ASSAY OF GLUTAMINE SYNTHETASE IN CEREBROSPINAL FLUID AS A SPECIFIC MARKER IN ALZHEIMER'S DISEASE

NICOLA OETTLE

Thesis submitted in fulfilment of the requirements for the Master's Degree in Medical Technology in the School of Life Sciences at the Cape Technikon

Chemical Pathology Laboratory
Groote Schuur Hospital and the
University of Cape Town
Cape Town

External Supervisor: Prof D McIntosh
Internal Supervisor: Mr E J Truter

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I declare that this thesis is my own work. It is being submitted for the Masters Degree in Medical Technology, to the Cape Technicon, Cape Town. It has not been submitted before for any diploma, degree or examination at any other tertiary institution.

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Nicola C Oettle

31 MARCH 1997

Date

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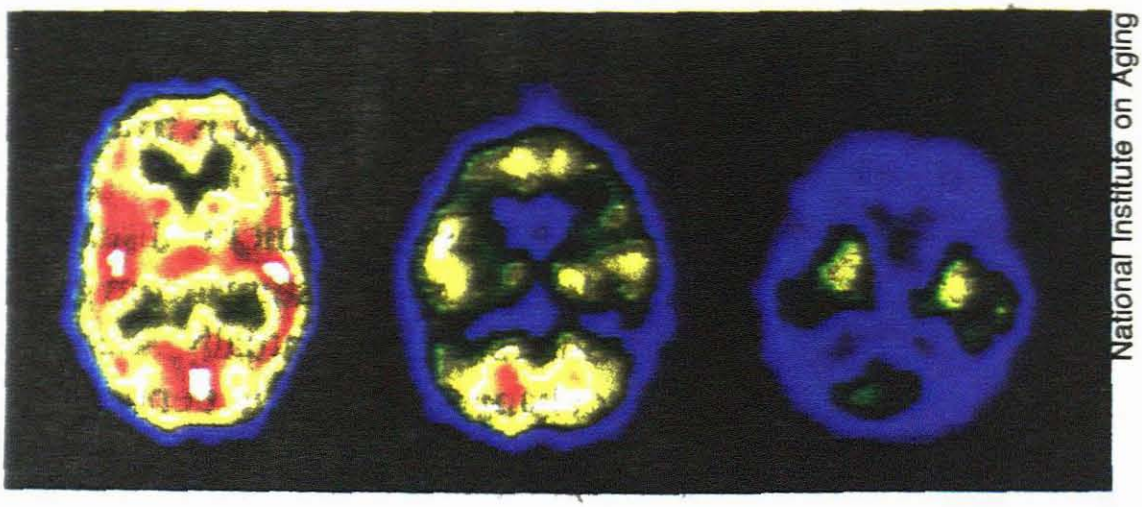

Positron emission tomography images of glucose uptake in the brain of a healthy older person (left) and patients with moderate (center) and severe (right) stages of Alzheimer's disease reflect neurodegeneration that accompanies the disease. Glucose uptake, the yardstick for measuring brain activity via glucose metabolism, decreases as the disease progresses. In these images, white and red colors indicate highest uptake; dark blue and darker colors, the lowest.

Brennan, M. 1997. Bringing back the memories. *Chemical and Engineering News*. 75(3): 29 - 35.

ABSTRACT

There is, at present, no recognised diagnostic biochemical marker of Alzheimer's Disease (AD). Recently, Gunnerson and Haley, (1992), reported that the presence of glutamine synthetase (GS) in cerebrospinal fluid (CSF) samples showed a 97% correlation with patients diagnosed as having AD. GS was detected by photolabelling with [γ^{32}P]2-azido-ATP or [γ^{32}P]8-azido-ATP and visualisation following sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

This study set out to reproduce Gunnerson and Haley's methodology for labelling sheep GS in CSF using [γ^{32}P]8-azido-ATP, to develop this assay or possibly another, using a fluorescent probe of ATP binding sites, into a robust procedure suitable for a routine diagnostic laboratory, and finally to assess whether the presence of GS in CSF is indeed a marker of AD.

The ATP analogue [γ^{32}P]8-azido-ATP was synthesised from 8-azido-ATP by an enzymatic isotope exchange method and purification on a DE-52 anion exchange column. Photolabelling was performed with a 150 watt Xenon lamp and standardised for illumination time, sample distance, and type of filter using isolated sarcoplasmic reticulum vesicles as a model system. The photolabelling method of Gunnerson and Haley (1992) was found to be problematical and the method was then modified in various ways and conditions for maximum specific labelling of

GS investigated.

The main methodological change was to eliminate the acid precipitation and resolubilisation steps just prior to loading onto the gel. The recovery of protein was found to be variable following acid precipitation and resolubilisation. Removing this step necessitated substituting potassium phosphate for sodium phosphate and loading high amounts of radioactivity onto the gels, but was found to be much simpler and more reproducible.

The specificity of GS photolabelling (reduced labelling of other proteins) was found to be increased by changing the buffer to ammonium bicarbonate and including 20% (v/v) glycerol. The labelling of GS was not influenced by the addition of $MgCl_2$ and was eliminated by the addition of EDTA. However, prior treatment with EDTA followed by the addition of $MgCl_2$ enhanced photolabelling 2-fold over that found without treatment with the chelator. The pH dependence of the reaction indicated a small but significant decrease in labelling at alkaline pH. ATP inhibited the reaction with a Ki of 0.1 mM at 10 μM [γ^{32}P]8-azido-ATP. GS exhibited a fall off in labelling with time (t1/2 for inactivation = 7 days at O and 20 °C). The optimal conditions for labelling of GS in CSF were determined and found to be 100 mM ammonium bicarbonate, pH 8.5, 2 mM EDTA, 20 mM MgCl2, 20% (v/v) glycerol, 10 μM [γ^{32}P]8-azido-ATP. Under these conditions, the labelling was highly specific for GS and the main

components labelled in Gunnerson and Haley's method, namely albumin and a protein of 28 kDaltons, were virtually undetectable when GS was present at a level of 10 μg/ ml and well labelled. Another photosensitive ATP analogue, namely [γ^{32}P]2',3'-O-(2,4,6-trinitrophenyl)-8azido-ATP (TNP-8N_3-ATP), was examined as a GS labelling agent. However, although it labelled GS well in the absence of CSF, it failed to label GS in its presence in a variety of conditions.

The widely used fluorescent probe of ATP binding sites, fluorescein-5'-isothiocyanate, was also investigated as a potential fluorescent labelling agent of GS. However, it failed to derivatise GS.

The CSF of two patients suspected of AD and two of dementia were examined for the presence of GS. No GS was detected in any of the samples.

In summary, the specificity of the GS photoaffinity labelling assay has been improved and a number of variables determined. The assay is ready to be implemented in a routine diagnostic laboratory. The efficiency of the assay as a diagnostic indicator of AD could not be confirmed, but only a very limited number of patients were analysed.

ABBREVIATIONS

AD	: Alzheimer's disease
AMPS	: Ammonium persulphate
CSF	: Cerebrospinal fluid
DMF	: Dimethylformamide
DTT	: Dithiothreitol
EDTA	: Ethylene diamine tetra-acetic acid
EGTA	: Ethyleneglycol-bis-tetraacetic acid
FITC	: Fluoroscein 5' isothiocyanate
G_3PDH	: Glyceraldehyde 3-P dehydrogenase
GS	: Glutamine synthetase
HEPPS	: N-[2-Hydroxyethyl]piperazine-N'-[3-propanesulfonic acid]
KPi/NaPi	: Potassium/Sodium phosphate
PCA	: Perchloric acid
PGA	: Phosphoglycerate tricyclohexylammonium
PGK	: Phosphoglycerate kinase
SDS-PAGE	: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SR	: Sarcoplasmic reticulum
TCA	: Trichloroacetic acid
TEMED	: Tetramethyl-ethyldiamine
TNP-$8N_3$-AMP/ATP	: 2',3'-o-(2,4,6-trinitrophenyl)-8-azido-adenosine 5'-monophosphate/triphosphate
TRIS	: Tris(hydroxymethyl)-methylamine
TMAH	: Tetramethylammonium hydroxide
$8N_3$-ATP	: 8-azido-adenosine 5'-triphosphate

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CHAPTER 1

INTRODUCTION

1.1 ALZHEIMER'S DISEASE

Alzheimer's disease (AD) was first described in 1907 by Alois Alzheimer, as a neuropathological disorder that caused progressive dementia (progressive loss of higher cerebral functions) with deterioration of memory and in advanced cases, all cognitive functions. The cognitive decline is thought to be the result of the synaptic loss and extensive neuronal cell death that occurs in the specific regions of the brain that have been shown to be involved in cognition and memory (Lacomis, *et al.*, 1993).

AD is of great medical importance, and has enormous impact, both socially and economically. Almost 1 in 10 individuals who survive beyond the age of 65 become affected by this disease and as individuals reach the age of 80 the prevalence increases to an estimated 19% (Evans, *et al.*, 1989). For individuals over 85 it becomes greater than 45%.

AD is linked to two characteristic histopathological lesions of the brain: (i) intracellular neurofibrillary tangles (NFT's), composed of abnormally phosphorylated proteins (tau proteins) involved in microtubule assembly and (ii) complex senile amyloid plaques, consisting mainly of a ~40-amino acid peptide, amyloid-ß (Aß), a proteolytic product of amyloid precursor protein (APP) (Muller-Hill & Beyreuther, 1989).

A number of different proteins are associated with the formation of fibrils from the amyloid peptides that lead to plaques. These have been termed "pathological chaperons" and may or may not be themselves components of the plaques. The pathological chaperons include amyloid P component, apolipoprotein E, proteoglycan, and alpha-1 antichymotrypsin (Wisniewski & Frangione, 1992).

1.1.1 Clinical Diagnosis

AD is the commonest example of dementia. However, dementia is associated with many clinical states, thus making the diagnosis a very complicated one.

In living persons and without histopathological investigation, a provisional diagnosis of AD can usually only be made after specific neurological and psychological testing and elimination of other causes of memory loss and dementia. Perhaps the single most important clinical sign of AD is progressive memory loss.

At present in the USA, as many as 25 to 40% of AD cases may be clinically incorrectly diagnosed (Rocca *et al.*, 1986). One reason is that the common neuropsychological tests are relatively insensitive to early stages of cognitive change and when a change is detected it is difficult to link it to AD (Petersen *et al.*, 1994). Another reason is that various unconscious conditions (e.g., delirium and coma), as well as psychiatric and neurological diseases (depression and

Parkinsonism), obscure symptoms of dementia and AD, at this early stage.

Many other dementias need to be excluded. These include (i) toxic causes (e.g. alcohol abuse), (ii) nutritional deficiencies (e.g vitamin B12), (iii) infections (e.g. encephalitis and syphilis), (iv) endocrinological disorders (e.g. myxoedema), (v) cancer (e.g. metastatic cerebral masses), (vi) traumatic causes (e.g. subdural haematoma), (vii) circulatory causes (e.g. cerebral aneurism) and others. Hence, various microbiological and chemical pathological testing needs to be done.

A technique which is proving very useful for the antemortem diagnosis of AD is a non-invasive imaging technique, positron emitting tomography (PET), which was developed to study human brain function in vivo. It is similar to the more familiar computed tomography (CT) scan, in that it involves the construction of three dimensional images of the brain. A major difference is that PET gives an indication of brain function and CT of structure.

The PET technique, as applied to humans, makes use of [^{18}F]2-deoxy-2-fluoro-D-glucose (FDG), which is an analog of glucose with a positron-emitting fluoro isotope with a half-life of 110 min. Thirty minutes after an intravenous administration of 5 to 10 mCi/mg FDG (time needed for plasma equilibration of FDG) serial scans are obtained showing the location and, with

time, the metabolic flux of the sugar. The procedure takes approximately 2 h. AD patients show a reduction in metabolic flux in certain areas of the brain compared with controls. It is one technique which seems to be able to provide positive evidence supporting the diagnosis of AD (Jagust & Eberling, 1991).

Earlier studies indicated that is was difficult to make a diagnosis of AD using PET alone since cortical atrophy complicated interpretation of the results (Ferris *et al.*, 1983; Herscovitch *et al.*, 1984). CT scanning was necessary to assess the extent of the cortical damage.

There is also the problem of knowing what represents a lowering of metabolic flux for a particular patient. Minoshima *et al.*, (1995) have found that only certain regions of the brain are defective in metabolising the glucose analogue in AD and that the pons region does not show any changes. The metabolism in this region of the brain could then be used as the control region to which the others could be compared, obviating the need to compare to normal aged control persons.

A new technique called frequency-shifted burst imaging, a form of nuclear magnetic imaging, might provide a noninvasive means of detecting changes in the brain from outside the skull (Dyun *et al.*, 1994). This technique images the brain in only a few minutes, is performed using an ordinary CT scanner, and

does not require administration of radioisotopes. It thus has advantages over the previously mentioned PET method, which takes many hours, needs specialized and expensive equipment and relies on the intravenous administration of a radioactive isotope. However the technique may only detect advanced stages of AD (Beardsley, 1995).

1.1.2. Biochemical features of Alzheimer's Disease

1.1.2.1. Amyloid-ß Peptide (Aß)

Aß is the principal component of the extracellular senile plaques which are characteristic of AD. It is a hydrophobic peptide of 39-41 amino acids that aggregates into an extended fibrillar beta-pleated structure. The peptide is a proteolytic digestion product of a much larger amyloid precursor protein (APP), a transmembrane glycoprotein with a large N-terminal domain situated extracellularly and a short C-terminal tail lying in the cytoplasm.

APP is normally processed by cleavages outside and within the Aß section of the molecule and close to the extracellular and intramembranous junction to yield large extracellular soluble polypeptides (110-140 kDaltons) and a membrane-embedded portion that undergoes further proteolytic cleavage and endocytosis (Weidemann *et al.*, 1989). Most of the soluble fragments produced are non-amyloidogenic because they do not contain all of the Aß section. However, in disease states that present with increased concentrations of APP (e.g. Down's syndrome), the overexpression of APP can increase the

secretion of Aß and hence lead to the formation of plaques (Hardy, 1992).

It is unclear how the excessive secretion of Aß leads to memory loss and dementia but the insoluble aggregates may be neurotoxic (Jarrett & Lansbury, 1993). There have been conflicting reports on the toxicity of Aß but it now appears that this is because the toxicity depends on the formation of the proper beta-sheet conformation, a process which takes several days (Simmons *et al.*, 1994). The reason for the toxicity of Aß aggregates is still a mystery, but there is evidence that they may form calcium channels (Arispe *et al.*, 1993), promote oxidative free radical formation and hence membrane damage (Behl *et al.*, 1994), or potentiate cytokine secretion which may control APP expression (Gitter *et al.*, 1995). Aß can be detected both extracellularly (in plaques, as well as in csf of both normal and AD patients, (Seubert *et al.*, 1992; Shoji *et al.*, 1992) and intracellularly. Its build up intracellularly may inhibit ubiquitin-dependent protein degradation and hence contribute to abnormal amounts of unwanted protein and neurofibrillary tangles (Gregori *et al.*, 1995).

The importance of the processing of APP and the production of abnormal amounts of Aß is provided by various familial ADs that have point mutations in the APP molecule (termed AD1). Mutation of valine-717 to isoleucine (located in the small intracellular tail and 3 residues beyond the final 43 amino

acid of Aß) is associated with early-onset AD (Crawford *et al.*, 1991). Two other changes in this valine (to phenylalanine or glycine) have also be identified in other families (Murrell *et al*, 1991: Chartier-Harlin *et al.*, 1991). A double mutation of lysine-670 to asparagine and methionine-671 to leucine (two amino acids immediately preceding the Aß sequence) in a Swedish family leads to classical AD. These mutations located just outside of the Aß peptide must promote the cleavages required for Aß excision from APP. All mutations in APP which result in AD are associated with an early onset of the disease and 100% penetrance (Schellenberg, 1995).

Other non-APP familial types of AD have been identified that result in increased production of Aß. Greater than 70% of early-onset AD has been linked to chromosome 14 and the gene identified as AD3 (Schellenberg *et al.*, 1992). It encodes a membrane protein with no homology to any protein of known function. Recently, a similar gene has been identified in chromosome 1 (Li *et al.*, 1995). Both proteins contain seven transmembrane segments and a common DNA binding motif (S/TPXX), suggesting that either they play a role in gene expression or possibly in joining chromatin to the nuclear membrane during cell division. It is not clear how these proteins increase Aß production but they may increase the expression of the APP gene or somehow, as a result of being membrane proteins, interfere with the normal proteolytic cleavage of APP.

Other amyloidogenic factors appear to act, not by increasing the amount of Aß, but rather by facilitating the formation of the beta-pleated fibrils from Aß. Perhaps the most important of these pathological chaperons is the E4 allele of the apolipoprotein E gene which is located on chromosome 19 and termed AD2. It has been linked by epidemiological and genetic studies to the late-onset form of familial AD (Strittmatter *et al.*, 1993; Corder *et al.*, 1993; Saunders *et al.*, 1993). Apo E binds Aß very tightly and causes spontaneous fibril formation, at least under in vitro conditions (Wisniewshi *et al.*, 1994).

Another amyloidogenic protein that appears to aid fibril formation is the protease inhibitor alpha1-antichymotrypsin. It has been found associated with amyloid plaques in vivo and promotes the polymerization of Aß into filaments (Abraham *et al.*, 1990).

There are other amyloidogenic factors that could play a role in the formation of Aß. Besides apo E and alpha1 antichymotrypsin, proteoglycan (Snow *et al*, 1990), the complement protein C1q (Rogers *et al.*, 1992), and a non-Aß component (Yoshimoto *et al.*, 1995) are present in plaques and bind Aß. Zn^{2+} and Al^{3+} have been implicated in the development of AD and bind Aß with high affinity and facilitate aggregation (Bush, 1994; Maclachlan *et al.*, 1991).

Neurotransmitters can regulate APP processing. The muscarinic agonist carbachol (analogue of acetyl choline) (Nitsch *et al.*, 1992) and glutamate (Lee *et al.*, 1995) activate the production of soluble non-amyloidogenic APPs.

1.1.2.2. Tau Protein

Besides extracellular senile plaques, the other typical feature of AD is the presence of intracellular neurofibrillary tangles, made up of paired helical filaments. The main component of the filaments is the microtubule-associated protein, termed tau. It is a rod shaped molecule rich in proline, glycine, serine and threonine, and contains 3-4 segments in the C-terminal portion, each of 31 or 32 amino acids, that bind microtubules (Lee *et al.*, 1988; Goedert *et al.*, 1988; Andreadis *et al.*, 1992). The N-terminal portion of the molecule can insertions of 29-54 amino acids contributing to at least 6 isoforms in adult brain (Goedert & Jakes, 1990). In AD, tau is abnormally phosphorylated (5-9 mol/mol protein instead of the normal 3-4) and as a consequence self-associates through the microtubule-binding segments to form filaments (Lee *et al.*, 1991; Crowther *et al.*, 1992, Mandelkow & Mandelkow, 1993). All isoforms that make up the filaments are abnormally phosphorylated and do not bind tubulin except after in vitro dephosphorylation. Tau is developmentally regulated, the fetal brain contains only one isoform which is phosphorylated to a greater extent than the adult equivalent. An extra phosphorylation site in the fetal form has been identified as Serine-202 and the same site is phosphorylated in the adult form in AD (Goedert *et al.*, 1993).

Abnormal phosphorylation of tau therefore prevents the normal assembly of microtubules. Microtubules are essential for a variety of cellular processes including neuronal growth and

cell division. Tau proteins, by virtue of not being degraded, are subject to nonenzymatic glycation to abnormally high levels and could provide a source of damaging reactive oxygen intermediates (Yan *et al.*, 1994).

It is not known whether the increased phosphorylation of tau in AD is the result of overactive kinases, the appearance of new kinases, or impaired phosphatase activity.

1.1.2.3 Possible biochemical markers of Alzheimer's Disease

At the present time there is no generally recognised biochemical marker for the antemortem diagnosis of AD. Such a test would be very useful. It would allow (i) early diagnosis, thus making it possible for early therapeutic treatment and early family counselling and planning, (ii) eliminate the need for extensive testing for other causes of memory loss and dementia, (iii) permit easier epidemiological studies on living populations, and (iv) allow the progress of the disease to be monitored.

Several groups have examined whether there are higher levels of Aß in the CSF of AD patients. However there is unanimous agreement that there is no significant different between AD and controls (Nakamura *et al.*, 1994; Palmert *et al.*, 1990; Wagner *et al.*, 1994).

The levels of tau protein in CSF from AD patients has also been investigated. Munroe *et al.* (1995) used dual monoclonal

antibody microplate immunoassays to compare the mean CSF tau concentrations in AD from three control groups (neurological, normal and other). They found higher tau concentrations in the AD group compared with the controls and concluded that the clinical diagnosis of AD may be aided by determination of tau proteins levels in CSF. Their study also showed that there was no correlation between tau concentrations and age, in agreement with the earlier findings of Vandermeeren *et al.*, (1993).

The presence of one particular isoform of apolipoprotein E, namely Apo-E4, appears to increase the risk of developing AD. Apo-E has 3 isoforms, namely Apo-E2, E3 and E4, differing by one amino acid each. Apo-E4 has an arginine at residue 112, Apo-E3 a cysteine and Apo-E2 has a cysteine at residue 112 as well as at residue 158 (Mahley, 1988). Epidemiological studies using genotyping has shown that having 2 copies of the Apo-E4 allele predisposes to AD generally before 70 years of age, and with no copies the average age is older than 85 (Weisgraber *et al.*, 1994). In a more specific breakdown of their results, they conclude that if a 78 year old person has 2 E4 alleles there is a 98% chance of having the disease, 1 E4 allele a 60% chance and with no E4 allele a 25% chance. If someone has mild memory loss, both Apo-E4 alleles and is in the correct age category, they have the highest risk of further progression of the disease.
Patients with Down's syndrome, who reach middle age, have been shown to develop a neuropathological condition identical to

AD. Down's patients are sensitive to drugs that block acetylcholine effects (Potter, 1991). Extending this observation to AD patients, Scinto *et al.*, (1994) have found that low concentrations of tropicamide, a commonly used pupil dilator, caused enhanced dilation of the pupils of patients with probable AD over that observed for control persons and suggested it might be used as a simple test of AD (Scinto *et al.*, 1994).

Gunnerson & Haley (1992) have found that high glutamine synthetase (GS) levels in csf are correlated with AD. They showed that GS levels are significantly raised in CSF of AD with a positive correlation of 97% (38 in 39) and a false positive of 2% (1 in 44). The authors measured the amount of GS in CSF by assaying the covalent, light-dependent incorporation of [γ^{32}P]8-azido-ATP into GS.

The levels of choline, homovanillic acid and 5-hydroxyindol acetic acid in the CSF of AD patients were not significantly different from non-demented patients or from patients with Parkinson's disease (Smith, *et al.*, 1991).

1.2 PHOTOAFFINITY LABELLING

Photoaffinity labelling was discovered over thirty years ago by Singh, *et al.* (1962). Since then it has evolved into a useful technique for studying molecular interactions in biological systems.

A schematic diagram of photoaffinity labelling is shown in Fig. 1. A photoactivatable but chemically inert ligand analogue is allowed to bind to a macromolecule, photolysis of the complex then leads to the generation of a highly reactive species that, by reacting (by insertion) rapidly with the immediate environment, labels the macromolecule irreversibly (covalently). This method of affinity labelling is based upon the fact that the binding of most biological ligands to their specific receptor sites involves a number of favourable interactions, that together make up the ligand-receptor recognition process. In favourable cases, this allows the introduction of photoactivatable groups into the ligand. Identification of the amino acids labelled can provide information about the position of binding sites in the primary structure of receptors or enzymes and the nature of the ligand-receptor/protein interaction.

The only chemical species potentially capable of insertion into C-H bonds are carbenes and nitrenes (Fleet *et al.*, 1969). Carbenes are generated by photolysis of diazoalkanes, diazirines, and alpha-keto diazo compounds and are extremely reactive. The alpha-keto diazo compounds are suitably stable but tend to undergo internal rearrangements that are inert. Nitrenes are generated by photolysis alkyl, acyl and aryl azides but due to various factors such as stability, reactivity, photoactivatable wavelengths, and ease of synthesis, only the latter are suitable as photoaffinity reagents (Knowles, 1972).

The first photoaffinity analog of ATP to be synthesized was 8-azido-ATP (Haley & Hoffman, 1974) and it was used to label Ca2+-ATPase of human red cell membranes. In another early study, it was used to label the ATP binding sites of the F_1-ATPase (Wagenvoord, 1977). Azidopurines are a category of aryl azides and are very often excellent nucleotide analogues because of the small size of the azido group.

Non-specific labelling can be a problem in photoaffinity labelling. It arises because of the generation of highly reactive species in the medium which could react with regions of the protein outside of the ligand binding site or with other proteins. The higher the ligand concentration during the labelling reaction (low-affinity probe), the greater the possibility of non-specific labelling. The use of radical scavengers has been used successfully to trap the reactive species in the medium, restricting the chemical reaction to the specific binding site (Garlardy *et al*, 1973). It is also helpful to perform photolabelling at high enzyme concentrations since this reduces the ligand excess and hence nonspecific labelling.

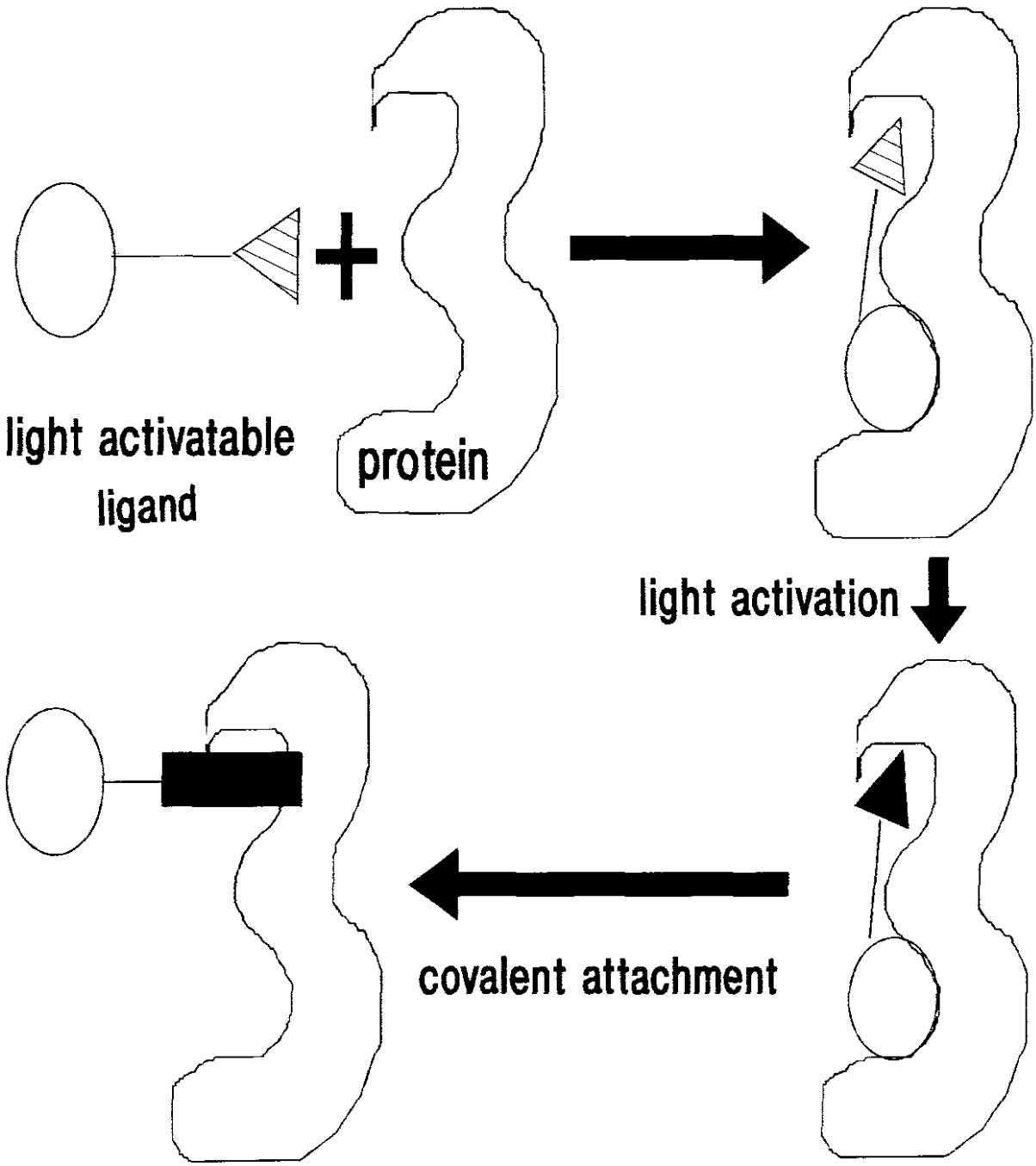


Fig. 1. Schematic representation of photoaffinity labelling.

1.2.1 Trinitrophenyl (TNP) Nucleotides

2'3'-O-(2,4,6-trinitrophenyl)-ATP, -ADP, -AMP are analogues of adenosine nucleotides that are useful due to the fact that they often exhibit higher affinity for ATP binding sites than ATP itself, their absorbance in the visible range, and their fluorescence. They have been useful in distinguishing different types of nucleotide binding sites of the Ca^{2+}-ATPase of sarcoplasmic reticulum (Dupont, 1982; Watanabe & Inesi, 1982; Bishop *et al.*, 1984; Berman, 1986; Davidson & Berman, 1987). The 8-azido derivatives specifically label lysine-492 at the active site (Seebregts & McIntosh, 1989; McIntosh et al., 1992; McIntosh & Woolley, 1994).

