FACILITATION OF HEAT SHOCK PROTEIN EXPRESSION IN BLOOD MONONUCLEAR CELLS BY ANTI-INFLAMMATORY -RHEUMATIC AGENTS

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DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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ABBREVIATIONS

AA	Adjuvant Arthritis
Ab	Antibody
AP	Alkaline Phosphatase
APC	Antigen Presenting Cells
APS	Ammonium Persulphate
ATP	Adenosine Triphosphate
BiP	Heavy Chain Binding Protein
BSA	Bovine Serum Albumin
DMF	N, N-Dimethylformamide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
eIF-2	Eukaryotic Initiation Factor-2
ER	Endoplasmic Reticulum
FCS	Foetal Calf Serum
FKBP	A PPI Related Protein
GroEL	Bacterial HSP60 Analogue
grp70	70kDa Glucose Regulated Protein
grp90	90kDa Glucose Regulated Protein
HBSS	Hanks' Balanced Salt Solution
HEPES	N-2-Hydroxyethylpiperazine-N-2-Ethanesulphonic Acid
hr(s)	Hour(s)
HS	Heat Shock
hsc70	Constitutively Expressed 70kDa Heat Shock Protein Analogue
HSE	Heat Shock Element
HSF	Heat Shock Factor
HSP	Heat Shock Proteins
HSP100	100kDa Heat Shock Protein Family
HSP65	65kDa Heat Shock Protein Family
hsp65	Heat Inducible 65kDa Heat Shock Protein (Mycobacterial)
HSP70	70kDa Heat Shock Protein Family
hsp70	Heat Inducible 70kDa Heat Shock Protein
HSP90	90kDa Heat Shock Protein Family
IgH	Immunoglobulin H
Kar2p	Yeast HSP70 analogue localised in the ER
kDa	kiloDalton(s)

mAb	Monoclonal Antibody
MHC	Major Histocompatibility Complex
min(s)	Minute(s)
MNC	Mononuclear Cells
Mr	Molecular Weight
mRNA	Messenger Ribonucleic Acid
NBT	4-Nitro Blue Tetrazolium Chloride
NK	Natural Killer
PBP	Peptide Binding Protein (a HSP70 member)
PBS	Phosphate-Buffered Saline
PDI	Protein Disulfide Isomerase(s)
PPI	Peptidyl-Prolyl *cis-trans* Isomerase(s)
PVDF	Polyvinylidene Fluoride
RA	Rheumatoid Arthritis
RUBISCO	Ribulose Bisphosphate Carboxylase Subunit Binding Protein of Chloroplast Stroma
SDS	Sodium Dodecylsulfate
SDS PAGE	Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis
snRNP	Small Nuclear Ribonucleoprotein(s)
SP	Stress Protein
SSA/SSB	Yeast HSP70 analogues localised in the cytosol
Ssc1p	Yeast HSP70 analogue localised in mitochondria
TBS	Tris-Buffered Saline
TEMED	N,N,N',N',-Tetramethylethylenediamine
TNF	Tumour Necrosis Factor (α or β)
5'UTR	5' Untranslated Region
WBC	White Blood Cells

THESIS SUMMARY

Heat shock protein (HSP) expression was initially recognised in the fruit fly, *Drosophila busckii*, after exposure to hyperthermia (Ritossa 1962, 1964). As HSP were further investigated, it became apparent their induction could be brought about by other stresses, such as chemicals and ionising radiation. The significance of HSP induction by cells after being exposed to a stress, relates to HSP function in repairing the damage caused by stress. Furthermore, by preinducing HSP, it is possible to demonstrate the protective effect that elevated cellular HSP have when the cell is subsequently exposed to a severe stress.

This thesis investigates the induction of HSP by some of the anti-inflammatory agents and antirheumatic agents used in the management of rheumatoid arthritis. Others have previously demonstrated the induction of HSP by the antirheumatic gold complex, auranofin. The work presented here investigated HSP induction in peripheral white blood cells cultured *in vitro*. The agents used included: aspirin, chloroquine, diclofenac, D-penicillamine, indomethacin, methotrexate, paracetamol and sodium salicylate. Under the selected experimental conditions the above agents, alone, did not demonstrate HSP induction in white blood cells. However, exposure of cells to the combination of agent and mild hyperthermia clearly demonstrated that most of the above agents influenced HSP induction and expression by lowering the temperature threshold of HSP induction and enhancing synthesis. Chloroquine, diclofenac, indomethacin and sodium salicylate also lowered the temperature at which hyperthermia inhibited total protein synthesis from 45°C to 43°C. Furthermore, the protective effect of HSP induced by a combination of hyperthermia and agent was demonstrated with D-penicillamine, indomethacin, paracetamol and sodium salicylate whereas, those agents alone or hyperthermia alone did not show the same level of protection.

I hypothesise that the exposure of white blood cells in the peripheral blood of patients with rheumatoid arthritis may induce HSP synthesis in those cells. Later when these cells enter the inflamed joint, where they encounter the stresses associated with inflammation (i.e. oxy radicals, proteolytic enzymes, cytotoxic cytokines, fever, etc), they are able to respond to and cope with the stresses by inducing sooner and synthesising more HSP. The synthesised HSP would function to protect and repair the cell's biological and immunoregulatory functions, resulting in a more appropriate immune response to help resolve the inflammatory stimulus. In addition, enhanced HSP synthesis in the cells of the joint (e.g. chondrocytes, osteocytes, cells of the synovial membrane etc.) would help protect these cells, resulting in the maintenance of normal tissue and hence, prevent the deformation of the joint as occurs in severe cases of rheumatoid arthritis.

The results described above also have potential relevance to the pathogenesis of Reye's Syndrome and also in the treatment of tumours. Reye's Syndrome affects mainly young children who have been treated with aspirin to relieve their fever. The pathogenesis of Reye's Syndrome includes damage to the liver, and my work suggests that this may be caused by the inhibition of total protein synthesis in liver cells by the combination of hyperthermia (43°C) and salicylate.

The use of hyperthermia in the treatment of tumours is an established procedure which enhances the effectiveness of other cytotoxic treatments like radiotherapy. Similarly the combination of cytotoxic agents and hyperthermia may be used to target the shutdown of tumour protein synthesis.

CHAPTER ONE

Introduction

1.1 HEAT SHOCK PROTEINS

1.1.1 Introduction

Throughout evolution, the heat shock proteins (HSP) have been one of the most conserved protein families. Normally, HSP are expressed by cells in small amounts for the purposes of carrying out housekeeping functions, but when cells are exposed to a stress they become expressed in large amounts. This induction of HSP by a stress has become known as the "heat shock response" or "stress response". The heat shock response has been found to occur in all organisms examined to date-from the most primitive organisms such as bacteria, fungi and yeast through to the more advanced multicellular organisms like plants and the highly developed vertebrates through to human kind. Incredibly, high levels of conservation exist between organisms and species in the function for specific HSP, meaning an analog of a specific HSP can be found in almost all other organisms.

HSP function to repair and/or protect cells from the effects of stress. Stresses inhibit cellular metabolism by damaging (denaturing) the proteins involved in the catalysis of the many cellular functions (Wynn *et al.* 1994; Parsell and Lindquist 1993; Nover 1991). HSP induced by stress restore damaged cellular functions by refolding denatured proteins or assisting in the synthesis and transport of new proteins to their proper locations (e.g. mitochondria, golgi apparatus, etc.) (Nover 1991). HSP induced by a mild stress, which does not significantly damage cells, will protect cells from a more severe stress by repairing damage immediately or possibly by preventing severe denaturation. Therefore, at the genetic level, HSP have evolved to be induced by the presence of a stress on a cell. Such a stress can take many forms. One of the earliest stresses discovered to induce the synthesis of HSP was hyperthermia, hence the name "heat shock proteins" (HSP). Later it was found that some HSP were also induced by stresses other than hyperthermia, and that some of these stresses induce proteins which were not inducible by hyperthermia. These other proteins were named "stress proteins" (SP).

There are many different HSP, and for convenience they have been categorised into major groups based on their molecular weight. These categories include the HSP families of the 70kDa, 65kDa, 83-90kDa, higher Mr HSP, lower Mr HSP and other stress proteins (e.g. enzymes, metallothioneins, and ubiquitins) (Healy *et al.* 1992). The categories were selected due to the lack of knowledge at the time about the exact function(s) of many of these proteins.

HSP genes possess important common characteristics. For example, all *HSP* genes have a heat shock responsive transcription element (heat shock element) in the 5' untranslated region (UTR) (Pelham 1982; Hickey *et al.* 1986; Hickey *et al.* 1989; Hosokawa *et al.* 1993). Most *HSP* genes in *Drosophila* and lower organisms and *HSP70* genes, in particular, lack introns (Lindquist 1986). These are discussed in more detail below.

The heat shock element (HSE) has been characterised and is involved in the induction of transcription of *HSP* genes after HS and a variety of other stresses (Perisic *et al.* 1989; Sorger 1991). After cells have been exposed to a stress the HSE interacts with a stress specific trans-acting factor, or the heat shock factor (HSF), which can lead to the transcription of the downstream protein coding region (Perisic *et al.* 1989; Benjamin *et al.* 1990). However, stress protein genes which are induced by other stresses, but not necessarily by hyperthermia, may not necessarily have a functional HSE in their 5'UTR.

Unlike the majority of genes found in higher life forms, the heat inducible genes, hsp70 in particular, lack introns (Hickey *et al.* 1986; Hickey *et al.* 1989; Hosokawa *et al.* 1993) in the HSP coding region. This allows for their uninhibited expression after severe hyperthermia as there is little need for post-transcriptional processing. In contrast, the induced expression of other genes is inhibited at the precursor mRNA processing stage. This is a direct result of hyperthermia disrupting the small nuclear ribonucleoproteins (snRNP), which are responsible for splicing out the intervening sequences from precursor mRNA (Wright-Sandor *et al.* 1989).

1.1.2 Heat inducibility of *HSP* genes: The role of HSF and the HSE

Although a large amount of work has already been performed on the elucidation of mechanisms involved in the regulation of *HSP* genes, a large amount still needs to be achieved. Up to now the *Drosophila* model has been the most comprehensively studied with regards to *HSP* gene regulation. However, minor differences have been found to exist in different organisms.

Control of *HSP* gene expression occurs primarily at the level of transcription (Pelham 1982; Goldenberg *et al.* 1988). In *Drosophila*, the *HSP* genes, *hsp70*, *hsp27* and *hsp26*, require HSF binding to the promoter region before transcription proceeds (Lis and Wu 1993; Rasmussen and Lis. 1993). Depending on the organism, HSF is coded by one or more genes. Of the two human *HSF* gene products,(Rabindran *et al.* 1991; Schuetz *et al.* 1991) HSF1 has been reported to be activated by hyperthermia and HSF2 is activated by hemin (haem) during erythroid differentiation (Sistonen *et al.* 1994). HSF are synthesised constitutively and stored in a latent form which does not bind specific DNA sequences (Westwood *et al.* 1991). The exception is in budding yeast, where the HSF is constitutively bound to the HSE (Sorger and Pelham 1988). However, further activation, of the factor is required for transcription (Jurivich *et al.* 1992; Hensold *et al.* 1990).

The activation of *Drosophila* HSF involves the conversion of HSF monomers to homotrimers (Sorger and Pelham 1988; Westwood and Wu 1993). Each HSF molecule has a conserved DNA binding domain and an alpha helical domain at the N-terminus. The alpha helical domain interacts with other HSF to produce a trimer. Trimerisation occurs when three individual alpha helices combine by a zipper type interaction or form a single triple stranded alpha helix (Rabindran *et al.* 1993; Peteranderl and Nelson 1992; Sorger and Nelson 1989). The formation of the trimer seems, thus far, to be an irreversible process. The HSF trimer demonstrates specific binding to HSE sequences (Sorger and Nelson 1989). The HSE consists of five base pair sequences, "nGAAn", arranged as consecutive inverted repeats, "nGAAnnTTCnnGAAn" (Perisic *et al.* 1989). For the HSF

trimer to bind to the HSE, the HSE must consist of a minimum of three consecutive inverted repeats (Perisic *et al.* 1989; Sorger 1991).

In the budding yeasts, *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, binding of HSF trimer to HSE is constitutive but insufficient to cause transactivation of the *HSP* genes. Transcription occurs only after the bound HSF has been phosphorylated by a serine/threonine kinase (Sorger 1990; Sorger and Pelham 1988).

Besides HSF, other transcription factors, most of them common to non-heat inducible initiation complexes, are also required for the initiation of transcription. The uninduced heat shock promoter is free of nucleosomes and has transcription factors already interacting with this region (Lee *et al.* 1992). RNA polymerase II is also part of the initiation complex, which although initiated, is stalled after synthesising a short transcript of approximately 25 bases in length (Giardina *et al.* 1992; Rougvie and Lis 1988; Lee *et al.* 1992). After the HSF trimer interacts with the promoter and has been modified by phosphorylation, RNA polymerase II is released to continue with transcription (Lee *et al.* 1992; Lis and Wu 1993). The pausing of RNA polymerase II is not restricted to heat shock genes. Pausing is common to other *Drosophila* genes and in humans occurs on the c-myc gene (Giardina *et al.* 1992; Krumm, *et al.* 1992). Unlike these other genes, RNA polymerase II, on *HSP* genes, is released when the HSF trimer binds and becomes modified (Lee *et al.* 1992).

The mechanisms involved in HSF activation are not known, although some have been proposed. Hsp70 has been shown to interact with HSF (Abravaya *et al.* 1992), hence, one proposition put forward suggests that hsp70 is involved in controlling HSF activity. In this scenario, hsp70, under normal conditions, sequesters HSF into an HSF-hsp70 complex (Rabindran *et al.* 1994; Mosser *et al.* 1993). Upon exposure to stress, hsp70 is sequestered by denatured proteins, causing a shift in the equilibrium from the HSF-hsp70 complex to the denatured protein-hsp70 complex, leaving HSF available for trimerisation and subsequent HSE binding. (Lis and Wu 1993; Abravaya *et al.* 1992) Another proposed mechanism suggests that the molecular surfaces on HSF monomers,

involved in inter-HSF interaction when HSF trimers are formed, only become accessible to other HSF molecules after exposure to stress or xenobiotics. The monomers would then combine into trimers and bind to the UTR of *HSP* genes (Rabindran *et al.* 1993).

1.1.3 HSP90

The HSP90 family refers to the heat shock proteins which not only have a molecular weight in the range of 90kDa but also significant functional and sequence similarities. Depending on the organism or species being studied, proteins of the HSP90 family have molecular weights which falls between 80kDa and 110kDa (Hightower and Nover 1991). Homologs of the vertebrate HSP90 have been reported in insects, plants, yeast and bacteria (Nover 1991). The glucose regulated proteins (grp) in the range of 90kDa (grp90) are also regarded as part of the 90kDa stress protein family. Grp90 proteins are induced by glucose deprivation (Welch *et al.* 1983; Sorger and Pelham 1987). Unlike hsp90s, which are predominantly found in the cytoplasm (Healy *et al.* 1992; Nover 1991), the grp90s are found predominantly in the endoplasmic reticulum and Golgi system (Nover 1991). The grp90s bind to poorly glycosylated cellular proteins in order to maintain protein solubility and to prevent interactions with other hydrophobic surfaces prior to glycosylation.

Under normal temperatures, hsp90 is maintained at relatively high levels but its synthesis is increased further by hyperthermia (Burdon 1993). In yeast, there exist two members of the *hsp90* genes, one of which is not inducible. Deletion of both members is lethal (Picard *et al.* 1990), suggesting that hsp90 plays an important role in stressed and unstressed cells.

Hsp90 has been found to interact with several steroid receptors along with hsp70 and other minor proteins (Picard *et al.* 1990; Perdew and Whitelaw 1990; Smith *et al.* 1990). Hsp90 binds to the receptor and maintains it in an inactive state (Healy *et al.* 1992). When the appropriate steroid binds to the receptor complex, hsp90 dissociates

and the steroid receptor then specifically binds to DNA to induce the steroid-inducible gene(s) (Burdon 1993).

Hsp90 also forms a complex with the haem-regulated protein kinase (known as haem-controlled inhibitor) (Rose *et al.* 1989). In the presence of haem, haem-regulated protein kinase is inactive, but in its absence, haem-regulated protein kinase is active and is involved in the phosphorylation of the alpha subunit of eIF-2 (eIF-2 phosphorylation blocks initiation of protein synthesis). Depending on the extent of eIF-2 phosphorylation, initiation of cellular protein synthesis becomes reduced or totally inhibited. Therefore, hsp90 can play a role to inhibit general cellular protein synthesis after severe heat stress and hence, divert cellular resources to HSP synthesis (Rose *et al.* 1989; Burdon 1993).

In cells transformed with Rous sarcoma virus, hsp90 demonstrates similar behaviour to that observed with steroid receptors. Hsp90 is found in a complex with the $pp60^{src}$ oncoprotein (Oppermann *et al.* 1981; Brugge *et al.* 1983). As a part of this complex $pp60^{src}$ does not display tyrosine kinase activity (Healy *et al.* 1992; Burdon 1993). When the complex reaches the plasma membrane it dissociates and $pp60^{src}$ attaches to the inside of the membrane and regains tyrosine kinase activity (Healy *et al.* 1992; Burdon 1993).

Therefore in unstressed cells, the HSP90 family are also implicated in important and diverse roles involving the regulation of other protein activities.

1.1.4 HSP70

The HSP70 family are well associated with the cellular stress response. In almost all cases of physiological stress, ranging from hyperthermia, chemicals and a wide range of other stressful conditions (ionising radiation, starvation, infection, amino acid analogues, viral infection, fever, inflammation, ischaemia, oxidative injury, heavy metals, etc.) HSP70 are induced (Liu *et al.* 1994; Donati *et al.* 1990; Welch *et al.* 1983; Welch *et*

al. 1992; Resendez *et al.* 1985; Olden *et al.* 1979). Depending on the nature and severity of the stress, HSP70 levels may increase up to 100 fold of the normal basal levels (Sorger 1991).

One of the effects of severe stress is the disruption of post-translational mRNA processing. The HSP70 mRNA escape this block in *Drosophila* and in a few other organisms, like some other HSP genes, by having evolved without introns (Lindquist 1986; Yost and Lindquist 1986; Bond 1988). This allows the HSP70 mRNA to be translated into protein immediately following the completion of transcription, and thus produces an immediate stress response, which in turn leads to a speedy cellular recovery.

Currently, it is understood that the major role of hsp70 involves catalysing the unfolding and correct refolding of either denatured, nascent or newly synthesised cellular proteins (Beckmann *et al.* 1990; Agard 1993; Craig *et al.* 1993; Wynn *et al.* 1994). For this purpose there are two types of *hsp70* genes - a constitutively expressed housekeeping gene and a stress-inducible gene (Gething and Sambrook 1992). Hsp70 has also been proposed to be involved in many other equally important cellular processes (e.g. disaggregation of inclusion bodies); facilitation of protein transport, possibly via the cytoskeleton (Tsang 1993); exporting/importing proteins into the mitochondria, endoplasmic reticulum, golgi bodies, lysosomes and the nucleus (Kassenbrock *et al.* 1988; Chirico *et al.* 1988; Deshaies *et al.* 1988; Ellis 1987).

Hsp70 consists of 2 domains, the C- and N- termini (Gething and Sambrook 1992). The C- terminus has considerable sequence variability between different hsp70 proteins. It has been proposed that the variability in the C- terminus reflects its ability to interact with a broad range of substrates (Rippmann *et al.* 1991; Tsang 1993). The N-terminus of approximately 44kDa or 2/3 of the molecule, unlike the C- terminus, is highly conserved over the entire HSP70 family and possesses ATPase activity (Flaherty *et al.* 1990; Flynn *et al.* 1989). The N- terminus also demonstrates structural similarities with the actin monomer and experiments have shown hsp70 to bind actin tightly (Flaherty *et al.* 1991). However, the interaction between actin and hsp70 differs func-

tionally from that between hsp70 and other proteins. It has been proposed that hsp70 interacts with actin resulting in the translocation of hsp70 along actin filaments, and any protein bound by the C- terminus of hsp70 would be transported along with hsp70 (Tsang 1993).

The folding of protein substrates by hsp70 into functional conformations requires ATP hydrolysis (Parsell and Lindquist 1993). In this case, energy released by ATP hydrolysis drives a series of conformational changes in the denatured protein which finally leads to the release of a functional protein (Parsell and Lindquist 1993). If hsp70 is unable to repair denatured protein, it usually remains bound to hsp70 until it is finally degraded by proteinases (Hershko and Ciechanover 1992).

Hsp70, like hsp90, is coded by multiple genes in several locations on the genome. The constitutively expressed housekeeping 73kDa HSP exists in relatively high concentrations (Nover 1991). Under normal conditions the 73kDa protein functions in the folding of newly synthesised proteins and in the translocation of proteins across organellular membranes (Nover 1991). However, it is possible that under stress this protein, along with the inducible hsp70, becomes involved in rescuing damaged cellular functions.

Hsp70 has also been implicated in having an important role in the functioning of the immune system. Two intronless genes, which are also inducible and identical, (*hsp70-1* and *hsp70-2*) and a third (*hsp70-hom*) which encodes a constitutively expressed non heat-inducible protein are located within the MHC locus (Healy *et al.* 1992). It has been proposed that HSP70 is involved with peptide production by protease degradation of proteins, transport of antigenic peptides, charging major histocompatibility complexes with peptides and in the assembly of antibodies (Munro and Pelham 1986 ; Chiang *et al.* 1989; Pierce *et al.* 1988; Srivastava *et al.* 1994). The length of antigenic peptides presented by the MHC molecules have been estimated to be 7 to 11 amino acids (Cerundolo *et al.* 1991; Guo *et al.* 1992). Similarly, Flynn *et al.* (1991) showed that peptides of 7 amino acids in length bound to the endoplasmic reticulum (ER) localised HSP70 more efficiently than shorter or longer peptides. This suggests that

during protease processing of antigenic proteins in the ER, hsp70 may play an important role by binding peptides of 7 amino acids in length and protecting them from further protease degradation. Later these peptides become associated with MHC molecules and are presented on the surface of the antigen presenting cells.

1.1.5 HSP60

HSP60 are a class of structurally related proteins of molecular weight between 57 and 64kDa and are members of the chaperonin family. They are primarily located in organelles of endosymbiotic origin (e.g. mitochondria and chloroplasts) and are found in the cytosol of all prokaryotes (GroEL in *E. coli*). (Kaufmann 1991; Cheng *et al.* 1990; Gething and Sambrook 1992) All of the *hsp60* genes are constitutively expressed but are also induced, up to 3 fold, by heat shock (Kaufmann 1991).

