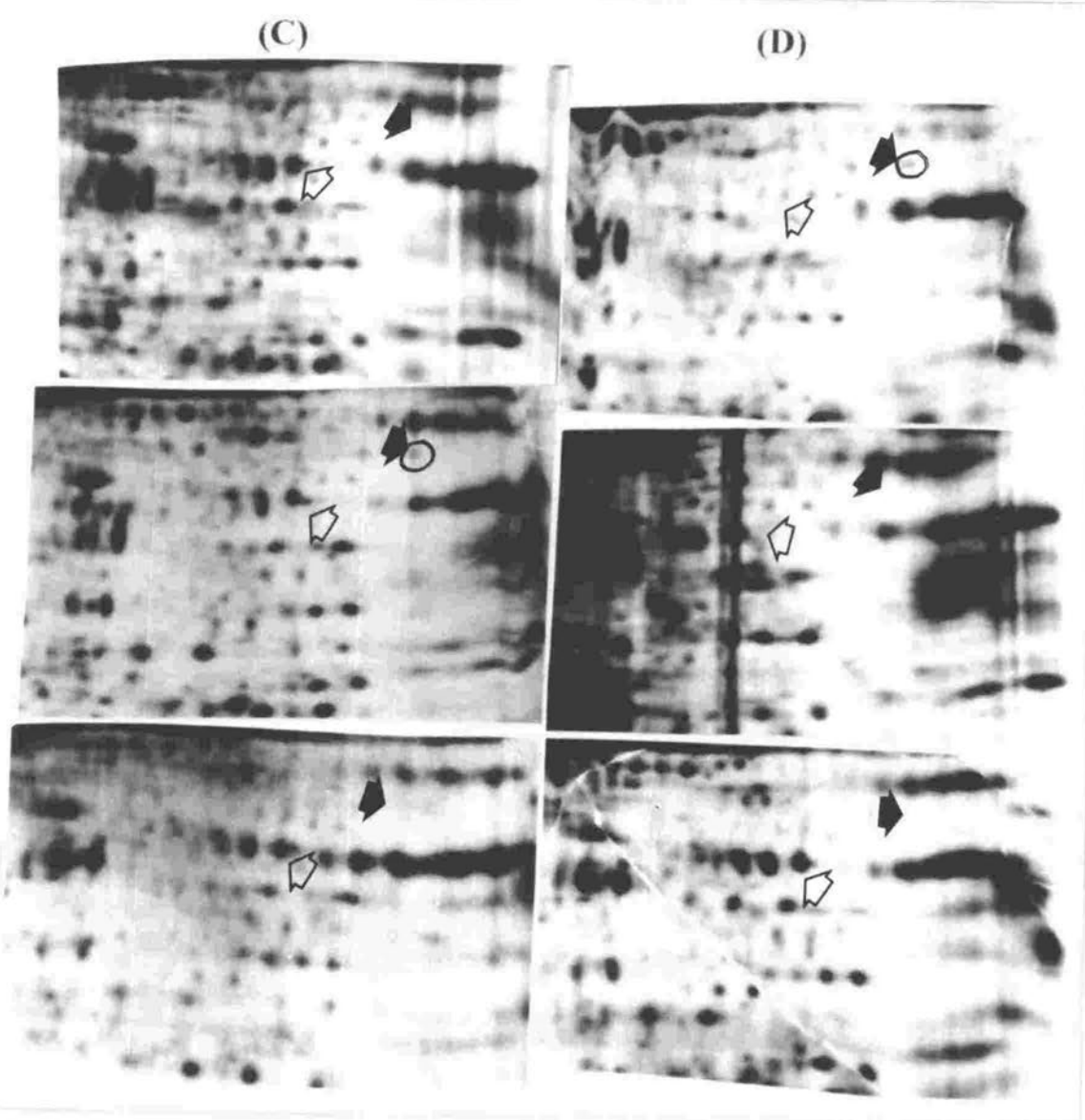


Figure 6.835. (C) Platelets obtained from three controls, and prepared in the absence of protease inhibitors.
(D) Platelets obtained from three controls, and prepared in the presence of protease inhibitors.

Several quantitative analyses were performed. Initially, platelet proteins prepared in the absence of protease inhibitors were compared between Alzheimer's patients and controls. Platelet proteins prepared in the presence of protease inhibitors were then compared between Alzheimer's patients and controls. Finally, platelet proteins prepared in the presence or absence of protease inhibitors were compared in the patient group and also in the control group.

Figure 6.842 shows the same region of a 2-dimensional PAGE gel (corresponding to platelet protein map region 4, Figure 6.840) for three Alzheimer's patients and three controls, for platelets prepared in both the presence and absence of protease inhibitors. This figure also gives histograms showing mean compositional VI values and standard deviations for proteins 32 (solid arrow) and 99 (hollow arrow) in Alzheimer's patients and controls. Protein 32 was significantly different ($P = 0.026$) between platelets prepared in the presence of protease inhibitors and platelets prepared in the absence of protease inhibitors in Alzheimer's patients. Protein 99 was also significantly different ($P = 0.035$) between platelets prepared in the presence of protease inhibitors and platelets prepared in the absence of protease inhibitors in Alzheimer's patients. Figure 6.842 illustrates some of the variation in spot position and intensity that was observed throughout this study, and gives an example of the range of values obtained after protein spot quantitation.

Figure 6.842. ***Variation in the same region of gel (platelet map region 4) for Alzheimer's patients and controls. Platelets prepared in the presence or absence of protease inhibitors are shown.***

Figure 6.842. (A) Platelets obtained from three Alzheimer's patients, and prepared in the absence of protease inhibitors.

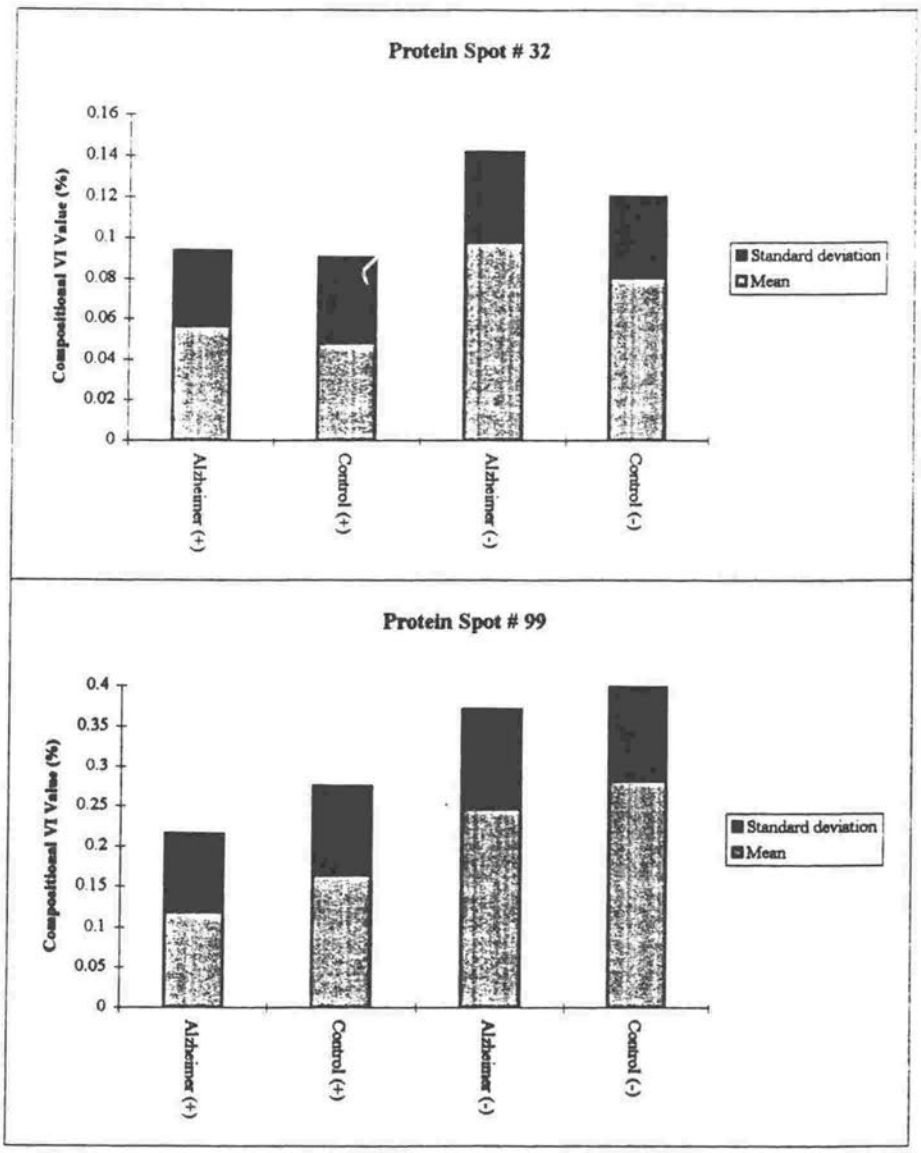
(B) Platelets obtained from three Alzheimer's patients, and prepared in the presence of protease inhibitors.

Figure 6.842 (contd.). Variation in the same region of gel (platelet map region 4) for Alzheimer's patients and controls. Platelets prepared in the presence and absence of protease inhibitors are shown.

Figure 6.842. (C) Platelets obtained from three controls, and prepared in the absence of protease inhibitors.

(D) Platelets obtained from three controls, and prepared in the presence of protease inhibitors.

Figure 6.842 (contd.). Variation in the abundances of two proteins for Alzheimer's patients and controls.



In the histograms, (+) refers to platelets prepared in the presence of protease inhibitors; (-) refers to platelets prepared in the absence of protease inhibitors. Results are for ten individuals in each group.

6.8321) Platelet proteins prepared in the absence of protease inhibitors.

Disease related proteolytic differences were searched for in Alzheimer's patients and controls. For the first quantitative analysis, platelet proteins prepared in the absence of protease inhibitors were compared between ten Alzheimer's patients and ten controls. The null hypothesis adopted for this analysis was that *the Alzheimer's patients and their controls (of both sexes) were members of the same population.* After testing, the hypothesis was rejected on the basis of finding six significant differences at the 95% confidence level between the 2 groups. These proteins are shown in the following table.

Table 6.832. Platelet proteins (prepared in the absence of protease inhibitors) found to differ significantly between ten Alzheimer's patients and ten matched controls.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.
10	0.481	0.042	Yes	ER-60 protease inhibitor
12	0.052	0.021	Yes	ER-60 protease inhibitor
24	0.848	0.032	Yes	Unknown
42	0.225	0.006	Yes	Serotransferrin
79	0.116	0.032	Yes	Unknown
83	0.252	0.021	Yes	Unknown

Sex-linked studies were then performed, where the groups were analysed according to sex. The following tests were performed.

1) Null hypothesis: *that the male Alzheimer's patients and the female Alzheimer's patients were members of the same population.* After testing, the hypothesis was rejected on the basis of finding nine significant differences between the two groups at the 95% confidence level. Table 6.833 lists those differences.

Table 6.833. Platelet proteins (prepared in the absence of protease inhibitors) found to differ significantly between five male Alzheimer's patients and five female Alzheimer's patients.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.
8	0.152	0.020	Yes	Unknown
13	0.832	0.016	Yes	Hb β chain
15	0.010	0.046	No	Unknown
25	0.086	0.023	No	Unknown
33	0.472	0.042	Yes	Unknown
66	0.599	0.024	Yes	HG3PD
85	0.294	0.001	Yes	Unknown
91	0.087	0.026	No	Unknown
95	0.026	0.017	No	Unknown

2) Null hypothesis: *that the male controls and the female controls were members of the same population.* After testing, the hypothesis was rejected on the basis of finding four significant differences at the 95% confidence level between the two groups. Table 6.834 lists these differences.

Table 6.834. Platelet proteins (prepared in the absence of protease inhibitors) found to differ significantly between five male controls and five female controls.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.
42	0.461	0.018	Yes	Serotransferrin
47	0.213	0.015	Yes	Unknown
71	0.569	0.038	Yes	Transcriptionally controlled tumour protein
89	0.788	0.001	Yes	Unknown

3) Null hypothesis: *that the male controls and the male Alzheimer's patients were members of the same population.* After testing, the hypothesis was rejected on the basis of finding six significant differences between the two groups at the 95% confidence level. Table 6.835 lists those proteins found to differ between the two groups.

Table 6.835. Platelet proteins (prepared in the absence of protease inhibitors) found to differ significantly between five male Alzheimer's patients and five male controls.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.
12	0.213	0.038	Yes	ER-60 protease inhibitor
25	0.124	0.020	Yes	Unknown
60	0.537	0.049	Yes	Unknown
68	<0.001	0.006	No	HG3PD
83	0.863	0.033	Yes	Unknown
85	0.008	0.032	No	Unknown

4) Null hypothesis: *that the female controls and the female Alzheimer's patients were members of the same population.* After testing, the hypothesis was rejected on the basis of finding one significant difference at the 95% confidence level between the two groups. Table 6.836 lists this protein.

Table 6.836. Platelet proteins (prepared in the absence of protease inhibitors) found to differ significantly between five female Alzheimer's patients and five female controls.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.
42	0.788	0.005	Yes	Serotransferrin

6.8322) Platelet proteins prepared in the presence of protease inhibitors.

Disease related changes in protein abundance/expression were searched for in Alzheimer's patients and controls. For the first quantitative analysis, platelet proteins prepared in the presence of protease inhibitors were compared between ten Alzheimer's patients and ten controls. The null hypothesis adopted for this analysis was that *the Alzheimer's patients and their controls (of both sexes) were members of the same population.* After testing, the hypothesis was rejected on the basis of finding six significant differences at the 95% confidence level between the 2 groups. These proteins are shown in Table 6.837.

Table 6.837. Platelet proteins (prepared in the presence of protease inhibitors) found to differ significantly between ten Alzheimer's patients and ten matched controls.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.
11	<0.001	0.022	No	Unknown
12	0.591	0.001	Yes	ER-60 protease inhibitor
17	0.189	0.026	Yes	Unknown
24	0.830	0.045	Yes	Unknown
30	0.121	0.042	Yes	Unknown
42	0.405	0.038	Yes	Serotransferrin

Sex-linked studies were then performed, where the groups were analysed according to sex. The following tests were performed.

1) Null hypothesis: *that the male Alzheimer's patients and the female Alzheimer's patients were members of the same population.* After testing, the hypothesis was rejected on the basis of finding nine significant differences between the two groups at the 95% confidence level. Table 6.838 lists those differences.

Table 6.838. Platelet proteins (prepared in the presence of protease inhibitors) found to differ significantly between five male Alzheimer's patients and five female Alzheimer's patients.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.
11	0.647	0.012	Yes	Unknown
13	0.488	0.040	Yes	Hb β chain
24	0.390	0.028	Yes	Unknown
25	0.008	0.019	No	Unknown
41	0.208	0.036	Yes	Pyruvate kinase-isozyme R
52	0.981	0.011	Yes	Unknown
91	0.193	0.027	Yes	Unknown
92	0.410	0.043	Yes	Unknown
94	0.242	0.039	Yes	Unknown

2) Null hypothesis: *that the male controls and the female controls were members of the same population.* After testing, the hypothesis was rejected on the basis of finding four significant differences at the 95% confidence level between the two groups.Table 6.839 lists these differences.

Table 6.839. Platelet proteins (prepared in the presence of protease inhibitors) found to differ significantly between five male controls and five female controls.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.
3	0.552	0.016	Yes	Unknown
15	0.174	0.027	Yes	Unknown
33	0.349	0.015	Yes	Unknown
89	0.717	0.004	Yes	Unknown

3) Null hypothesis: *that the male controls and the male Alzheimer's patients were members of the same population.* After testing, the hypothesis was rejected on the basis of finding six significant differences between the two groups at the 95% confidence level. Table 6.840 lists those proteins found to differ between the two groups.

Table 6.840. Platelet proteins (prepared in the presence of protease inhibitors) found to differ significantly between five male Alzheimer's patients and five male controls.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.
10	0.207	0.033	Yes	ER-60 protease inhibitor
11	0.048	0.012	No	Unknown
12	0.860	0.011	Yes	ER-60 protease inhibitor
24	0.375	0.029	Yes	Unknown
53	0.152	0.031	Yes	Unknown
86	0.171	0.048	Yes	Unknown

4) Null hypothesis: *that the female controls and the female Alzheimer's patients were members of the same population.* After testing, the hypothesis was upheld on the basis of finding three significant differences at the 95% confidence level between the two groups. Table 6.841 lists those proteins.

