MITOCHONDRIAL DNA IN ALZHEIMER'S DISEASE
MITOCHONDRIAL DNA IN ALZHEIMER'S DISEASE:
EXAMINATION USING IN SITU HYBRIDIZATION

By

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A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree
Master of Science

McMaster University
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MASTER OF SCIENCE (1995)  
MCMASTER UNIVERSITY
(Biochemistry)  
Hamilton, Ontario

TITLE: Mitochondrial DNA in Alzheimer’s Disease: Examination using in situ hybridization

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NUMBER OF PAGES: ix, 200
Abstract:

Mitochondria are intracellular organelles responsible for oxidative phosphorylation. They contain their own DNA which encodes some components involved in oxidative phosphorylation. Mitochondrial DNA is very susceptible to mutations. Mitochondrial abnormalities have been observed in several disorders of muscle and brain. Alzheimer's disease is a form of dementia characterized by the formation of numerous neuritic plaques and neurofibrillary tangles. There is evidence suggesting a possible role for mitochondrial abnormalities in Alzheimer’s disease.

The goal of this project was to determine if there were quantitative changes in mitochondrial DNA content in large neurons from Alzheimer’s disease patients, compared to age-matched control patients. The relative mitochondrial DNA content per unit area was assessed in brain sections from Alzheimer’s disease subjects and age-matched control subjects using in situ hybridization to mitochondrial DNA. The results were not conclusive due to technical concerns with the in situ hybridization technique which are discussed.
Acknowledgements

I am deeply indebted to all of the wonderful people at McMaster University, the Hamilton Regional Cancer Centre, and elsewhere, whose unwavering support and guidance meant so much to the progress and completion of this project.

This thesis is dedicated to my husband Gary Gin who, through his patient support and generous spirit, was able to give me strength that I could not have found within myself.
Abstract

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Abbreviations

β-amyloid: amyloid β-protein or A4
A.D.: Alzheimer's Disease
A4\textsubscript{695} (β-amyloid fragment 695 amino acids in length)
A4\textsubscript{751} (β-amyloid fragment 751 amino acids in length)
ApoE: apolipoprotein E
APP: amyloid preprotein
APTEX: 3-aminopropyl-triethoxy-silane
BCIP: 4-bromo-5-chloro-3-indolylphosphate
bp: base pairs
Ci: curie
COX: cytochrome c oxidase
CPEO: chronic progressive external ophthalmoplegia
CPM: counts per minute
E.M.: Electron microscopy
EDTA: ethylenediamine tetraacetic acid
FAD: familial form of A.D.
PBS: fetal bovine serum
g: gram
HSP 60: heat shock protein 60
ISH: in situ hybridization
KSS: Kearns-Sayre Syndrome
L: litre
LHON: Leber's Hereditary Optic Neuropathy
M: molar
MELAS: mitochondrial encephalopathy, lactic acidosis and stroke-like episodes
MEM: Minimal essential medium
MERRF: myoclonic epilepsy with ragged red fibres
MeV: mega electron volt
mm: millimetre
MPP\textsuperscript{+}: (1-methyl-4-phenylpyridinium)
MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtDNA: mitochondrial DNA
n: nano
NBT: nitroblue tetrazolium
ND: NADH dehydrogenase
nt: nucleotide
P.D.: Parkinson's disease
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PHF: paired helical filaments
RPM: rotations per minute
RPMI: Roswell Park Memorial Institute
SDS: sodium dodecyl sulphate
Tris: Tris(hydroxymethyl)aminomethane
μ: micro
°C: degrees Celsius
Chapter 1
Introduction

A) Mitochondria and mitochondrial DNA

Mitochondria are organelles found in virtually all eukaryotic cells. They are the site of oxidative phosphorylation, as well as the site of synthesis of certain lipids. The number of mitochondria per cell varies between tissues. Each mitochondrion contains several copies of its own mitochondrial DNA (mtDNA). Human mtDNA is a 16,569 bp covalently closed circular molecule (Figure 1). It codes for 13 proteins involved in oxidative phosphorylation (NADH dehydrogenase subunits 1, 2, 3, 4L, 4, 5, 6; cytochrome b, cytochrome c oxidase subunits I, II, III; ATPase 6, ATPase 8) as well as for 22 tRNAs and 2 rRNAs required for the expression of mitochondrial genes\(^1,2,3\). DNA polymerase γ is primarily responsible for the replication of mtDNA.\(^4,5,6\) The mitochondrial volume in cells doubles during the cell cycle, so that each daughter cell contains the same mitochondrial volume.\(^7\) The mechanisms regulating this increase in volume are not fully understood. In non-cycling cells mitochondrial components turn over with half lives ranging from about 13 days (mtDNA), to about 40 days (cardiolipin).\(^8\) The expression
of mitochondrial genes in normal mammalian muscle has been shown to be proportional to their copy number, and mitochondrial DNA copy number has been shown to increase in response to increased energy demands in this system.  

Figure 1: Map of human mitochondrial DNA showing the approximate location of mitochondrial genes. Mutations and deletions associated with some mitochondrial disorders are shown in the interior of the map. The location of the point mutation sometimes correlated to A.D. is also shown. Amino acid abbreviations refer to DNA regions encoding the mitochondrial tRNA's specifying these amino acids; OH: origin of heavy strand replication; 12S: mitochondrial 12S rRNA gene; ND: NADH dehydrogenase subunit (the number following ND indicates the subunit number encoded); OL: origin of light strand replication; CO: cytochrome c oxidase subunit (the number following CO indicates the subunit encoded); ATPase: ATP synthase subunit (the number following ATPase indicates the subunit encoded); Cyt.b: cytochrome b; MERRF: myoclonic epilepsy with ragged red fibres; MELAS: mitochondrial encephalopathy, lactic acidosis and stroke-like episodes; LHON: Leber's hereditary optic neuropathy; KSS: Kearns-Sayre syndrome; CPEO: chronic progressive external ophthalmoplegia.  

1, 2, 3, 25, 36, 54, 115
Mitochondrial DNA is extremely susceptible to mutations resulting from oxidative damage by intermediates of oxidative phosphorylation because mitochondria lack an excision repair system for their DNA\textsuperscript{10}. The rate of mutation of mitochondrial DNA has been estimated to be ten times the rate of nuclear DNA\textsuperscript{11}. As almost all of the sequences in mitochondria encode either protein or RNA species necessary for normal cell functions, mtDNA mutations can be expected to alter mitochondrial function, and in sufficient quantities, to alter cell or tissue phenotype. The accumulation of mitochondrial DNA mutations over the lifetime of an individual has been postulated to lead to an age-related decline in oxidative phosphorylation capacity\textsuperscript{12}. An age-related decline in respiratory rate and respiratory enzyme activity per gram of mitochondrial protein has been observed in skeletal muscle biopsies\textsuperscript{13}.

In addition to arising spontaneously, mitochondrial mutations can be inherited. Since mitochondria are normally inherited only from the ovum, mtDNA mutations are maternally inherited\textsuperscript{14}. Mutations in mitochondrial DNA have been found in a variety of disorders. These mutations may be homoplasmic (mutation present in all copies of mtDNA) or heteroplasmic (mutation present in only a fraction of the mtDNA molecules). If inheritance is heteroplasmic then the fertilized egg will contain both mutated and wild-type mtDNA's and the segregation
of the mutated molecules into progenitor cells for different tissues may determine if the mutation will be expressed, and the level to which it will be expressed. Mitochondrial DNA molecules containing mutations but retaining the origins of replication, may be preferentially amplified. This is observed in mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS). Preferential amplification of mutant forms of mtDNA would allow an age-related decline in oxidative phosphorylation potential in cells which originally contained a low enough proportion of mutated mtDNA to be viable during the early stages of development and differentiation. Mutations arising or accumulating during development might become significant in terminally differentiated tissues, in which there is no ongoing selection against compromised cells. This could allow the accumulation of mutated mtDNA's during mitochondrial turnover. The large number of mitochondria per cell and the presence of multiple mtDNA molecules per mitochondrion allows for different genotypes within neighbouring tissues and even neighbouring cells, depending on the segregation of mitochondria containing mutated mtDNA's during development.

The relationship between quantitative mitochondrial changes and mitochondrial energy metabolism in cells has been examined. In normal neurons and type I muscle cells
mitochondrial DNA levels and the expression of mitochondrially encoded genes are proportional to mitochondrial energy metabolism.\textsuperscript{9,186,200} These studies have examined the effect of induced changes in energy metabolism on the cellular levels of mitochondrial components including mtDNA. This is in contrast to altering the levels of mtDNA in cells and assaying the effect of these changes on energy metabolism. Experiments conducted in this manner suggested that mitochondria can maintain normal levels of function despite the reduction of their mtDNA content to less the half of normal levels.\textsuperscript{185} A key difference between these studies is the source of the cells examined. The manipulation of mtDNA levels was performed on taper liver tumour cells. The normal oxidative demands of these cells would be considerably lower than one would expect for neurons or type I muscle cells. It is therefore possible that, while some tissues maintain a considerable excess of mitochondrial components, those tissues (such as brain) which have high oxidative demands require all their mitochondrial components for normal function. It is equally possible that neurons and type I muscle cells do not normally use their full complement of mitochondrial components, but do maintain a fixed excess of these components in case of severe energetic demands. This could lead to the upregulation of mitochondrial components in these cells following increased oxidative demands. Thus the exact
relationship between levels of mitochondrial components and mitochondrial energy metabolism in normal cells is not clear. When changes in mitochondrial components and mitochondrial energy metabolism are observed they are in the same direction; however, the extent of variation in mitochondrial components required to elicit an assayable change in mitochondrial function is not clear.

II) Mitochondrial disorders

Many of the mitochondrial disorders observed to date are myopathies or encephalomyopathies (effecting muscle and/or the central nervous system). This is not at all surprising, as the energy requirements of these tissues are high, and one would expect compromised oxidative phosphorylation capability in these cells to be readily apparent. Myopathies are subdivided into four groups for classification. These four groups are: inflammatory myopathies, muscular dystrophies, inherited metabolic and congenital myopathies, and acquired metabolic and toxic myopathies. Mitochondrial myopathies are classified with the inherited metabolic and congenital disorders.\textsuperscript{16,17,65} Other members of this subgroup of myopathies include disorders of glycolysis, such as McArdle's syndrome.\textsuperscript{16,17,65}
Mitochondrial disorders share some common histological changes including the formation of Ragged Red Fibres (accumulations of aggregated abnormal mitochondria in degenerating muscle fibres), paracrystalline mitochondrial inclusions and unusual alterations in mitochondrial number, size, and cristae arrangement (Figure 2). Some ultrastructural changes in mitochondria can be induced experimentally in normal muscle by infusion with uncouplers of oxidative phosphorylation\(^\text{16}\); however the presence of many ragged red fibers is diagnostic for mitochondrial myopathies\(^\text{16}\).

Several mitochondrial disorders will be presented including two linked to mitochondrial tRNA mutations: myoclonic epilepsy with ragged red fibres (MERRF), and mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS); three linked to large mtDNA deletions: chronic progressive external ophthalmoplegia (CPEO), Kearns-Sayre syndrome (KSS), and the Pearson marrow-pancreas syndrome; and two linked to a point mutation in a mitochondrial oxidative phosphorylation protein subunit: Leber’s hereditary optic neuropathy (LHON), and Leigh’s syndrome. In addition, evidence suggesting a role for mitochondrial abnormalities in Parkinson’s disease will be discussed.
Figure 2: Common histological features of mitochondrial disorders. A) ragged red fibres in degenerating muscle (bar: 50 μm); B) paracrystalline inclusions in mitochondria (bar: 2 μm) (adapted from 17)
MERRF is associated with functional defects in mitochondrial oxidative phosphorylation complexes I and IV.\(^1\) Symptoms of disease are usually first observed in children and young adults. The disease phenotype has been linked to a mtDNA mutation in the region coding for tRNA\(^{Lys}\)\(^2\) (Figure 1). This mutation has also been observed in the asymptomatic aged.\(^2\) Such a mutation could interfere with the accurate and efficient translation of mitochondrially encoded proteins. MERRF shows strong maternal inheritance when the mtDNA mutation is present at high levels. When only low levels of mutated mtDNA are present in family members, inheritance appears sporadic. MERRF patients and their maternal relatives maintain some wild type mtDNA and show an increase in the severity of symptoms with increasing age.\(^2\) Clinically this disorder may lead to mitochondrial proliferation, increased mtDNA content, spontaneous myoclonic jerking, neurosensory hearing loss, muscle weakness, cardiomyopathy, and dementia.\(^2\) Neuronal degeneration is seen primarily in the cerebellum, brain stem, and spinal cord.\(^2\)

MELAS is a fatal disorder usually observed in infants and toddlers. Maternal transmission of this disorder has been observed.\(^1\)\(^,\)\(^2\)\(^3\)\(^,\)\(^2\)\(^4\) This disorder has been linked to a point mutation in the mitochondrial tRNA\(^{Leu}\) gene (Figure 1).\(^2\)\(^5\) The homoplasmic form of this mutation causes serious defects in
oxidative phosphorylation and mitochondrial protein synthesis.\textsuperscript{26} This mutation has been shown \textit{in vitro} to impair transcript termination after the 16S rRNA gene; this leads to an incorrect ratio of rRNA to other mitochondrial transcripts.\textsuperscript{27} A replicative advantage of the mutant mtDNA has been observed to allow the rapid movement from heteroplasmy to a nearly homoplasmic form of the disease.\textsuperscript{26} Clinically this disorder may lead to an increase in both size and number of mitochondria, reduced activity of oxidative phosphorylation enzymes, multifocal necrosis throughout the brain, mineral deposits in the basal ganglia, and neuronal loss (primarily in the cerebellum).\textsuperscript{23}

Kearns-Sayre syndrome (KSS) and chronic progressive external ophthalmoplegia (CPEO) appear to be related disorders. KSS is a distinct form of CPEO, young patients with CPEO may develop the full KSS later in life.\textsuperscript{28} The clinical features of each are described, followed by a discussion of the apparent genetic factors responsible for these disorders.

Chronic progressive external ophthalmoplegia's pathological features can include paresis of the extraocular eye muscles, cardiac conduction defects sometimes accompanied by fatty infiltration, fibrosis and giant mitochondria in conducting fibres and elsewhere in the myocardium. Abnormal mitochondria containing vacuoles, abnormal cristae, and
sometimes inclusion bodies are observed. Ragged red fibres are observed in muscle of affected individuals. Renal dysfunction, usually due to a proximal tube defect, is sometimes observed. Other less common dysfunctions can include diabetes mellitus (usually not observed until after glucocorticoid treatment), proportionate short stature, scoliosis, and high arched palate. Partial complex III functional deficiency has been reported in this disorder. Symptoms are first observed in adults, but biochemical and ultrastructural alterations may be visible in teenagers or young adults. The type of histopathological abnormalities observed are the same in subjects exhibiting pathology and asymptomatic offspring of symptomatic subjects, but the changes are less pronounced in asymptomatic offspring.

This disorder can be transmitted autosomally or be inherited through mitochondrial DNA deletions. Maternal transmission of CPEO to offspring is eight times more frequent than paternal transmission, and about half of all CPEO patients have large deletions in their mtDNA.

KSS is defined as a disorder beginning before the age of 20 with ophthalmoplegia, atypical pigmentary degeneration of the retina, and heart block. An increase in mitochondrial size and number is observed in patients suffering from this disease. In addition, ragged red fibres and mitochondria with
paracrystalline inclusions are observed in muscle.\textsuperscript{34,35} KSS can be transmitted autosomally or be linked to heteroplasmic mtDNA deletions.\textsuperscript{34,35} Almost all KSS patients have large deletions in their mitochondrial DNA.\textsuperscript{33}

There appear to be several potential causes for KSS and CPEO. Both can be transmitted autosomally, but the majority of cases appear to be linked to mtDNA mutations either arising spontaneously or transmitted to the offspring via mutated mtDNA's in the ovum.\textsuperscript{32} The exact mtDNA region deleted in KSS and CPEO varies, but the majority of deletions are in the region between the origin of replication of the heavy strand and the origin of replication of the light strand, and including the cytochrome oxidase subunit III gene (Figure 1).\textsuperscript{36} About 40\% of deletions found in both KSS and CPEO are at the same site. This region is bounded by a 13 base pair direct repeat at nt 8470-8482 and at nt 13447-13459. Homologous recombination between the direct repeat regions is suspected to be the mechanism by which deletion occurs.\textsuperscript{33} The deletion of the DNA between these repeats results in the loss of the genes encoding cytochrome oxidase subunit III, NADH dehydrogenase subunits 3, 4L, and 4, ATPase subunit 6, and the loss of part of the genes encoding ATPase subunit 8 and NADH dehydrogenase subunit 5.\textsuperscript{1} Deletions in mtDNA can often be found in many tissues of the body in patients with KSS or CPEO, suggesting that the mutation is either inherited, or
occurs spontaneously in the zygote prior to organogenesis.\textsuperscript{37}

The autosomal form of these diseases may represent more than one genetic defect. An autosomal dominant form of CPEO has been reported in which the affected individuals suffer from large deletions in mtDNA. As mtDNA deletions themselves are not autosomally transmitted, this suggests that in this pedigree there is a mutation in a nuclear gene responsible for the replication or repair of mtDNA.\textsuperscript{38,39} In addition to this type of situation, it is possible that a mutation in a nuclearly encoded gene for a mitochondrial protein could lead to mitochondrial dysfunction similar to that observed in KSS and CPEO. Autosomally transmitted KSS\textsuperscript{34} and CPEO\textsuperscript{38,40} appear clinically the same as the non-autosomal forms of these disorders.

The Pearson marrow-pancreas syndrome is a haematopoietic disorder appearing in early infancy. It is characterized by pancytopenia with vacuolization of bone marrow precursors and exocrine pancreatic dysfunction.\textsuperscript{41} The activity of mitochondrial oxidative phosphorylation enzymes is decreased in this disorder.\textsuperscript{42} Examination of mtDNA from Pearson’s marrow-pancreas syndrome reveals large mtDNA deletions.\textsuperscript{43,44} A majority of unrelated subjects had a 4978 bp deletion in their mtDNA\textsuperscript{43} in the same region as the deletion found in KSS and CPEO\textsuperscript{36}. The remaining subjects also had mtDNA deletions
in the same general area as those found in KSS and CPEO (ie. deleting parts of the region encoding the NADH dehydrogenase subunit 4 and 5 genes). In all cases the region deleted was bounded by direct repeat sequences in the wild-type mtDNA sequence.

The classification of mitochondrial disorders is not yet refined. The distinction between Kearns-Sayre syndrome and CPEO is based on clinical features and age of onset. The same mitochondrial DNA deletion appears capable of inducing either KSS, CPEO, or Pearson's marrow-pancreas syndrome. In addition, this same mutation has been shown to accumulate with age in subjects not exhibiting mitochondrial or neurological disorders. For this reason, the KSS/CPEO mutation is also sometimes referred to as the common deletion. As deleted mtDNA's can be observed in many tissues of the body in KSS and CPEO, tissue distribution can not be the sole factor determining the disease state which will develop in individuals suffering from these mtDNA deletions. The ratio of deleted mtDNA to wild type mtDNA in cells could potentially affect the disease course. However, a previous correlative study of patients suffering from CPEO (including some with KSS) found no relationship between the activity of respiratory chain enzymes in muscle and mtDNA deletion size, localization, or ratio to wild type mtDNA. It is probable that other
factors, genetic or environmental, can contribute to disease progression.

In order to accurately assess the role different factors play in the pathogenesis of these disorders, good analysis of the molecular and biochemical lesions in diseased pedigrees is needed. Such studies have been undertaken, including assessment of the proportion of deleted mtDNA and an assessment of mitochondrial enzyme function in tissue. This has suggested a link between focal cytochrome oxidase deficiency and the presence of mtDNA deletions, and a link between age of disease onset and the size of mtDNA deletions. This study was limited to the examination of mtDNA from muscle, preventing the examination of a potential relationship between tissue distribution of deleted mtDNA and disease phenotype. In one study in which the tissue distribution of deleted mtDNA was examined, the proportion of deleted mtDNA to wild type was found to be the highest in skeletal muscle (50%) and lowest in the smooth muscle (4%), with brain, heart, liver, and kidney falling between these extremes. Unfortunately, this study considered only mtDNA and did not examine enzyme activities, or attempt any correlation to clinical symptoms. Longitudinal studies examining molecular changes accompanying the development of disease phenotype in identified KSS pedigrees would also be helpful in this regard. Such a study would facilitate the examination of the factors
critical in the development of disease phenotype. This could also be investigated by the identification of mutations linked to the autosomal forms of these disorders. This might provide evidence relating clinical symptoms to deficiencies in genes encoding components of one oxidative phosphorylation complex. Once such factors were determined it would be possible to investigate the pathways by which specific molecular defects lead to the disease phenotype.