1.3 GLUTAMINE SYNTHETASE

1.3.1 Detoxification role

GS is a key metabolic enzyme that aids in the removal (detoxification) of ammonia from the body. It also provides glutamine which is a source of nitrogen in many biosynthetic reactions. It plays an essential role in inactivation of the synapse signal carried by the neurotransmitter glutamate. Changes in the activity or expression of GS in AD could alter Glutamate metabolism and/or neuronal impulse conduction.

GS

Glutamate + NH_3 + ATP <----> glutamine + ADP + Pi.

GS catalyses the formation of glutamine from glutamate, ammonia and ATP. The role of GS in the elimination of ammonia from the body is shown diagrammatically in Fig. 2. In

peripheral tissues, an ammonium ion is attached to alpha-ketoglutarate to form glutamate which in turn uses another ammonium ion to form glutamine. This is transported to the liver where it is converted back to glutamate by the enzyme glutaminase, and the released ammonium ion is linked to alpha-ketoglutarate to also form glutamate. These two glutamate molecules are converted to the Urea Cycle intermediates carbamyl phosphate and aspartate which yield urea, containing 2 equivalents of ammonia.

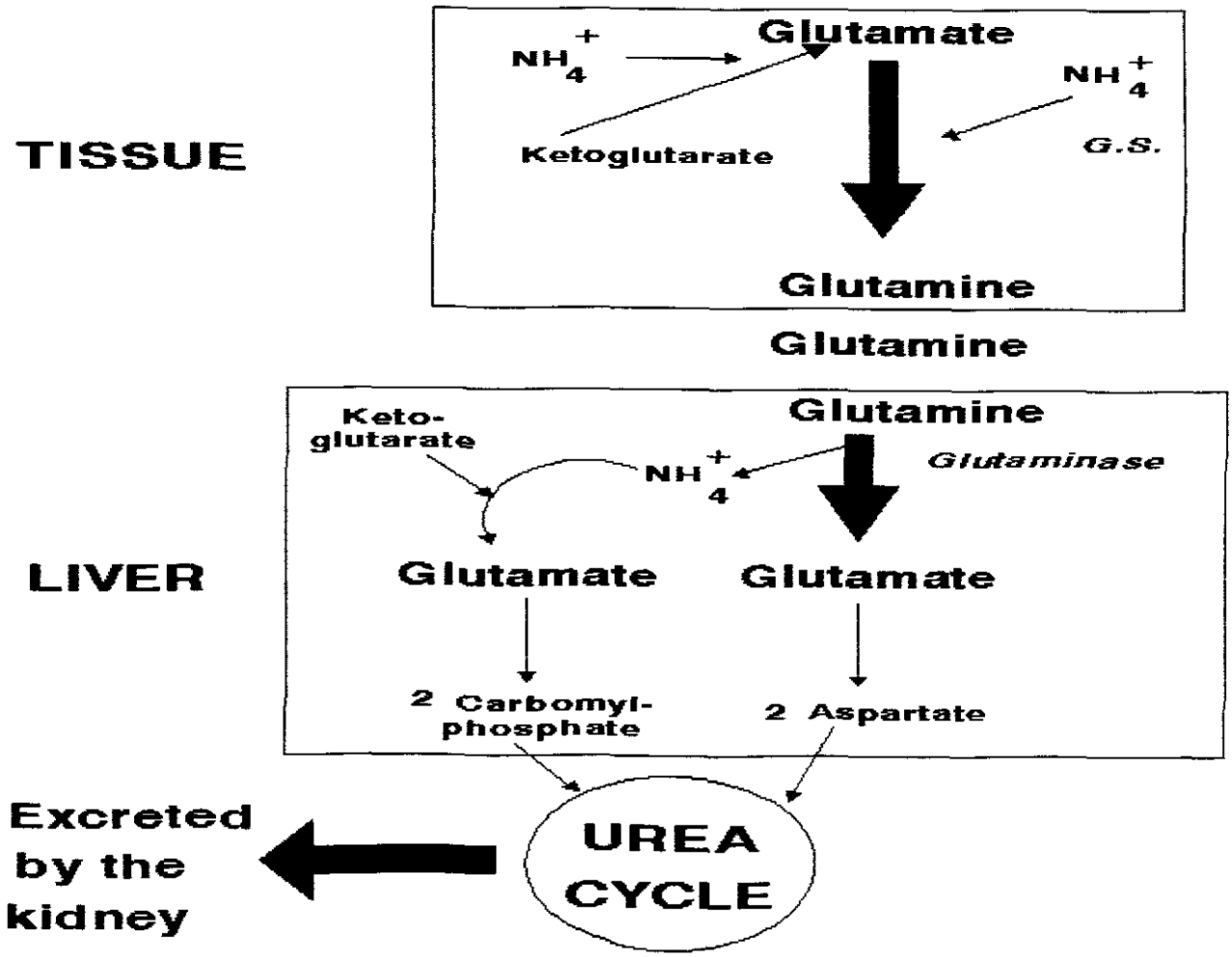


Fig. 2. Role of glutamine synthetase in the detoxification of ammonia.

1.3.2 Structure and reaction mechanism

Bacterial GS has 12 identical subunits (dodecamer) (Valentine *et al.*, 1968) and eukaryotic GS has 8 (octomer) (Ronzio *et al.*, 1969)) (Fig. 3). The reactions catalysed by the two enzymes are the same. The X-ray crystal structure of the enzyme from Salmonella typhimurium has been determined to 3.5-A resolution and shows that the active sites are situated at each side-to-side interface (Almassy *et al.*, 1986; Yamashita *et al.*, 1989). Each active site contains 2 Mn^{2+} ions. The E. coli and S. typhimurium enzymes are now known to 2.8-A (Liaw, Jun & Eisenberg, 1993).

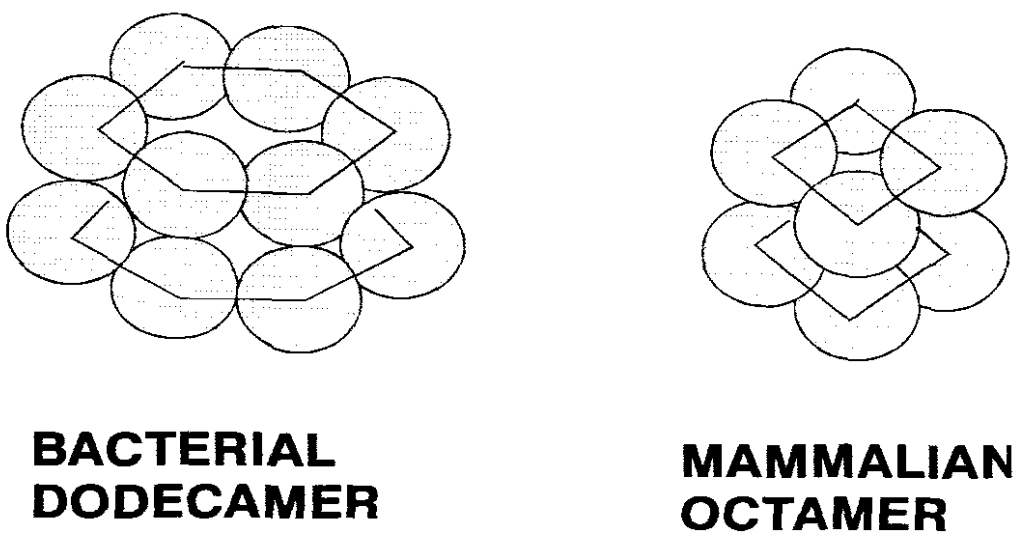


Fig. 3. **Bacterial and mammalian glutamine synthetase**

The two divalent cation sites have differing affinities for metal ions. The higher affinity site (n1) binds Mn^{2+} or Mg^{2+} and induces a conformational change in the structure such that it becomes catalytically active and aids in the binding of glutamate (Shapiro & Ginsburg, 1968; Hunt & Ginsburg, 1980). The lower affinity site (n2) plays a role in binding ATP.

A structural model for the reaction mechanism of GS of *S. typhimurium*, based on five crystal structures of enzyme-substrate complexes has been presented (Liaw & Eisenberg, 1994). A diagrammatic representation of the mechanism is shown in Fig. 4.

1. ATP binds first to the top of the funnel-shaped active cavity, near to Mn2+ (n2).
2. Glutamate then binds adjacent to the second Mn^{2+} (n1) at the bottom of the active site near a flexible loop. Glu attacks the γ-phosphorus atom of ATP to produce γ-glutamyl phosphate and ADP.
3. The presence of ADP (but not ATP) is thought to stabilize the γ-glutamyl phosphate, so enhancing the binding of the third substrate, NH^{+4}. This ion undergoes deprotonation and allows the resulting active product, ammonium, to attack the γ-glutamyl phosphate, forming a tetrahedral intermediate.
4. Due of the interaction of the positive charge on the γ-amino group of the intermediate with the negative charge on the side chain of glutamate, the glutamate binding loop becomes stabilized, so closing the path of glutamate entry through the bottom of the active site funnel.
5. Phosphate then leaves through the top of the active site and a proton from the γ-amino group of the tetrahedral intermediate is lost, to yield glutamine.
6. The absence of electrostatic interaction between glutamate and the product glutamine permits the segment to open, allowing glutamine to leave through the bottom of the

active site and glutamate to enter from the top for the next catalytic cycle.

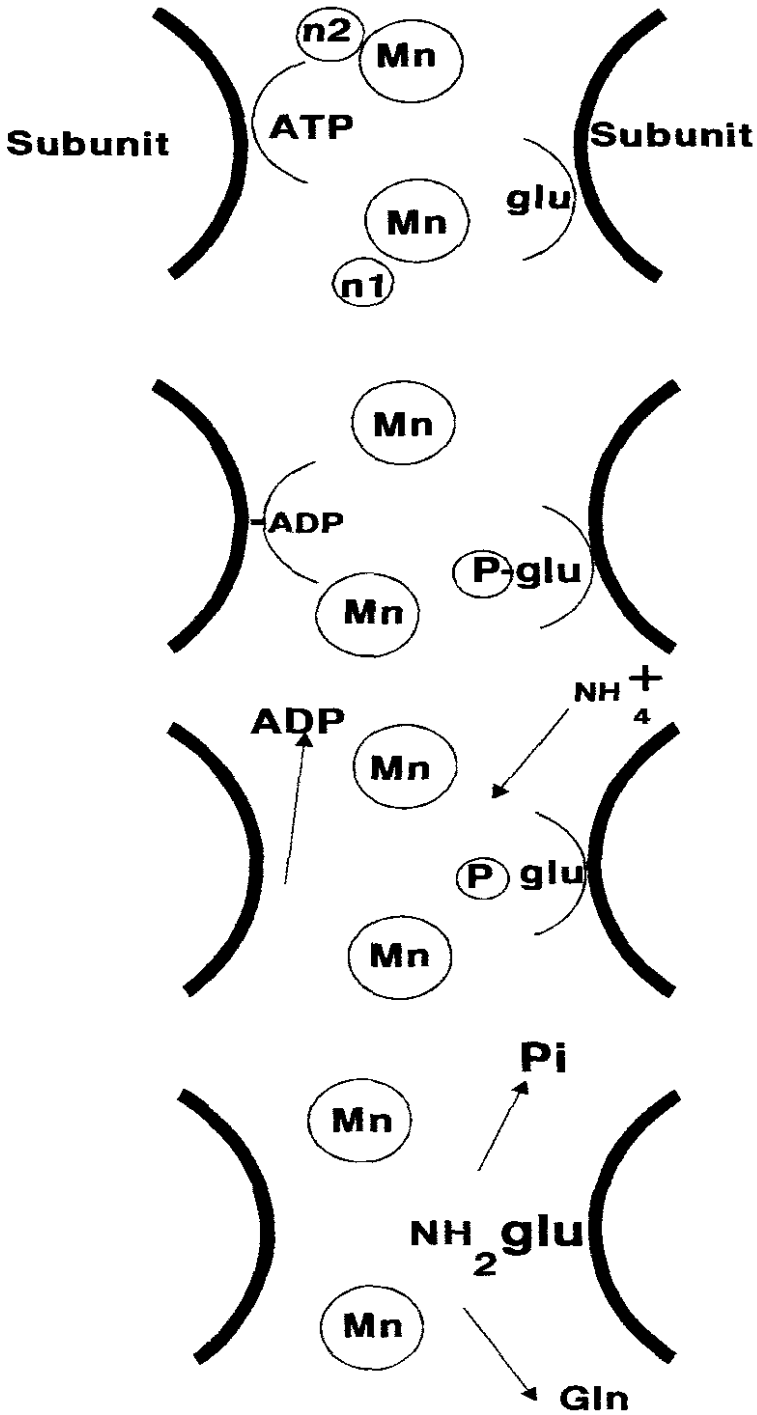


Fig. 4. Reaction mechanism of glutamine synthetase

1.4 SARCOPLASMIC RETICULUM (SR)

Calcium is widely used in regulation in biological systems and it's concentration in cells is strictly controlled. The presence of energy dependent calcium transport proteins located in the cell membrane maintains the low intracellular concentration against a high extracellular concentration. An example is the Ca^{2+} ATPase of cellular membranes which maintains low intracellular Ca^{2+} levels by pumping Ca^{2+} out of cells. The sarcoplasmic reticulum is a specialised intramembranous system of skeletal muscle, which contains large amounts of a similar Ca^{2+} pump that serves to relax muscle by pumping Ca^{2+} into the lumen of the reticulum and away from the myofibrils in the sarcoplasm.

Homogenisation of skeletal muscle results in the formation of vesicles of sarcoplasmic reticulum which can be isolated in pure form by differential centrifugation. Protein makes up 60 - 70 % of SR vesicles by weight with the balance being lipid (Seebregts, 1989). The 110 kDalton Ca^{2+}-ATPase accounts for 70 - 90 % of the protein component of preparations (Tada *et al*, 1978, and the present study) and calsequestrin for about 10 - 20 % (MacLennan & Wong, 1971).

Various pilot studies using SR as the model system, together with [γ^{32}P]8-azido-ATP, were performed to determine optimal labelling experimental conditions. These included obtaining optimal irradiation times and concentration dependence of labelling.

1.5 Aims

This study seeks to:

1. reproduce and possibly improve on Gunnerson and Haley's assay with a view to making the assay robust enough for a routine diagnostic laboratory in the antemortem diagnosis of AD.

2. extend Gunnerson and Haley's study and establish whether GS levels in CSF are a reliable indication of AD and whether GS can be useful in distinguishing between forms of dementia.

3. if the results are positive, implement the assay in the routine investigation of suspected cases of AD.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

ATP (disodium salt), GS (from sheep brain), Coomassie Brilliant Blue and Pyronin-Y (tracking dye) were from Sigma Chemical Company. γ^{32}Pi and high performance autoradiographic film were from Amersham International. Pre-swollen microgranular DE-52 was obtained from Whatman Industries and the PEI-Cellulose-F TLC plates from Merck Laboratory Supplies. Other analytical grade chemicals were obtained from BDH Chemicals LTD.

2.2 SYNTHESIS AND PURIFICATION OF HIGH SPECIFIC ACTIVITY [γ^{32}P]-8-AZIDO-ADENOSINE 5'-TRIPHOSPHATE

8-azido-ATP was obtained from stocks previously synthesized in our laboratory. Briefly the synthesis was as follows:
ATP was brominated in the 8 position on the adenine ring according to the method of Ikehara and Uesugi, (1969). Bromine (Br) was displaced with an azido group at 70 °C in organic solvent by the reaction with triethylammonium azide according to the method of Schafer *et al.* (1978b). The final concentration of 8-azido-ATP was determined using an extinction coefficient of 15.9 millimolar at 280 nm. The [γ^{32}P]8-azido-ATP synthesis was carried out approximately every 4 weeks according to the method of Glynn and Chappel, (1964), modified slightly by using a DE-52 anion exchange column for purification. With the use of DE-52, less hydrochloric acid

(HCl) is needed to elute the [γ^{32}P]8-azido-

ATP from the column, thereby decreasing the quantity of TRIS needed to neutralise the final mixture. TRIS interferes with the absorbance spectrum of 8-azido-ATP, and makes the determination of the concentration of nucleotide difficult. ^{30}Pi <-> ^{32}Pi exchange was performed with 200 mM Tris.Cl, 12 mM $MgCl_2$, 0.1 M NaOH, 0.5 M cysteine, 15 mM 8-azido-ATP, 0.1 M 3-phosphoglycerate tricyclohexylammonium, 31 mg/ml glyceraldehyde $_{3-}$P dehydrogenase, 6 mg/ml phosphoglycerate kinase and approximately 1 mCi γ^{32}Pi over 1 hour at room temperature (appendix 7.3.1). The progress of the reaction was monitored using thin layer chromatography with PEI-Cellulose-F plates. AMP, ADP and ATP were used as standard markers. The radioactivity of the appropriate spot was measured using a Berthoid Radio-Isotope Proportional Scanner. The reaction mix (1 ml) was applied to a DE-52 column (2 x 0.5 cm) prewashed with water. The column was washed with water, and acidified with 5 ml 10 mM HCl (appendix 7.1.7). The [γ^{32}P]8-azido-ATP was then eluted with 7 ml 30 mM HCl (appendix 7.1.7) into a freeze drying flask on ice and neutralised with 1 M TRIS (appendix 7.1.1). The mixture was freeze dried overnight and the residue reconstituted with 1 ml cold water. The purity and specific activity were measured on diluted aliquots. The mixture was then aliquoted out into usable volumes, snap frozen and stored at -20°C.

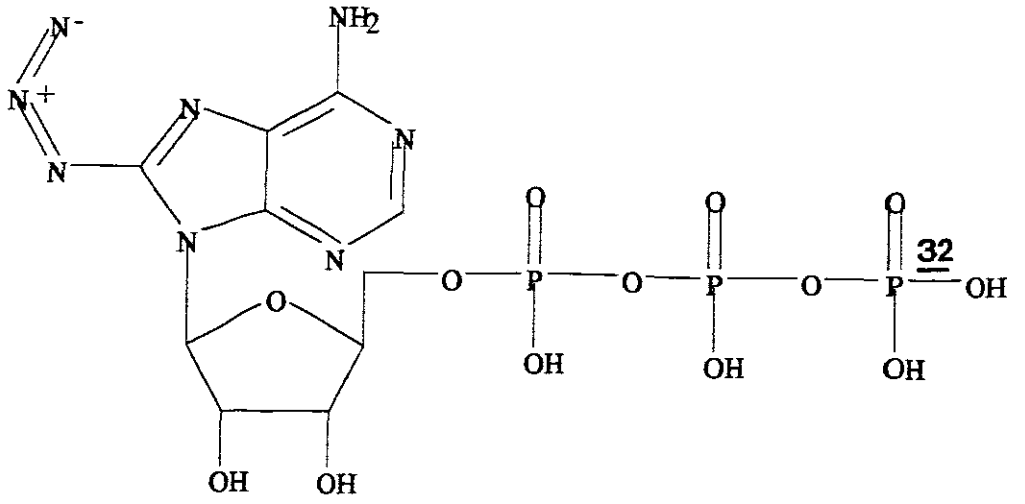


Fig. 5. Structure of [γ^{32}P]8-azido-adenosine 5'-triphosphate

2.3 SYNTHESIS OF [γ^{32}P]TRINITROPHENYL-8-AZIDO-ADENOSINE 5'-TRIPHOSPHATE

[γ^{32}P]TNP-8-azido-ATP was synthesised in two steps. Firstly γ^{32}P was incorporated into the γ-phosphate of 8N3-ATP using the Glynn and Chappel method (1964). The trinitrophenol group was introduced according to the method of McIntosh and Woolley, (1994).

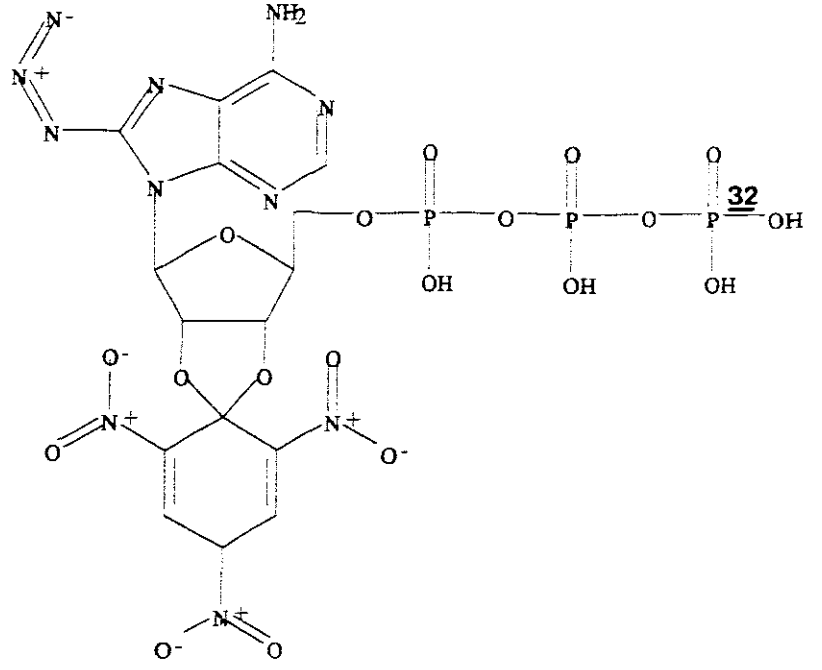


Fig. 6. Structure of [γ^{32}P]trinitrophenyl-8-azido-adenosine 5'-triphosphate

2.4 SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS PAGE)

SDS PAGE of photolabelled SR vesicles and GS was carried out according to the method of Laemmli, (1970). The running gel contained either 7 or 12 % acrylamide (appendix 7.6.8), depending on the protein preparation, and the stacking gel 4 % acrylamide (appendix 7.6.9). The photolabelled protein samples were mixed in an appropriate volume of solubilizing solution, 2 % SDS, 0.5 % (v/v) 2-mecaptoethanol and a few grains of bromophenol blue (appendix 7.6.7), then loaded onto the gel. Gels were electrophoresed at 4°C at a constant current of 30 mA for 3 hours or until the tracking dye reached a few cm from the bottom of the glass plates. For staining, the gels were removed and fixed in 30 % (v/v) methanol + 10 % (v/v) acetic acid (appendix 7.6.11) for 30 minutes, then stained in 0.1 % (w/v) Coomassie Brilliant Blue in 30 % (v/v) methanol + 10 % (w/v) trichloroacetic acid (appendix 7.6.10) overnight. Destaining was carried out in a couple of washes in 30 % (v/v) ethanol + 10 % (v/v) acetic acid (appendix 7.6.12) followed by clarification in a single wash of 7 % (v/v) acetic acid (appendix 7.6.13). Finally the gels were dried on an Ephortec Gel Dryer.

For autoradiography, unstained gels were rinsed in water for approximately 5 minutes, placed on filter paper, covered with a plastic wrap and dried. The gels were placed in an autoradiograph cassette together with high performance autoradiography film and then placed at -80°C for an appropriate time.

2.5 STORAGE OF L-GLUTAMINE SYNTHETASE

L-GS (EC 5.3.1.2) from sheep brain was obtained as a lyophilized powder. It contained approximately 5% (w/v) protein/80 mg solid (Lowry, *et al.*, 1951) and approximately 95% (w/v) buffer salts (potassium phosphate, sodium citrate and magnesium acetate). The powder (100 units) was reconstituted with 800 µl water, aliquoted into Eppendorf tubes (50 µg protein/tube), freeze dried and stored at -80°C.

2.6 SARCOPLASMIC RETICULUM

SR vesicle preparations were obtained from stocks in our research group. Briefly they were prepared by differential centrifugation of back and hind leg muscle homogenates from fasted rabbits according to the method of Champeil *et al* (1985). The final resuspension medium for the vesicles was 0.3M sucrose + 5 mM Hepes, pH 7.4. Aliquots were frozen in liquid nitrogen and stored at -80°C. The protein concentration was determined by absorbance at 280 nm in 1 ml 50 mM NaPi, pH 7.0 (appendix 7.2.2) and 1% SDS (appendix 7.6.1). A conversion factor of 1 absorbance unit/mg of protein was used, a value which had been determined in the laboratory based on protein determinations using the Lowry method (1951) with bovine serum albumin as standard.

Protein composition was checked by SDS PAGE (as in 2.4) and generally Ca^{2+}-ATPase (MW 110 000) made up greater than 70 % of the total protein.

2.7 PHOTOAFFINITY LABELLING ASSAY

2.7.1 Standard methodology

Protein suspensions (usually 0.1 mg/ml in a volume of 100 µl) were incubated for 60 seconds at room temperature, in a suitable buffer system, before the addition of radiolabelled nucleotide. This solution was then irradiated in quartz cuvettes using a Xenon lamp and two toluene filters, positioned in front and behind the sample cuvette, for a predetermined time. The irradiated solution was mixed in a solubilising solution and finally subjected to SDS PAGE. The incorporation of radiolabel on the gel was viewed with autoradiographs and the quantitation determined by means of an Instantimager Electronic autoradiograph by Packard.

2.7.2. Gunnerson and Haley's methodology (1992)

10 - 15 µl CSF sample was mixed with 10 µM [γ^{32}P]8-azido-ATP (appendix 7.5.1) in 50 µl of 25 mM potassium phosphate buffer, pH 7.0, (appendix 7.2.1) at room temperature for 30 seconds. Irradiation was performed over 45 seconds with a hand-held 254-nm UV lamp (I = 5000 µW/cm^2). The protein was precipitated with 3.5% PCA (appendix 7.1.8), centrifuged and the pellet resolubilzed in a buffer containing 10% SDS, 3.6M urea, 162 mM DTT, pyronin-Y as a tracking dye and 20 mM Tris.HCl, pH 8.0, (appendix 7.6.14). Solubilized photolabelled samples were subjected to SDS-PAGE using a 10% separating gel (appendix 7.6.8) with a 4% stacking gel (appendix 7.6.9) according to the method of Laemmli (1970).

CHAPTER THREE

RESULTS

3.1 SYNTHESIS OF [γ^{32}P]8-AZIDO-ADENOSINE 5'-TRIPHOSPHATE

Usually, 100% of $[32P]_{Pi}$ was incorporated into the nucleotide. The purity of the neutralised [γ-^{32}P]8-azido-ATP is shown in Fig. 7A. The absorption spectrum of the product, from which the specific activity was calculated, is shown in Fig. 7B. There was some interference in the spectrum from the absorption of the Tris which was used to neutralise the HCl eluant. However, this interference was not enough to introduce significant errors into the measurement at 280 nm.

A starting radioactivity of 2 mCi $[32P]_{Pi}$ was convenient and, with 226 nmol 8-azido-ATP, this resulted in an initial specific activity of approximately 20 x 10^6 cpm/nmol.

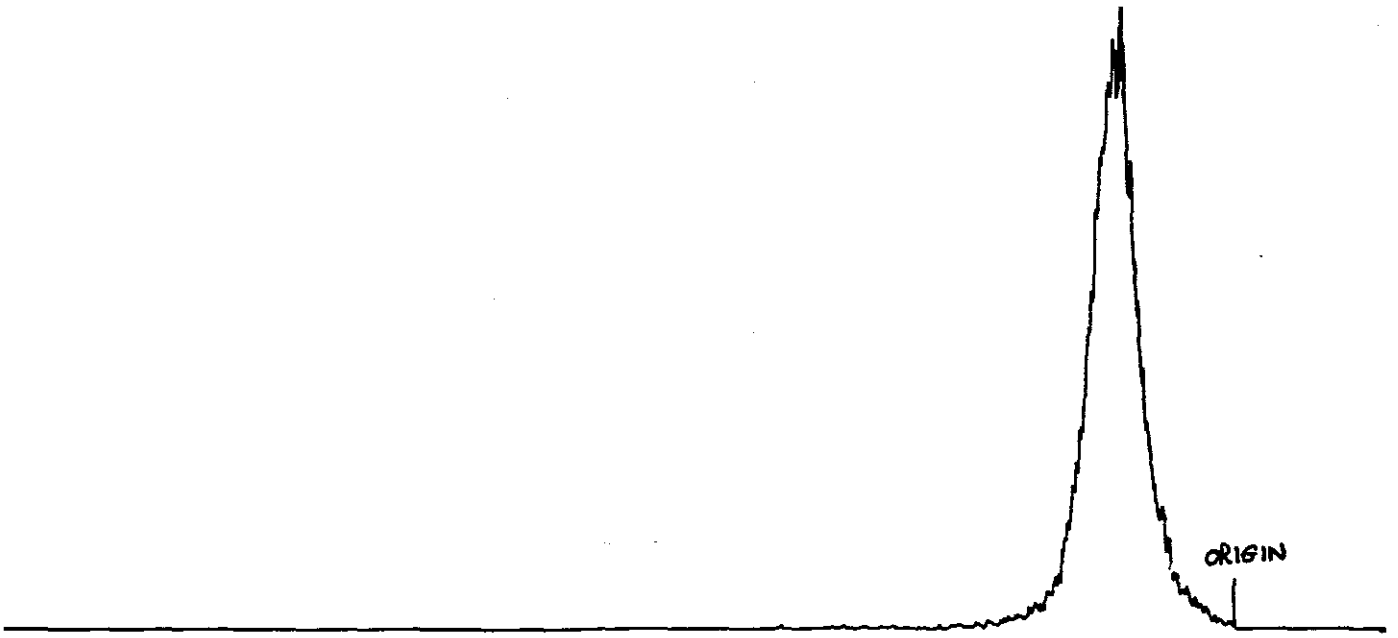


Fig. 7A. **Radioactivity profile of [γ^{32}P]8-azido-adenosine 5'-triphosphate, separated by thin layer chromatography.** A single peak is seen in the position corresponding to 8-azido-ATP.