Table 6.841. Platelet proteins (prepared in the presence of protease inhibitors) found to differ significantly between a sample of five female Alzheimer's patients and five female controls.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.
15	0.347	0.008	Yes	Unknown
43	0.514	0.037	Yes	Serotransferrin
75	0.346	0.032	Yes	Unknown

6.8323) Quantitative differences in platelet proteins prepared in the presence and absence of protease inhibitors.

Proteolytic processing was examined for both patients and controls. For this analysis, platelet proteins prepared both in the presence and in the absence of protease inhibitors were compared for Alzheimer's patients and for their controls. Initially, data for the platelets prepared in the presence of protease inhibitors was pooled for each group and compared to the pool of data obtained for the platelets prepared in the absence of protease inhibitors for that group. The null hypothesis for each study was that *the platelet proteins prepared in the presence of protease inhibitors and the platelet proteins prepared in the absence of protease inhibitors (for both sexes) did not differ significantly in abundance.* For the patient and control groups, this hypothesis was rejected after testing on the basis of finding significant differences at the 95% confidence level between the platelets prepared in the presence of protease inhibitors and those prepared in the absence of protease inhibitors. Table 6.842 lists those proteins that were found to be significantly different in the Alzheimer's patients, and Table 6.843 lists those proteins that were found to be significantly different in the control group.

Table 6.842. Platelet proteins in Alzheimer's patients found to differ significantly in abundance when proteins prepared in the presence of protease inhibitors were compared to proteins prepared in the absence of protease inhibitors.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.
12	0.130	0.019	Yes	ER-60 protease inhibitor
13	0.235	0.009	Yes	Hb β chain
14	0.006	0.029	No	Hb α chain
15	0.443	0.032	Yes	Unknown

Table 6.842 (contd.). Platelet proteins in Alzheimer's patients found to differ significantly in abundance when proteins prepared in the presence of protease inhibitors were compared to proteins prepared in the absence of protease inhibitors.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.
16	0.453	0.015	Yes	Unknown
18	0.027	0.039	No	Unknown
32	0.085	0.026	No	Unknown
37	0.341	0.047	Yes	Unknown
40	0.321	0.036	Yes	Pyruvate kinase -isozyme R
57	0.112	0.024	Yes	Unknown
62	0.251	0.045	Yes	Unknown
72	0.143	0.017	Yes	Unknown
85	0.083	0.049	No	Unknown
86	0.020	0.023	No	Unknown
87	0.227	0.036	Yes	Unknown
96	0.525	0.009	Yes	Unknown
97	0.081	0.012	No	Unknown
98	0.015	0.029	No	Unknown
99	0.052	0.035	No	Unknown

Table 6.843. Platelet proteins in the control group found to differ significantly in abundance when proteins prepared in the presence of protease inhibitors were compared to proteins prepared in the absence of protease inhibitors.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.
7	0.987	0.009	Yes	Unknown
10	0.004	0.044	No	ER-60 protease inhibitor
15	0.002	0.005	No	Unknown
16	0.068	0.031	No	Unknown
17	0.310	0.038	Yes	Unknown
24	0.043	0.009	No	Unknown
27	0.690	0.029	Yes	Unknown
30	0.003	0.007	No	Unknown
31	0.301	0.013	Yes	Unknown
39	0.013	0.011	No	Unknown
40	0.059	0.011	No	Pyruvate kinase-isozyme R
54	0.231	0.021	Yes	Unknown
58	<0.001	0.020	No	Unknown
97	0.005	0.042	No	Unknown

Sex-linked studies were then performed, where the patient group and the control group were analysed according to sex. The following tests were performed.

1) Null hypothesis: *that the platelet proteins prepared in the presence of protease inhibitors and the platelet proteins prepared in the absence of protease inhibitors in male controls did not differ significantly in abundance.* The hypothesis was rejected after testing due to finding eight significant differences at the 95% confidence level. Table 6.844 lists these proteins.

Table 6.844. Platelet proteins in male controls found to differ significantly in abundance when proteins prepared in the presence of protease inhibitors were compared to proteins prepared in the absence of protease inhibitors.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.
15	0.062	0.009	No	Unknown
16	0.169	0.046	Yes	Unknown
24	0.100	0.045	Yes	Unknown
40	0.526	0.009	Yes	Pyruvate kinase-isozyme R
53	0.082	0.049	No	Unknown
58	0.023	0.046	No	Unknown
63	0.060	0.032	No	Unknown
67	0.003	0.025	No	HG3PD

2) Null hypothesis: *that the platelet proteins prepared in the presence of protease inhibitors and the platelet proteins prepared in the absence of protease inhibitors in male Alzheimer's patients did not differ significantly in abundance.* The hypothesis was rejected after testing due to finding twelve significant differences at the 95% confidence level. Table 6.845 lists these proteins.

Table 6.845. Platelet proteins in male Alzheimer's patients found to differ significantly in abundance when proteins prepared in the presence of protease inhibitors were compared to proteins prepared in the absence of protease inhibitors.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.
8	0.647	0.016	Yes	Unknown
11	0.231	0.034	Yes	Unknown

Table 6.845 (contd.).

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.
13	0.645	0.034	Yes	Hb β chain
16	0.022	0.020	No	Unknown
47	0.739	0.020	Yes	Unknown
49	0.141	0.046	Yes	Unknown
74	0.363	0.011	Yes	Unknown
85	0.003	0.038	No	Unknown
86	0.012	0.045	No	Unknown
96	0.046	0.014	No	Unknown
97	0.242	0.014	Yes	Unknown
99	0.218	0.037	Yes	Unknown

3) Null hypothesis: *that the platelet proteins prepared in the presence of protease inhibitors and the platelet proteins prepared in the absence of protease inhibitors in female controls did not differ significantly in abundance.* The hypothesis was rejected after testing due to finding five significant differences at the 95% confidence level. Table 6.846 lists these proteins.

Table 6.846. Platelet proteins in female controls found to differ significantly in abundance when proteins prepared in the presence of protease inhibitors were compared to proteins prepared in the absence of protease inhibitors.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.
7	0.635	0.029	Yes	Unknown
30	0.006	0.035	No	Unknown
31	0.842	0.029	Yes	Unknown
42	0.239	0.041	Yes	Serotransferrin
100	0.290	0.023	Yes	Unknown

4) Null hypothesis: *that the platelet proteins prepared in the presence of protease inhibitors and the platelet proteins prepared in the absence of protease inhibitors in female Alzheimer's patients did not differ significantly in abundance.* The hypothesis was rejected after testing due to finding eight significant differences at the 95% confidence level. Table 6.847 lists these proteins.

Table 6.847. Platelet proteins in female Alzheimer's patients found to differ significantly in abundance when proteins prepared in the presence of protease inhibitors were compared to proteins prepared in the absence of protease inhibitors.

Spot I.D.	Prob. F Value.	Prob. *t* Value.	Equal Variance.	Protein Name.
13	0.185	0.027	Yes	Hb β chain
14	0.502	0.043	Yes	Hb α chain
20	0.708	0.028	Yes	Unknown
21	0.466	0.006	Yes	Unknown
24	0.284	0.049	Yes	Unknown
41	0.064	0.041	No	Pyruvate kinase-isozyme R
97	0.337	0.035	Yes	Unknown
98	0.018	0.035	No	Unknown

6.84) Discussion.

Two-dimensional PAGE was used to separate ^{14}C-labelled platelet proteins of ten Alzheimer's patients and ten age and sex matched controls. Table 6.848 summarises the results of the quantitative study of variation in abundance among 100 proteins. The table gives ratios of mean abundances for proteins found to differ significantly between the groups.

Table 6.848. Significant differences (* 0.01 < P < 0.05; ** 0.001 < P < 0.01) in abundance of platelet proteins prepared in the presence (+) or absence (-) of protease inhibitors for ten Alzheimer's patients A, and ten matched controls C.

Spot Number	Ratio of Mean Abundances [1]			
	A(+)/A(-)	A(+)/C(+)	A(-)/C(-)	C(+)/C(-)
7				** (0.473)
10			* (0.575)	* (0.571)
11		* (0.587)		
12	* (0.498)	** (0.151)	* (0.248)	
13	** (0.426)			
14	* (0.432)			
15	* (0.496)			** (0.436)
16	* (0.489)			* (0.495)
17		* (2.321)		*(0.459)
18	* (0.559)			
24		* (2.502)	* (1.835)	** (0.446)
27				* (0.481)
30		* (2.328)		** (0.331)
31				* (0.512)
32	* (0.577)			
37	* (0.522)			
39				* (0.429)

1 Numbers in brackets are a measure of the relative mean abundances of each protein in the two populations which are being compared.

Table 6.848 (contd.). Significant differences (* 0.01 < P < 0.05; ** 0.001 < P < 0.01) in abundance of platelet proteins prepared in the presence (+) or absence (-) of protease inhibitors for ten Alzheimer's patients A, and ten matched controls C.

Spot Number	Ratio of Mean Abundances [1]			
	A(+)/A(-)	A(+)/C(+)	A(-)/C(-)	C(+)/C(-)
40	* (0.518)			* (0.505)
42		* (0.497)	** (0.425)	
54				* (0.470)
57	* (0.492)			
58				* (0.576)
62	* (0.627)			
72	* (0.324)			
79			* (2.047)	
83			* (2.923)	
85	* (0.465)			
86	* (0.439)			
87	* (0.542)			
96	** (0.441)			
97	* (0.356)			* (0.509)
98	* (0.522)			
99	* (0.485)			

1 Numbers in brackets are a measure of the relative mean abundances of each protein in the two populations which are being compared.

Of those proteins shown to differ significantly, six are known proteins that were identified on the Swiss-2D PAGE platelet map, and named in the S.P. protein database. The proteins were ER-60 protease inhibitor, Hb α and β chains, pyruvate kinase (isozyme R), and serotransferrin.

ER-60 protease inhibitor, spot 10, differed significantly ($P = 0.042$) between the platelets prepared in the absence of protease inhibitors for Alzheimer's patients and controls (Figure 6.844), and also between control platelets (both sexes) prepared in the absence of protease inhibitors and control platelets prepared in the presence of protease inhibitors ($P = 0.044$). This protein was found to differ significantly between Alzheimer's patients and their controls only in the absence of protease inhibitors. The mean compositional VI values for patients and controls were 0.40% and 0.70% respectively. This protein was not seen to alter in abundance /expression in Alzheimer's disease. This implied that the proteolytic processing of this protein was enhanced in Alzheimer's disease, possibly due to an increased protease concentration. No consistent sex linked pattern was observed for this protein.

ER-60 protease inhibitor, spot 12 differed significantly between the platelets prepared in the presence of protease inhibitors and those prepared in the absence of protease inhibitors for the Alzheimer's patient group ($P = 0.019$), between the platelets prepared in the presence of protease inhibitors for the Alzheimer's patients and controls ($P = 0.001$), and between the platelets prepared in the absence of protease inhibitors for the Alzheimer's patients and controls ($P = 0.021$). The protein was more abundant in the control group (Figure 6.843). As this protein differed significantly between Alzheimer's patients and controls in samples prepared in the presence of protease inhibitors, and between samples prepared in the absence of protease inhibitors, it implied that the abnormality was not the result of abnormal proteolytic processing in Alzheimer's disease, assuming that this protein was affected by the inhibitor cocktail used. No consistent sex linked pattern was observed for this protein.

ER-60 protease inhibitor inhibits ER-60, a recently identified serine protease found in the ER (Urade *et al.*, 1992). The abundance of ER60-protease inhibitor protein always decreased in

Alzheimer's patients. ER membranes have been shown to accumulate in platelets in Alzheimer's disease (Zubenko, !990).

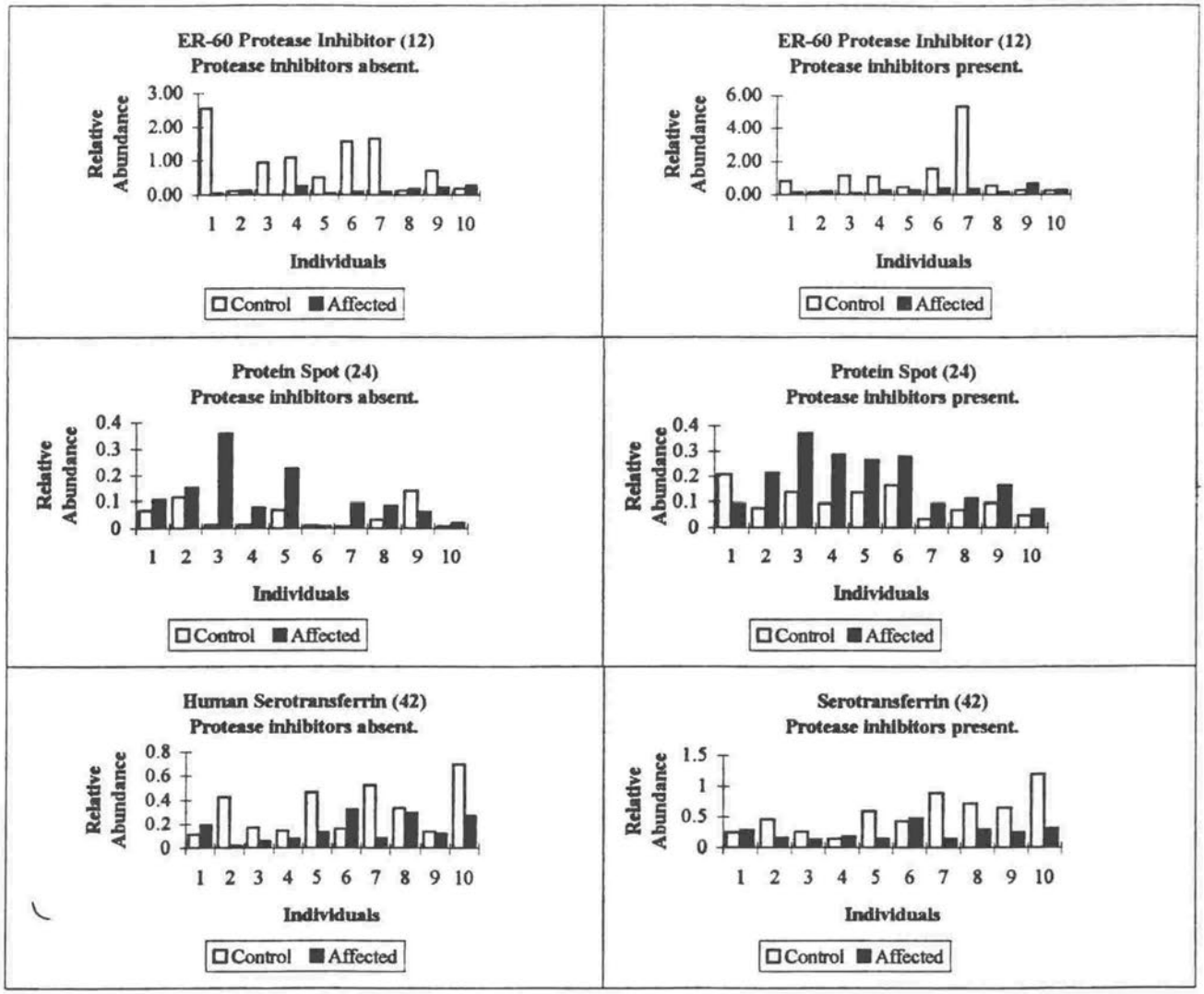
Hb α and β chains (spots 13 and 14) were found to differ significantly (P = 0.029 and P = 0.009) when platelets prepared in the presence of protease inhibitors were compared to those prepared in the absence of protease inhibitors in Alzheimer's patients. The proteins had reduced levels in the samples prepared in the presence of protease inhibitors. As these proteins were not seen to differ significantly in any of the other comparisons performed however, it is difficult to assess the clinical significance of these observations. Hb α and β chains were not studied in the plasma of Alzheimer's patients, as the proteins were not clearly resolved.

Pyruvate kinase (isozyme R), spot 40, was significantly different between platelets prepared in the absence of protease inhibitors and platelets prepared in the presence of protease inhibitors for Alzheimer's patients (P = 0.036) and controls (P = 0.011). In both cases, the protein had higher abundances in the samples prepared in the absence of protease inhibitors. However, as this protein difference was not detected between the Alzheimer's patients and controls, it may be of little diagnostic use.