Leigh's syndrome (also known as subacute necrotizing encephalomyelopathy) is fatal disorder striking in infancy or early childhood. Clinical symptoms include psychomotor retardation and brainstem abnormalities. It is characterized pathologically by focal necrosis in the spinal cord, medulla, pons, midbrain, and thalamus.\textsuperscript{49} Cytochrome \textit{c} oxidase deficiency in many tissues has been observed in this disorder.\textsuperscript{50,51,52} This deficiency appears to be generalized for many cytochrome oxidase subunits including both nuclearly and mitochondrially encoded subunits. Decreased transcription of cytochrome oxidase mRNA's from both nuclearly and mitochondrially encoded genes has been observed in brain tissue from some patients.\textsuperscript{52} An increase in mtDNA content in tissue (relative to nuclear DNA content) has been observed in this disorder.\textsuperscript{52} Two modes of genetic transmission appear to be involved in the inheritance of Leigh's syndrome. In some pedigrees it appears autosomal recessive\textsuperscript{50}, and a nuclear DNA
mutation has been identified linked to some cases of Leigh’s syndrome. However, a sporadic form of Leigh’s syndrome is also observed. Sporadic Leigh’s syndrome has been linked to a mutation in the mitochondrially encoded ATPase subunit 6 gene (Figure 1). The mutation occurs at nt 8993 and alters the codon specifying a highly conserved leucine. This mutation was observed to be heteroplasmic, and present in high abundance in both muscle and blood. The mutation has also been observed in lower abundance in a patient’s asymptomatic sister, and mother. The molecular mechanism by which this mutation leads to reduced cytochrome c oxidase expression has not yet been determined.

LHON is a maternally inherited disease affecting young adults. The major clinical symptom of LHON is optic nerve degeneration leading to blindness; however cardiac dysrhythmia and minor neurologic abnormalities are sometimes observed. LHON has been linked to a missense mutation at mtDNA nucleotide 11778 (Figure 1). This region encodes the NADH dehydrogenase subunit 4 gene. This mutation converts a highly conserved arginine to a histidine. In addition to the ND4 mutation, several other mtDNA mutations have been identified, which appear to be responsible for a minority of cases of LHON. Mutations leading to LHON can be classified as Type I or Type II, reflecting the degree to which a carrier is at risk of developing the clinical
symptoms of LHON. The clinical development of LHON may result from the combination of several mutations, and may also be affected by additional genetic (nuclear or mitochondrial) or environmental factors.  

Parkinson's Disease

Parkinson's disease (P.D.) is a progressive syndrome primarily afflicting older adults. It is characterized by gait disturbances, stooped posture, slowed voluntary movement, and rigidity. It can be distinguished clinically from other parkinsonian disorders by a good and sustained response of affected subjects to levodopa. Pathological features of P.D. include the degeneration of pigmented and other brainstem nuclei, particularly the substantia nigra, and the formation of neuronal eosinophilic Lewy inclusion bodies. Disease becomes clinically apparent with a loss of about 50% of pigmented cells from the nigra compacta and a loss of 80% of dopamine from the putamen. The causative agents in P.D. have not been identified.

There is evidence supporting a role for mitochondrial abnormalities in P.D. Interest in a role for mitochondria in the pathogenesis of P.D. arose when it was discovered that the compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces a form of Parkinsonism clinically indistinguishable from the idiopathic disorder. MPTP is oxidized to MPP⁺ (1-methyl-4-phenylpyridinium) which accumulates in mitochondria.
MPP+ inhibits NADH dehydrogenase in mitochondria, leading to rapid ATP depletion in the affected cells. An increase in lipid peroxidation in the substantia nigra of P.D. subjects has also been observed. This could be the result of aberrations in mitochondrial oxidative phosphorylation.

The genetics of Parkinson's disease are not entirely clear. While it does appear to cluster in families, a similar concordance rate is observed for monozygotic and dizygotic twins. This suggests that some factor beyond nuclear mutation(s) is required to allow disease onset. A heteroplasmic mitochondrial DNA mutation predisposing towards P.D. could generate such a transmission pattern, either alone, or acting in conjunction with other nuclear or environmental factors.

A number of groups have investigated the activities of mitochondrial enzyme complexes in P.D. patients. Decreased activity of mitochondrial complex I has been observed in the substantia nigra, muscle, lymphocytes, and platelets. Complex II deficiency was also reported in platelets, and muscle. Complex IV deficiency in muscle has also been reported. The question of mitochondrial enzyme activities in P.D. is still controversial, however. Some researchers have found no significant differences in muscle mitochondrial enzyme activities from P.D. and control subjects. The issue of mtDNA abnormalities in P.D. is
not much clearer. An increase in the "common" (4977 bp KSS deletion) mtDNA deletion in the substantia nigra of P.D. subjects has been reported.\textsuperscript{79} However, no significant increase in mtDNA deletions in muscle was observed for P.D. subjects compared to age-matched controls.\textsuperscript{77,80} It is possible that a tissue specific deletion of mtDNA could be involved in the pathogenesis of some cases of P.D.

Abnormal mitochondria containing paracrystalline inclusions (similar to those described earlier for mitochondrial disorders) (Figure 2) have been observed in a subject with P.D. and A.D.\textsuperscript{81} Such changes can however be induced secondarily in otherwise healthy tissue by treatment with uncouplers of oxidative phosphorylation, or by excessive exercise.\textsuperscript{18} This leaves open the possibility that mitochondrial changes in P.D. are secondary to other non-mitochondrial causative lesions. Considerable work in this area is required before it will be known if mitochondrial abnormalities play a role in P.D., much less if they are causative agents.

Future research in this field would be aided by a clearer knowledge of the consistency with which mitochondrial aberrations are observed in the substantia nigra of P.D. subjects. This would be made possible by investigations of mtDNA abundance and size and the relative expression levels of different mRNA's encoding mitochondrial proteins in the
substantia nigra of P.D. subjects. If abnormalities were found in P.D. subjects compared to age-matched control subjects these results could be compared to respiratory enzyme activities in the substantia nigra of P.D. subjects. If the decrease in NADH dehydrogenase activity in the substantia nigra correlated with mtDNA or RNA abnormalities in this region, this would support a possible role for mitochondrial abnormalities in the etiology of P.D. However, if the functional NADH dehydrogenase deficiency did not correlate with abnormalities in mtDNA or RNA in this region, then this would suggest that the mitochondrial enzyme defect observed was secondary to some non-mitochondrial lesion. Additional information could be obtained from the level of expression of nuclearly and mitochondrially encoded mRNA’s for mitochondrial proteins. If a nuclearly encoded mRNA (for a mitochondrial protein) was most affected, this would suggest that the lesion might be in a nuclear gene, and might not be accompanied by a mtDNA mutation.
Alzheimer’s Disease (A.D.) is a form of dementia characterized clinically by memory impairment and the formation of numerous neuritic plaques and neurofibrillary tangles. The primary component of neurofibrillary tangles appears to be paired helical filaments of hyperphosphorylated microtubule associated protein Tau, and the major component of neuritic plaques appears to be amyloid β-protein (also known as A4 or β-amyloid). There are two distinct forms of Alzheimer’s disease: the familial form, and the sporadic form. The familial form of A.D. (FAD) shows Mendelian inheritance patterns. No clear inheritance pattern is observed in sporadic A.D., although it does tend to cluster within families. In both forms of A.D. the region most affected is the basal forebrain (Figure 3). There is currently no definitive antemortem diagnostic marker for A.D. A.D. in vivo is characterised by decreased glucose metabolism in specific regions of the brain, most noticeably in the parietotemporal association cortex, and later in the frontal lobe. This pattern is typical of A.D., and aids in distinguishing it from other forms of dementia. A.D. is diagnosed on autopsy based on the density of neuritic plaques and neurofibrillary tangles found in the forebrain. The
diagnosis is complicated by the fact that the brains of the non-diseased aged also contain these structures.\textsuperscript{65}

\textbf{Figure 3:} A: Midline sagittal section of the human brain showing the cingulate gyrus and the cerebellum, both examined in this project. B: Coronal section of the brain (viewed from the front) showing the hippocampus. (adapted from 217)
Neurofibrillary tangles of the type occurring in A.D. patients are also found in boxers suffering from dementia pugilistica ("punch drunkenness"). The distribution of neurofibrillary tangles is different in these individuals.\textsuperscript{85}

Alzheimer's disease specifically affects certain cells while apparently sparing others. The major cell group affected is pyramidal neurons with a cross sectional area larger than 90 $\mu m^2$. In the temporal lobe, the effects of A.D. are very specific, "disconnecting" the hippocampus from other brain regions by destroying the relatively few cells in the hippocampus and entorhinal cortex that relay messages to or from the hippocampus.\textsuperscript{86} The density of neuritic plaques and severity of dementia correlate with deficits in choline acetyl transferase.\textsuperscript{87,88}

Currently, most hypotheses suggest the causative agent in A.D. to be linked to either the accumulation of $\beta$-amyloid in affected areas of the brain and the formation of neuritic plaques; or the formation of neurofibrillary tangles by hyperphosphorylated Tau proteins. There is also growing evidence in support of a role for apolipoprotein E (ApoE) in A.D. ApoE is deposited with $\beta$-amyloid in neuritic plaques, and a mutation in the apo E gene maps to the same region of chromosome 19 as do some cases of A.D.\textsuperscript{89,90,91} Some, but not all forms of familial A.D. are linked to mutations in the
In addition, the expression of the amyloid precursor protein is not limited to the brain regions affected by A.D. suggesting that it is not sufficient to cause A.D. A more detailed description of β-amyloid in A.D. follows.

The protein from which β-amyloid is generated is the amyloid preprotein (APP). The expression of normal APP appears to be regulated by many factors. Increased APP mRNA expression has been observed following heat shock, and in vivo following induced focal cerebral ischemia. The APP gene lacks a typical TATA box. It has been shown to contain promoter regions similar to typical housekeeping gene promoter regions. In addition it has several regions homologous to promoter regions: a) a region with homology to AP-1 binding sites; b) a region homologous to the heat-shock control element binding protein site; c) six copies of a 9 base-pair G/C rich element; and d) two Alu-type repetitive sequences. In addition to this, five potential binding sites resembling the Hox-1.3 consensus binding sequence have been identified upstream of the APP gene. Two of these sites specifically bound Hox-1.3 in a band shift assay. These data suggest that the regulation of APP is complex and that its expression can be induced by a wide variety of cell stresses, as well as other factors.
The amyloid precursor protein is a type 1 transmembrane protein. The cytoplasmic tail of the normal amyloid precursor protein (APP) is 47 residues long. Aberrant processing of APP appears to lead to the production of β-amyloid. The C-terminus of the β-amyloid 4.5 kDa peptide is located within the transmembrane-domain of the APP. The intact β-amyloid peptide can be generated by processing of APP in the endosomal-lysosomal pathway following recycling. In addition to this, a calcium-activated serine protease has been partially purified from the brains of A.D. patients and normal aged individuals which cleaves APP at the N-terminus of the β-amyloid region in vitro. Normal processing of APP results in cleavage of APP by the APP secretase at residues 15 and 17 within the β-amyloid region. This prevents the deposition of β-amyloid in tissues in which normal processing occurs. In A.D. subjects, β-amyloid deposits have been detected in non-neuronal tissues and blood. This presents the possibility that β-amyloid may be derived from a precursor present in the circulating blood.

The expression and processing of the amyloid precursor protein is altered in A.D. There are alternately spliced forms of the APP mRNA, and the relative abundance of these forms is changed in A.D. Two forms of APP differ by an insert of 57 amino acids which is highly homologous to the Kunitz
family of protease inhibitors. The A₄₆₉₅ (β-amyloid fragment 695 amino acids in length) form which does not contain the Kunitz inhibitor domain is normally restricted to expression in neuronal cells. In contrast to this, the A₄₇₅₁ (β-amyloid fragment 751 amino acids in length) form containing the Kunitz inhibitor domain is ubiquitously expressed.¹⁰³ In normal subjects, the expression of A₄₇₅₁ is much higher than the expression of A₄₆₉₅ in the hippocampus. This changes in A.D., where frontal cortex expression of A₄₇₅₁ is nearly normal, but expression of A₄₆₉₅ is selectively lost.¹⁰⁴ A role for the protease inhibitor domains contained in some forms of APP has been suggested for the regulation of APP processing. It has been proposed that a relative increase in the expression of the mRNA encoding the protease inhibitors could lead to inhibition of normal APP processing by APP secretase, and allow aberrant cleavage, forming β-amyloid.

Clearly it is essential to determine if β-amyloid deposition is primary or secondary to A.D. pathology. To investigate this fact many groups have looked at β-amyloid toxicity both in vitro and in vivo. The cytotoxicity of β-amyloid has been demonstrated in vitro, although toxicity appears to absolutely require aggregation of β-amyloid subunits, a condition that varies with the age of the stock solution, and the solvent used.¹⁰⁵ The in vivo data are not
so clear. Transgenic mice expressing the complete APP gene suffer from deposition of \( \beta \)-amyloid in the cortex and hippocampus, however these animals do not show neurofibrillary tangle formation or neurodegeneration.\(^{106}\) The toxicity of \( \beta \)-amyloid peptides \textit{in vitro} has been linked to alterations in calcium homeostasis. \( \beta \)-amyloid has been shown to be capable of forming ion channels in bilayer membranes.\(^{107}\) \( \beta \)-amyloid peptides increase the sensitivity of cultured neurons to toxicity by either calcium ionophores, or excitatory amino acids, and to increased intracellular calcium levels. These effects are not seen in calcium free medium.\(^{108}\)

This brings us to the "amyloid cascade hypothesis" of A.D. This hypothesis suggests that \( \beta \)-amyloid is the primary cause of Familial Alzheimer's Disease. The major points of the hypothesis are: The presence of \( \beta \)-amyloid in neurons alters calcium homeostasis. This causes an upregulation of calcium-dependant enzymes. (The phosphorylation of Tau can be calcium-dependant.\(^{109}\)) This results in the hyperphosphorylation of Tau, leading to the formation of paired helical filaments and neurofibrillary tangles.\(^{110}\)

This hypothesis suggests that A.D. can be triggered by mutations in the APP gene, or other factors which trigger \( \beta \)-amyloid deposition. Some cases of early onset A.D. have been linked to mutations in the APP gene on chromosome 21.
IV) General hypothesis: There is a quantitative change in mitochondria in Alzheimer's Disease.

V) Background to the hypothesis

Evidence exists to support hypotheses for a role of mitochondrial aberrations in the causation of A.D. These hypotheses are based on the premise that mitochondrial mutations can accumulate throughout life, and that in the later stages of life this can lead to a significant reduction in the oxidative phosphorylation capacity of cells. Neuronal death could either result as a direct consequence of impaired mitochondrial energy metabolism, or impaired energy metabolism could serve as a trigger to other cellular processes which
could lead to neuronal death. It is possible that several distinct mechanisms exist for the pathogenesis of A.D. Mitochondrial energy metabolism can be impaired by many factors. Genetically, mitochondrial proteins are encoded in both the mtDNA and the genomic DNA, so a wide range of expression patterns could be observed, depending on the gene affected.

Reports have linked some cases of A.D. to a point mutation in the mitochondrial gene encoding NADH dehydrogenase subunit II at nucleotide 5460 (Figure 1). The pathological significance of this finding remains unclear, however several potential explanations exist. NADH dehydrogenase is a component of the electron transport chain in mitochondria. A point mutation in NADH dehydrogenase subunit IV is known to cause Leber's hereditary optic neuropathy (a mitochondrial disorder). It is possible that a mutation resulting in a reduced capacity of the mitochondria to undergo oxidative phosphorylation could induce an energy crisis within the cell, leading eventually to neuronal death.

It is also possible to postulate a role of mitochondrial DNA abnormalities in some cases of familial A.D. A nuclear DNA mutation predisposing toward specific deletions of mitochondrial DNA has been reported. This mutation is
responsible for an autosomal dominant mitochondrial myopathy.\textsuperscript{39} A similar situation can be envisioned for A.D. in which a mutation in a nuclear gene coding for an RNA or protein product required for accurate mtDNA replication could result in specific errors in mtDNA replication. This could result in a heteroplasmic mtDNA population which would move toward a homoplasmic mutant population with increasing age. Alternately, a nuclear mutation could alter or prevent the expression of a nuclearly encoded mitochondrial protein, resulting in a mitochondrial disorder without any mitochondrial DNA mutation. Such a disorder could show a Mendelian inheritance pattern.

A disorder resulting from a heteroplasmic mutation in mtDNA would not show an easily recognised maternal inheritance [as is observed in disorders resulting from homoplasmic mutations], but might appear to be sporadic. A heteroplasmic mutation would be consistent with the 50\% concordance seen for A.D. in monozygotic twins.\textsuperscript{119} If each twin did not receive the same mitochondrial genetic endowment from the oocyte, or if the affected mitochondria segregated differently during development, the genotype of the cells in the central nervous system would be different, resulting in the expression of a different phenotype in each twin.

Two major suspect enzymes in A.D. are cytochrome c oxidase and NADH dehydrogenase. Both of these enzymes are
comprised of several subunits, some of which are encoded in the mitochondrial DNA, and some encoded in the nuclear DNA. A mutation in a subunit of either of these genes could severely impair the ability of affected mitochondria to undergo oxidative phosphorylation. Genetic evidence for a role of NADH dehydrogenase has been presented\textsuperscript{115,116}, only circumstantial evidence currently exists for a role of cytochrome c oxidase. No large deletions or insertions have been found in the mtDNA encoded subunits of cytochrome c oxidase in A.D.\textsuperscript{120} Predominantly cortical symptoms are observed in other disorders resulting from cytochrome c oxidase deficiencies such as Steely hair disease and Alper's disease.\textsuperscript{121,122} The tissue specificity of this effect may be related to the level of cytochrome c oxidase expression and the isozyme expressed in different regions of the brain. There is evidence to suggest expression of tissue-specific isozymes of cytochrome c oxidase.\textsuperscript{123} The distribution of cytochrome c oxidase in the brain is not uniform, it has a very complex pattern of distribution.\textsuperscript{124} Regions of the brain usually involved in A.D. have a much higher expression of an mRNA homologous to the mitochondrial gene encoding of subunit I of cytochrome c oxidase than do non-affected regions of the brain.\textsuperscript{125,126} Thus, the effects of a mutation in this mitochondrial gene might be much more apparent in the regions affected by A.D.
Several observations support a relationship between impaired mitochondrial function and A.D. Mitochondrial uncoupling has been observed in mitochondria from cultured skin fibroblasts of A.D. patients. Mitochondrial antigens (recognised by anti-PHF or Alz-50 antibodies) can be induced on normal cells by treatment with m-chlorophenylhydrazone, an uncoupler of oxidative phosphorylation. This suggests that these antigens may be markers for mitochondrial malfunction in affected cells. Treatment with uncouplers also leads to a collapse of the cytoskeleton of HeLa cells similar to the collapse observed in cells affected by A.D. A deficiency in cytochrome c oxidase activity in platelet mitochondria and brain homogenates from patients suffering from A.D. has been observed. Diethyldithiocarbamate (a copper chelator) depresses cytochrome c oxidase activity and produces an amnesic state in rats. A.D. cells show direct symptoms of mitochondrial damage such as: the presence of distorted mitochondria, alterations in cellular Ca\(^2+\) homeostasis; and decreases in the activities of NADH-tetrazolium blue reductase, 2-glutarate dehydrogenase, and pyruvate dehydrogenase. Recently a decrease in the mRNA levels for mitochondrially encoded subunits of cytochrome c oxidase was reported in brain tissue from A.D. subjects. No
change was observed for subunits encoded in the nuclear DNA.\textsuperscript{146}

The question which arises is how the evidence suggesting a role for mitochondrial aberrations in A.D. can be harmonized with the apparent involvement of $\beta$-amyloid in the causation of some cases of A.D. The hypothesis has been presented by M.F. Beal that impairment of energy metabolism can lead to excitotoxic neuronal death.\textsuperscript{147} This argument is based on the observation that glutamate neurotoxicity can result from the reduction of intracellular energy levels.\textsuperscript{148} This is postulated to occur because the reduction of intracellular energy levels leads to a reduction in the resting potential across the neuronal membrane by reducing the activity of the sodium-potassium ATPase pump and allowing the intracellular accumulation of sodium. This allows the relief of the voltage dependant Mg\textsuperscript{2+} block of the NMDA glutamate receptor channel.\textsuperscript{151} This causes the opening of voltage-dependant calcium channels and leads to an increase in intracellular calcium. The ability of the cell to sequester or excrete calcium is compromised by decreased energy metabolism in the cell and decreased potential across the mitochondrial membrane. This could result in increased intracellular calcium, similar to the effect seen \textit{in vitro} with $\beta$-amyloid\textsuperscript{107}, and possibly leading to the calcium-dependant hyperphosphorylation of Tau, as seen in A.D.\textsuperscript{109} In addition, increased calcium levels could
activate the calcium-activated protease postulated to cleave APP to give rise to the N-terminus of the amyloid β-protein.\textsuperscript{149} No significant increase in glutamate levels are observed in the hippocampus of A.D. subjects\textsuperscript{150}, however studies have indicated that normal levels of glutamate are sufficient to cause excitotoxicity in neurons in which energy metabolism has been impaired.\textsuperscript{151} This suggests a mechanism of neurotoxicity through impaired mitochondrial energy metabolism. Thus, calcium-induced cytotoxicity could be induced by impaired mitochondrial metabolism, aggregation of β-amyloid, or a combination of both.
B) Technical introduction

There are many potential methods for studying mitochondrial abnormalities in fixed post-mortem brain tissue. Different techniques are better suited for studying different types of abnormalities (e.g. mtDNA quantity, mitochondrial size, mitochondrial protein expression). I was primarily concerned to look for differences in the quantity of mitochondrial components in A.D. brain sections as compared to age-matched control subjects. I was limited to the use of fixed tissues, precluding the use of functional assays for mitochondrial components. Several major techniques exist and are in common use for the quantitative (or semi-quantitative) assessment of mitochondria or mitochondrial components in fixed tissue sections. I shall consider these techniques in relation to their suitability for use in the examination of my hypothesis.