The functional hsp60 complex is made up of fourteen 60kDa monomers, arranged in a double 7-mer ring which has a sevenfold rotational symmetry (Kaufmann 1991; Cheng *et al.* 1990). The hsp60 14-mer binds unfolded proteins on its surface and catalyses protein folding and assembly of multimeric proteins via an ATP dependent process, similar to that of hsp70 (Cheng *et al.* 1989; Ostermann 1989). Evidence suggests that the hsp60 complex also binds to, and stabilises, regions of protein secondary structure during folding. Although both hsp60 and hsp70 stabilise the conformation of folding intermediates to prevent aberrant structures, the function of hsp70 is thought to differ from hsp60, in that, hsp70 binds to proteins which have less secondary structure (Gething and Sambrook 1992).

The hsp60 complex is also essential for self assembly. Newly translated hsp60 precursors are imported into organelles, like the mitochondria, where they are proteolytically processed. The hsp60 monomers are assembled into active 14-mer complexes in 5-10min (Cheng *et al.* 1990). If hsp60 is depleted in genetically manipulated cells, all the cells eventually die. However, the cells will survive if returned to normal growth before

the total depletion of hsp60. In addition to a functional hsp60 complex, mitochondrial hsp70 (mhsp70) has been found to interact with newly imported hsp60 (Hallberg *et al.* 1993). Therefore, both proteins may be necessary for assembly of the hsp60 complex.

The mitochondrial hsp60 may also maintain other protein molecules in a non-native form for subsequent transportation across the inner mitochondrial membrane. This conclusion was reached as proteins normally targeted to mitochondrion compartments exhibit some targeting or processing defects in the absence of hsp60 function (Hallberg *et al.* 1993; Cheng *et al.* 1989).

1.1.6 HSP30

This family of proteins includes enzymes with antioxidant potential like superoxide dismutase and haem oxygenase (Healy *et al.* 1992). Oxidative injury can be caused by exposure to heavy metals, carcinogens, xenobiotics, etc. and occurs in medical conditions such as inflammation, ischaemia/reperfusion (heart failure, acute renal failure, and stroke). Under these conditions, highly reactive toxic oxygen species are generated causing oxidative damage to proteins and/or nucleic acid and lipid peroxidation (Healy *et al.* 1992). Superoxide dismutase is induced by heat in bacteria but not in human cells, while haem oxygenase is induced by oxidant injury in humans and in rats also by hyperthermia (Healy *et al.* 1992). During phagocytosis, superoxide dismutase and haem oxygenase are expressed by monocytes and macrophages to protect themselves from the highly reactive oxygen species generated (Healy *et al.* 1992; Clerget and Polla 1990).

1.1.7 HSP and Development

In germ cells, from a variety of organisms, HSP expression changes during different stages of development. For example, in mouse testis two different *HSP70* genes (*hsp70.1* and *hsp70.2*)[1] show unique patterns of expression. The *hsp70.1* transcript like the *hsp70.2* transcript is restricted to the germinal compartment. However, *hsp70.2* is

1. *hsp70.1* and *hsp70.2* give rise to transcripts of 3.5kb and 2.4kb in length and their expression is restricted to the germinal compartment (the compartment where spermatozoa are produced) of the mouse testis unlike the abundant hsp70 (2.7kb transcript) which is expressed in somatic tissues. *hsp70.1* and *hsp70.2* should not be confused with the *hsp70-1*, *hsp70-2* and *hsp70-hom* genes which have been mapped to the MHC Class III region.

specifically expressed during the meiotic prophase (Zakeri and Wolgemuth 1987; Zakeri *et al.* 1988; Krawczyk *et al.* 1988). Similarly, in human testis, a *HSP70* (designated *73T*) is expressed during prophase and in post-meiotic germ cells but is not inducible by HS(Zakeri *et al.* 1990).

The HS response is also suppressed during certain early stages of development. In the mouse embryo prior to the blastocyst stage, HS does not induce HSP expression (Morange *et al.* 1984). Similarly, at the 16-cell stage of the rabbit embryo, HS is unable to induce HSP synthesis (Heikkila *et al.* 1985). HS of embryos also induces developmental malformations. Embryos are most sensitive to HS-induced malformations during the earlier stages of development, before organogenesis. After organogenesis, malformations induced by HS are not as severe (German, 1984; Lary (1986). However, by preinducing HSP expression in embryos with a well timed mild HS, the severity of malformations caused by a severe HS can be reduced (Mirkes 1987; Walsh *et al.* 1987).

As yet, the exact functions of HSP in stressed and nonstressed germ cells and embryos are not known, but studies suggest that these HSP function in the usual manner by mediating protein-protein interactions and protein transport (Ananthan *et al.* 1986; Pelham 1985; Pelham 1988; Pelham 1986).

1.2 CHAPERONES

1.2.1 Introduction

Chaperones are protein catalysts which interact with polypeptide substrates for the purpose of inducing conformational changes and in some instances to block unproductive protein-protein interactions (Ellis 1987; Wynn, *et al.* 1994). Through this process chaperones help damaged or nascent protein molecules fold into a native conformation and help assemble protein monomers into multisubunit complexes (Ellis 1987; Parsell and Lindquist 1993). Some of the chaperones are also involved in the process of protein transportation across the membranes of organelles or across cellular membranes in the

case of secreted proteins. A chaperone may differ slightly or be identical to a specific HSP, or SP, both in function or amino acid sequence, but unlike the inducible HSP, chaperones are constitutively expressed during normal conditions (e.g. hsp70 and its constitutively expressed analogue hsc70). Not all chaperones are HSP and therefore not all are heat- or stress-inducible. Chaperones and their analogues are found in all living organisms and include the: HSP90, HSP70, HSP60 families, proline isomerases and the disulphide isomerases (Craig *et al.* 1993).

Other types of peptides which also catalyse the folding of protein molecules, are the pro-regions of pre-proteins (Agard, 1993). Both amino and carboxy terminal pro-regions are possible and these are later cleaved from the molecule to produce a mature protein. Pro-regions are common to many bacterial and eukaryotic proteases and are required for the proper folding of the proteases (Silen and Agard 1989; Ohta *et al.* 1991; Baker *et al.* 1992). However, pro-regions do not suppress protein aggregation like some of the chaperones (Agard, 1993). Experimentally, by using a protease lacking its pro-region, it can be shown that in its unfolded conformation the protease is stable without any aggregation or folding. However, on the separate addition of the pro-region, rapid folding of the protease follows (Baker *et al.* 1992). Unlike some of the chaperones, the pro-regions only promote forward folding and do not require nucleotide tri-phosphate hydrolysis to drive the reaction (Agard, 1993).

1.2.2 HSP90 Chaperones

HSP90 stabilise a variety of target proteins in an inactive or unassembled state. Hsp90 stabilises the unfolded conformation, therefore blocking activation by steric influence of tyrosine kinases and steroid hormone receptors and other proteins until they are properly localised. In the case of the glucocorticoid receptor, hsp90 maintains the receptor in an inactive state by remaining bound to the receptor. Binding of the hormone results in the release of hsp90 from the receptor complex and conversion of the receptor

from a non DNA binding to DNA binding form (Scherrer *et al.* 1990; Pratt 1990; Huchison *et al.* 1992). Hsp70 is also present in the hsp90-receptor complex and may play a role in receptor folding (Hutchinson *et al.* 1994). Other functions of hsp90 include the facilitation of protein folding such as denatured citrate synthase and the Fab fragment of monoclonal antibodies (Gething and Sambrook 1992; Craig *et al.* 1993).

1.2.3 HSP70 Chaperones

The HSP70 family, as chaperones, are primarily involved in the folding, unfolding and maintaining an extended conformation while preventing aggregation of a polypeptide prior to its transport across a membrane (Beckmann *et al.* 1990). Not only do HSP70 hydrolyse ATP to induce conformational changes on themselves and their protein substrates, but ATP hydrolysis is also required for the release of most polypeptides from HSP70. However, the association can be prolonged if the polypeptide has an aberrant conformation or cannot be assembled into its appropriate oligomeric complex (Gething and Sambrook 1992). The HSP70 chaperones have been well investigated in the yeast model *(Saccharomyces cerevisiae)*. The yeast HSP70 analogues are the Ssc1p, which reside primarily in the mitochondria, Kar2p, which reside in the ER, and the SSA and SSB members, which reside in the cytosol (Giardina *et al.* 1992; Hendrickand Hartl 1993; Parsell and Lindquist 1993). Inactivation of Ssc1p (temperature sensitive yeast mutants), can demonstrate blocks in mitochondrial import. For example, it was found that precursor proteins destined for the mitochondria were inserted into the mitochondrial membrane but were not translocated across the membrane (Kang *et al.* 1990). This suggested that Ssc1p was required for the efficient import of proteins into the mitochondria (Kang *et al.* 1990). Mitochondrial import of proteins is proposed to occur by the following pathway: partial insertion across both outer and inner membranes, cleavage of the pre sequence, mHsp70/Ssc1p interaction with the polypeptide and (by an unknown mechanism) transport of the polypeptide across the membrane. As the protein enters the

mitochondrion, hsp70/Ssc1p molecules bind to the incoming protein, inducing a directional movement and preventing the protein from moving backwards (Deshaies *et al.* 1988; Kang *et al.* 1990; Neupert *et al.* 1990).

In vitro the addition of the cytosolic hsp70/SSA to a suspension of mitochondria and preproteins results in import of these proteins into the mitochondria (Deshaies *et al.* 1988). Alternatively, import into mitochondria can be demonstrated when the proteins are denatured with urea before presenting them to the mitochondria (Kang *et al.* 1990). This observation implies that cytoplasmic hsp70/SSA changes the protein's conformation to a translocation competent conformation allowing it to be thread through the membrane and imported into the mitochondrion (Craig *et al.* 1993).

The endoplasmic reticulum (ER) localised HSP70 (grp78) is known as the immunoglobulin heavy chain binding protein (BiP) (Munro and Pelham 1986). In yeast Kar2p is the ER analogue of the mammalian BiP (Craig *et al.* 1993). BiP associates with unassembled immunoglobulin heavy chain molecules and other proteins (Hass and Wabl 1983). The association of BiP with proteins is also disrupted by ATP addition but not by non hydrolysable ATP analogues (Munro and Pelham 1986). As in yeast mitochondria, where Ssc1p is required for polypeptide import, Kar2p is required for import of polypeptides into the ER (Kassenbrock *et al.* 1988; Craig *et al.* 1993).

In the *Saccharomyces cerevisiae* cytosol, localised HSP70 are derived from a multigene family comprised of six genes. These genes are divided into two subfamilies, the *SSA* and the *SSB* families. There are four members in the *SSA* subfamily: *SSA1*, *SSA2*, *SSA3* and *SSA4* (Craig *et al.* 1993). The *SSA* subfamily are essential for cell viability as they are involved in protein transport, the heat shock response, and other functions. There are two members in the *SSB* family: *SSB1* and *SSB2* (Craig *et al.* 1993). The SSB proteins are constitutively expressed but also inducible by cold (Craig and Jacobsen 1985). They are found associated with ribosomes and are involved in protein synthesis (Craig *et al.* 1993). Mutants in both *SSB* genes grow slowly but the growth rate is decreased even further compared to wild type if the temperature is reduced (Craig and Jacobsen 1985).

1.2.4 HSP60 Chaperones

The HSP60 family of chaperones includes the bacterial GroEL protein and the ribulose bisphosphate carboxylase subunit binding protein of chloroplast stroma (RUBISCO) (Craig *et al.* 1993). GroEL, like HSP60 are arranged as complexes of two rings, side by side, each made of seven monomers (Ellis and Van Der Vies 1991). One or two protein substrate molecules bind to each hsp60/GroEL complex near the large cavity of the 14 subunit protein. Unfolded proteins complexed with hsp60 are protease sensitive. When ATP is added it causes the release of the peptide and the peptide becomes protease resistant (Craig *et al.* 1993). The hsp60 complex binds to, and stabilises regions of protein secondary structure during folding, allowing regions, which take longer to fold,

more time to fold (Craig *et al.* 1993). Evidence suggests hsp70 and hsp60 function sequentially in protein folding (Craig *et al.* 1993). In bacteria GroEL binds unfolded peptides directly, later GroES binds to the GroEL-peptide complex. GroES influences the release of the peptide by modulating ATP hydrolysis (Craig *et al.* 1993). The hsp60/GroEL complex is important in the binding of unfolded polypeptide precursors before their export as secretory proteins, in the assembly into oligomeric complexes and help in the folding and assembly of polypeptides translocated into organelles (Gething and Sambrook 1992).

In the eukaryotic cytosol there have been no hsp60/GroEL identified by specific anti-hsp60 antibodies. However protein complexes with a double doughnut appearance have been identified with the electron microscope (Hendrick and Hartl 1993). These complexes are similar to those having ATPase activity in *Pyrodictium occultum*, an organism which thrives in temperatures above 50°C (Phipps *et al.* 1991). Also a related protein, thermophilic factor 55kDa (TF-55), is synthesised in large amounts, by *Sulfolobus shibatae*, after heat shock. TF-55 assembles into a complex consisting of two rings arranged side by side each comprised of eight TF-55 monomers (Trent *et al.* 1991). TF-55,

like hsp60/GroEL, binds unfolded proteins but not their native counterparts and has a weak ATPase activity. TF-55 is homologous to the eukaryotic protein TCP1 which is not a heat shock protein (Hendrick and Hartl 1993). Mutants in *tcp1* fail to assemble normal mitotic spindles in *Saccharomyces cerevisiae* at non permissive temperatures (Ursic and Culbertson 1991). TRiC (TCP1 ring complex) has been purified from mammalian cells (Ursic and Culbertson 1991; Hendrick and Hartl 1993). TRiC is comprised of the 60kDa TCP1 and 5 to 7 other proteins (Mr 50 to 68kDa) arranged in two rings, stacked side by side, having eightfold symmetry (Ursic and Culbertson 1991; Frydman *et al.* 1992; Gao *et al.* (1992; Lewis *et al.* 1992; Yaffe *et al.* 1992). TRiC interacts with unfolded polypeptides and requires ATP before release of the bound polypeptide substrate is possible (Hendrick and Hartl 1993).

Therefore, HSP60 are another well conserved family of proteins with homology between species and in cells of the same species. All HSP60 homologues display functional and structural similarities and play important housekeeping roles in unstressed organisms.

1.2.5 Proline Isomerases & Disulphide Isomerases

The protein disulfide isomerases (PDI) and peptidyl-prolyl *cis-trans* isomerases (PPI) also play a role in protein folding. Although little is known about most of the PDIs and PPIs, it is known that FKBP60, a PPI related protein, is a heat shock protein of 56kDa (hsp56) (Sykes *et al.* 1993; Sanchez 1990). PPIs catalyse *cis-trans* isomerisation of X-Pro peptide bonds to accelerate the slow phase of folding of several proteins which depend on proline isomerisation (Stamnes and Zuker 1990; Lang *et al.* 1987; Takahashi *et al.* 1989; Schonbrunner *et al.* 1991). Meanwhile, the PDIs can be found in the lumen of the ER of eukaryotic cells catalysing the formation of disulfide bonds in reduced proteins or newly synthesised proteins (e.g. immunoglobulins) (Noiva and Lennarz 1992; Craig *et al.* 1993).

Thecyclophilinsand the FKBPs, are related to but distinct from PPIs. Cyclophilins and FKBP catalyse the same chemical reaction, prolyl *cis-trans* isomerisation or proline rotamerization (Schreiber 1991; Schreiber and Crabtree 1992). The prolyl peptide bond has partial double bond character. Since the *trans* isomer is favoured for all amino acids except proline because of steric interactions between side chains, *cis* or *trans* isomers can prevail (Heitman *et al.* 1992; Liu *et al.* 1990). Even the ribosomes synthesise the peptide bond as the *trans* isomer (Heitman *et al.* 1992). *Trans* to *cis* isomerization may occur during or after translation. During *in vitro* folding, isomerisation can sometimes be the rate limiting step during protein folding (Schmid *et al.* 1992; Fischer and Schmid 1990). Cyclophilins and FKBP both catalyse isomerisation and accelerate the folding of some proteins. Cyclophilins accept a variety of Ala-Xaa-Pro-Phe peptides as substrates whereas FKBP prefers peptides in which Xaa is a hydrophobic amino acid (Craig *et al.*1993; Heitman *et al.* 1992).

Therefore proline and disulphide isomerases, although not necessarily inducible by hyperthermia or stress, have functional similarity to the HSP chaperones in their ability to help proteins fold into their correct conformation. However there exists one major difference: chaperones generally catalyse conformational changes without disturbing covalent bonds, whereas disulphide isomerases catalyse disulphide bond formation.

1.3 THE IMMUNE SYSTEM AND HSP

1.3.1 The MHC Locus

In higher eukaryotes, related genes expressed specifically for a certain purpose or function are usually organised in clusters, called loci. In mammals, the immune system's major histocompatibility complex (MHC) genes are divided into three classes, MHC Class I, II and III, and for man, mouse and rat, all three groups are found adjacent to each other on the same chromosome. The Class I and Class II genes encode cell surface molecules which generally consist of two polypeptide chains. Class III genes map between

the Class II and Class I genes and include members of the complement, tumour necrosis factor (TNF) and two *hsp70* genes to name just a few (Spies *et al.* 1989; Sargent *et al.* 1989). The location of these *hsp70* genes suggests that hsp70 plays an important role for the proper functioning of the immune system (as discussed in more detail below).

1.3.2 HSP in Antigen Processing and Presentation

Class I and II MHC molecules are polymorphic cell surface glycoproteins which bind peptides extremely tightly. The role of MHC molecules is to display antigenic peptides derived from foreign globular proteins by a protease degradation pathway on the cell surface. Once on the cell surface the MHC-peptide complex is accessible to and recognised by T cell receptors resulting in the activation of cytotoxic and helper T cells. T cell recognition of MHC Class I-peptide complexes results in cytotoxic T cell responses, whereas recognition of MHC Class II-peptide complexes elicits helper T cell responses. The MHC Class I molecules normally present peptides derived from intracellular protein antigens, such as viral proteins, on virally infected cells. MHC Class II molecules usually present peptides derived from internalised exogenous protein antigens (Cresswell 1990).

In humans, the *MHC* genes are adjacent to each other on the short arm of chromosome 6 (Dunham *et al.* 1987; Carroll *et al.* 1987). The MHC is divided into three regions: the MHC Class I, MHC Class II and, MHC Class III. The *MHC Class I* genes are encoded at the telomeric end, the *MHC Class II* genes at the centromeric end while the *MHC Class III* genes occupy a central region of approximately 1000kbp (Dunham *et al.* 1987; Davies and Bjorkman 1988; Trowsdale 1987). The MHC class III region encodes members of the complement cascade, some cytokines, TNFα and TNFβ and three *hsp70* [1] genes (Dunham *et al.* 1987; Carroll *et al.* 1987; Carroll *et al.* 1984; Sargent *et al.* 1989). Presently, it is not clear why *hsp70* genes should be part of the MHC locus. However, as discussed below, members of the hsp70 family play important roles as part of the immune system, from the assembly of antibodies to the processing of antigens.

1. Two of the three *hsp70* genes mapped to the MHC Class III region are inducible (*hsp70-1* and *hsp70-2*) while the third (*hsp70-hom*) is not inducible. See also page 9.

Also, hsp70 genes are linked to the MHC of all mammals that have been examined (Salter-Cid *et al.* 1994). Therefore, the occurrence of *hsp70* genes within the MHC is likely to have a strategic purpose rather than being purely coincidental.

The peptide binding proteins (PBP) of Mr72 and 74kDa (PBP72/74) are found expressed on the surface of B cells and macrophages, but not on the surface of T cells, natural killer (NK) cells, or fibroblasts (VanBuskirk *et al.* 1989; Manara *et al.* 1993). However, PBP72/74 has been isolated from cellular lysates of B cells, T cells and fibroblasts (Lakey *et al.* 1987). The PBP72/74 expressed on cellular surfaces bind exogenous peptides but not to exogenous globular proteins (Lakey *et al.* 1987), which require internalisation and proteolytic processing before presentation by MHC Class II molecules. Antibodies raised against PBP72/74 have been shown to block the formation of PBP72/74-peptide complexes on the cellular surface by preventing peptide accessibility and hence, preventing the stimulation of T cells (Kaufmann 1991). By binding exogenous peptides and presenting them directly to T cells, PBP72/74 capitalises on the protease-degraded state of some exogenous proteins, partly eliminating the need for the more lengthy process of protein internalisation and processing by antigen presenting cells (APC) (Lakey *et al.* 1987).

PBP72/74 is also localised to cellular endosomes where it is likely to bind peptides of protease-processed extracellular proteins. PBP72/74 has been proposed to play a role in preventing total protease degradation of peptides and subsequently to act as a chaperone in transporting the peptides to, and helping to load them on to, MHC molecules (Kaufmann 1991).

Antibodies specific to hsp70 bind to the PBP72/74 which are expressed on the surface of APC (Manara *et al.* 1993) . Because of this observation and other similarities between members of the HSP70 family and PBP72/74, VanBuskirk (1989) and coworkers further characterised[1] Like HSP70, PBP72/74 bound to ATP-agarose and could be eluted with ATP or an appropriate peptide solution. PBP72/74 also released bound peptides on addition of ATP and is recognised by anti-HSP70 monoclonal antibodies on Western

1.Should read: Because of this observation and other similarities between members of the HSP70 family and PBP72/74, VanBuskirk (1989) and coworkers further characterised PBP72/74.

blots (Vanbuskirk *et al.* 1989 ; Lakey *et al.* 1987) . This evidence strongly suggests that PBP72/74 is a member of the HSP70 family and that HSP70 analogues are a vital part of the immune system.

1.3.3 HSP and the Assembly of Antibodies

Antibodies are composed of four polypeptide chains, two identical light chains (IgL) and two identical heavy chains (IgH). Each chain is synthesised separately and assembled post-translationally with the three other chains in the ER to produce an antibody. The chains are in part held together by inter-chain disulfide bonds. In addition each chain has regularly spaced intra-chain disulfide bonds and the heavy chain is glycosylated.