Unidentified protein (Spot 24) was significantly different between Alzheimer's patients and controls for platelets prepared in the absence of protease inhibitors (P = 0.021) and for platelets prepared in the presence of protease inhibitors (P = 0.001). The protein was more abundant in Alzheimer's patients than in controls (Figure 6.843). As this difference was observed for platelets prepared in the absence of protease inhibitors and for platelets prepared in the presence of protease inhibitors, it implied that the abnormality was not the result of abnormal proteolytic processing in Alzheimer's disease.

Serotransferrin, protein spot 42, was significantly different between Alzheimer's patients and controls in platelets prepared in the presence (P = 0.038) or absence (P = 0.006) of protease inhibitors. This protein decreased in relative abundance in Alzheimer's disease (Figure 6.843). As

Figure 6.843. ***Platelet proteins prepared in the absence or presence of protease inhibitors found to differ significantly between ten Alzheimer's patients and ten matched controls.***

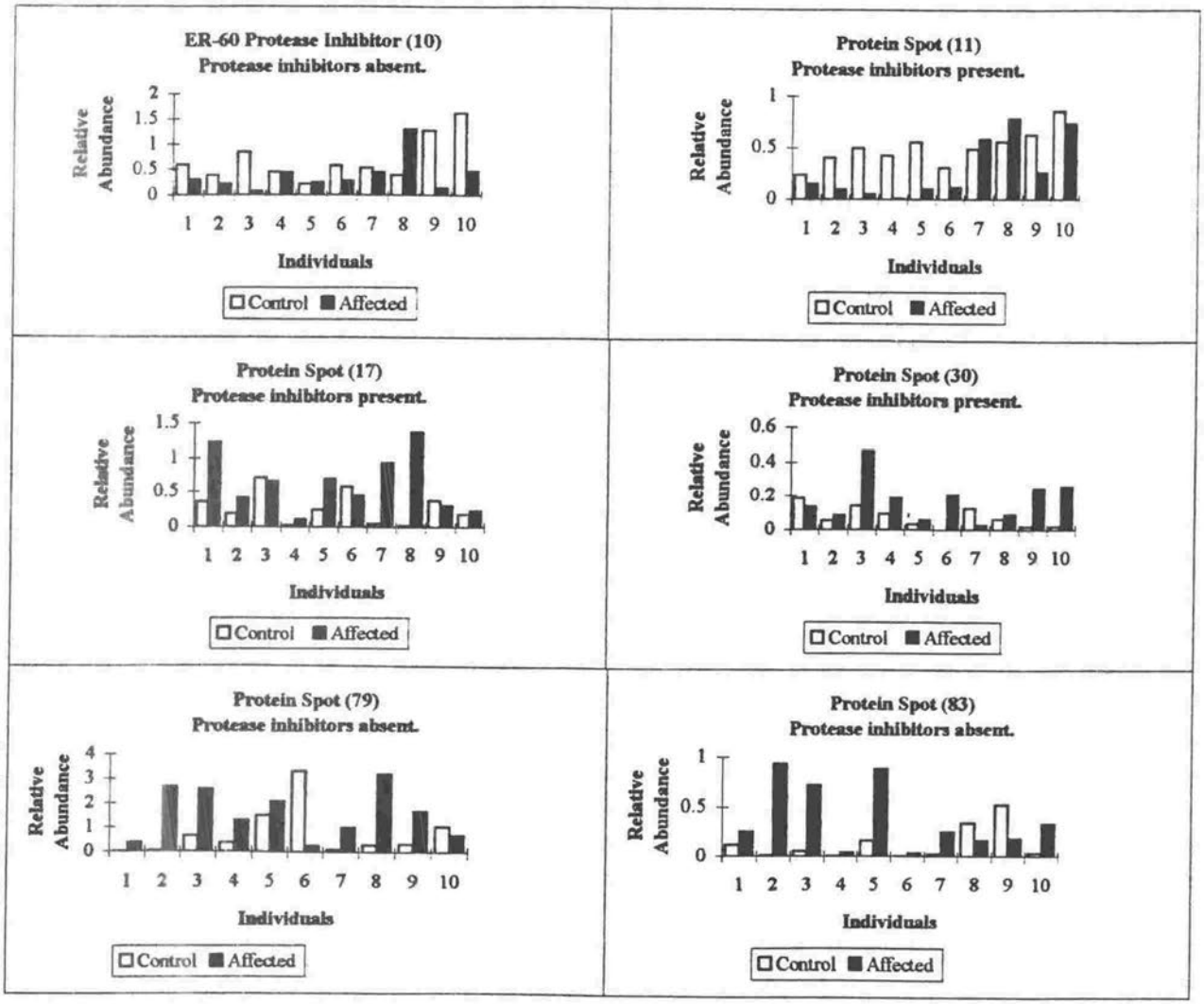


the difference was detected in platelets prepared in the absence or presence of protease inhibitors, it did not appear that the difference was due to an aberrant proteolytic event. As iron has been shown to have a potential physiological role in controlling amyloid precursor protein processing (Bodivitz *et al.*, 1995), this observation may be of clinical significance. Serotransferrin could not be analysed on 2-dimensional PAGE plasma gels, as it was not resolved from other major plasma proteins such as albumin.

There were several possible disease linked proteins that could not be identified. Two unidentified proteins were found to significantly differ between Alzheimer's patients and their controls only in the absence of protease inhibitors. These proteins were protein spot 79 and protein spot 83. The relative abundance of proteins 79 and 83 increased in Alzheimer's patients (Figure 6.844). The average compositional VI values for patients and controls for spot 79 were 1.57 % and 0.77 %. The average compositional VI values for patients and controls for protein spot 83 were 0.38 % and 0.13%. As no significant disease related increase in abundance/expression was detected for either of these proteins, it implied that the proteolytic processing of these proteins was reduced in Alzheimer's disease, possibly due to a reduced protease concentration.There were however substantial differences between individuals (Figure 6.844).

Three unidentified proteins were significantly different between Alzheimer's patients and matched controls only in the presence of protease inhibitors.These proteins were protein spots 11, 17, and 30. As none of these proteins was observed to be significantly different between platelets prepared in the absence of protease inhibitors for Alzheimer's patients and controls, it implied that these differences were disease related changes in abundance/expression. Protein spot number 11 decreased in relative abundance in Alzheimer's patients (Figure 6.844). Average compositional VI values for patients and controls for this protein were 0.29 % and 0.50 %. Protein spots 17 and 30 increased in relative abundance in Alzheimer's patients (Figure 6.844). The average compositional VI values for patients and controls for protein spot 17 were 0.64 % and 0.28 %. The average compositional VI values for patients and controls for protein spot 30 were 0.17 % and 0.08 %.

Figure 6.844. ***Platelet proteins prepared in the absence or presence of protease inhibitors found to differ significantly between ten Alzheimer's patients and ten matched controls.***



When differences in abundance were examined for platelet proteins prepared in the presence or absence of protease inhibitors in both Alzheimer's patients and controls, many differences were observed. The Alzheimer's group showed nineteen significant differences between the samples prepared in the absence of protease inhibitors and those prepared in the presence of protease inhibitors. The control group showed fourteen significant differences. The differences in both groups showed both increases and decreases in relative abundance between samples prepared in the absence of protease inhibitors and those prepared in the presence of protease inhibitors. The two groups shared four significant protein differences. They were protein spots 15, 16, 40 (pyruvate kinase-isozyme R), and 97. For both groups, all of these proteins decreased in relative abundance between samples prepared in the presence of protease inhibitors, compared to those prepared in the absence of protease inhibitors. This indicated that these proteins may have been cleavage products of larger proteins. When sex linked analyses were performed, there were differences between males and females from both the control and patient groups. The were no consistent differences between males and females in the two groups however.

It is difficult to assess the significance of the significant protein changes observed here, as very few of the proteins involved have been identified. It is clear however, that complex changes in protein processing occurred in Alzheimer's disease.

Chapter 7. Summary and Conclusions.

7.1) 2-Dimensional PAGE and Quantitative Densitometry.

High resolution 2-dimensional PAGE is a powerful and efficient technique for analysing individual proteins within complex biological mixtures. The gels used in this study allowed detection of up to 500 proteins within the approximate pI range of 4.0 to 8.5, and within the approximate molecular weight range of 13,000 to 165,000. Although the resolution of the procedure used in this study was generally high, the procedure did have some limitations. It was only capable of resolving a subset of the total protein species present within a complex mixture. Proteins falling outside the pI and mw ranges, proteins insoluble in the sample preparation, proteins with very similar pI and mw values, and low abundance proteins would not have been detected.

The goal of this study was to search for disease associated changes in protein abundance in patients with MS or Alzheimer's disease. An accurate method for measuring protein abundance was thus necessary for this study. To achieve this, an investigation was made to determine the optimum method of obtaining and analysing protein abundance data. One initial observation made in this study was that the relationship between protein load and VI values was different for a number of proteins. There may be more than one type of Coomassie blue binding site on proteins. It is also possible that the proteins studied exhibited different degrees of denaturation in the electrophoretic conditions used in this project. The level of denaturation may then have determined the number of accessible dye binding sites. It is also possible that protein load influences denaturation differently between different proteins. The observations made here are consistent with those of Cogden *et al.* (1993) and Chial *et al.* (1993), who showed kinetically that binding sites with different affinities for Coomassie blue may exist. These investigators were using assay conditions that differed from the electrophoretic conditions in this study however.

One important observation made in this initial study was that the reproducibility of gel quantitation was not great enough to allow VI values from different gels to be compared directly.

Several methods of gel "calibration" were investigated. Of these, conversion of VI values into compositional data yielded the greatest reproducibility. Standard deviations over means (SD/M %) for replicate measurements of individual plasma proteins were in the range 1.8 % to 7.0 % for compositional data. The greatest variation occurred for proteins that were either small, were poorly resolved, or were in complex or inconsistent backgrounds. Statistical analysis was done on logratio transformants of compositional data. The advantages of using compositional analysis were that it enabled different gels to be reliably compared, and that it made the original VI values amenable to analysis using powerful multivariate statistical techniques. In general, when variances were equal, significant differences ($0.01<P<0.05$) occurred between pairs of proteins whose abundances differed by approximately 1.2 to 1.4 fold. More significant differences ($0.001<P<0.01$) required an approximately 1.6 fold difference in abundance, and highly significant differences ($0.0001<P<0.001$) required at least a 1.8 fold difference in abundance. A more detailed description of the statistical procedures used and an evaluation of their performance is given in the Appendix.

7.2) The Search for Protein Abnormalities in Neurological Diseases.

2-Dimensional SDS PAGE was used to search for protein abnormalities in samples from patients with MS or Alzheimer's disease. Two types of abnormalities were sought. First, I searched for qualitative differences between the disease and control states. The tissues in these searches included plasma from patients with MS or Alzheimer's disease, CSF from patients with MS, and platelets from patients with Alzheimer's disease.

For all of the qualitative searches, the initial approach involved pooling samples, and searching for differences between pooled samples using visual inspection of the gels or fluorograms. When abnormalities were detected between the pooled samples, they were further examined by studying the individual members making up the pool in order to confirm the difference, and assess the extent of variation among individuals. There were several advantages in using a pooling technique. First, as many samples were analysed simultaneously, the cost and time for the initial analysis was also much reduced. Second, by pooling the samples, any variation

in the samples not related to the disease would be diluted by the other samples in the pool that did not possess that difference. This would minimise random protein differences in the pooled samples. Also, any protein differences introduced into the pools as the result of incorrect diagnosis would be minimised. One disadvantage of using pools is that a protein may only have been present in a proportion of the individual samples. Such an abnormality would arise if disease sub-types existed. If such an abnormality was only minor, it may have been diluted by the other samples in the pool to the extent that it became unobservable.

In almost every instance in this study, qualitative differences between pooled samples were not consistent when the individuals making up the pool were examined. Thus, the differences detected in the pools may have represented analytical differences, they may have represented the presence of disease sub-types, or they may have represented diagnostic inaccuracy. For several members of the MS affected female group however, a consistent qualitative abnormality was detected (Figures 4.215 to 4.219). This protein was isolated and a partial sequence analysis identified the protein as human haptoglobin $\alpha 2$. Although this observation is interesting, it fails to meet the criteria for marker proteins discussed in Chapter 1, because haptoglobins are not disease specific, and also because the abnormality was not detected in the majority of MS patients. It is likely that the protein was present in all of the patients, but was at a concentration too low to be reliably detected by Coomassie blue. The abnormality therefore had little diagnostic utility for MS.

Quantitative densitometry was used to obtain relative abundance data for the proteins separated in the qualitative study. Unlike for the qualitative study however, pooled samples could not be used, as they were not amenable to statistical analysis. Differences in relative abundance of various proteins were observed in all of the studies that I performed. In the analysis of plasma proteins, many proteins were seen to alter in relative abundance in MS and Alzheimer's disease. Almost all of the protein changes observed involved APRs., and so were of little diagnostic utility. In Alzheimer's disease, orosomucoid, Hpα, Hpβ, apo D, RBP, and a group of unidentified proteins were significantly different ($P < 0.01$) between the Alzheimer's patients and a group of age and sex matched controls. Of these abnormalities, I have found no literature reference to

Alzheimer's disease associated changes in the plasma levels of orosomucoid, apo D, or RBP. This study did not support the observations of Wood *et al.* (1993) who suggested that increased plasma α2-macroglobin levels were associated with Alzheimer's disease. Giometto *et al.* (1988) reported that plasma levels of α2-macroglobin, acid glycoprotein, and Hp were similar in Alzheimer's patients and controls. The current study however, showed increased plasma levels of Hp in Alzheimer's disease. In agreement with Giometto *et al.* (1988), I found no significant change in the plasma levels of α2-macroglobin or acid glycoprotein in Alzheimer's disease. The results obtained in this study are consistent with inflammatory mechanisms being important in Alzheimer's disease (Aisen *et al.*, 1994) .

In MS, plasma levels of orosomucoid, Hpα, Hpβ, apo A1 and apo D, IgG, IgJ, and a group of unidentified proteins were found to be significantly different ($P < 0.01$) between a group of MS patients and their age and sex matched controls. These observations are consistent with those of Dowling *et al.* (1976) that showed transient increases in plasma orosomucoid levels in patients with MS. The observations are also consistent with the increased HDL (apo A) plasma levels that Reider *et al.* (1970) observed in patients with MS, although I found no evidence of the increased LDL (apo B) that Reider *et al.* observed. I could also find no evidence of the novel proteins that Wu *et al.* (1979) found associated with MS. Wu *et al.* detected these proteins in serum using 1-dimensional gradient PAGE, although the proteins could not be observed using uniform (5% and 7%) gels. Wu's observations have not been further confirmed in the literature however. The observed increase in apo A1 plasma levels is not consistent with the observations of Gelman *et al.* (1988), who concluded that the plasma levels of apo A1 remained unchanged in a group of MS patients when compared to healthy controls. The IgG oligoclonal banding reported by Mehta (1991) was also occasionally seen in some MS patients in this study. However, the changes in α2-macroglobin reported by Back *et al.* (1992) were not observed. I have found no reference in the literature to the MS related changes in the plasma levels of Hpα, Hpβ, apo D, or IgJ that were observed in this study. It is possible that these plasma proteins have not yet been studied by others. The unidentified plasma proteins that were found to be significantly different in both MS and Alzheimer's disease in this study, may have been APRs. The fact that there were

both increases and decreases in plasma levels of known or suspected APRs in both diseases supports the presence of inflammation in both MS and Alzheimer's disease as suggested by McRae *et al.*(1993).

Differing plasma levels of various APRs were seen for both diseases. It is possible that these differences were great enough to support clinical diagnosis, although a much larger study would be needed to assess this. Such a study would also need to include a variety of other neurological diseases. Changes in the APR profile over time may also be useful in prognostic assessment, but again, a larger study would be required before this could be assessed.

Several differences were also detected between the CSF proteins of MS patients and their controls. As in the plasma studies, all of the protein changes detected involved APRs, and so were of limited diagnostic utility. The proteins significantly different ($P < 0.05$) between the control and MS patients were Apo A1, Hpβ, fibrinogen β, AT, and actin. Orosomucoid was not seen to be significantly increased in concentration between MS patients and controls as reported by Vrethem *et al.* (1987). The results obtained in the current study were also inconsistent with the observations of Gelman *et al.* (1988) who showed that there was no significant difference in the CSF levels of apo A1 between MS patients and controls. Wildenauer *et al.* (1991) showed an increased concentration of a fibrin fragment that was loosely correlated with various neurological diseases, including MS. These changes were not detected in this project, although significant changes were observed for fibrinogen, the proteolytic precursor of fibrin. I have found no literature reference to altered CSF Hp levels in MS. However, CSF Hp levels have been shown to increase in Alzheimer's disease and vascular dementia (Johnson *et al.*, 1992; Mattila *et al.*, 1994). Again no literature reference to altered CSF levels of AT, fibrinogen, or actin in MS could be found. A preliminary study was also made of the co-abundance of proteins in the CSF and plasma of patients with MS and their controls. The results from this study suggested a complex pattern of protein processing in the disease state. No obvious disease related patterns could be detected.