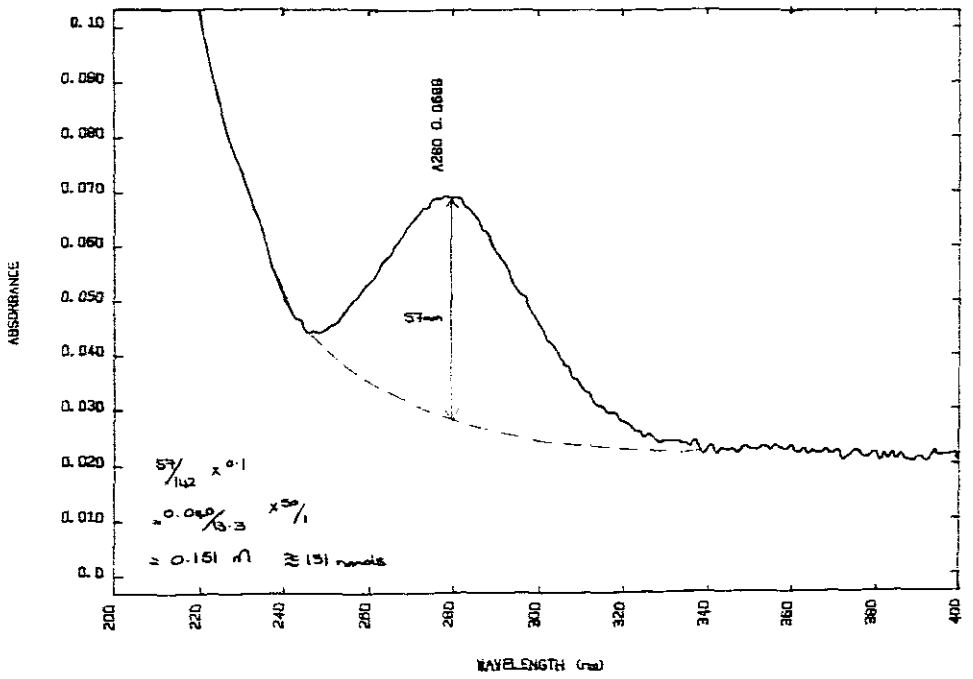


Fig. 7B. **Absorption spectrum of radioactive [γ^{32}P]8-azido-adenosine 5'-triphosphate.** The 0.040 change in absorption at 280 nm, together with a dilution factor (1:50), and molar extinction coefficient of 8-azido-ATP (13.3), permitted the final concentration to be determined.

3.2 SARCOPLASMIC RETICULUM VESICLE PROTEIN COMPOSITION

SR vesicles were used as a model protein photolabelling system since it has been well characterised in this laboratory. The protein composition of 3 different preparations, is shown in Fig 8. The principal protein is the Ca^{2+}-ATPase of 110 kDa.

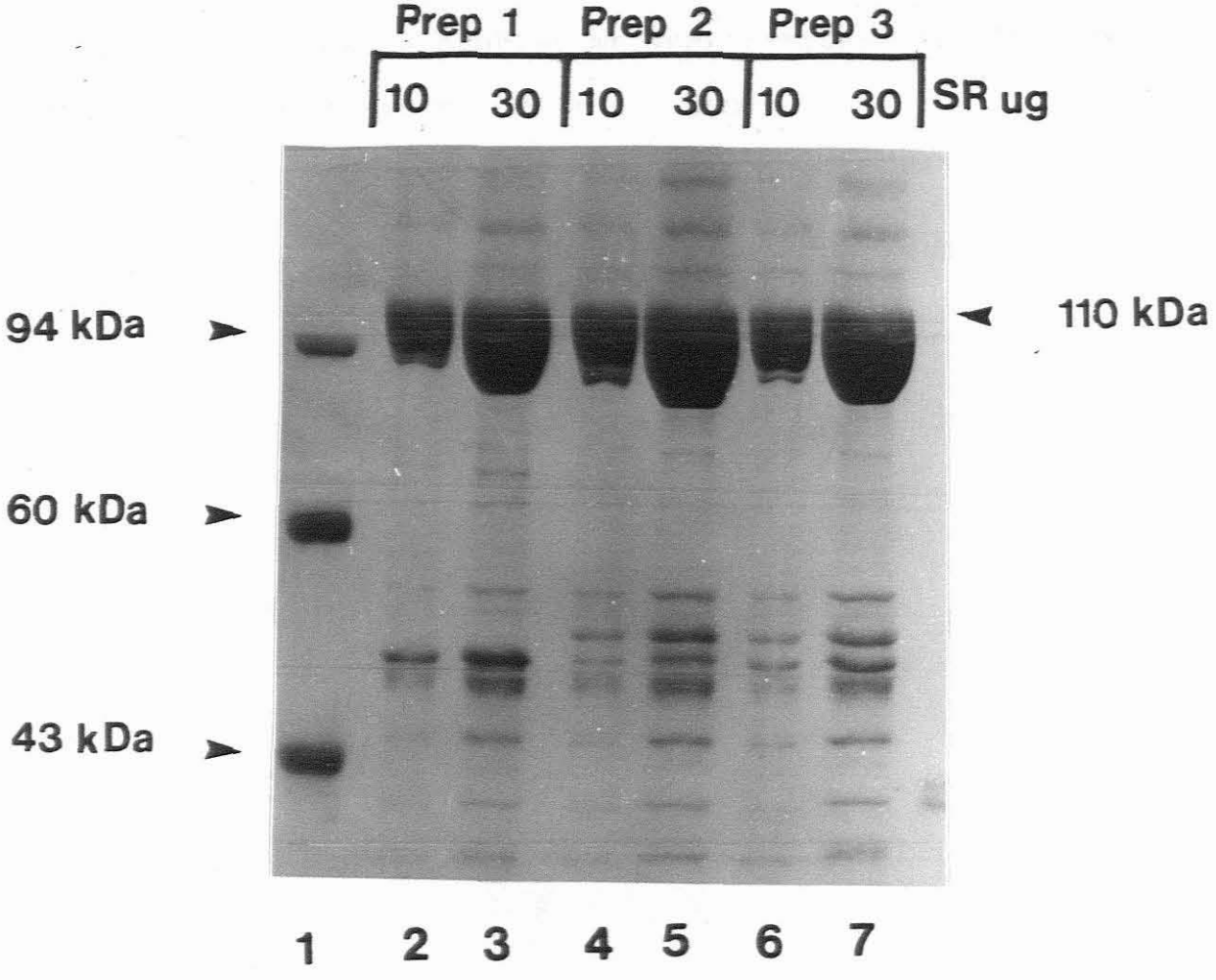


Fig. 8. Protein composition of sarcoplasmic reticulum vesicle preparations. SR vesicles from 3 different preparations (10 and 30 μg each) were subjected to 10% SDS PAGE and stained with Coomassie Brilliant Blue, (lanes 2 - 7). The markers used were, phosphorylase-B (94 kDaltons), Bovine Serum Albumin (60 kDaltons) and Oval- albumin (43 kDaltons), lane 1. (appendixes 7.6.8 & 7.6.10).

3.3 CEREBROSPINAL FLUID AND GLUTAMINE SYNTHETASE PROTEIN COMPOSITION

The protein composition of CSF and the sheep brain GS obtained from Sigma is shown in Fig. 9. The principal protein in CSF is albumin (60 kDaltons). Several other proteins are also evident. GS is seen as a doublet at approximately 43 kDaltons. The reason for two forms of GS is not known.

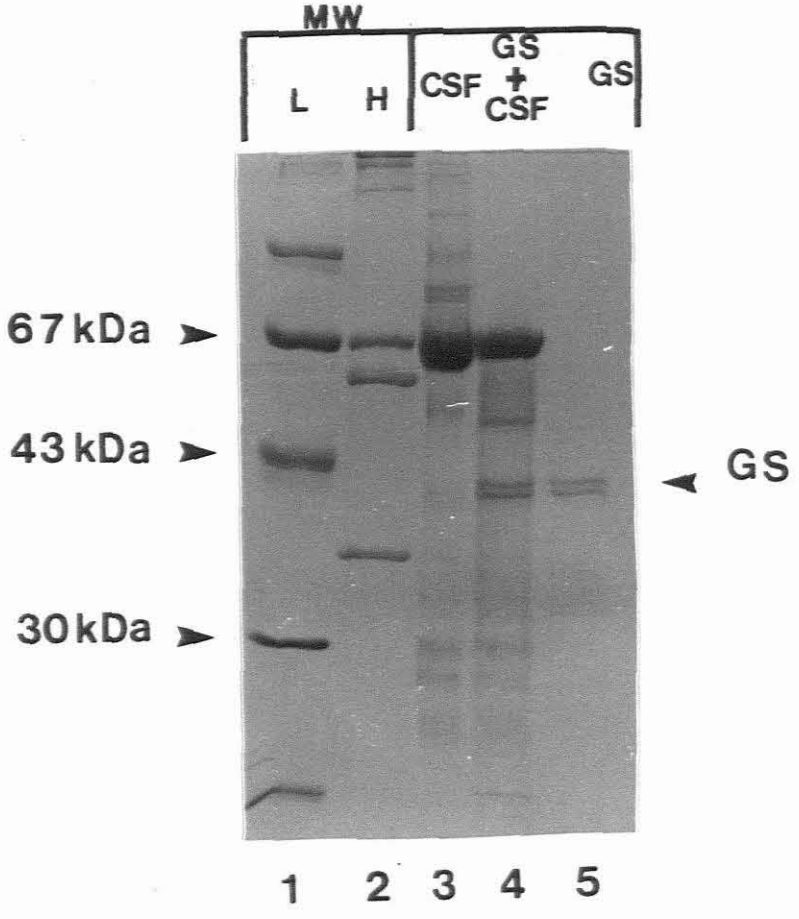


Fig. 9. **Cerebrospinal fluid and commercial glutamine synthetase protein compositions.** Markers used were: Albumin, 67 kDa; Ovalalbumin, 43 kDa; Carbonic anhydrase and 30 kDa; Soybean trypsin inhibitor (LMW). Albumin; 67 kDa (HMW). These are shown in lane 1 and 2. Lane 3 is 15 µl CSF, lane 4 is 15 µg GS + 15 µl CSF and lane 5 is 15 µg GS.

3.4 OPTIMISATION OF PHOTOLABELLING

3.4.1 Characteristics of the Xenon lamp

The time dependence of photolysis of 8-azido-ATP in water using toluene filters (cut off 290 nm) positioned in the front and at the back of the sample cuvette is shown in Fig. 10. There was a time dependent decrease in the peak at 280 nm, from which it could be ascertained that photolysis of the nucleotide under these conditions was 95% complete in 1 min (inset).

The time dependence of photolabelling of SR vesicles is shown in Fig. 11. EDTA is included in the medium as 8-azido-ATP is a good substrate for the Ca^{2+}-ATPase and probably other enzymes in the preparation. There is a close correlation between the extent of photolabelling and the degree of photolysis (Fig. 10 inset)

However, labelling is not very efficient under maximal conditions, with only 4 nmoles ATPase/mg of the active ATP being labelled (ie: only $^{1}/_{10}$).

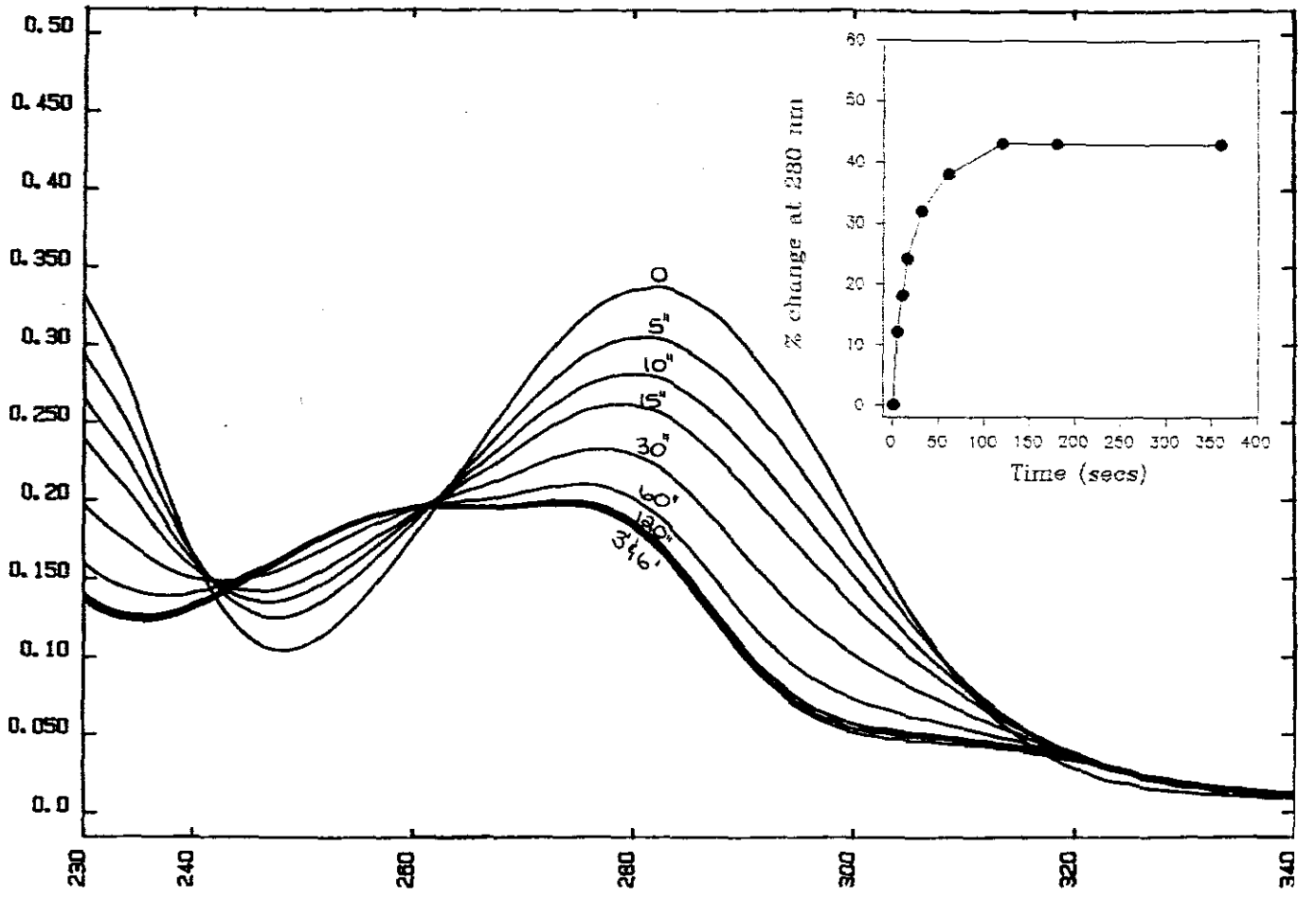


Fig. 10. **Time dependent changes in the spectrum of 8-azido-adenosine 5'-triphosphate following irradiation.** 8-azido-ATP (0.02 mM) was irradiated in water, between 2 toluene filters, at accumulative times starting at zero and increasing to 6 min.

Inset: Graphic representation of the % change in absorbance against time of the photolysis of 8-azido-ATP.

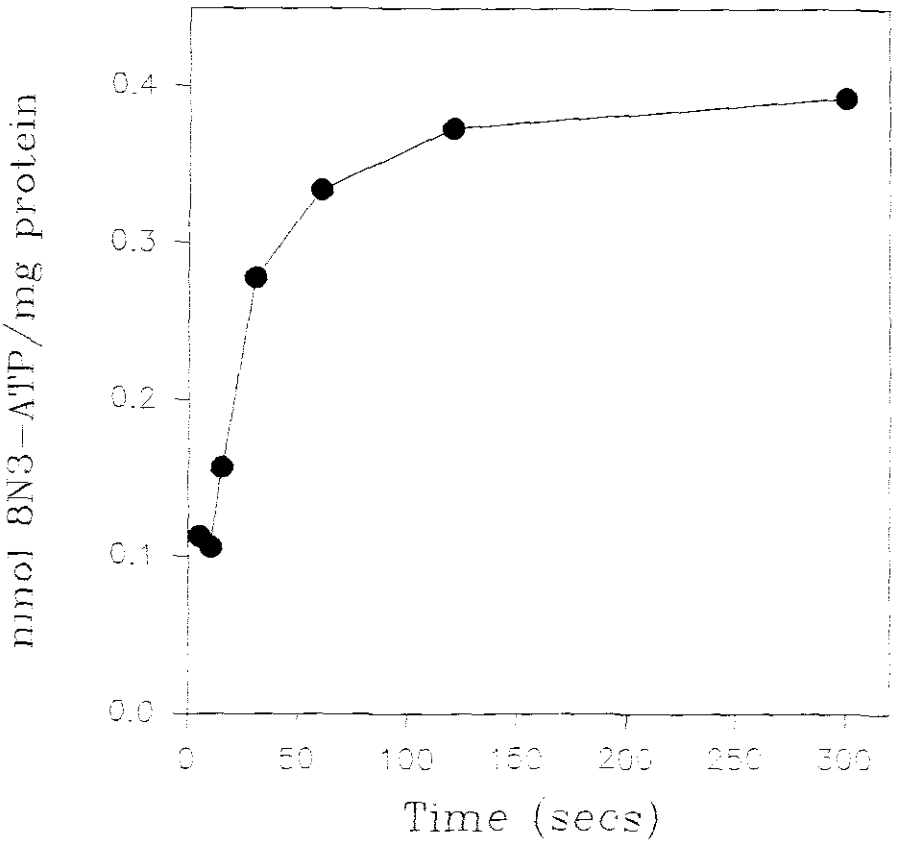


Fig. 11. **Time dependence of photolabelling of SR vesicles.** SR vesicles (0.2 mg/ml), were irradiated in 50 mM HEPPS-TMAH/0.5 mM EDTA, pH 8.5, 20 % (v/v) glycerol and 20 μM [^{32}P]8-azido-ATP. Aliquots were filtered at timed intervals through 2 glass fibre filters and their radioactivity assayed in a scintillation counter. (appendixes 7.4.2; 7.2.5; 7.1.7 & 7.5.1).

3.5. PHOTOAFFINITY LABELLING OF GLUTAMINE SYNTHETASE

Photolabelling of sheep brain GS alone and in the presence of human CSF, in addition to 5 control human CSF samples, is shown in Fig. 12. These experiments were performed by the method and under the conditions of Gunnerson and Haley (1992) except for the omission of the precipitation and resolubilisation steps. GS (50 ug/ml) is prominently labelled in the presence and in the absence of CSF (lanes 1 and 2 respectively). Albumin and a protein of 28 kDaltons are also well labelled, similar to what was found by Gunnerson and Haley (1992). In the first of the control patient samples a band corresponding to that of GS is visible. This CSF was xanthochromic (contaminated by blood). The other 4 samples do not exhibit labelling at the position expected for GS.

While the labelling of GS was clearly visible at this concentration, it was considered that low levels of GS may not be detected because of the large amount of presumably non-specific labelling of other proteins. The labelling of albumin is of particular concern because it migrates not far from GS in the gel. Longer exposure times cause the radioactivity from this band to overlap with the GS band as can be seen in lane 3 of Fig. 10. It is also possible that the binding of 8-azido-ATP to these other proteins could

sequester or use up the probe so that less is available for binding and reaction with GS. For these reasons conditions were explored which may provide more specific labelling of GS.

It was unclear whether the conditions used by Gunnerson and Haley (1992) were optimal for photolabelling of GS. Introduction of an assay into the routine diagnostic laboratory also requires that the main parameters which may affect the assay have been elucidated. Accordingly, we explored possible variables which may affect the labelling of GS.

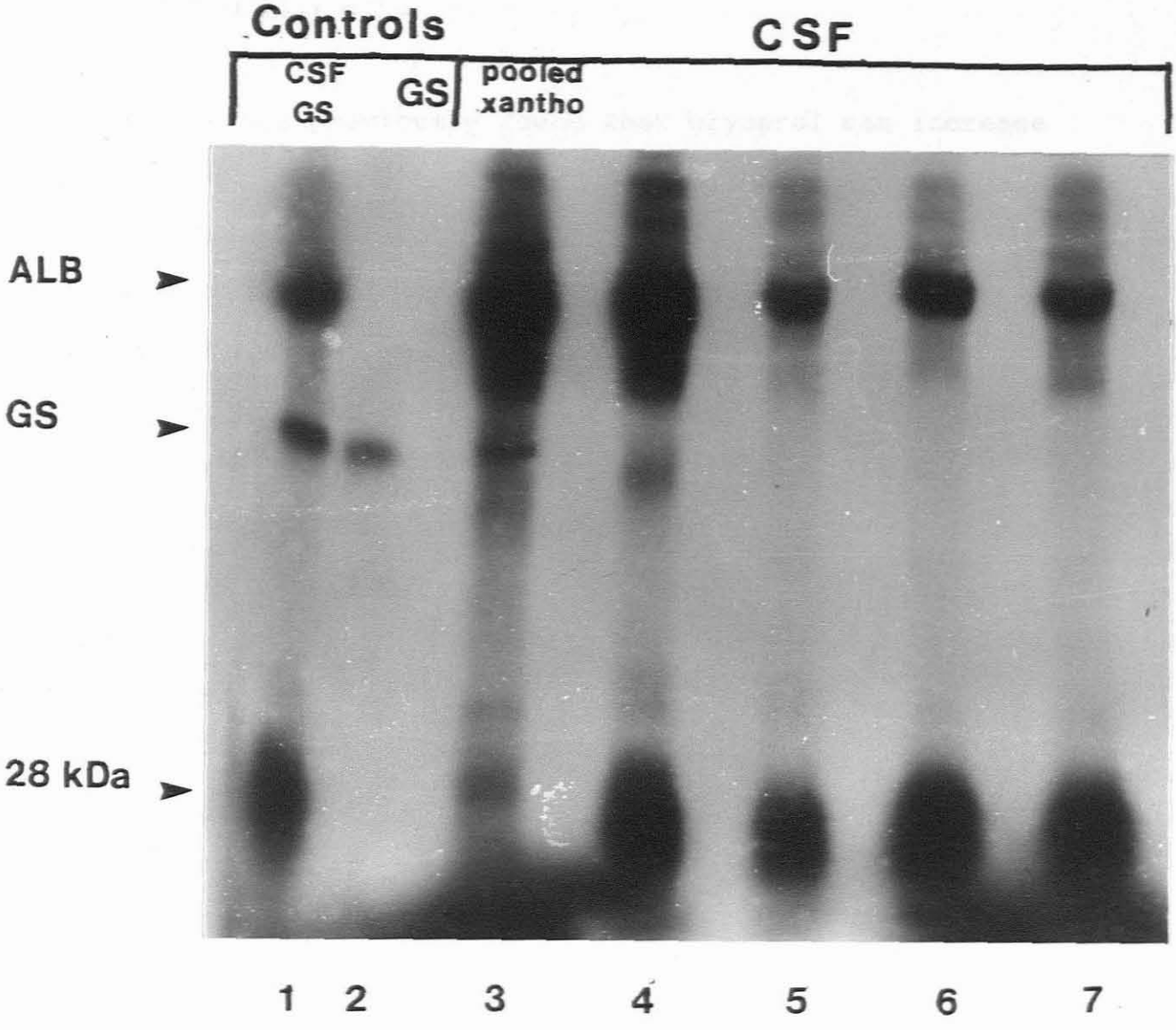


Fig. 12. **Photolabelling using Gunnerson and Haley's method.** Lanes 3 to 7 are CSF samples (15 µl/lane) that were irradiated in 50 mM potassium phosphate, pH 7.0 and 10 µM [γ-^{32}P]8-azido-ATP. Controls used were 15 µl CSF and 50 µg/ml GS (lane 1) and 50 µg/ml GS alone (lane 2). (appendixes 7.2.1; 7.5.1 & 7.4.1)

3.5.1. Effect of glycerol

Our laboratory has previously found that glycerol can increase the specificity of azido labelling of sarcoplasmic reticulum Ca2+-ATPase (unpublished findings). The influence of glycerol was therefore tested on the labelling of GS and CSF proteins and the results are shown in Fig. 13. In these experiments the potassium phosphate buffer of Gunnerson and Haley (1992) was changed to sodium phosphate as elimination of the protein precipitation step caused the potassium ion to interact with the sodium dodecyl sulphate in the gel buffer. The change in buffer had no effect on the labelling pattern as can be seen if the first three lanes of the gel in Fig. 13 are compared with the first two lanes of Fig. 12. Note that the autoradiograph of Fig. 13 is not as exposed as that in Fig. 12 and the GS is barely visible in the former. As can be seen in Fig. 13, 20 % (v/v) glycerol (appendix 7.1.7), if preincubated with the buffer overnight had no effect on the labelling of GS or the other proteins in CSF (compare lanes 4, 5, and 6 with lanes 1, 2, and 3). However, if the preincubation time was shortened there was a marked lowering of the labelling of albumin and the 28 kDalton protein and an enhancement of the labelling of GS (lanes 7, 8, and 9). The time dependence of this effect is shown in Fig. 14. Least labelling of albumin and the 28 kDalton species occurred at the shortest times of preincubation.

In all subsequent experiments, except where indicated, glycerol was added to the buffer just prior to irradiation.

which are eliminated by incubation with the buffer. In all subsequent experiments, except where indicated, glycerol was added to the buffer just prior to irradiation.

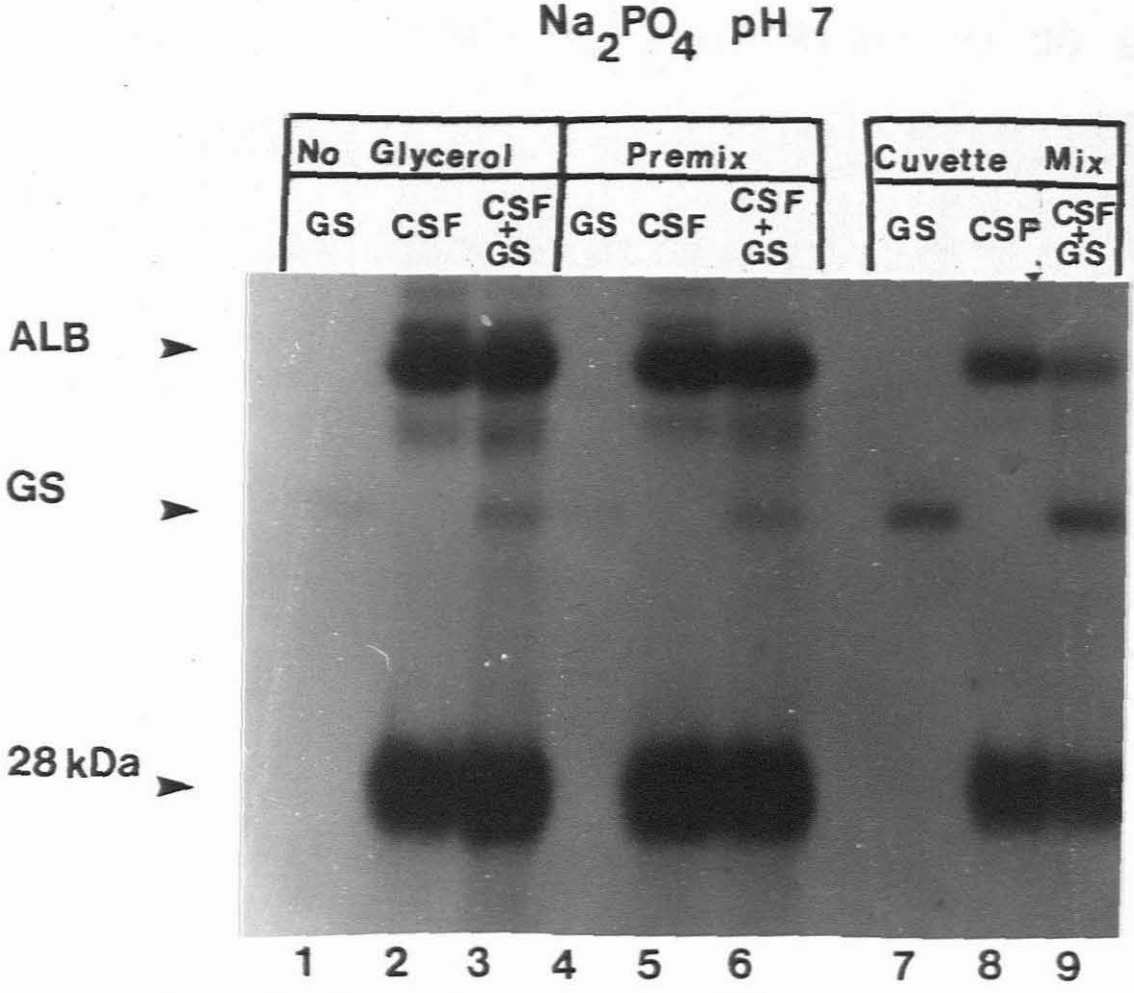


Fig. 13. **Effect of glycerol on photolabelling on cerebrospinal fluid and glutamine synthetase.** Sheep brain GS (50 μg/ml) and 5 μl CSF were irradiated in 25 mM sodium phosphate, pH 7.0, with two 20% glycerol time conditions. Control results in lanes 1, 2 and 3 are in the absence of glycerol. Lanes 4, 5 and 6 show the results obtained when glycerol is preincubated in the buffer overnight. Results in lanes 7, 8 and 9 are with the addition of the glycerol mixed with buffer approximately 30 secs before irradiation. (appedixes 7.4.1; 7.3.3 & 7.1.7).