Precipitation of IgH results in the coprecipitation of a 78kDa protein which was named immunoglobulin heavy chain binding protein (BiP) (Hass and Wabl 1983). BiP was found in the soluble fraction of the ER and associated with the immunoglobulin heavy chain only if it had not already associated with a light chain (Munro and Pelham 1986; Kassenbrock *et al.* 1988). In the absence of BiP, IgH aggregates and therefore forms inclusion bodies. Extensive build-up of protein inclusion bodies normally results in cell death. BiP prevents aggregation by maintaining IgH in a soluble form until it becomes incorporated into an antibody molecule (Kohler 1980). It was later discovered that BiP is identical to the grp78 stress protein (Munro and Pelham 1986; Hass, and Meo 1988). BiP-protein complexes, like the grp78-protein complexes, are disrupted on exposure to ATP; BiP also binds proteins which are malfolded, under-glycosylated, or contain thiol groups (Munro and Pelham 1986).

1.3.4 Immunisation Against Tumours

Tumours arising from non-viral infections are the result of genetic mutations occurring spontaneously or induced by environmental conditions. Mutations in the

promoter regions of a gene can cause over-expression or under-expression of a gene product. This may give rise to a tumour cell while not necessarily giving rise to any mutant protein within the cell. Mutations in the coding sequence of genes may give rise to altered protein structure. Structural changes in proteins, in particular proteins associated with cellular growth control, can give rise to a tumour cell. Not all mutations result in tumours as some proteins do not influence cellular tumourgenicity. However, in situations where tumours, have resulted from mutations causing protein structural changes, immunotherapy can be directed towards the aberrant protein(s) to help eliminate the tumour cells.

The discovery and characterisation of tumour-specific antigens is of great interest. A tumour-specific antigen would allow the use of immunisation as a treatment against growth and spread of cancer. Up to now, two major antigenic differences have been identified which distinguish tumours from normal tissue. These are (i) normal tissue antigens expressed at considerably elevated levels and, (ii) constitutively expressed antigens which normally are expressed during specific stages of development (Old 1981; Kaufmann 1991). Such antigens serve well as tumour markers for the purpose of diagnosis and tumour identification but, because they are not unique to tumours, their use in immunisation has the potential of inducing autoimmunity against healthy tissues.

Although a universal tumour-specific antigen has not yet been found, studies have demonstrated that mice immunised with inactivated tumour cells were immune to tumour development when challenged with live tumour cells (Srivastava 1993). In this study, inbred mice with tumours induced by chemical carcinogens were cured by surgically removing the tumour. When cells of that tumour were later reinjected into the mice, those mice demonstrated resistance towards tumour formation. However, naive mice, injected with the tumour cells, developed tumours. Immunity was specific to only the tumour which the mice had been exposed to and not other tumours. This specificity even applies to tumours induced by the same carcinogen in the same inbred colony of mice and of the same histological origin (Srivastava 1993).

Specific proteins from tumours have been purified and tested for their ability to immunise against tumour formation. In these studies immunisation of mice and rats with HSP prevented the development of tumours when challenged with live tumour cells (Udono and Srivastava 1993). Udono and Srivastava (1993) have isolated hsp70 from a chemically induced tumour in mice and used this preparation to immunise naive mice against the same tumour. Immunogenicity was dose dependent and tumour specific. Hsp70 isolated from normal tissue did not have the ability to protect mice when challenged with live tumour cells. However, when the hsp70 preparation, which was isolated from tumour tissue, was treated with ATP (followed by the removal of low molecular weight peptides), the resulting intact hsp70 had lost its ability to immunise against tumour formation (Udono and Srivastava 1993). In this procedure, the added ATP was hydrolysed by the hsp70 ATPase followed by the subsequent release of the peptides bound by the hsp70. Udono and Srivastava suggest that the immunogenicity of the original HSP70 preparation is not due to the hsp70 molecule alone but more likely due to the peptides associated with hsp70 (Udono and Srivastava 1993).

Members of the HSP90 family, gp96/grp94, have also demonstrated an ability to immunise against tumour formation in mice. Once again immunisation with gp96 was tumour specific and worked only if the gp96 has been isolated from tumours and not from normal tissue (Blachere *et al.* 1993; Li and Srivastava 1993; Srivastava 1993). The sequence of gp96 cDNA derived from tumour and normal tissue was examined and found to contain no tumour specific mutations. However, post-translational modifications to gp96, such as glycosylation are still a possibility. But gp96 also binds peptides and when these peptides were removed, gp96 also lost its ability to immunise mice against introduced tumours (Srivastava 1993).

Immunisation experiments on animals, so far, have demonstrated stringent tumour specificity. HSP prepared from one tumour do not immunise animals against other tumours. This observation can be explained by the statistical probability of chemically induced mutations in genetic DNA. Chemically induced mutations resulting in

tumours are likely to occur in different genes or locations within the same gene. Therefore the mutant proteins from different tumours will show diversity and immunisation with one protein will not necessarily protect against tumours which carry a different mutation.

Isolating HSP from tumours suitable for immunotherapy has been argued to have advantages over other methods of immunisation against cancer. Since tumour antigens, like other peptides, are carried by the HSP chaperones, immunising with the peptide-HSP complex makes isolating tumour antigens simpler and elicits a stronger immune response than with the peptide alone. Also immunising with purified HSP reduces the risk of transmitting infection between individuals, although the possibility of inducing autoimmunity needs to be investigated (Udono and Srivastava 1993; Li and Srivastava 1993; Srivastava 1993; Jaattela 1993; Kaufmann 1991).

1.4 RHEUMATOID ARTHRITIS AND HSP

1.4.1 Introduction

Rheumatoid arthritis (RA) is a chronic, systematic, inflammatory disorder involving the joints, which eventually results in the destruction of the joint and severe deformity. The cause of RA is as yet unknown however, the pathogenesis of rheumatoid arthritis involves white blood cells entering the affected joints. Once in the joint, the white blood cells release, and cause other cells to release, cytotoxic agents such as cytokines, proteolytic enzymes (hyaluronidase, collagenase, elastase, etc), oxy-radicals, prostaglandins, etc (Lacour *et al.* 1990). As the inflammation progresses, localised fever develops and the joint also swells with an increased amount of synovial fluid. This results in an increased intra-articular pressure, which prevents the proper circulation of blood through the joint, resulting in hypoxia (Blake *et al.* 1989). In addition, white blood cells also release chemotactic agents, inducing more white blood cells to enter the joint and therefore perpetuate the inflammation. Hence, new white blood cells entering an

inflamed joint are confronted with a hostile intra-articular environment. In addition, the cells of the joint (synovial membrane cells, chondrocytes of the cartilage, osteocytes of the bone) are continuously subjected to these stresses which results in the formation of abnormal tissues and deformation of the joint. Joints affected by RA have also been shown to have elevated HSP expression (Bernstein 1989; VanEden 1991), suggesting that the stress levels are physiologically relevant.

1.4.2 HSP in Auto-Immunity

One popular rational, for the pathogenesis of RA, proposes the involvement of auto-reactive T lymphocytes which arise from the cross reaction of the mycobacterial hsp65 with human hsp65 analogues (Van Eden 1991; Karlsson-Parra *et al.* 1990; Evans *et al.* 1990; De Graeff-Meeder *et al.* 1990). It was proposed that individuals infected with mycobacteria, develop T cell clones which react with the mycobacterial hsp65 antigen and that these clones then cross-react with the HSP in the joints. This conclusion was supported by some experiments which demonstrated mycobacterial hsp65 reactive T lymphocytes in the joints and peripheral blood of RA sufferers (Gaston *et al.* 1989; Danieli *et al.* 1992).

Furthermore, this view has been supported by animal studies using the adjuvant-induced arthritis (adjuvant arthritis) model in rats. Adjuvant arthritis (AA) can be induced in rats by inoculation with emulsified *Mycobacterium tuberculosis* in Freund's adjuvant (oil) (Cromartie *et al.* 1977). Rats with AA were found to have T cells which cross-reacted with a mycobacterial hsp65 epitope and a cartilage proteoglycan epitope (Cohen *et al.* 1985). When these T cells were isolated and cloned and then injected into irradiated rats (rats which had their immune systems destroyed by radiation), the rats went on to develop an arthritis which resembled AA.

However, as research into this particular area of autoimmunity progressed, more inconsistencies arose. In the first instance, mycobacterial infections are common. Most

individuals have been predisposed to some degree of mycobacterial infection during their lifetime and yet most do not go on to develop RA. Furthermore, mycobacterial hsp65 reactive T cells have also been isolated from overwise healthy individuals, suggesting, that the occurrence of hsp65 reactive T cells is a more general phenomenon in the population rather than specifically linked to individuals with autoimmune disease (Munk *et al.* 1989; Kumararatne *et al.* 1990). Also a study by Res *et al.* (1990), which examined mononuclear cells from RA and non-RA individuals, led them to conclude that the occurrence of mononuclear cells with an enhanced reactivity to mycobacterial and other bacterial antigens was common to chronic inflammation and not restricted to mononuclear cells from inflamed joints (Res *et al.* 1990). Another dilemma arose when it was shown that the anti-rheumatic gold salts, which are widely used in RA treatment, induced HSP synthesis in human peripheral blood MNC and in mammalian cell lines (Caltabiano *et al.* 1986, 1988). These agents were inducing the very proteins which were considered by some to be targets for specific T cells but yet these same agents are beneficial in the treatment of RA (Van Den Broek *et al.* 1989).

Another inconsistency was demonstrated when rats immunised with purified preparations of mycobacterial hsp65 did not develop AA but rather developed resistance to the induction of AA by the crude mycobacterial adjuvant preparation (Lopez-Guerrero *et al.* 1994; Golden *et al.* 1991). Furthermore, Lopez-Guerrero *et al.* (1994), by using a recombinant human hsp60 vaccine, demonstrated a therapeutic effect on already established AA.

Therefore the above contradictory evidence suggests that still more work needs to be done in this area to help resolve the role of hsp65/hsp60 in autoimmunity.

AIMS OF THESIS

In rheumatoid arthritis (RA) the affected joints are slowly eroded and deformed by the disease. The insighting stimulus which causes RA is not known. However, the hostile environment of the joint further aggravates the condition by causing further damage to the joint tissues. There is already a large number of mononuclear cells (monocytes and lymphocytes) as well as polymorphonuclear cells in the joint which contribute to the stressful environment by releasing more stressful agents but fresh white blood cells (WBC) still continue to enter the joint. These WBC are confronted and damaged by the stresses located in the joint. These fresh WBC become stimulated and inappropriately release more cytotoxic agents and hence perpetuate the inflammatory condition.

Others have already demonstrated the induction of HSP by the anti-rheumatic gold salts, while other anti-inflammatory/rheumatic agents alone did not demonstrate HSP induction or otherwise have not been investigated for HSP expression. This thesis is based on the hypothesis that some of the anti-inflammatory/rheumatic agents used in the treatment of rheumatoid arthritis induce or facilitate HSP synthesis in the peripheral blood white cells (WBC) while they are still in the circulation. Therefore when WBC move into the joint, their metabolic functions are protected or, the WBC are better prepared to respond to the stress by synthesising more HSP. The elevated levels of HSP would function to protect the regulatory mechanisms of WBC metabolism from damage and allow WBC to respond appropriately to resolve the inflammation. In addition, facilitation of HSP synthesis in the tissues of the joint would help protect them better from the stresses and reduce the amount of abnormal tissue formation and therefore reduce the level of joint deformation.

The aim of this thesis is to determine: (a) whether or not the anti-inflammatory/ rheumatic agents selected for this thesis induce or facilitate *HSP* expression in *in vitro* cultured MNC and, (b) whether or not the increased levels of HSP, induced by the anti-inflammatory/rheumatic agent(s), protect normal MNC protein synthesis from a

subsequent exposure to a severe heat shock which would normally inhibit normal protein synthesis.

CHAPTER TWO

Materials & Methods

2.1 MATERIALS

2.1.1 Chemicals and Reagents

All chemicals and reagents used were of the highest purity (analytical grade) unless stated otherwise.

Acrylamide (Ultrograde)	LKB-Produkter AB Bromma, Sweden
Ammonium Chloride (NH_4Cl)	Ajax Chemicals, Australia
Anti-Heat Shock Protein 70 Mouse Monoclonal Antibody (IgG) (clone 3A3)	Affinity BioReagents, USA
Anti-Heat Shock Protein 90 Mouse Monoclonal Antibody (IgG) (clone 3B6)	Affinity BioReagents, USA
Anti-Mouse IgG (H+L) Alkaline Phosphatase Conjugated Antibody	Promega Corporation, Australia
APS (Ammonium Persulphate)	BioRad Laboratories Pty. Ltd, Australia
Aspirin (Acetylsalicylic Acid)	Sigma Chemical Company, U.S.A.
Benzylpenicillin Sodium	Commonwealth Serum Laboratories Ltd, Australia
Biotinylated Standards (low molecular weight range)	BioRad Laboratories Pty. Ltd, Australia
Bis-acrylamide (Ultrograde)	LKB-Produkter AB Bromma, Sweden
Bromophenol Blue Sodium	Sigma Chemical Company, U.S.A.
BSA (Bovine Serum Albumin)	Sigma Chemical Company, U.S.A.
Coomassie Blue R250	LKB-Produkter AB Bromma, Sweden
Chloroquine (Diphosphate salt)	Sigma Chemical Company, U.S.A.
Cronex MRF33 Blue Video Imaging Film	Du Pont, U.S.A.
Diclofenac Sodium	Sigma Chemical Company, U.S.A.
DMF (N, N-Dimethylformamide)	Ajax Chemicals, Australia
EDTA (Ethylenediaminetetraacetic Acid)	Sigma Chemical Company, U.S.A.
Electrophoresis Calibration Kit (low molecular weight standards)	Pharmacia LKB Biotechnology Inc. U.S.A.
FCS (Foetal Calf Serum)	Commonwealth Serum Laboratories Ltd, Australia
Folic Acid (Cell Culture Tested)	Sigma Chemical Company, U.S.A.

Glacial Acetic Acid	BDH Chemicals, Australia
Glycerol	Ajax Chemicals, Australia
Glycine	Ajax Chemicals, Australia
Hanks' Balanced Salt Solution (10X liquid without sodium bicarbonate and phenol red)	Gibco Laboratories U.S.A.
Hydrochloric acid (HCl) 11.45M	BDH Chemicals, Australia
HEPES (N-2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid)	BDH Chemicals, Australia
Indomethacin	Merck Sharp & Dohme
L-methionine [35S] (Cat # 51001H05)	ICN Biochemicals Australasia, Australia
Lymphoprep (Sodium Metrizoate/Ficoll)	Nycomed, Norway
Magnesium Chloride ($MgCl_2$)	Sigma Chemical Company, U.S.A.
Methanol (HiPerSolv)	BDH Chemicals, Australia
Methotrexate ((+)Amethopterin)	Sigma Chemical Company, U.S.A.
Millex-GS 0.22µm filter unit	Millipore, U.S.A.
NBT (4-Nitro blue tetrazolium chloride)	Boehringer Mannheim Biochemica, Australia
Paracetamol (4-Acetamidophenol)	Sigma Chemical Company, U.S.A.
D-Penicillamine	Eli Lilly, Australia
pH-indicator strips (Neutralit pH 5-10 Art. 9533)	E. Merck, Germany
Potassium Chloride (KCl)	Ajax Chemicals, Australia
Potassium di-Hydrogen Orthophosphate (KH_2PO_4)	Ajax Chemicals, Australia
Prostaglandin A_1	Sigma Chemical Company, U.S.A.
PVDF (polyvinylidene fluoride) membrane	BioRad Laboratories Pty. Ltd, Australia
RPMI powder (Methionine Deficient)	Commonwealth Serum Laboratories Ltd, Australia
RPMI powder (without Sodium Bicarbonate)	ICN Biochemicals Australasia, Australia
[35S] L-methionine (Cat # 51001H05)	ICN Biochemicals Australasia, Australia
Salicylate (Sodium)	Sigma Chemical Company, U.S.A.
SDS (Sodium-Dodecyl-Sulphate)	Sigma Chemical Company, U.S.A.
Sodium Azide (NaN_3)	Sigma Chemical Company, U.S.A.

Sodium Chloride (NaCl)	Ajax Chemicals, Australia
Sodium Hydrogen Carbonate ($NaHCO_3$)	Ajax Chemicals, Australia
di-Sodium Hydrogen Orthophosphate (Na_2HPO_4)	Ajax Chemicals, Australia
Sodium Hydroxide (NaOH)	Ajax Chemicals, Australia
Sterivex-GS 0.22μm filter unit with filling bell	Millipore, U.S.A.
Streptavidin-Alkaline Phosphatase Conjugate	BioRad Laboratories Pty. Ltd, Australia
Streptomycin Sulphate	Sigma Chemical Company, U.S.A.
TEMED (N,N,N',N',-Tetramethylethylenediamine)	BioRad Laboratories Pty. Ltd, Australia
Triton X-100	BioRad Laboratories Pty. Ltd, Australia
Trypan Blue	Commonwealth Serum Laboratories Ltd, Australia
Vitamin B_{12} (Cell Culture Tested)	Sigma Chemical Company, U.S.A.
Whatman 3MM Chromatography Paper	Whatman International Ltd, England
X-Phosphate (5-bromo-4-chloro-3-indolyl phosphate toluidinium Na_2)	Boehringer Mannheim Biochemica, Australia

2.1.2 Solutions and Buffers

The composition of the solutions and buffers used is given below:

APS (Ammonium Persulfate) 10%

Compound	Concentration	Quantity
Ammonium Persulfate	approx. 440mM	0.02g
H_2O		200µl
Prepared fresh on the day.		

Culture Medium RPMI, Complete

Compound	Concentration	Quantity
RPMI 1640 powder	1X	10.418g
HEPES	25mM	5.95g
$NaHCO_3$	21mM	1.80g
Streptomycin Sulphate	0.5mg/ml	0.5g
Benzylpenicillin Sodium	0.03mg/ml	0.03g
NaOH solution		adjust to pH 7.2
FCS (Heat inactivated)	Add only when 2.5% (v/v) FCS media is required	25ml
H_2O		final volume 1000ml
Filter sterilise through a Sterivex-GS 0.22µm filter unit with filling bell.		

Destaining Solution

Compound	Concentration	Quantity
Methanol	45% (v/v)	225ml
Glacial Acetic Acid	10% (v/v)	50ml
H_2O		final volume 500ml

EDTA 4.5%, pH 7.4

Compound	Concentration	Quantity
EDTA	4.5% (w/v)	45g
NaOH solution		adjust to pH 7.4
H_2O		final volume 1000ml

Electrophoresis Buffer

Compound	Concentration	Quantity
Trizma Base	$50E10^{-3}$mM	0.006g
Glycine	0.37mM	0.028g
SDS	0.1% (w/v)	1g
H_2O		final volume 1000ml

Hanks' Buffered Salt Solution (HBSS)

Compound	Concentration	Quantity
Hanks' Buffered Salt Solution (10X)	1X	100ml
$NaHCO_3$ (0.5M Solution)		adjust to pH 7.2
H_2O		final volume 1000ml

Lysis Buffer

Compound	Concentration	Quantity
Trizma Base	10mM	0.012g
Triton X-100 (10%)	1% (v/v)	1ml
SDS (10%)	0.1% (v/v)	0.1ml
NaCl	150mM	0.088g
HCl solution		adjust to pH 7.6
H_2O		final volume 10ml

Magnesium Chloride Solution (1M)

Compound	Concentration	Quantity
$MgCl_2$	1M	9.25g
H_2O		final volume 100ml
Autoclave for 20min at 121°C.		

Methionine Deficient RPMI Cell Culture Medium

Compound	Concentration	Quantity
RPMI 1640 powder without $NaHCO_3$, folic acid & vitamin B_{12}	1X	10.418g
HEPES	25mM	5.95g
$NaHCO_3$	21mM	1.80g
Folic acid	0.002mM	0.001g
Vitamin B_{12}	$4E10^{-6}$mM	0.005mg
Streptomycin Sulphate	0.5mg/ml	0.5g
Benzylpenicillin Sodium Salt	0.03mg/ml	0.03g
NaOH		adjust to pH 7.2
H_2O		final volume 1000ml

Filter sterilise through a Sterivex-GS 0.22µm filter unit with filling bell.
Immediately before use add 0.130mCi of ^{35}S L-methionine (approx 1100 Ci/mmole) to every ml of medium.

NBT Solution

Compound	Concentration	Quantity
4-Nitro blue tetrazolium chloride (NBT)	90mM	75mg
DMF 70% (v/v)		1ml

PBS

Compound	Concentration	Quantity
NaCl	137mM	8.0g
KCl	2.7mM	0.2g
Na_2HPO_4	10.0mM	1.44g
KH_2PO_4	1.8mM	0.24g
HCl		adjust to pH 7.2
H_2O		final volume 1000ml
Autoclave for 20min at 121°C.		

PBS Blocking Solution

Compound	Concentration	Quantity
BSA	15mg/ml	3.0g
PBS	1X	200ml
10% NaN_3	0.02% (w/v)	400µl

Red Blood Cell Lysis Buffer

Compound	Concentration	Quantity
NH_4Cl	155mM	4.15g
$NaHCO_3$	10mM	0.42g
EDTA	0.1mM	0.0186g
$NaHCO_3$ solution		adjust to pH 7.2
H_2O		final volume 500ml

Filter sterilise through a Sterivex-GS 0.22µm filter unit with filling bell
Must be prepared fresh and checked for correct pH.

Resolving Gel Acrylamide

Acrylamide:Bisacrylamide 36:1

Compound	Concentration	Quantity
Acrylamide	30% (w/v)	60g
Bisacrylamide	0.83% (w/v)	1.66g
H_2O		final volume 200ml

SDS 10% Solution

Compound	Concentration	Quantity
SDS	10% (w/v)	10g
H_2O		final volume 100ml

Autoclave for 20min at 121°C.

SDS Sample Buffer (2X)

Compound	Concentration	Quantity
Trizma Base	12mM	0.0726g
SDS	6% (w/v)	3g
Glycerol	20% (w/v)	10ml
Bromophenol blue	0.03% (w/v)	0.015g
HCl solution		adjust to pH 6.8
H_2O		final volume 50ml

Before use add 0.1ml of β-mercaptoethanol to every 1ml of loading buffer.

Sodium Chloride Solution (1M)

Compound	Concentration	Quantity
NaCl	1M	5.84g
H_2O		final volume 100ml

Autoclave for 20min at 121°C.

Solution A

Compound	Concentration	Quantity
Trizma Base	3M	36.33g
HCl solution		adjust to pH 8.8
H_2O		final volume 100ml

Autoclave for 20min at 121°C.

Solution B

Compound	Concentration	Quantity
Trizma Base	500mM	6.055g
HCl solution		adjust to pH 6.8
H_2O		final volume 100ml
Autoclave for 20min at 121°C.		