A quantitative study of platelet proteins was also carried out. When platelet proteins

prepared in the absence of protease inhibitors were examined, several significant differences were observed. The proteins significantly different ($P < 0.05$) between Alzheimer's patients and controls were ER-60 protease inhibitor (protein spot 10), serotransferrin , and several unidentified proteins. When platelet proteins prepared in the presence of protease inhibitors were examined, several differences were seen. Those proteins found to differ significantly ($P < 0.05$) between controls and Alzheimer's patients were ER-60 protease inhibitor (protein spot 11) and several unidentified proteins. ER-60 protease inhibitor may be linked to platelet abnormalities in Alzheimer's disease through the role of ER-60 protease in phosphoinositide metabolism in platelets. Platelet serotransferrin levels may be significant given the renewed interest in the role of iron in Alzheimer's disease. However, no reference has been made in the literature to altered platelet levels of ER-60 protease inhibitor or serotransferrin in Alzheimer's disease.

Using PCR-mediated mRNA amplification, Schlossmacher *et al.* (1992) showed that APP was present in human platelets. Platelet APP was not contained in the Swiss-2DPAGE database however, so could not be easily identified on the 2-dimensional PAGE platelet protein separations made in this study. However, serum APP has a pI of 8.9 and a mass of 11.6 kDa. Such a protein would not have been resolved on the gels used in the current study. Although it is difficult to assess the significance of the other platelet protein changes observed in this study, due to the limited number of platelet proteins that have been identified in the Swiss-2DPAGE platelet database, the results obtained do add further support to the suggestion of Inestros *et al.* (1993) that platelets may provide a systemic marker for Alzheimer's disease.

7.3) Avenues for Further Investigation.

Although the sample sizes used in this study make it difficult to interpret the results in context of the wider affected populations, they do clearly illustrate one point. They show that with larger sample sizes, and with the recent developments in immobilised pH gradient gels and automated gel spot matching and quantitation, there is good potential to produce useful information

regarding the disease process in both Alzheimer's disease and MS. Such information could provide an insight into the pathology of the diseases at the molecular level, and so would have many potential uses. The observations made in this study are consistent with inflammatory processes occurring in both MS and Alzheimer's disease. Recently, Aisen *et al,* (1994) has suggested that anti-inflammatory drugs may be of benefit in treating Alzheimer's disease.

If a disease specific protein abnormality was found for either Alzheimer's disease or MS, its suitability as a marker protein would need to be assessed. This would necessitate determining the specificity of the abnormality, and its prevalence within the patients with the disease. If the abnormality met the criteria for being a marker protein, and if it was also found in an easily accessible biological fluid or tissue, then one application of the abnormality would be to use it as the basis of an ELISA-based laboratory test for the disease. If the abnormality took the form of a protein polymorphism associated with the disease, then the role of the allele in the disease could be investigated. It would be important to know whether the allele was directly involved in the pathogenesis of the disease, or whether it was genetically linked to a nearby locus involved in disease pathogenesis. In the case of a genetic linkage, the purified protein (abnormality) could serve as a template for the construction of a DNA probe to investigate the second locus.

Given the large social and economic costs associated with both MS and Alzheimer's disease, protein markers for these diseases would be of great benefit. It would therefore be useful to continue searching for such markers.

Appendix 1.

Statistical analysis of quantitative data.

The relative abundance data obtained from the studies performed in this project were recorded as percentages. For each set of quantitations made for a single gel, the abundances were summed and the abundance of each protein was expressed as a percentage (X) of the total. Measurements such as this belong to a class of multivariate observations called compositional data. Compositional data are distinguished from other types of experimental data by the imposition of constraints on the range of possible values. Each measurement must be non-negative, and the sum of one complete set of measurements over each experimental unit must be a fixed constant. This constant is usually 1, 100%, or 10^6 ppm. The complete set of measurements on one experimental unit is called a composition. The statistical analysis of compositional data is a relatively new field, which has only been described in detail so far by Aitchison (1986).

Aitchison (1986) proposed the "logratio" transformation for compositional data. He showed that if, for each experimental unit, the measurements are all divided by the first, last, or any other measurement on a fixed position in the variable list, then the resultant set of ratios is invariant to the transformation into subcompositions. Therefore, the relative abundances of the components are invariant. Aitchison also showed that if a composition is repeatedly "perturbed" so that its components drift randomly, but their sum remains fixed at 1, 100%, or 10^6 ppm., then the natural logarithm of the ratio described above would be the sum of a large number of small random changes. The set of logratios perturbed in this way follows the multivariate normal distribution. This presents a strong argument for making a logratio transformation of compositional data if one wants to take advantage of the power of multivariate statistical tests. In this project, interest was focused more on the variation of individual proteins rather than the variation of the total abundance of proteins. Aitchison's theory reduces then to the special case of the abundances X and 100-X, (since X is a percentage). The appropriate logratio is $\mathrm{Log}_n [X/(100-X)]$. The logratio can then be shown to be the sum of a fixed part and a collection of random errors that should have a

normal distribution. This allows the valid application of the *t*-test for comparing independent pairs of samples from more than two populations.

Statistical analysis of variation was then done on the logratio data. The SAS and Excel statistical packages were used to analyse variation between different experimental groups. Variation was assessed using 2-tailed *t*-tests.

For all of the analyses performed, the normality and the equal variance criterion was checked for the logratio transforms. Normality was assessed using an analysis of residuals. The reason that a residual analysis was used, is that it allowed the M.S. and Alzheimer's data to be pooled, (at least for the plasma studies) thus increasing sample size. Residuals were calculated using Excel, using a one way ANOVA (pooled disease against pooled controls). Residuals are defined as shown below:

$$e_{ij} = Y_{ij} - \overline{Y_j}$$

where e_{ij} is the residual of the i^{th} variate for the variable j; Y_{ij} is the absolute value of the i^{th} variate, and $\bar{Y}_j$ is the mean of variates for the variable j.

The residuals were standardised by dividing by the standard deviation. If the relative abundance of a plasma protein is normally distributed in the disease and control subpopulations, then the standardised residuals should be normally distributed about a mean of zero. The normality of the distributions was assessed first by plotting frequency and cumulative frequency distributions for the standardised residuals for each protein. For normal distributions, the cumulative frequency distribution plot should be sigmoidal. Although graphical tests of normality are subjective, they readily show if the distribution (normal or not) is skewed, etc. To decide if the residual distributions were significantly different from normal distributions, a χ^2 test was used. This test was based on the standard deviation ratio for a normal distribution:

2.5 : 13.5 : 34 : 34 : 13.5 : 2.5 (- -2, -2 - -1, -1 - 0, 0-1, 1-2, 2+ σ respectively).

The χ^2 value was calculated as shown below:

$$\chi^2_{\alpha,5} = \sum_{i=1}^{\kappa} \frac{(fi - Fi)^2}{Fi}$$

where fi is the observed frequency, and Fi is the expected frequency.

Probability values were obtained from a table of the Cumulative Distribution of Chi-square contained in Snedecor *et al.*, (1989).

When the logratio transform for a protein was found not to be distributed normally, the original compositions were transformed via angular transformation. In this transformation, compositional values were converted to the arcsine of their square root. The arcsine value can be in degrees or radians. I used degrees. The transformation is shown below:

$$X' = \sin^{-1} \sqrt{x}$$

where X' is the transformed compositional value, and x is the original compositional value.

For proportions between 0.00 to 1.00 (ie. percentages between 0 and 100%), the transformed values range from 0° to 90°. Zero proportions were counted as 1/(4n), as recommended by Snedcor (1989). The transformed distributions were assessed for normality as described previously.

When *t*-Tests were performed, 2-tailed tests were used. When a protein was found to differ significantly between the two samples being tested, a test was performed to determine if the samples being tested had equal variance for the given protein (this is a requirement for the *t*-test). The test used to determine this was the variance ratio test. The null hypothesis (H_o) was:

$$H_0 : \sigma_1^2 = \sigma_2^2$$

This null hypothesis asked what was the probability of taking two samples from two populations having identical variances and having the two sample variances be as different as s_1^2 and s_2^2. If the probability was low (≤ 0.05), the validity of H_0 was rejected. If the probability of H_0 is greater than α, it was said that there was insufficient evidence to conclude that the variances of the two populations are different. The variance ratio was calculated as shown below:

$$F_{s,\alpha(2)\nu 1,\nu 2} = \frac{S_1^2}{S_2^2}$$

For this ratio, the larger sample variance was termed s_1^2. The F_s value was then located in the F-distribution , and probability values assigned to it.

Whenever critical values were required for a statistical test, and the appropriate table did not contain a value for the appropriate degrees of freedom, linear interpolation was used to estimate the probability value.

When a protein did not have a normal distribution, or the variances of a pair of proteins being compared was significantly different, a non-parametric test was used to assess if the two samples were significantly different. The test used for this was the Wilcoxon Two Sample Test. The H_0 for the test was that the two samples came from populations having identical distributions. The method used for the analysis is given below.

All observations were pooled and ranked from low to high. Average ranks were given in the case where variates had the same value. The ranks were then assigned to their respective variable and summed. The Wilcoxon statistic (C) was calculated as follows:

$$C = n_1 n_2 + \frac{n_2 (n_2 + 1)}{2} - \sum_{i=1}^{n_2} R$$

Where R is ranks and n_2 is the sample with the smallest size

The C statistic was compared with $n_1 n_2$ - C. The greater of the two values was chosen as the test statistic, U_s. This value was compared to the critical values for $U_{\alpha,n1,n2}$ as given in a table of Values of the Mann-Whitney U distribution (eg. Zar, 1974). In those cases where n_1 was greater than 20, as in the pooled experiments, the following value was calculated:

$$t_s = \frac{(U_s - \frac{n_1 n_2}{2})}{\sqrt{n_1 n_2 \frac{(n_1 + n_2 + 1)}{12}}}$$

where the denominator 12 is a constant.

This expression is distributed (approximately) as a normal deviate. The significance of t_s was obtained from a table of critical values of the t-Distribution against the values of $t_{\alpha[\infty]}$ (for a 2-tailed test). In those instances where tied values occurred, the above formula was modified as follows (after Sokal *et al.*, (1969)):

$$t_s = \frac{\left(U_s - \frac{n_1 n_2}{2} \right)}{\sqrt{\left(\frac{n_1 n_2}{(n_1 + n_2)(n_1 + n_2 - 1)} \right) \left(\frac{(n_1 + n_2)^3 - (n_1 + n_2 - \sum_{i=1}^{m} T_j}{12} \right)}}$$

In the above equation, $\sum_{i=1}^{m} T_j$ is a function of t_j, the number of variates tied in the j^{th} group of ties (the t is not related to the Student t). The function is : $T_j = t_j^3 - t_j$. The T_j values were obtained from those tabled in Sokal *et al.*, (1969).

All of the results presented in this thesis were obtained for logratio transforms, despite whether the equality of variance and normality criteria were satisfied. In those instances where these criteria were not met, the above methods were used to assess the validity of the results obtained for the logratio data. Always, the conclusions were the same. I think that this clearly illustrates the robustness and rigour of the logratio based method of analysis for quantitative 2-dimensional PAGE.

MS Patient Summary.

The following table summarises the major clinical symptoms of the individuals included in the MS study. Due to patient confidentiality, detailed descriptions of each patient are not included, although detailed records of each patient are kept by Wellington Hospital.

Table A2.1. MS Patient Summary.

	Sample Code	Age	Major Clinical Symptom	Hospital Number
Control Males				
1	C008	26	Headache	DZF8571
2	C014	42	Back Pain	FFJ5558
3	C017	31	Back Pain	BAB2952
4	C018	26	Back Pain	EXW9221
MS Males				
1	M003	27	Brain Stem Lesion	FGF7761
2	M007	36	Brain Stem Lesion	AAM8358
3	M016	41	Cervical Cord Lesion	FGB1880
4	M024	45	Brain Stem Lesion	BQJ3870

Table A2.1(contd.). MS Patient Summary.

	Sample Code	Age	Major Clinical Symptom	Hospital Number
Control Females				
5	C002	54	Stroke	FHP6330
6	C009	22	Depression	FGG7473
7	C011	16	Back Pain	FHW4106
8	C015	47	Anxiety	AYZ5958
9	C019	68	Occular Palsy	BQP3320
10	C020	40	Neck Pain	CVP0742
MS Females				
5	M002	37	Optic Neuritis	FGF8806
6	M004	37	Cervical Cord Lesion	AFJ4642
7	M006	39	Hemianopsia	DXA3417
8	M013	42	Brain Stem Lesion	CZW3802
9	M014	36	Cervical Cord Lesion	DJB2930
10	M019	36	Brain Stem Lesion	EAS2203

References.

Adinolfi M., and Adinolfi A. (1976) Ontogeny of human plasma proteins: detection of the onset and site of synthesis using genetic markers and in vitro cultures. In Allison A. (Ed.) *Structure and Function of Plasma Proteins* (Vol. 1) pp. 23-26 Plenum Publishing, New York.

Ai K.Z., Vermuyten K., DeDeyn P.P., Lowenthal A., and Karcher D. (1989) A serum protein involved in aging. *Molecular Chemical Neuropathology* **11:** 131-141

Aisen P.S., and Davis K.L. (1994) Inflammatory mechanisms in Alzheimer's disease: implications for therapy. *American Journal of Psychiatry* **151:** 1105-1113

Aitchison J. (1986) *The Statistical Analysis of Compositional Data.* Chapman and Hall, London

Alafuzoff I., Adolfsson R., Bucht G., Jellum E., Mehta P.D., and Winblad B. (1986) Isoelectric focusing and two-dimensional gel electrophoresis in plasma and cerebrospinal fluid from patients with dementia. *European Neurology* **25:** 285-289

Allen G. (1986) Sequencing of Proteins and Peptides Elsevier, Amsterdam

Altschul S.F., Gish W., Miller W., Myers E.W., and Lipman D.J. (1990) Basic local alignment search tool. *Journal of Molecular Biology* **215:** 403-410

Amaducci L., and Lippi A. (1990) Diagnosis and care of Alzheimer's disease patients: A European perspective. *Advances in Neurology* **51:** 7-13

Ameglio F., Giannarelli D., Cordiala-Fei P., Pietravelle M., Alemanno L., Paone G., Amicosante M., Saltini C., and Bisetti A. (1994) Use of discriminant analysis to assess disease activity in pulmonary tuberculosis with a panel of specific and nonspecific serum markers. *American Journal of Clinical Pathology* **101:** 719-725

Ames G.F.-L., and Nikaido K. (1976) Two-dimensional electrophoresis of membrane proteins. *Biochemistry* **15:** 616-623

Anderson N.L., Wiltsie J.J., Li C.Y., Willard-Gallo K.E., Tracy R.P., Young D.S., Powers M.T., and Anderson N.G. (1983) Analysis of human leukemic cells using two-dimensional electrophoresis. I. Results of a pilot study. *Clinical Chemistry* **29:** 762-767

Anderson N.L., and Anderson N.G. (1991) A two-dimensional gel database of human plasma proteins. *Electrophoresis* **12:** 883-906

Anderson N.L., and Anderson N.G. (1977) High resolution two-dimensional electrophoresis of human plasma proteins. *Proceedings of the National Academy of Science (USA)* **74:** 5421-5425

Appel R.D., Sanchez J.C., Bairoch A., Golaz O., Miu M., Vargas J.R., and Hochstrasser D.F. (1993) SWISS-2DPAGE: A database of two-dimensional gel electrophoresis images. *Electrophoresis* **14:** 1232-1238

Arcone R., Gualandini G., and Ciliberto G. (1988) Identification of sequences responsible for acute-phase induction of human C-reactive protein. *Nucleic Acids Research* **16:** 3195-3207

Arnaud P., Miribel L., and Roux A.F. (1988) Alpha 1-acid glycoprotein. In Colowick S.P., and Kaplan N.O. (Eds.) *Methods in Enzymology* **163:** 418-430

Back S., and Alhadeff J.A. (1992) Differential isoform profiles of α_2-macroglobin from plasma of patients with chronic-progressive or relapsing-remitting multiple sclerosis. *Clinica Chemica Acta* **211:** 27-36