I) Electron microscopy

Electron microscopy (E.M.) is a commonly used technique in the diagnosis and pathological examination of mitochondrial disorders. It can give qualitative information on mitochondrial structure and location\(^{152,153,154,155,156,157}\) (Figure 2) as well as quantitative information on mitochondrial cross sectional surface area\(^{158,159}\). As I was primarily interested in quantitative data, my application of
this technique would have been to measure mitochondrial surface area within sections from A.D. and control subjects.

The major strength of this method is its ability to provide mitochondrial localization information, in addition to information on the relative mitochondrial volume in tissues. The use of E.M. would have allowed me to distinguish between mitochondria contained within the neuron of interest, and those mitochondria immediately proximal to the neuron of interest, but actually belonging to another neuron, forming a synapse with the neuron of interest. In addition to this, E.M. would have allowed me to more accurately determine the sample population I was studying. As neurons die there are many potential morphological changes which they could undergo. There might be a change in the volume of the cell body, thereby altering the number of mitochondria per unit area, while not increasing the actual number of mitochondria per cell. In addition, in the late stages of neuronal death, one might see the breakdown of mitochondria into smaller sub-mitochondrial particles. These changes would have been evident under E.M., and neurons grossly abnormal in these ways could have been omitted from the study. One must understand that the tissue available is primarily from subjects in the late stages of A.D. One would expect to see many late-stage changes in this tissue. Using E.M. it might be possible to
reduce the number of neurons examined showing gross morphological alterations. This approach is weakened by the possibility that neurons which remain normal late in the disease course may represent a distinct population of neurons which may not be strongly affected in A.D.

There are two major drawbacks to the use of E.M. in this study. The first is the difficulty in obtaining suitably fixed specimens from enough subjects. In order to accurately measure mitochondrial area using E.M., the tissue must be fixed for E.M. either prior to death, by perfusion (used in animal studies\textsuperscript{158}, not possible for human tissues), or immediately after death\textsuperscript{156,159}. Autopsy tissue is not routinely processed in this manner (at the hospital from which I obtain my samples), so limited samples were available to us. Tissue not fixed specifically for E.M. immediately post-mortem will show artifacts due to degradation during fixation, or artifacts from incorrect embedding for E.M. sections. Such artifacts can include the loss of good mitochondrial morphology and can influence apparent mitochondrial cross sectional surface area. Due to mitochondrial effects in Parkinson's disease\textsuperscript{72,76}, and potentially other neurological disorders, I limited my A.D. samples to subjects with no known neurological conditions other than A.D. The limited number of samples from which to draw, and the stringency of my subject
selection criteria, combined to make it extremely difficult to obtain enough suitable samples for analysis by E.M.

The second drawback to using E.M. is the large number of micrographs required to obtain significant results. Previous use of this method to study mitochondrial cross sectional surface area\(^{158}\) has required the examination of 100 micrographs (5100 \(\mu\text{m}^2\)) per subject or sample. While this is feasible for small-scale studies, my long term goal was to look for changes common throughout the population of A.D. patients. This required that the technique I chose be easily applied to samples of ten to twenty patients per group.

The limited number of suitable samples available and the labour-intensive nature of analysis indicated that E.M. was not the most appropriate method for my purposes.

II) Immunohistochemistry for mitochondrial proteins

There are two major methods for quantifying mitochondrial protein levels using immunohistochemistry. These methods differ primarily in the technique used to visualize the bound antibody. If one has access to tissue which has been processed correctly for E.M., then one can use gold labelling to visualize antibody binding. This has the advantage of allowing one to assess the localization of the antigen within the mitochondrion, and to determine if cellular morphology appears normal.
Tissue fixation should be optimized for the antigen of interest, however fixation in 1% glutaraldehyde in 0.1 M phosphate buffer has proven suitable for two mitochondrial proteins, one located in the mitochondrial membranes, the other in the matrix\textsuperscript{160}. Lowicryl K4M was shown to be a suitable embedding agent and post-fixation osmium tetroxide was omitted.\textsuperscript{160,161} Sections were mounted on nickel grids having a carbon-coated Parlodion film.\textsuperscript{160} Immunolabelling steps were performed by floating the grids on drops of solution. The sections were exposed to the appropriate antibody, washed, and exposed to gold-conjugated protein A.\textsuperscript{160,161} Sections were stained with uranyl acetate and alkaline lead citrate and observed under E.M.

This is a powerful technique, however it suffers from many of the limitations discussed later for immunohistochemistry assessed by light microscopy. The E.M. method of evaluating the results of immunohistochemistry allows one to determine intracellular localization and cell morphology more accurately than is possible with conventional immunohistochemistry. The major limitation of this technique as applied to my system is the requirement for tissue preserved especially for E.M. immediately post mortem. There were not enough suitable samples available to me for me to use this method in my initial investigations.
Immunohistochemistry viewed by light microscopy is frequently used in the diagnosis and examination of mitochondrial disorders. This method does not allow the spacial resolution afforded by E.M., nor does it allow the researcher to assess subtle subcellular changes in the neuron of interest. One of the major advantages of using immunohistochemistry to examine mitochondrial proteins is the information it can potentially provide about relative expression levels of protein subunits encoded in the nuclear DNA as compared to the mitochondrial DNA. This has been used in previous studies to suggest the potential nature of the defect in some mitochondrial disorders. The second major advantage of immunohistochemistry assessed under the light microscope is the relative ease of qualitative analysis. There are two major disadvantages to the use of immunohistochemistry as a measure of mitochondrial components. The first is the difficulty in obtaining consistent quantifiable results for relative levels of the target protein from different samples. This method is typically used to compare the relative abundance of different proteins in the same section, or as a semiquantitative technique to compare protein expression in different tissues fixed and processed in parallel with each other. While it is possible to quantify signal from immunohistochemical sections, this still requires that the sections be treated in parallel.
Factors such as fixation time, elapsed time between death and tissue fixation, and the fixative used can affect the signal observed for different antigens. The effects may be different for different antigens in the same tissue section, making it difficult to standardize signals in order to control for variations in tissue handling and treatment. As these are factors I was not able to control for in the human sections, this technique was not appropriate for my purposes.

The second disadvantage of the use of immunohistochemistry to measure mitochondrial abundance is the possibility of selective depletion of one protein subunit in the disease state, while the other subunits are present in normal quantities. This difficulty can be overcome if one knows the subunits most likely to be affected in a given disorder. However, if one does not know which subunits are likely to be affected one can obtain misleading results. Thus, in order to obtain reliable results with this technique, it would be prudent to use antibodies to several different protein subunits in serial sections to ensure that any differences in staining intensity between A.D. subjects and controls are consistent for antibodies against several different targets. This technique was not selected for use in my study, primarily because of potential difficulties in comparing quantitative results between subject samples.
III) DNA isolation

The isolation of total DNA from tissue, and subsequent quantification, has been used to quantify mtDNA in some tissues (primarily muscle or isolated blood components). This method has been advanced in recent years by the introduction of the polymerase chain reaction (PCR), and the use of quantitative PCR for this purpose. There is one major limiting factor to both these methods. This is the fact that when one homogenizes tissue for DNA extraction, one can not be selective in precisely which cells one homogenizes. A.D. primarily affects large neurons. These are found predominately in layers three and five of the cortex. Methods in which one can work with tissue sections allow one to restrict investigation to this population of neurons. This is not possible when working with homogenates. In addition to the contamination of the sample by small neurons, there are also glial cells in brain which would be a source of contamination in a homogenate. Previous studies have shown tissue-specific differences in mitochondrial DNA content, therefore the presence of these contaminating cells could substantially affect the results and compromise the significance of the experiment. In addition to this, I am limited to small quantities of fixed tissues. Such tissues are not suitable for large-scale DNA extraction for the
purposes of direct Southern or slot-blotting. It is possible to use PCR to amplify regions of DNA from sections fixed in 10% neutral buffered formalin (as are most of the sections to which I have access). The maximum size of template from which one can expect to get good amplification (for a section fixed for 8 days prior to embedding) is 500 bp.\(^{173}\) This is adequate to allow detection of mitochondrial DNA; however, due to the frequency of deletions in mtDNA, especially in the aged\(^{171,175,176}\) it would be necessary to amplify at least two distinct regions to ensure that the results obtained reflected the true copy number of mtDNA in that sample, and were not influenced by the presence of a heteroplasmic mtDNA deletion in the region to be amplified. Quantitative PCR is a technique still in its infancy. Several methods have been developed to help ensure representative results.\(^{170,172}\) A combination of these with an internal standard could likely give representative data for the quantity of a target mtDNA sequence in a target tissue. However, as addressed at the beginning of this section, the primary problem is that the homogenate would contain both my target tissue (large neurons) and many other cells of various origins. This represents a substantial source of contamination. This method was determined to not be the most appropriate for application to my system.
IV) \textit{In situ} hybridization

\textit{In situ} hybridization can be used to quantify mtDNA levels in tissues of interest. The relative stability of mtDNA in preserved tissue makes this technique resistant to some of the limitations found with the quantification of proteins by immunohistochemistry. An increase in mtDNA content has been reported for several mitochondrial disorders, making this a useful marker for potential mitochondrial involvement.\textsuperscript{22,35,52} Such experiments will be considered in more detail later. There are various protocols for the use of \textit{in situ} hybridization for the quantification of mtDNA. They differ primarily in the method used to label the probe and the method used to visualize probe binding.

i) Electron-microscopy combined with \textit{in situ} hybridization

Electron-microscopy can be used to assess the results of \textit{in situ} hybridization using biotinylated mtDNA probes, thus allowing the quantification of mtDNA within distinct cells in tissue. These probes are visualized by immunocytochemistry using a colloidal-gold conjugated marker, and the gold particles are quantified under E.M. This method offers some of the advantages already discussed in relation to E.M. in that it is possible to identify individual mitochondria in these sections and ensure that the signal one sees is in fact localized over mitochondria.\textsuperscript{177} In order to get accurate
measurements of mitochondrial surface area in these sections, one would have to have well-preserved tissue processed especially for E.M. The difficulties encountered due to autolysis when trying to use autopsy tissue in electron microscopy are discussed in the next section.

Previous researchers looking at mitochondrial RNA localization have found that tissue dissected in paraformaldehyde (4%) and gluteraldehyde (0.1%) and embedded in Lowicryl K4M was suitable. Ultrathin sections for use with E.M. were picked up on carbon-parlodion-coated nickel grids\textsuperscript{178} or acetone-treated naked gold grids\textsuperscript{177}. Hybridization was to biotin-labelled DNA [note that some limitations of biotin labelling will be discussed later]. Signal was visualized by reacting the grids to rabbit anti-biotin antibodies, followed by reaction with gold complexed goat anti rabbit antibodies\textsuperscript{177} or Protein A-gold complex\textsuperscript{178}. Hybridization was performed by floating the grids on drops of hybridization solution. Following washing of the tissue, the sections were stained with uranyl acetate and observed under E.M.\textsuperscript{177,178}

This method offers some of the advantages of E.M., but in order to obtain these advantages, one must have specimens preserved specifically for E.M. In addition, other researchers have found that the visualization of biotin-labelled DNA can lead to higher background in mitochondria
than is obtained for digoxigenin-labelled DNA.\textsuperscript{179} This can be overcome by labelling the DNA with digoxigenin, as will be discussed later. \textit{In situ} hybridization visualized using E.M. is a powerful technique which can potentially provide very important data on mtDNA abundance and localization. However, this technique still requires that one have access to tissue prepared especially for E.M. immediately post-mortem. I did not have access to a sufficient number of suitable samples for this to be a viable option in my situation.

ii) Biotin- and digoxigenin-labelled mtDNA probes

It is possible to label a mtDNA probe with biotin and visualize the binding of this probe to mtDNA in cells under the light microscope. The localization of the bound probe is visualized colorimetrically using alkaline phosphatase conjugated to streptavidin. One advantage of this method is the relative ease with which qualitative data can be obtained by comparing staining intensity over cells. Unfortunately, a special image processor and software are required to obtain good semi-quantitative measurements from colorimetric signals.\textsuperscript{165} Another disadvantage of the use of enzyme-conjugation as a means of visualizing probe binding is the loss of good spatial resolution in the sample. The colour is produced by a diffusible substrate reacting with an immobilized enzyme. While the staining certainly localizes
near the site of probe binding, there is always going to be some diffusion. The localization properties of the colour product of nitroblue tetrazolium (commonly used to visualize alkaline phosphatase conjugate location) are discussed in the next section. A disadvantage specific to the use of biotin as the labelling agent for the mtDNA probe is the high level of background staining observed in mitochondria when the streptavidin-alkaline phosphatase conjugate is added. This is believed to occur because mitochondria are rich in endogenous biotin which binds the avidin complex. The intensity of background staining varies somewhat between samples, likely due to differences in fixation.

Digoxigenin labelling relies on the incorporation of digoxigenin-labelled dNTP’s into the probe molecule. The incorporated digoxigenin is then detected using (typically) an alkaline phosphatase conjugated anti-digoxigenin antibody. It gives a similar signal intensity, and similar advantages and disadvantages as biotinylated probes. The major practical difference between the two methods is that the use of digoxigenin prevents the high cytoplasmic background staining seen due to endogenous biotin in mitochondria.

In samples in which the background staining from endogenous biotin is low, the combination of a digoxigenin-labelled mitochondrial DNA probe, and a biotin-labelled nuclear DNA probe (conjugated to a different reporter enzyme,
eg. one with peroxidase and one with alkaline phosphatase) could provide an internal standard within each cell to help control for small differences between section thicknesses, etc. In the absence of a good system for quantifying staining intensity, however, the value of such an internal control is reduced. The difficulty in quantifying staining intensity could be overcome in part by visualizing the bound probes using fluorescently conjugated secondary molecules (common fluorochromes used include rhodamine and aminomethyl-coumarin acetic acid). The intensity of fluorescence could then be quantified. One major limitation of this technique is that it is not readily applicable to paraffin-embedded sections, due to autofluorescence of tissue. The long-term goal of this project was to look for changes present in the A.D. population in general. This would have required access to a large number of archival samples. The vast majority of such samples are preserved in paraffin blocks, making fluorescence in situ hybridization unsuitable for analysis of these samples.

iii) In situ polymerase chain reaction

The combination of the principles of the polymerase chain reaction (PCR), with in situ hybridization is a recent development which allows the localization of low copy number DNA or RNA sequences in cells or tissue sections which might not otherwise be detected by in situ hybridization alone. The
principle of the method is the same as for conventional PCR, however the template DNA is not isolated from its host tissue. Rather, the reaction is performed on top of (or around) the tissue section containing the target. This method has been applied as a semiquantitative tool for the assessment of low-copy number transcripts.\textsuperscript{182} This technique is applicable to the detection of specific DNA sequences in paraffin sections.\textsuperscript{183} Mitochondrial DNA is relatively abundant in cells and therefore for my purposes this method does not seem necessary, or even potentially beneficial. One of the strengths of this technique is reported to be the specificity (and reduction of background) one can achieve by using labelled (typically biotinylated) dNTP's during the amplification process.\textsuperscript{182,183} In theory this reduces background by eliminating the need to probe the amplified DNA with a secondary (labelled) DNA probe. Ideally, provided that the primers used for PCR are sufficiently specific, the only DNA amplified (and labelled during amplification) will be the target. The amplified DNA containing the label (eg. biotin), could then be visualized by the addition of a reporter molecule (eg. avidin conjugated alkaline phosphatase). Unfortunately, the incorporation of labelled dNTP's does not always appear to be highly specific. Significant non-specific labelling has been observed for this system when used on samples containing dying cells.\textsuperscript{184} It appears that the
fragmented DNA in such cells can serve as primers to initiate incorporation of labelled nucleotides into non-target DNA. As a significant proportion of the cells of interest in A.D. sections are dying, this could present a significant problem for my application. The solution to this difficulty for in situ PCR is to use unlabelled dNTP's in the amplification step, and then to probe the amplified target DNA with a labelled probe (by standard in situ hybridization methods). This is useful when the copy number of the target DNA is limiting and it could not be detected without amplification, however it does add a processing step, and it makes it more difficult to quantify the original amount of target DNA present in the tissue. As the copy number of mtDNA in neurons is not limiting for conventional in situ hybridization, and in situ PCR can not offer an increase in sensitivity (and would be more difficult to quantify), this method is not appropriate for my purposes.

iv) $^{35}$S-labelled mtDNA probes

The use of radiolabelled probes to mtDNA is a common method for the quantitation of mtDNA in cells. $^{35}$S is the preferred radioisotope because its comparatively low emission energy (0.17 MeV as compared to 1.7 MeV for $^{32}$P) allows good intracellular localization of probe binding. The major advantages of the use of $[^{35}\text{S}]$dATP-labelled probes are
the ability to get some sense of the intracellular localization of probe binding, and the ability to quantify probe binding. The relative signal between similar sections from the same experiment can be determined by counting the number of silver grains over individual cells and comparing the average number of grains per unit area.\textsuperscript{187,188} Depending on the probe used, \textit{in situ} hybridization can be made somewhat resistant to the effects of deletions in the mtDNA of some subjects. Certain regions of mtDNA are more subject to deletion than others\textsuperscript{171,175}, so the most reliable results would likely be obtained by avoiding these regions. In addition, the use of two probes to different regions of mtDNA should give the same signal ratio (A.D. vs. control). If the ratios are markedly different it is likely that one of the probes used is specific for a region deleted or grossly mutated in one or several subjects.

There are several disadvantages to the use of $[^{35}\text{S}]\text{dATP}$-labelled probes shared by every other histological method discussed except those involving electron microscopy. The first of these is the inability to distinguish a signal coming from mtDNA within the neuron of interest, but near the cell membrane, from the signal due to mtDNA in a neighbouring neuron forming a synapse with the neuron of interest. It is not possible to clearly distinguish two such cells under the light microscope without additional staining. The second
major disadvantage with the use of radioisotopic in situ hybridization is the potential effect of Alzheimer's disease related changes on cellular morphology. It is possible that mitochondria could break down into submitochondrial particles during the later stages of neuronal death in A.D. This breakdown could lead to the separation of a mitochondrion's copies of mtDNA over a larger area within the degenerating neuron. Probe fragments bound to separated mtDNA's might give a signal easier to see and count as distinct grains, than would have been the case when the mitochondrion was intact and the probe fragments were bound to mtDNA's close to one another. During quantitative analysis of in situ hybridization one attempts to count individual silver grains, however one does see grains of varying sizes, suggesting that more signal may have gone in to forming one grain than another. This would be expected to average out over an experiment where samples from all groups had mtDNA spaced in the same sort of manner, however if one sample consistently had mtDNA's spread over a larger area due to the formation of small sub-mitochondrial particles, this sample might have a greater number of silver grains, each with a smaller average size. A similar difficulty has been noted with biotin and digoxigenin labelled probes conjugated to alkaline phosphatase.205,206
Another limitation of in situ hybridization (and other non-E.M. histological techniques) as applied to this system is the possibility of neuronal volume changes due to A.D. Changes in cellular volume have been reported in A.D. These changes typically lead to reduced neuronal volume, but swelling has also been observed.\textsuperscript{189} As described in the section on E.M., this could lead to a change in mtDNA per unit area, without there being a true increase in mitochondrial DNA per cell. This question could be addressed by considering the average neuron size in A.D. and control sections; however, a preferential loss of large neurons has been observed in A.D.\textsuperscript{190,191} Therefore, a slight decrease in the average size of neurons assayed in A.D. samples compared to controls could be attributed to the selective depletion of large neurons in this population, without significant shrinkage of the neurons assayed. This difficulty complicates the analysis of results which are most easily expressed as signal per unit area. The quantification of a second component in the neuron as an internal standard would allow one to overcome this challenge by expressing the mtDNA signal as mtDNA signal as a percent of control signal.