Stacking Gel Acrylamide
Acrylamide:Bisacrylamide 20:1

Compound	Concentration	Quantity
Acrylamide	4% (w/v)	6g
Bisacrylamide	0.2% (w/v)	0.3g
H_2O		final volume 150ml

Staining Solution

Compound	Concentration	Quantity
Coomassie Brilliant Blue (R250)	2.5% (w/v)	1.25g
Methanol	45% (v/v)	225ml
Glacial Acetic Acid	10% (v/v)	50ml
H_2O		final volume 500ml

Substrate Buffer

Compound	Concentration	Quantity
Trizma pH 9.5 (1M Solution)	0.1M	10ml
NaCl (1M Solution)	0.1M	10ml
$MgCl_2$ (1M Solution)	0.1M	10ml
H_2O		final volume 100ml
Prepare fresh on the day and add 45µl of NBT solution and 35µl of X-phosphate solution per 10ml.		

TBS

Compound	Concentration	Quantity
Trizma Base	20mM	2.42g
NaCl	500mM	29.22g
HCl		adjust to pH 7.5
H_2O		final volume 1000ml
Autoclave for 20min at 121°C.		

TBS Blocking Solution

Compound	Concentration	Quantity
BSA	15mg/ml	3.0g
TBS	1X	200ml
10% (w/v) NaN_3	0.02% (w/v)	400µl

Transfer Buffer

Compound	Concentration	Quantity
Trizma Base	25mM	0.303g
Glycine	192mM	1.44g
H_2O		final volume 100ml
Unadjusted pH 8.3		
Autoclave for 20min at 121°C.		

Triton X-100 Solution (10%)

Compound	Concentration	Quantity
Triton X-100	10%	10ml
H_2O		final volume 100ml

Trizma pH 9.5 (1M Solution)

Compound	Concentration	Quantity
Trizma Base	1M	12g
HCl		adjust to pH 9.5
H_2O		final volume 100ml
Autoclave for 20min at 121°C.		

X-Phosphate Solution

Compound	Concentration	Quantity
5-bromo-4-chloro-3-indolyl phosphate, toluidinium Na_2	125mM	50mg
DMF		1ml

2.1.3 Drug Supplemented Culture Media

All drugs were dissolved in RPMI medium without FCS supplement (to avoid complex formation between FCS proteins and drugs, thus preventing the reduction of free drug in solution) at room temperature (20°C-24°C) with the exception of indomethacin and prostaglandin A_1 which were dissolved in DMF (N,N-dimethylformamide) before the addition to RPMI. With the exception of aspirin and methotrexate, all of the other drugs mentioned here did not generally require media pH adjustment, as the buffering capability of the RPMI medium was sufficient to maintain the pH in the range of 7.0-7.2. The pH of the solutions was monitored during the adjustment procedure by observing the colour of the phenol red in the medium and by withdrawing 25µl from the medium and applying it to Merck pH-indicator strips (range pH 5-10). pH adjustments, if required, were made by adding the appropriate solution of NaOH or HCl (HCl concentrations: 1M or 0.1M, NaOH concentrations: 5M, 1M or 0.1M), depending on the difference between the actual pH and the desired pH. The volumes added and removed, during pH adjustment, were kept to a minimum in order to avoid significant changes in the final drug concentration. This method of pH adjustment was preferred over the use of a pH probing electrode as it eliminated any possibility of contamination of solutions. All solutions were prepared fresh on the day of their use.

Aspirin Medium

Compound	Concentration	Quantity
Aspirin	22mM	0.0396g
RPMI culture medium	1X	9ml
Adjust pH with NaOH solution		pH 7 - pH 7.2
RPMI culture medium	1X	final volume 10ml
Filter sterilise through a Millex-GS 0.22µm filter unit.		

Chloroquine Medium

Compound	Concentration	Quantity
Chloroquine	0.8mM	0.0041g
RPMI culture medium	1X	9ml
Adjust pH with NaOH solution if required to		pH 7-pH 7.2
RPMI culture medium	1X	final volume 10ml
Filter sterilise through a Millex-GS 0.22µm filter unit.		

Diclofenac (Sodium) Medium

Compound	Concentration	Quantity
Diclofenac (Sodium)	1.0mM	0.0032g
RPMI culture medium	1X	9ml
Adjust pH with $NaHCO_3$ solution if required to		pH 7-pH 7.2
RPMI culture medium	1X	final volume 10ml
Filter sterilise through a Millex-GS 0.22µm filter unit.		

Indomethacin Medium

Compound	Concentration	Quantity
Indomethacin	0.6mM	0.0021g
DMF	0.2%	20μl
RPMI culture medium	1X	9ml
Adjust pH with NaOH or HCl solution if required to		pH 7-pH 7.2
RPMI culture medium	1X	final volume 10ml

Add the DMF to indomethacin solid and dissolve the solid before adding the aqueous components.
Filter sterilise through a Millex-GS 0.22μm filter unit.

Methotrexate Medium

Compound	Concentration	Quantity
Methotrexate	10mM	0.0454g
RPMI culture medium	1X	9ml
Adjust pH with NaOH solution		pH 7 - pH 7.2
RPMI culture medium	1X	final volume 10ml

Because methotrexate is unstable under alkaline conditions care was used to ensure that pH was always $<$ pH 7.2. As methotrexate is more soluble at higher pH, pH adjustments were made and the pH monitored during dissolution of the solid.
Filter sterilise through a Millex-GS 0.22μm filter unit.

Paracetamol Medium

Compound	Concentration	Quantity
Paracetamol	20ml	0.0302g
RPMI culture medium	1X	9ml
Adjust pH with NaOH solution if required		pH 7-pH 7.2
RPMI culture medium	1X	final volume 10ml

Filter sterilise through a Millex-GS 0.22μm filter unit.

D-Penicillamine Medium

Compound	Concentration	Quantity
D-Penicillamine	25mM	0.0373g
RPMI culture medium	1X	9ml
Adjust pH with $NaHCO_3$ solution, if required, to		pH 7-pH 7.2
RPMI culture medium	1X	final volume 10ml

Filter sterilise through a Millex-GS 0.22μm filter unit.

Prostaglandin A_1 Medium

Compound	Concentration	Quantity
Prostaglandin A_1	4μg/ml (12E10^{-6}M)	40μg
DMF	0.05%	5μl
RPMI culture medium	1X	final volume 10ml

Filter sterilise through a Millex-GS 0.22μm filter unit.

Salicylate (Sodium) Medium

Compound	Concentration	Quantity
Salicylate (Sodium)	17mM	0.0272g
RPMI culture medium	1X	9ml
Adjust pH with NaOH solution if required		pH 7-pH 7.2
RPMI culture medium	1X	final volume 10ml

Filter sterilise through a Millex-GS 0.22μm filter unit.

2.2 METHODS

2.2.1 Blood

Fresh venous blood was collected by venipuncture of the antecubital vein of consenting healthy subjects using a sterile, disposable 21 gauge winged infusion set with flexible tubing and rubber septum. Blood was drawn into a sterile disposable syringe(s) containing the appropriate volume of 4.5% EDTA solution as the anticoagulant (2ml 4.5% EDTA:10ml blood). Immediately after the blood collection, the syringe was inverted several times to ensure the uniform mixing of the EDTA solution and blood.

2.2.2 Mononuclear cell (MNC) preparation

The isolation of MNC was carried out at room temperature under sterile conditions by the following procedure:

(a) Blood was transferred into sterile 50ml centrifuge tubes (Corning 25330-50, U.S.A.) and centrifuged at 100g for 10min to obtain a buffy coat.

(b) The supernatant (containing platelets) was discarded, two volumes of Hanks' buffered salt solution (HBSS) added and mixed well by inverting.

(c) The mixture was underlaid with 15ml of Lymphoprep (density gradient) and centrifuged at 500g for 30min.

(d) As much as possible of the supernatant layer was removed, without disturbing the interface.

(e) The MNC were collected from the interface between the remaining upper supernatant and the Lymphoprep layer and diluted with 2.5 vols of HBSS.

(f) Centrifuged at 500g for 10min and the supernatant disposed.

(g) Washed by resuspending in 20ml of fresh HBSS and recentrifuged as in (f).

(h) Resuspended in RPMI supplemented with 2.5% FCS ready for culture.

2.2.3 Initial preincubation of MNC

After isolation, the MNC were allowed to acclimatise to the *in vitro* culture conditions. Cells were incubated overnight (16 to 18 hours) at a density between 2.5E10^6 and 3.5E10^6 cells/ml in RPMI 1640 medium with 2.5% foetal calf serum at 37°C in a humidified (95%), CO_2 (5%) incubator. The viability of MNC was determined by trypan blue exclusion. MNC cultures not showing considerable aggregation of cells and showing a greater than 90% viability were used in experiments.

2.2.4 Heat shock treatment

The cell cultures were aliquotted (1ml) into sterile 1.5ml microfuge tubes before harvesting by pulsing for 1 to 2sec in a Phoenix Clements microfuge which was set on the slow speed of 6500rpm (under these conditions 6500rpm was not acquired by the rotor). The media in each individual tube was replaced with RPMI media without FCS and containing added anti-rheumatic agents or no agents and the cells then resuspended. The cells were incubated at 37°C for 30min (unless otherwise stated) to allow the agents time to interact with the cells. Tubes were then exposed to different temperatures, ranging from 37°C to 45°C, for 15min (unless otherwise stated) by placing in a temperature regulated circulating water bath (Techne TE-8J, England: specified temperature variation ± 0.05). The temperature was calibrated by a total immersion thermometer (TOT IMM NF 9294860 Zeal, England). Cell cultures were centrifuged and the media was then replaced (cells were washed to remove all traces of agents) with RPMI medium not containing agents.

2.2.5 ^{35}S Metabolic labelling

Metabolic radiolabelling was selected over immunodetection as it is compatible with the detection of *de novo* synthesised proteins whereas immunodetection detects both existing as well as *de novo* synthesised proteins. Cells requiring metabolic labelling were harvested by centrifugation as described in "(2.2.4) Heat shock treatment". The media was gently removed by pipette and the cell pellet once again subjected to centrifugation to collect the remaining media at the bottom of the tube. The remaining media was removed and

replaced with 500µl of RPMI 1640 deficient in L-methionine but supplemented with ^{35}S labelled L-methionine. The cells were resuspended and incubated at 37°C for 2 hours to allow for HSP expression. Experiments were terminated by harvesting the cells by centrifugation, removing the media, recentrifuging, removing any remaining media and lysing the cells with equal volumes (75µl) of lysis buffer and SDS (2X) sample buffer. If SDS PAGE (Sodium-Dodecyl-Sulphate Poly-Acrylamide Gel Electrophoresis) analysis was not to be performed within 3 days of experiment termination, 75µl of lysis buffer was added before freezing at -70°C. Later, immediately before SDS PAGE analysis, 75µl of SDS loading buffer was added. *(Freezing the samples at -70°C in lysis buffer alone produced more satisfactory results as there was less protein degradation and hence produced clear sharp protein bands on one dimensional SDS PAGE, as opposed to samples frozen with both lysis and SDS (2X) sample buffers added).*

2.2.6 Sample preparation for SDS PAGE

At this stage all samples contained an equal number of lysed cells (between $2.5E10^6$ and $3.5E10^6$ cells) in a total volume of 150µl. Samples were usually analysed within 5 days by SDS PAGE. Immediately before loading samples onto SDS polyacrylamide gels, the samples and protein standards, were denatured by placing the 1.5ml microfuge tubes containing the samples into a boiling water bath for 10min. Sample tubes were totally sealed during boiling to prevent uneven evaporation between individual samples. After denaturation, samples were centrifuged in a Phoenix Clements microfuge for 15min at 13,000rpm. Aliquots loaded onto the SDS polyacrylamide gel were withdrawn from the top of the sample to avoid collecting any insoluble material from the bottom. Samples were loaded by equal volumes hence achieving a situation where equal cell numbers were loaded per well. Using the BioRad Protein Assay Kit I, it was determined that loading by equal volumes also resulted in loading by equal protein implying that cell numbers remained constant between individual samples. Loading of samples by equal radioactive counts was not used as different drug treatments differentially inhibited total cellular protein synthesis.

2.2.7 SDS PAGE (Sodium-Dodecyl-Sulphate Poly-Acrylamide Gel Electrophoresis)

One dimensional SDS PAGE was used for the separation and resolution of cellular proteins. The proteins of interest were in the molecular weight range of 20-130kDa. For proteins in this range, the polyacrylamide resolving gel was prepared to a concentration of 13% with a ratio of acrylamide:bis-acrylamide of 36:1. The stacking gel was prepared to a concentration of 5% with a ratio of acrylamide:bis-acrylamide of 20:1. Gel cocktails were prepared from individual stock solutions immediately before the pouring of a gel as this method gave best results and the stock solutions could be stored for long periods (6 months). Gels were used within three hours of being poured. The composition of the resolving and stacking gels is given below:-

Resolving gel (one gel)

Component	Quantity
Resolving gel acrylamide	1300μl
H_2O	1240μl
Solution A	430μl
SDS 10%	30μl
TEMED	4μl
APS 10%	20μl

Stacking gel (one gel)

Component	Quantity
Stacking gel acrylamide	750μl
Solution B	240μl
SDS 10%	8μ
TEMED	4μl
APS 10%	10μl

Analytical SDS PAGE was performed using the BioRad Mini-PROTEAN II System. In general, the preparation of SDS gels and electrophoresis was carried out as described in the manufacturer's manual. Polyacrylamide gels were prepared using spacers having a thickness of 0.5mm and 10 well combs. Resolving gels were poured to a height which was 10-15mm below the bottom of the wells and overlaid with deionized water and then allowed to set. Each gel layer was allowed a minimum of 30min to set before the next step. Once the stacking gels had been poured and set, the combs were removed and the wells washed with deionized water followed by the removal of the water from the wells.

Gels were first loaded with low molecular weight range protein standards (electrophoresis calibration kit; Pharmacia U.S.A.), followed by 25µl of experimental sample per well. The samples and protein standards were overlaid with SDS electrophoresis buffer before filling the upper buffer chamber. The electrode and gel assembly was lowered into the buffer tank which was prefilled to the appropriate level with 1X SDS electrophoresis buffer. Electrophoresis was carried out as per instructions until the indicator dye had eluted from the bottom of the gel.

2.2.8 Autoradiography

After one dimensional SDS PAGE separation with radiolabelled protein samples was completed, gels were removed from between the glass plates and the stacking gels were cut away and discarded. The resolving gels were placed into containers with sufficient staining solution to allow the gel to submerge and move freely. Coomassie blue protein staining was carried out with gentle agitation on a rocking platform for 60min. Subsequently, gels were removed and destained in two changes of destaining solution for 30min each. Staining allowed the visualisation of the separation of the protein samples and the Pharmacia low molecular weight range protein standards. The destained gels were placed on Whatman 3mm chromatography paper and covered with thin plastic film before drying for 60min at 80ºC on a heated vacuum slab gel drier (BioRad, Mode 1125B).

The plastic film was removed from the gel before insertion of the Whatman paper with the gel into an X-ray cassette and exposing the gel to a single sided X-ray film (Cronex MRF 33). The X-ray film was placed over the gel with the emulsion side against the gel and the entire cassette stored at -70ºC to enhance the efficiency of exposure to the radioactive decay. Exposures were between 3 to 5 days duration. Where samples relating to a single experiment could not be loaded on the same gel, all gels relating to the same experiment were exposed to film for identical durations. All exposed films were developed under identical conditions using a DuPont QC1-R/T automated X-ray film processor. The developed films were then superimposing over the dried gels to allow for the correct placement of the protein standards. The position of the protein standards was then marked on the film with a fine black felt tipped pen.

2.2.9 Densitometry

When quantitative results were required, autoradiographs were analysed by densitometry using the Beckman DU 650 Spectrophotometer equipped with a Gel Slit Accessory (0.2mm slit) and an Autoradiograph Holder Accessory.

Full length scans of each lane were made and the density of each lane was then corrected for width variation. This was necessary as the outer most lanes on most gels were wider than the inner lanes due to sideways diffusion of protein samples during electrophoresis. Therefore due to sample diffusion, the wider lanes appeared less dense. Lane width corrections were made by measuring the width of each lane and calculating a lane width correction factor for individual lanes. The narrowest lane was assigned a correction factor equal to 1 while the calculated value for wider lanes was <1. Densities of each lane were then corrected by multiplying the measured density by the inverse of the width correction factor. Band densities were expressed in arbitrary values which represented the area under the curve.

Density increases of induced HSP bands were estimated by first normalising total protein synthesis between all lanes to compensate for decreases resulting from inhibition of normal protein synthesis. Density increase of an induced HSP band was expressed as the ratio of the HS induced band density over the control band density.

2.2.10 Western Blot Transfer

The following protocol was developed by the modification of previously published methodologies and from the manufacturer's instructions. Electrotransfer was performed using the BioRad Mini Trans-Blot Electrophoretic Transfer Cell. Reduced and denatured protein samples were separated by one dimensional SDS PAGE as follows:

(a) BioRad biotinylated SDS PAGE protein standards were diluted 1:20 in equal volumes of lysis buffer and 2X SDS loading buffer.

(b) Tubes containing the biotinylated standards were heated in a boiling water bath for 10min along with the experimental samples.

(c) Ten μl of the diluted biotinylated standards were loaded into the appropriate wells alongside the experimental samples on the gel and electrophoresis performed as described in "(2.2.7) SDS PAGE".

(d) After electrophoresis the gel was recovered and the stacking gel discarded from the top. The orientation of the resolving gel was marked by nicking the top right hand corner.

(e) The gel was equilibrated in transfer buffer by submerging it in sufficient buffer to allow the gel to move freely in solution. Soaking with gentle agitation on a rocking platform was carried out for 15min.

(f) Precut PVDF transfer membranes were presoaked for 5min in 100% methanol by slowly sliding the membrane into the methanol at a 45° angle, to displace air from the membrane.

(g) The PVDF transfer membranes were removed from the methanol and excess methanol allowed to drain. The membranes were then inserted into transfer buffer and submerged with gentle agitation for 15min.

(h) Whatman 3mm chromatography paper, precut to slightly larger dimensions than the gel, and the fibre pads supplied with the electrotransfer apparatus, were presoaked for 15min by submerging in transfer buffer.

(i) For electrotransfer, the apparatus was assembled with a fibre pad on the bottom followed by Whatman paper, the polyacrylamide gel, the PVDF transfer membrane, Whatman paper and finally a fibre pad. During assembly care was taken to exclude air bubbles, especially from between the gel and transfer membrane.

(j) Electrotransfer was performed with the gel on the side of the negative electrode at 70V; 350-750mA for 2hrs with cooling and circulating transfer buffer.

(k) On completing electrotransfer the gel assembly was disassembled and the transfer membrane was removed and submerged in PBS.

2.2.11 Immunodetection

(a) Blotted membranes were blocked for 60min in PBS blocking solution with gentle agitation on a rocking platform.

(b) Membranes were removed from the blocking solution and placed into a heat sealed plastic bag containing 10ml of PBS blocking solution with the primary antibody[1] diluted to the appropriate concentration. The primary antibody was allowed to bind for 60min at room temperature with gentle agitation.

(c) The membranes were removed from the plastic bag and washed twice for 5min in PBS with gentle agitation and then once in TBS for 5min.

(d) Membranes were removed from the washing solution and placed into a sealed plastic bag containing 10ml of TBS blocking solution with the alkaline phosphatase conjugated secondary antibody[2] diluted to the appropriate concentration. The secondary antibody was allowed to bind for 60min at room temperature with gentle agitation.

(e) The membranes were removed from the plastic bag and washed twice for 5min in TBS with gentle agitation.

(f) Where biotinylated protein standards were used, the membranes were removed from the washing solution and sealed in a plastic bag containing 10ml of TBS blocking solution with alkaline phosphatase conjugated streptavidin diluted to the appropriate concentration. The binding of biotin by the streptavidin conjugate was carried out for 30min at room temperature.

(g) The membranes were removed from the plastic bag and washed twice for 5min in TBS with gentle agitation.

(h) Membranes were then washed for 1min in substrate buffer before placing into substrate buffer supplemented with substrate and applying gentle agitation until the desired intensity of the bands and protein standards was achieved.

1. The primary antibody is as given in section 2.1: mouse anti-heat shock protein 70 monoclonal (IgG) antibody.

2. The secondary antibody is as given in section 2.1: anti-mouse IgG (H+L) alkaline phosphatase conjugated antibody.

2.2.12 FCS Heat inactivation

The following procedure is recommended by the manufacturing supplier of FCS:

(a) The serum was removed from storage (-70°C) and allowed to thaw out and equilibrate to 37°C in a water bath, the container was then inverted several times to ensure the proper mixing of the serum.

(b) An identical container to that of the FCS container (or similar) was filled with an identical volume of water and allowed to equilibrate to 37°C.

(c) A thermometer was placed into the bottle of water and then both the container with the water and the FCS were placed into a preheated and circulating water bath set at 60°C.

(d) Both the water and the serum were mixed at regular intervals until the thermometer reached 56°C. The FCS was allowed to incubate for 30min from this point on with regular mixing.

(e) The FCS was removed from the water bath and aliquotted (30ml) into sterile containers and once again stored at -70°C. Aliquots were removed as required.

CHAPTER THREE

Heat Shock Protein Induction in Human Mononuclear Cells

3.1 INTRODUCTION

Research into HSP and SP has established several fundamental characteristics of these proteins, relating to their synthesis and how they are induced. Although most types of stress induce a common set of HSP, there are some proteins which are induced by some stresses and not by others. Furthermore, the HSP and SP profile induced by a particular stress may vary from one organism to another and between one tissue type and another in the same organism.

Others have already demonstrated the induced expression of HSP and SP in white blood cells in a variety of different species. For example, Rodenhiser *et al.* (1985) identified proteins of the molecular weight 110kDa, 100kDa, 90kDa, 70kDa, 65kDa and 26kDa as those which are induced by hyperthermia in human, rabbit and mouse lymphocytes. Joslin *et al.* (1991) showed that human mononuclear phagocytes have marked increases in HSP70 and HSP90 synthesis and to a much lesser extent increases in BiP and grp94 synthesis after exposure to heat shock, but they observed no increase in the constitutively expressed 70kDa stress protein (hsc70). Similarly, Guerriero and Raynes (1990) reported that lymphocytes from species other than human, also demonstrated a strong inducibility of hsp70. However, differences in the amount of hsp70 synthesised varied between the bovine, equine, ovine and chicken species under identical conditions, ranging from a 3.75 fold increase in equine lymphocytes to over 20 fold in chicken lymphocytes. HSP profiles between the different species were also found to vary after heat shock, with equine lymphocytes demonstrating the least number of different induced HSP and bovine lymphocytes the greatest number (Guerriero and Raynes 1990). Thus, while a certain degree of variability in HSP expression can be expected from peripheral white blood cells depending on the species, the synthesis of several stress proteins like hsp90 and hsp70 seem to be common in all species.