Baker H.F., Ridley R.M., Duchen L.W., Crow T.J., and Bruton C.J. (1993) Experimental induction of beta-amyloid plaques and cerebral angiopathy in primates. *Annals of the New York Academy of Sciences* **695:** 228-231

Barinaga M. (1994) Possible new test found for Alzheimer's Disease. *Science* **266:** 973

Barrett T., and Gauld H.T. (1973) Tissue and species specificity of non-histone chromatin proteins. *Biochimica et Biophysica Acta* **294:** 165-171

Bates D. (1990) Dietary lipids and multiple sclerosis. *Upsala Journal of Medical Science (Suppl. WRH)* **48:** 173-187

Baumann H., Wom K.-A., and Jahreis G.P. (1987) Distinct sets of acute phase plasma proteins are stimulated by separate human hepatocyte-stimulating factors and monokines in rat hepatoma cells. *The Journal of Biological Chemistry* **262:** 9756-9768

Beeley J.A., Khoo K.S., and Lamey P.-J. (1991) Two-dimensional electrophoresis of human parotid salivary proteins from normal and connective tissue disorder subjects using immobilised pH gradients. *Electrophoresis* **12:** 493-499

Benditt E.P., Eriksen N., and Meek R.L. (1988) Serum Amyloid A protein. In Colowick S.P., and Kaplan N.O. (Eds.) *Methods in Enzymology* **163:** 510-523

Bever C. (1994) The current status of studies of aminopyridines in patients with multiple sclerosis. *Annals of Neurology* **36:** s118-s121

Binzer M., Forsgren L., Holmgren G., Drugge U., and Fredrikson S. (1994) Familial clustering of multiple sclerosis in a northern Swedish rural district. *Journal of Neurology, Neurosurgery, and Psychiatry* **57:** 497-499

Bodovitz S., Falduto M.T., Frail D.E., and Klein W.L. (1995) Iron levels modulate alpha-secretase clevage of amyloid precursor protein. *Journal of Neurochemistry* **64:** 307-315

Bondareff W., Mountjoy C.Q., Wischik C.M., Hauser D.L., LaBree L.D., and Roth M. (1993) Evidence of subtypes of Alzheimer's disease and implications for aetiology. *Archives of General Psychiatry* **50:** 350-356

Bowen D.M. (1990) Treatment of Alzheimer's disease: Molecular pathology versus neurotransmitter based therapy. *British Journal of Psychiatry* **157:** 327-330

Bradford M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72:** 248-254

Breithaupt T.B., Nystrom I.E., Hodges D.H. Jr., and Babitch J.A. (1978) Composition of an isoelectric focusing gel yeilding a broad pH gradient. *Analytical Biochemistry* **84:** 579-582

Brody J.A. (1982) An epidemiologist's views on senile dementia; facts and fragments. *American Journal of Epidemiology* **115:** 155-162

Brown W.T., Dutkowski R., and Darlington G.J. (1981) Localisation and quantitation of human superoxide dismutase using computerised 2D gel analysis. *Biochemical and Biophysical Research Communications* **102:** 675-681

Burgess-Cassler A., Johansen J.J., and Kendrick N.C. (1992) Two-dimensional gel analysis of serum apolipoprotein A-I isoforms. *Applied Theoretical Electrophoresis* **3:** 41-45

Burgess-Cassler A., Johansen J.J., Santek D.A., Ide J.R., and Kendrick N.C. (1989) Computerised quantitative analysis of Coomassie Blue stained serum proteins separated by two-dimensional electrophoresis. *Clinical Chemistry* **35:** 2297-2304

Cantrell, S.J., Babitch J.A., and Torres S. (1981) Protein load effects on the pH gradient of isoelectric focusing in polyacrylamide gels. *Analytical Biochemistry* **116:** 168-173

Carleton-Gajdusek D., Beyreuther K., Brown P., Cork L.C., Cunningham D.D., Frangione B., Gibbs C.J., Goldfarb L.G., Goldgaber D., Hsaio K.K., Koo E.H., Martin L.J., Masters C.L., Odenwald W.F., Price D.L., Prusiner S.B., Ruddle F.H., Safar J., Scangos G., Schmechel D.E., Shashikant C.S., Shlichta P.J., Sisodia S.S., Trapp B.D., Unterbeck A., Van Nostram W.E., Violet S.M., Walker L.C., and Wirak D. (1991) Regulation and genetic control of brain amyloid. *Brain Research Reviews* **16:** 83-114

Carmela A.R., Driscoll J., Potter H., Van Nostrand W.E., and Tempst P. (1991) A calcium activated protease from Alzheimer's disease brain cleaves at the N-terminus of the amyloid β-protein. *Biochemical and Biophysical Research Communications* **174:** 790-796

Chan-Palay V. (1990) Neuronal communication breakdown in neurotransmitter systems in Alzheimer's and Parkinson's dementias. *Journal of Neurocytology* **19:** 802-807

Chial H.J., and Splittgerber A.G. (1993) A comparison of the binding of Coomassie brilliant blue to proteins at low and neutral pH. *Analytical Biochemistry* **213:** 362-369

Clark P.M.S., Kricka L.J., and Whitehead T.P. (1980) Pettern of urinary proteins and peptides in patients with rheumatoid arthritis investigated with the ISO-DALT technique. *Clinical Chemistry* **26:** 201-204

Clarke M.S.F., and West D.C. (1991) The identification of proliferation and tumor-induced proteins in human endothelial cells: A possible target for tumor therapy. *Electrophoresis* **12:** 500-508

Clauberg M., and Joshi J.G. (1993) Regulation of serine protease activity by aluminium: implications for Alzheimer's disease. *Proceedings of the National Academy of Sciences (USA)* **90:** 1009-10012

Clemetson K.J., McGregor J.L., James E.J., Dechavanne M., and Luscher E.F. (1982) Characterisation of the platelet membrane glycoprotein abnormalities in Bernard-Soulier syndrome and comparrison with normals by surface labeling techniques and high resolution two-dimensional electrophoresis. *Journal of Clinical Investigation* **70:** 304-311

Cogdon R.W., Muth G.W., Splittgerber A.G. (1993) The binding interaction of Coomassie blue with proteins. *Analytical Biochemistry* **213:** 407-413

Comings D.E. (1979) Pc-1 Duarte, a common polymorphism of a human brain protein, and its relationship to depressive disease and multiple sclerosis. *Nature* **277:** 28-32

Corrigan F.M., Crichton J.S., Ward N.I., and Horrobin D.F. (1992) Blood tin concentrations in Alzheimer's disease. *Biological Psychiatry* **31:** 749-750

Corrigan F.M., Van Rhijin A.G., Ijomah G., McIntyre F., Skinner E.R., Horrobin D.F., and Ward N.I. (1991) Tin and fatty acids in dementia. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **43:** 229-238

Cowan E., Pierce M., and McFarlin D. (1991) HLA-DR and DQ allelic sequences in multiple sclerosis patients are identical to those found in the general population. *Human Immunology* **32:** 203-210

Danon M.J., Giometti C.S., Manaligod J.R., Peruena O.H., and Skosey J.L. (1981) Adult onset nemaline rods in a patient treated for suspected dermatomyositis: study with two-dimensional electrophoresis. *Archives of Neurology* **38:** 761-766

Davies P. (1991) Therapy for Alzheimer's disease: Choosing a target. *Clinical Neuropharmacology* **14:** s24-s33

Deber C.M., and Reynolds S.J. (1991) Central nervous system myelin: Structure, function, and pathology. *Clinical Biochemistry* **24:** 113-134

Decker M.W., and McGaugh J.L. (1991) The role of interactions between the cholinergic system and other neuromodulatory systems in learning and memory. *Synapse* **7:** 151-168

Delamarche C., Berger F., Gallard L., and Pouplard-Barthelaix A. (1991) Aging and Alzheimer's disease: protease inhibitors in cerebrospinal fluid. *Neurobiology of Aging* **12:** 71-74

Delincee H., and Radola B.J. (1978) Determination of isoelectric points in thin layer isoelectric focusing. *Analytical Biochemistry* **90:** 609-614

DeStrooper B., and Van Leuven F. (1993) α2-Macroglobin and subacute-phase response in Alzheimer's disease. *Immunology Today* **14:** 143-144

Diezel W., Kopperschläger G., and Hofmann E. (1972) An improved procedure for protein staining in polyacrylamide gels with a new type of Coomassie brilliant blue. *Analytical Biochemistry* **48:** 617-621

Dowling P.C., and Cook S.D. (1976) Disease markers in acute multiple sclerosis. *Archives of Neurology* **33:** 668-670

Duff K., and Hardy J. (1995) Mouse model made. *Nature* **373:** 476-477

Dunbar B.S. (1987) *Two dimensional electrophoresis and immunological techniques* Plenum Press, New York

Dunn M.J., and Burghes A.H.M. (1983) High resolution two-dimensional polyacrylamide gel electrophoresis I. Methodological procedures. *Electrophoresis* **4:** 97-116

Dunnett S. (1991) Cholinergic grafts, memory and ageing. *Trends in Neurosciences* **14:** 371-376

Duthie G.G., Arthur J.R., and James W.P. (1991) Effects of smoking and vitamin E on blood antioxidant status. *American Journal of Clinical Nutrition* **53:** s1061-s1063

Eagger S.A., Levy R., and Sahakian B.J. (1991) Tacrine in Alzheimer's disease. *The Lancet* **337:** 989-992

Easty D.J., Patel K., Otto W.R., Dunn M.J., Kiil J., and Evans D.J. (1991) A study of protein synthesis in cells cultured from involved psoriatic skin. *Electrophoresis* **12:** 579-584

Ebers G. (1994) Genetics and multiple sclerosis: an overiew. *Annals of Neurology* **36:** s12-s14

Ebers G.C., Bulman D.E., and Sadovnick A.D. (1986) A population based study of multiple sclerosis in twins. *The New England Journal of Medicine* **315:** 1638-1642

Edwards J.J., Anderson N.G., Tollakson S.L., von Eschenbach A.C., and Guevara J. Jr. (1982) Proteins of human urine. II. Identification by two-dimensional electrophoresis of a new candidate marker for prostatic cancer. *Clinical Chemistry* **28:** 160-163

Edwards J.J., Tollakson S.L., and Anderson N.G. (1981) Proteins of human semen I. Two-dimensional mapping of human seminal fluid. *Clinical Chemistry* **27:** 1335-1340

Elovaara I., Maury C.P., and Palo J. (1986) Serum amyloid A protein, albumin and prealbumin in Alzheimer's disease and in demented patients with Down's syndrome. *Acta Neurologica Scandinavica* **74:** 245-250

Endler A.T. (1987) High resolution two-dimensional electrophoresis of cerebrospinal fluid in patients with neurological diseases. *Journal of Clinical Chemistry and Clinical Biochemistry* **25:** 61-70

Ernst R., and Hay J.W. (1994) The US economic and social cost of Alzheimer's disease revisited. *American Journal of Public Health* **84:** 1261-1264

Farrer L.A., Myers R.H., Connor L., Cupples L.A., and Growdon J.H. (1991) Segregation analysis reveals evidence of a major gene for Alzheimer's disease. *American Journal of Human Genetics* **48:** 1026-1033

Fibiger H.C. (1991) Cholinergic mechanisms in learning, memory, and dementia: A review of recent evidence. *Trends in Neurosciences* **14:** 220-223

Fisher A., Brandeis R., Karton I., Pittel Z., Dachir S., Sapir M., Grunfeld Y., Levy A., and Heldman E. (1990) AF102B: Rational treatment strategy for Alzheimer's disease: Recent advances. *Advances in Neurology* **51:** 257-259

Fitten L.J., Perryman K.M., Gross P.L., Fine H., Cummins J., and Marshall C. (1990) Treatment of Alzheimer's disease with short and long term oral THA and lecithin: A double-blind study. *American Journal of Psychiatry* **147:** 239-242

Flaten T.P. (1989) Mortality from dementia in Norway, 1969-83. *Journal of Epidemiology and Community Health* **43:** 285-289

Forbes E.F., Hayward L.M., Agwini N. (1992) Aluminium and Alzheimer's disease. *Canadian Medical Association Journal* **146:** 1534-1540

Ford H.C. (1985) Abnormalities of serum and plasma components in patients with multiple sclerosis. *Clinical Biochemistry* **18:** 3-13

Foster A.C. (1990) Physiology and pathophysiology of excitatory amino acid neurotransmitter systems in relation to Alzheimer's disease. *Advances in Neurology* **51:** 97-101

Franceschi M., Comola M., Piattoni F., Gaulandri W., and Canal N. (1990) Prevalence of dementia in adult patients with trisomy 21. *American Journal of Medical Genetics* **7:** 306-308

Francis P.T., Palmer A.M., Sims N.R., Bowen D.M., Davison A.N., Esir M.M., Neary D., Snowden J.S., and Wilcock G.K. (1985) Neurochemical studies of early-onset Alzheimer's disease: Possible influence on treatment. *The New England Journal of Medicine* **313:** 7-11

Franzén B., Iwabuchi H., Kato H., Lindholm J., and Auer G. (1991) Two-dimensional polyacrylamide gel electrophoresis of human lung cancer: Qualitative aspects of tissue preparation in relation to histopathology. *Electrophoresis* **12:** 509-515

Fretland D.J. (1992) Potential role of prostaglandins and leukotrienes in multiple sclerosis and experimental allergic encaphalomyelitis. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **45:** 249-257

Friedman A., al-Sabbagh A., Santos L.M., Fishman-Lobell J., Polanski M., Das M.P., Khoury S.J., Weiner H.L. (1994) Oral tolerance: a biologically relevant pathway to generate peripheral tolerance against external and self-antigens. *Chemical Immunology* **58:** 259-290

Fuller G.M., Bunzel R.J., and Nesbit J.E. (1988) Fibrinogen. In Colowick S.P., and Kaplan N.O. (Eds.) *Methods in Enzymology* **163:** 474-485

Fung W.-P., and Schrieber G. (1987) Structure and expression of the genes for major acute phase α_1-protein(thiostatin) and kininogen in the rat. *The Journal of Biological Chemistry* **262:** 9298-9308

Games D., Adam D., Alesandrini R., Barbour R., Berthelette P., Blackwell C., Carr T., Clemens J., Donaldson T., Gillespie F., Guido T., Hagoplin S., Johnson-Wood K., Khan K., Lee M., Leibowitz P., Leiberburg I., Little S., Masliah E., McConlogue L., Montoya-Zavala M., Mucke L., Paganini L., Penniman E., Power M., Schenk D., Seubert P., Snyder B., Sorlano F., Tan H., Vitale J., Wadsworth S., Wolozin B., and Zhoa J. (1995) Alzheimer-type neuropathology in transgenic mice overexpressing V717F βamyloid precursor protein. *Nature* **373:** 523-527

Gajdusek D.C., Beyreuther K., and Wirak D. (1991) Regulation and genetic control of brain amyloid. *Brain Research Reviews* **16:** 83-114

Gandy S., and Greengard P. (1992) Amyloidogenesis in Alzheimer's disease: Some possible therapeutic opportunities. *Trends in Pharmacological Sciences* **13:** 108-113

Ganong W. (1985) *Review of Medical Physiology (Twelfth Edition).* Lange Medical Publications, California

Garrels J. (1983) Quantitative two-dimensional gel electrophoresis of proteins. In Colowick S.P., and Kaplan N.O. (Eds.) *Methods in Enzymology* **100:** 411-423

Gauldie J., Richards C., Harnish D., Lansdorp P., and Baumann H. (1987) Interferon β_2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. *Proceedings of the National Academy of Sciences* (USA) **84:** 7251-7255

Gehring M.R., Shiels B.R., Northemann W., deBruijn M.H., Kan C.C., Chain A.C., Noonan D.J., and Fey G.H. (1987) Sequence of rat liver α_2-macroglobin and acute phase control of its messenger RNA. *The Journal of Biological Chemistry* **262:** 446-454

Gelman B.B., Rifai N., Christenson R.H., and Silverman L.M. (1988) Cerebrospinal fluid and plasma apolipoproteins in patients with multiple sclerosis. *Annals of Clinical Laboratory Science* **18:** 46-52

Gentleman S., and Roberts G. (1992) Risk factors in Alzheimer's disease. *British Medical Journal* **304:** 118-119

Gerard N.P., Hodgest M.K., Drazen J.M., Weller P.F., and Gerard C. (1989) Characterization of a receptor for C5a anaphylatoxin on human eosinophils. *Journal of Biological Chemistry* **264:** 1760-1766

Giblett E. (1974) Haptoglobin. In Allison A.C., (Ed.) (1976) *Structure and Function of Plasma Proteins* (Vol. 1) pp55-72 Plenum Publishing, New York.