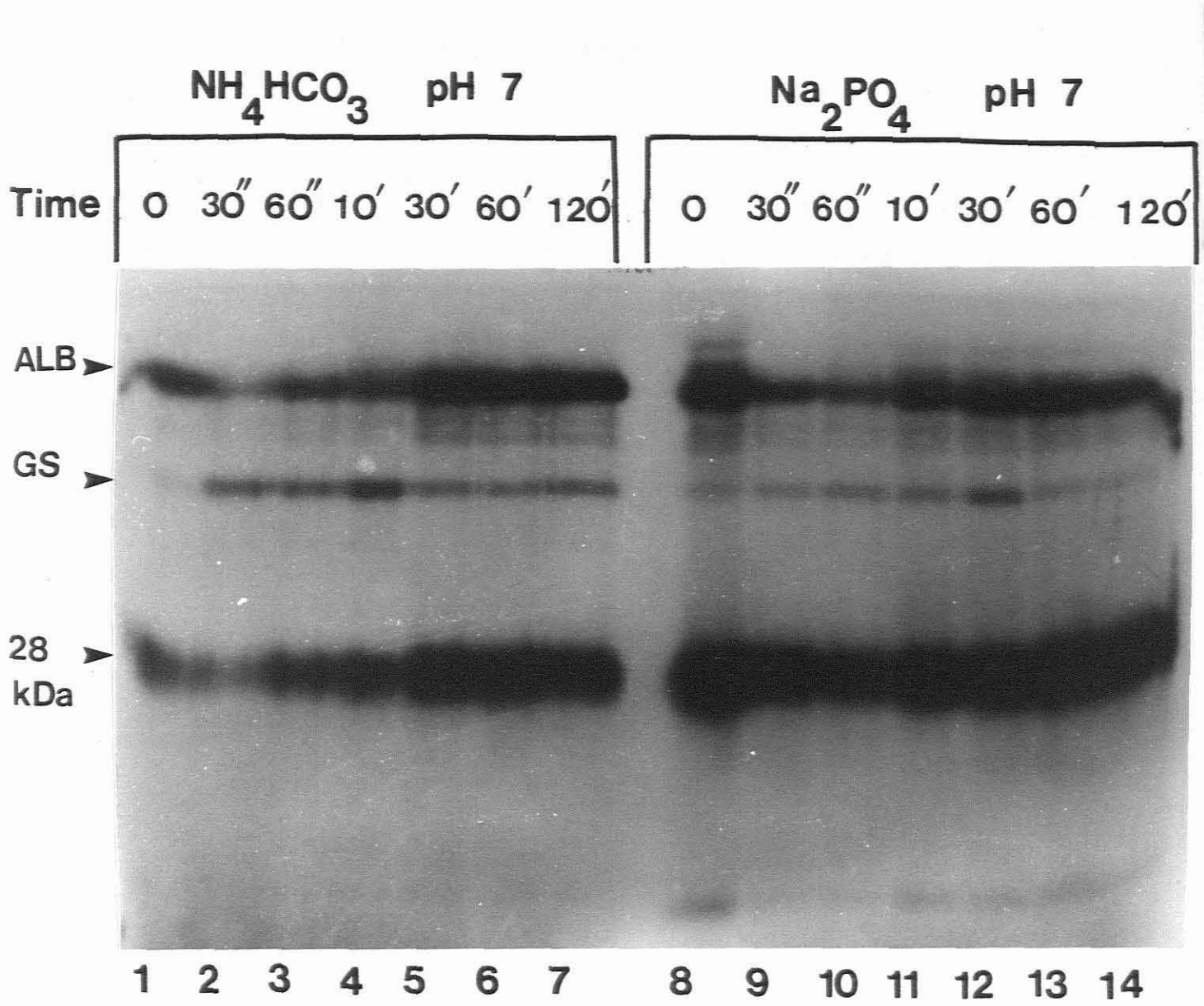


Fig. 14. **The time dependence of the glycerol effect.** Csf (5 µl) and GS (50 µg/ml), in the presence of increasing incubation time with 20% glycerol (30 sec to 120 min), were irradiated in two different buffers (25 mM ammonium bicarbonate in lanes 1 to 7, and sodium phosphate in lanes 8 to 14, pH 7.0) and separated on a 12% SDS PAGE. Lanes 1 and 8 are the zero time points (no glycerol). (appendixes 7.4.1; 7.1.7; 7.3.6 & 7.3.3).

3.5.2 Effect of ammonium bicarbonate

Ammonium ion is a substrate of the GS-catalysed reaction. It normally binds after ATP has phosphorylated the glutamate (Eisenberg, 1994). Its binding is enhanced by ADP. If there is a reciprocal effect, ammonium ions may increase the affinity of the enzyme for nucleotide or alter its position at the active site such that there is an increase in labelling. We also considered that phosphate may compete with 8-azido-ATP for the active site and diminish labelling.

Accordingly, we examined whether substituting 25 mM ammonium bicarbonate (appendix 7.3.6) for 25 mM sodium phosphate (appendix 7.3.3) could enhance labelling of GS and perhaps lower the non-specific labelling. The results are shown in Fig. 15. The effect of glycerol in combination with the ammonium bicarbonate change is also shown. In the first 3 lanes labelling was performed with ammonium bicarbonate and glycerol premixed overnight. The labelling should be compared with that obtained with sodium phosphate in lanes 7, 8, and 9. Clearly the labelling of GS is more pronounced and that of albumin and the 28 kDalton protein diminished when ammonium bicarbonate is used. Shortening the exposure time of the glycerol to the buffer is, as has been shown above, very effective in enhancing the labelling of GS and lowering the non-specific labelling (Compare lanes 3 and 6, and 9 and 12). Lane 6 shows that the combination of $NH^{+}_{4}HCO_{3}$ and fresh glycerol results in GS being the most prominently labelled band in GS + CSF mixtures. The labelling of albumin and the 28 kDalton component is virtually eliminated.

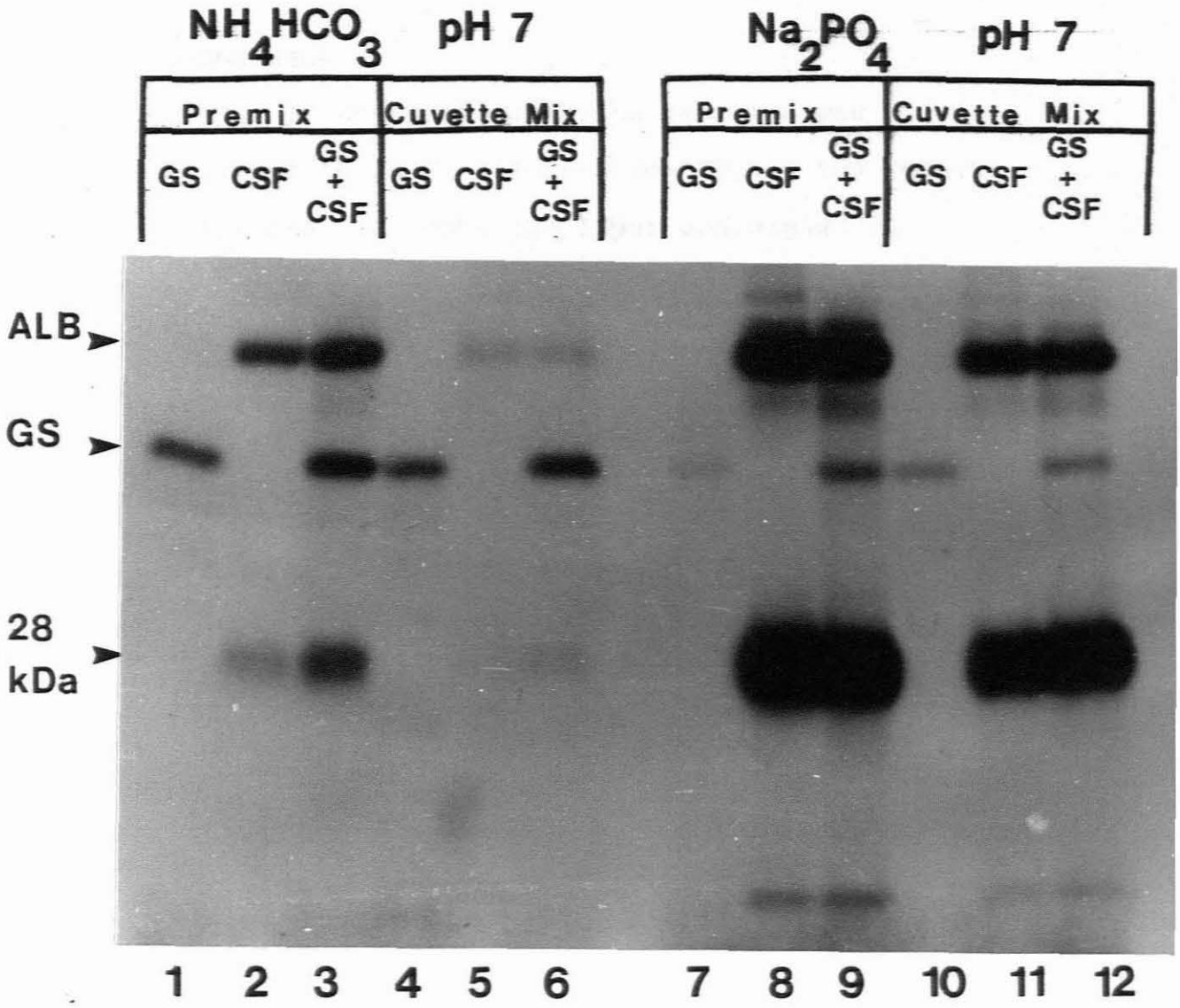


Fig. 15. **A comparison of photolabelling in the presence of ammonium or sodium ions.** GS (lanes 1 and 4) and CSF (lanes 2 and 5) were photolabelled individually and together (lanes 3 and 6) in ammonium bicarbonate pH 7.0, (25 mM), with 10 μM [γ^{32}P]8-azido-ATP. Lanes 1, 2 and 3 are the results from overnight incubation in 20 % glycerol and lanes 4, 5 and 6 are the results from an approximate 30 sec preincubation in the buffer. Lanes 7 to 12 are a repeat of the above conditions with the only change being the buffer, to 25 mM sodium phosphate, pH 7.0. (appendixes 7.3.6; 7.5.1; 7.1.7 & 7.3.3).

3.5.3. pH dependence

The pH dependence of the photolabelling reaction was determined at 2 and 10 uM [γ-32P]8-azido-ATP and the results are shown in Fig 16A. At both the lower and higher concentrations of nucleotide there was a decrease in the labelling at more alkaline pH values, although the effect was slightly more prominent at the lower concentration.

We experienced problems with maintaining the $NH^{+}_{4}HCO_{3}$ buffer at neutral pH (close to a pH shift within 24 h). It is much more stable at pH 8.5 (pH maintained for at least a week), which is the pH of the solution before HCl is added, and in all subsequent experiments the pH was kept at pH 8.5.

Fig 16B is the graphic representation of the pH dependence.

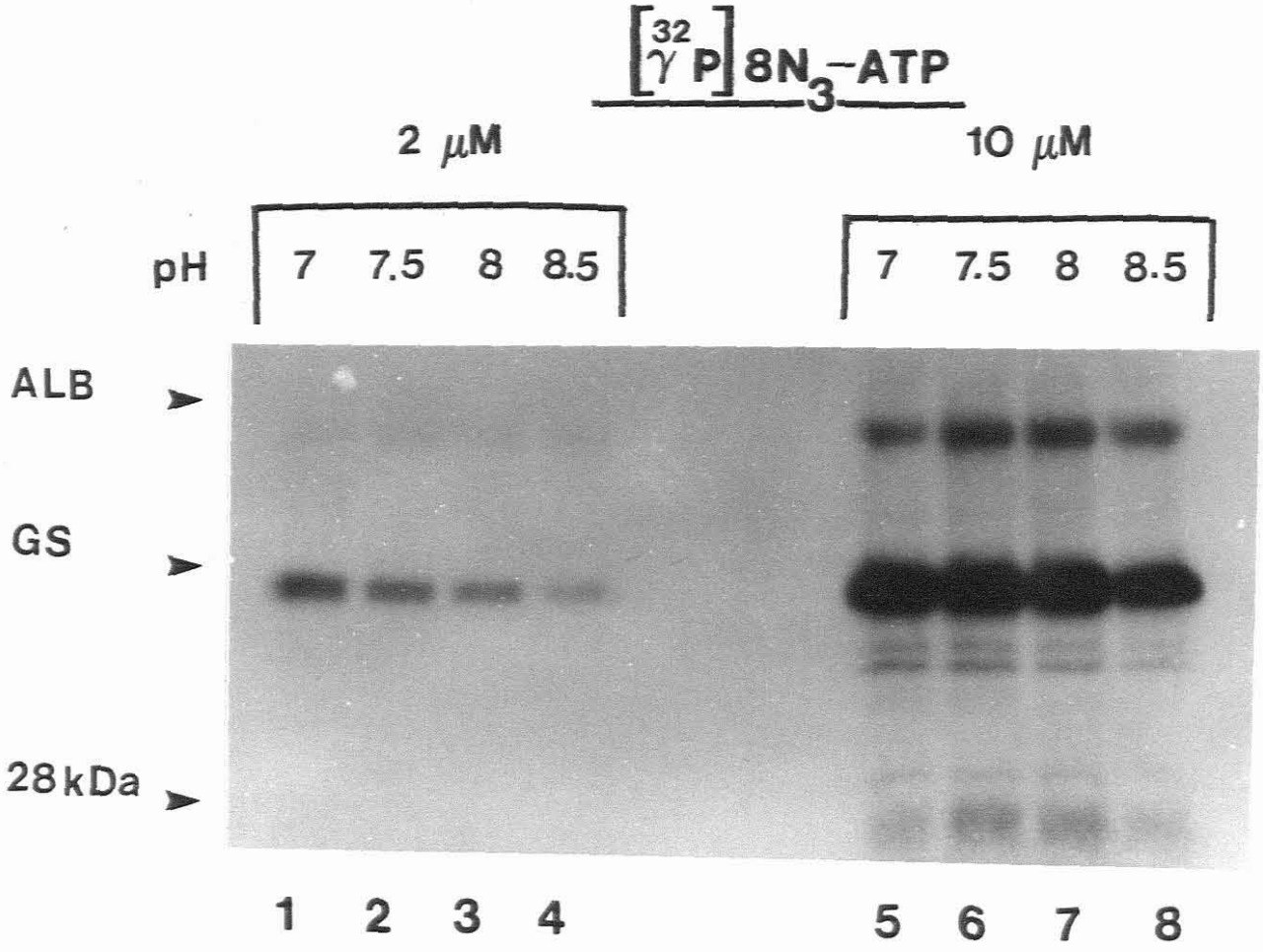


Fig. 16A. **pH dependence of photolabelling.** Csf and GS were irradiated in 100 mM ammonium bicarbonate and 20% glycerol at pH's 7.0, 7.5, 8.0 and 8.5, and subjected to 12% SDS PAGE. Lanes 1 to 4 correspond to the increasing alkalinity respectively when photolabelled with 2 μM [γ^{32}P]8-azido-ATP, as do lanes 5 to 8 with 10 μM [γ^{32}P]8-azido-ATP. (appendixes 7.2.6; 7.1.7; 7.6.8 & 7.5.1).

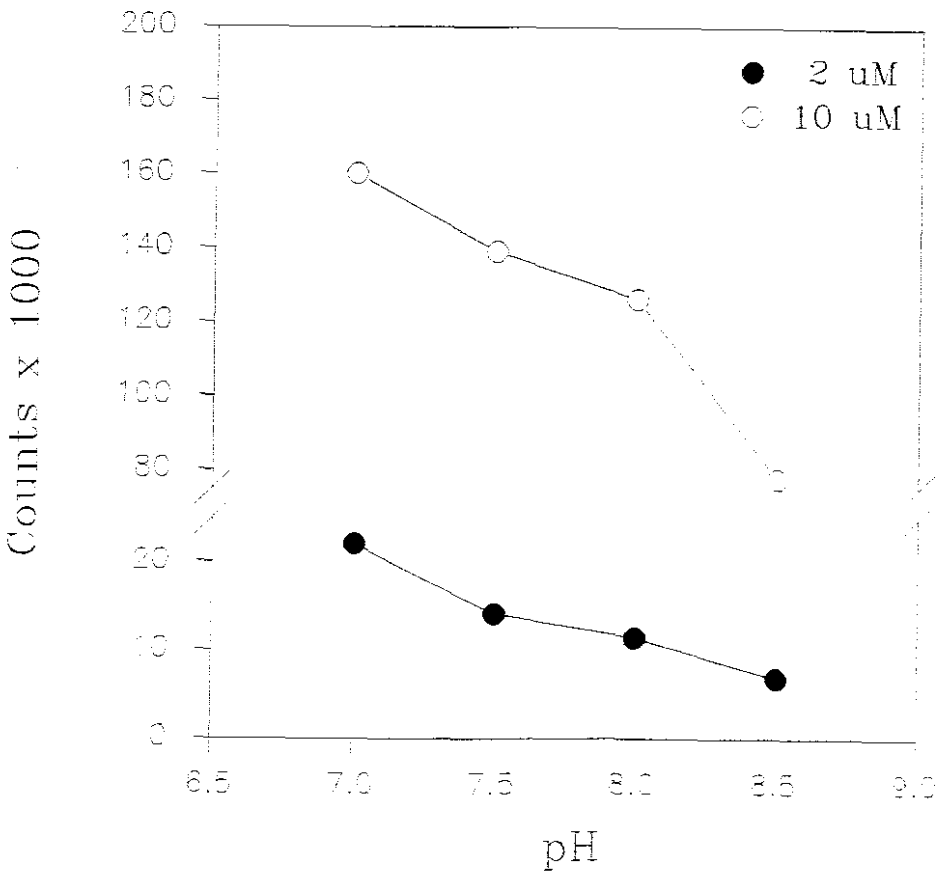


Fig. 16B. **Graphical representation of the pH dependence of photolabelling.** Counts from both concentrations of nucleotide obtained from the quantitation of Fig 16A plotted against the pH.

3.5.4. Effect of Magnesium and Ethylene diamine tetra-acetic acid

GS is dependent on divalent cations (Mn^{2+} is the natural cation but it can be replaced by Mg^{2+}) for the stabilisation of the native octomeric form of the enzyme (Wilk *et al.*, 1969) and for catalysis (Hunt & Ginsberg., 1972). It was therefore of interest to determine the effect of Mg^{2+} on labelling of GS. Gunnerson and Haley (1992) did not add Mg^{2+} to their irradiation medium. EDTA is a commonly used anticoagulant in the collection of blood and we also considered what its effect might be. The effects of Mg^{2+} and EDTA are shown in Fig. 17A. In the first lane the extent of labelling obtained in the absence of both Mg^{2+} and EDTA is shown. The second lane shows that the addition of 20 mM Mg^{2+} (appendix 7.1.2) has no effect. Irradiation in the presence of 2 mM EDTA (appendix 7.1.11) and no Mg^{2+} (lane 3) eliminated the labelling of GS. Addition of Mg^{2+} to the enzyme preincubated with EDTA (approximately 30 sec) caused the photolabelling to reappear as soon as the concentration of Mg^{2+} was greater than that of the EDTA. The concentration of Mg^{2+} at which the half maximal effect occurred is approximately 4.2 mM (Fig. 17B), which, after subtracting the concentration of EDTA, yields a $K_{0.5}$ value of 2.2 mM. This is equal to the $K_{0.5}$ value for activation of the enzyme (Allison, 1977).

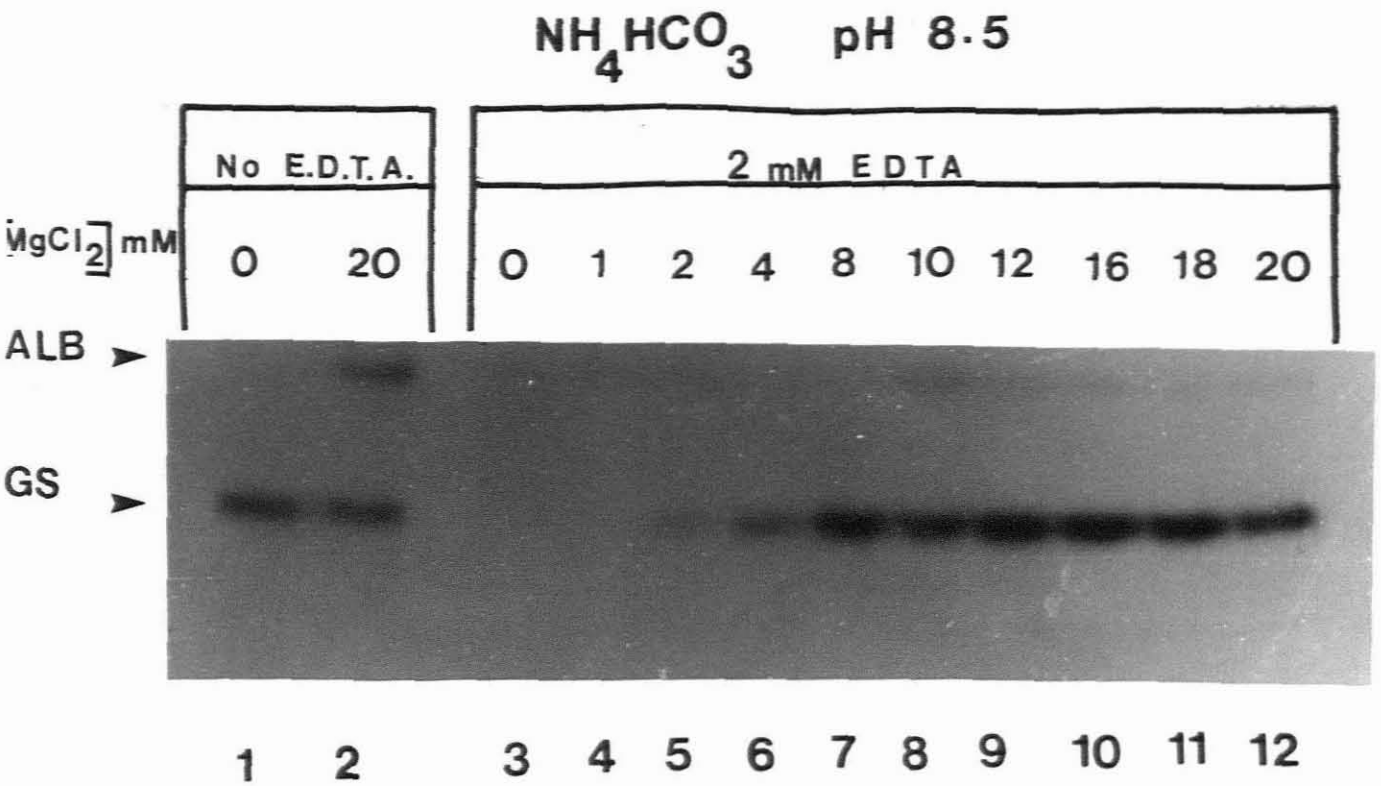


Fig. 17A. **Mg^{2+} and EDTA effects on photolabelling.** Lanes 3 to 12 are 100 mM ammonium bicarbonate, pH 8.5 initially mixed with 20% glycerol, followed by the addition of 5 µl CSF, 50 µg/ml GS, 2 mM EDTA and then the desired concentration of Mg^{2+}. Finally 10 µM [γ^{32}P]8-azido-ATP was added just before irradiation. The controls followed the same ordered additions, with the exceptions of lane 1 (no Mg^{2+} or EDTA) and lane 2 (no EDTA). (appendixes 7.2.6; 7.1.7; 7.4.1; 7.1.11 & 7.5.1).

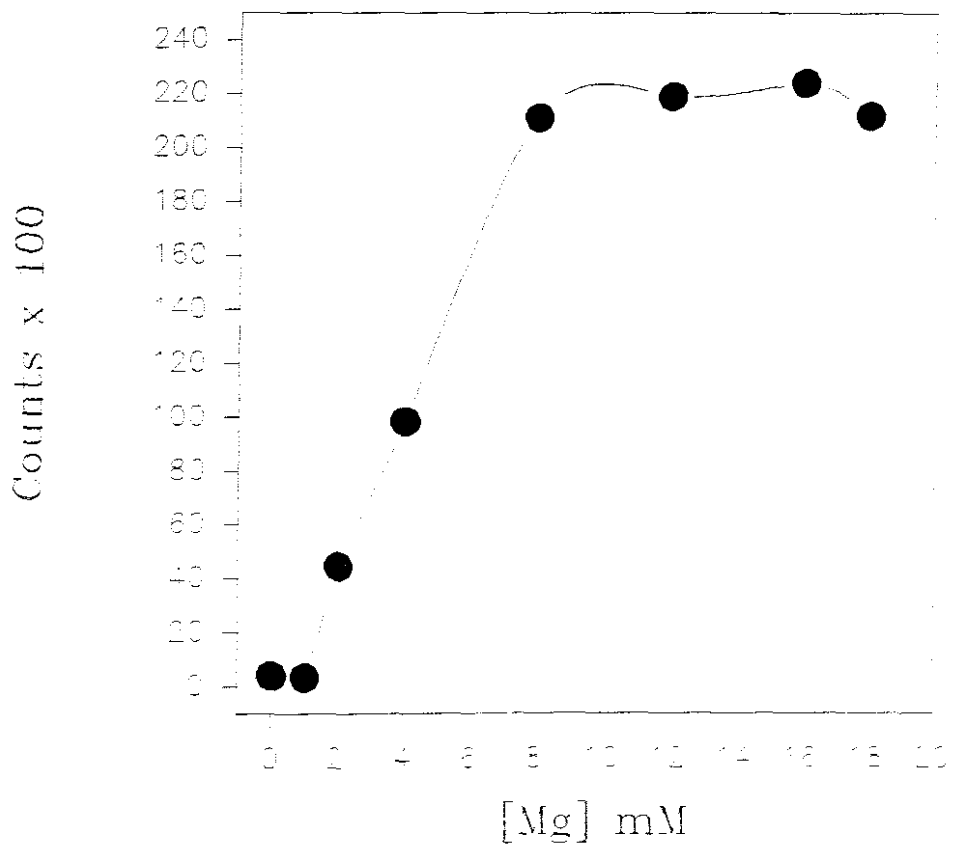


Fig. 17B. **Mg^{2+} concentration dependence of photolabelling.**

3.5.5. Adenosine 5'-triphosphate (ATP)

Under conditions of this experiment, the 8-N_3-ATP and ATP are presumed to compete for the substrate binding site on GS according to the following reactions:

$$N_3ATP + E \quad E.N_3 \quad \text{and} \quad ATP + E \quad E.ATP$$

The ATP binding site of GS is known to be a low affinity site with a Kd of approximately 0.3 mM (Allison, 1977). The effect of increasing concentrations of ATP on the photolabelling in the absence and presence of csf at 10 uM [γ-32P]8-azido-ATP is shown in Fig. 18A. Half-maximal inhibition of photolabelling in the absence of CSF was approximately 0.25 mM ATP. The "true" Ki for inhibition would be a bit lower than this but is still close to the Kd quoted above.

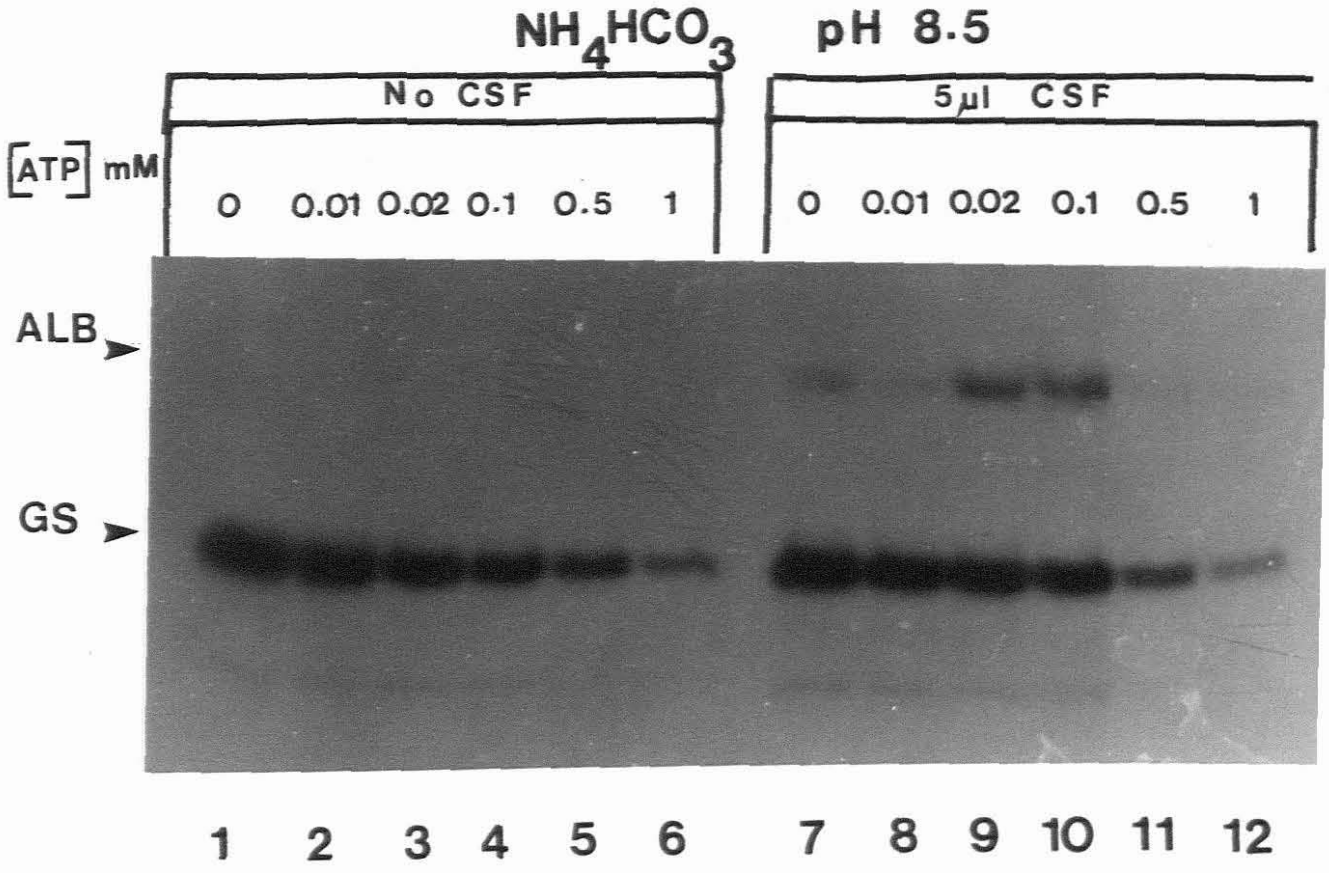


Fig. 18A. **Effect of adenosine 5'-triphosphate on photolabelling.** Using optimal conditions determined, increasing ATP concentrations (0, 0.01, 0.02, 0.1, 0.5 and 1 mM) (appendix 7.1.5) were added to irradiation samples in the absence and presence of 5µl CSF. Lanes 1 to 6 and 7 to 12 respectively.