In vitro cultured human peripheral blood mononuclear cells (MNC) were used for the work presented in this thesis. To better understand the expression and induction of HSP in MNC, this chapter deals with the characterisation of HSP expression, as detected by metabolic labelling with ^{35}S methionine of MNC cultured under a variety of conditions. A specific monoclonal antibody, which recognises the

inducible hsp70 as well as the constitutive hsc70 proteins, was also used for a positive identification of these 70kDa stress proteins using Western Blots of total cellular proteins separated by one dimensional SDS PAGE. Although the induction of other HSP, besides hsp70, was not studied in detail in this thesis, hsp70 was selected as a typical marker for HSP induction because of its synonymity with the stress response regardless, in most cases, of the type of stress (e.g. hyperthermia, UV radiation, osmotic stress, chemical teratogens, bacterial toxins, etc.) (Li 1983; Liu *et al.* 1992; Varela *et al.* 1992; Koller *et al.* 1993; Mirkes *et al.* 1994) or the organism being studied. Several publications addressing the synthesis of HSP in peripheral white blood cells report the HSP70 family as one of the most abundantly synthesised stress proteins after exposure to heat, erythrophagocytosis, mitogen stimulation or other stresses.(Joslin *et al.* 1991; Clerget and Polla 1990; Guerriero and Raynes 1990; Ghuassemi *et al.* 1991; Hansen *et al.* 1991; Kamwanja *et al.* 1994; Maridonneau-Parini *et al.* 1988; Mosser and Martin 1992; Polla 1991)

A correlation between elevated levels of cellular hsp70 and enhanced cell survival after exposure to stress, has been experimentally demonstrated (Angelidis *et al.* 1991). Conversely, by inhibiting HSP synthesis, cells become more susceptible to stress damage. For example, Johnston *et al.* (1988) transfected a Chinese hamster ovary cell line with a plasmid containing the HSE sequence. When the plasmid was induced to a high copy number, it inhibited the induction of HSP, after HS, in the transfected cells which resulted in these cells being thermosensitive. Inhibition of HSP synthesis was due to endogenous HSF binding to the introduced HSE, thus leaving insufficient HSF available to bind to endogenous HSE of the *hsp70* and the other *HSP* genes. This suggests that HSP induction was required to establish thermotolerance (Johnston and Kucey 1988). More specifically, with respect to hsp70, Riabowol *et al.* (1988) demonstrated a lack of thermotolerance after injecting anti-hsp70 monoclonal antibodies into cells. The excess antibody bound to free and newly synthesised hsp70 in the cytoplasm of the injected cells and prevented hsp70 from establishing thermotolerance (Riabowol *et al.* 1988). Furthermore, Liu *et al.* (1992) transfected Rat-1 cells, a rat fibroblast cell line, with the human *hsp70* gene which had been inserted into a DNA plasmid expression vector. The transfected cells

demonstrated constitutive expression of the introduced *hsp70* gene and were protected against damage when exposed to stress (Liu *et al.* 1992). These studies demonstrated the importance of hsp70 as part of the stress response in rescuing and protecting cells from the damage caused by stress. Furthermore, thermotolerance can also be conferred on cells by pre-exposure to other types of stress as demonstrated by Haveman *et al.* (1986) and Li and Hahn (1978). In their experiments, mammalian cells were exposed to chemical agents which induced HSP synthesis and protected the cells from damage when exposed to a subsequent HS. These results suggested that the HSP which are necessary to protect cells from HS, and perhaps other stresses, can be induced by different inducers and will protect a cell against a specific stress like HS. The findings by Haveman *et al.* (1986) and Li and Hahn (1978) support the hypothesis on which this thesis is based and that is the presumption that HSP induction and synthesis, facilitated by anti-inflammatory agents, can help protect human white blood cells from the harmful effects of a second exposure to stress.

3.2 RESULTS

3.2.1 Induction and Synthesis of HSP

Human MNC cultured in RPMI medium were exposed to a heat shock (HS) treatment of 42°C for 45min while control cells were maintained at 37°C. After HS treatment the media was replaced by methionine-deficient RPMI supplemented with ^{35}S L-methionine and the cells were incubated for a further 2 hours at 37°C. During this time all newly synthesised proteins including HSP and other cellular proteins were radiolabelled with ^{35}S. Cells were then lysed and cellular proteins separated on one dimensional SDS PAGE (Sections 2.2.6 and 2.2.7). The SDS polyacrylamide gels were stained with coomassie blue and autoradiographed (Section 2.2.8).

Careful comparison of the coomassie blue stained HS-treated cellular protein pattern to that of the control cellular protein pattern showed no apparent differences between the two protein synthesis patterns, indicating that coomassie blue staining is not sufficiently sensitive to detect the synthesis of newly induced synthesised cellular proteins. However, comparison of *de novo* protein synthesis between the HS and control cells, from the autoradiograph, showed clear induction of seven proteins

in the HS cells (Fig. 3.1a) *(The sensitivities of coomassie blue staining and autoradiography for detecting HSP induction are compared in Section 5.3).* The most intensely induced protein band migrated at a similar rate to that of the 67kDa protein standard. The HS cells also showed an overall reduction in total cellular protein synthesis during the ^{35}S methionine labelling period following the HS. This is reflected by the lower amount of radiolabel incorporated into the other non-induced proteins. This result was consistent with the reported inhibition of normal cellular protein synthesis during more severe HS (Lindquist 1981). Densitometric analysis of the autoradiograph estimated the HS treatment inhibited normal protein synthesis by ≈ 65%, while relative density increased for the induced protein bands ranged from 1.6 fold up to 4.5 fold (Fig. 3.1b).

3.2.2 HSP Synthesis: A Temperature Profile

In Section 3.2.1 an arbitrary temperature (42°C) and exposure period (45min) were selected to demonstrate the behaviour of cellular protein synthesis under HS conditions. In this section HS induced HSP expression was further characterised by varying the severity of HS while the duration of the HS remains fixed. By constructing a temperature profile a clear indication of how human MNC protein synthesis responds to different levels of HS was established. In later chapters, the information gained here, allowed the selection of a HS temperature which gave the precise level of HSP induction required in human MNC.

The control MNC were maintained at 37°C, while the HS MNC were exposed to a single HS temperature ranging from 40°C to 46°C for 30min. MNC were then metabolically radiolabelled as described in 2.2.5, MNC and their lysates were further processed as described in Sections 2.2.6 to 2.2.8, and the resulting autoradiograph analysed by densitometry.

From the autoradiograph in Fig.3.2a, it can be seen that HSP synthesis increased as HS temperature increased from 40°C to 42°C without significant visible differences in normal cellular protein synthesis. MNC exposed to 43°C and up to 46°C showed a clear visible reduction in normal cellular protein synthesis with increasing HS temperature, with inhibition becoming almost complete at 45°C. At 43°C, while

normal protein synthesis showed significant inhibition, HSP synthesis remained strong. However, at 44°C, HSP synthesis was also reduced and almost completely inhibited at 45°C and 46°C. Once again, these changes in protein synthesis were not visible on the coomassie blue stained polyacrylamide gel.

3.2.3 The Changing Profile of HSP Synthesis: With Respect to Heat Shock Duration

As seen in Section 3.2.2, the extent of HSP induction by HS, in human MNC, can be controlled by using a different HS temperature. This section investigates how MNC respond to a fixed HS temperature while varying the duration of the HS. The HS temperature of 42°C was chosen because, as demonstrated in Section 3.2.2, it induced a wide range of HSP and also induced these proteins to high levels without significantly affecting normal cellular protein synthesis. The MNC in this section were either incubated at the control temperature of 37°C or heat shocked at 42°C. MNC were incubated at 42°C and equal volume aliquots were withdrawn at different time intervals corresponding to: 30min, 1hr, 2, 3, 4, 5, 6 and 7hrs. Care was taken to fully resuspend the MNC before withdrawing each aliquot, therefore ensuring aliquots contained equal cell numbers. The MNC were radiolabelled and processed as described in Sections 2.2.5 to 2.2.8. Control MNC were radiolabelled and processed as described in Sections 2.2.5 to 2.2.8 only after incubation at 37°C for 7hrs.

Fig. 3.3 shows an autoradiograph of MNC proteins separated by SDS PAGE from the experiment described in the above paragraph. After 30min at 42°C the MNC showed a clear induction of five proteins, this number increased to six proteins after 1hr at 42°C and the amounts of the existing five proteins increased slightly. After 3hrs at 42°C the HSP profile did not change, however, normal protein synthesis and HSP synthesis began to decline. At 3hrs at 42°C right through to 6hrs at 42°C normal protein synthesis and HSP synthesis continued to decline, until 7hrs at 42°C when total protein synthesis was almost completely inhibited. This result suggests that MNC exposed to a 42°C HS, for periods longer than one hour, experienced disruption to protein synthesis by hyperthermia and that the HSP induced and synthesised during and after this HS were unable to prevent this. Therefore, in order to achieve a

high level of HSP induction, without significant disruption to total protein synthesis, MNC should be incubated at 42°C between 30min to 1hr.

3.2.4 Protein Synthesis in Methionine Deficient Media

As discussed in Sections 1.1.4 and 1.2.1 of the Introduction, HSP are induced by aberrant or unfolded proteins or, when large amounts of nascent proteins accumulate within the cell because their synthesis cannot be completed, or because a stimulus has caused a sudden and large increase in protein synthesis resulting in the need for more HSP chaperones to help the new proteins fold.

This thesis extensively utilised aqueous solutions of ^{35}S L-methionine, added to methionine deficient RPMI 1640, when metabolically radiolabelling *de novo* protein synthesis in MNC. Media insufficiently supplemented with ^{35}S L-methionine or with methionine, which has oxidised, would have been expected to cause a block in the elongation of the polypeptide chain and hence, a build-up of incompletely synthesised proteins, resulting in HSP induction and synthesis. Since L-methionine stored improperly in the laboratory or during transportation is susceptible to oxidation, this section investigated the behaviour of protein synthesis in MNC cultured in medium totally deficient in L-methionine. Characteristic changes in protein synthesis, observed in this section, were used to identify experiments where oxidised ^{35}S L-methionine and insufficient amounts of ^{35}S L-methionine were used.

MNC were incubated at either the control temperature of 37°C or at the HS temperature of 46°C for 30min. Control MNC were either: (Fig. 3.4a lane 2) cultured in complete RPMI for 30min at 37°C before the medium was replaced with methionine-deficient medium containing no added radiolabel and then incubated for a further two hours at 37°C, or (Fig. 3.4a lanes 3 & 4) cultured in complete RPMI for 30min at 37°C before the medium was replaced with methionine-deficient medium supplemented with ^{35}S L-methionine and then incubated for a further two hours at 37°C. The MNC exposed to HS were either: (Fig 3.4a lane 5) cultured in complete RPMI for 30min at 46°C before replacing the medium with methionine-deficient media, containing no added radiolabel, and then incubated for a further two hours at 37°C, or (Fig. 3.4a lane 6) cultured in complete RPMI for 30min at 46°C before replacing the

medium with methionine-deficient medium supplemented with ^{35}S L-methionine and then incubated for a further two hours at 46°C, or (Fig. 3.4a lane 7) cultured in complete RPMI for 30min at 46°C before replacing the medium with methionine-deficient medium supplemented with ^{35}S L-methionine and then incubated for a further two hours at 37°C. The cells were then processed as described in Sections 2.2.6 to 2.2.8.

All lanes on the coomassie blue stained SDS PAGE appearing in Fig. 3.4a, were loaded with equal volumes of sample with the exception of lane 3, which was loaded with approximately one half of the volume. The protein standards, in lanes 1 & 8, and the MNC proteins, in lanes 2 to 7, show clearly focused and distinct band patterns. Lane 2, loaded with MNC incubated at 37°C without methionine, when compared to lanes 3 & 4, loaded with MNC incubated at 37°C with methionine, shows a strongly induced band migrating in the vicinity of the 67kDa protein standard. The difference in sample volume loaded on lane 3 is proportionally reflected by the coomassie blue staining. Once again, the MNC incubated without methionine but exposed to 46°C, lane 5, shows a strongly induced band migrating in the vicinity of the 67kDa protein standard, whereas this band is absent in MNC exposed to 46°C and incubated in medium supplemented with methionine (lanes 6 & 7).

Fig. 3.4b shows the autoradiograph corresponding to the coomassie blue stained gel in Fig. 3.4a. The lower amount of protein loaded in lane 3 was accordingly reflected by the lower amount of ^{35}S L-methionine detected by the film. Proteins from MNC incubated without ^{35}S L-methionine (lanes 2 and 5) were not detected by the film. The cells exposed to HS at 46°C, lanes 6 & 7, demonstrated total *de novo* protein synthesis inhibition as indicated by the lack of ^{35}S L-methionine incorporation detected by the film.

The results, in this section, suggest that it was possible, after coomassie blue staining of a SDS polyacrylamide gel, to identify two or more lanes where a large difference in the amount of loaded protein occurs. This was useful, should an accidental gel loading error had occurred, in helping to distinguish between MNC cultures which had a lower (or higher) rate of normal protein synthesis, as detected by autoradiography, and a lane showing less radioactive proteins due to a reduction

in protein sample loading. In addition, coomassie blue staining of a gel was also used to identify cultures which were excessively deficient in methionine by the appearance of the characteristic protein band observed in Fig. 3.4a.

3.2.5 Positive Identification of hsp70 and hsc70 Proteins

The use of SDS PAGE to separate ^{35}S L-methionine radiolabelled MNC cellular proteins, produces a consistent and distinctly recognisable protein pattern, where the migratory position of any protein, relative to all the other cellular proteins within the same lane or different lanes on the same or other gels can easily be recognised. Hence, provided the hsp70 protein band has been positively identified, the hsp70 band can then be located on other gels. Loading molecular weight protein standards or loading pure hsp70 in lanes adjacent to MNC lysates provides only an approximate indication of hsp70 position therefore, detection of hsp70 on Western blots, of SDS PAGE separated MNC proteins, using a specific monoclonal antibody to hsp70 was necessary.

In this section the position of hsp70 and hsc70 relative to other MNC proteins was positively identified after separation by SDS PAGE. MNC were either, incubated at the control temperature of 37°C or exposed to a multiple HS by incubating at 43°C for 15min, then allowing cells to synthesise HSP for 2hrs at 37°C before exposure to 43°C for another 15min and radiolabelling cellular protein synthesis at 37°C for 2hrs, as described in Section 2.2.5. The MNC lysates were processed, separated by SDS PAGE and Western blotted as described in Sections 2.2.6, 2.2.7, 2.2.10 and 2.2.11. Autoradiographs were also produced from the Western blot membrane as described for the dried SDS gels in Section 2.2.8.

The Western blots appearing in Fig. 3.5a are of SDS PAGE separations of MNC proteins electrophoresed for the same length (upper blot) of time or approximately twice as long (lower blot) as described in Section 2.2.7. The extended electrophoresis period was used to improve the separation between the hsc70 protein and the inducible hsp70, which differ in molecular weight by approximately 2kDa. The anti-HSP70 mouse monoclonal antibody (clone 3A3) clearly detected both hsc/hsp70 proteins in the MNC exposed to HS as seen in the lower Western blot appearing in

Fig. 3.5a. The positions of both the constitutively expressed hsc70 protein (upper band) and the inducible hsp70 (lower band) are easily compared to the relative position of the 66.2kDa biotinylated molecular weight protein standard. Heat shocked MNC, clearly demonstrated an increased level of the inducible hsp70 band when compared to the control MNC. The position of hsp70, relative to the 66.2kDa biotinylated standard and the other MNC proteins, were then compared to the overlaid autoradiographs in Fig. 3.5b. Fig. 3.5c shows the autoradiographs separately. From the Western blots and the autoradiographs appearing in the lower half of Fig. 3.5a to 3.5c it is clear that the lower of the two bands detected by the antibody was induced by HS- the same band detected in the 70kDa range by X ray film during autoradiography. The induced band was therefore hsp70, while the band above it was hsc70.

Gels in this section were loaded with equal volumes of samples in each lane as described in Section 2.2.6 hence, an equal number of cells was loaded per lane. Normally, as observed in Sections 3.2.2 and 3.2.3, MNC which were exposed to higher HS temperatures or to a HS at lower temperatures for extended periods showed reduced amounts of normal protein synthesis compared to unstressed cells. However, autoradiographs appearing in Fig. 3.5c showed that the lanes containing proteins from MNC exposed to HS have higher levels of protein synthesis compared to the unstressed MNC. This may be due to an overall higher than normal rate of synthesis of new proteins, which are required to replace cellular proteins irreversibly damaged by the multiple HS treatment, and possibly the HSP induced by the first HS treatment functioning to protect the ribosomes and associated mechanisms from disruption during the second HS.

3.3 SUMMARY AND DISCUSSION

3.3.1 Summary of Results

In this chapter, HSP induction by HS in MNC was characterised. In Section 3.2.1 an arbitrary temperature of 42°C for 45min was used to HS MNC and was found to induce seven different proteins bands as detected by autoradiography. But these

same proteins were not detectable on the coomassie blue stained gel. The most abundantly induced band out of the seven bands, migrated at a similar rate to that of the 67kDa protein molecular weight protein standard. The effects of HS severity on MNC was investigated in Sections 3.2.2 and 3.2.3. HS severity was modulated by either, varying the temperature or, by varying the duration of the HS. In these experiments, however, only six proteins were induced by HS with maximum HSP induction occurring after incubating MNC at 42°C for approximately 30min to 1hr. Normal protein synthesis started to show inhibition after exposure to, ≥ 43°C for 30min or, 42°C for ≥ 2hrs and the inhibition increased as stress conditions became more severe. Total protein inhibition was complete at 45°C for 30min or 42°C after 7hrs.

Up to this point no visible signs of HSP induction were observed on coomassie blue stained SDS gels. Only the omission of ^{35}S L-methionine, from the labelling medium during the 2hrs which cells were metabolically labelled, produced a strong visible induction of one protein band migrating in the vicinity of the 67kDa molecular weight protein standard. This protein was induced in both the control and HS treated MNC even though autoradiography showed that the HS treatment inhibited total protein synthesis in MNC.

Finally the use of an anti-HSP70 mouse monoclonal antibody (clone 3A3), in Section 3.2.5 to probe Western blots, clearly identified the hsc/hsp70 protein band on autoradiographs in relation to the other MNC proteins. By extending the duration of SDS PAGE, a clear separation between the hsc70 and the hsp70 proteins, was achieved, as observed on the antibody probed Western blot in Figs. 3.5a to 3.5c. The autoradiograph of this Western blot detected and identified the faster migrating band as the hsp70 which was induced by HS in MNC.

3.3.2 Discussion

The induction of the same six proteins induced by HS in MNC, in Sections 3.2.1 to 3.2.3, was consistently reproducible. However, Fig. 3.1a shows the induction of seven proteins by HS in MNC. The seventh protein in Fig. 3.1a is indicated by the arrow labelled 'd' and migrates with an apparent molecular weight between 70 and

90kDa. The induction of this protein by HS in Section 3.2.1 and not in any of the other Sections remains a mystery. Polla (1991) reported a stress protein with an apparent molecular weight of 84kDa induced in human monocytes/macrophages by opsonised sheep red blood cells. It will be interesting to see if exposure of MNC to anti-inflammatory agents also causes the induction of this protein in a reproducible manner. The results presented in Sections 3.2.1 to 3.2.3 and 3.2.5 are consistent with HS treatment of peripheral white blood cells reported by Joslin, *et al.* (1991), Guerriero and Raynes (1990) and, Ciavarra and Simeone (1990a) who observed increases in hsp70 and hsp90 to be well above all other induced proteins. In particular, Joslin, *et al.* (1991) reported an increase in hsp70 synthesis and relatively no change in the constitutively expressed hsc70 protein. Likewise, I have demonstrated the strong induction, by HS, in MNC of two proteins migrating on SDS PAGE where the hsp90 and hsp70 are expected.

The separation of hsc70 and hsp70 by SDS PAGE, in Section 3.2.5, was consistent with the generally reported difference in molecular weight of ≈2kDa between these two proteins. The use of autoradiography to detect *de novo* protein synthesis in combination with the immunodetection of hsc70 and hsp70, with a specific monoclonal antibody to hsc/hsp70, identified the lower band of the hsc/hsp70 doublet as that of the inducible hsp70. The Western blot analysis presented in Section 3.2.5 supports the findings of Ciavarra and Simeone (1990a; 1990b) who reported that T lymphocytes not exposed to HS contained high levels of hsc70 but following HS, hsp70 synthesis increased considerably.

The use of a specific monoclonal antibody against the hsc/hsp70 proteins in Section 3.2.5 not only detected the induction of hsp70 but positively identified the migratory position of hsp70 relative to the 66.2kDa molecular weight protein standard on Western blot and relative to other MNC proteins appearing on autoradiographs. It would have been predicted that hsp70 would migrate slower than the 66.2kDa biotinylated protein standard, however this was not the case. As the Western blots reveal, hsp70 migrated below the level of the 66.2kDa biotinylated protein standard. This unexpected phenomenon may be due to the difference in composition between the protein standards and the MNC protein samples. The

molecular weight protein standards are composed of a relatively simple protein combination whereas, MNC protein samples are composed of a complex myriad of proteins which all migrate in close proximity to each other. This may affect the migratory rate of the individual proteins.

In conclusion, human MNC exposed to HS respond by synthesising a reproducible and characteristic HSP profile with the possible exception of a protein in the 80kDa region. Hsp70 which was chosen as the marker for HSP induction and was positively identified by a specific monoclonal antibody against the hsc/hsp70 proteins and, as expected, found to be induced in MNC after HS. The amount of hsp70 synthesised (as detected by autoradiography and quantitated by densitometry) after HS depended on the severity of the HS.