Giometti C., and Anderson N.G. (1984) Protein changes in activated human platelets. *Clinical Chemistry* **30:** 2078-2083

Giometto B., Argentiero V., Sanson F.J., Ongaro G., and Tavolato B.H. (1988) Acute phase proteins in Alzheimer's disease. *European Neurology* **28:** 30-33

Goldman D., Merril C.R., Polinski R.J., and Ebert M.H. (1982) Lymphocyte patterns in Huntington's disease: quantitative analysis by use of two-dimensional electrophoresis and computerised densitometry. *Clinical Chemistry* **28:** 1021-1025

Goldman D., Merril C.R., and Ebert M.H. (1980) Two-dimensional gel electrophoresis of cerebrospinal fluid proteins. *Clinical Chemistry* **26:** 1317-1322

Goldstein S., and Reivich M. (1991) Cerebral blood flow and metabolism in aging and dementia. *Clinical Neuropharmacology* **14:** s34-s44

Goldstein G.W., and Betz A.L. (1986) The blood brain barrier. *Scientific American* **225:** 70-79

Goodin D.S. (1991) The use of immunosuppressive agents in the treatment of multiple sclerosis. *Neurology* **41:** 980-985

Gordon J. (1981) Structure and function of platelets. In Gordon J. (Ed.) *Platelets in Biology and Pathology (Vol. 2)*: pp1-17 Elsevier/ North-Holland Biomedical Press

Gorelick P.B., and Bózzola F.G. (1991) Alzheimer's disease. *Postgraduate Medicine* **89:** 231-240

Gorg A. (1991) Two-dimensional electrophoresis. *Nature* **349:** 545-546

Granner D.K. (1988) Thyroid Hormones. In Murray R.K., Granner D.K., Mayes P.A., and Rodwell V.W. (Eds.) *Harper's Biochemistry (Twenty First Edition)* pp496-502 Prentice-Hall Publishing, California

Grasso M.G., Cutrupi F., Bernardi S., Trabace S., Pozzilli C., Cappellacci S., and Fieschi C. (1992) HLA determinants in familial multiple sclerosis. *Neuroepidemiology* **11:** 85-89

Grover P.K., Resnick M.I. (1993) Two-dimensional analysis of proteins in unprocessed human urine using double stain. *Journal of Urology* **150:** 1069-1072

Guyton A. (1986) *Textbook of Medical Physiology (Seventeenth Edition).* Igaku-Shoin Saunders, New York

Guze B.H., Hoffman J.M., Mazziotta J.C., Baxter L.R., and Phelps M.E. (1992) Positron emission tomography and familial Alzheimer's disease: A pilot study. *Journal of the American Geriatric Society* **40:** 120-123

Hajimohammadreza I., and Brammer M. (1990) Brain membrane fluidity and lipid peroxidation in Alzheimer's Disease. *Neuroscience Letters* **112:** 333-337

Hames B. (1981) An introduction to polyacrylamide gel electrophoiresis. In Hames B. and Rickwood D. (eds.) (1981) *Gel electrophoresis of proteins: A practical approach* IRL Press, Oxford.

Hansen T.M. (1994) Biochemical markers in rheumatoid arthritis. *Nordic Medicine* **109:** 228-229

Harbige L.S.,Jones R., Jenkins R., Fitzgerald G., Forti A., and Budowski P. (1990) Nutritional management in multiple sclerosis with reference to experimental models. *Upsala Journal of Medical Science* **48:** 189-207

Hardy J.A., and Mullan M. (1992) In search of the soluble. *Nature* **359:** 268-269

Hardy J.A., and Higgins G.A. (1992) Alzheimer's disease: The amyloid cascade hypothesis. *Science* **256:** 184-185

Harik S.I, and Kalaria R.N. (1991) Blood-brain barrier abnormalities in Alzheimer's disease. *Annals of the New York Academy of Sciences* **640:** 47-52

Harrington M.G., Aebersold R., Martin B.M., Merril C.R., and Hood L. (1993) Identification of a brain specific human cerebrospinal fluid glycoprotein, beta-trace protein. *Applied Theoretical Electrophoresis* **3:** 229-234

Harrison H.H., Miller K.L., Dickinson C., and Daufeldt J.A. (1992) Quality assurance and reproducibility of high resolution two-dimensional electrophoresis and silver staining in polyacrylamide gels. *Clinical Chemistry* **97:** 97-105

Hashizume M., Kitano S., Yamaga H., and Sugimachi K. (1988) Haptoglobin to protect against renal damage from ethanolamine oleate sclerosant. *Lancet* (8606) **2:** 340-341

Hedo C.C., Aken'ova Y.A., Okpala I.E., Durojaiye A.O., and Salimonu L.S. (1993) Acute phase reactants and severity of homozygous sickle cell disease. *Journal of Internal Medicine* **233:** 467-470

Heinrich P.C., Castell C.V., and Andus T. (1990) Interleukin-6 and the acute phase response. *Biochemical Journal* **256:** 621-635

Heiss W.D., Szelies B., Kessler J., and Herholz K. (1991) Abnormalities of energy metabolism in Alzheimer's disease studied with PET. *Annals of the New York Academy of Sciences* **640:** 65-71

Hendrie H.C., Hall K.S., Hui S., Unverzagt F.W., Yu C.E., Lahiri D.K., Sahota A., Farlow M., Musick B., and Class C.A. (1995) Appolipoprotein E genotypes and Alzheimer's disease in a community study of elderly African Americans. *Annals of Neurology* **37:** 118-120

Hieber A.D., and Christie D.L. (1993) Characterisation of glycoprotein II from bovine adrenal medullary chromafin granules. Identification of components representing the secretory vesicle counterparts of the lysosomal-associated membrane glycoproteins (lamp-1 and lamp-2). *Journal of Biological Chemistry* **268:** 11073-11078

Higginbotham J.W., Smith J.S.C., and Smith O.S. (1991) Quantitative analysis of two-dimensional protein profiles of inbred lines of maize (*Zea mays* L.). *Electrophoresis* **12:** 425-431

Hintzen R.Q., Polman C.H., Lucas C.J., and van Lier R.A.W. (1992) Multiple sclerosis: immunological findings and possible implications for therapy. *Journal of Neuroimmunology* **39:** 1-10

Hoyer S. (1991) Abnormalities of glucose metabolism in Alzheimer's disease. *Annals of the New York Academy of Sciences* **640:** 53-58

Hoyer S., Nitsch R., and Oesterreich K. (1990) Ammonia is endogenously generated in the brain in the presence of presumed and verified dementia of Alzheimer type. *Neuroscience Letters* **117:** 358-362

Hughes R.A.C. (1991) Prospects for the treatment of multiple sclerosis. *Journal of the Royal Society of Medicine* **84:** 63-65

Humphery-Smith I., des Francs-Small C.C., Ambart-Bretteville F., and Remy R. (1992) Tissue specific variation of pea mitochondrial polypeptides detected by computerised image analysis of two-dimensional electrophoresis gels. *Electrophoresis* **13:** 168-172

Hwanghee B.L., Yoo O.J., and Lee M.H. (1992) Serum α1-antitrypsin in patients with hepatocellular carcinoma. *Clinica Chimica Acta* **206:** 225-230

Inestrosa N.C., Alarcon R., Arriagada J., Donoso A., and Alvarez J. (1993) Platelets of Alzheimer's patients: increased counts and subnormal uptake and accumulation of ^{14}C 5-hydroxytryptamine. *Neuroscience Letters* **163:** 8-10

Ishiura S. (1991) Proteolytic cleavage of the Alzheimer's disease amyloid A4 precursor protein. *Journal of Neurochemistry* **56:** 363-369

Jacobs L., Goodkin D.E., Rudick R.A., and Herndon R. (1994) Advances in specific therapy for multiple sclerosis. *Current Opinions in Neurology* **7:** 250-254

Jentoft N., and Dearborn D.G. (1979) Labelling of proteins by reductive methylation using sodium cyanoborohydride. *Journal of Biological Chemistry* **254:** 4359-4365

John H.A., Purdom I.F. (1989) Elevated plasma levels of haptoglobin in Duchenne muscular dystrophy: electrophoretic variants in patients with a severe form of the disease. *Electrophoresis* **10:** 489-493

Johnson G., Brane D., Block W., van Kammen D.P., Gurklis J., Peters J.L., Wyatt R.J., Kirch D.G., Ghanbari H.A., and Merril C.R. (1992) Cerebrospinal fluid protein variations in common to Alzheimer's disease and schizophrenia. *Applied Theoretical Electrophoresis* **3:** 47-53

Jorm A.F., Henderson A.S., and Jacomb P.A. (1989) Regional differences in mortality from dementia in Australia: An analysis of death certificate data. *Acta Psychiatra Scandinavica* **79:** 179-185

Kahana E., Zilber N., Abramson J.H., Biton V., Leibowitz Y., and Abramsky O. (1994) Multiple sclerosis: genetic versus environmental aetiology. *Journal of Neurology* **241:** 341-346

Kalaria R.N. (1992) The blood-brain barrier and cerebral microcirculation in Alzheimer's disease. *Cerebrovascular and Brain Metabolism Reviews* **4:** 226-260

Kalmovarin N., Friedrichs W.E., O'Brien H.V., Linehan L.A., Bowman E.H., and Yang F. (1991) Extrahepatic expression of plasma protein genes during inflammation. *Inflammation* **15:** 396-379

Kaltschmidt E., and Wittman H.G. (1970) Ribosomal proteins VII. Two-dimensional polyacrylamide gel electrophoresis for finger printing of ribosomal proteins. *Analytical Biochemistry* **36:** 401-412

Kanof P.D., Mohs R.C., Gross J., Davidson M., Bierer L.M., and Davis K.L. (1991) Platelet phospholipid synthesis in Alzheimer's disease. *Neurobiology of Aging* **12:** 65-69

Katzman R. and Saitoh T. (1991) Advances in Alzheimer's disease. *The FAESB Journal* **5:** 278-286

Katzman R. (1986) Alzheimer's disease. *The New England Journal of Medicine* **314:** 964-973

Kawai M., Crass P., Richey P., Tobaton M., Lowery D.E., Gonzalez-DeWhitt., Greenburg B.D., Gambetti P., and Perry G. (1992) Subcellular localization of amyloid precursor protein in senile plaques of Alzheimer's disease. *American Journal of Pathology* **104:** 947-958

Kida E., Golabek E.E., Wisniewski T., and Wisniewski K.E. (1994) Regional differences in apolipoprotein E immunoreactivity in diffuse plaques in Alzheimer's disease brain. *Neuroscience Letters* **167:** 73-76

Kinnunen E., Juntunen J., and Ketonen L. (1988) Genetic susceptibility to multiple sclerosis. A co-twin study of a nationwide series. *Archives of Neurology* **45:** 1108-1111

Koj A., Gauldie J., Regoeczi E., Sauder D.N., and Sweeney G.D. (1984) The acute phase response of cultured rat hepatocytes. *Biochemical Journal* **224:** 505-514

Koj A. (1976) The acute phase response. In Allison A.C. (ed.) (1976) *Structure and Function of Plasma Proteins* (Vol. 1) Plenum Publishing, New York

Kokmen E., Naessens J.M., and Offord K.P. (1987) A short test of mental status: description and preliminary results. *Mayo Clinic Proceedings* **62:** 281-87

Kuick R.D., Hanash S.M., Chu E.H.Y., and Strahler J.R. (1987) A comparison of some adjustment techniques for use with quantitative spot data from two-dimensional gels. *Electrophoresis* **8:** 199-204

Kurtzke J.F. (1991) Multiple sclerosis: changing times. *Neuroepidemiology* **10:** 1-8

Kurtzke J.F. (1993) Epidemiological evidence for multiple sclerosis as an infection. *Clinical Microbiology Reviews* **6:** 382-427

Kushner I. (1988) The acute phase response: an overview. In Colowick S.P., and Kaplan N.O. (Eds.) *Methods in Enzymology* **163:** 373-383

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **237:** 680-685

Lantos P.L., Luthert P.J., Hanger D., Anderton B.H., Mullan M., and Rossor M. (1992) Familial Alzheimer's disease with the amyloid precursor protein position 717 mutation and sporadic Alzheimer's disease have the same cytoskeletal pathology. *Neuroscience Letters* **137:** 221-226

Lapin A., and Feigl W. (1991) A practicable two-dimensional electrophoresis of urinary proteins as a useful tool in medical diagnosis. *Electrophoresis* **12:** 472-477

Lee P.N. (1994) Smoking and Alzheimer's disease: a review of the epidemiological evidence. *Neuroepidemiology* **13:** 131-144

Lee V. M-Y., Balin B.J., Otvos L.Jr., Trojanowski J.Q. (1991) A68: A major subunit of paired helical filaments and derivatized forms of normal tau. *Science* **251:** 675-678

Levenson R.M., Anderson G.M., Cohn J.A., and Blackshear P.J. (1990) Giant two-dimensional gel electrophoresis: Methodological update and comparison with intermediate-format gel systems. *Electrophoresis* **11:** 269-279

Levenson R.M., Anderson G.M., Cohn J.A., and Blackshear P.J. (1989) Insulin stimulated protein tyrosine phosphorylation in intact cells evaluated by giant two-dimensional gel electrophoresis. *The Journal of Biological Chemistry* **264:** 19984-19993

Li Q.X., Berndt M.C., Bush A.I., Rumble B., Mackenzie I., Friedhuber A., Beyreuther K., and Masters C.L. (1994) Membrane associated forms of the beta A4 amyloid protein precursor of Alzheimer's disease in human platelet and brain: surface expression on the activated human platelet. *Blood* **84:** 133-142

Lindberg C., Andersen O., Vahlne A., Dalton M., and Runmarker B. (1991) Epidemiological investigation of the association between mononucleosis and multiple sclerosis. *Neuroepidemiology* **10:** 62-65

Lindvall O. (1991) Prospects of transplantation in human neurodegenerative diseases. *Trends in Neurosciences* **14:** 376-384

Lopez O.L., Boller F., Becker J.T., Miller M., and Reynolds C.F. (1990) Alzheimer's disease and depression: neuropsychological impairment and progression of the illness. *American Journal of Psychiatry* **147:** 855-860

Lowell C.A., Potter D.A., Stearman R.S., and Morrow J.F. (1986) Structure of the murine serum amyloid-A gene family. *The Journal of Biological Chemistry* **261:** 8442-8452

Lustbader J.W., Arcoleo J.P., Birken S., and Greer J. (1983) Hemoglobin binding site on haptoglobin probed by selective proteolysis. *Journal of Biological Chemistry* **258:** 1227-1234

Maeda N. (1991) DNA polymorphisms in the controlling region of the human haptoglobin genes: a molecular explanation for the haptoglobin 2-1 modified phenotype. *American Journal of Human Genetics* **49:** 158-166

Maes M., Scharpe S., Van Grootel L., Uyttenbroeck W., Coorman W., Cosyns P., and Suy E. (1992) Higher α1-antitrypsin, haptoglobin, ceruloplasmin and lower retinol binding protein plasma levels during depression: Further evidence for the existence of an inflammatory response during that illness. *Journal of Affective Disorders* **24:** 183-192

Maes M., Scharpe S., Bosmans E., Vanderwoude M., Suy E., Uyttenbroeck W., Coorman W., Vandervorst C., and Raus J. (1992a) Disturbances in acute phase plasma proteins during melancholia: additional evidence for the presence of an inflammatory process during that illness. *Progress in Neuropsychpharmacology and Biological Psychiatry* **16:** 501-515

Mann D.M.A. Jones D., South P.W., Snowden J.S., Neary D. (1992) Deposition of amyloid β protein in non-Alzheimer dementias: Evidence for neuronal origin of parenchymal deposits of β protein in neyrodegenerative disease. *Acta Neuropathologica* **83:** 415-419

Mann D.M.A., Jones D., Snowden J.S., Neary D., and Hardy J. (1992a) Pathological changes in the brain of a patient with familial Alzheimer's disease having a missense mutation at codon 717 in the amyloid precursor protein gene. *Neuroscience Letters* **137:** 225-228

Manuelidis E.E., and Manuelidis L. (1991) Search for a transmissible agent in Alzheimer's disease: Studies of human buffy coat. *Current Topics in Microbiology and Immunology* **172:** 275-280

Martin R., McFarland H.F., and McFarlin D.E. (1992) Immunological aspects of demyelinating diseases. *Annual Review of Immunology* **10:** 153-187