In summary, the major disadvantage of in situ hybridization is one shared by all the histological techniques discussed except E.M. In A.D. brain one has many populations of cells. It is possible to select large neurons under light
microscopy, and restrict oneself to neurons in a particular region, but one can not know for certain what the intracellular state of that cell was just prior to fixation. I was interested in studying mitochondrial changes occurring well prior to neuronal death. In A.D., large populations of neurons are dying all the time, and it is difficult to ensure that these neurons are not assessed. This difficulty could be overcome in part by performing immunohistochemistry on each section in addition to in situ hybridization. By using antibodies to paired helical filaments (PHF) (components of neurofibrillary tangles and neuritic plaques) one could identify potential problem areas and assess mitochondrial changes in cells not already showing signs of advanced A.D. specific degeneration.

V) Summary and rationale for the selection of technique

From the techniques discussed above, it was necessary to select one method for preliminary investigation of potential mitochondrial abnormalities in A.D. As previously discussed, I was interested in obtaining quantitative data. That left me with the question of what mitochondrial component or feature to quantify that would provide the most accurate measure of mitochondrial components in these cells, while remaining manageable for analysis purposes.
Electron microscopy would have allowed detailed assessments of mitochondrial area cross sectional in neurons. In conjunction with colloidal-gold in situ hybridization, it would have allowed the measurement of mtDNA levels in these cells, and in conjunction with immunogold immunohistochemistry it would have allowed the measurement of relative mitochondrial protein levels in these cells. However, little suitable tissue was available to us. In addition, the investment in E.M. costs and analysis time for the large-scale application of this technique was formidable. For these reasons, E.M. was not selected for use.

Immunohistochemistry assessed under light microscopy was a more practical method for my purposes, but suffered from difficulties in obtaining quantifiable results from the tissue available to us, and potential problems resulting from altered protein expression and degradation in A.D. cells. For this reason, immunohistochemistry was not chosen as the primary means of investigating my hypothesis.

DNA isolation and either PCR or direct Southern or dot blotting did not allow the study of the distinct subpopulation of cortical cells in which I was interested. In situ PCR did not offer an increase in specificity (over conventional in situ hybridization) in this system, and would have made quantification more complex.
In situ hybridization to mitochondrial DNA using a [35S]dATP-labelled probe was determined to be the best method for the evaluation of my hypothesis. The decision to use in situ hybridization was based on several factors:

a) The signal from a radiolabelled probe can be easily quantified by counting individual silver grains using a light microscope. This is a time-consuming process, but the equipment was available to me for this type of analysis.

b) Mitochondrial DNA is relatively stable, and would not be expected to suffer significant degradation during the handling process typical for autopsy material. Thus, it would be possible to use sections from routine clinical material. This would allow me to be very stringent in my selection criteria for suitable subjects.

c) In situ hybridization using a radiolabelled probe is easily applicable to both paraffin embedded and frozen tissue sections. While minor protocol adjustments might be required to optimize conditions, this method could be applied to archival material in paraffin. The vast majority of clinical material is preserved in this way, so it was to my advantage to select a technique which would allow me to use this material.

The decision of which reporter system to use for the visualization of probe was based on the ease of quantification
of the signal. The use of enzyme-conjugates recognizing either biotin or digoxigenin on the probe DNA would have introduced difficulties in quantifying probe binding. The use of a fluorescently-conjugated reporter would have limited me to looking at frozen sections, thus limiting me to a small fraction of the available archival material. The decision to use $[^{35}\text{S}]\text{dATP}$-labelled probes for \textit{in situ} hybridization was made because of ease of quantification and suitability of this technique for use on paraffin-embedded sections.

There are some limitations of \textit{in situ} hybridization as applied to this system:

i) In cases where there is a synapse between two neurons in a section, the resolution of \textit{in situ} hybridization viewed by light microscopy is not sufficient to allow one to distinguish which neuron the signal came from, if the mitochondria are close to the cell membrane. This limitation exists for both A.D. and control sections, however, and would not normally be expected to significantly affect results.

ii) The information one can gain about the disease stage of the neuron one is studying is limited. In A.D. large neurons are dying at an accelerated rate. Thus, if one selects large neurons randomly from a section of A.D. brain, one is likely to select more neurons in the advanced stages of neuronal degeneration than one would in a normal, age-matched brain
section. As I was interested in mitochondrial abnormalities preceding neuronal death (perhaps even predisposing neurons to die) I did not want to sample neurons already well along the path to degeneration. This limitation exists for all the methods discussed except E.M., where one could assess neuronal subcellular morphology prior to analysis. This limitation can be overcome somewhat by performing immunohistochemistry with an antibody against paired helical filaments in the same sections used for in situ hybridization. This would not interfere with manual quantification of silver grains using an [³⁵S]dATP-labelled mtDNA probe, and it would allow one to avoid cells showing advanced A.D. changes.

iii) One limitation which exists for attempts to quantify many cellular components in autopsy material is the effect of the agonal state (the condition of the patient immediately prior to death) on the levels of cellular components. Major factors determining the agonal state of the patient include hypoxia, pyrexia, coma, and dehydration.¹⁹² The possibility of hypoxia is especially critical when examining mitochondrial RNA or mtDNA levels. The levels of mitochondrially encoded proteins, mitochondrial RNA and mtDNA are significantly reduced by transient ischemia.¹⁹³⁻²⁰³ It should be possible to minimize the effect of agonal state by selecting only those subjects whose condition did not deteriorate significantly until immediately prior to death. The cellular component
assayed will also affect the degree to which agonal state differences affect the observed results. Ischemia-induced changes in mitochondrial DNA in forebrain are not observed until 2 days after the induction of transient ischemia. This is in contrast to both mitochondrial RNA and protein which began to show decreased levels within 3 hours of ischemia.\textsuperscript{193}

iv) The final major limitation of \textit{in situ} hybridization in this system relates to potential changes in the mitochondria of neurons prior to morphological changes related to A.D. The copy number of mtDNA within cells can vary in response to oxidative demands.\textsuperscript{9} It must be remembered when assessing mitochondrial changes using \textit{in situ} hybridization to mtDNA, that changes in the level of mtDNA in a cell may, but do not necessarily, reflect changes in other mitochondrial components. This question could be addressed on a small scale using E.M to measure mitochondrial area in tissues also examined with \textit{in situ} hybridization, or on a larger scale using immunohistochemistry to several mitochondrial proteins to determine if the trend observed for mtDNA was reflected in other mitochondrial components as well.

Overall, \textit{in situ} hybridization is a good tool for the examination of mitochondrial changes in A.D.; however it can not give all the answers on its own. At some point it will become necessary to use a second, and possibly a third technique to determine the exact nature of the changes
observed (if any) using *in situ* hybridization to mitochondrial DNA.

**VI) Review of papers performing *in situ* hybridization to mitochondrial DNA and factors affecting the use of *in situ* hybridization on tissue**

**i) Factors affecting the use of *in situ* hybridization on tissue**

Several groups have used *in situ* hybridization to study quantitative changes in mtDNA in tissues. The methods used share many common features; however there are some differences. Factors such as the source of the tissue examined and the method of fixation can effect the treatment of tissue necessary for *in situ* hybridization. The nucleic acid probe used and its method of visualization will also effect aspects of the *in situ* hybridization technique. In this section I will discuss the major factors affecting tissues to be used for *in situ* hybridization and how other groups have performed *in situ* hybridization to mtDNA.

The fixation of tissue alters both the microscopic appearance of that tissue, and its suitability for analysis by certain methods. The type of fixative used in most *in situ* examinations of mtDNA is formaldehyde. Formaldehyde solutions are typically used at a concentration of 4% formaldehyde in a salt buffer (known as neutral buffered formalin). The
fixative properties of formaldehyde are linked primarily to its ability to cross link proteins. Some reversible cross linking of nucleic acids is also observed.\textsuperscript{194} Formaldehyde fixation can lead to some shrinkage in the fixed tissue (about 10% in liver). The majority of shrinkage observed in formalin-fixed, paraffin-embedded tissues is actually due to the process of embedding in paraffin which can cause liver tissue shrinkage of 15%.\textsuperscript{195} While one would not expect shrinkage artifact to affect absolute signal intensity, it might have an effect on signal intensity expressed as signal (grains or percent area) as a percent of total cell area. Some methods (including mine) have used signal per unit area as a measure of signal intensity. Thus for experiments such as these it is imperative that the tissue samples examined received very similar fixation and embedding treatments to minimize the differences in cell areas resulting from shrinkage artifact. One means to overcome this to some extent would be to probe sections for a second stable cellular component which could be used as an internal control. Signal could then be expressed as mtDNA signal as a percent of the signal obtained from the internal control. The exact nature of the internal control selected would depend on the tissue studied and the pathogenic changes expected in that tissue.

The use of paraffin to embed tissue can lead to difficulties if the material is not processed with sufficient
care. The surface of paraffin-embedded tissue may become pitted when cutting the tissue sections. This can lead to holes in the sections. These holes could potentially complicate the recognition of individual cell boundaries in the regions adjacent to the holes. The difficulties associated with tissue pitting can be minimized if care is taken in the preparation and analysis of these tissues. The majority of cellular lipids are lost during the processing of tissue for paraffin-embedding. This does not prevent the localization of approximate cellular boundaries. While paraffin-embedding can cause damage preventing the localization of signal within the ultrastructure of cells, it will normally preserve sufficient morphology to allow the localization of signal to within individual cells. The use of frozen tissue sections avoids some of these difficulties, but introduces some of its own. A major concern with the use of frozen sections is the formation of holes in the tissue resulting from the formation of large ice crystals during freezing. Frozen sections may be fixed either prior to freezing and sectioning, or after sectioning. Tissues fixed prior to sectioning do not adhere well to slides when sectioned. This difficulty can be partially overcome by the use of treated slides (for example APTEX coated) and the use of extreme care during all stages of sample processing. This is particularly challenging for in situ hybridization
where the post-hybridization removal of the coverslips can lead to the loss of material from the slides. Thus, there are potential difficulties with the use of either frozen or paraffin-embedded tissue sections for in situ hybridization. However both have been used successfully for in situ hybridization and have allowed the localization of signal to individual cells.\textsuperscript{196,200,201}

Neutral buffered formalin appears to be a good fixative for tissues destined for examination using in situ hybridization to DNA assayed under the light microscope. This assessment is based on two lines of evidence. The first is the preservation of DNA in formalin solutions. These experiments indicated that formalin fixation preserved DNA fragment length both in tissues and in solution.\textsuperscript{197} The second type of investigation supporting the use of formalin fixation looked at the signal strength and localization from biotin-labelled oligonucleotide probes used for in situ hybridization. The strongest and best localized signal (determined by subjective analysis) was obtained from tissues fixed in formalin.\textsuperscript{198} Other fixatives investigated were Bouin's solution, B-5, Omnifix, and ethanol. Thus, it appears that formalin fixation is satisfactory for the preservation of DNA in tissues to be examined by in situ hybridization.

The time between the removal of the tissue (or the death of the patient) and the fixation of the tissue is critical to
the preservation of that tissue. Some tissues are more prone to undergo rapid autolysis than others. Tissues of the central nervous system undergo rapid autolysis and must be fixed promptly after death to preserve subcellular morphology. Subcellular changes occurring during autolysis include condensation, fragmentation, or lysis of nuclei, swelling of the cytoplasm, and the eventual conversion of the tissue to a granular homogeneous mass. The mitochondria in cells undergoing autolysis may display a rounded shape, condense, swell, or rupture. Clearly these changes are undesirable in tissues to be studied by in situ hybridization. The degree to which autolytic changes interfere with the interpretation of data obtained from tissue sections depends in part on the magnification at which the sections are to be studied. Cells which appear normal under the light microscope may show signs of autolysis when viewed under the electron microscope. Thus, material destined for examination under the electron microscope must be obtained and fixed immediately post mortem. While this is possible with human biopsy material and animal tissues it is very difficult for human material where a post mortem interval of twelve hours before autopsy is common.

Several different types of probes to mtDNA were used in these papers. The majority of papers used DNA probes, although there were two papers in which RNA sense strand
probes to cytochrome c oxidase subunit I were used. While one paper reports results using a total human mtDNA probe, the majority used probes encoding between 0.8 and 3 kbp of mtDNA. Several investigators looking at mtDNA in clinical material used more than one mtDNA probe. They used one probe complementary to a mtDNA region within the common deletion region and one outside this region. The purpose of this was to assess the relative proportion of wild type to mutant DNA in individual muscle fibres. The different probes were used in serial sections from each subject and the degree of signal reduction in the samples hybridized using the probe complementary to the deleted region of mtDNA was assessed. The methods of signal quantification will be discussed later.

The method used to label the probe may be radioactive or non-radioactive. In the papers reviewed here the two radioisotopes used for probe labelling were $^{35}$S and $^{32}$P. As discussed previously, $^{35}$S labelling allows better localization of probe binding. This is due to the lower emission energy of $^{35}$S. The one paper in which probes were labelled using $[^{32}\text{P}]$dATP did not attempt to localize probe binding within individual cells. Several groups have used $^{35}$S-labelled probes to demonstrate localization of signal within cells. No attempt has been made at the light microscope level to localize signal from mtDNA within specific intracellular compartments. Reports of signal localization were restricted
to very general areas within cells (i.e. the periphery of the cell versus the centre), and within cortical layers. Where adequate figures are provided in the paper one can generally observe the signal localization described.

The use of non-radioactive probes for in situ hybridization to mtDNA is not common, but has been employed. The paper of Muller-Hocker et. al 196 reports the use of a digoxigenin-labelled probe, visualized using alkaline phosphatase bound to an anti-digoxigenin antibody. No attempt was made to determine signal localization in these sections.196 The alkaline-phosphatase reaction with NBT/BCIP has been shown to give an assayable intracellularly localized signal; however this was observed for low copy number targets.205,206 In the case of hybridization to mtDNA it is likely that the high copy number of the target DNA leads to loss of spacial resolution within the cell. It is still possible to distinguish signal arising from one cell from signal arising from a neighbouring cell.196 Biotin-labelled probes have been used in in situ hybridization to mtRNA assessed under the electron microscope. In this case the location of probe binding was visualized using colloidal gold, as opposed to an enzyme reaction with a diffusible substrate. The material examined was tissue culture cells and surgically removed tissue fixed immediately with glutaraldehyde and
paraformaldehyde and embedded in Lowicryl. This was necessary to preserve the morphology of the tissue sufficiently for examination under the electron microscope.\textsuperscript{177}

The apparent localization of an enzyme in tissue sections can be affected by two major processes. The first is diffusion of the enzyme itself to secondary sites within the tissue of interest. The enzyme can dissociate preferentially from certain locations giving the appearance of "improved" localization at the remaining sites.\textsuperscript{207} This is more likely to play a major role in histochemical procedures in which the affinity of the enzyme for its site within the tissue has not been rigorously challenged prior to the addition of substrate. In the case of \textit{in situ} hybridization visualized by enzyme conjugates it is standard procedure to wash the probed tissue prior to the addition of enzyme conjugate, and then wash the enzyme-conjugated tissue section in substrate buffer alone prior to the addition of substrate. This wash should remove most of the diffusible enzyme in the sample, preventing the majority of non-specific signal resulting from diffused enzyme.

The second major factor affecting apparent signal localization from enzyme-conjugated probes is the nature of the coloured product. The product must localize predominately in the immediate vicinity of the enzyme. Two major factors can affect this. The first is the solubility of the reaction...
product in the reaction medium. One wants to use a reagent which will give a reaction product that is insoluble in the aqueous reaction medium. In addition, solubility in lipids is undesirable as this may compromise the specificity of signal localization. The structure of the precipitate is also important as only those reaction products forming small discrete deposits will allow good localization of signal. The second feature affecting the localization of the coloured product is its substantivity. Substantivity is a measure of the affinity of a product for proteins. Reaction products with high substantivity will show improved localization due to protein binding resulting in decreased solubility. A major reagent type used in assaying the presence of immobilized enzymes used in in situ hybridization is the tetrazolium salts. Tetrazolium salts can be reduced to coloured formazan products, the deposition of which can be observed under the light microscope. The tetrazolium salt most commonly used in in situ hybridization experiments is nitroblue tetrazolium (NBT). The formazan produced by the reduction of NBT has several advantages over the formazan products of other tetrazolium salts used in histochemistry. The formazan product of NBT is strongly coloured and microcrystalline allowing good localization. It is insoluble in lipids and binds strongly to proteins, increasing its insolubility and hence its localization. It does however have one weakness.
Nitroblue tetrazolium tends to form deposits at lipid-aqueous interfaces. The deposited product appears to be bound to proteins in the lipid membrane, rather than to the lipids themselves. This deposition should not interfere with the localization of signal at the light microscope level. Non-isotopic in situ hybridization frequently uses a digoxigenin or biotin-labelled nucleic acid probe which is visualized using an alkaline-phosphatase conjugated antibody (or in the case of biotin avidin or streptavidin may be used). The location of probe binding is then determined by the addition of the nitroblue tetrazolium - 5-bromo-4-chloro-3-indole phosphate enzyme substrate solution. In this application the use of alkaline phosphatase and NBT allows good intracellular signal localization when the targets sites are limited in number. Some difficulties in determining exact subcellular localization can occur when numerous target sites are adjacent to one another as the signals from each target tend to blend together.

ii) Analysis of the methods used in the detection of mitochondrial DNA in tissue sections using in situ hybridization

The tissue used for in situ hybridization and subsequent analysis is crucial to the success of the assay and its analysis. As discussed previously, the fixative used and the
embedding method employed will both affect the suitability of
the tissue for analysis. The vast majority of papers
discussed here report findings from formaldehyde fixed frozen
tissue sections. This appears to be the best method currently
available for the fixation of tissue for use with in situ
hybridization. Most papers report the fixation of
cryosectioned tissue with 3 or 4 % paraformaldehyde at room
temperature. The duration of fixation is not
reported in all cases, but 5 to 10 minutes is typical. This
is a much shorter duration of fixative treatment than is
typically employed for tissue blocks obtained on autopsy. The
fixation of frozen sections after sectioning allows rapid
fixation because the formaldehyde can penetrate the thin
tissue section much more rapidly than is possible for large
blocks of tissue. Of the papers reviewed, there were two in
which this method of tissue treatment was not employed. The
first was the paper of Collins et. al in which tissue
sections were fixed in methanol prior to in situ
hybridization. Methanol fixation preserves DNA well, however
it does not cross link protein as do the aldehyde fixatives.
This leaves the section more accessible to probes, but more
vulnerable to damage or loss during handling. It is
interesting to note that the average probe size used in this
experiment was quite large (1000 to 3000 bp) compared to the
suggested size of 100 bp. The use of methanol as a
fixative may have allowed the use of such a large probe. The remaining steps in the in situ hybridization assay were very similar to the methods used by others.

The paper of Muller-Hocker et al. reports the use of paraffin-embedded tissue. The basic steps of in situ hybridization employed in these experiments were very similar to those used with frozen sections. There were two additions to the normal pretreatment procedure. The first was the removal of paraffin from the sections prior to treatment with proteinase K. The second change to the pretreatment was the acetylation of the tissue following treatment with proteinase K. Acetylation was performed to reduce background signal resulting from probe binding to basic proteins in the tissue section. The method of probe labelling and visualization used was different in these sections, as a non-isotope labelling method was used. The probe was labelled with digoxigenin-nucleotides. Following hybridization and washing of tissue the slides were incubated with alkaline phosphatase conjugated to an anti-digoxigenin antibody. Probe binding was assessed by the formation of the coloured formazan product of NBT over the region of the tissue section containing bound probe.

Several methods of probe labelling were employed in the papers examined. The most common technique employed ³⁵S-labelled nucleotide incorporation into the probe. There was
one paper in which \(^{32}\text{P}\)-labelling was employed, however in this case no attempt was made to localize signal within cells.\(^ {202}\)

As previously mentioned, the paper of Muller-Hocker et. al\(^ {196}\) reports results obtained using digoxigenin labelled probes. All papers examined which reported results using radiolabelled DNA probes used either the random primer method or nick translation to incorporate the labelled nucleotide. The average size of the labelled probes is not reported. Two papers report the use of RNA probes instead of DNA probes.\(^ {174,186}\)

RNA probes have the advantage of being single stranded (thereby preventing the loss of available probe by the reannealing of complementary probe strands), and of easy removal of unbound probe following hybridization by treatment of the tissue sections using RNase. An additional advantage of the use of single stranded probes is that if one uses the sense strand in the hybridization it should not bind to RNA remaining in the tissue section. This should increase the signal specificity for the DNA target. While the use of RNA probes clearly offers some advantages in terms of signal specificity and reduction in background, it should not fundamentally alter the results obtained from \textit{in situ} hybridization.