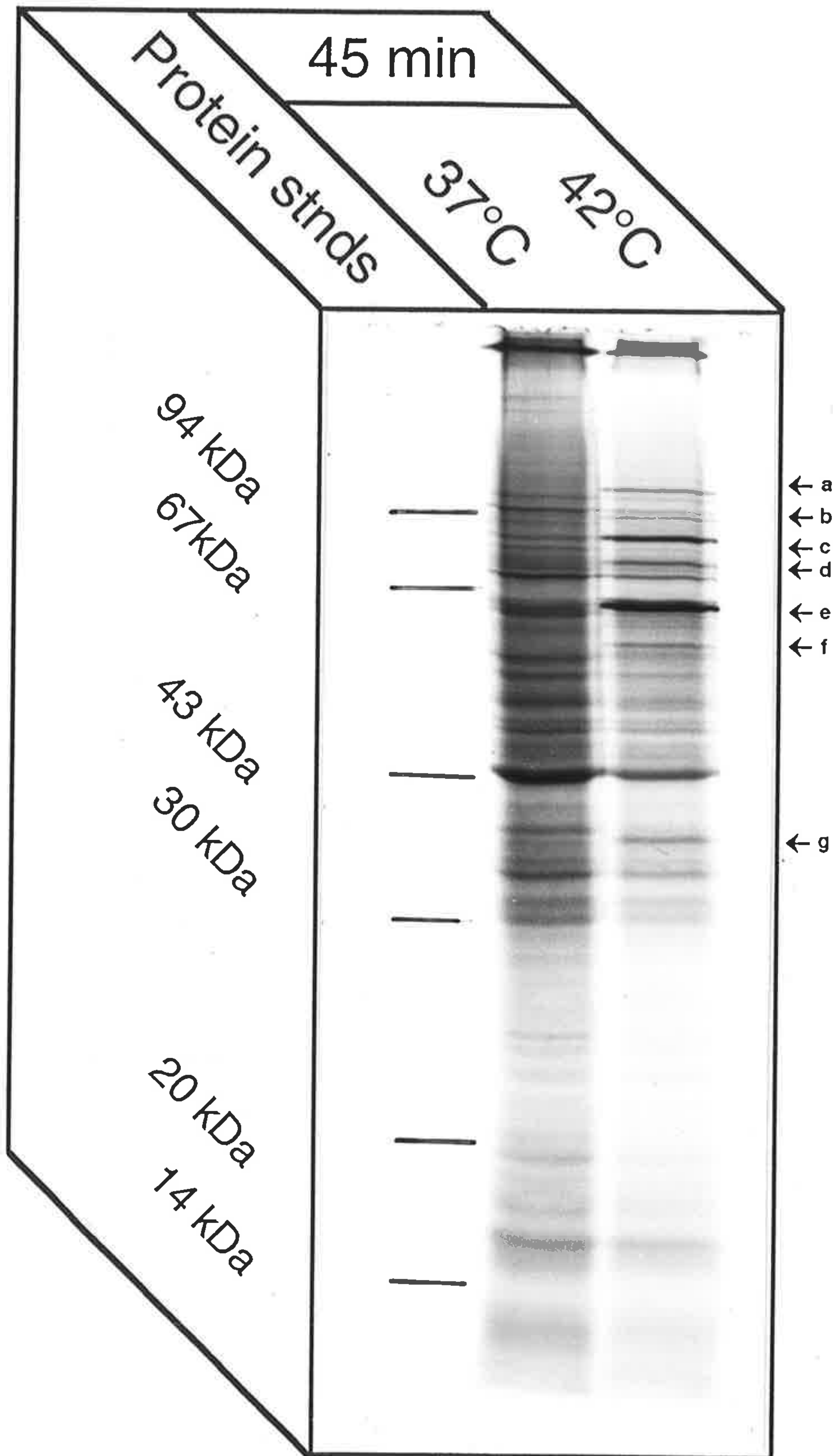


Figure 3.1a shows an autoradiograph of unstressed and HS-stressed MNC at 42°C separated by SDS PAGE. The arrows, labelled from a to g, indicate the position of the proteins induced by HS.

Figure 3.1a

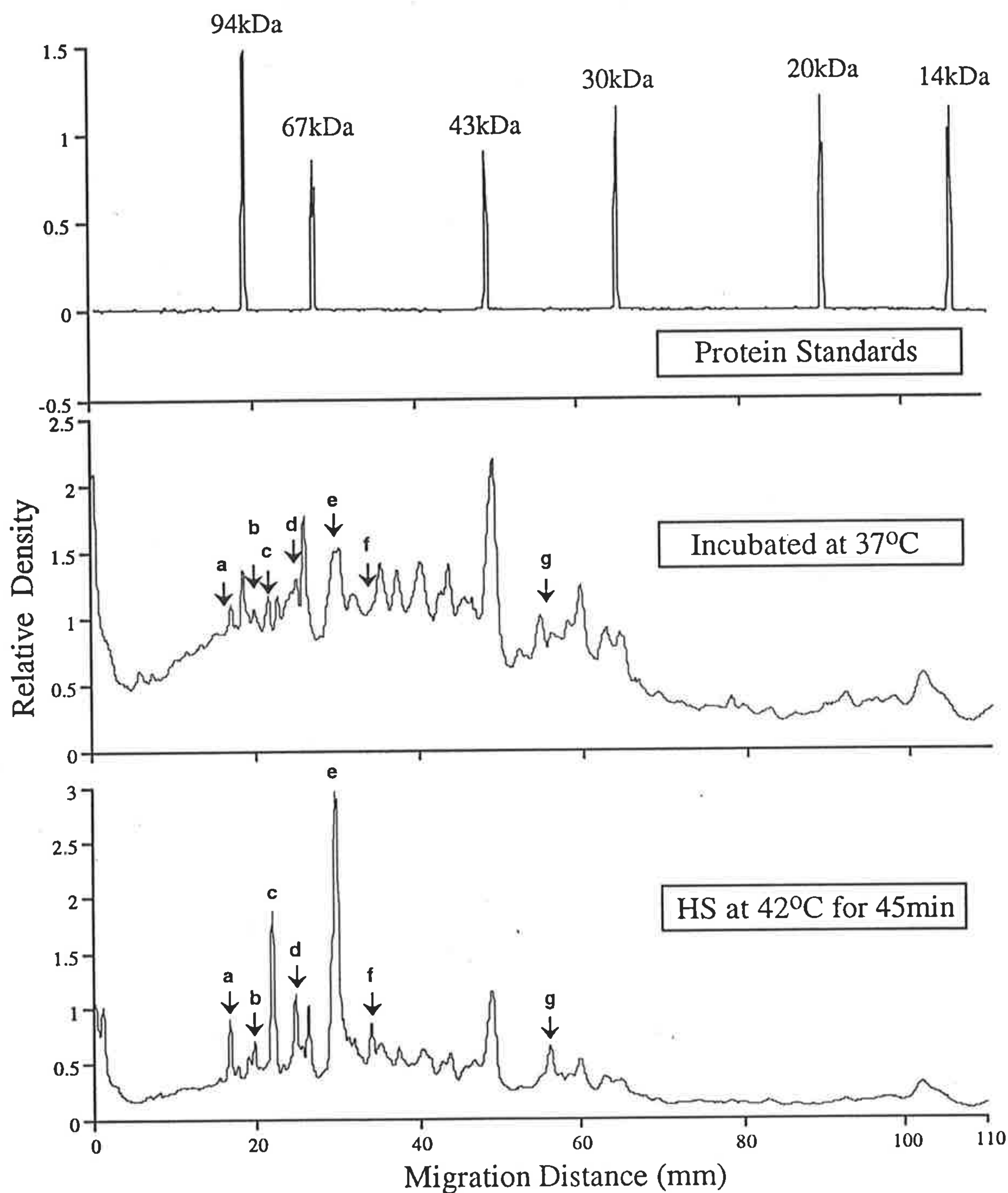


Figure 3.1b shows the densitometric scan of the autoradiograph in Fig 3.1a. The top scan is of the protein standards ranging from ≈ 94kDa to ≈ 14kDa. The middle and bottom scans are of MNC treated at 37°C and 42°C respectively for 45min. The arrows labelled from a to g indicate the position of the corresponding induced protein bands, after HS at 42°C. Density increases of the induced protein bands, relative to the control cells, were calculated from the densitometric data and expressed in terms of fold increase and are as follows: a ≈ 4.5, b ≈ 1.6, c ≈ 4.1, d ≈ 3.3, e ≈ 3.5, f ≈ 3.7 and g ≈ 3.7.

Figure 3.1b

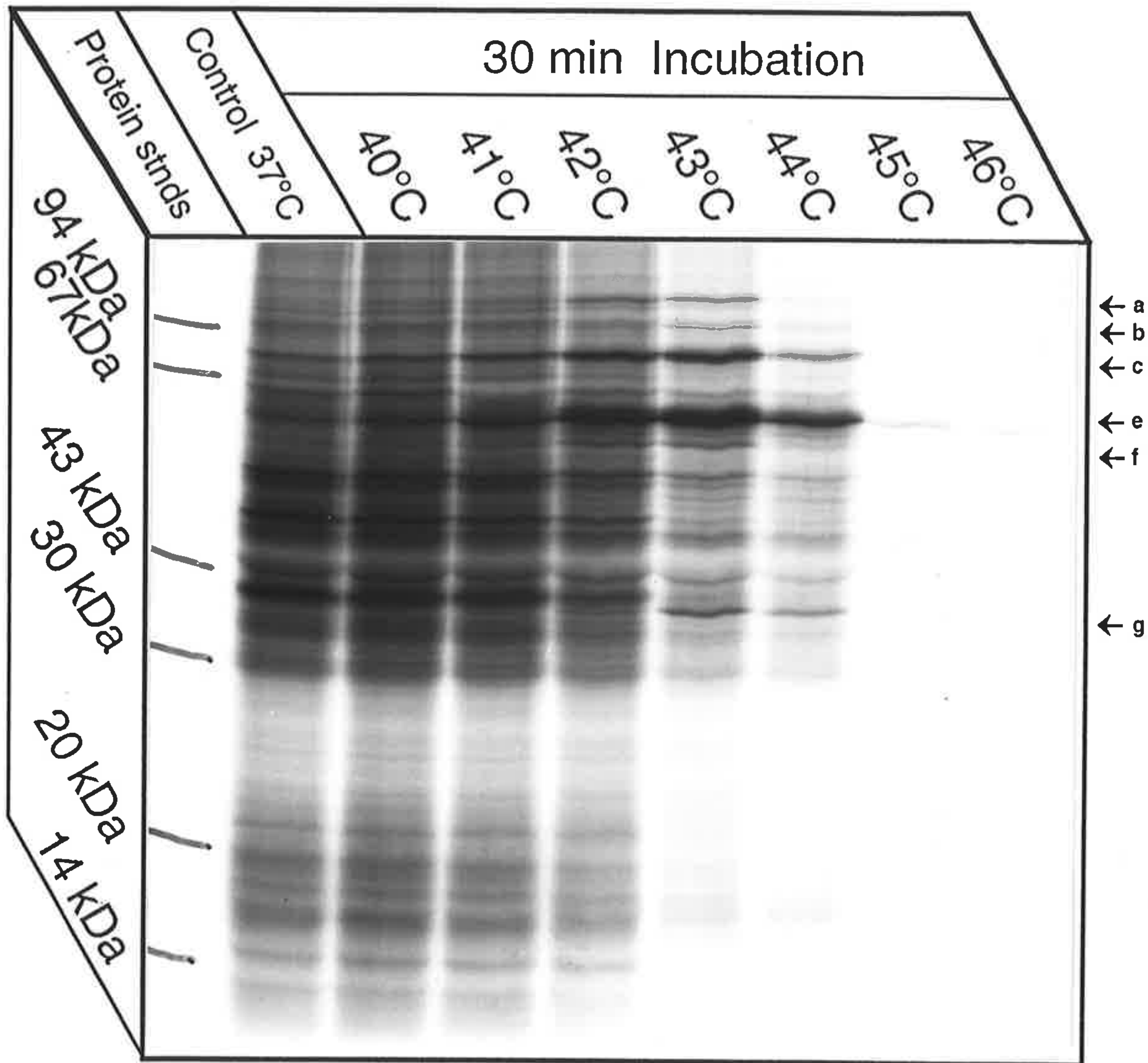


Figure 3.2a shows an autoradiograph of MNC proteins, after exposing the cells to HS at 40°C - 46°C, separated by SDS PAGE. The arrows, labelled from a to g, indicate the approximate positions of the proteins induced by HS. All six of these proteins (a to g) are seen in MNC which have been incubated at 42°C and 43°C. However, protein d which appears in Figs. 3.1a and 3.1b is absent.

Figure 3.2a

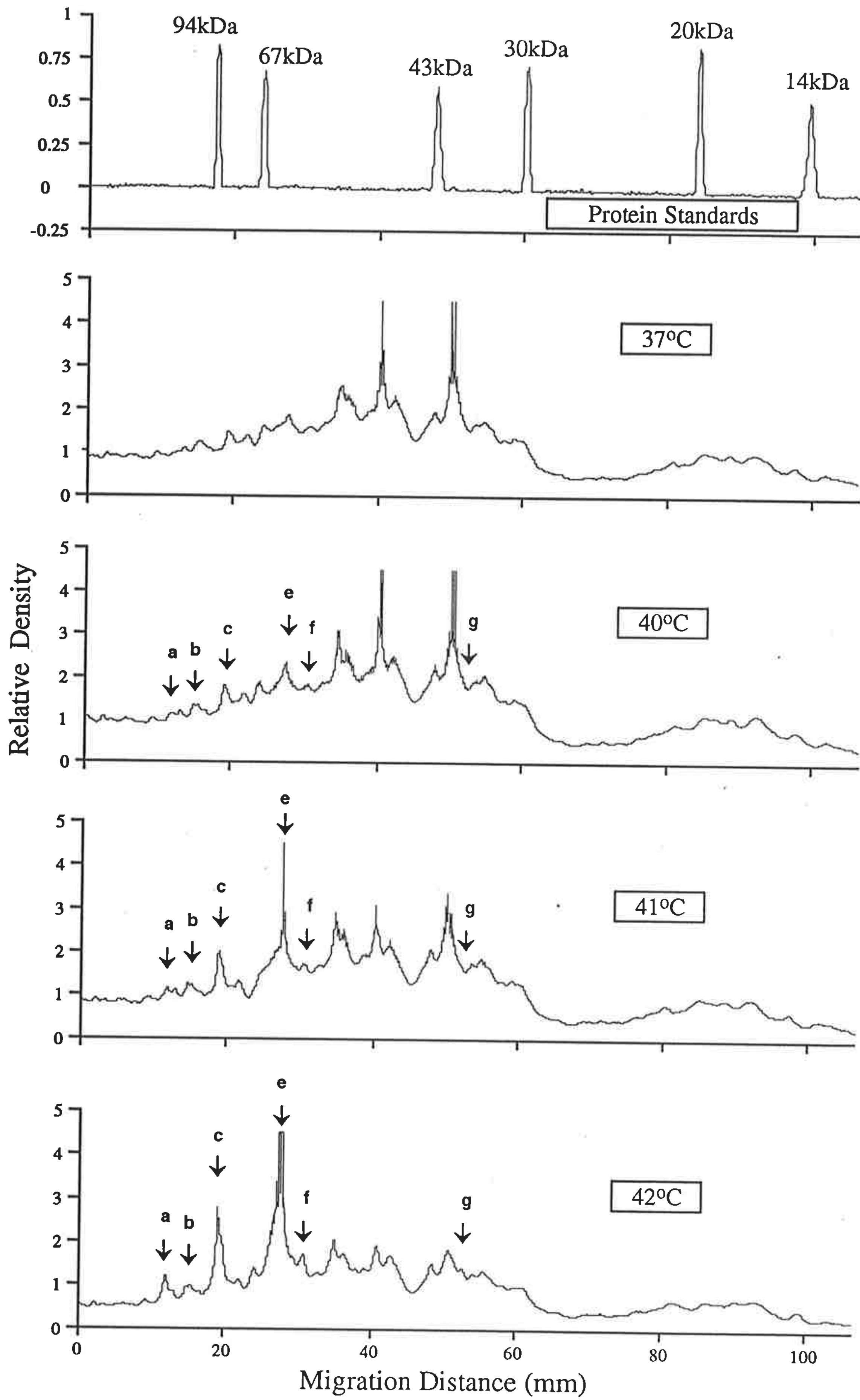


Figure 3.2b

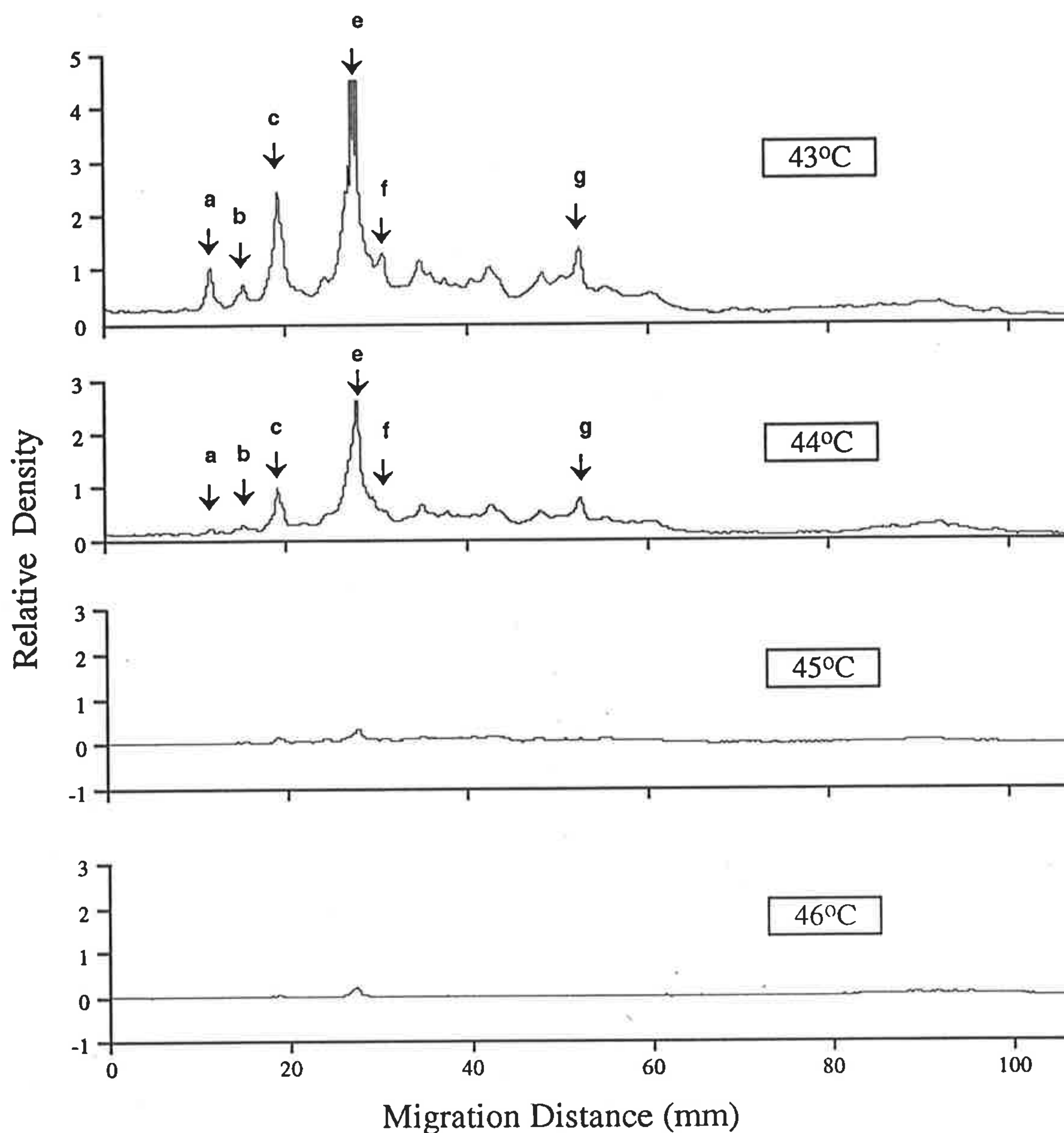


Figure 3.2b shows the densitometric scans of the autoradiograph in Fig 3.2a. The first scan (previous page) shows the positions of the protein standards. The next scan down shows the control cells incubated at 37°C. The following scans are of cells exposed to heat shock ranging from 40°C up to 46°C for 30min. The arrows labelled from a to g mark the relative positions of the HS-induced proteins shown in figure 3.2a.

Figure 3.2b (continued)

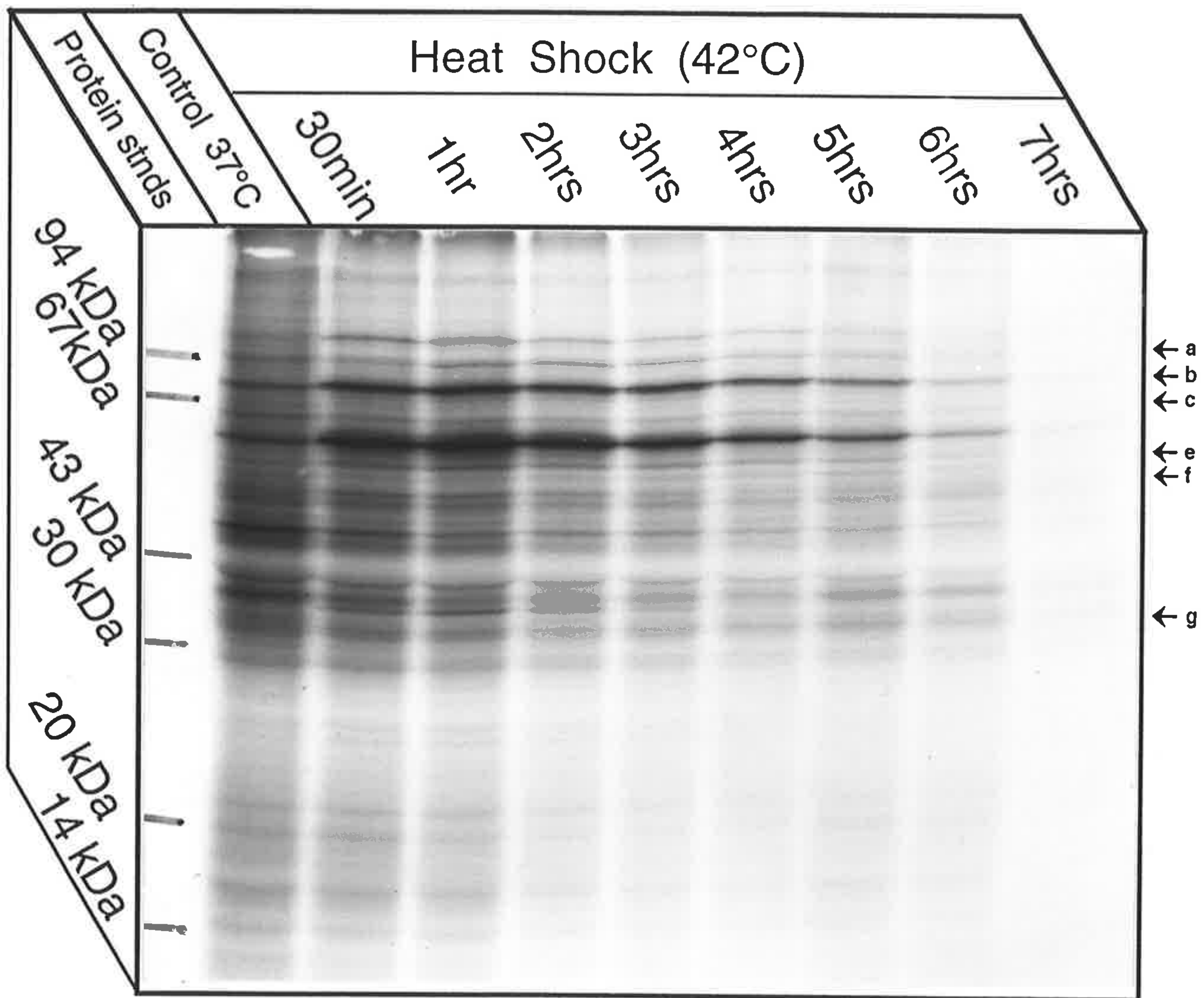


Figure 3.3a shows an autoradiograph of MNC proteins, after the cells were exposed to HS for 30min to 7hrs, as separated by SDS PAGE. The arrows, labelled from a to g, indicate the approximate position of the proteins induced by HS at 42°C for 1hr. Once again protein band d, which appears in Figs. 3.1a and 3.1b, is absent.