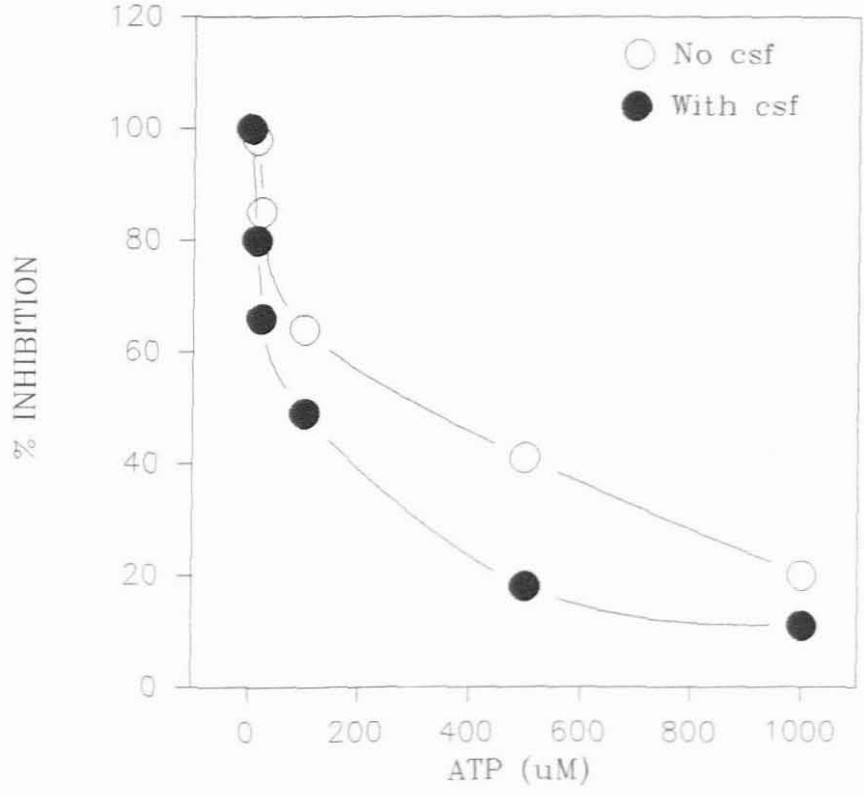


Fig. 18B. **ATP concentration dependence of the inhibition of photolabelling.**

3.5.6. Effect of Sodium Azide (NaN_3)

NaN_3 is a commonly used inhibitor of bacterial growth. We considered whether this salt could be used to preserve CSF samples. The effect of increasing concentrations of NaN_3 on photolabelling is shown in Fig. 19. Concentrations of NaN_3 of up to 2 % had no effect on the labelling (please ignore lane 2, we suspect that there was an error in sample loading).

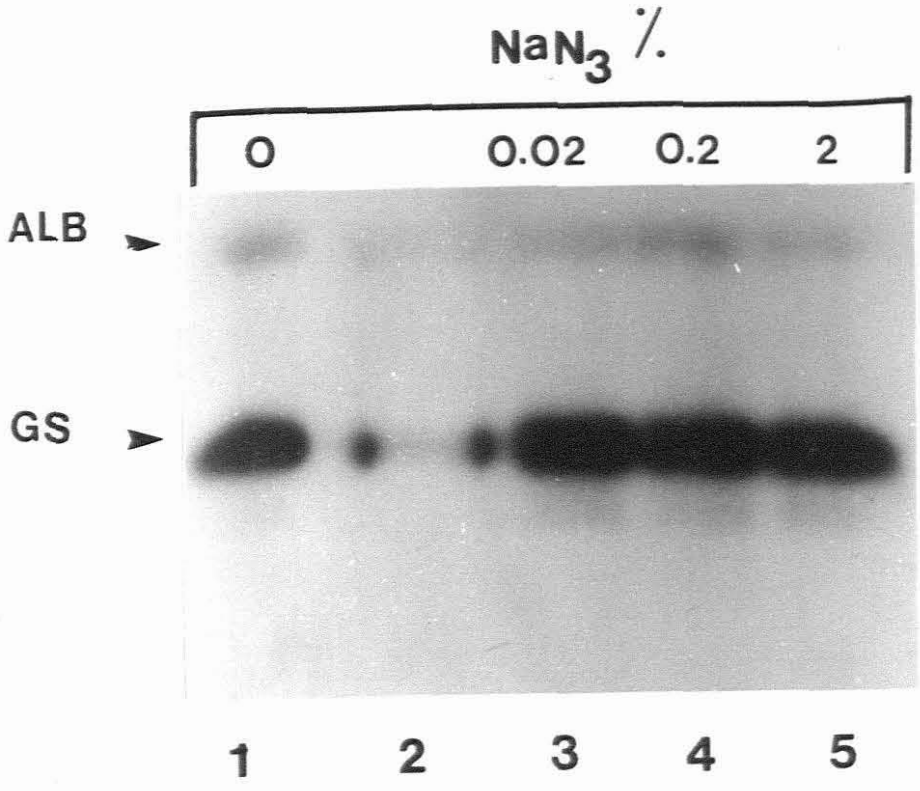


Fig. 19. **Effect of sodium azide on photolabelling.** Csf and GS samples were photolabelled in 100 mM ammonium bicarbonate/2 mM EDTA/20 mM Mg^{2+}, pH 8.5, 20 % glycerol and 10 μM [γ^{32}P]8-azido-ATP. Lane 1 is with no sodium azide, lane 3 with 0.02 %, lane 4 with 0.2 % and lane 5 with 2 %. (appendixes 7.3.4; 7.1.7; 7.5.1 & 7.1.13).

3.5.7. Concentration dependence of [γ^{32}P]8-azido-adenosine 5'-triphosphate photolabelling

The dependence of photolabelling on the concentration of 8-azido-ATP was investigated and the results are shown in Fig. 20A. Quantitation of the bands indicates that there is a linear dependence on concentration of probe, suggesting that the site has a low affinity for the nucleotide.

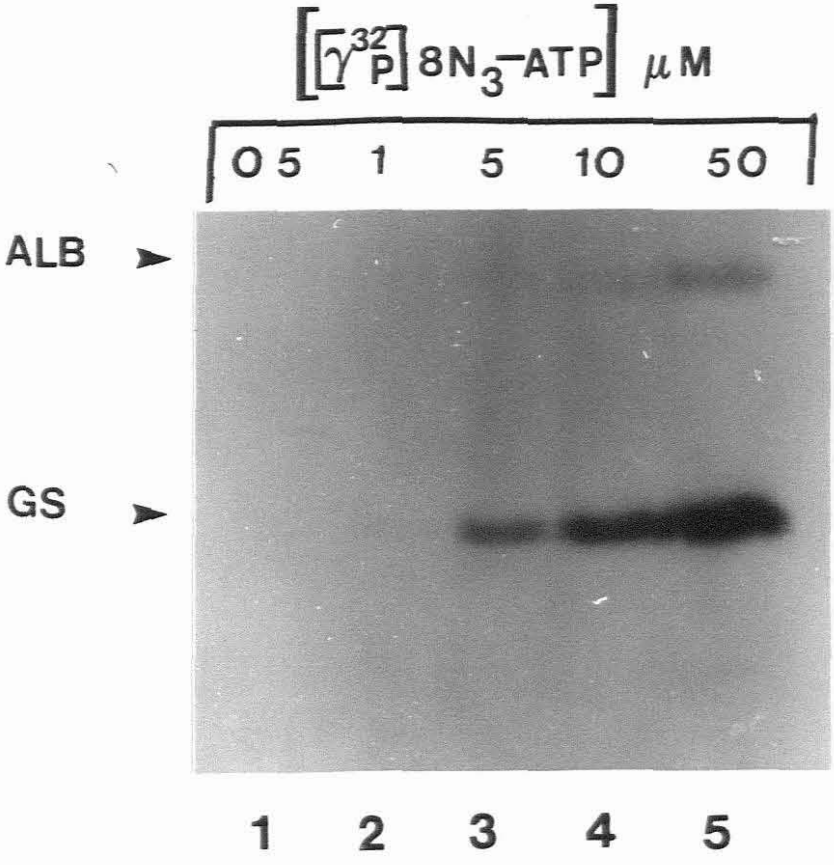


Fig. 20A. **Effect of [γ^{32}P]8-azido-adenosine 5'-triphosphate concentration on photolabelling.** GS (50 μl/mg) in the presence of 5 μl CSF was irradiated using optimised conditions, with increasing concentrations of [γ^{32}P]8-azido-ATP, from 0.5 μM (lane 1) to 50 μM (lane 5). (appendixes 7.4.1; 7.3.4 & 7.5.1).

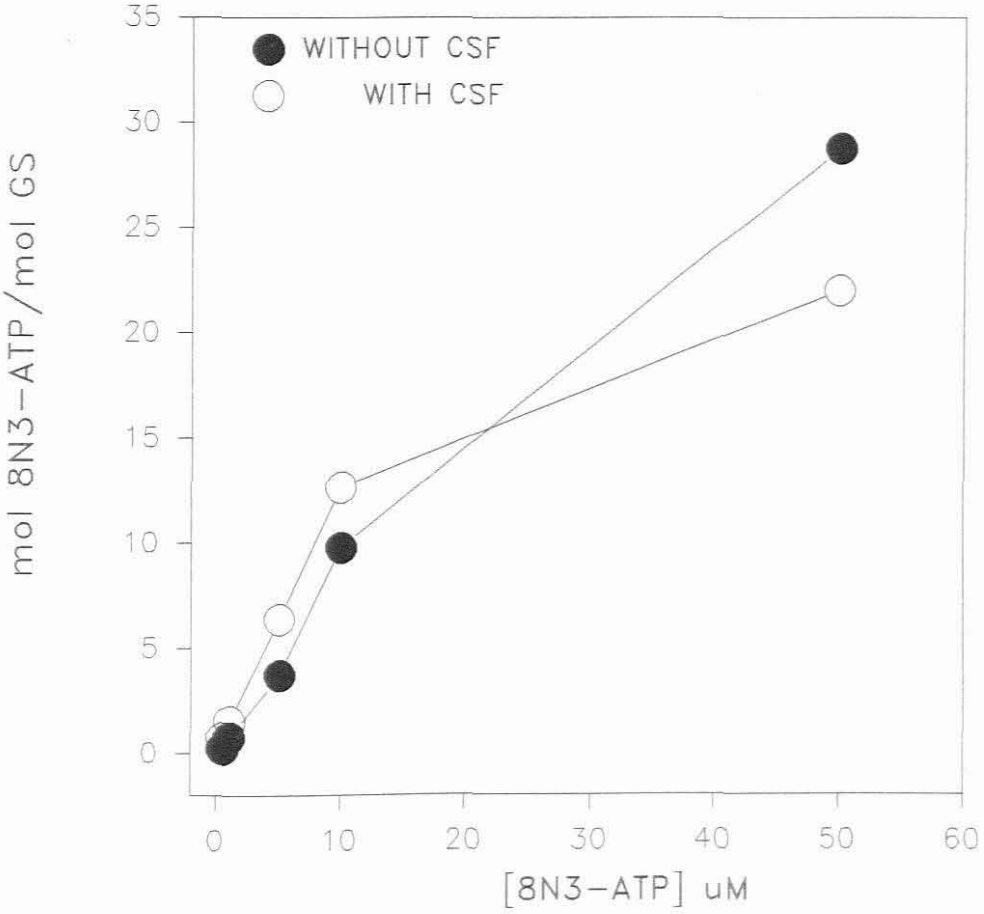


Fig. 20B. **Graphic representation of Fig 20A.**

3.5.8. Photolabelling as a function of glutamine synthetase concentration

The dependence of photolabelling on the level of GS in CSF is shown in Fig. 21. There is a linear dependence on GS concentration in the range 1-100 ug/ml.

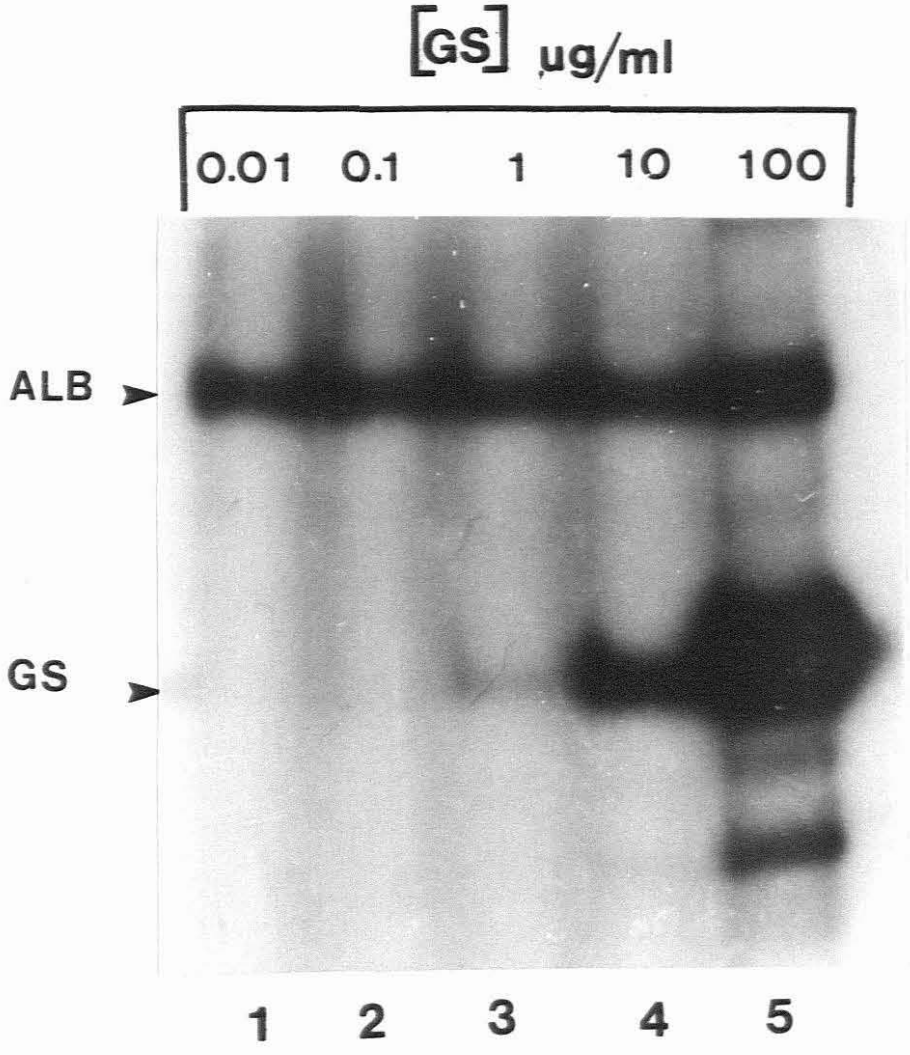


Fig. 21. **Effect of glutamine synthetase concentration on photolabelling.** Increasing concentrations of GS, 1 - 100 µg/ml, seen in lanes 1 - 5 respectively, were irradiated under optimal conditions with 10 µM [γ^{32}P]8-azido-ATP and subjected to 12% SDS PAGE. (appendixes 7.4.1; 7.3.4; 7.5.1 & 7.6.8).

3.5.9. Thermal stability of Glutamine Synthetase

The stability of GS in CSF is of interest in case the history of the handling of patient samples is not known. Accordingly, preincubations of GS in CSF were performed at three different temperatures (0°C, 20oC and 37°C) in the presence and absence of 0.02 % sodium azide (to prevent bacterial growth). Aliquots were snap frozen at timed intervals and stored at -80 oC until all the samples had been collected and then the photolabelling was performed. Only the results of the labelling of GS in the absence of sodium azide are shown as in its presence the results were similar. The results are shown in Fig. 22. (? The labelling of GS declined exponentially and similarly with increasing times of preincubation at 0°C and 20 °C, with a $t_{1/2}$ value of approximately 200 h). Preincubation at 37 °C resulted in a progressive increase in labelling until 50 h and then declined rapidly to zero thereafter (Fig 23). Fig 24 shows a graphical representation of all the temperatures together.

We conclude that it does not matter whether the CSF sample is placed on ice or kept at room temperature after collection, but it should not be stored for more than a few hours like this before being frozen. The result of preincubation at 37 °C is peculiar and possible explanations will be put forward in the "Discussion".

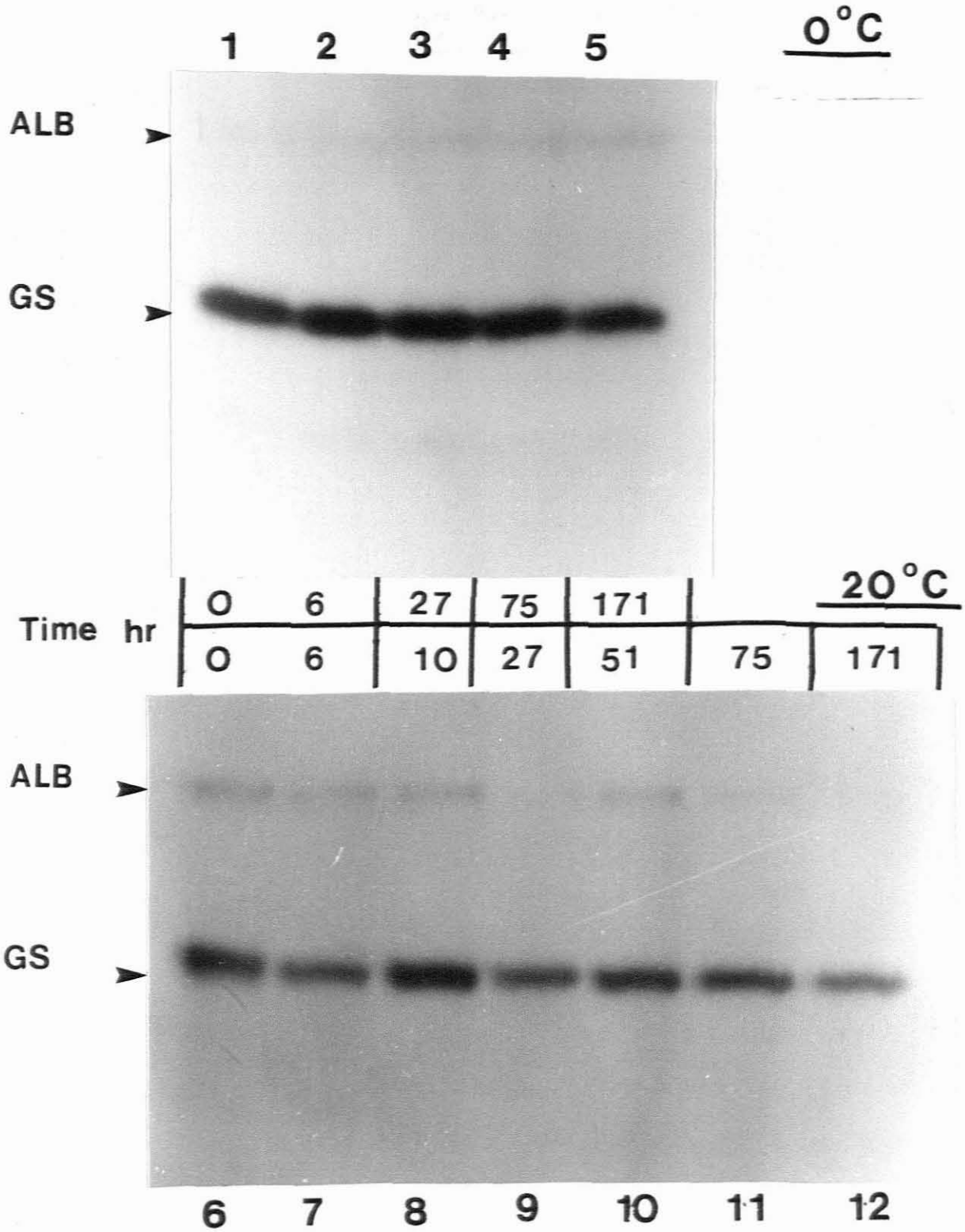


Fig. 22. **Effect of preincubation at 0°C and 20°C on photolabelling.** Timed aliquots of 50 ug GS photolabelled under optimal conditions with 10 um [γ^{32}P]8-azido-ATP and subjected to 12% SDS PAGE. Lanes 1 - 5 (0°C) and 6 -12 (20°C). (appendixes 7.4.1; 7.5.1 & 7.6.8).

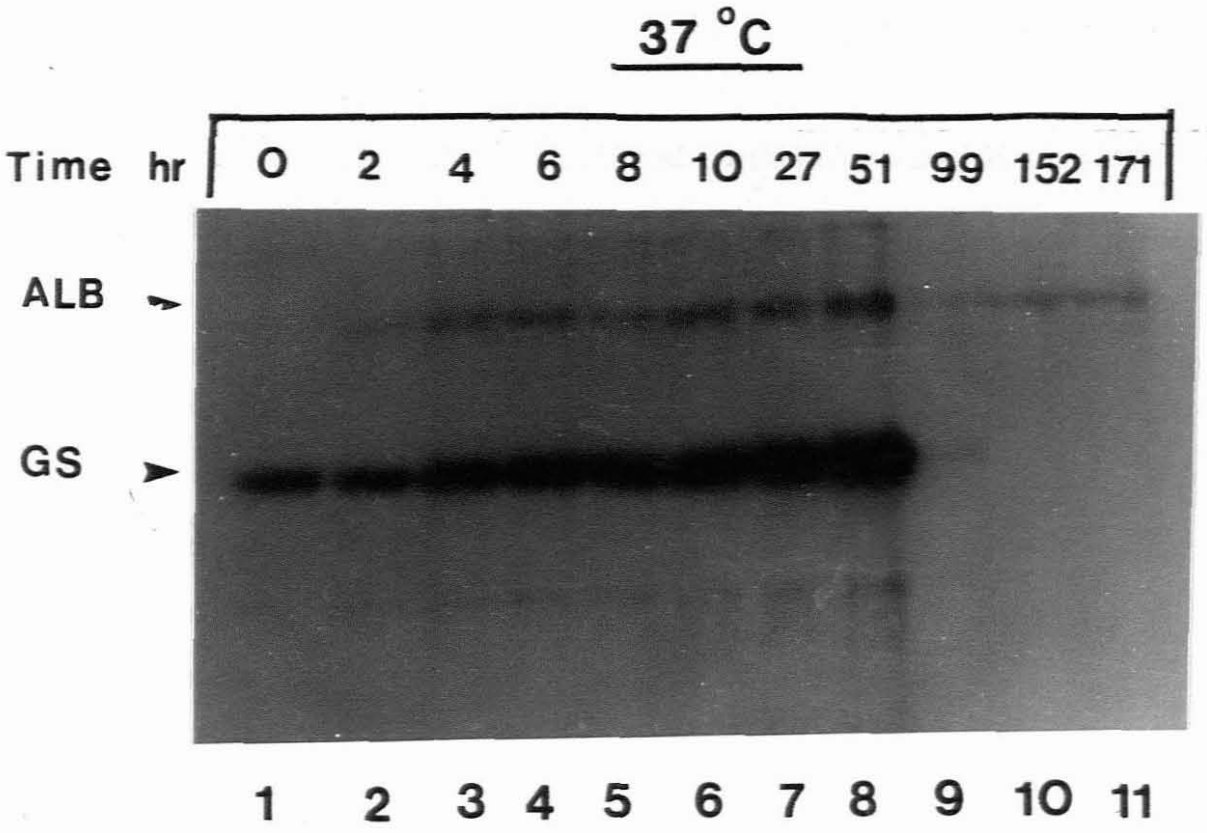


Fig. 23. **Effect of preincubation at 37°C on photolabelling.** Timed aliquots of the 37°C incubations photolabelled under optimal conditions. Lanes 1 - 11 show the times from 0 - 171 hours.

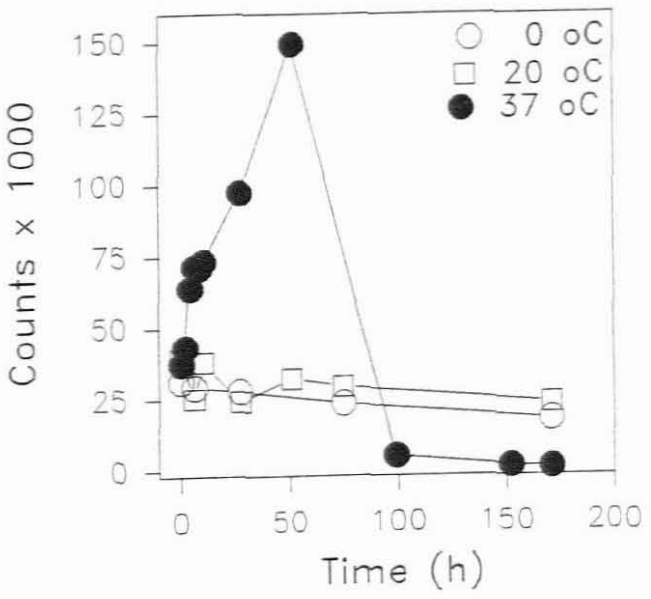


Fig. 24. **Graphical representation of the effect of GS stability in CSF on photolabelling.** Percentage GS remaining after 0°C, 20°C and 37°C incubations against time. Results are from figures 21, 22 and 23.

3.6 PHOTOLABELLING WITH [γ^{32}P]TRINITROPHENYL-8-AZIDO-ADENOSINE 5'-TRIPHOSPHATE

[γ-^{32}P]TNP-8-azido-ATP is a fluorescent derivative of 8-azido ATP with a trinitrophenyl moiety attached onto the two hydroxyl groups of the ribose. Its interaction with the Ca2+-ATPase of sarcoplasmic reticulum and its specific photolabelling of Lys-492 has been well characterised by our laboratory (Seebregts & McIntosh, 1987; McIntosh et al., 1992, McIntosh & Woolley, 1994). It was of interest to examine whether the probe exhibited a high specificity for GS, as it does for the Ca2+-ATPase.

3.6.1. Gunnerson and Haley's method (1992)

Using Gunnerson and Haley's conditions as described in 2.7.2, we substituted 10 μM [γ^{32}P]8-azido-ATP (appendix 7.5.1) with 1 μM [γ^{32}P]TNP-8-azido-ATP (appendix 7.5.2) and photolabelled in the presence and absence of 15 μl CSF. Fig 25 shows the enormous labelling of albumin and the 28 kDalton protein obtained after photolabelling CSF + GS. The albumin and 28 kDa proteins seemed to have bound all the available radiolabel, with the result that there was very little then available for the labelling of GS. This conclusion was substantiated by the fact that, when GS was irradiated in the absence of CSF under the same conditions, labelling of GS was achieved. This also occurred with the [γ^{32}P]8-azido-ATP experiments as described in 3.5.