The degree of pretreatment of tissue sections prior to hybridization was determined by several factors. These will have included the type of tissue examined (and how it was
fixed) and the probe used in hybridization. All of the papers examined pretreated the tissue sections with RNase prior to hybridization. The exact concentration and treatment time varied somewhat between groups, with the least rigorous treatment consisting of treatment with 50 µg/mL RNase for 30 minutes at 37 °C\textsuperscript{196,200,203} and the most rigorous consisting of 100 µg/mL RNase for 1 hour at 37 °C\textsuperscript{201,204}. Pretreatment of sections with proteinase K, while common, was not always performed. A typical treatment was 5 µg/mL proteinase K for 10 minutes. No proteinase K pretreatment was performed in the experiments in which RNA probes were used for hybridization.\textsuperscript{174,186} This may reflect a difference in the ability of RNA probes to enter tissue.

Most of the papers examined used essentially the same hybridization method. This was the method of Mita et al\textsuperscript{200}. In this method the probes and the tissue were denatured separately. The probe was denatured by heating and the tissue was denatured by heating in the presence of 70% formamide. The tissue sections were dehydrated through ethanol and the denatured probe, dissolved in the hybridization solution, was applied to the denatured tissue section. The hybridization solution used contained: 50% formamide, 600 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.12% polyvinylpyrrolidone, 0.12% ficoll, 0.6% BSA, 10% dextran sulphate, 10 mM DTT, and denatured salmon sperm DNA at 200 µg/mL. Even within papers
referencing the methods of Mita et. al there was some variation in the exact concentration of components in the hybridization solution. The concentration of salmon sperm DNA, EDTA, bovine serum albumin, ficoll, and polyvinylpyrrolidine were often altered by different groups. These are all components involved in blocking potential non-specific probe binding to the target tissue. The concentration of these reagents required to reduce non-specific signal to acceptable levels will be determined by factors specific to each probe and tissue. Thus it is not surprising that different papers report the use of different concentrations of these components. Some groups prehybridized the tissue sections in the hybridization solution (usually without DTT and dextran sulphate) for one or two hours prior to denaturing the tissue and hybridization. The purpose of this was to saturate the non-specific binding sites on the target tissue prior to the addition of probe. While this could reduce background, the value of the prehybridization must be reduced substantially by the removal of the prehybridization solution and denaturation of the tissue prior to hybridization. This is likely the reason that many groups did not prehybridize the tissue sections but relied solely on the ability of blocking agents to reduce non-specific binding during hybridization. Some groups used 2 x standard sodium citrate (SSC) buffer in place of NaCl alone in the
hybridization solution. The use of 2 x SSC gives 0.6 M Na⁺ in the solution, the same concentration used by Mita et. al. When SSC is used there is no need to use Tris-HCl in the solution as a buffer. The hybridization temperature used ranged from 37 °C to 44 °C, with most groups using temperatures near 42 °C. One would expect some variation in hybridization temperature due to differences in the probes used. The formamide concentration was usually maintained at 50%, although in the case of the experiments using RNA probes it was reduced to 40%. These experiments also used a high salt concentration (4 x SSC giving 1.2 M Na⁺ in solution) and a fairly low hybridization temperature (39 °C). This is contrary to what one would expect to find for RNA probes to a DNA target. RNA-DNA hybrids are more stable than equivalent DNA-DNA hybrids and are less susceptible to denaturation by formamide. The figures provided in these papers are not adequate to allow the assessment of signal specificity at the cellular level. In those experiments which used 35S-labelled DNA probes the activity of the probe in the hybridization solution was 1 x 10⁴ CPM per μL (where it was reported).

The majority of papers reported two posthybridization washes, the first for one hour in 2 x SSC at room temperature (similar salt concentration to the hybridization solution, but lower temperature), and the second wash in low salt (0.2 or 0.1 x SSC) at 50 °C for 1 to 3 hours. The second wash was
considerably more stringent than the hybridization conditions due to the increase in temperature and the decrease in salt concentration. The two papers in which RNA probes were used, and one paper in which DNA probes were used reported posthybridization washes in 50% formamide and 2 x SSC, rather than salt solutions alone.\textsuperscript{174,186,201} The paper in which DNA probes were used reported that the slides were washed three times for three minutes each in 50% formamide, 2 x SSC at 45 °C and then three times for three minutes each in 2 x SSC alone. These sections had been hybridized at 37 °C so these wash conditions are considerably more stringent than the hybridization conditions. The papers in which RNA probes were used reported that the slides were washed at 39 °C in 50% formamide and 2 x SSC. These wash conditions are at the same temperature as was the hybridization, however the salt concentration has been decreased by half and the formamide concentration was increased by 10%. This will result in more stringent conditions in the posthybridization wash than were present during the hybridization.

The negative controls run with the experiments were of two main types. A non-specific labelled DNA probe was hybridized to tissue sections to determine the degree of non-specific probe binding to the target tissue. Typically the non-specific DNA used was a purified plasmid. In addition to this, some papers also used non-denatured target (or, rarely,
non-denatured probe) DNA to assess the level of background signal. The use of non-denatured target DNA is useful because it provides a means of distinguishing specific signal from mtDNA from specific signal arising from the binding of the mtDNA probe to mtRNA remaining in the section. In the papers in which this control was run no significant signal was reported from non-denatured target tissue. The use of non-denatured probe on denatured target tissue does not seem like a particularly useful negative control.

The exposure time for autoradiography varied somewhat between papers, probes, and photographic emulsion varieties. Exposure times for $^{35}$S-labelled probes were typically about 14 days.

A number of the papers examined report quantitative changes in mitochondrial DNA. The majority of papers do not report any formal counting or densitometric method of analysis. This is despite the fact that such methods exist and have been shown to provide reliable quantitative or semiquantitative measures of relative signal levels. Despite the lack of formal quantification in most of these papers, the quantitative changes which they report do appear to be present. This assessment is based on the figures provided in the papers themselves (if one trusts that they are representative). The purpose of most of these papers is to demonstrate a change in mitochondrial DNA content in
individual muscle fibres. They are not concerned to quantify the magnitude of this change, so long as it appears real and consistent. The sections examined in each experiment were treated together under the same conditions and should therefore be comparable to one another. The reported differences in signal intensity (and signal localization where reported) are supported by the figures provided in the papers. While in some ways it might be more reassuring to have numerical values for the relative signal levels obtained in these experiments it is not necessary to the goal of these papers. The paper in which alkaline-phosphatase was used to assess probe binding reported a quantitative difference in signal intensity, however no formal densitometric analysis method was reported.¹⁹⁶ Once again, this figures provided in the paper support the results reported. One paper reported the use of a formal method to assess the signal intensity. In this case, the relative intensity of the signal was determined by counting the silver grains visible over a fixed area of individual muscle fibres in serial sections. The purpose of this paper was to assess the relative ratio of mtRNA signal to signal from mtDNA encoding that transcript. Therefore, while relative mtDNA levels were determined they were not the final goal of this paper.²⁰⁴
C) Objective

To determine if there is a quantifiable change in mitochondrial DNA in neurons from Alzheimer's disease subjects compared to age-matched control subjects.
Chapter 2

Methods

A) In situ Hybridization

The methods used for radioactive in situ hybridization were similar to those used by Mita et al.\textsuperscript{200} with several modifications. There were differences in the types of target tissue used and their processing. Frozen sections were fixed prior to sectioning rather than after. The sections used in my experiments were fixed for a much longer period of time. In addition I examined paraffin-embedded sections and cytospins. The procedures used in handling these sections are described. The concentration of proteinase K employed in my experiments was higher than that used by Mita et al.\textsuperscript{200}. The hybridization buffer employed in my experiments was similar to the one used by Mita et al., however I used a higher concentration of DTT and Denhardt’s solution. The posthybridization washes in my experiments were shorter in duration and lower in temperature than those reported by Mita et al. The potential significance of these changes to my results are considered in the discussion. The methods used for non-radioactive in situ hybridization were those provided with the Gibco-BRL in situ hybridization system.
I) Preparation of slides containing target DNA

a) Human Brain Sections

Brain sections (cerebellum and anterior cingulate) from A.D. and age-matched control subjects (formalin fixed, frozen, mounted on charged glass slides) were the generous gift of Dr. D. Munoz (Director of Neuropathology at the University of Western Ontario). Dr. Munoz screened potential subjects, and selected appropriate sections. Dr. Munoz also taught me how to locate and identify large neurons to study in these sections.

Formalin fixed human forebrain tissue was a kind gift of Dr. J. McGuire at the Hamilton General Hospital. Tissue was washed in 10% sucrose, then 30% sucrose until it was denser than the sucrose solution. At this point it was frozen and sectioned. Fifteen micron sections were mounted on 3-aminopropyl-triethoxy-silane (APTEX)-derivatized glass slides.

b) Rat tissue sections

Paraffin embedded sections of formalin-fixed rat tissue sections were mounted on APTEX-derivatized glass slides. Kaye Suyama prepared and mounted these histological sections.

c) Preparation of cytospins

HL60 cells were grown in suspension in RPMI containing 10% FBS (fetal bovine serum), 100 units per mL penicillin G sodium, 100 μg/mL streptomycin sulphate, 0.25 μg/mL amphotericin B
(Gibco BRL). Ten mL of cell suspension was removed and the cell density was determined. The cell concentration was adjusted to $2 \times 10^5$ cells/mL with sterile PBS (phosphate buffered saline). Cells ($4 \times 10^4$ cells per slide) were immobilized to APTEX coated slides using a Cytospin centrifuge (Shandon). Cells were spun at $1.5 \times 10^4$ RPM for 5 minutes. Immediately after immobilization the slides were fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature. They were then dehydrated through graded ethanol, and placed in a sealed box with desiccant. The slides were stored at -70 °C until required.

II) Preparation of DNA probes
a) Preparation of radiolabelled probes
i) Probes for \textit{in situ} hybridization

The probes labelled were: lambda DNA (\textit{Hind} III digested) (New England Biolabs), a 1.4 kbp fragment encoding the carboxyl terminal portion of heat shock protein 60 (HSP 60) (a kind gift from Dr. R.S. Gupta, Department of Biochemistry, McMaster University)\textsuperscript{214}; and the full length murine mitochondrial DNA probe, pAMI (obtained from Dr. D.A. Clayton, Department of Pathology, Stanford University). The probes were labelled using nick translation with the standard method provided by Gibco (cat.#8160SB). $[{\textsuperscript{35}}S]$dATP (New England Nuclear) was used to radiolabel 200 ng of DNA in each reaction. The final
concentration of dATP in the reaction was 0.33 μM. dCTP, dGTP, and dTTP were present in the reaction at a final concentration of 20 μM. The reaction was allowed to proceed at 15 °C for 1 hour and then EDTA was added to a final concentration of 27 mM to stop the reaction. The samples were precipitated by the addition of ammonium acetate to a final concentration of 1.25 M and cold absolute ethanol to a final concentration of 67%. The percent incorporation for each probe was determined as described for the preparation of the $^{32}$P-labelled probe for autoradiography. The percent incorporation was found to be approximately 60% for lambda DNA and 75% for hsp 60 DNA. Calculations for counts added during hybridization were based on incorporated counts only.

ii) Mitochondrial DNA probe for Southern analysis

The mitochondrial DNA probe used was pAMI, obtained from Dr. D.A. Clayton at Stanford University. This probe contains full length murine mitochondrial DNA, cut with Hae II. The mitochondrial DNA probe was inserted into the vector pACYC177 at the Hae II cut site. The total construct size was 20235 bp. The probe DNA was labelled using $[^{32}\text{P}]$dATP (New England Nuclear) and the random primer method of labelling. 25 ng of pAMI DNA was denatured by boiling and snap cooling on ice. dTTP, dCTP, and dGTP were added to a final concentration of 20 μM each. The reaction buffer used was that provided by Gibco with the labelling kit (Gibco BRL cat.#8187SA). $[^{32}\text{P}]$dATP was
added to a final concentration of 0.33 μM. Klenow fragment was added and the labelling reaction was allowed to proceed for 1 hour at room temperature. After an hour, EDTA (pH 7.5) was added to a final concentration of 18 mM to stop the reaction. One microliter of the reaction solution was placed on each of two Whatman DE51 filters. One filter was then placed on a vacuum filter apparatus and washed once with 30 mL each of 0.5 M Na₂HPO₄, sterile distilled water, and cold absolute ethanol. Each filter was placed into a scintillation vial containing aqueous counting solution, and the degree of radioactivity retained on each filter was determined. The percent incorporation was determined by comparing the radioactivity retained on the washed filter to the radioactivity on the unwashed (total) filter. It was determined that the percent incorporation was approximately 75% (4.3 x 10⁴ CPM/5.8 x 10⁴ CPM), and that the total incorporated counts available to be hybridized to the blotting membrane were approximately 2.3 x 10⁶ CPM.

b) Preparation of biotin conjugated probes
The following probes were labelled using biotin-nucleotides: lambda DNA, pBR322 DNA (both from New England Biolabs), pAMI (mitochondrial probe), adenovirus type 2 DNA (Sigma), and the 1.4 kbp fragment of the hsp 60 probe. The probes were
labelled using Gibco's BioNick labelling system (cat.#18247-015). The reaction was carried out following the procedures recommended by Gibco BRL. Biotin-14-dATP was used to label the probe DNA. All dNTP's were present in the reaction at a final concentration of 20 $\mu$M; 10 $\mu$M of the dATP was biotin conjugated, the other 10 $\mu$M was not. Unincorporated dNTP's were removed by two precipitations using ammonium acetate (1.25 M final concentration) and cold ethanol (67% final concentration).

III) Preparation of tissue and cytospins for in situ hybridization

Paraffin sections were deparaffinized by two five minute washes in xylene, followed by hydration through graded ethanol. All tissue sections were washed for 5 minutes in PBS, and then refixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature. The slides were then washed twice for 5 minutes each in PBS. The exact pretreatment conditions used varied with the probe used and the tissue examined. This was necessary due to differences in probe size with the different labelling methods employed, and due to differences in target DNA location (cytoplasmic or nuclear) and accessibility in cells or tissues.

a) Tissue to be probed with $^{35}$S-labelled mitochondrial DNA and pBR322 probes
Once the slides were washed in PBS they were incubated in a solution containing 50 μg/mL RNase A (Sigma) and 3 μg per mL proteinase K (Sigma) in 0.5 M sodium chloride, 10 mM Tris-HCl pH 8 for 30 minutes at room temperature. The slides were then rinsed for 5 minutes in PBS. The slides were dehydrated through graded ethanol and allowed to air dry at room temperature.

Negative control slides treated with DNase were pretreated in the manner described above. However, these slides were not dehydrated following treatment with proteinase K. Instead, they were covered with DNase solution (Sigma) in 50 mM sodium acetate pH 6.5, 10 mM magnesium chloride, 2 mM calcium chloride, and incubated at 37 °C overnight under siliconized coverslips. The following day the coverslips were removed and the slides were washed for 4 x 5 minutes in 0.5 x SSPE (standard sodium phosphate EDTA buffer containing 75 mM NaCl, 5 mM NaH₂PO₄, 0.5 mM EDTA, pH 7.4), and then dehydrated through graded ethanol. The concentration of DNase used routinely was 0.5 mg/mL.

b) Tissue to be probed with ³⁵S-labelled hsp 60 DNA probe or biotin-labelled probes

Following the wash in PBS, slides were incubated with 40 μg/mL RNase A (in 2 x SSC containing 300 mM sodium chloride, 30 mM sodium citrate, pH 7.0) at 37 °C for 1 hour. They were then
rinsed for 5 minutes in PBS. Following this, the slides were treated with 40 μg/mL proteinase K in PBS at 37°C. Treatment time was optimized for signal intensity and specificity. HL60 cells probed with either [35S]dATP-labelled hsp 60 probes or [35S]dATP-labelled lambda DNA probes were treated for 30 minutes. All slides probed with biotin-labelled probes were treated for 15 minutes. Proteinase K treatment was followed by two 5 minute rinses in PBS. The slides were dehydrated through graded ethanol and air dried at room temperature.

IV) Hybridization

a) [35S]dATP-labelled mitochondrial DNA probe used in preliminary experiment with A.D. and control samples

[35S]dATP-labelled mitochondrial DNA probe was denatured by boiling for 5 minutes, and snap-cooled on ice. Tissue sections were denatured by heating at 95 °C for 5 minutes, and snap cooling on ice. Denatured probe was added to hybridization buffer (50% formamide, 2 x SSC (pH 7), 10% dextran sulphate, 10 X Denhardt’s solution (containing bovine serum albumin at 2 mg/mL, ficoll at 2 mg/mL, and polyvinylpyrrolidone at 2 mg/mL), 0.2 mg/mL herring DNA, 100 mM dithiotreitol) to a final activity of 1.2 x 10^4 counts per minute per μL of hybridization solution. Fifty μL of this solution were placed on each tissue section and a siliconized coverslip was carefully placed on top. The
slides were transferred directly to 42 °C and were incubated in a humid chamber at 42 °C overnight.

b) $^{35}$S-dATP-labelled probes used in 1994

Fifty μL of hybridization buffer solution (50% formamide, 2 x SSC (pH 7), 10% dextran sulphate, 10 X Denhardt’s solution, 0.2 mg/mL herring DNA, 100 mM dithiotreitol) containing 6 x $10^5$ CPM of $^{35}$S-dATP-labelled probe were placed on each section. A siliconized coverslip was placed carefully over each section, with no visible bubbles under the coverslip. The target DNA and probe DNA were denatured together by heating at 95 °C for 5 minutes. The slides were then incubated in a humid chamber at 42 °C overnight.

c) Biotinylated probes

Fifty μL of hybridization solution (50% formamide, 2 x SSC, 10% dextran sulphate, 1 X Denhardt’s solution (containing bovine serum albumin at 0.2 mg/mL, ficoll at 0.2 mg/mL, and polyvinylpyrrolidone at 0.2 mg/mL), 0.1 M sodium phosphate, pH 6.5) containing eight ng of probe DNA from the biotin labelling reaction was placed on each tissue section. A siliconized coverslip was placed carefully over each section, with no visible bubbles under the coverslip. The tissue and probe were denatured together by heating the covered slide to 95 °C for five minutes. Following denaturation, the covered slides were placed in a humid chamber and incubated at
42 °C overnight. This change in denaturation procedure was made in an effort to increase the efficiency of probe binding to target DNA.\textsuperscript{215}

V) Post hybridization washes

The stringency of post-hybridization washes were optimized in the later experiments to give improved signal to background ratios. The average size and base composition of the probes used in the hybridization will have affected the conditions required. DTT was used to wash slides hybridized with \([^{35}S]\)dATP-labelled probes to reduce the \(^{35}S\) in the hybridization solution and diminish non-specific binding. Formamide was included in some cases to increase washing stringency.

a) \([^{35}S]\)dATP-labelled hsp 60 and lambda DNA probes

The slides were washed for 5 minutes in 4 x SSC, 10 mM DTT at room temperature to remove coverslips. They were then washed for 30 minutes in 50% formamide, 2 x SSC, 10 mM DTT at 50 °C. They were washed once for 5 minutes in 4 X SSC at room temperature, and then dehydrated through graded ethanol containing 300 mM ammonium acetate. All dark room work was performed under D2 red filtered safelights only. The slides were dipped in Kodak NBT-2 photographic emulsion, and allowed to air dry for one hour. They were then transferred to a light-tight box and stored with desiccant at 4 °C for up to
two weeks. Sample slides were prepared for individual
development to determine exposure times.

b) $[^{35}\text{S}]d\text{ATP}$-labelled mtDNA and pBR322 DNA probes
The slides were washed twice for 10 minutes each at room
temperature in 2 X SSPE containing 0.1% SDS to remove the
coverslips. The slides were then washed twice for 10 minutes
at room temperature each in 0.1 X SSPE containing 0.1% SDS.
They were rinsed twice in 2 X SSPE at room temperature and
then dehydrated through graded ethanol containing 300 mM
ammonium acetate. All dark room work was performed under D2
red filtered safelights only. The slides were dipped in Kodak
NBT-2 photographic emulsion, and allowed to air dry for one
hour. They were then transferred to a light-tight box and
stored with desiccant at 4 °C for up to two weeks. Sample
slides were prepared for individual development to determine
exposure times.

c) Biotinylated probes
The slides were washed for 5 minutes in 0.2 X SSC at room
temperature to remove coverslips. They were then washed twice
for 15 minutes each in 0.2 X SSC at room temperature.