Figure 3.3a

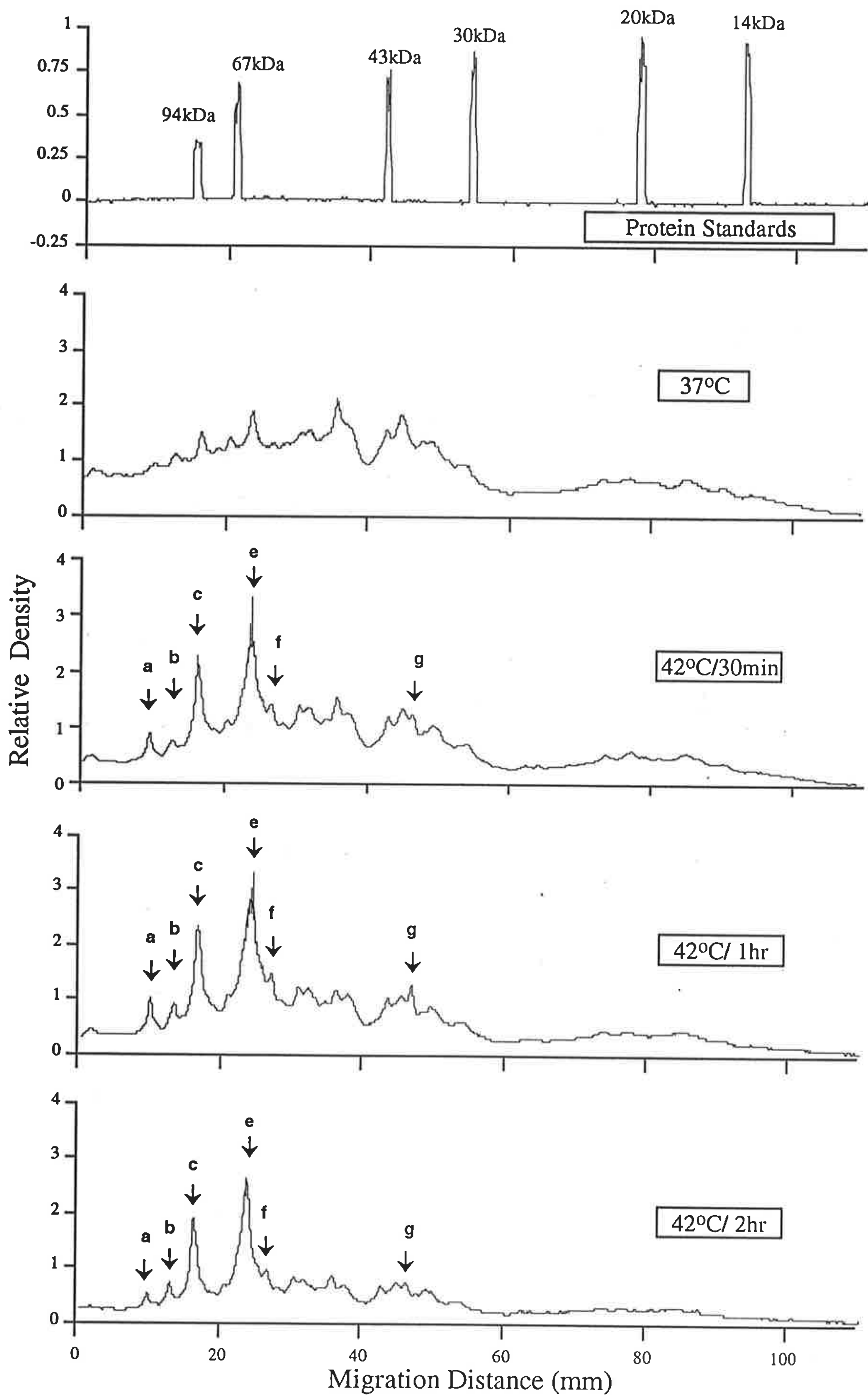


Figure 3.3b

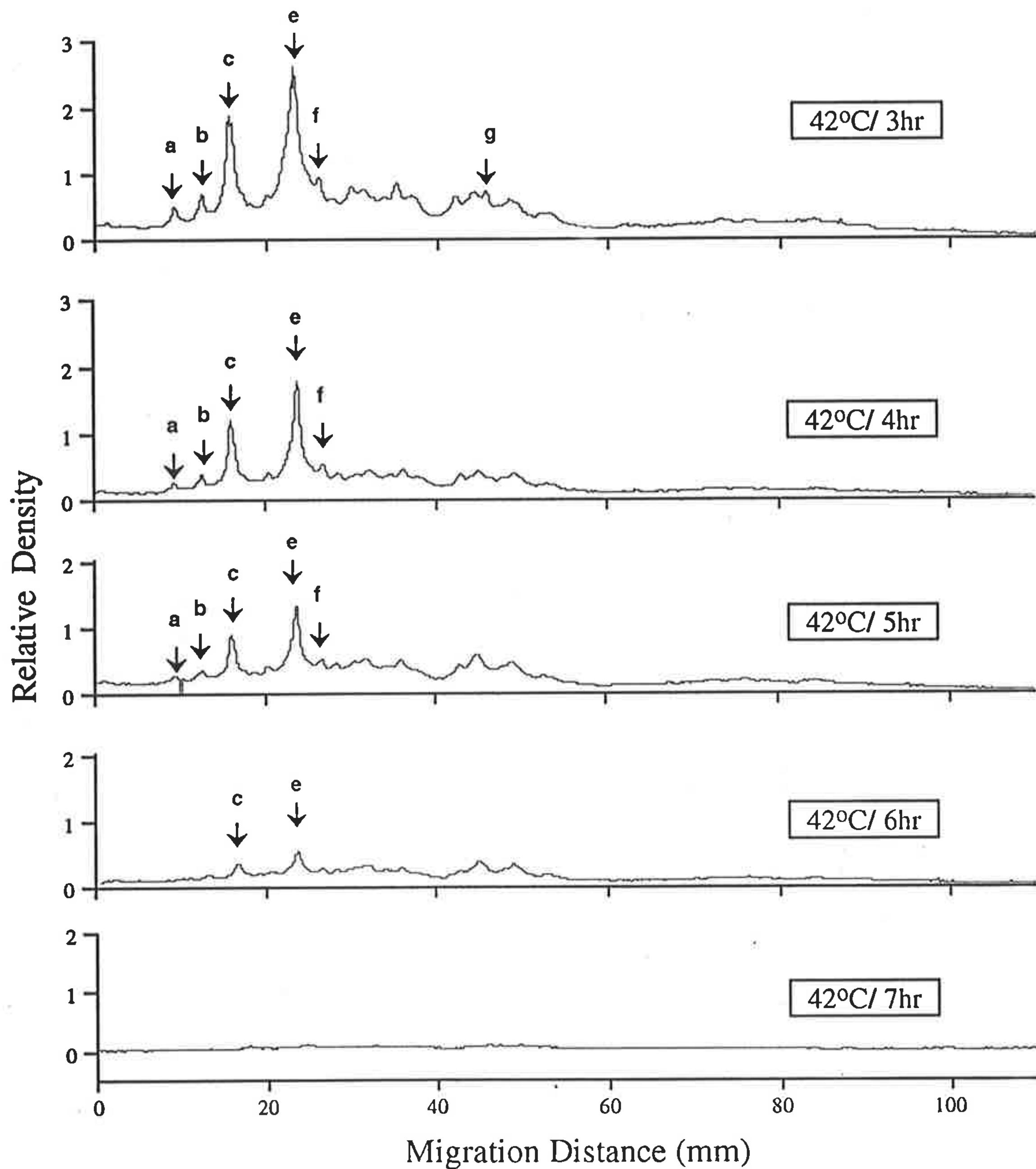


Figure 3.3b shows the densitometric scans of the autoradiograph in figure 3.3a. The first scan (previous page) shows the positions of the protein standards. The next scan down shows the control cells incubated at 37°C for 7hrs. The following scans are of cells exposed to a heat shock of 42°C from 30min up to 7hrs. The arrows labelled from a to g mark the relative positions of the HS induced proteins shown in figure 3.3a.

Figure 3.3b (continued)

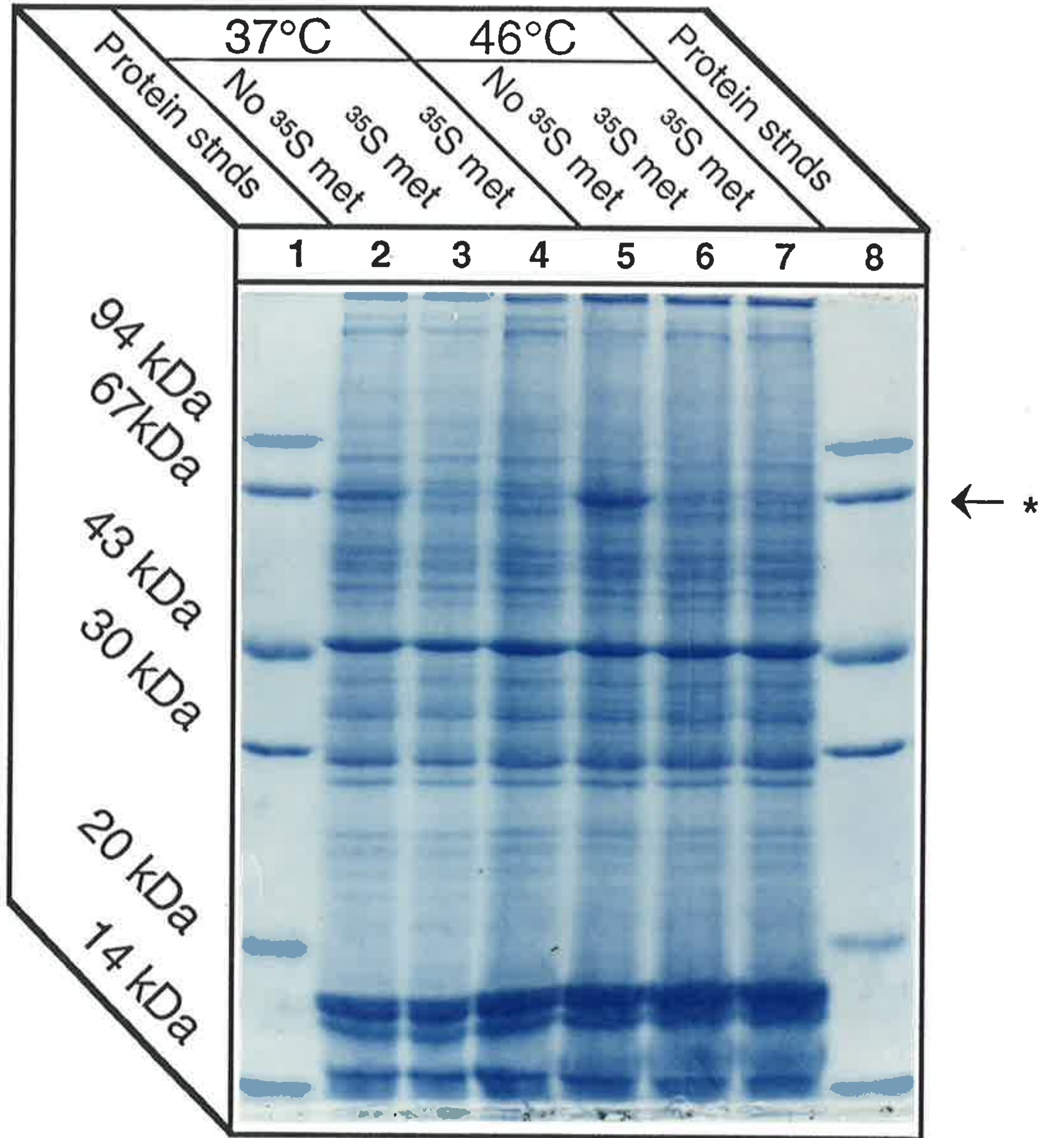


Figure 3.4a shows the coomassie blue stained poly acrylamide gel of MNC, exposed to HS incubated in methionine deficient media or complete media, proteins as separated by SDS PAGE. The arrow labelled with * indicates the position of the protein band induced in MNC incubated in media without methionine.

Figure 3.4a

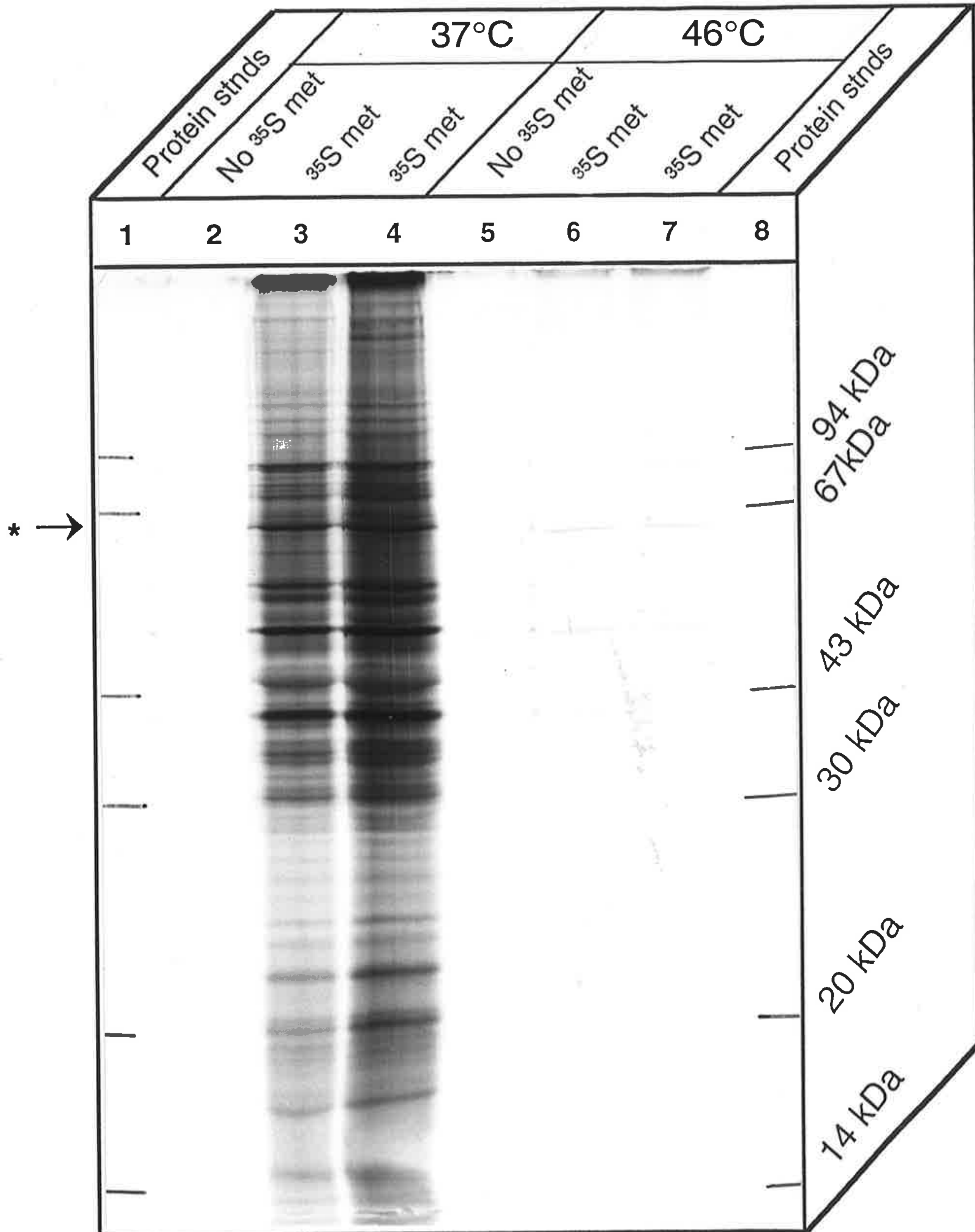


Figure 3.4b shows the autoradiograph obtained from the SDS polyacrylamide gel appearing in figure 3.4a.

Figure 3.4b

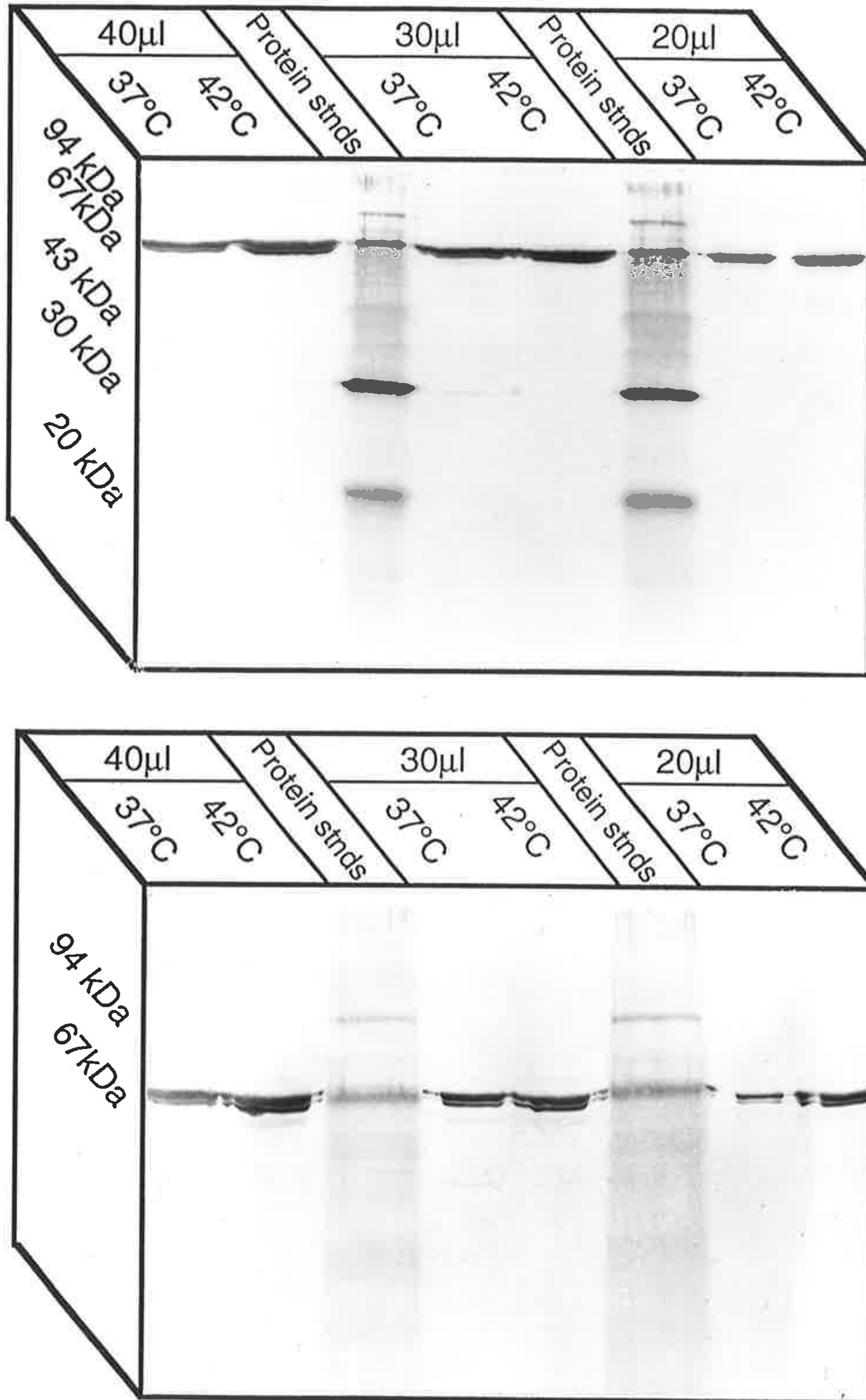


Figure 3.5a shows two Western blots of MNC proteins after exposure to a HS of 42°C and then separated by SDS PAGE. The top blot is of a SDS PAGE which has been electrophoresed for the standard length of time whereas, the bottom blot is of SDS PAGE electrophoresed for approximately twice as long. Both blots have been probed with the anti-heat shock protein 70 mouse monoclonal antibody (IgG) clone 3a3 which is specific for both the hsc70 and hsp70 proteins. In the lower blot, two distinct bands are detected by this antibody whereas the separation in the upper blot is insufficient to resolve the two bands.