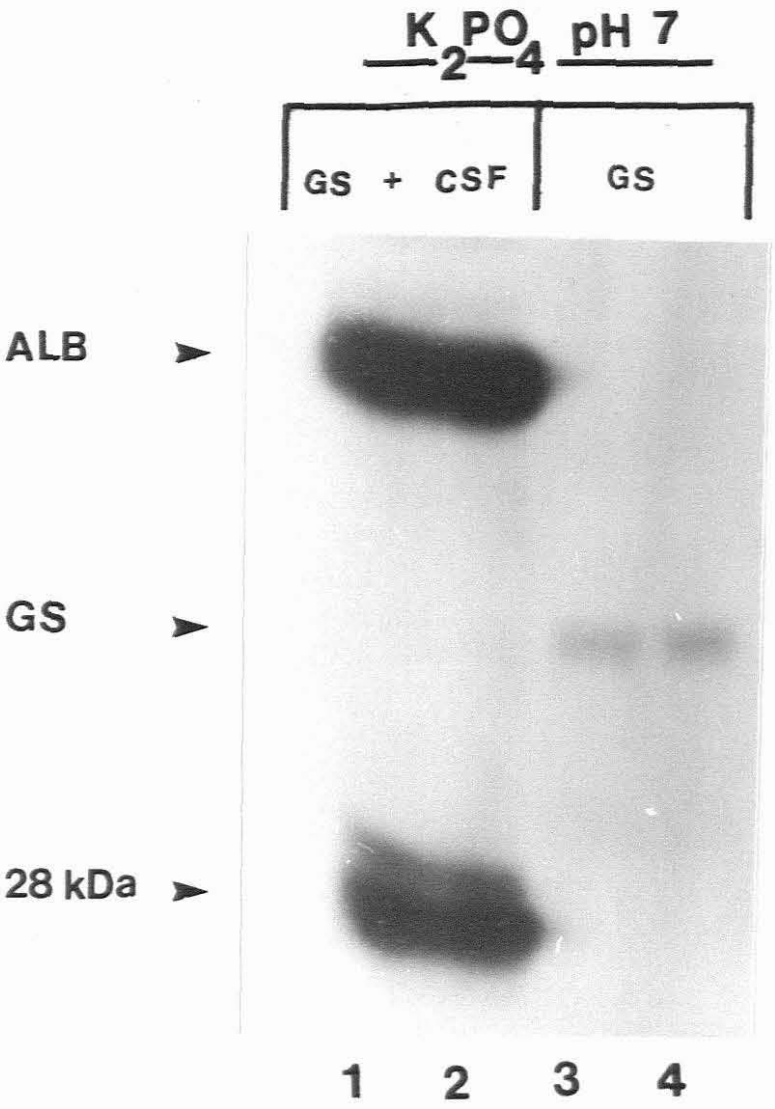


Fig. 25. **Photolabelling with trinitrophenyl-8-azido-adenosine 5'-triphosphate.** 50 μg GS in the presence (lanes 1 and 2) and in the absence of 15 μl CSF (lanes 3 and 4) was irradiated with 1 μM [γ^{32}P]TNP-8N_3-ATP in a 25 mM potassium phosphate buffer, pH 7.0. (appendixes 7.4.1; 7.5.2 & 7.3.2).

3.6.2. Buffer, pH and glycerol effects

With the knowledge that a change in buffers from potassium phosphate to ammonium bicarbonate as well as the addition of 20 % glycerol dramatically increased the labelling of GS in the presence of CSF, when photolabelling with [γ^{32}P]8-azido-ATP, these conditions were also experimented with using 1 uM [γ^{32}P]TNP-8-azido-ATP.

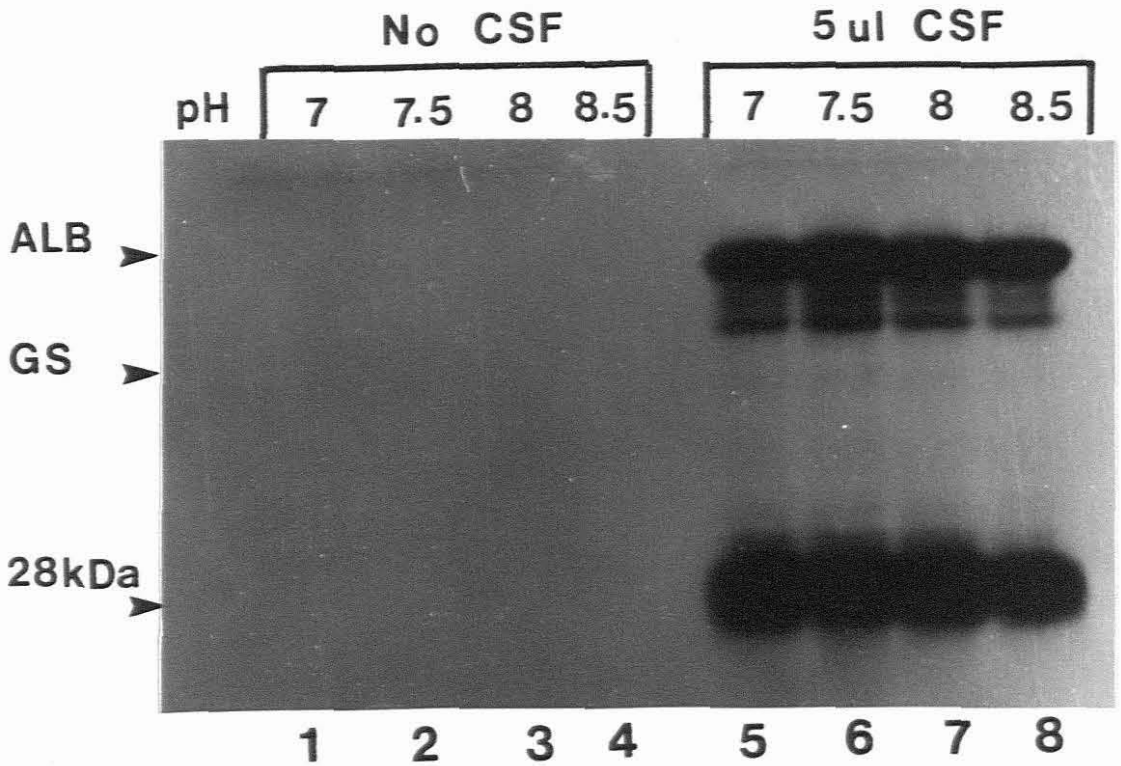


Fig. 26. **pH dependence of photolabelling using [γ^{32}P] trinitrophenyl-8-azido-adenosine 5'-triphosphate.** GS was irradiated in 100 mM ammonium bicarbonate/ 20% (v/v) glycerol, pH's 7.0, 7.5, 8.0 and 8.5, in the presence and absence of 5 ul CSF. The gel was subjected to 12% SDS PAGE and finally autoradiographed. Lanes 2 - 5 show the above conditions in the absence of CSF and lanes 7 - 10 in the presence of CSF. (appendixes 7.2.6; 7.1.7 & 7.6.8).

The results seen in Fig 26, show no comparison to the labelling effect as seen in the [γ^{32}P]8-azido-ATP experiments. The albumin and 28 kDa proteins are not eliminated, and the GS was not labelled well (lanes 7 - 10). The only effect that can be clearly seen is the very slight drop in labelling of both proteins, (albumin and the unkown protein) as the pH becomes slightly more alkaline.

3.6.3. Concentration dependence of [γ_{32}P]trinitrophenyl-8-azido-adenosine 5'-triphosphate and adenosine 5'-triphosphate protection studies

Knowing that an ammonium bicarbonate buffer and the addition of glycerol has no improved effect on the labelling of GS when using [γ^{32}P]TNP-8-azido-ATP, we then returned to the potassium phosphate (pH 7.0) buffer and 10 μg GS in the absence of CSF. Linear labelling can be seen in Fig 27, lanes 1 - 4, when using a TNP-8-azido-ATP range of 0.1 - 5 μM, with 1 μM being sufficient to produce acceptable GS labelling.

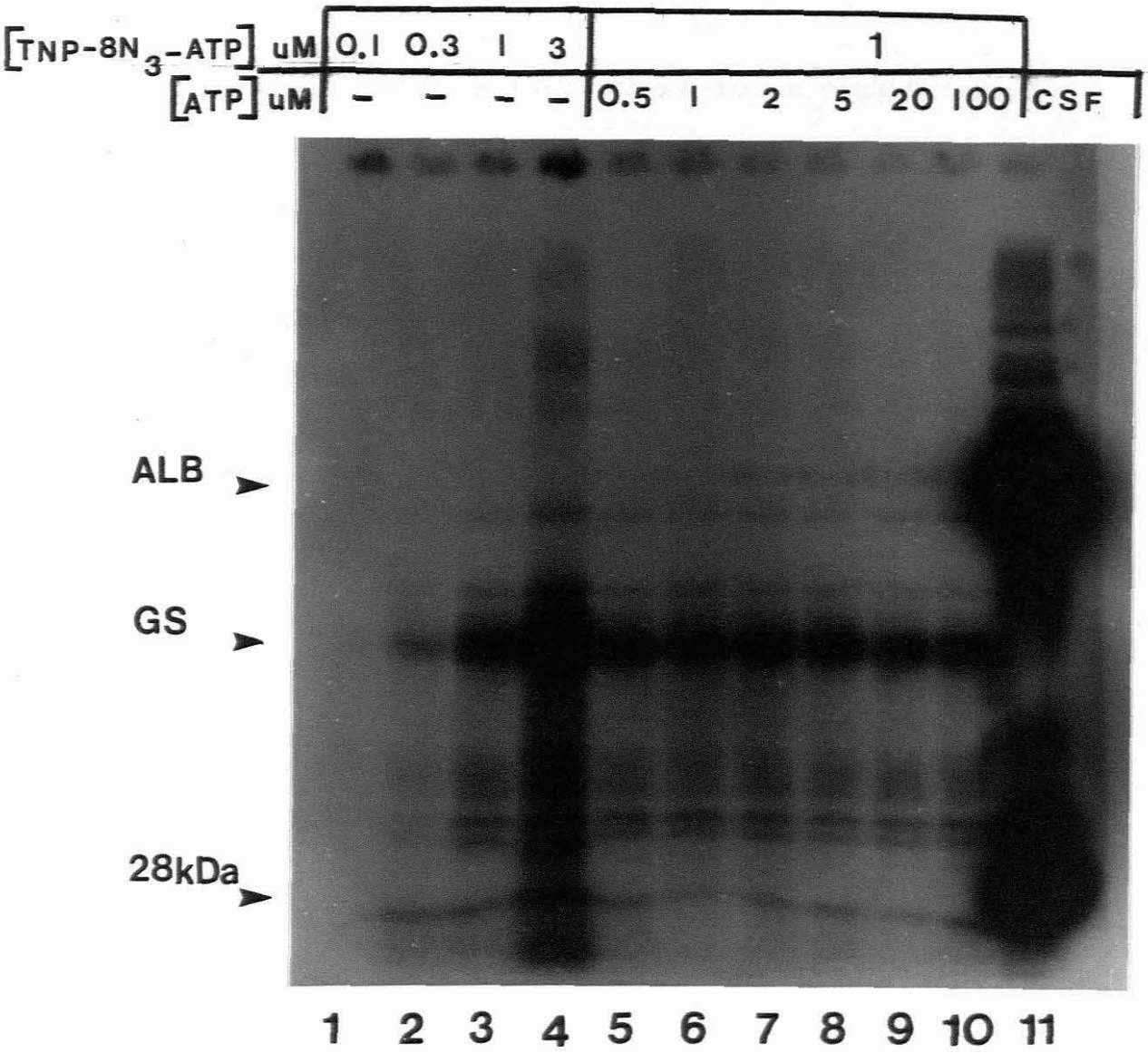
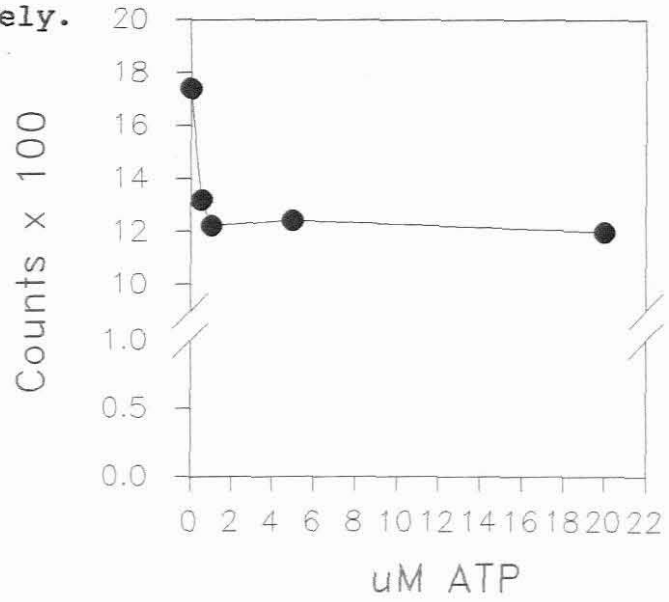


Fig. 27. **Effect of trinitrophenyl-8azido-adenosine 5'-triphosphate concentration on photolabelling and adenosine 5'-triphosphate concentration dependence of the inhibition of photolabelling.** 10 μg GS in the absence of CSF was irradiated with increasing concentrations of μM [γ^{32}P]TNP-8N_3-ATP (0.1 - 3), lanes 1 - 4. Lanes 5 - 10 show the photolabelling of 10 μg GS in the absence of CSF with 1 μM [γ^{32}P]TNP-8N_3-ATP and increasing concentrations of μM ATP (0.5 - 100). A control of 15 μl CSF irradiated with 1 μM [γ^{32}P]TNP-8N_3-ATP is shown in lane 11. (appendixes 7.5.2 & 7.1.5).

.so in Figure 27, lanes 5 - 10, there is no significant ATP
rotection in the range 0.5 - 100 μM. Lane 11 is 15 ul CSF
iotolabelled and shows the massive albumin and unknown
rotein labelling. Linear plots of ATP and TNP-8-azido-ATP
oncentration against counts are shown in Figures 28 and 29
espectively.



ig. 28. **Graphical representation of ATP protection with 1 ug**
S.

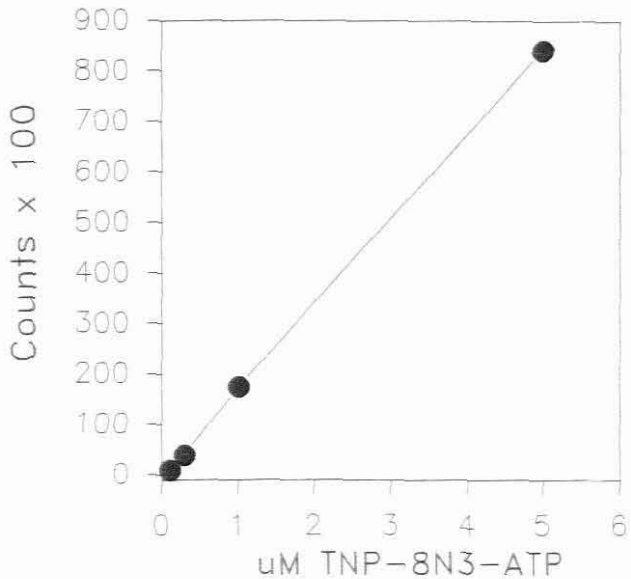


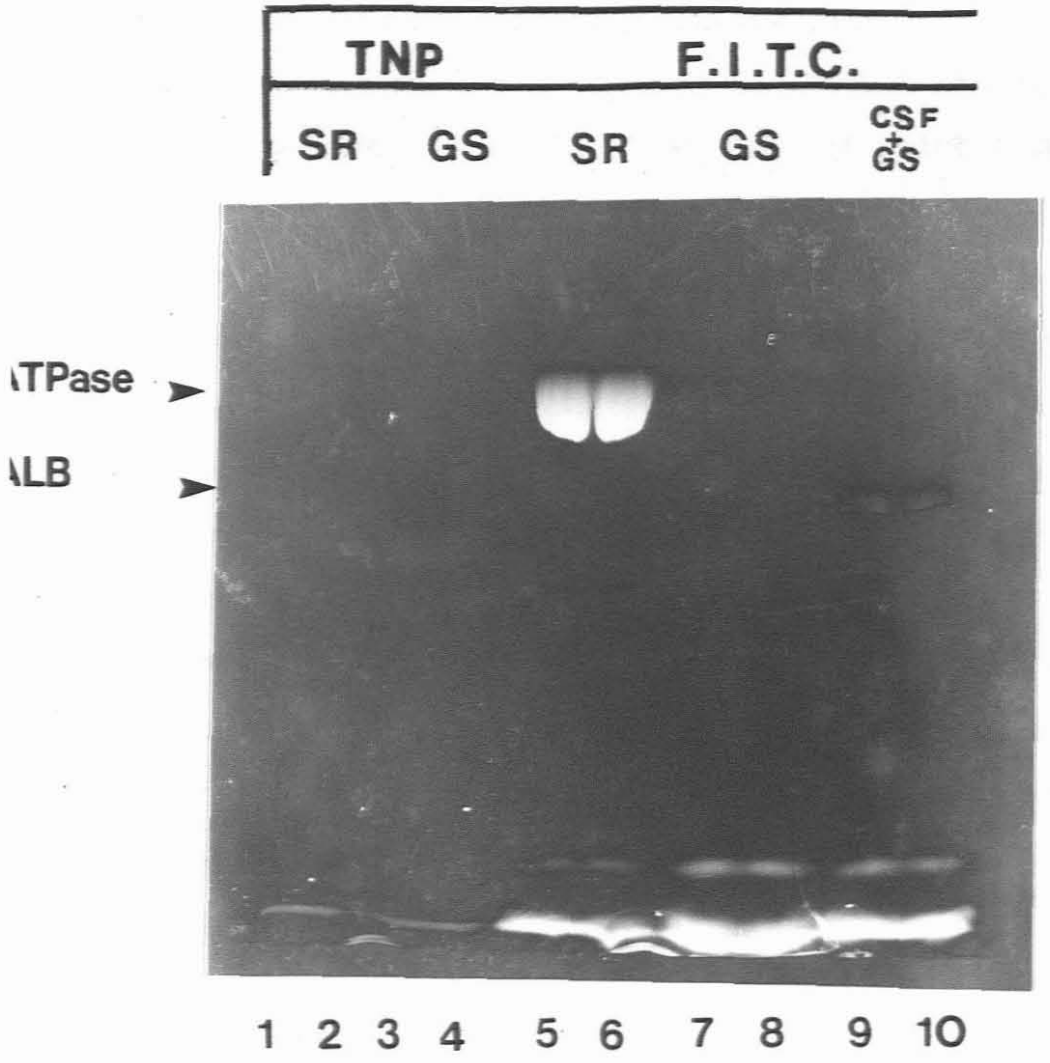
Fig. 29. **TNP-8-azido-ATP concentration dependence effect on**
photolabelling.

7. FLUORESCENT LABELLING OF GLUTAMINE SYNTHETASE

uorescein-5'-isothiocynate (FITC) is a lysine specific uorescent probe that has been previously shown to label the P binding site of Ca-ATPase of SR (Pick et al, 1980). If TC specifically labelled the ATP site of GS, there could be vantages over radiolabelled 8-N_3-ATP. One of the main vantages would be the elimination of the use of dioactivity. Others include increasing the turn around time results, decreasing the expense involved and finally creasing the availability of the investigation to more ople. TNP-8N_3-AMP is also fluorescent and we considered that may be possible to visualise the photo-dependent labelling GS by fluorescence.

ıe TNP-8N_3-AMP reaction was done in a glycerol buffer by .xing 2 μM TNP-8-azido-AMP with 0.2 mg/ml SR (control) ıppendixes 7.5.2 & 7.4.2 respectively) and 2 μM TNP-8-azido-IP with 10 μg GS (test)(appendixes 7.5.2 & 7.4.1 ɜspectively). ß-Mecaptoethanol was added to all mixtures ˙ter 60 seconds incubation at room temperature and samples ɜre then subjected to SDS PAGE using a 9 % separating gel, ɔr 4 hours at 30 mA. The gel was then viewed under a UV ıght and photographed for a permanent record.

ıgure 30 shows that only the Ca^{2+}-ATPase of SR is fluorescent ɔllowing the reaction with FITC. There was no fluorescence ɜsociated with GS with either probe.



ig. 30. FITC and TNP-8N_3-AMP labelling of GS and SR. (All ests were done in duplicate). The FITC reaction was performed n a FITC buffer at pH 7.5, by mixing 2 mg/ml SR with 25 μM (lanes 5 and 6) and 10 μg GS with 25 uM FITC (lanes 7 and), as well as 10 ug GS + 15 ul CSF with 25 μl FITC (lanes 9 nd 10). Lanes 1 and 2 show the effect of labelling of SR and anes 3 and 4, GS with TNP-8N_3- AMP. (appendixes 7.2.4; .4.2 & 7.4.1).

3.8. PHOTOLABELLING OF SUSPECTED ALZHEIMER'S DISEASE PATIENT CEREBROSPINAL FLUID WITH [γ^{32}P]8-azido-ATP

A total of 11 CSF samples were received. 8 from Professor S. Louw of the Department of Geriatrics, GSH, of which 2 were diagnosed as possible AD patients based on clinical criteria, 3 with ? dementia, 2 with memory loss and 1 with early cognitive impairment. The remaining 3 samples were obtained from the Department of Microbiology, GSH; 1 ? meningitis, 1 ? encephalitis and the final one was a pooled xanthochromic (yellow in colour) csf. The labelling of the two possible AD samples by the method of Gunnerson and Haley (1992) is shown in Fig 31 and under our optimised conditions in Fig 32. In neither case could labelling of GS be detected in the patients samples. The xanthochromic CSF specimen was also irradiated to observe what labelling it produced. The latter showed an extra protein, situated just above the commercial GS (internal control).

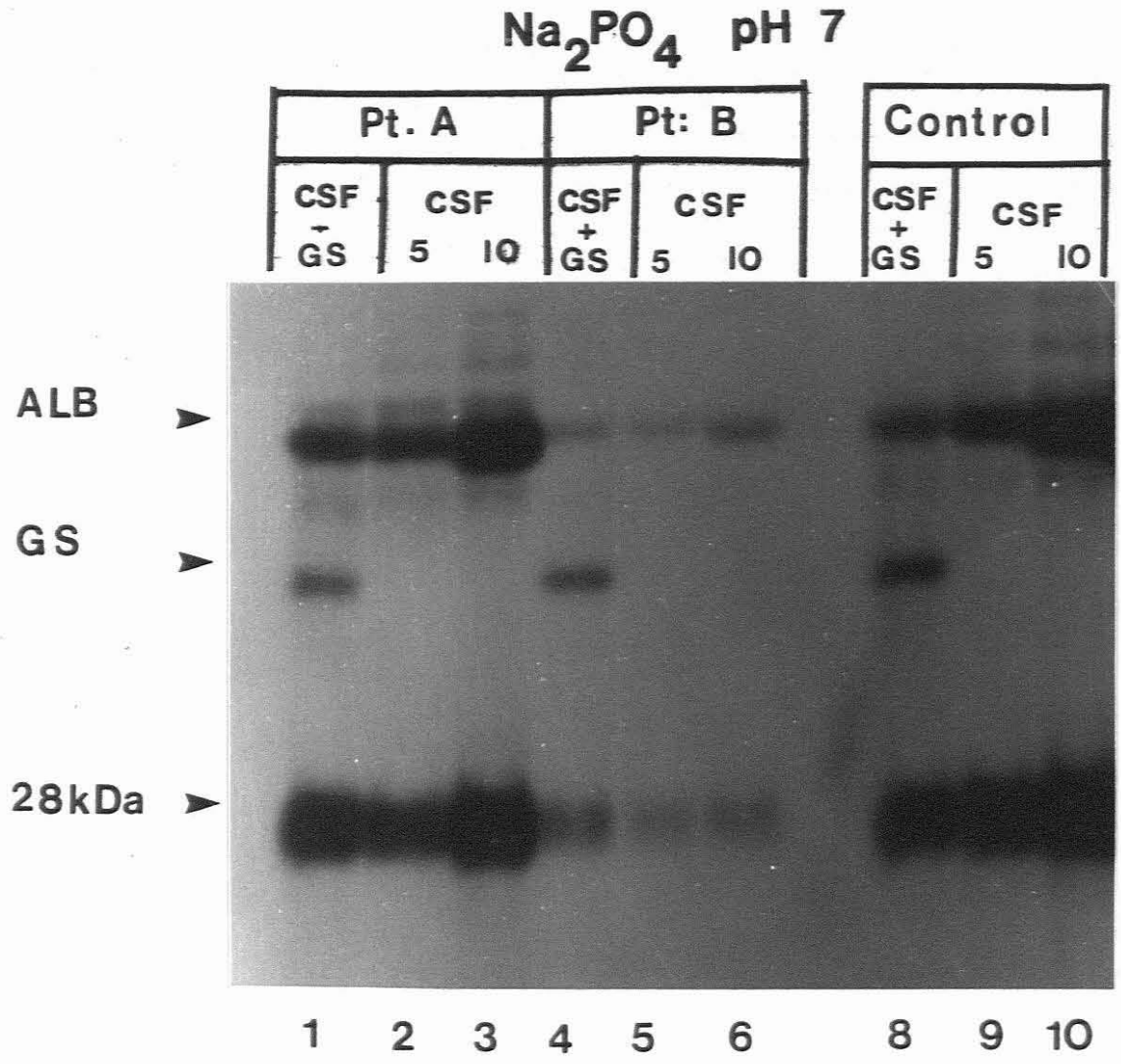


Fig. 31. **Gunnerson and Haley's method using suspected AD patients CSF.** 5 and 10 µl csf samples from patients A and B were photolabelled under Gunnerson and Haley's method, lanes 1 - 6 (the only exception was the use of sodium phosphate and not potassium phosphate), with lanes 1 and 4 containing 10 µg commercial GS as the internal control. The external control is in lanes 8 - 10.

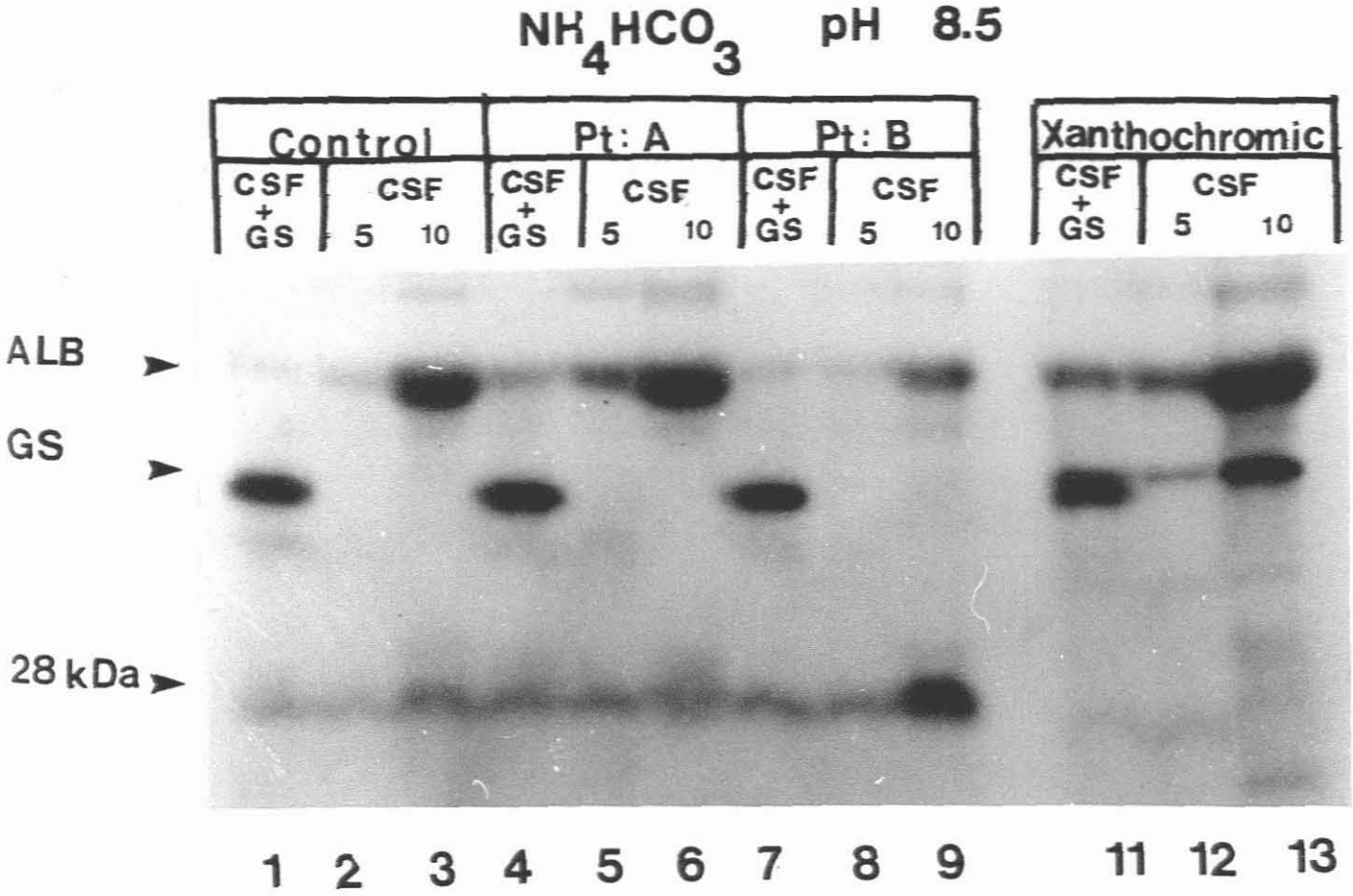


Fig. 32. **Our methodology using suspected Alzheimer disease patients cerebrospinal fluid.** An external control (EC), patients A and B and a xanthochromic CSF were photolabelled under the optimal conditions, using 5 and 25 ul. Lanes 1 - 3 is the EC; 4 - 6 patient A; 7 - 9 patient B and 11 - 13 xanthochromic csf. All samples had internal controls (IC); lanes 1, 4, 7 and 11 respectively.

3.9. EFFECT OF BLOOD CONTAMINATION IN CEREBROSPINAL FLUID ON PHOTOLABELLING

To determine whether xanthochromia in a CSF sample would interfere in the methodology by producing false positives, we tried to produce the effect by lysing blood in CSF and photolabelling with 10 μM [γ^{32}P]-8N_3-ATP (appendix 7.5.1). The original method of Gunnerson and Haley (1992) was used. A control (no blood contamination) and 5 tests (varying degrees of blood lysis) were irradiated for 60 seconds in a potassium phosphate buffer, pH 7.0, (appendix 7.3.2) with 1 μg GS.