Martin-Loeches M., Gil P., Jimenez F., Exposito F.J., Miguel F., Cacabelos R., and Rubia F.J. (1991) Topographic maps of brain electrical activity in primary degenerative dementia of the Alzheimer-type and multi-infarct dementia. *Biological Psychiatry* **29:** 211-223

Martyn C.N., Cruddas M., and Compston D.A. (1993) Symptomatic Epstein-Barr virus infection and multiple sclerosis. *Journal of Neurology, Neurosurgery, and Psychiatry* **56:** 167-168

Martyn C.N and Pippard E.C. (1988) Usefullness of mortality data in determining the geography and time trends of dementia. *Journal of Epidemiology and Community Health* **42:** 134-137

Masters C.L., and Beyreuther K. (1990) Protein abnormalities in neurofibrillary tangles: Their relation to the extracellular amyloid deposits of the A4 protein in Alzheimer's disease. *Advances in Neurology* **51** 151-161

Matheka H.D., Enzmann P.J., Bachrach H.L., and Migl B. (1977) The influence of sodium dodecyl sulphate from different sources on the separation of virus proteins in polyacrylamide gel electrophoresis. *Analytical Biochemistry* **81:** 9-17

Matsubara E., Hirai S., Amari M., Shoji M., Yamaguchi H., Okamoto K., Ishiguru K., Harigaya Y., and Wakabayashi K. (1990) Alpha 1-antichymotrypsin as a possible biochemical marker for Alzheimer-type dementia. *Annals of Neurology* **28:** 561-567

Matsuyama S.S., and Jarvik L.F. (1989) Hypothesis: Microtubules, a key to Alzheimer's disease. *Proceedings of the National Academy of Sciences* (USA) **86:** 8152-8156

Mattila K.M., and Frey H. (1994) Alzheimer brain proteins investigated by two-dimensional gel electrophoresis with immobilized pH gradients in the first dimension. *Electrophoresis* **15:** 721-725

Mattila K.M., Pirtilla T., Blennow K.A., Wallin A., Viitanen M.N., and Frey H. (1994a) Altered blood-brain-barrier function in Alzheimer's disease? *Acta Neurologica Scandinavica* **89:** 192-198

Mattson M.P. (1992) Effects of microtubule stabilization and destabilization on tau immunoreactivity in cultured hippocampal neurons. *Brain Research* **582:** 107-118

Mauch E., and Kornhuber H.H. (1993) Immunosuppressive therapy of multiple sclerosis with mitoxantrone. *Fortschre Neurologre and Psychiatrie* **61:** 410-417

Mavra M.M., Luxton R., Keir G., and Thompson E.J. (1992) Oligoclonal immunoglobin D in the cerebrospinal fluid of neurologic patients. *Neurology* **42:** 1244-1245

Maytin E.V., Colbert R.A., and Young D.A. (1985) Early heat shock proteins in primary thymocytes. *The Journal of Biological Chemistry* **260:** 2384-2392

McAlpine D., Lumsden C.F., and Acheson E.D. (eds.) (1972) *Multiple Sclerosis: A Reappraisal.* Williams and Wilkans, Baltimore.

McConkey E.H. (1979) Double label autoradiography for comparison of complex protein mixtures after gel electrophoresis. *Analytical Biochemistry* **96:** 39-44

McDonald W.I. (1986) The mystery of the origin of multiple sclerosis. *Journal of Neurology, Neurosurgery, and Psychiatry* **49:** 113-123

McGeer P.L., and Rogers J. (1992) Anti-inflammatory agents as a therapeutic approach to Alzheimer's disease. *Neurology* **42:** 447-449

McKhann G., Drachman D., Folstein M., Katzman R., Price D., and Stadlan E.M. (1984) Clinical diagnosis of Alzheimer's disease: Report of the NINCDS-ARDA work group under the auspices of Department of Health and Human Services Task Force on Alzheimer's disease. *Neurology* **34:** 939-944

McLean B.N., Zeman A.Z.J., Barnes D., and Thompson E.J. (1993) Patterns of blood-brain barrier impairment and clinical features in multiple sclerosis. *Journal of Neurology, Neurosurgery, and Psychiatry* **56:** 356-360

McLeod J.G., Hammond S.R., Hallpike J.F. (1994) Epidemiology of multiple sclerosis in Australia. *Medical Journal of Australia* **160:** 117-122

McLeod J.G. (1982) Multiple sclerosis: A review. *The Australian and New Zealand Journal of Medicine* **12:** 302-308

McRae A., Dahlstrom A., Polinski R., and Ling E.A. (1993) Cerebrospinal fluid microglia antibodies: potential diagnostic markers for immune mechanisms in Alzheimer's disease. *Behavioural Brain Research* **57:** 225-34

Means G.E., and Freeney R.E. (1968) Reductive alkylation of amino groups in proteins. *Biochemistry* **7:** 2192-2201

Mehta P. D. (1991) Diagnostic usefulness of cerebrospinal fluid in multiple sclerosis. *Critical Reviews in Clinical Laboratory Sciences* **28:** 233-245

Mendez M.F., Martin R.J., Smyth K.A., Whitehouse P.J. (1990) Psychiatric symptoms associated with Alzheimer's disease. *Journal of Neuropsychiatry and Clinical Neurosciences* **2:** 28-33

Merril C. (1990) Gel staining techniques. In Colowick S.P., and Kaplan N.O. (Eds.) *Methods in Enzymology* **182:** 477-488

Mesulam M.M., and Geula C. (1990) Shifting patterns of cortical cholinesterases in Alzheimer's disaese: Implications for treatment, diagnosis, and pathogenesis. *Advances in Neurology* **52:** 235-240

Meyer T.S., and Lamberts B.L. (1965) Use of Coomassie brilliant blue R250 for the electrophoresis of microgram quantities of parotid saliva proteins on acrylamide gel strips. *Biochemica et Biophysica Acta* **107:** 144-145

Miller D.H., Hammond S.R., McLeod J.G., Purdie G., Skegg D.C.G. (1990) Multiple sclerosis in Australia and New Zealand: Are the determinants genetic or environmental? *Journal of Neurology, Neurosurgery, and Psychiatry* **53:** 903-905

Möller E., Böhme J., Valugerdi M.A., Ridderstaid A., and Olerup O. (1990) Speculations on mechanisms of HLA associations with autoimmune diseases and the specificity of "autoreactive" T lymphocytes. *Immunological Reviews* **118:** 7-19

Moreira H.W., and Naoum P.C. (1990) Serum haptoglobin types in patients with haemoglobinopathies. *Hereditas* **113:** 227-231

Morimatsu M., Syuto B., Shimada N., Fujinaga T., Yamamoto S., Saito M., and Naiki M. (1991) Isolation and characterisation of bovine haptoglobin from acute phase sera. *The Journal of Biological Chemistry* **266:** 11833-11837

Moshage H.J., Roelofs H.M., van Pelt J.F., Hazenberg B.P., van Leeuwen M.A., Limburg P.C., Aarden L.A., and Yap S.H. (1988) The effect of interleukin-1, interleukin-6 and its interrelationship on the synthesis of serum amyloid A and C-reactive protein in primary cultures of adult human hepatocytes. *Biochemical and Biophysical Research Communications* **155:** 112-117

Mullan M., Tsuji S., Miki T., Katsuya T., Naruse S., Kaneko K., Shimuzu T., Kojima T., Nakano T., and Ogihara T. (1993) Clinical comparison of Alzheimer's disease in pedigrees with the codon 717 val →ile mutation in the amyloid precursor protein gene. *Neuroepidemiology of Aging* **14:** 407-419

Müller A., Henn W., and Unteregger G. (1991) Nuclear and DNA binding proteins in human brain tumors. *Electrophoresis* **12:** 515-522

Müller-Hill B. (1989) Molecular biology of Alzheimer's disease. *Annual Review of Biochemistry* **58:** 287-307

Nemeroff C.B., Bissette G., Slotkin T.A., Seidler F.J., Miller B.E., and Ghanbari H. (1991) Recent advances in the neurochemical pathology of Alzheimer's disease. *Annals of the New York Academy of Sciences* **640:** 193-196

Neuhoff V., Arold N., Taube D., and Ehrhardt W. (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **9:** 255-262

Newman S.C. and Bland R.C. (1987) Canadian trends in mortality from medical disorders, 1965-1983. *Acta Psychiatra Scandinavica* **76:** 1-7

Nguyen N.Y., and Crambrach A. (1977) Natural pH gradients in buffer mixtures: formation in the absence of strongly acidic and basic anolyte and catholyte, gradient steepening by sucrose, and stabilization by high buffer concentrations in the electrolyte chambers. *Analytical Biochemistry* **79:** 462-469

Noonan D.J., Isakov N., Theofilopoulos A.N., Dixon F.J., and Altman A. (1987) Protein kinase C-activating phorbol esters augment expression of T cell receptor genes. *European Journal of Immunology* **17:** 803-807

Noseworthy J.H (1991) Therapeutics of multiple sclerosis. *Clinical Neuropharmacology* **14:** 49-61

Nurden A.T., Kunicki T.J., Dupuis D., Soria C., and Caen J.P. (1982) Specific protein and glycoprotein deficiencies in platelets isolated from two patients with the Grey platelet syndrome. *Blood* **59:** 709-718

O'Farrell P.Z., Goodman H.M., and O'Farrell P.H. (1977) High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12:** 1133-1142

O'Farrell P.H. (1975) High resolution two-dimensional electrophoresis of proteins. *The Journal of Biological Chemistry* **250:** 4007-4021

Oliviero S., Morrone G., and Cortese R. (1987) The human haptoglobin gene: Transcriptional regulation during development and acute phase induction. *The EMBO Journal* **6:** 1905-1912

Olney J.W. (1990) Excitotoxic amino acids and neuropsychiatric disorders. *Annual Review of Pharmacology and Toxicology* **30:** 47-71

Olson L. (1993) NGF and the treatment of Alzheimer's disease. *Experimental Neurology* **124:** 5-15

Paganini-Hill A., and Henderson V.W. (1994) Oestrogen deficiency and risk of Alzheimer's disease in women. *American Journal of Epidemiology* **140:** 256-261

Pandey J.P., Goust J.M., Salier J.P., and Fudenburg H.H. (1981) Immunoglobin G heavy chain (Gm) allotypes in multiple sclerosis. *Journal of Clinical Investigation* **67:** 1797-1800

Pappola M.A.,Omar R.A., Kim K.S., and Robakis N.K. (1992) Immunohistochemical evidence of antioxidant stress in Alzheimer's disease. *American Journal of Pathology* **140:** 621-628

Parker W.D. Jr., Mahr N.J., Filley C.M., Parks J.K., Hughes D., Young D.A., and Cullum C.M. (1994) Reduced platelet cytochrome C oxidase activity in Alzheimer's disease. *Neurology* **44:** 1086-1090

Parnetti L., Rebold G.P., Santucci A., Gaita A., Brunetti M., Ceccetti R., and Senin O. (1994) Platelet MAO-B activity as a marker of behavioural characteristics in dementia disorders *Aging* **6:** 201-207

Pawlowski T. (1989) Microheterogeneity of α1-acid glycoprotein in detection of intercurrent infection in patients with rheumatiod arthritis. *Arthritis Rheumatology* **32:** 347-51

Payami H., Montee K., and Kaye J. (1994) Evidence for familial factors that protect against dementia and outweigh the effect of increasing age. *American Journal of Human Genetics* **54:** 650-657

Pearce J.M.S. (1983) Dementia. *Medicine International* **30:** a3070-a3073

Pearson R.C., Esiri, M.M., Hiorns, R.W., Wilcock, G.K., and Powell, T.P. (1985) Anatomical correlates of the distribution of the pathological changes in the neocortex in Alzheimer's disease. *Proceedings of the National Academy of Sciences (USA)* **82:** 4531-4534

Pericak-Vance M.A., Bebout J.L., Gaskell P.C., Yamaoka L.H., Hung W.-Y., Alberts M.J., Walker A.P., Bartlett R.J., Haynes C.A., Welsh K.A., Earl N.L., Heyman A., Clark C.M., and Roses A.D. (1991) Linkage studies in familial Alzheimer disaese: Evidence for chromosome 19 linkage. *American Journal of Human Genetics* **48:** 1034-1050

Pirttila T., Mattila K., and Frey H. (1991) CSF proteins in neurological disorders analyzed by immobilized pH gradient isoelectric focusing using narrow pH gradients. *Acta Neurologica Scandinavica* **83:** 34-40

Potter H. (1991) Alzheimer disease and Down syndrome: chromosome 21 nondisjunction may underlie both disorders. *American Journal of Human Genetics* **48:** 1192-1200

Powel D. and Courtice T. (1986) *Alzheimer's disease: A giude for families.* Addison and Wesley Publishing Co., Massachusetts

Powers W.J., Perlmutter J.S., Videen T.O., Hersovitch P., Griffeth L.K., Royal H.D., Siegal B.A., Morris J.C., and Berg L. (1992) Blinded clinical evaluation of positron emission tomography for diagnosis of probable Alzheimer's disease. *Neurology* **42:** 765-770

Price D.L. (1986) New perspectives on Alzheimer's disease. *Annual Review of Neuroscience* **9:** 489-512

Prodouz K.N., Habraken J.W., and Moroff G. (1990) Resistance of platelet proteins to effects of ionizing radiation. *Journal of Laboratory Clinical Medicine* **116:** 766-770

Putnam F.W. (1975) Haptoglobin. In Putnam F.W. (Ed.) *The Plasma Proteins: Structure, Function and Genetic Control (Vol II)* pp2-51, Academic Press, New York

Radola B.J. (1980) Ultrathin-layer isoelectric focusing in 50-100 μm polyacrylamide gels on silanized glass plates or polyester films. *Electrophoresis* **1:** 43-56

Ransohoff R.M. (1992) T-cell receptor germline genes and multiple sclerosis susceptibility. *Neurology* **42:** 714-718

Rasmussen H.H., Mertz E., Mann M., Roepstorff R., and Celis J.E. (1994) Identification of transformation sensitive proteins recorded in human two-dimensional gel protein databases by mass spectrometric peptide mapping alone and in combination with microsequencing. *Electrophoresis* **15:** 406-416

Ray A., Tatter S.B., May L.T., and Sehgal P.B. (1988) Activation of the human "β_2-interferon/hepatocyte-stimulating factor/interleukin-6" promotor by cytokines, viruses, and second messenger agonists. *Proceedings of the National Academy of Sciences (USA)* **85:** 6701-6705

Raymond S. (1964) Acrylamide gel electrophoresis. *Annals of the New York Academy of Sciences* **121:** 350-365

Reisberg B. (1990) Alzheimer disease: the clinical syndrome; diagnosis and eitiological importance. *Acta Neurologica Scandinavica* **82:** 2-4

Reisberg B., Ferris S.H., de Leon M.J., and Crook T. (1982) The global deterioration scale for assessment of primary degenerative dementia. *American Journal of Psychiatry* **139:** 1136-1139

Rieder H.P., and Peyer C. (1970) Sex differences in the serum lipoprotein relations of normal persons and multiple sclerosis patients. *Clinica Chemica Acta* **30:** 305-310

Righetti P.G., Tudor G., and Gianazza E. (1982) Effect of 2-mercaptoethanol on pH gradients in isoelectric focusing. *Journal of Biochemical and Biophysical Methods* **6:** 219-227

Rilbe H. (1977) Isoelectric focusing. In Radola and Graesslin (eds.) (1977) *Electrofocusing and Isotachophoresis* de Gruyler, Berlin

Rock G., Labow R.S., and Adams G.A. (1988) Storage of human platelet concentrates for transfusion. *Progress in Clinical Biological Research* **283:** 673-704

Rogers J., and Morrison, J.H. (1985) Quantitative morphology and regional and laminar distributions of senile plaques in Alzheimer's disease. *Journal of Neuroscience* **5:** 2801-2808

Rohwer R.G. (1992) Alzheimer's disease transmission: Possible artifact due to intercurrent illness. *Neurology* **42:** 287-288

Rosenburg R.N., Ivy N., Kirkpatrick J., Bay C., Nyhan W.L., and Baskin F. (1981) Joseph disease and Huntington's Disease: protein patterns in fibroblasts and brain. *Neurology* **31:** 1003-1014

Rosenmann E., Kreis L., Thompson R.G., Dobbs M., Hamerton J.L., and Wrogemann K. (1982) Analysis of fibroblast proteins from patients with Duchenne muscular dystrophy by two-dimensional electrophoresis. *Nature* **298:** 563-565