VI) Development of slides
a) Development of slides probed with $[^{35}\text{S}]d\text{ATP}$-labelled
probes, and slides containing $[^{35}\text{S}]\text{methionine}$-labelled U20s
osteosarcoma cells
Slides were allowed to warm to room temperature before opening the desiccated box in which they were stored. The slides were developed under D2 safelights. They were placed in Kodak D-19 developer for two minutes, rinsed in tap water for 30 seconds, and transferred to fixer for five minutes. They were then washed in gently running tap water for 15 minutes at room temperature. They were stained with haematoxylin for two minutes, and washed in tap water. They were dehydrated by two washes in absolute ethanol, and cleared in xylene. They were then mounted using Permount (Fisher).

b) Colour development for biotin-labelled probes

The visualization of probe binding for the biotin-labelled probes was performed using the reagents and conditions provided with the Gibco *in situ* hybridization and detection system (cat.# 8250SA). After post-hybridization washing the slides were incubated with blocking solution (50 mg/mL protein in 100 mM Tris-HCl pH 7.8, 150 mM sodium chloride, 0.2 mg/mL sodium azide) at room temperature for 15 minutes. They were then incubated with streptavidin-alkaline phosphatase conjugate (4 μg/mL conjugate in 90 mM Tris-HCl, 135 mM magnesium chloride, 9 mg/mL bovine serum albumin, 0.18 mg/mL sodium azide, pH 7.5) at room temperature for 15 minutes. Following this they were washed twice for 15 minutes each in Tris buffered saline. The slides were washed for 5 minutes in alkaline substrate buffer (100 mM Tris-HCl, 150 mM sodium
chloride, 50 mM magnesium chloride, pH 9.5) at room temperature. The slides were then transferred to alkaline substrate buffer containing 0.3 mg/mL NBT nitroblue tetrazolium and 0.17 mg/mL 4-bromo-5-chloro-3-indolylphosphate and incubated at 37 °C for up to 2 hours. Slides within an experiment were always incubated for the same period of time, together. They were then rinsed in distilled water and mounted using GVA mount (Ziamed).

VII) Quantification of signal density from $[^{35}S]$dATP and $[^{35}S]$methionine-labelled probes

Cells were identified using oil immersion light microscopy. Grains of silver from the autoradiograph were counted over the area of the cell around and including the nucleus. The area of the cell in which grains were counted was measured. The density of signal in each cell was expressed as grains per unit area of cell assessed. For the preliminary experiment performed using A.D and control sections, three patients per group were examined. Sections from the anterior cingulate gyrus and the cerebellum were examined for each patient. Thirty cells per brain region were quantified for silver grains per unit area.
B) Trouble Shooting

I) Test of photographic emulsion

I wished to test the effectiveness of the photographic emulsion and to calculate the average number of disintegrations required to form one silver grain in the photographic emulsion used for the in situ hybridization assay. In order to do this I labelled cells with $^{35}$S methionine, fixed them to slides, and coated with emulsion. The formation of silver grains in the emulsion was assessed for three different time points.

$^{35}$S methionine labelling of U20s osteosarcoma cells

U20s cells were grown as a subconfluent monolayer in $\alpha$ MEM containing 10% FBS, 100 units per mL penicillin G sodium, 100 $\mu$g/mL streptomycin sulphate, 0.25 $\mu$g/mL amphoterin B (Gibco BRL). They were washed twice with serum-free RPMI, and then incubated at 37 °C for 15 minutes in serum-free RPMI. The medium was removed, and replaced by serum-free RPMI containing 0.1 mCi/mL of $^{35}$S methionine (ICN). The cells were incubated for 2 hours at 37 °C. Following this incubation, the medium was removed and the monolayer was washed twice with ice-cold PBS. The PBS was removed and the cells were incubated with 10 X trypsin-EDTA (Gibco BRL) for two minutes. Medium containing 10% FBS was added, and the detached cells were removed. The cells were pelleted by centrifugation at 1000 RPM for 5 minutes. The pelleted cells were washed once with PBS and
repelleted. The cells were resuspended in PBS and counted. A known number of cells were placed in a scintillation vial containing aqueous scintillation cocktail, and the average activity per cell was determined. Cytospins of $4 \times 10^4$ cells per slide were prepared as described for the HL60 cells. The dried slides were dipped in Kodak NTB-2 emulsion, stored, and developed as described for the slides probed with $[\text{\textsuperscript{35}S}]d\text{ATP}$-labelled probes.

II) Quantification of the average number of disintegrations required to form one silver grain in NTB-2 emulsion under the normal autoradiography conditions

Slides containing immobilized U20s cells labelled with $[\text{\textsuperscript{35}S}]\text{methionine}$ were coated with Kodak NTB-2 emulsion and allowed to dry at room temperature. Two slides were developed 30 minutes after dipping (just dry), two more were developed an hour after that, and a third pair were developed the next day. The average number of grains per cell was determined for each development time. Then the average number of grains formed per minute between the first and second, and the second and third development time were determined. This was compared to the known average counts per minute per cell, and the average number of counts per grain was calculated.
III) Determination of mtDNA probe specificity

In order to determine the specificity of the mitochondrial DNA probe for mtDNA it was necessary to obtain a fraction enriched in mtDNA from mouse liver, restriction digest it, and assess probe binding to separated mtDNA fragments by autoradiography. The identity of the probe was also investigated by restriction enzyme mapping using Hae II.

a) Isolation of mitochondria from liver

Seven C57 black mice were sacrificed and 7.05 g of liver were obtained. These livers were chopped into pieces and homogenized using a glass and teflon motorized homogenizer. The homogenizing buffer used was ice cold 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.2. The samples were homogenized until no intact pieces of liver were visible (4 to 5 slow strokes at low speed). The homogenate was diluted up to 40 mL using homogenizing buffer. The sample was aliquoted into three fifteen mL tubes and centrifuged at 750 x g at 4 °C for ten minutes to sediment the nuclei. The supernatant was removed from these tubes, leaving the pellet and the fat layer (floating on top of the supernatant). The supernatant was transferred to Beckman quick seal tubes and centrifuged at 1 x 10⁴ x g for ten minutes in a Beckman floor model ultracentrifuge with a 70.1 Ti rotor, precooled to 4 °C. For DNA extraction the pellet
from the 10,000 x g spin was resuspended in homogenization buffer.

b) Mitochondrial DNA extraction
The 10,000 x g pellet was resuspended in homogenization buffer and SDS solution was added to a final concentration of 1% SDS. One-half millilitre aliquots were placed in microcentrifuge tubes. One-half millilitre of phenol:chloroform:isoamyl alcohol solution (25:24:1) was added to each sample, and the tubes were mixed gently for several minutes. The samples were spun at high speed in a microcentrifuge for 2 minutes, and the aqueous layer was carefully removed and transferred to clean tubes. This was repeated once with phenol:chloroform:isoamyl alcohol, and twice more with just chloroform:isoamyl alcohol. NaCl was added to the final aqueous layer to a final concentration of 0.2 M NaCl, and 1 mL of cold absolute ethanol was added to each sample. The samples were mixed gently and then centrifuged at high speed in a microfuge for 2 minutes. The pellet from this spin was washed with room temperature 70% ethanol and allowed to dry.

c) Restriction digestion of isolated DNA
Precipitated DNA was resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. Restriction digestion was performed using BamH I and Hae II obtained from New England Biolabs. Reactions were
performed in a final volume of 20 μL containing 20 units of restriction enzyme and approximately 15 ng of DNA. The reactions were performed using the conditions and buffers supplied with each enzyme. Digests were incubated at 37 °C overnight.

d) Separation of digested DNA fragments
A 1% agarose gel was prepared containing 0.5 μg ethidium bromide per mL. The samples from the restriction digest of the mitochondrial DNA were loaded and the gel was allowed to run at 100 volts for 2 hours. It was then photographed under ultraviolet illumination to reveal the location of the DNA bands.

e) Southern blotting of separated mitochondrial DNA fragments
The DNA was blotted to the nylon membrane (GeneScreen) using the LKB VacuGene Blotting System. The DNA was depurinated, denatured, and blotted on the vacuum apparatus according to the manufacturer's instructions for blotting high molecular weight DNA. The nylon transfer membrane was rinsed briefly in 20 X SSC, blotted on number 1 Whatman filter paper, wrapped loosely in aluminum foil, and baked at 65 °C for 1 hour to irreversibly bind the DNA.
f) Prehybridization and hybridization of transferred DNA
A prehybridization/hybridization buffer was prepared containing: 0.5 M NaH₂PO₄, 15% deionized formamide, 7% SDS, 10% BSA, 66 μg/mL herring DNA. This solution was warmed to 60 °C in a hybridization oven. The nylon membrane containing the immobilized DNA was placed in the hybridization tube with the prehybridization solution and allowed to prehybridize overnight.

g) Hybridization of blotting membrane with probe
The ³²P-labelled mitochondrial DNA probe was denatured by boiling for 5 minutes, then set on ice. 500 μL of prehybridization/hybridization solution was added to the denatured probe and the diluted probe was added to the prehybridization/hybridization solution in the hybridization tube. Care was taken to ensure that concentrated probe did not come into contact with the membrane until it was diluted in the prehybridization / hybridization solution. Once the probe was added, the membrane was allowed to hybridize at 60 °C overnight.

h) Washing the DNA transfer membrane and autoradiography
The hybridization solution was removed from the hybridization tube and 30 mL of 150 mM NaH₂PO₄, 1% SDS was added. This was incubated at 60 °C for 15 minutes. This solution was removed
and 30 mL of fresh 150 mM NaH$_2$PO$_4$, 1% SDS was added and incubated for 30 minutes at 65 °C. This solution was removed and this step was repeated once. Then the membrane was washed once at 50 °C in 30 mL of 30 mM NaH$_2$PO$_4$, 0.1% SDS for 30 minutes. After 30 minutes the membrane was removed from the hybridization tube, wrapped in saran wrap, and placed in an autoradiography cassette with Kodak X-Omat film overnight. The film was allowed to expose overnight at -70 °C, then the cassette was removed from the freezer and allowed to warm to room temperature. Once the film was at room temperature, the film was removed and processed using a Kodak automatic processor.

i) SDS-PAGE and gel staining
Samples were preserved from the major steps of the preparation of the mitochondrially enriched fraction. A 12% SDS-PAGE gel was prepared. Samples containing approximately 100 µg of total protein from each sample were diluted with loading buffer containing 100 mM dithiothreitol and heated at 95 °C for 5 minutes. The samples were loaded and the gel was run at 50 volts overnight. The gel was stained for 30 minutes in Coumassie Blue, destained for 30 minutes, then transferred to distilled water containing 0.02% sodium azide. The gel was photographed four days later.
j) Restriction digestion of pAMI (mitochondrial DNA probe) with *Hae* II

One hundred ng of pAMI DNA was digested for 2 hours at 37 °C with 10 units of *Hae* II (New England Biolabs), using the conditions and buffers supplied with the enzyme. The fragments were separated on a 1% agarose gel containing 0.5 µg/mL of ethidium bromide. The DNA standard marker used was lambda DNA digested with *Hind* III (New England Biolabs). The gel was run at 75 volts for 2 hours and photographed under ultraviolet illumination.
Chapter 3

Results

I) Preliminary data from in situ hybridization to mitochondrial DNA in A.D. and age-matched control subjects

Neurons typical of those studied from the anterior cingulate gyrus and the cerebellum are shown in Figures 5 and 6, respectively. In situ hybridization analysis of formalin fixed frozen brain sections revealed a significant increase in signal per unit area from A.D. patient sections compared to control patient sections in both brain regions examined (P < 0.05) (Table I, Figure 4). Background was determined by counting the number of grains per unit area in regions of each slide outside the tissue section. Background signal represented about 15% of raw signal from the anterior cingulate gyrus sections, and about 34% of raw signal from the cerebellum sections. The absolute background level was not significantly different between tissue or subject types. The average background grain density was subtracted from the average grain density over the neurons examined. The only negative control run in this experiment was unprobed sections, to control for problems with light leaks or chemical
interactions of the emulsion with components of the tissue sections. No significant signal was observed from these slides.

Table I: Silver grains per nm² in sections probed in situ with a mitochondrial DNA probe. A.D. and control subjects were examined. The regions examined were the anterior cingulate gyrus (ACG), and the cerebellum (CER).

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Diagnosis</th>
<th>Grains per nm² ± s.d.</th>
<th>ACG</th>
<th>CER</th>
</tr>
</thead>
<tbody>
<tr>
<td>91-170</td>
<td>AD</td>
<td>470 ± 100</td>
<td>370 ± 110</td>
<td></td>
</tr>
<tr>
<td>91-19</td>
<td>AD</td>
<td>360 ± 70</td>
<td>220 ± 70</td>
<td></td>
</tr>
<tr>
<td>90-148</td>
<td>AD</td>
<td>450 ± 150</td>
<td>300 ± 90</td>
<td></td>
</tr>
<tr>
<td>90-198</td>
<td>Control</td>
<td>230 ± 70</td>
<td>130 ± 100</td>
<td></td>
</tr>
<tr>
<td>91-185</td>
<td>Control</td>
<td>210 ± 50</td>
<td>180 ± 140</td>
<td></td>
</tr>
<tr>
<td>90-228</td>
<td>Control</td>
<td>290 ± 70</td>
<td>130 ± 50</td>
<td></td>
</tr>
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Figure 4: Average signal from sections of Alzheimer's disease and control tissue hybridized with mitochondrial DNA.
Figure 5: Typical neurons from the anterior cingulate gyrus of an A) Alzheimer's Disease Subject; B) Age-Matched Control Subject. Sections were probed with [35S]dATP labelled mitochondrial DNA probe. i) focused on cell; ii) focused on silver grains. C) Outlined version of A indicating the approximate location of the visible cell boundary and the silver grains.
Figure 6: Typical neurons from the cerebellum of an (A) Alzheimer's Disease Subject, (B) Age-Matched Control Subject. Sections were probed with [35S]dATP labelled mitochondrial DNA probe. i) focused on cell; ii) focused on silver grains.
II) DNase negative control for *in situ* hybridization to mitochondrial DNA in paraffin sections

DNase pretreatment was attempted as a negative control to demonstrate the specificity of the mitochondrial DNA probe. These experiments were performed on paraffin sections, as it was my intention to pursue further investigations of my hypothesis on this type of section. Photographs of representative neurons from rat brain sections treated with various concentrations of DNase are shown in Figure 7. It was determined that 0.1 μg/μL of DNase (Figure 7B) was sufficient to significantly reduce visible chromatin staining by haematoxylin, and that 1 μg/μL of DNase (Figure 7C) was sufficient to eliminate it. The signal observed regardless of the DNase pretreatment did not appear localized over individual cells and appeared non-specific.

III) Determination of relative signal from different rat tissues:

DNase pretreatment is not routinely used as a negative control for *in situ* hybridization to mitochondrial DNA. For this reason, I was concerned that perhaps the DNase treatment was not acting to degrade and remove the mitochondrial DNA (possibly due to packaging of the mtDNA within the cell). To test whether the signal from the probe was reflecting the level of mitochondrial DNA in the tissue examined,
Figure 7: Rat brain paraffin sections treated with DNase to determine effect on signal following hybridization with $^{35}$S dATP labelled mitochondrial DNA probe. A) No DNase, B) 0.1 $\mu$g/$\mu$L DNase, C) 1 $\mu$g/$\mu$L DNase.
hybridization to three different rat tissues was performed and the signal ratios obtained were compared to the values expected from the literature\textsuperscript{158}. Rat liver, kidney, and brain were examined. The signal obtained in this experiment did not show good localization over individual cells. The signal ratios obtained were 3:1:1 (liver: kidney: brain). This did not agree with the expected ratio of 23:2:1.\textsuperscript{158} The expected ratio was determined based on the densitometric quantitation of signal intensity from isolated DNA probed with a specific mtDNA probe. Differences between this technique and in situ hybridization could account for the dissimilarities in the results. Thus, it is not possible to draw firm conclusions from this data. Representative fields are shown in Figure 8.

IV) In situ hybridization on frozen human brain sections

While in situ hybridization should work as well on paraffin sections as on frozen sections, the pretreatment conditions required for good signal could vary. The switch to rat brain sections had been made due to their increased availability, and their suitability for hybridization with this probe. I chose to return to frozen human sections (similar to those used in the preliminary study) to see if the in situ hybridization system would work under these conditions. The negative control used in this experiment was pBR322. There is very little signal visible on these sections, and the signal intensity observed was much lower
Figure 8: Rat liver (A), kidney (B), and brain (C) paraffin sections hybridized with mitochondrial DNA probe to determine relative signal intensity from different tissues.
than that observed from the preliminary experiment (Figure 9A; see Figures 5 and 6 for comparison). There was no significant difference in the signal intensity observed from the slides probed with the mitochondrial DNA probe pAM!, or with the non-specific DNA probe pBR322.

Figure 9: *In situ* hybridization to frozen human brain sections. The probes used were (A) the mitochondrial DNA probe pAM!, and (B) the negative control probe pBR322. Additional results from similar experiments are displayed in Appendix figure A-1.
Troubleshooting:

V) Test of photographic emulsion sensitivity

I was concerned to determine if the photographic emulsion was consistently recording signals due to \(^{35}\text{S}\) disintegration. In order to do this, cells were labelled with \([^{35}\text{S}]\)methionine and immobilized on slides. A representative cell from each time point is displayed in Figure 10. The average number of counts per cell was determined prior to immobilization. An average of 1.5 counts per minute per cell was calculated. When these slides were developed silver grains were visible, localized over individual cells. The average number of grains formed per cell per minute was determined to be 0.26 for the time period between the development of the first and second pair of slides (75 minutes). The average number of grains formed per cell per minute was determined to be 0.25 for the time period between the development of the second and third pair of slides (1252 minutes). Thus, the formation of silver grains appears to be linearly related to exposure time (and therefore number of disintegrations) in this range. Representative cells from each time period are shown in Figure 10. From this data it is possible to calculate the average number of counts required to form one silver grain in the NBT-2 emulsion under my conditions. The activity of the cells was determined to be 1.5 CPM per cell, and the grains per minute per cell were
Figure 10: Test of Kodak NTB-2 photographic emulsion sensitivity. Slides were developed after drying. A: Time 0, B: Time 75 minutes, C: Time: 1252 minutes.
determined to be 0.26 grains per minute per cell. From this
data one can determine that approximately six counts were
required to form each silver grain. This would give a maximum
of approximately 120 grains per cell in the preliminary
experiment (see Appendix B).
VI) Test of mitochondrial DNA probe (pAMI) specificity to mitochondrial DNA

a) Restriction mapping of mitochondrial DNA probe (pAMI)

pAMI was digested with *Hae* II. The expected band sizes are 16295 bp, 1430 bp, and 2510 bp. The expected bands were observed with *Hae* II digestion. The uncut sample ran as three bands representing uncut covalently closed circular DNA, nicked circular DNA, and full length linearized DNA. Figure 11 shows a photograph of the gel.

![Image of gel showing bands at 23, 4.3, and 2 kb](image-url)

**Figure 11:** Restriction digest of pAMI cut with *Hae* II. Fragments of 16295 bp, 2510 bp, and 1430 bp were expected from this digestion.
b) Isolation of a mitochondrially enriched fraction from mouse liver

A SDS-PAGE gel was run with samples from the initial liver homogenate (lane 1), the supernatant from the 750 x g spin (lane 2), the pellet from the 1 x 10^4 x g spin (saved as the mitochondrially enriched fraction) (lane 3), and the supernatant from the 1 x 10^4 x g spin (lane 4). Figure 12 shows a photograph of the stained gel. One can see that the initial 750 x g spin did not selectively remove a lot of protein bands visible on the gel. While the bands from the later fractions are much sharper, the major bands remain the same for the initial homogenate and the supernatant from the 750 x g spin. This could be the result of incomplete lysis of organelles in the sample from the original homogenate.
Figure 12: SDS-PAGE of samples from different stages of the preparation of a mitochondrially enriched fraction from mouse liver. Lane 1: unfractionated liver homogenate; lane 2: supernatant from 750 x g spin; lane 3: pellet from the 1 x $10^4$ x g spin; lane 4: supernatant from the 1 x $10^4$ x g spin. The pellet from the 1 x $10^4$ x g spin was retained for DNA extraction.
c) Restriction digest of isolated DNA

DNA was isolated from the pellet from the 10,000 x g spin and digested with *BamH I* (lane 3), and *Hae II* (lane 4). The undigested pAMI probe was run in lane 1 as a positive control for the hybridization, and *Hind III* digested lambda DNA was run in lane 2 as a size standard, and to indicate the degree of non-specific binding exhibited by the pAMI probe during hybridization. A photograph of the gel is displayed in Figure 13. The bands on the original photograph are faint and do not reproduce well. The linearized mtDNA band (16295 bp) expected from *Hae II* digestion is observed on the original gel but is extremely difficult to see in the reproduction. The high molecular mass fragments expected from the *Bam HI* digestion can be observed on both the original photograph and the reproduction. The approximate location of these bands is indicated to the right of the figure. The presence of a large quantity of RNA in the gel obscured the lower molecular mass bands, and this portion of the gel has not been displayed.
Figure 13: Agarose gel separation of restriction enzyme digested DNA fragments. Lane 1: undigested pAM! probe, lane 2: lambda DNA digested with Hind III, lane 3: BamH I digest of DNA isolated from the mitochondrially enriched fraction, lane 4: Hae II digest of DNA isolated from the mitochondrially enriched fraction.