Fig 33, lane 1 (control) shows the expected labelling of albumin, GS and the unknown 28 kDa protein. Lanes 2 - 6, increasing presence of blood, show these proteins with different labelling intensities, due to much more albumin being present from the blood contamination, resulting in a decline in GS labelling.

GS can be seen clearly in lanes 2 and 3 (trace and 1+ blood presence respectively), and then from lane 4 it becomes difficult to detect the band. Lane 5 starts to show a drop in labelling of albumin and finally in lane 6 no GS can be seen and the labelling of the other significant proteins have almost disappeared. At no point was any other protein in the vicinity of sheep GS seen.

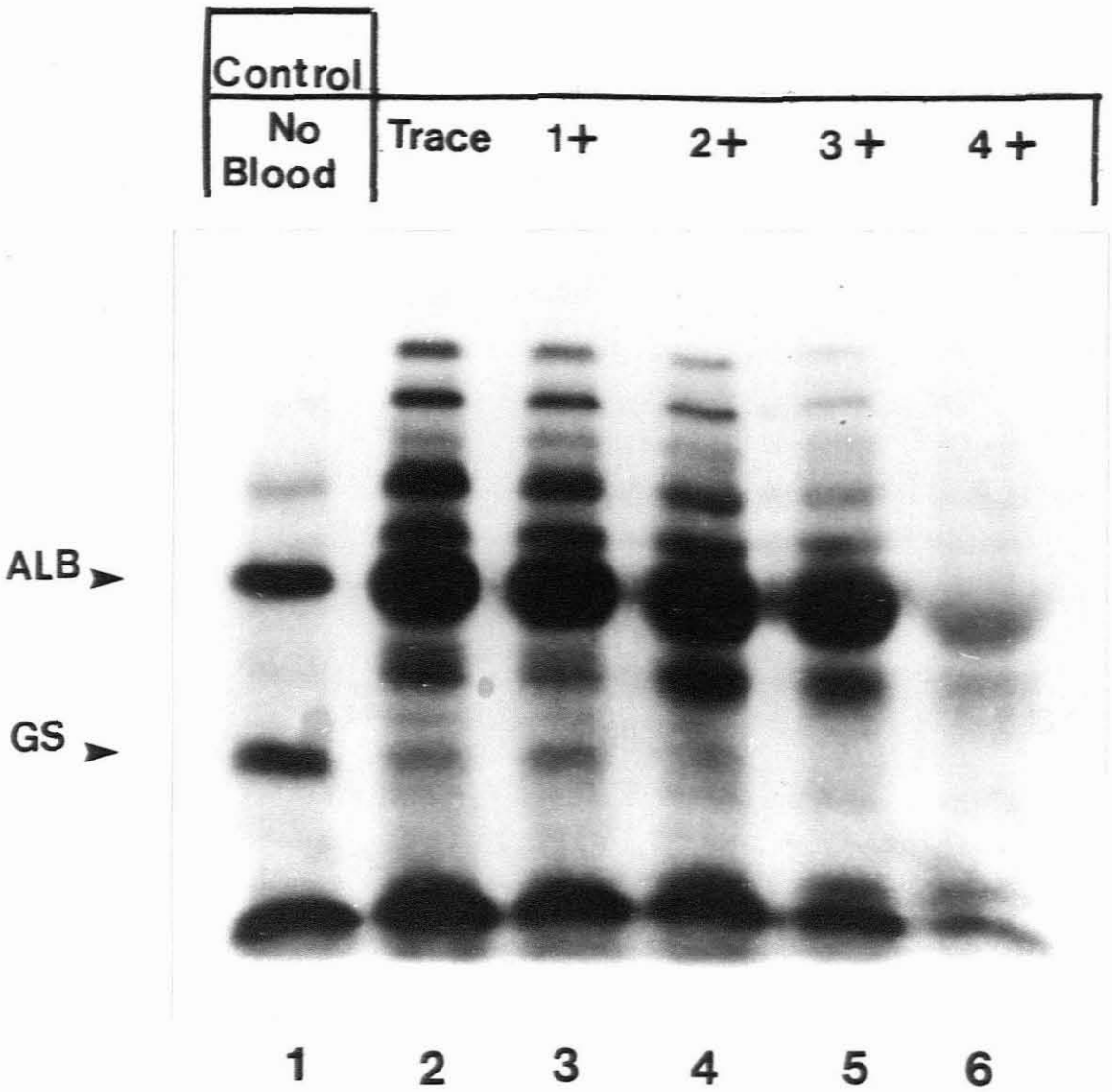


Fig. 33. **Photolabelling of blood contaminated CSF.** A control (lane 1) with no blood and 5 tests (lanes 2 - 6) with increasing concentrations of blood, trace to 4+ respectively, were irradiated by the Gunnerson and Haley method (5 µl CSF, 1 µg sheep GS, 10 µM [γ^{32}P]8N_3-ATP and 25 mM potassium phosphate, pH 7.0). (appendixes 7.3.2; 7.4.1 & 7.5.1)

CHAPTER 4

DISCUSSION

In this study we have reproduced and improved on the existing assay of GS in CSF published by Gunnerson and Haley (1992). We estimate that the specificity of photoaffinity labelling, and hence the sensitivity is increased at least an order of magnitude. Non-specific labelling of albumin and an unknown 28 kDa protein, the most prominently labelled proteins using Gunnerson and Haley's methodology, was virtually eliminated. The critical changes introduced included the use of fresh 20 % (v/v) glycerol, switching from a potassium phosphate buffer to ammonium bicarbonate, inclusion of Mg^{2+}, and a preincubation at 37 °C.

4.1 8-AZIDO-ADENOSINE 5'-TRIPHOSPHATE

Gunnerson and Haley used both [γ^{32}P]8N_3-ATP and [γ^{32}]2N_3-ATP to label GS in their experiments. They showed that both were equally effective at labelling GS. Our choice to use [γ^{32}P]8N_3-ATP was primarily based on availability, as our laboratory is already actively using [γ^{32}P]8-azido-ATP in other research programmes and there is a stock of nonradioactive nucleotide. Due to a special interest of our laboratory in the mechanism of Ca^{2+}-ATPase of SR, SR vesicles are also available. The labelling of the Ca^{2+}-ATPase with 8-azido-ATP served as a convenient model system for optimising photolysis conditions (distance from lamp, irradiation time, filters). All the latter parameters would be directly applicable to the labelling of GS in CSF.

4.2 CEREBROSPINAL FLUID AND GLUTAMINE SYNTHETASE

Csf contains similar proteins as blood plasma, but in different concentrations. Protein concentrations in CSF are generally lower than those in blood. The proteins in CSF are derived from blood components or the brain. Proteins identified in CSF include albumin, transferrin, apolipoprotein A-I, beta 2-microglobulin and prealbumin. The only protein we positively identified was albumin. Sheep GS, included in human CSF at a level of 250 μg/ml could be seen, following SDS-PAGE and Coomassie Blue staining, as a doublet running just below the albumin band. We do not know the reason for the doublet in this preparation. Labelling with 8-azido-ATP never revealed a doublet.

4.3 GLYCEROL EFFECT

The greatest increase in specificity of the labelling reaction (ie: virtually eliminating the labelling of albumin and other proteins) came about as a result of the addition of 20% (v/v) glycerol. The effect is strange as fresh glycerol was much more effective. Preincubation of glycerol in the irradiation medium for longer than 5 minutes diminished the effect. It is unclear how glycerol exerts this effect. An unstable component in the solvent may be acting as a radical scavenger. It is difficult to guess what this scavenger could be. Several studies have suggested that the specificity of azido labelling can be improved by sulphydryl reducing agents, eg. ß-mecaptoethanol (Maasen, & Möller, 1974.). It is possible that there is a small amount of an unknown reducing agent in the

glycerol that is sensitive to oxidation by dissolved air in the buffers, and hence changed with time.

4.4 AMMONIUM BICARBONATE

The use of ammonium bicarbonate as buffer increased GS specificity several fold, compared with potassium phosphate, by apparently both increasing 8-azido-ATP labelling of GS as well as decreasing the labelling of albumin and other proteins. The increased GS labelling may be rationalised in either of three ways, (i) increase in affinity for 8-azido-ATP, (ii) increase in efficiency of labelling, (iii) a decrease in non-specific labelling and hence allowing more 8-azido-ATP to be available for labelling GS.

(i) Affinity

The Kd for ATP binding for the formation of glutamine is 0.3 mM (Allison, 1977). Labelling of GS with 8-azido-ATP was linear up to 50 μM, compatible with a low affinity site. Since the concentration of 8-azido-ATP is 10 μM during irradiation, only a small proportion of active sites can be occupied by the nucleotide at any one time. This means that probably only 10% or so of active sites are derivatized during the course of irradiation. If NH^+_4 increased the affintiy of the ATP site for 8-azido-ATP, enhanced labelling could result.

(ii) Efficiency

The efficiency of the nitrene reaction with its target amino acid depends on the intrinsic reactivity of each species (eg:

strong or weak nucleophile), their environment (eg: presence of water), and distance apart. A favourable change in any of these parameters through conformational change in the protein and active site could enhance labelling.

Both of the above, (i) and (ii), require conformational change induced by NH_4HCO_3. NH^+_4 is a substrate of the GS for the synthesis of glutamine. However, it is not clear from mechanistic studies whether a binding site for NH^+_4 exists in the absence of glutamate. Several studies, including that concerned with elucidation of the crystal structure (Liaw & Eisenberg, 1994) suggest that substrate binding is ordered and glutamate-P and ADP are necessary for the NH^+_4 binding site to develop. The created binding pocket appears to involve conserved residues Asp-50 and Glu-327 (Liaw & Eisenberg, 1994; Wray & Fisher, 1988). On the other hand studies pointing to random binding of substrates (Rhee, et al, 1976 and Timmons, et al, 1974).

Bild and Boyer (1980), proposed that catalytic cooperativity occurs between subunits, and is promoted by the binding of NH_4^+ to the catalytic site of one subunit. This was determined by measuring the exchange of oxygen between [^{18}O]glutamate and phosphate per molecule of glutamine formed, at various NH_4^+ concentrations. It is unclear whether the NH^+_4 cooperativity effect can be induced in the absence of the other substrates.

(iii) Non-specific labelling

The apparent decrease in non-specific labelling of albumin and the 28 kDa protein may be due to:

(a) bicarbonate ions competing with 8-azido-ATP for non-specific sites on albumin and the 28 kDa protein. This however does not explain why phosphate in not as effective as bicarbonate.

(b) NH_4HCO_3 and not potassium phosphate, acting as a radical scavenger. It seems possible that the nitrene radical could react with either NH^+_4 or HCO^-_3.

4.5 DIVALENT CATION EFFECT

Two divalent cations, both Mn^{2+} in the physiological state, are needed per active site for catalysis. One site, termed n1, exhibits a higher affinity for divalent cations compared with the other, n2, site. The higher affinity is attributed to 3 negatively charged Glu residues, while the lower one to 2 Glu residues. (Abbel et al, 1995). Functionally, the n1 site is closely associated with the catalytic binding of glutamate, and the n2 site with ATP binding. (Hunt & Ginsberg, 1980). When the tight, n1 site, is occupied by Mg^{2+} or Mn^{2+}, the enzyme undergoes a conformational change allowing the enzyme to convert from a catalytically inactive form to a catalytically active form (Hunt, & Ginsberg, 1972).

In phosphoryl transfer reactions, metal ions have both a catalytic and orientating role (Herschlag, & Jencks, 1989).

Besides these roles, the divalent cations also stabilize the protein against subunit dissociation. The dissociation in EDTA is enhanced in the presence of urea or at alkaline pH (7.9) (Woolfolk, & Stadtman, 1967).

We have shown that divalent cations are an absolute requirement for photolabelling and that the prior removal of divalent cations enhanced magnesium dependent labelling. It is worth pointing out that the irradiation medium of Gunnerson and Haley contained no added divalent cation.

4.5.1. Magnesium requirement

Reasons for the Mg^{2+} dependency may be:

(a) that it is required for ATP to bind, by acting as a substrate (MgATP).

(b) that it is an essential requirement for the correct orientation of the nucleotide so that the azido group is positioned alongside its target amino acid.

(c) for the correct conformation of the protein, thus allowing the azido group of the nucleotide to interact with the target amino acid.

4.5.2. Enhanced labelling

Removal of divalent cations with EDTA eliminated labelling and when Mg^{2+} was re-introduced, labelling was not only regained, it was increased. This enhancement may be due to other divalent cations, like Ca^{2+} or Mn^{2+}, being bound to the commercially isolated/purified enzyme. The bound cation(s)

could prevent the principle nucleotide species ($Mg8N_3$-ATP) from binding and hence labelling. An alternative explanation may be that Mg^{2+}, and not Ca^{2+}/Mn^{2+}, aligns the nucleotide or target amino acid closer to the azido group. The concentration of Mg^{2+} required to regain maximal labelling in the presence of 2 mM EDTA, namely approximately 5 mM, suggests a low affinity site.

EDTA is commonly present in vacutainer tubes used for the collection of blood and CSF. Our results show that it is important that Mg^{2+} is added in excess over EDTA for labelling. More studies are needed to determine the stability of GS in EDTA.

4.5.3 Labelled amino acid

The crystal structure of GS from *Salmonella typhimurium* suggests that conserved nucleophiles Arg-344, Arg-355, His-271 and Ser-273 are in close proximity to the n2 site and probably ligate ATP (Liaw & Eisenberg, 1994). Anyone of these residues in sheep GS could react with the nitrene. The nonpolar amino acids are relatively unreactive to nitrenes (Knowles, 1972).

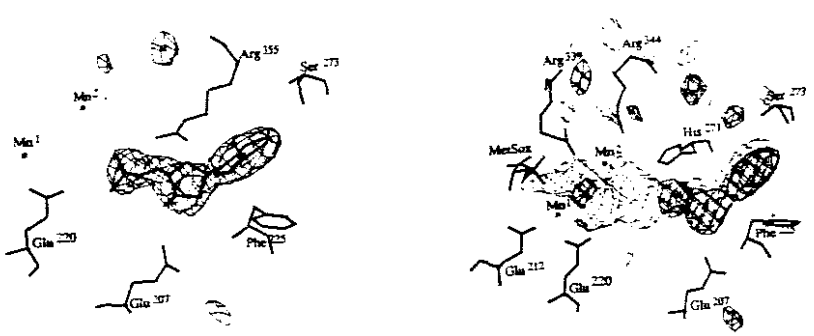


Fig. 34. **Diagrams representing the orientation of the amino acid residues of glutamine synthetase.** (Obtained from Liaw, Jun and Eisenberg, 1994).

4.6 ADENOSINE 5'-TRIPHOSPHATE

The observed $K_{0.5}$ for the inhibition by ATP calculated from our experimental work was 0.25 mM. The published K_d (Allison, 1977) for the binding of ATP to GS in the absence of competing ligands was shown to be 0.3 mM. This suggests that under the conditions of our assay, ATP competitively inhibits binding of 8-N_3-ATP and in addition, at the concentration of the probe used (10 μM) there was no effect on ATP binding. This suggests that the K_d for the equilibrium binding of the probe is much greater than 10 μM. This conclusion is in keeping with our data, that with an increase in concentration of 8-N_3-ATP up to 50 μM, no saturation was observed. 8-azido-ATP and ATP appear to bind with similar affinity.

4.7 SODIUM AZIDE

In the collection of many biological specimens from patients there are certain recommendations that need to be adhered to for the preservation of these samples to enable accurate results to be obtained. One example is the determination of blood glucose levels. The addition of sodium fluoride is the commonly used preservative and inhibitor of kinases, eliminating the chance for the red blood cells present to utilise the available glucose in the sample. Sodium azide is an inhibitor commonly used for preventing bacterial growth. With this in mind we looked at the effect that sodium azide may have on the activity of GS.

We conclude that upto 2 % sodium azide had no adverse effect on the activity of GS. This inhibitor of bacterial growth may be the most suitable for preservation of GS in CSF.

4.8 8-AZIDO-ADENOSINE 5'-TRIPHOSPHATE CONCENTRATION DEPENDENCE

We found linear labelling of GS with up to 50 μM [γ^{32}P]8N_3-ATP, in line with a low affinity ATP site. As there was no saturation, we can not determine the K0.5 value for labelling. However, knowing the concentration of GS present, as well as the specific activity of 8-azido-ATP used, it is possible to calculate the moles of GS derivatized.

4.9 GLUTAMINE SYNTHETASE CONCENTRATION DEPENDENCE

The linear dependence referred to above indicates that the amount of counts incorporated into the GS band is directly proportional to the amount of GS in the CSF. This simplifies quantitation.

4.10 THERMAL STUDIES

Since samples collected from patients may not be able to be frozen immediately, we considered it necessary to determine the effect that temperature could have on the stability of GS, both with sodium azide and without. There was little change in the labelling of GS with time over 7 days in the presence or absence of sodium azide, at 0°C and 20°C.

In contrast, at 37° there is a linear increase in labelling up to 50 hours and then a sudden decrease. The explanation of

this latter effect could be due to possible bacterial contamination. It was also seen in the presence of sodium azide, but occurred after 4 days and not 2. It is possible that sodium azide was unstable and broken down after 4 days at 37 °C.

The increase in labelling seen over 2 days is puzzling. One explanation may be that the GS is gradually reaching a new conformation causing an increase in the affinity for 8-azido-ATP of the intact octamer. A second reason could be that the enzyme slowly dissociates at 37°C over time, first into 2 tetramers, then 4 dimers and finally into 8 monomers, and with each dissociation step more ATP binding sites are made available.
Since the 8 active sites of the octamer are located between subunits it is possible that dissociation causes the exposure of 16 half sites which may each be labelled. Alternatively, binding may be highly negatively cooperative in the octamer, binding of one nucleotide lowers the affinity of the other 7 sites, and this is eliminated in the dissociated subunits. Catalytic cooperativity between subunits has been reported (Bild & Boyer, 1980).

4.11 SENSITIVITY

The enhancement in specificity and labelling of GS through all the changes discussed above greatly increased the sensitivity of the assay. In a direct analysis of the amount of GS that could be detected, we determined that 1 μg GS/ml was fairly

easily visualised on the autoradiograph (usually 100 μg GS/ml was used). We estimate that the sensitivity of the assay has been increased up to 2 orders of magnitude.

4.12 [γ^{32}P]TRINITROPHNEYL-8-AZIDO-ADENOSINE 5'-TRIPHOSPHATE LABELLING

TNP-nucleotides generally bind to ATP binding sites with high affinity than ATP itself. TNP-8azido-ATP has been successfully used as a photoaffinity label of the Ca2+-ATPase of sarcoplasmic reticulum (Seebregts & McIntosh, 1987; McIntosh et al., 1992, McIntosh & Woolley, 1994). We found that TNP-8azido-ATP labelled GS, but in the presence of CSF the labelling was poor and albumin and a 28 kDa protein were strongly labelled. We reason that the strong labelling of albumin and the other protein could be due to these latter proteins binding all the available radiolabelled nucleotide, thus leaving minimal amounts for the labelling of GS. However we were unable to increase the specificity of GS labelling with this probe, using the identical conditions that we has developed with [γ^{32}P]8N_3-ATP. Glycerol and ammonium bicarbonate had no effect at all in eliminating labelling the non-specific proteins. In fact, using the new methodology eliminated labelling of GS in the absence of CSF, suggesting that the labelling of GS with Gunnerson and Haley's methodology was non-specific. This was confirmed by the lack of any ATP protection. The TNP moiety apparently prevents nucleotide binding to GS. It appears that TNP-8-azido-ATP has a very high affinity for albumin and the 28 kDa protein, because glycerol

had no effect on labelling of these proteins. Intriguingly, the results suggest that those two proteins have a TNP-8N_3-ATP binding site.

4.13 FLUORESCENT LABELLING

FITC is a general reagent used for derivitizing lysine residues in ATP binding sites. The conjugated system substitutes for the adenine moiety. We found that FITC does not label GS efficiently.

4.14 PATIENT SAMPLES

Unfortunately, due to ethical reasons, we were unable to obtain enough CSF samples from suspected AD patients to confirm the correlation between the presence of GS with AD. We used both Gunnerson and Haley's and our own improved method to run patients samples and in neither case did we find any indication of the presence of a band corresponding to the commercial sheep GS.

Xanthochromic CSF arises from blood metabolites and protein degradation products appearing in the CSF. This type of specimen is of interest since GS from blood could also contaminate CSF and contribute to false positives. Rather unexpectedly, we did see labelling of a protein at approximately 43 kDa, lying immediately above the sheep GS band. Could it be that this band is the human GS and that its amino acid sequence differs very slightly from that of sheep, thus producing a protein band on our SDS-PAGE of a slightly

higher molecular weight? We investigated the possibility that the band arose from contamination by red blood cells, by trying to label an artificially lysed CSF sample. Since no band corresponding to the one seen in the xanthochromic CSF, we could only conclude that the presence of "fresh" blood in CSF does not introduce false positives into the method. However in the case of post-mortem samples where blood plasma and cellular contents could mix with CSF over time, this issue could become important. It is therefore of vital importance that when investigating the presence of GS in CSF that the conditions of sample taking are known. (ie: if the sample happens to be a ventricular one from a post mortem, the time between death and taking the post mortem sample, must be known). This will determine how long there has been for CSF contamination from blood to take place.

CHAPTER 5

FUTURE STUDIES

5.1 Quantitation by filtration

The huge increase in specificity of GS photolabelling obtained through optimisation of conditions, allows us to consider it possible to measure GS in CSF without separation from other proteins. Therefore the development of a method that would determine the amount of photolabelling as a function of GS concentration in CSF, followed by precipitation of proteinaceous material with perchloric acid and filtration would therefore be advantageous. This would eliminate the very time consuming SDS-PAGE step.

5.2 Sample study

To get a better working sample population study running using Gunnerson and Haley's and our modified methodologies. This study should include CSF samples from apparent normal individuals (control), AD patients under both post- and ante-mortem conditions and other dementing illnesses.

5.3 Contamination

Comparing ante- and post-mortem CSF samples would be one way to try and determine whether there is any possibility of contamination of CSF occurring after death, either from blood (sample taking) or from the leakage of other enzymes.

CHAPTER 6

CONCLUSION

We have reproduced an assay for GS described by Gunnerson and Haley (1992) and greatly improved on it. The assay is robust enough to be implemented into a routine diagnostic laboratory. With the small sample population tested, we are however unable to confirm or deny that GS in CSF is useful as an ante-mortem marker for the diagnosis of AD or for distinguishing between forms of dementia.

CHAPTER 7

APPENDIX

7.1 Stock solutions

7.1.1. 2 M TRIS

121.14 g in 400 ml water, pH to 8.0 with TMAH and make the final volume up to 500 ml. Store at 4°C.

1 M Tris: 50 ml 2 M + 50 ml water.

200 mM Tris/$MgCl_2$: 5 ml 2 M Tris + 6 ml 1 M $MgCl_2$ + 45 ml water, pH to 8.0 and then make up to 50 ml with water.

7.1.2. 1 M $MgCl_2$

20.33 g in 100 ml water. Store at 4°C.

20 mM $MgCl_2$: 2 ml 1 M + 98 ml water.

7.1.3. 0.1 M NaOH

0.4 g in 100 ml water. Store at room temperature (RT).

7.1.4. 0.5 M Cysteine (free base)

0.61 g in 10 ml water. Aliquot and store at -4°C.

7.1.5. 0.1 M (10 mM) ATP (disodium salt)

0.605 g (0.061 g) Na-ATP in 5 ml water. pH to 7.0 with TMAH and then make up to 10 ml. Aliquot stocks and store -4°C.

The following amounts are all in 100 µl:

1.0 mM ATP: 10 µl 10 mM

0.5 mM ATP: 5 µl 10 mM

0.1 mM ATP: 10 µl 1 mM

0.02 mM ATP: 20 µl 0.1 mM

0.01 mM ATP: 10 µl 0.1 mM

7.1.6. 1 M Lithium

21.2 g Lithium Chloride in 500 ml water. Store at 4°C.

7.1.7. 1 M HCl

50 ml of a 10 M into 450 ml water. Store at RT.

10 mM HCl: 1 ml 1 M + 99 ml water.

30 mM HCl: 3 ml 1 M + 97 ml water.

7.1.8. 3.5 % PCA

7 ml 100 % PCA up to 200 ml water. Store at 4°C.

7.1.9. 10 M Urea

3 g Urea dissolved in 5 ml water.

7.1.10. 162 mM DTT

0.0250 g in 1 ml buffer. Prepare fresh.

7.1.11. 100 mM EDTA

3.72g in 80 ml water, pH to 7.0 with TRIS and make the final volume up to 100 ml. Store at 4°C.

2 mM EDTA: 1 ml 100 mM + 49 ml water.

7.1.12. 100 mM EGTA

3.804 g in 80 ml water, pH to 7.0 with TMAH and make the final volume up to 100 ml. Store at 4°C.

7.1.13. 20 % Sodium Azide

0.2 g in 1 ml water. Store at 4°C.

The following amounts are in 100 µl:

2 % NaN_3: 10 µl 20 %.

0.2 % NaN_3: 10 µl 2 %.

0.02 % NaN_3: 10 µl 0.2 %.

7.1.14. 0.1 M PGA

97 mg in 2 ml water. Store at -4°C.

7.1.15. 50 mg/ml G_3PDH

Obtained from Sigma. Store at 4°C.

7.1.16. 6 mg/ml PGK

Obtained from Sigma. Store at 4°C.

7.1.17. 20 % Glycerol

20 µl in 100 µl buffer. Store at RT.

7.1.18. 1 mM FITC

Dissolve 0.0078 g FITC in 20 ml DMF. Store at 4°C.

7.2 Stock buffer solutions

7.2.1. 50 mM Potassium Phosphate (double strength)

0.871 g K_2HPO_4 (base) in 100 ml water and 0.680 g KH_2PO_4 (acid) in 100 ml water. Once dissolved, take 50 ml base and pH to 7.0 with acid. Store at 4°C.

7.2.2. 50 mM Sodium Phosphate (double strength)

0.71 g Na_2HPO_4 (base) in 100 ml water and 0.60 g NaH_2PO_4 (acid) in 100 ml water. Once dissolved, take 50 ml base and pH to 7.0 with acid. Store at 4°C.

7.2.3. 300 mM Ammonium Bicarbonate/6 mM EDTA/60 mM $MgCl_2$ (triple strength)

1.19 g NH_4HCO_3 + 3 ml 100 mM EDTA + 3 ml 1 M $MgCl_3$ in 40 ml water, pH to 8.5 with TMAH and make to a final volume of 50 ml. Store at 4°C.

7.2.4. FITC Buffer: 30 mM Mops/1 mM EGTA/2 mM $MgCl_2$/25 µM FITC

1.26 g Mops + 1 ml 100 mM EGTA + 200 µl 1 M $MgCl_2$ + 2.5 ml 1 mM FITC in 80 ml water, pH to 7.5 with TMAH. Make final volume up to 100 ml. Store at 4°C.

7.2.5. 50 mM Hepps/0.5 mM EDTA

0.63 g Hepps + 0.25 ml 100 mM EDTA + 40 ml water. pH to 8.5 with TMAH and make final volume up to 50 ml with water.

7.2.6. 100 mM NH_4HCO_3

0.40 g NH_4HCO_3 + 40 ml water. pH to appropriate pH required with either HCl or TMAH and make final volume up to 50 ml.

7.3 Working buffer solutions

7.3.1. [γ^{32}P]8N-ATP synthesis enzyme incubation medium

250 µl	200 mM TRIS/12mM $MgCl_2$
51 µl	0.1 M NaOH
2 µl	0.5 M Cysteine
17 µl	226 nmols $8N_3$-ATP
5 µl	0.1 M PGA
5 µl	50 mg/ml G_3PDH (1:5 dilution)
4.3 µl	6 mg/ml PGK (1:5 dilution)

7.3.2. 25 mM Potassium Phosphate

50 µl 50 mM potassium phosphate, pH 7.0 (appendix 7.2.1) in a total irradiation volume of 100 µl. Keep on ice.

7.3.3. 25 mM Sodium Phosphate

50 µl 50 mM sodium phosphate, pH 7.0 (appendix 7.2.2) in a total irradiation volume of 100 µl. Keep on ice.

7.3.4. 100 mM Ammonium Bicarbonate/2 mM EDTA/20 mM $MgCl_2$

33 µl 300 mM ammonium/6 mM EDTA/60 mM $MgCl_2$, pH 8.5 (appendix 7.2.3) stock buffer in a total irradiation volume of 100 µl. Keep on ice.

7.3.5. FITC Buffer

1000 µl of stock buffer, pH 7.5 (appendix 7.2.4) per incubation. Keep on ice.

7.3.6. 25 mM Ammonium Bicarbonate

0.099 g NH_4HCO_3 + 40 ml water. pH to 7.0 with HCl. Store at 4°C.

7.4 Protein solutions

7.4.1 100 µg/ml GS

Add 10 µl stock GS (stored at -80°C) to 100 µl irradiation medium.

10 µg/ml GS: 10 µl 100 µg/ml

1 µg/ml GS: 10 µl 10 µg/ml

0.1 µg/ml GS: 10 µl 1 µg/ml

0.01 µg/ml GS: 10 µl 0.1 µg/ml

7.4.2 0.2 mg/ml (2 mg/ml) SR

Mix 5.6 µl (56 µl) of a 35.9 mg/ml stock SR (stored at -80°C) with 994.4 µl buffer. Keep on ice.