Roth M. (1980) Senile dementia and its borderlands. In Cole J. and Barrett J. (eds.) (1980) *Psychopathology in the Aged* Raven, New York

Roth M. (1993) Advances in Alzheimer's disease. A review for the family physician. *Journal of Family Practice* **37:** 593-607

Rowland L.P. (1991) Cerebrospinal fluid: blood-brain barrier, brain edema and hydrocephalus. In Kandel E. (Ed.) *Principles of Neural Science (third edition)*: 1050-1061 Elsevier, New York

Rumble B.A., Beyruther K., and Masters C.L. (1991) The molecular pathology of Alzheimer's disease. *Today's Life Science* **April:** 24-31

Sadovnick A.D., and Ebers G.C. (1993) Epidemiology of multiple sclerosis: a critical overview. *Canadian Journal of Neurological Science* **20:** 17-29

Sarchielli P., Trequattrini A., Usai F., Murasecco D., and Galli V. (1993) Role of viruses in the etiopathogenesis of multiple sclerosis. *Acta Nuerologica* **15:** 363-381

Savage M.J., Iqbar M., Loh T., Trusko S.P., Scott R., and Siman R. (1994) Cathepsin G: localization in human cerebral cortex and generation of amyloidogenic fragments from the beta amyloid precursor protein. *Neuroscience* **60:** 607-619

Scharpe S.L., Vanhoof G.C., De Meester I.A., Handricks D.F., van Sande M.E., Muylle L.M., and Yaron A. (1991) Exopeptidases in human platelets: an indication for proteolytic modulation of biologically active peptides. *Clinica Chimica Acta* **195:** 125-131

Scheele G.H. (1981) Human pancreatic cancer: analysis of proteins contained in pancreatic juice by two-dimensional isoelectric focusing/sodium dodecyl sulphate gel electrophoresis. *Cancer* **47:** 1513-1515

Schlossmacher M.G., Ostaszewski B.L., Hecker L.I., Celi A., Haass C., Chin D., Lieberburg I., Furie B.C., Furie B., and Selkoe D.J. (1992) Detection of distinct isoform patterns of the beta amyloid precursor protein in human platelets and lymphocytes. *Neurobiology of Aging* **13:** 421-434

Schoenberg B.L. (1988) Epidemiology of vascular and multi-infarct dementia. In Meyer J.S. and Lechner H. (eds.) (1988) *Vascular and Multi-Infarct Dementia* Futura, New York pp13-22

Schupf N., Kapell D., Lee J.H., Ottman R., and Mayeux R. (1994) Increased risk of Alzheimer's disease in mothers of adults with Down's syndrome. *Lancet* **344** (8919): 353-356

Selkoe D.J. (1990) Deciphering Alzheimer's disease: The amyloid precursor protein yields new clues. *Science* **248:** 1058-1060

Shapira J. (1994) Research trends in Alzheimer's disease. *Journal of Gerontology Nursing* **20:** 4-9

Sharief M.K., and Thompson E.J. (1991) Intrathecal immunoglobin M synthesis in multiple sclerosis. *Brain* **114:** 181-195

Shurbaji M.S., Kuhajda F.P., Pasternack G.R., and Thurmond T.S. (1991) Expression of haptoglobin-related protein in primary and metastatic breast cancers. *American Journal of Clinical Pathology* **96:** 238-242

Siu A.L. (1991) Screening for dementia and investigating its causes. *Annals of Internal Medicine* **115:** 122-132

Skegg D.C.G. (1991) Multiple sclerosis: nature or nurture? *British Medical Journal* **302:** 247-248

Skegg D.C.G., Corwin P.A., Craven R.S., Malloch J.A., and Pollock M. (1987) Occurrence of multiple sclerosis in the north and south of New Zealand. *Journal of Neurology, Neurosurgery, and Psychiatry* **50:** 134-139

Skinner M.K., and Griswold M.D. (1983) Fluorographic detection of radioactivity in polyacrylamide gels with 2,5-diphenyloxazole in acetic acid and its comparrison with existing procedures. *Biochemical Journal* **209:** 281-284

Slunga L., Johnson O., Dahlen G.H., and Eriksson S. (1992) Lipoprotein (a) and acute phase proteins in acute myocardial infarction. *Journal of Clinical Laboratory Investigation* **52:** 95-101

Snedecor G. and Cochran W. (1989) *Statistical Methods.* Ames: Iowa State University Press

Sokal R. and Rohlf F. (1969) *Biometry: The Principles and Practice of Statistics in Biological Research.* Freeman Publishing, San Fransico

Sparks D.L., Hunsaker J.C., Scheff S.W., Kryscio R.J., Henson J.L., and Markesbery W.R. (1990) Cortical senile plaques in coronary artery disease, aging, and Alzheimer's disease. *Neurobiology of Aging* **11:** 601-607

Spurkland A., Rønningen K.S., Vandvik B., Thorsby E., and Vartdal F. (1991) HLA-DQB1 genes may jointly determine susceptibility to develop multiple sclerosis. *Human Immunology* **30:** 69-75

St. Groth S.F., Webster R.G., and Datyner A. (1963) Two new staining procedures for quantitative estimation of proteins on electrophoretic strips. *Biochemica et Biophysica Acta* **71:** 377-383

Steel D.M., and Whitehead A.S. (1991) Heterogenous modulation of acute phase reactant mRNA levels by interleukin-1β and interleukin-6 in the human hepatoma cell line PLC/PRF/5. *Biochemical Journal* **277:** 477-482

Stern Y., Gurland B., Tatemichi T.K., Tang M.X., Wilder D., and Mayeux R. (1994) Influence of education and occupation on the incidence of Alzheimer's disease. *Journal of the American Medical Association* **271:** 1004-1010

Stewart G.D., Van Leuren M.L., Galt J., Kurachi S., and Buraczynska M.J. (1989) Molecular structure of human chromosome 21. *Annual Review of Genetics* **23:** 409-423

Strittmatter W.J., Saunders A.N., and Schmechel D. (1993) Apoliporotein E: high avidity binding to β-amyloid and increased frequency of type 4 allele in late onset famillial Alzheimer's disease. *Proceedings of the National Academy of Science (USA)* **90:** 1977-1981

Sun G.Y., and Lin T.-N. (1990) Dynamic turnover of mouse brain phospholipids during normal aging and response to ischemia. *Upsala Journal of Medical Science* **48:** 209-223

Swingler R.J and Compston D.A.S. (1992) The morbidity of multiple sclerosis. *Quarterly Journal of Medicine* **83:** 325-337

Swingler R.J., and Compston D.A.S. (1986) The distribution of multiple sclerosis in Britain. *Journal of Neurology, Neurosurgery, and Psychiatry* **49:** 1115-1124

Tal M., Siberstein A., and Nusser E. (1985) Why does Coomassie brilliant blue R interact differently with different proteins? *The Journal of Biological Chemistry* **260:** 9976-9984

Taylor J., Giometti C.S. (1992) Use of principal components analysis for mutation detection with two-dimensional electrophoresis protein separations. *Electrophoresis* **13:** 162-168

Terry R., Katzman R., and Bick L. (eds.) (1978) *Alzheimer's Disease: Senile Dementia and Related Disorders*. Raven, New York

Thalman I., Kohut R.I., Ryu J., Comegys T.H., Senarita M., and Thalmann R. (1994) Protein profile of human perilymph: in search of markers for the diagnosis of perilymph fistula and other inner ear disease. *Otolaryngology, Head and Neck Surgery* **111:** 273-280

Timmons S., and Hawiger J. (1989) Isolation of human platelets by albumin gradient and gel filtration. In Colowick S.P., and Kaplan N.O. (Eds.) *Methods in Enzymology* **169:** 11-21

Tohgi H., Abe T., Takahashi S., and Kimura M. (1992) A selective reduction of excitatory amino acids in cerebrospinal fluid of patients with Alzheimer type dementia compared with vascular dementia of the Binswanger type. *Neuroscience Letters* **141:** 5-8

Tournier-Lasserve E., and Bach J.F. (1993) The immunogenetics of myasthenia gravis, multiple sclerosis and their animal models. *Journal of Neuroimmunology* **47:** 103-114

Tracy R.P., and Young D.S. (1984) Clinical applications of two-dimensional electrophoresis. In J. Celis and R. Bravo (Eds.) *Two-Dimensional Gel Electrophoresis of Proteins: Methods and Applications* Academic Press, Orlando 193-241

Tracy R.P., Currie R.M., Kyle R.A., and Young S.D. (1982a) Two-dimensional gel electrophoresis of serum specimens from patients with monoclonal gammopathies. *Clinical Chemistry* **28:** 900-907

Tracy R.P., Wold L.E., Currie R.M., and Young D.S. (1982b) Patterns of normal colon mucosa and colon adenocarcinoma compared by two-dimensional electrophoresis. *Clinical Chemistry* **28:** 915-919

Tracy R.P., Hill H.D., Currie R.M., and Young D.S. (1982c) Two-dimensional gel electrophoresis of urinary proteins from renal transplant recipients. *Clinical Chemistry* **28:** 1580

Tzourio C., Alpérovitch A., Mäccario J., and Dartigues J.-F. (1991) Is dementia of the Alzheimer type a purely genetic illness? A modelling approach. *Neuroepidemiology* **10:** 288-296

Uddhammer A., Rantapaa-Dahlqvist S., and Nilsson T.K. (1992) Plasminogen activator inhibitor and von Willebrand factor in polymyalgia rheumatica. *Clinical Rheumatology* **11:** 211-215

Urade R., and Kito M. (1992) Inhibition by acidic phospholipids of protein degradation by ER-60 protease, a novel cysteine protease, of endoplasmaic reticulum. *FEBS letters* **312:** 83-86

Van Leeuwen M.A., van der Heijde D.M., van Rijswijk M.H., Houtman P.M., van Riel P.L., van de Putte L.B., and Limburg P.C. (1994) Interrelationship of outcome measures and process variables in early rheumatoid arthritis. *Journal of Rheumatology* **21:** 425-429

Van Rensburg S.J., Carstens M.E., potocnik F.C., Aucamp A.K., and Taljaard J.J. (1993) Increased frequency of the transferrin C2 subtype in Alzheimer's disease. *Neuroreport* **4:** 1269-1271

Vrethem M., Ohman S., von Schenck H., Forsberg P., and Olsson J.E. (1987) Comparison of concentration of orosomucoid in serum and cerebrospinal fluid in different neurological diseases. *Acta Neurologica Scandinavica* **75:** 328-331

Wallace C.J., Seland T.P., and Fong T.C. (1992) Multiple sclerosis: The impact of MR imaging. *American Journal of Roentgenology* **158:** 849-857

Walton, Lord of Detchant (1992) Alzheimer's disease and the environment. *Journal of the Royal Society of Medicine* **85:** 67-70

Waxman S.G. (1982) Membranes, myelin, and the pathophysiology of multiple sclerosis. *The New England Journal of Medicine* **306:** 1529-1533

Weil J., and Epstein C.J. (1979) The effect of trisomy 21 on the patterns of polypeptide synthesis in human fibroblasts. *American Journal of Human Genetics* **31:** 478-488

Weiner H.L., Friedman A., Miller A., Khoury S.J., al-Sabbagh A., Santos L., Sayegh M., Nussenblatt R.B., Trentham D.E., and Hafler A. (1994) Oral tolerance: immunological mechanisms and treatment of animal and human organ specific autoimmune diseases by oral administration of autoantigens. *Annual Review of Immunology* **12:** 809-837

Weiner H.L., Mackin G.A., Matsui M., Orav E.J., Khoury S.J., Dawson D.M., and Hafler D.A. (1993) Double blind pilot trial of oral tolerization with myelin antigens in multiple sclerosis. *Science* **259:** 1321-1324

Whalen R.G., and Ecob M.S. (1982) Two-dimensional electrophoretic analysis of muscle contractile proteins in patients with idiopathic scoliosis. *Clinical Chemistry* **28:** 1036-1040

Wheeler T.W., Loong P.C., Jordan T.W., Ford H.C. (1986) A double label 2-dimensional gel electrophoresis procedure specifically designed for serum or plasma protein analysis. *Analytical Biochemistry* **159:** 1-7

Wheeler T., and Ford H.C. (1988) A search for protein abnormalities in erythrocyte membranes and platelets from patients with multiple sclerosis using double-label two-dimensional electrophoresis. *Journal of Neurological Sciences* **88:** 151-159

Wheeler T. (1988) The development of a double-label two-dimensional electrophoretic procedure and its use in a search for blood abnormalities in neurological diseases. *Ph.D. Thesis.* Victoria University of Wellington.

White A. (1978) The major nutrients. In White, Handler, and Smith (Eds.) *Principles of Biochemistry*: Elsivier, New York

Wiederkehr F., Büeler M.R., Wacker M., and Vonderschmitt D.J. (1989) Study of human cerebrospinal fluid proteins by size exclusion-high performance liquid chromatography and two-dimensional gel electrophoresis. *Electrophoresis* **10:** 480-488

Wiederkehr F., Büeler M.R., Wacker M., and Vonderschmitt D.J. (1985) Two-dimensional gel electrophoresis of cerebrospinal fluid proteins from patients with various neurological diseases. *Clinical Chemistry* **31:** 1537-1542

Wildenauer D.B., Körschenhausen D., Hoechtlen W., Ackenheil M., Kehl M., and Lottspeich F. (1991) Analysis of cerebrospinal fluid from patients with psychiatric and neurological disorders by two-dimensional electrophoresis: Identification of disease associated polypeptides as fibrin fragments. *Electrophoresis* **12:** 487-492

Williams J.G., and Penman S. (1975) The messenger RNA sequences in growing and resting mouse fibroblasts. *Cell* **6:** 197-206

Wilson C. (1983) Staining procedures on gels: comparisons of dyes and procedures. In Colowick S.P., and Kaplan N.O. (Eds.) *Methods in Enzymology* **91:** 236-254

Windholz M. (Ed.) (1976) *The Merck Index: An encyclopedia of chemicals and drugs (ninth edition)* Merck and Co., Inc. New Jersey

Wood J.A., Wood P.L., Ryan R., Graff-Radford N.R., Pilapil C., Robitaille Y., and Quirion R. (1993) Cytokine indices in Alzheimer's temporal cortex: no changes in mature IL-1 beta or IL-1RA but increases in the associated acute phase proteins IL-6, alpha 2-macroglobin and C-reactive protein. *Brain Research* **629:** 245-252

Wu J-Y., Welch K.M.A., and Duncan C.C. (1979) Abnormal protein patterns in multiple sclerosis. *Neurochemistry Research* **4:** 811-819

Wurtman R.J. (1987) Dietary treatments that affect brain neurotransmitters. Effects on calorie and nutrient intake. *Annals of the New York Academy of Sciences* **499:** 179-190

Yankner B.A., and Mesulam, M.M. (1991) β-Amyloid and the pathogenesis of Alzheimer's disease. *The New England Journal of Medicine* **325:** 1849-1857

Yiangou M., Ge X., Carter K.C., Papaconstantinou J. (1991) Induction of several acute phase protein genes by heavy metals: A new class of metal-responsive genes. *Biochemistry* **30:** 3798-3806

Young D.A. (1984) Advantages of separations on "giant" two-dimensional gels for the detection of physiologically relevant changes in the expression of protein gene products. *Clinical Chemistry* **30:** 2104-2108

Yu C.E., Payami H., Olson J.M., Boehnke M., Wijsman E.M., Orr H.T., Kukull W.A., Goddard K.A., Nemens E., and White J.A. (1994) The apolipoprotein E/CI/CII gene cluster and late onset Alzheimer's disease. *American Journal of Human Genetics* **54:** 631-42

Yun M., Wu W., Hood L., and Harrington M. (1992) Human cerebrospinal fluid database: edition 1992. *Electrophoresis* **13:** 1002-1013

Zar J. (1974) *Biostatistical Analysis.* Prentice-Hall, New Jersey

Zeineh R.A., and Kyriakidis G. (1987) High resolution qualitative and quantitative comparison of autoradiograms of 2-D gel electrophoresis by computer-aided soft laser scanning densitometry. *American Laboratory News* **19:** 63-67

Zubenko G.S. (1990) Significance of increased platelet membrane fluidity in mental disorders of Late-life. *Upsala Journal of Medical Science* **48:** 225-244

Zubenko G.S., Farr J., Stiffler J.S., Hughes H.B., and Kaplan B.B. (1992) Clinically silent mutation in the putative iron-responsive element in exon 17 of the β-amyloid precursor protein gene. *Journal of Neuropathology and Experimental Neurology* **51:** 459-463