Figure 14: Autoradiograph of the Southern blot from the gel in Figure 10, probed with the mitochondrial DNA probe pAM!. Lane 1: undigested pAM! probe, lane 2: lambda DNA digested with Hind III, lane 3: BamH I digest of DNA isolated from the mitochondrially enriched fraction, lane 4: Hae II digest of DNA isolated from the mitochondrially enriched fraction.
d) Southern blot of isolated DNA fragments

The DNA separated on the gel described in c) was blotted to a nylon membrane and probed with \[^{32}P\]dATP-labelled mtDNA (pAMI) probe. The autoradiograph from this blot displayed the strong signal expected from the pAMI positive control (lane 1), and the lack of visible signal expected from the lambda DNA bands (lane 2). Bands of the expected size were observed for both the \textit{BamH} I (lane 3), and the \textit{Hae} II (lane 4) digests of mitochondrial DNA. Some smearing of signal down the length of the lanes was observed. This was not sufficient to obscure the restriction digest bands on the film. It is likely that some DNA degradation occurred during the isolation process, leading to a continuum of mtDNA fragment lengths which bound probe, giving a smeared signal on the autoradiograph. The autoradiograph is displayed in Figure 14. The reproduction of this film does not clearly reproduce the presence of two distinct bands near 7 kbp in the \textit{BamH} I digested lane. These bands are readily apparent on the original film. These likely represent the 8350 bp and the 6892 bp bands expected from this digest.
VII) *In situ* hybridization of a $[^{35}S]$dATP-labelled hsp 60 probe to HL60 cells

Cells probed with the hsp 60 DNA probe showed signal localized predominately over the nucleus (Figure 15). This is what one would anticipate if the probe was binding to nuclear genes for HSP 60. There were approximately 6 times more grains per unit area over the haematoxylin stained nuclei than over non-stained regions of the slide. No strong localized signal was observed for cells probed with the non-specific lambda DNA probe. The overall level of background was rather high in this experiment; however there is clearly increased signal localized around the nucleus (blue) in the cells probed with hsp 60 DNA. Representative cells are displayed in Figure 15 and Appendix A Figure A-2.
Figure 15: HL60 cells probed with $^{35}$S]dATP labelled (A) HSP 60 DNA probe, or (B) lambda DNA probe. The cells were counterstained with haematoxylin. (C) Outlined version of A and B indicating approximate nuclear boundaries and silver grains.
VIII) In situ hybridization of a biotin labelled adenovirus type 2 DNA probe to adenovirus type 2 infected HeLa cells

This experiment was run as a positive control for the biotin labelling method and the in situ hybridization method. Paraffin sections containing HeLa cells (some, but not all of which were infected with adenovirus) probed with biotin-labelled adenovirus type 2 DNA showed nuclear signal in some, but not all cells. This is consistent with the results predicted by Gibco, the supplier of these sections. Representative cells are displayed in Figure 16.

Figure 16: Adenovirus infected HeLa cells probed with biotin-labelled adenovirus DNA probe. Not all cells were infected with adenovirus.
IX) In situ hybridization of a biotin-labelled mitochondrial DNA probe to HL60 cells

Cells probed with the mitochondrial DNA probe pAMI showed strong signal. Representative cells are displayed in Figure 17. No significant signal was observed from cells probed using biotin-labelled pBR322 as a negative control. The cells were not counterstained.

![A](image1.png) ![B](image2.png)

Figure 17: HL60 cells probed with biotin labelled mitochondrial DNA probe (A), or biotin labelled pBR322 (B) as a negative control. The cells were not counterstained. Additional cells are displayed in Appendix figure A-3.
X) **In situ** hybridization of a biotin-labelled mitochondrial DNA probe to human brain tissue

Tissue probed with the mitochondrial DNA probe pAMI showed strong signal over distinct cell bodies. No distinct signal was observed from sections probed with the nonspecific DNA probe pBR322. Representative neurons are displayed in Figure 18.

![Image A](imageA.png) ![Image B](imageB.png)

**Figure 18:** *In situ* hybridization to frozen human brain sections with biotin labelled probes. The probes used were (A) the mitochondrial DNA probe pAMI, and (B) the negative control probe pBR322. The sections were not counterstained. Additional results from similar experiments are displayed in Appendix figure A-4.
Chapter 4
Discussion

A) Discussion of results

The preliminary data from the in situ hybridization experiment using \([^{35}\text{S}]\text{dATP}\)-labelled mtDNA probe suggested an increase in mtDNA content per unit area in A.D subjects over age-matched control subjects (Figure 4). This encouraged me to pursue in situ hybridization analysis of this system further. As discussed in the introduction, an increase in mitochondrial DNA content has been observed in some mitochondrial disorders. My preliminary results suggesting an increase in mtDNA content in A.D. brain tissues were therefore consistent with a possible role for mitochondrial abnormalities in A.D. In addition, the fact that the increase in mtDNA content was observed in both the cingulate and the cerebellum was intriguing.

If the apparent increase in mtDNA content was simply a secondary effect of the A.D. disease process, then one would expect to see increased mtDNA content in the cingulate (which is affected by A.D.) but not in the cerebellum (which is not a region believed to be significantly affected by A.D.\(^65\)). My preliminary data suggested an increase in both regions. This
apparent increase in mtDNA content in both brain regions could suggest a primary role for mitochondrial DNA abnormalities in A.D. Specific cortical effects of widespread mitochondrial abnormalities have been observed previously.  \textsuperscript{121,122}

Following my initial experiment using frozen tissue sections, I switched to paraffin-embedded sections due to greater sample availability. Attempts to develop a DNase negative control for \textit{in situ} hybridization to mtDNA in paraffin sections were not successful (Figure 7). This may have been due to the ineffectiveness of the \textit{in situ} technique in general during this time period. As no positive control was run at that time it is not possible to determine this definitively.

Following the DNase experiment I wished to assess the \textit{in situ} hybridization system to see if it was working in the paraffin embedded sections. In order to determine if the observed signal correlated to expected mtDNA abundance in the target (paraffin-embedded) tissue, \textit{in situ} hybridization to different tissues was performed. A comparison of relative signal intensities from various tissues (Figure 8) did not yield the ratio of signal intensities expected if the signal was due primarily to binding to mtDNA, suggesting that the \textit{in situ} hybridization system was not working with these sections. This raised the question of whether the difficulty lay in the \textit{in situ} hybridization procedure (e.g. reagents, probes) or in
the application of this procedure to paraffin-embedded tissue. In order to address this question I decided to return to in situ hybridization in frozen (rather than paraffin-embedded) tissue sections and to prepare a new mtDNA probe stock from stock obtained from Dr. Clayton’s laboratory. Experiments using frozen tissue sections and the new probe stock were performed. These did not lead immediately to positive results (see Figures 9 and A-1). It became clear that there was a problem with the in situ hybridization procedure which had to be resolved.

The identity and specificity of the mitochondrial DNA probe obtained from Dr. Clayton was examined. The identity of the mitochondrial DNA probe was confirmed by restriction mapping (Figure 11). The digestion pattern obtained matched the predicted pattern for this probe. The specificity of the mitochondrial DNA probe was assayed by modified Southern blotting of mitochondrial DNA, probing the blot with the $^{32}$P-labelled mitochondrial DNA probe, and subsequent autoradiography (Figure 14). This indicated that the mitochondrial DNA probe recognized mitochondrial DNA and bound it with much greater affinity than it bound the lambda DNA ladder. The results from individual steps in the preparation of the mitochondrial DNA enriched fraction are discussed at the end of this section.
The signal produced by the photographic emulsion used in the autoradiography of the in situ hybridized sections was examined for intensity following exposure to radioisotope. The effectiveness of the photographic emulsion was confirmed by the assessment of signal intensity (silver grain production) after different exposure times to \([^{35}\text{S}]\)methionine-labelled cells (Figure 10). From this experiment I determined that the photographic emulsion was detecting signal from \([^{35}\text{S}]\)dATP-labelled cells and that the signal recorded by the emulsion was linear in the range I was using.

I wished to confirm the effectiveness of the reagents used in the in situ hybridization experiment to determine if contamination of one or more solutions, or the incorrect preparation of a solution was interfering with the success of the assay. A positive control DNA probe was used to ensure that specific signal could be obtained using the in situ hybridization stock solutions. The probe used was a 1.4 kbp fragment encoding the carboxyl terminus of human HSP 60, obtained from Dr. Gupta.\(^{214}\) Hybridization of this probe to HL60 cells immobilized by cytospinning and treated with RNase gave nuclearly localized signal (as would be expected for this probe). From these results I concluded that the reagents used in the in situ hybridization experiment were adequate to allow specific signal using \([^{35}\text{S}]\)dATP-labelled probes.
Further investigations into the effectiveness of the in situ hybridization system were conducted using biotin-labelled probes. The decision to pursue further investigations using biotin-labelled probes was made because the location of biotin-dATP-labelled probes can be visualized the same day as the post-hybridization washes. This is much faster than the one to two week period routinely required for signal development with $[^{35}\text{S}]$dATP-labelled probes.

In situ hybridization experiments using biotin-labelled mitochondrial DNA probe indicated that it was possible to get specific signal from brain sections and isolated cells probed in situ with biotin-labelled mtDNA probe. Strong signal was observed in cells probed with the mitochondrial DNA probe. Representative cells are displayed in Figure 17 and in Appendix A (Figure A-3). Only very low levels of signal were observed with the negative control non-specific DNA probe.

The pattern of signal observed in brain sections probed with biotin-labelled mtDNA probe (Figure 18, A-4) differed substantially from the pattern of signal seen for the $[^{35}\text{S}]$dATP-labelled mtDNA probe used in the initial in situ hybridization experiment (Figure 6). As discussed in the introduction, it is not possible to determine sub-cellular signal localization using this system. However, one can determine if the signal is localized inside or outside of distinct cell bodies. The signal from the biotin-labelled
probe was much more strongly localized to within distinct neurons than was the case for the earlier experiment. This can be observed from the signal intensity observed within the distinct neuronal cell body compared to areas outside distinct cell bodies. The tissue hybridized with the biotin-labelled probe shows strong signal (blue-purple staining) within the neuronal cell body, yet very little signal outside (Figure 18). This is in contrast to the signal localization observed in the tissue probed with the \[^{35}\text{S}]dATP-labelled probe. In this case (Figure 6) one sees substantial signal (silver grains) outside of neuronal cell bodies. In some areas the signal intensity observed outside the cell body is similar to the intensity observed within the cell body.

The exact brain region assayed in these two experiments is different, however both experiments were performed on forebrain sections. The sensitivity of biotin-nucleotide-labelled probes is not as high as for \[^{35}\text{S}]dATP-labelled probes, however this alone can not account for the difference in signal localization in these experiments. The signal observed using the original \[^{35}\text{S}]dATP-labelled mtDNA probe outside distinct neurons is similar to that obtained inside some distinct neurons. If this signal was specific for mtDNA, then one would expect to see substantial signal from the biotin-labelled mtDNA probe outside the boundaries of distinct neurons. The presence of neuronal processes and glia
in the regions outside distinct neurons makes it possible that one would observe specific signal from these regions. The sections examined in the experiment using \(^{35}\text{S}\)dATP-labelled probes were stained with haematoxylin. This stains nuclei strongly and also lightly stains the boundaries of large neuronal cell bodies. It does not visibly stain the cytoplasm of glia, nor will it stain axons or dendrites. As these structures do contain some mitochondria, one would expect to see some specific signal from regions not stained with haematoxylin.

It is necessary to determine which pattern of signal localization is most likely to represent specific probe binding. The probes used in these experiments were prepared from different probe stocks and were labelled differently for \textit{in situ} hybridization. The probe labelled with biotin is from the same stock as the probe tested for specificity to mtDNA by hybridization to isolated mtDNA and autoradiography. The preferential binding of this probe to mtDNA has been established. In addition, the recent \textit{in situ} hybridization experiment to mtDNA using biotin-labelled probes included a non-specific DNA probe as a negative control, this was not done for the preliminary experiment. This leaves open the possibility of high non-specific signal in the preliminary experiment using the original mtDNA probe. These concerns by no means prove that the probe used in the preliminary
experiment exhibited non-specific binding; however this probe was not examined as closely as was the probe used for the later in situ hybridization experiment using biotin-labelled probe. The signal localization observed from the later probe stock (labelled with biotin) is more consistent with the expected mitochondrial DNA distribution in forebrain. Mitochondrial DNA has been shown to be preferentially concentrated in the cell bodies of forebrain neurons\textsuperscript{174}. Thus, while one can not definitively state which pattern of signal localization best represents mtDNA localization in these sections, it seems probable that the more recent probe (used in these experiments labelled with biotin), most accurately reflects this. Had a negative control probe been used in the preliminary experiment this would have facilitated the resolution of this question.

The specificity of the mtDNA probe (pAMI) used in the later in situ hybridization experiments was determined by hybridization to purified mtDNA blotted to a nylon membrane. The preparation of a mitochondrially enriched fraction was performed by differential centrifugation. The mitochondrially enriched fraction was not purified further because the goal of the purification was simply to obtain a preparation enriched in mitochondria relative to nuclei. There was no need to remove other cytoplasmic organelles from this preparation as
DNA was to be extracted, and nuclei and mitochondria are the only cellular compartments containing DNA. Figure 12 shows the major protein bands obtained from each step of the purification. Most of the major protein bands were not removed by the initial low speed spin intended to remove nuclei and unlysed cells. The medium speed spin used to spin down the mitochondrially enriched pellet resulted in the separation of the major protein bands between the pellet and the supernatant. Most bands appear in both fractions, but significant enrichment in one fraction over the other is evident for most bands.

This was not intended to be a highly purified mitochondrial preparation, and it likely was not. The agarose gel used to separate the mtDNA fragments generated by digestion with restriction enzymes shows distinct bands in the size ranges expected for mitochondrial DNA (Figure 13). The autoradiograph (Figure 14) of the gel shown in Figure 13, probed with the mitochondrial DNA probe, shows clear preferential binding of the mtDNA probe (pAM!) to the isolated mtDNA over other DNA (the lambda DNA standard) or RNA components in the blot. Thus it appears that this probe shows specificity for mtDNA.
B) Analysis of difficulties encountered with the in situ hybridization system

The preliminary data obtained using the in situ hybridization system for mitochondrial DNA appeared promising. This early data was obtained using formalin-fixed frozen human brain sections. In order to continue this path of investigation in a larger sample population, it was necessary to use paraffin-embedded sections. For this reason, the decision was made to pursue all further experiments, including the development of an appropriate negative control, in paraffin sections. When at first in situ hybridization did not appear to be working in these sections, (low signal, signal similar in negative control slides and experimental slides), it was unclear whether the problem lay in the use of paraffin sections, or in the hybridization experiment itself. In addition to the use of paraffin sections, the batch of mitochondrial DNA probe used in the later experiments was different. The use of a positive control, consisting of a DNA probe known to bind its target strongly in paraffin-embedded sections, would have facilitated the determination of the nature of the difficulty in these experiments.

After determining that I was not obtaining specific signal from the mitochondrial DNA probe on paraffin sections, I returned to fixed frozen sections (as were used in the preliminary experiments) to see if in situ hybridization would
work in this system. I also received a new mitochondrial DNA probe stock (pAMI) from Dr. Clayton's laboratory, and prepared fresh probe. In situ hybridization under these conditions did not appear to work. There was no significant difference in signal intensity between the sections probed with the mitochondrial DNA probe and the pBR322 negative control probe.

These results prompted me to examine different aspects of the in situ hybridization method, which could be contributing to the overall ineffectiveness of the technique. The first factor investigated was the photographic emulsion. This was investigated to ensure that signal was observed due to the radioactive decay of $^{35}$S in cells. This proved to be the case, and I determined that the photographic emulsion was not a problem in this system.

The next factor I examined was the specificity of the mitochondrial DNA probe for mitochondrial DNA. This was examined by the purification of mtDNA and modified Southern blotting of this DNA followed by probing with the mtDNA probe. These results indicated that the mitochondrial DNA probe was specific for mtDNA.

In order to test the in situ hybridization method it was necessary to use a positive control DNA probe. The 1.4 kbp hsp 60 DNA probe was selected for this purpose. Binding of this probe to a nuclear target in HL60 cells was observed. No significant nuclear binding of the non-specific DNA probe was
observed. This suggested that under appropriate conditions using these reagents it was possible to get specific probe binding in situ with \([^{35}\text{S}]\text{dATP}-\text{labelled probe.}\]

Further investigations on the effectiveness of in situ hybridization were performed using biotin-labelled probes. The use of a biotin-labelled probe against mtDNA gave a strong signal in both HL60 cells, and frozen human brain sections. There is one curious result, however. If one considers the signal localization from the preliminary in situ hybridization experiment on brain, one sees substantial signal outside the boundaries of any individual neuron; yet when brain was probed with the new (biotin-labelled) mtDNA probe, the vast majority of the signal appeared localized within the boundaries of distinct neuronal cell bodies. We know from the experiment in which this new probe was used against HL60 cells, and from the Southern blotting data, that this probe recognizes mtDNA much more strongly than, for instance, nuclear DNA. Thus this probe appears to bind primarily to mtDNA concentrated within neuronal cell bodies in forebrain sections. This is consistent with previous findings.\(^{174}\) This draws into question the validity of the preliminary data obtained using the \([^{35}\text{S}]\text{dATP}-\text{labelled mtDNA probe. No negative control was run in the preliminary experiment to control for the effects of non-specific probe binding. Background was calculated only based on signal present outside the region of}} \)
the slide containing tissue. No attempt was made to control for signal resulting from non-specific probe binding. Thus it is possible that most of the signal seen both inside and outside distinct cell boundaries in these sections represents non-specific binding. It is possible to estimate the maximum number of grains one would expect to find over a single neuron in the preliminary experiment. One must estimate the maximum number of copies of mtDNA likely to be present in the cell body of an average large neuron in control brain. It is possible to estimate that this number will not exceed 1000 copies per cell body. The experiment in which cells were labelled with \[\text{[}^{35}\text{S]}\text{methionine}\) (Figure 10) indicated that approximately 6 counts were required for the formation of a single silver grain in the photographic emulsion. Based on this information and the activity of the probe used in the preliminary experiment it is possible to estimate that a maximum of about 120 grains per cell could be attributable to specific signal (see Appendix B for calculations). As the average signal observed per neuron in the control samples was in excess of 200 grains per cell, this suggests that a considerable proportion of the observed signal was not due to specific binding. This too must be viewed with some caution however. It is not unheard of for long double stranded probes such as the one employed in this experiment to form a crosshybridized network with one another by the binding of
complementary regions. This can result in a concentration of signal in excess of that which one would predict by calculating the maximum signal from the saturation of binding sites in the target tissue. The fact that an increase in signal was observed for the A.D. sections relative to the age-matched control sections could be the result of any number of factors. It could reflect an increase in mtDNA density in these tissues. If, however, the majority of signal in these tissues was due to binding to some non-mtDNA component in these sections, the increased signal could be the result of such binding.

The possibility exists that the in situ hybridization system using the \(^{35}\text{S}\)dATP-labelled mtDNA probe did not work very well in tissue in the first place. Thus the preliminary data obtained from this experiment should be regarded with extreme reservations. Any number of technical factors could have generated these results, even if the probe was not binding to mtDNA with good specificity.

If one presumes for a moment that the in situ hybridization system was working to an extent during the preliminary experiment, it then remains to be determined what factor(s) were responsible for its subsequent failure, and eventual revival. The first point to be considered is that my recent experiments used a biotin-labelled mtDNA probe and did not attempt to demonstrate the effectiveness of the in situ
hybridization method using a \(^{35}\text{S}\)dATP-labelled mtDNA probe. Thus it is possible that differences in the probe labelling or visualization methods for \(^{35}\text{S}\)dATP probes as compared to biotin probes could account for the recent success with the biotin-labelled mtDNA probe in tissue sections.

In addition, I did not determine if \textit{in situ} hybridization with the mtDNA probe would work on paraffin sections using my original method. This makes it difficult to determine precisely when the \textit{in situ} technique stopped working. All one can know for certain is that it was not working when I returned to frozen sections. It seems probable that it was not working on paraffin either, but without knowing that it can give good specific signal under these conditions, this can not be proven.

Many factors were checked throughout the course of this work, in an effort to restore the \textit{in situ} system. Reagent stocks were destroyed and made up fresh, and enzymes were reordered and old supplies discarded. These changes alone were not sufficient to lead to positive \textit{in situ} hybridization results with the mtDNA probe.