7.5 Photoaffinity probes

7.5.1 50 µM [γ^{32}P]8N_3-ATP

33.5 µl from a 150 µM stock into 100 µl irradiation medium. The following amounts are all into 100 µl.

20 µM [γ^{32}P]8N_3-ATP: 13.4 µl 150 µM

10 μM [γ^{32}P]8N_3-ATP: 6.7 μl 150 μM

5 μM [γ^{32}P]8N_3-ATP: 3.4 μl 150 μM

1 μM [γ^{32}P]8N_3-ATP: 10 μl 10 μM

0.5 μM [γ^{32}P]8N_3-ATP: 5 μl 10 μM

7.5.2 10 μM [γ^{32}P]TNP-8N_3-ATP

10 μl from a 100 μM stock into 100 μl irradiation medium. The following amounts are in 100 μl

3 μM [γ^{32}P]TNP-8N_3-ATP: 3 μl 100 μM

2 μM [γ^{32}P]TNP-8N_3-ATP: 2 μl 100 μM

1 μM [γ^{32}P]TNP-8N_3-ATP: 10 μl 10 μM

0.3 μM [γ^{32}P]TNP-8N_3-ATP: 10 μl 3 μM

0.1 μM [γ^{32}P]TNP-8N_3-ATP: 10 μl 1 μM

7.6 SDS Electrophoresis gel stocks

7.6.1. 10 % SDS

10 g SDS in 80 ml water. Make the final up to 100 ml. Store at RT.

1 % SDS: 10 ml 10 % + 90 ml water.

7.6.2. Running Gel Buffer

187.5 ml 2 M TRIS.HCl (pH 8.6) + 10 ml 10 % SDS + 52.5 ml water. Check that the pH is 8.6. Store at 4°C.

7.6.3. Stacking Gel Buffer

25ml 2 M TRIS + 4 ml 10 % SDS + 71 ml water. Adjust pH to 6.8 with HCl. Store at 4°C.

7.6.4. 30 % Arcylogel 5 Premix

30 g Acrylogel 5 Premix in 15 ml distilled water and then make up to a final volume of 100 ml. Store at RT in a dark bottle.

7.6.5. 100 mg/ml AMPS

0.1 g AMPS in 1 ml water. Prepare fresh.

7.6.6. TEMED

Obtained from Sigma and stored at 4°C.

7.6.7. 0.2 M Tris.Cl, 8 M Urea and 2 % SDS Solubilizing Buffer

40 ml stacking gel buffer + 20 ml 10 % SDS + 40 ml water + as much urea as possible + a few grains of bromophenol blue (or pyronin-Y). Store at RT. Just before use take 960 µl of the above solution and add 40 µl ß-mecaptoethanol (4 %).

7.6.8. 7, 10 and 12 % SDS Running Electrophoresis Gel

7, 10 and 12 ml respectively of the 30 % Acrylogel 5 Premix (appendix 7.6.4) + 7.5 ml Running buffer (appendix 7.6.2). Make up to 30 ml with water. Add 150 µl 100mg/ml AMPS (appendix 7.6.5) and 18 µl TEMED (appendix 7.6.6) immediately before use. Prepare fresh.

7.6.9. 4 % SDS Stacking Electrophoresis Gel

4 ml 30 % Acrylogel 5 Premix (appendix 7.6.4) + 1.875 ml stacking buffer (appendix 7.6.3). Make up to 7.5 ml with water. Add 35 µl 100 mg/ml AMPS (appendix 7.6.5) and 5 µl TEMED (appendix 7.6.6) just before use. Prepare fresh.

7.6.10. Coomassie Blue Stain

2.5 g Coomassie Blue powder, 0.1 % (w/v) + 750 ml Methanol, 30 % (v/v) + 250 g TCA, 10 % (w/v). Make up to 2.5 L with water. Store on the floor at RT.

7.6.11. 30 % (v/v) Methanol + 10 % (v/v) Acetic Acid

150 ml methanol + 50 ml acetic acid in 300 ml water. Store on the floor at RT.

7.6.12. 30 % Ethanol (v/v) + 10 % (v/v) Acetic Acid

150 ml ethanol + 50 ml acetic acid in 300 ml water. Store on the floor at RT.

7.6.13. 7 % Glacial Acetic Acid

35 ml Acetic Acid in 450 ml water. Store on the floor at RT.

7.6.14. Gunnerson and Haley's Resolubilizing Buffer

1 ml 2 M Tris + 10 g SDS + 2.5 g DTT + 35 ml 10 M Urea + a few grains Pyronin-Y. Dissolve in 80 ml water and make final volume up to 100 ml.

CHAPTER 8

REFERENCES

Abbel, L., Schineller, J., Keck, P. & Villafranca, J.(1995). Effect of Metal-Ligand Mutations on Phosphoryl Transfer Reactions Catalyzed by *Escherichia coli* Glutamine Synthetase. *Biochemistry*. 34: 16695 - 16702.

Abraham, C., Shirahama, T & Potter, H. (1990). Alpha 1-antichymotrypsin is associated solely with amyloid deposits containing the beta-protein. Amyloid and cell localization of alpha 1-antichymotrypsin. *Neurobiol. Aging*. 11(2): 123 - 129.

Allison, R., Todhunter, J. & Purich, D. (1977). Steady state and equilibrium exchange kinetic studies of the sheep brain glutamine synthetase reaction. *J. Biol. Chem.* 252(17): 6046 - 6051.

Almassy, R., Janson, C., Hamlin, R., Xuong, N. & Eisenberg, D. (1986). Novel subunit-subunit interactions in the structure of glutamine synthetase. *Nature*. 323(6086): 304 - 309.

Andreadis, K., Brown, M. & Kosik, K. (1992). Structure and novel exons of the human tau gene. *Biochemistry*. 31(43): 10626 - 10633.

Arispe, N., Rojas, E. & Possard, H. (1993). Alzheimer's disease amyloid ß-protein forms calcium channels in bilayer membranes: blockade by tromethamine and aluminium. *Proc. Natl. Acad. Sci.* 90: 567 - 571.

Beardsley, T. (1995). Putting Alzheimer's to the tests. Several new techniques may detect the disease. *Sci. Amer.* 272(2): 12 -13.

Behl, C., Davis, J., Lesley, R. & Schubert, D. (1994). Hydrogen peroxide mediates amyloid protein toxicity. *Cell.* 77: 817 - 827.

Berman, M. (1986). Absorbance and fluorescence properties of 2',3'-O-(2,4,6-trinitrophenyl)-ATP 5'-triphosphate bound to coupled and uncoupled Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* 261(35): 16494 - 16501.

Bild, G. & Boyer, P. (1980). Subunit interaction during catalysis: Ammonium ion modulation of catalytic steps in the *E. Coli.* glutamine synthetase reaction. *Biochemistry.* 19: 5774 - 5781.

Bishop, J., Johnson, D. & Berman, M. (1984). Transient kinetic analysis of turnover-dependent fluorescence of 2',3'-O-(2,4,6-trinitrophenyl)-ATP bound to Ca^{2+}-ATPase of sarcoplasmic reticulum. *J. Biol. Chem.* 259(24): 15163 - 15171.

Bush, A., Pettingell, W., Paradis, M. & Tanzi, R. (1994). Modulation of A beta adhesiveness and secretase site cleavage by zinc. *J. Biol. Chem.* 269(16): 12152 - 12158.

Champeil, P., Guillain, F., Venien, C. & Gingold, M. (1985). Interaction of magnesium and inorganic phosphate with calcium-deprived sarcoplasmic reticulum adenosinetriphosphatase as reflected by organic solvent induced perturbation. *Biochemistry*. 24(1): 69 - 81.

Chartier-Harlin, M., Crawford, F., Houlden, H., Warren, A., Hughs, D., Fidani, L., Goate, A., Rossor, M., Roques, P., Hardy, J. & Mullan, M. (1991). Early-onset Alzheimer's disease caused by mutations at codon 717 of the beta-amyloid precursor protein gene. *Nature*. 353(6347): 844 - 846.

Corder, E., Saunders, A., Strittmatter, W., Schmechel, D., Gaskell, P., Small, G., Roses, A., Haines, J. & Pericak-Vance, M. (1993). Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's Disease in late onset families. *Science*. 261: 921 - 923.

Crawford, F., Hardy, J., Mullan, M., Goate, A., Hughs, D., Fidani, L., Roques, P., Rossor, M. & Chartier-Harlin, M. (1991). Sequencing of exons 16 and 17 of the beta-amyloid precursor protein gene in 14 families with early onset Alzheimer's disease fails to reveal mutations in the beta-amyloid sequence. *Neurosc. Lett.* 133(1): 1 - 2.

Crowther, R., Olesen, O., Jakes, R. & Goedert, M. (1992). The microtubule binding repeats of tau protein assemble into filaments like those found in Alzheimer's disease. *FEBS Lett.* 309(2): 199 - 202.

Davidson, G. & Berman, M. (1987). Phosphoenzyme conformational states an nucleotide-binding site hydrophobicity following thiol modification of the Ca^{2+}-ATPase of sarcoplasmic reticulum from skeletal muscle. *J. Biol. Chem.* 262(15): 7041 - 7046.

Dupont, Y. (1982). Low temperature studies of the sarcoplasmic reticulum calcium pump. Mechanism of calcium binding. *Biochim. Biophys. Acta.* 688(1): 75 - 87.

Duyn, J., Mattay, V., Sexton, R., Sobering, G., Barrios, F., Liu, G., Frank, J., Weinberger, D. & Moonen, C. (1994). 3-dimensional functional imaging of human brain using echo-shifted FLASH MRI. *Mag. Res. in Medicine.* 32(1): 150 - 155.

Evans, D., Funkenstein, H., Albert, M., Scherr, P., Cook, N., Chown, M., Herbert, L., Hennekens, C. & Taylor, J. (1989). Prevalence of Alzheimer's disease in a community population of older persons. *J. Am. Med. Assoc.* 262(18): 2551 - 2556.

Ferris, S., de Leon, M., Wolf, A., George, A., Reisberg, B., Christman, D., Yonekura, Y. & Fowler, J. 1983. Positron emission tomography in dementia. *Advances in Neurology.* 38: 123 - 129.

Fleet, G., Porter, R. & Knowles, J. (1969). Affinity labelling of antibodies with aryl nitrene as reactive group. *Nature.* 224: 511 - 512.

Garlardy, R., Craig, L. & Printz, M. (1973). Benzophenone Triplet: A new photochemical probe of biological ligand-receptor interactions. *Nature.* 242: 127 - 128.

Gitter, B., Cox, L., Russell, E. & Patrick, C. (1995). Amyloid ß-peptide potentiates cytokine secretion by interleukin-1ß-activated human astrocytoma cells. *Proc. Natl. Acad. Sci.* 92: 10738 - 10741.

Glynn, I. & Chappel, J. (1964). A simple method for the preparation of ^{32}P-labelled adenosine triphosphate of high specific activity. *Biochem. J.* 90: 147 - 149.

Goedert, M., Wischik, C., Crowther, R., Walker, J. & Klug, A. (1988). Cloning and sequencing of the cDNA encoding a core protein of the paired helical filament of Alzheimer disease: identification as the microtubule-associated protein tau. *Proc. Natl. Acad. Sci.* 85(11): 4051 - 4055.

Goedert, M. & Jakes, R. (1990). Expression of separate isoforms of human tau protein: correlation with the tau pattern in brain and effects on tubulin polymerization. *EMBO. J.* 9(13): 4225 - 4230.

Goedert, M., Jakes, R., Crowther, R., Six, J., Lübke, U., Vandermeeren, M., Cras, P., Trojanowski, J. & Lee, V. (1993). The abnormal phosphorylation of tau protein at Ser-202 in Alzheimer disease recapitulates phosphorylation during development. *Proc. Natl. Acad. Sci.* 90: 5066 - 5070.

Gregori, L., Fuchs, C., Figueiredo-Pereira, M., Van Nostrand, W. & Goldgaber, D. (1995). Amyloid ß-protein inhibits Ubiquitin-dependent protein degradation *in vitro*. *J. Biol. Chem.* 270: 19702 - 19708.

Gunnerson, D. & Haley, B. (1992). Detection of glutamine synthetase in the cerebrospinal fluid of Alzheimer diseased patients: a potential diagnostic biochemical marker. *Proc. Natl. Acad. Sci.* 89: 11949 - 11953.

Haley, B. & Hoffman, J. (1974). Interaction of a photo-affinity ATP analog with cation-stimulated adenosine triphosphatases of human red cell membranes. *Proc. Natl. Acad. Sci.* 71(9): 3367 - 3371.

Hardy, J. (1992). Framing beta-amyloid. *Nat. Genet.* (1)4: 233 - 234.

Herschlag, D. & Jencks, W. (1989). The effect of Mg^{2+}, hydrogen bonding, and steric factors on rate and equilibrium constants for phosphoryl transfer between carboxylate ions and pyridines. *J. Am. Chem. Soc.* 112: 1942 - 1950.

Herscovitch, P., Gado, M., Mintun, M. & Raichle, M. (1984). The necessity for correcting of cerebral atrophy in global positron emission tomography measurements. *Neural. Sci.* 11: 93 -97.

Hunt, J. & Ginsberg, A. (1972). Some kinetics of the interaction of divalent cations with glutamine synthetase from *E. Coli*. Metal ion induced conformational changes. *Biochem.* 11: 3723 - 3735.

Hunt, J. & Ginsberg, A. (1980). Mn^{2+} and substrate interactions with glutamine synthetase from *Escherichia coli*. *J. Biol. Chem.* 255(2): 590 - 594.

Ikehara, M. & Uesugi, S. (1969). Studies on nucleosides and nucleotides. Synthesis of 8-bromoadenosine nucleotides. *Chem. Pharm. Bull.* 17(2): 348 - 354.

Jagust, W. & Eberling, J. (1991). MRI,CT,SPECT,PET: their use in diagnosing dementia. *Geriatrics.* 46(2): 28 - 35.

Jarrett, J. & Lansbury, P. (1993). Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? *Cell.* 73(6): 1055 - 1058

Knowles, J. (1972). Biological Receptor-site labelling. *Acc. Chem. Res.* 5: 155 - 160.

Laconis, D., Chad, D. & Smith, T. (1993). Myopathy in the elderly: evaluation of the histopathologic spectrum and the accuracy of clinical diagnosis. *Neurology.* 43(4): 825 - 828.

Laemmli, U. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227: 680 - 685.

Lee, G., Cowan, N. & Kirschner, M. (1988). The primary structure and heterogenecity of tau proteins from mouse brain. *Science.* 239: 285 - 288.

Lee, V., Balin, B., Otvos, L. & Trojanowski, J. (1991). A68: a major subunit of paired helical filaments and derivatized forms of normal tau. *Science.* 251(4994): 675 - 678.

Lee, R., Wurtman, R., Cox, A. & Nitsch, R. (1995). Amyloid precursor protein processing is stimulated by metabotropic glutamate receptors. *Proc. Natl. Acad. Sci.* 92: 8083 - 8087.

Li, J., Junli, M. & Potter, H. (1995). Identification and expression analysis of a potential familial Alzheimer disease gene on chromosome 1 related to AD3. *Proc. Natl. Acad. Sci.* 92: 12180 - 12184.

Liaw, S., Villafranca, J. & Eisenberg, D. (1993). A model of oxidative modification of glutamine synthetase, based on crystal structures of Mutant H269N and the oxidized enzyme. *Biochemistry.* 32(31): 7999 - 8003.

Liaw, S., Jun, G. & Eisenberg, D. (1993). Extending the diffraction limit of protein crystals: the example of glutamine synthetase from Salmonella typhimurium in the presence of its cofactor ATP. *Protein. Sci.* 2(3): 470 - 471.

Liaw, S. & Eisenberg, D. (1994). The structural model for the reaction mechanism of glutamine synthetase, based on five crystal structures of enzyme-substrate complexes. *Biochemistry.* 33(3): 675 - 681.

Liaw, S., Jun, G. & Eisenberg, D. (1994). Interactions of nucleotides with fully unadenylylated glutamine synthetase from *Salmonella typhimurium*. *Biochemistry.* 33(37): 11184 - 11188.

Lowry, O., Rosebrough, N., Farr, A. & Randall, R. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193: 265 - 275.

MacLennan, D. & Wong, P. (1971). Isolation of a calcium-sequestering protein from sacroplasmic reticulum. *Proc. Natl. Acad. Sci.* 68(6): 1231 - 1235.

Mahley, R. (1988). Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science.* 240(4852): 622 - 630.

Mandelkow, E.M. & Mandelkow, E. (1993). Tau as a marker for Alzheimer's disease. *Trends in Biochem. Sci.* 18(12): 480 - 483.

Massen, J. & Möller, W. (1974). Identification by photo-affinity labelling of the proteins in *E. Coli.* ribosomes involved in Elongation factor G-dependent GDP binding. *Proc. Natl. Acad. Sci.* 71: 1277 - 1280.

McIntosh, D., Woolley, D. & Berman, M. (1992). 2',3'-O-(2,4,6-trinitrophenyl)-8-azido-AMP and -ATP photolabel Lys-492 at the active site of sacrcoplasmic reticulum Ca(2$^+$)-ATPase. *J. Biol. Chem.* 267(8): 5301 - 5309.

McIntosh, D. & Woolley, D. (1994). Catalysis of an ATP analogue untethered and tethered to lysine 492 of sarcoplasmic reticulum Ca(2$^+$)-ATPase. *J. Biol. Chem.* 269(34): 21587 - 21595.

McLachlan, D., Dalton, A., Kruck, T., Bell, M., Smith, W., Kalow, W. & Andrews, D. (1991). Intramuscular desferrioxamine in patients with Alzheimer's disease. *Lancet.* 337(8753): 1304 - 1308.

Minoshima, S., Frey, K., Foster, N. & Kuhl, D. (1995). Preserved pontine glucose metabolism in Alzheimer disease: a reference region for functional brain image (PET) analysis. *J. of Comp. Ass. Tomog.* 19(4): 541 - 547.

Muller-Hill, B. & Beyreuther, K. (1989). Molecular biology of Alzheimer's disease. *Annual. Rev. Biochem.* 58: 287 - 307.

Munroe, W., Southeick, P., Chang, L., Scharre, D., Echols, C., Fu, P., Whaley, J. & Wolfert, R. (1995). Tau protein in cerebrospinal fluid as an aid in the diagnosis of Alzheimer's disease. *Annals of Clin. and Lab. Sci.* 23(3): 207 - 217.

Murrell, J., Farlow, M., Ghetti, B. & Benson, M. (1991). A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. *Science.* 254(5028): 97 - 99.

Nakamura, T., Shoji, M., Harigaya, Y., Watanabe, M., Hosoda, K., Cheung, T., Shaffer, L., Golde, T., Younkin, L. & Younkin, S. (1994). Amyloid beta protein levels in cerebrospinal fluid are elevated in early-onset Alzheimer;'s disease. *Ann. Neurol.* 36(6): 903 - 911.

Nitsch, R., Slack, B., Wurtman, R. & Growdon, J. (1992). Release of Alzheimer amyloid precursor derivatives stimulated by activation of muscarinic acetylcholine receptors. *Science.* 258: 304 - 307.

Palmert, M., Usaik, M., Mayeux, R., Raskind, M., Tourtellotte, W. & Younkin, S. (1990). Soluble derivatives of the beta amyloid precursor in cerebrospinal fluid: alterations in normal aging and in Alzheimer's disease. *Neurol.* 40(7): 1028 - 1034.

Petersen, R., Smith, G., Ivnik, R., Kokmen, E. & Tangalos, E.(1994). Memory function in very early Alzheimer's disease. *Neurol.* 44(5): 867 - 872.

Pick, U. & Karlish, S. 1980. Indications for an oligomeric structure and for conformational changes in sarcoplasmic reticulum Ca^{2+}-ATPase labelled selectively with fluorescein. *Biochim. Biophys. Acta.* 626(1): 255 - 261.

Potter, H. (1991). Review and hypothesis: Alzheimer disease and Down syndrome--chromosome 21 nondisjunction may underlie both disorders. *Amer. J. Hum. Genet.* 48(6): 1192 - 1200.

Rhee, S., Chock, P. & Stadtman, E. (1976). Mechanistic studies of glutamine synthetase from *E.Coli*. An integrated mechanism for biosynthesis, transferase, ATPase reaction. *Biochimie.* 58: 35 - 49.

Rocca, W., Amaducci, L. & Schoenberg, B. (1986). Epidemiology of clinically diagnosed Alzheimer's disease. *Annals. Neurol.* 19(5): 415 -424.

Rogers, J., Cooper, N., Webster, S., Shultz, J., McGeer, P., Styren, S., Civin, W., Brachova, L., Bradt, B. & Ward, P. (1992). Complement activation by beta-amyloid in Alzheimer's Disease. *Proc. Natl. Acad. Sci.* 89: 10016 - 10020.

Ronzio, R., Rowe, W., Wilk, S. & Meister, A. (1969). Preparation and studies on the characterization of sheep brain glutamine synthetase. *Biochemistry.* 8: 2670 - 2674.

Saunders, A., Schmoder, K., Brutner, J., Benson, M., Brown, W., Goldfarb, L., Goldgaber, D., Manwaring, M., Szymanski, M., McCrown, N., Dole, K., Schmechel, D., Strittmatter, W., Pericak-Vance, M. & Roses, A. (1993). Apolipoprotein E 4 allele distribution in late onset Alzheimer's disease and in other amyloid-forming diseases. *Lancet.* 342: 710 - 711.

Schafer, G., Onur, G., Edelman, K., Bickel-Sandkotter, S. & Strotmann, H. (1978b). Energy transfer inhibition in photosynthesis 3'-aryl-N_3-ADP, an ADP analog. *FEBS. Lett.* 87(2): 318 - 322.

Schellenberg, G., Bird, T., Wijsman, E., Orr, H., Anderson, L., Nemens, E., White, J., Bonnycastle, L., Weber, J., Alonso, M., Potter, H., Heston, L. & Martin, G. (1992). Genetic linkage evidence for a familial Alzheimer's Disease locus on chromosome 14. *Science.* 258: 668 - 671.

Schellenberg, G. (1995). Genetic dissection of Alzheimer disease, a heterogeneous disorder. *Proc. Natl. Acad. Sci.* 92(19): 8552 - 8559.

Scinto, L., Daffer, K., Dressler, D., Ransil, B., Rentz, D., Weintraub, S., Mesulam, M. & Potter, H. (1994). A potential noninvasive neurobiological test for Alzheimer's disease. *Science.* 266(5187): 1051 - 1054.

Seebregts, C. (1989). Photoaffinity labelling the nucleotide sites of the sarcoplasmic reticulum Ca^{2+}-ATPase. *Ph. D. thesis, University of Cape Town, S.A.*

Seebregts, C. & McIntosh, D. (1989). 2',3'-O-(2,4,6-trinitrophenyl)-8-azido-adenosine mono-,di-, and triphosphates as photoaffinity probes of the Ca^{2+}-ATPase of sarcoplasmic reticulum. Regulatory/superfluorescent nucleotides label the catalytic site with high affinity. *J. Biol. Chem.* 264(4): 2043 - 2052.

Seubert, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Davis, D., Sinha, S., Scholssmacher, M., Whaley, J., Swindlehurst, C., McCormack, R., Wolfert, R., Lieberburg,I. & Schenk, D. (1992). Isolation and quantification of soluble Alzheimer's-peptide from biological fluids. *Nature.* 359: 325 - 327.

Shapiro, B. & Ginsburg, A. (1968). Effects of specific divalent cations on some physical and chemical properties of glutamine synthetase from Escherichia coli. Taut and relaxed enzyme forms. *Biochem.* 7(6): 2153 - 2167.

Shoji, M., Golde, T., Ghiso, J., Cheung, T., Estus, S., Shaffer, L., Cai, X., McKay, D., Tintner, T., Frangione, B. & Younkin, S. (1992). Production of the Alzheimer amyloid beta protein by normal proteolytic processing. *Science.* 258: 126 - 129.

Simmons, L., May, P., Tomaselli, K., Rydel, R., Fuson, K., Brigham, E., Wright, S., Lieberburg, I., Becher, G., Brems, D. & Li, W. (1994). *Secondary structure of amyloid peptide* correlates with neurotoxic activity *in vitro. Mol. Pharmacol.* 45: 373 - 379.

Singh, A., Thornton, E. & Westheimer, F. (1962). The photolysis of diazo-acetylchymotrypsin. *J. Biol. Chem.* 237: 3006 - 3008.

Smith, C., Carney, J., Starke-Reed, P., Oliver, C., Stadtman, E., Floyd, R. & Markesbery, W. (1991). Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer's disease *Proc. Natl. Acad. Sci.* 88(23): 10540 - 10543.

Snow, A., Mar, H., Nochlin, D., Sekiguchi, R., Kimata, K., Koike, Y. & Wright, T. (1990). Early accumulation of heparin sulfate in neurons and in the beta-amyloid protein-containing lesions of Alzheimer's disease and Down's syndrome. *Am. J. Pathol.* 137(5): 1253 - 1270.

Stritmatter, W., Saunders, A., Schmechel, D., Pericak-Vance, M., Enghild, J., Salvesen, G. & Roses, A. (1993). Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer's Disease. *Proc. Natl. Acad. Sci.* 90: 1977 - 1981.

Tada, M., Yamamoto, T. & Tonomura, Y. (1978). Molecular mechanism of active calcium transport by sarcoplasmic reticulum. *Physiol. Rev.* 58(1): 1 - 79.

Timmons, R., Rhee, S., Luterman, D. & Chock, P. (1974). Mechanistic studies of glutamine synthetase from *E. Coli*. Fluorometric identification of a reactive intermediate in the biosynthetic reaction. *Biochemistry*. 13: 4479 - 4485.

Valentine, R., Shapiro, B. & Stadtman, E. (1968). Regulation of glutamine synthetase. XII. Electron microscopy of the enzyme from *E. Coli*. *Biochem.* 7: 2143 - 2152.

Vandermeerden, M., Mercken, M., Vanmechelen, E., Six, J., van de Voorde, A., Martin, J. & Cras, P. (1993). Detection of tau proteins in normal and Alzheimer's disease cerebrospinal fluid with a sensitive sandwich enzyme-linked immunosorbent assay. *J. Neurochem.* 61(5): 1828 -1834.

Wagenvoord, R., Van der Kraan, I. & Kemp, A. (1977). Specific photolabelling of beef-heart mitochondrial ATPase by 8-azido-ATP. *Biochim. Biophys. Acta.* 460(1): 17 - 24.

Wagner, S., Peskind, E., Nochlin, D., Provow, S., Farrow, J., Pandian, M., Cleveland, M., Ito, R. & Farlow, M. (1994). Decreased levels of soluble amyloid beta-protein precursor are associated with Alzheimer's disease in concordant and discordant monozygous twin pairs. *Ann. Neurol.* 36(2): 215 - 220.

Watanabe, T. & Inesi, G. (1982). The use of 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate for studies of nucleotide interaction with sarcoplasmic reticulum vesicles. *J. Biol. Chem.* 257(19): 11510 - 11516.

Weidenmann, A., Konig, G., Bunke, D., Fisher, P., Salbaum, M., Masters, C. & Beyteuther, K. (1989). Identification, biogenesis and localization of precursors of Alzheimer's disease A4 amyloid protein. *Cell.* 57(1): 115 - 126.

Weisgraber, K., Roses, A. & Strittermatter, W. (1994). The role of apolipoprotein E in the nervous system. *Current Opinion in Lipidology*. 5(2): 110 - 116.

Wilk, S., Meister, A. & Haschemeyer, R. (1969). Studies on the subunit structure of ovine brain glutamine synthetase. *Biochemistry*. 8(8): 3168 - 3174.

Wisniewski, T. & Frangione, B. (1992). Apolipoprotein E: a pathological chaperon protein in patients with cerebral and systemic amyloid. *Neurosci. Lett.* 135: 235 - 238.

Wisniewski, T., Castano, E., Golabek, A., Vogel, T. & Frangione, B. (1994). Acceleration of Alzheimer's fibril formation by apolipoprotein E *in vitro*. *Am. J. Pathol.* 145(5): 1030 - 1035.

Woolfolk, C. & Stadtman, E. (1967). Regulation of Glutamine Synthetase. IV. Reversible Dissociation and Inactivation of Glutamine Synthetase from *Escherichia Coli* by the Concerted Action of EDTA and Urea. *Arch. Biochem. and Biophys.* 122: 174 - 189.

Wray, L. & Fisher, S. (1988). Cloning and nucleotide sequence of the Streptomyces coelicolor gene encoding glutamine synthetase. *Gene*. 71: 247 - 256.

Yamashita, M., Almassay, R., Janson, C., Cascio, D. & Eisenberg, D. (1989). Refined atomic model of glutamine synthetase at 3.5 A resolution. *J. Biol. Chem.* 264(30): 17681 - 17690.

Yan, S., Chen, X., Schmidt, A., Brett, J., Godman, G., Zou, Y., Scott, C., Caputo, C., Frappiet, T., Smith, M., Yen, S. & Stern, D. (1994). Glycated tau protein in Alzheimer disease: A mechanism for induction of oxidant stress. *Proc. Natl. Acad. Sco.* 91(16): 7787 - 7791.

Yoshimoto, M., Iwai, A., Kang, D., Otero, D., Xia, Y. & Saitoh, T. (1995). NACP, the precursor protein of the non-amyloid ß/A4 protein (Aß) component of Alzheimer disease amyloid, binds Aß and stimulates Aß aggregation. *Proc. Natl. Acad. Sci.* 92: 9141 - 9145.