I had always used Sigma's Ultrapure formamide in the hybridization solution, however in later experiments I further deionized this ultrapure preparation with mixed resin. This should not have been required, as this was an Ultrapure product, and always appeared clear and colourless. However,
as the breakdown products of formamide can degrade nucleic acids, it seemed a reasonable precaution. Once again, while contamination of formamide could potentially have played a role in the difficulties with the \textit{in situ} system, it could not have been the only problem, because even with deionized Ultrapure formamide, the system did not work at first. I also checked my RNase and proteinase K stocks for DNase activity, and found no significant activity. In short, it does not appear that the problem was solely with the reagents used for \textit{in situ} hybridization. The hybridization solution used with the \(^{35}\text{S}\)dATP-labelled probes was slightly different than that used with the biotin-labelled probes. However, these solutions were prepared from the same stock reagents. As \textit{in situ} hybridization worked with the \(^{35}\text{S}\)dATP-labelled hsp 60 probe using the same hybridization solution used for the \(^{35}\text{S}\)dATP-labelled mtDNA probe, the hybridization solution was not likely the problem.

If one compares my \textit{in situ} hybridization method using \(^{35}\text{S}\)-labelled probes to the methods commonly employed in the literature, one will observe some differences. The extent of fixation of the tissue I studied was greater than the tissue studied in most of the papers reviewed. This difference exists because I was using autopsy material which is regularly fixed in formalin for a week or more. I used a more rigorous proteinase K treatment than was used in any of the papers
reviewed. As I discussed earlier, the degree of proteinase K treatment reflects many things, one of which is the extent of fixation-linked crosslinking in the target tissue. Clearly it would have been advantageous to try several different proteinase K treatments to determine which gave the strongest signal for in situ hybridization. The pretreatment of tissues with RNase was similar between my experiment and those in the literature. The hybridization buffer I used was very similar to those used in the literature. I used a higher concentration of DTT and Denhardt's solution (polyvinylpyrrolidone, Ficoll, and BSA) than did other groups, and I did not use EDTA as did some groups. These are components which were used in varying quantities by many groups. As I discussed in the introduction, these components are used in the reduction of background signal and would therefore be expected to vary in concentration for different probes and tissues. I did not optimize the concentration of these components in my hybridization solution. While this likely had an effect on the degree of non-specific binding in my experiment, it should not have eliminated specific binding. The concentration of radioactive counts in the [³⁵S]dATP-labelled probe solution used per slide was very similar to the concentrations reported in the literature. I used 1.2 x 10⁴ CPM/μL of hybridization buffer and the papers reviewed used 1 x 10⁴ CPM/μL of hybridization buffer. The posthybridization
washes used in my preliminary experiment were similar to those described in some of the papers reviewed, but there were significant differences. The duration and temperature of posthybridization washing were reduced in my experiment compared to those reported in the literature. I also included 0.1% SDS in the wash solution whereas other groups did not. These differences are a potential source of non-specific signal in this experiment.

The degree to which the probe contained incorporated $^{35}$S$d$ATP was determined by measuring the percent incorporation of $^{35}$S$d$ATP into the labelled probe. The percent incorporation data (see Methods) indicates that probe labelling with $^{35}$S$d$ATP was not the only factor affecting hybridization success. The percent incorporation of $^{35}$S$d$ATP into the probe was not checked in some of the earlier experiments; however, even when incorporation was shown to be good, no specific signal was obtained from the in situ hybridization experiment. This suggests that this was not the difficulty in this system.

I suspect that the key factor influencing the success of the in situ hybridization assay was the ability of the labelled probe to gain access to and bind the target DNA. There are two major factors to consider here. One is the effect of proteinase K treatment in unmasking the DNA target, and the second is the size of the labelled probe. These
factors provide a possible explanation of why the experiments using the biotin-labelled mtDNA probe worked, while earlier experiments on sections from the same block did not work using the \[^{35}\text{S}]\text{dATP}-labelled mtDNA probe. The proteinase K treatment of the sections probed with biotin-labelled mtDNA probe was more rigorous than for sections probed with \[^{35}\text{S}]\text{dATP}-labelled mtDNA probe. In addition, and potentially more important, the average (theoretical) probe size for the biotin-labelled probes was smaller than for the \[^{35}\text{S}]\text{dATP}-labelled probes (100 bp as compared to 1000 bp). This difference was due to the relative DNase I and Klenow titre in the nick translation labelling reaction. In both cases, a nick translation system from Gibco BRL was used to generate labelled probes. However, the biotin-nucleotide labelling system was specially designed to generate short probes (between 50 and 300 bp) for use with \textit{in situ} hybridization. The \[^{35}\text{S}]\text{dATP} labelling system employed was designed for general purpose use, giving probes with an average size around 1000 bp. The larger probe size limits the access of the probe to its target DNA. This, in combination with the reduced proteinase K treatment of the \[^{35}\text{S}]\text{dATP}-labelled sections, could be expected to result in significantly lower specific signal from these sections. Such signal could be sufficiently low to not be detectable above background.
The more complex question is why, if the *in situ* hybridization system was working in the first place, it stopped working. In light of the recent data for *in situ* hybridization to mtDNA using the biotin-labelled probe (relating to signal localization), I am not at all certain that the *in situ* hybridization system was working well in the preliminary experiment. That being so, any number of small changes could have decreased its specific binding below the detectable level. Once again, I suspect the probe size and target accessibility. Variations in the activity of different proteinase K stocks could have resulted a different degree of unmasking of target DNA in later experiments. A similar situation applies to the nick translation system employed to label the [\(^{35}\)S]dATP probe. The enzymes in these kits are only stable for 6 months (to be safe), therefore it was necessary to use a different kit to label the probe for the later experiments than had been used for the preliminary experiment. The exact titre of DNase activity to Klenow fragment activity varies from lot to lot, thereby affecting probe size. An increase in average probe size could have caused a decrease in specific signal observed. If my hypothesis is correct, and the *in situ* system was not working very well in tissue to begin with, such changes could potentially eliminate all detectable specific signal.
C) Reevaluation of the merits of \textit{in situ} hybridization for the quantitative investigation of mitochondrial changes in Alzheimer's disease

It is essential to distinguish between difficulties encountered due to technical problems within a technique and difficulties encountered due to the application of a technique to a question to which it is not suited. The difficulties encountered in these investigations reflected technical problems within the technique, and do not reflect on the suitability of this technique, correctly applied, to the study of quantitative mitochondrial changes in A.D. The rationale for using \textit{in situ} hybridization to study this system was explained in detail in the latter portion of the Introduction. The results from the experiments performed during the course of this project serve to emphasize technical considerations which must be dealt with when using this technique, but they do not invalidate the arguments presented in support of the use of \textit{in situ} hybridization in this context.

From the outset it was stated that the value of \textit{in situ} hybridization in this project was the information it could provide on relative mtDNA densities in A.D. and control tissues. It can only provide this information when applied under carefully controlled conditions. The same is true for any technique used. The use of good negative and positive
controls is essential to gain meaningful information from the careful application of any technique.

There are some experimental design factors which clearly warrant greater consideration than they initially received. The nature of the negative control used must be considered in detail. The negative control used in the later *in situ* hybridization experiments was a non-specific DNA probe. This is useful because it allows one to estimate what proportion of the observed signal is due to probe binding to non-target sites in the tissue section. The degree of non-specific DNA binding observed can be affected by the DNA sequence of different probes, therefore one must be careful when quantifying background signal using this technique. The use of a negative control composed of DNase pretreated target tissue probed with the normal (positive) probe would avoid the need to consider background signal variations due to probe sequence, as the positive probe could be used on the DNase treated tissue. If the RNase pretreatment is sufficient to remove any RNA in the tissue section, then any cytoplasmic signal in positive sections should be due to mtDNA. A DNase control would not address the question of non-specific probe binding to nuclear DNA. Such binding, however, would be localized over the nucleus.

RNase pretreatment in these experiments was based on typical treatments used in the literature; the extent of
RNase pretreatment required to remove RNA from the sections prior to hybridization was not experimentally determined for these tissue sections. This could have been checked for each lot of RNase by treating sections for different durations, followed by hybridization with a single stranded probe complementary to a high abundance mRNA. The treatment required to eliminate signal due to binding of probe to RNA could be determined by the treatment duration required to eliminate cytoplasmic signal in these sections.

One of the concerns outlined in the Introduction of the in situ hybridization technique was the inability (using this technique alone) to assess if the changes observed using in situ are occurring early in the disease state, or whether they are merely late-stage changes occurring in neurons which are near the end of the disease path. While this limitation will always exist for in situ experiments performed without a second means of assessing the intracellular status of the neuron studied, this limitation can be overcome somewhat by including a diseased-control group in the study. This group would be composed of individuals who suffered from neurological disease that is not likely linked to mitochondrial abnormalities. Creutzfeldt-Jakob disease is an excellent example of a disease similar to A.D. in its pathological features, while not linked etiologically to mitochondrial abnormalities. The purpose of such a diseased-
control group would be to control for nonspecific effects of neurological disease on mitochondrial DNA density.

As outlined in the Introduction, there are several ways in which one could overcome the limitations of in situ hybridization alone by combining it with a second or third technique to ensure that the neurons studied appear morphologically normal, and are not exhibiting signs of advanced neurodegeneration.

Despite the challenges encountered during the course of this project, I still believe that in situ hybridization was the most prudent choice for preliminary investigations into quantitative mitochondrial aberrations in A.D. Correctly applied, in situ hybridization with a \([^{35}\text{S}]\text{dATP}\)-labelled probe to mtDNA would have provided information regarding the relative mtDNA content per unit area in large neurons from A.D. patients compared to controls. This information would have been fairly straightforward to quantify (unlike methods relying on enzyme conjugates and staining intensity for a signal). Had a clear trend been visible in a study of fifteen or twenty A.D. patients and a similar number of controls, then I could have turned to a second and third technique to refine my observations. I believe that a small-scale electron microscopic investigation of large neurons from A.D. and control subjects, coupled with colloidal-gold in situ hybridization, would have been very useful to address the
question of the effect of A.D changes on mitochondrial DNA density, and to determine, on a small scale, if the changes observed with [\textsuperscript{35}S]dATP-labelled probes \textit{in situ} were dependant on changes in neuronal morphology (i.e. do normal looking neurons still show alterations in mitochondrial DNA density, or does this appear to occur only after other disease related changes?). E.M. would also allow the comparison of mitochondrial DNA density and mitochondrial cross-sectional area. This would help to address the question of whether observed changes in mtDNA density reflected overall mitochondrial changes, or were limited to mtDNA. As discussed earlier, there are limitations to the number of samples one can reasonably study under E.M., both because of limited sample availability, and the expense of the technique itself. Thus, while E.M. would be an excellent technique to refine earlier results, \textit{in situ} hybridization using [\textsuperscript{35}S]dATP-labelled probes is far more practical for preliminary investigations.
D) Future directions

A good starting point for future research in the area of mtDNA density abnormalities in A.D. would be to attempt to determine if such abnormalities can be detected using *in situ* hybridization under well controlled conditions. Ideally this would involve the inclusion of a diseased control group in addition to the age-matched control group. A second concern for these experiments would be to try to limit the degree to which changes are assessed in neurons which are already showing gross morphological changes associated with A.D. As discussed in the introduction, the use of immunohistochemistry to detect paired helical filaments in neurons also subjected to *in situ* hybridization would allow the exclusion from analysis of neurons already showing gross A.D. linked changes. This would help to limit the analysis to changes occurring early in the disease process. It is possible that changes would not be observed prior to observable morphological changes in affected neurons.

The method and tissue sections employed in these experiments would have to be carefully selected. *In situ* hybridization to mtDNA has been performed on both paraffin embedded and frozen tissue sections. The use of paraffin embedded sections can complicate analysis, however. The processing involved in embedding tissue in paraffin can lead to increased shrinkage of tissue. In addition, if one wished
to perform immunohistochemistry on these sections in combination with *in situ* hybridization the use of paraffin embedded sections could complicate this. Many antigenic sites are lost in paraffin embedded material. For these reasons I would favour the use of frozen sections.

The suitability of the tissue for examination by electron microscopy is another factor to be considered. In order to obtain meaningful results for the *in situ* hybridization experiment it would be necessary to examine tissues from many different patients. It has been proposed that the specificity of the signal for mtDNA could be examined by examining hybridized section by electron microscopy to determine if the observed signal was localized over mitochondria. In order to do this it is necessary that the tissue examined be sufficiently intact to allow the recognition of mitochondria within the section. As discussed in the introduction, tissues of the central nervous system are particularly prone to undergo autolysis. This process can lead to the total loss of visible subcellular structure, preventing the analysis of individual subcellular regions by electron microscopy. This difficulty can be overcome by perfusion and/or immediate fixation of the tissue following death or biopsy. However this is not possible with human autopsy samples. A post mortem interval of 12 hours is typical before tissues are removed on autopsy. Human brain biopsies are very rare,
therefore tissue for these experiments must be obtained on autopsy. Clearly the need for tissue from a large number of subjects is in conflict with the desire to confirm the intracellular localization of the signal from the probe using electron microscopy. There is no completely satisfactory resolution to this difficulty. In some hospitals in the United States autopsy material is obtained within two hours after the patients death, however the ethics of this practice have been questioned. If brain tissue fixed within 2 hours of death was available to us, it might be possible to assess signal localization at the E.M. level in these samples. This is not common practice for this type of investigation. Probe specificity is typically determined by Southern blotting, and the specificity of signal in *in situ* hybridization is typically determined by the use of a non-specific nucleic acid probe. This is not the most rigorous way of ensuring probe specificity, however it is the accepted procedure in this field. If one was unable to obtain tissue from a large number of subjects preserved for E.M. several options remain. It should be possible to obtain correctly fixed and embedded tissue from one or two subjects. One could perform *in situ* hybridization to these sections and assess signal localization at the E.M. level. It would then be necessary to trust that the differences in tissue handling in the other sections not prepared for E.M. did not affect signal localization. The
second option does not address the question of localization, but uses instead a unique negative control. The human ovarian carcinoma cell line 2008 has a derivative which contains no mitochondrial DNA, known as the Et-3 cell line. One could immobilize Et-3 cells on each slide containing brain tissue to be examined. One would not expect to see any specific signal from the Et-3 cells. These cells contain nuclear DNA, so any non-specific binding to nuclear DNA or nuclearly encoded RNA would still be observed in these cells.

The method of in situ hybridization employed in these experiments should be based on the method of Mita et al. This method is commonly used in the literature to assess quantitative changes in mitochondrial DNA abundance. A small probe specific for the D-loop region of mitochondrial DNA should be used. A mutation deleting the D-loop region will not accumulate in cells, as this mutation eliminates the heavy strand origin of replication. This probe should be labelled using $^{35}$S-labelled nucleotides and quantified by counting grains in the photographic emulsion. There are several ways of expressing the signal levels observed in tissues. In the preliminary experiment the signal level was expressed as grains per unit area. This has the advantage of allowing one to compensate for differences in the assayable area in neurons. One potential difficulty with this method discussed in the introduction, is the potential for changes in neuronal
area resulting from the A.D. disease process. An option to expressing signal as grains per unit area is to express it as grains per cell. This prevents difficulties due to changes in neuronal volume. Unfortunately, the cross sectional area of neurons assessed varies considerably between cells. One would expect to count more grains over a neuron with a large visible cross-sectional surface area than over a smaller one. Thus expressing signal as grains per cell is also an imperfect measure. One way of overcoming this challenge is to quantify the presence of another cellular component. One would wish to choose a component which is not likely to be altered in the disease process. One could then express signal as mtDNA signal as a percent of control signal. If the control component assayed was evenly distributed throughout the cell and was not altered in the disease process then this would allow and accurate measure of mtDNA signal, despite any possible A.D. related changes in neuronal size. In order to use this method, one would first have to prove that the control component was not altered in the disease state and was present in constant quantities in tissues from different individuals.

If quantitative changes in mtDNA density were detected using *in situ* hybridization as described above, the next step would be small-scale analysis using electron-microscopy and colloidal-gold *in situ* hybridization. Ideally such a study
would consider ten random neurons per subject and include five subjects per group. The groups studied would include A.D. patients, age-matched control patients, and neurologically diseased control patients. Factors studied would be the relative mtDNA density in neurons from A.D. and control subjects, the relative mitochondrial cross sectional area in neurons from A.D. and control subjects, and the extent of A.D.-like morphological alterations present in neurons studied in A.D. subjects. The purpose of this second study would be to determine if changes observed with $[^{35}\text{S}]$dATP-labelled probes to mtDNA reflected early-stage changes in A.D., and to see if such changes reflected a change in mitochondrial area in these neurons. Information on the morphology of mitochondria in neurons from A.D. subjects could also be obtained from such a study.
Chapter 5
Conclusions

This project has raised some interesting questions relating to the technical aspects of in situ hybridization to mitochondrial DNA. This experience has forced the careful examination of the various steps involved in this technique. Several key areas of concern have been identified with regard to the controls required to obtain consistent and reliable results. Primary areas of concern are: ensuring probe specificity, optimizing the extent of proteinase K and RNase treatment, and optimizing the average size of the probe used to detect target DNA. Careful monitoring of these factors to reduce variation between experiments should improve the results obtained.
Additional Figures

A

Figure A-1: In situ hybridization using $^{35}\text{S}\text{dATP}$-labelled probes to frozen human brain sections. A, C, E: sections probed with the mitochondrial DNA probe pAMI. B, D, F: sections probed with the nonspecific probe pBR322.
Figure A-1

C
Figure A-1
D
Figure A-1
Figure A-2: HL60 cells probed in situ with: (A) $[^{35}S]$dATP labelled HSP-60 DNA probe; (B) $[^{35}S]$dATP labelled lambda DNA probe. The cells were counterstained with haematoxylin.
Figure A-2
A
Figure A-2

B
Figure A-2

B
Figure A-3: HL60 cells probed in situ with biotin-nucleotide labelled probes. (A) Cells probed with the mitochondrial DNA probe pAMI. (B) Cells probed with pBR322.
Figure A-3

A
Figure A-3
Figure A-3
Figure A-4: Human brain tissue probed in situ with a biotin-nucleotide labelled probe. (A) mitochondrial DNA probe; (B) negative control probe pBR322. The sections were not counterstained.
Figure A-4

B
Appendix B

Calculation of maximum number of grains per cell in the preliminary in situ hybridization experiment:

Calculation of the average number of counts required to form a single silver grain in NBT-2 photographic emulsion under experimental conditions:

The number of cells per mL was determined and the average counts per cell were determined prior to cytopsinning the cells. The immobilized cells were coated with NTB-2 emulsion and the average number of grains per cell was determined by counting under oil immersion. The time difference between the development of different sets of slides was calculated and the observed grains per minute per cell were compared to the predicted counts per minute per cell.

<table>
<thead>
<tr>
<th>minutes:</th>
<th>0</th>
<th>75</th>
<th>1327</th>
</tr>
</thead>
<tbody>
<tr>
<td>delta min.</td>
<td></td>
<td>75</td>
<td>1252</td>
</tr>
<tr>
<td>grains/cell</td>
<td>63</td>
<td>82</td>
<td>391</td>
</tr>
<tr>
<td>delta grains</td>
<td>19.6</td>
<td>309</td>
<td></td>
</tr>
<tr>
<td>est. counts</td>
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<td>112</td>
<td>1990</td>
</tr>
<tr>
<td>grains/min.</td>
<td>0.26</td>
<td>0.25</td>
<td></td>
</tr>
</tbody>
</table>

The average CPM/cell was determined to be 1.5 CPM/cell. Therefore approximately 6 counts are required to form a single silver grain in the NBT-2 emulsion under experimental conditions.
Calculation of the maximum number of specific counts bound to mtDNA in the preliminary in situ hybridization experiment:

The mitochondrial DNA probe was added in excess, therefore the signal depends on the availability of specific binding sites (mtDNA copy #) and probe activity. If we estimate a maximum of 1000 copies of mtDNA per neuronal cell body, then 1000 complete copies of the probe can bind per cell.

1 µg of mtDNA probe was labelled with [35S]dATP. This represents approximately $6 \times 10^{10}$ copies of mtDNA labelled. The 1 µg of probe was labelled to $6 \times 10^6$ CPM/µg or 0.0001 CPM/copy.

Therefore if each cell binds 1000 complete mitochondrial DNA probes one can calculate the signal as:

$$(0.0001 \text{ CPM/copy}) \times (1000 \text{ copies/cell}) = 0.1 \text{ CPM/cell}$$

The slides were exposed photographic emulsion for 5 days prior to developing. Thus the exposure time was approximately 7200 minutes.

$$0.1 \text{ CPM/cell} \times 7200 \text{ minutes} = 720 \text{ counts per cell in five days}$$

As I was able to determine that on average 6 counts were required for the formation of one silver grain in the emulsion, this represents a maximum of about (720 counts per cell / 6 counts per grain) 120 specific grains per cell in this experiment.
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