Figure 3.5a

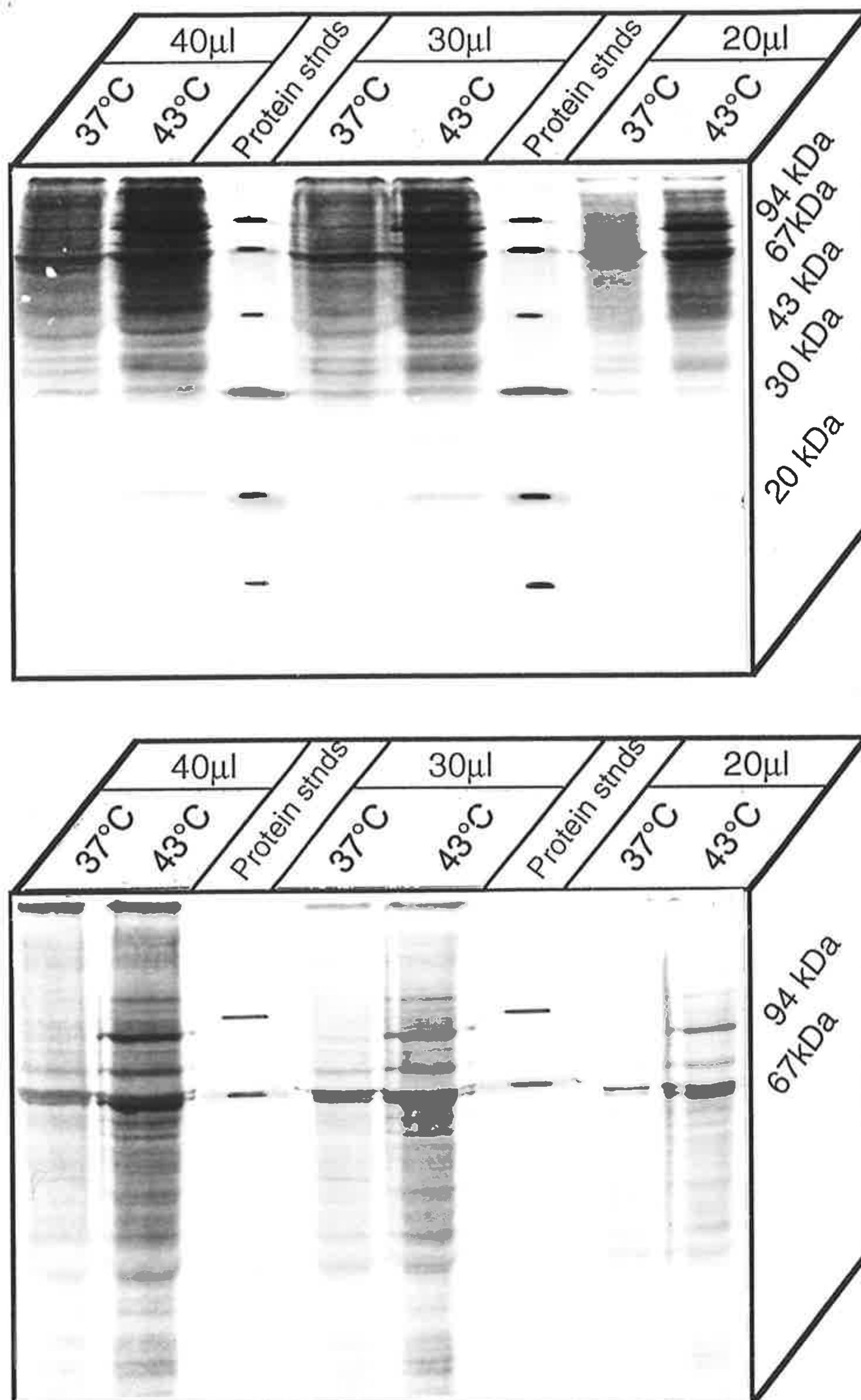
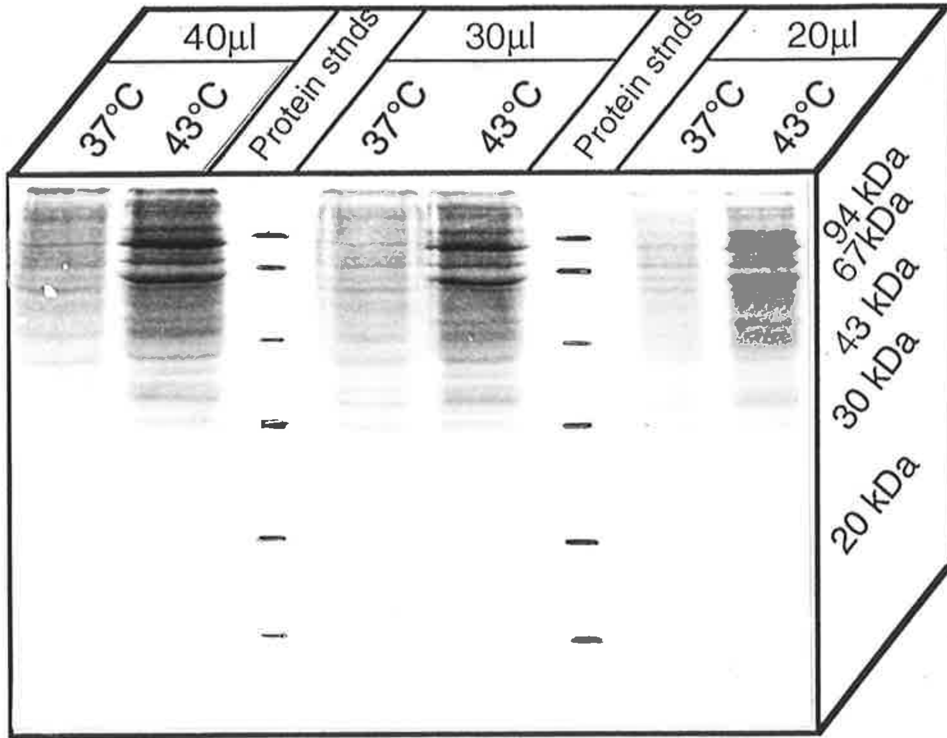


Figure 3.5b shows the two Western blots appearing in figure 3.5a overlaid by the autoradiographs produced from these blots. One of the induced protein bands on the bottom autoradiograph coincides exactly with the faster migrating band detected by the antibody, therefore positively identifying this band on the autoradiograph as hsp70. The slower migrating band, hsc70, on the bottom blot is not detected in the autoradiograph. The relative position of the hsp70 to the 66.2kDa biotinylated molecular weight protein standard on the Western blots and other MNC proteins on the autoradiograph is clearly identified by this method.

Figure 3.5b



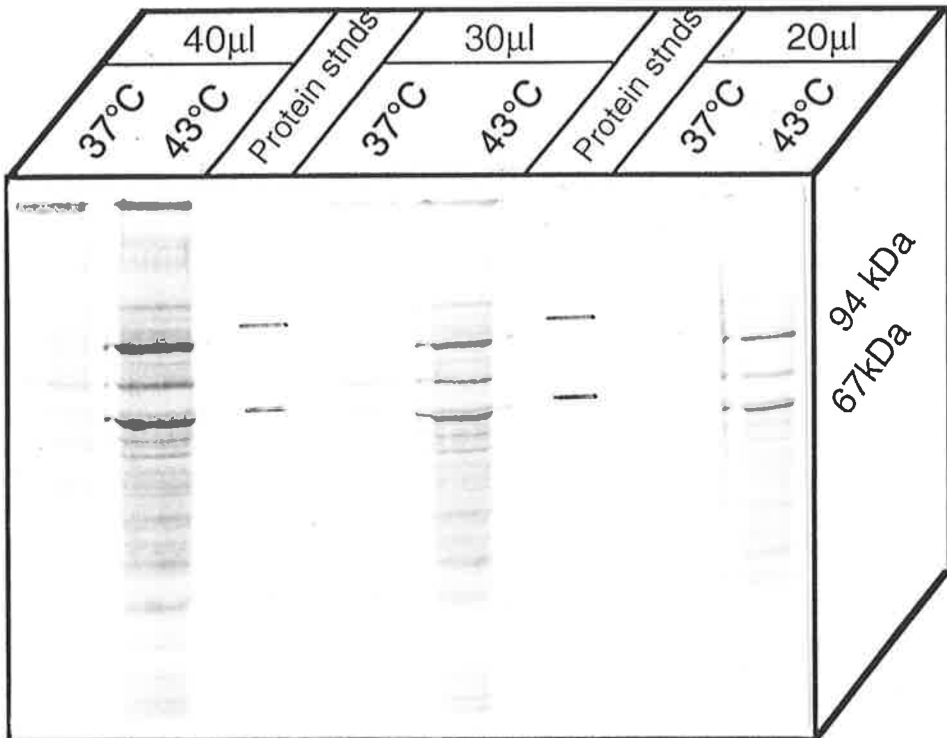


Figure 3.5c shows the two autoradiographs of the corresponding Western blots appearing in figure 3.5a.

Figure 3.5c

CHAPTER FOUR

Anti-inflammatory Agents: Resolving Concentrations for HSP Induction in Human Mononuclear Cells

4.1 INTRODUCTION

In my initial experiments, not presented here, I tested a variety of anti-inflammatory/ rheumatic agents with MNC. However none of these agents, except for auranofin, induced HSP expression. As discussed in Section 1.1.2 of the introduction, induction of HSP synthesis is primarily regulated through the HSF which, after HS, becomes activated and acts as a transcription factor. Activation of HSF is possibly a two step process, firstly: HSF acquires the ability to specifically bind DNA and secondly, HSF becomes phosphorylated (Sorger and Pelham 1988; Hensold *et al.* 1990; Jurivich *et al.* 1992). Zimarino and Wu (1987) demonstrated that HSF, in *Drosophila* cells, can be induced, by sodium salicylate, to specifically bind the HSE. Jurivich (1992) also reported that transcription from the *hsp70* gene, after salicylate treatment, did not occur despite the HSF binding to the HSE. Jurivich *et al.* (1992) suggested that the absence of transcription may be due to the absence of second stage activation of HSF (e.g. phosphorylation), even though first stage activation was induced by salicylate. Furthermore, the activation of HSF binding by salicylate, did not interfere with the induction of *HSP* gene transcription after a subsequent HS. Therefore, agents like salicylate, which activate specific HSF binding to DNA, may prepare cells for HSP expression by reducing the activation pathway to a single step process. Other anti-inflammatory agents may also have a similar affect on HSF-DNA binding without inducing transcription. Therefore, in this chapter, the potential of salicylate and other agents to facilitate HSP induction, in MNC, was investigated.

4.2 DOES SALICYLATE FACILITATE HSP INDUCTION IN MNC?

My preliminary experiments demonstrated that salicylate on its own did not induce HSP synthesis in MNC. However, in view of the fact that salicylate induces the formation of the HSE-HSF complex without increasing the expression of HSP (Jurivich *et al.* 1992), I designed an experiment to determine whether a combination of pretreating MNC with salicylate and exposure to a mild HS induced or affected (facilitated) HSP expression. In these experiments medium with sodium salicylate was prepared, as

described in Section 2.1.3, to a stock concentration of 50mM and diluted serially to obtain 25, 12, 6.0, 3.0, 1.0, 0.25 and 0.06mM solutions. MNC were either exposed to medium without salicylate or medium containing salicylate and incubated at 37°C for 30min before incubating at 39°C for 15min. The cells were then radiolabelled and processed as described in Sections 2.2.5 to 2.2.8. The autoradiograph of MNC proteins separated by SDS PAGE appears in Fig. 4.1. From the autoradiograph, the first signs of induced HSP expression occurred at 25mM . At 50mM, HSP expression was further increased. There was no evidence of total protein synthesis inhibition at any concentration of salicylate.

These experiments were then extended to determine the extent of the salicylate effect on HSP expression over a range of temperatures. A fixed salicylate concentration of 15mM and temperatures ranging from 37°C to 43°C were chosen. MNC were either incubated with 15mM salicylate or without salicylate for 10min at 37°C before incubating for a further 15min at 37°C or exposed to a HS of 39°C, 41°C or 43°C for 15min. The MNC cultures were then processed as described in Sections 2.2.5 to 2.2.8. The corresponding autoradiograph appears in Fig. 4.2. From the figure, MNC incubated without salicylate did not demonstrate HSP expression until incubated at 41°C. HSP expression increased even further in MNC incubated at 43°C. However, in salicylate treated MNC, HSP expression occurred when cells were exposed to the lower temperature of 39°C; at 41°C HSP expression increased even further. However, after salicylate-treated MNC were incubated at 43°C, normal protein synthesis was almost completely inhibited and HSP synthesis had completely disappeared.

These results demonstrate that salicylate alone, at 37°C, does not induce HSP expression in MNC. However, when MNC were exposed to a combination of salicylate followed by a HS, HSP expression was strongly facilitated. HSP synthesis was not only induced in salicylate-treated MNC at a much lower temperature, but was also increased at those temperatures which can alone induce HSP expression. At 43°C, normal protein synthesis and HSP synthesis was inhibited in salicylate treated MNC. This effect was similar to that of MNC exposed to more extreme HS conditions (e.g. 45°C) as was demonstrated in Chapter Three.

4.3 EFFECTS OF OTHER ANTI-INFLAMMATORY/ RHEUMATIC AGENTS ON THE FACILITATION OF HSP INDUCTION

4.3.1 Aspirin

A stock solution of aspirin was prepared in culture medium, as described in Section 2.1.3, to a concentration of 50mM. The 50mM stock was then serially diluted to obtain 25, 12, 6, 3, 1, 0.25 and 0.06mM concentrations. Equal MNC numbers were resuspended in medium without aspirin and all of the above media preparations containing aspirin. The MNC were then incubated at 37°C for 30min before incubating at 39°C for an additional 15min. The aspirin containing medium was removed from the MNC, replaced with ^{35}S methionine labelling medium and the MNC radiolabelled and processed as described in Sections 2.2.5 to 2.2.8. The autoradiograph of MNC proteins separated by SDS PAGE appears in Fig. 4.3. From the autoradiograph, HSP induction after MNC exposure to 39°C occurred when the concentration of aspirin was 25mM and became greater when the concentration of aspirin was increased to 50mM. Furthermore, there was no evidence that aspirin, at concentrations used in this experiment, caused any inhibition of total protein synthesis inhibition in MNC.

4.3.2 Chloroquine

A stock solution of chloroquine was prepared in culture medium, as described in Section 2.1.3, to a concentration of 20mM and diluted serially to obtain 15, 10, 5, 2.5, 1.2 and 0.6mM solutions. MNC were either exposed to medium without chloroquine or medium containing chloroquine and incubated at 37°C for 30min followed by an incubation at 39°C for 15min. The cells were then radiolabelled and processed as described in Sections 2.2.5 to 2.2.8. The autoradiograph of MNC proteins separated by SDS PAGE appears in Fig. 4.4. From the autoradiograph, there does not seem to be any distinctly recognisable HSP induction in MNC after exposure to 39°C, although a light band at approximately 70kDa appears after MNC have been incubated in medium containing 0.6 and 1.2mM chloroquine. With 1.2mM chloroquine, total protein

synthesis inhibition started to take effect under these cell culture conditions and protein inhibition became complete at concentrations ≥2.5mM.

4.3.3 Diclofenac

A stock solution of diclofenac was prepared in culture medium, as described in Section 2.1.3, to a concentration of 5mM and diluted serially to obtain 2.5, 1.2, 0.6, 0.3, 0.15 and 0.08mM solutions. MNC were either, exposed to medium without diclofenac, or medium containing diclofenac and incubated at 37°C for 30min before incubating at 39°C for 15min. The cells were then radiolabelled and processed as described in Sections 2.2.5 to 2.2.8. The autoradiograph of MNC proteins separated by SDS PAGE appears in Fig. 4.5. The induction of HSP began at the diclofenac concentration of 1.2mM and became stronger in the presence of 2.5mM diclofenac. At 5mM diclofenac, HSP synthesis continued and inhibition of total protein synthesis, although not complete, was considerable.

4.3.4 Indomethacin

A stock solution of indomethacin was prepared in culture medium, as described in Section 2.1.3, to a concentration of 5mM and diluted serially to obtain 5, 1, 0.5, 0.2, 0.1 and 0.05mM solutions. MNC were either, exposed to medium without indomethacin, or medium containing indomethacin. Because indomethacin concentration was the first to be optimised, the preincubation conditions had initially been at 37°C for 10min rather than 37°C for 30min. However, when the conditions were later changed to 37°C for 30min, there was no change in MNC protein synthesis. After preincubation, MNC were incubated at 39°C for 15min and then radiolabelled and processed as described in Sections 2.2.5 to 2.2.8. The autoradiograph of MNC proteins separated by SDS PAGE appears in Fig. 4.5. HSP synthesis in MNC was seen to occur once the indomethacin concentration in the medium reached 0.5mM, and continued and increased as the concentration of indomethacin went higher. At 1mM indomethacin, total protein synthesis inhibition began to set in and was complete at 5mM indomethacin.

Because indomethacin solid is difficult to dissolve in aqueous cell culture medium, indomethacin was first dissolved in a small amount of DMF before diluting in aqueous solution (Section 2.1.3). The final concentration of DMF in the cell culture medium, also supplemented with indomethacin, did not exceed 0.2% (v/v). To determine if DMF had an effect on MNC, MNC were also exposed to a range of DMF concentrations. A stock concentration of 5% (v/v) DMF was prepared and diluted serially to obtain 4, 3, 2, 1, 0.1, 0.05, and 0.01%(v/v). MNC were then incubated either without DMF or with DMF at 37°C for 10min followed by incubation at 37°C or 39°C for 15min. The autoradiograph of MNC proteins separated by SDS PAGE also appear in Fig. 4.4. No HSP induction in DMF-treated cells was seen until the DMF concentration reached 1%, where only very slight induction seemed to occur. A more distinct induction was observed in cells exposed to DMF concentrations of ≥2%. 5% DMF not only demonstrated HSP induction but also a partial inhibition of normal protein synthesis.

4.3.5 Methotrexate

Methotrexate, although not an anti-inflammatory agent, is effective in the treatment of rheumatoid arthritis. Methotrexate is an antimetabolite, antineoplastic agent that inhibits folate metabolism. However, during rheumatoid arthritis treatment, methotrexate, is administered to patients in significantly lower doses as compared to patients who are treated for cancer.

A stock solution of 2mM methotrexate was prepared, as described in Section 2.1.3, in culture medium and diluted serially to obtain solutions of 1.0, 0.5, 0.25, 0.12, 0.06, 0.03, and 0.01mM. MNC were incubated in media either without or with methotrexate, for 30min at 37°C before incubating for an additional 15min at 39°C. MNC were radiolabelled and processed as described in Sections 2.2.5 to 2.2.8. From Fig. 4.7, which shows the corresponding autoradiograph for methotrexate treated MNC, there does not appear to be any induction of specific protein bands. In later experiments, Section 5.2.5, 10mM methotrexate was used, as higher concentrations failed to dissolve completely in the culture medium. Even at 10mM, methotrexate was found to have little effect on both HSP expression and on total protein synthesis.

4.3.6 Paracetamol

Paracetamol is another non anti-inflammatory agent. Its use in treating rheumatoid arthritis is primarily concerned with controlling arthralgia and pyrexia.

A stock solution of 50mM paracetamol was prepared as described in Section 2.1.3, in culture medium and diluted serially to obtain solutions of 25, 12, 6.0, 3.0, 1.0, 0.25, and 0.06mM. MNC were incubated in medium either without or with paracetamol, for 60min at 37°C before incubating for an additional 15min at 39°C. MNC were radiolabelled and processed as described in Sections 2.2.5 to 2.2.8. The autoradiograph in Fig. 4.8 shows a very slight change in normal protein synthesis in MNC exposed to 12mM paracetamol but, at 25mM paracetamol, there was a strong induction of HSP which was also present at 50mM. However, at 50mM, paracetamol also caused a partial inhibition of normal protein synthesis.

4.3.7 D-Penicillamine

A stock solution of D-penicillamine was prepared in culture medium, as described in Section 2.1.3, to a concentration of 20mM and diluted serially to obtain 15, 10, 5.0, 2.5, 1.2 and 0.6mM. MNC were either exposed to medium with or without D-penicillamine and incubated at 37°C for 30min before incubating at 39°C for 15min. The cells were then radiolabelled and processed as described in Sections 2.2.5 to 2.2.8. The autoradiograph of MNC proteins separated by SDS PAGE appears in Fig. 4.9. From the autoradiograph there was no evidence of any HSP induction and synthesis above the normal cellular levels, nor was there any evidence of total protein synthesis inhibition at any of the concentrations used.

4.4 DISCUSSION

In this chapter (Section 4.2), a suitable concentration for salicylate was first estimated and then used to demonstrate that salicylate facilitates the induction of HSP synthesis in MNC exposed to the combination of salicylate and HS. In Section 4.3, the concentrations for other anti-inflammatory/rheumatic agents were also estimated for the purpose of investigating their ability to facilitate HSP synthesis in MNC as was demonstrated in Section 4.2.2 using salicylate. As discussed in the introduction, Section 4.1, the criteria for choosing a concentration for each agent, is that the concentration was sufficient to cause HSP induction in MNC while not interfering with normal protein synthesis. Concentrations high enough to cause inhibition of normal protein synthesis in MNC would eventually, as expected, prove toxic to the cells not only under *in vitro* conditions, but also under *in vivo* conditions. However, because the experimental procedure utilised short incubation periods in the presence of anti-inflammatory agent, unlike *in vivo* where MNC are exposed for considerably longer to drug plasma concentrations, the concentration of each agent required to induce HSP synthesis may differ from the therapeutic concentrations.

Salicylate, indomethacin and diclofenac demonstrated similar and interesting effects on normal protein synthesis as well as on HSP synthesis. Therefore, these agents may have applications in other areas of medicine (e.g., cancer therapy). These agents could be used, in combination with hyperthermia, to inhibit all protein synthesis in tumour cells, including HSP synthesis. This would render the tumour cells unable to respond to the cytotoxic stress of radiation therapy and chemotherapy, hence improving the success rate of cancer therapy.

To summarise, the concentrations of each anti-inflammatory/rheumatic agent for HSP induction were optimised and the selected concentrations are given in Table 4.3. The optimum selected concentration for salicylate was 17mM. This is similar to the concentration range used by Jurivich (1992) and coworkers, who studied the effects of salicylate on the formation of the HSE-HSF complex in HeLa cells. They found that, although salicylate concentrations of 2 to 30mM did not induce HSP synthesis, 20mM salicylate did activate the specific HSE binding activity of HSF to the same extent as a HS of 42°C.

Table 4.3

Agent	Selected Concentration
Aspirin	22mM
Chloroquine	0.8mM
Diclofenac	1.0mM
Indomethacin	0.6mM
Methotrexate	10mM
Paracetamol	20mM
D-Penicillamine	20mM
Salicylate	17mM
DMF	0.2%(v/v)

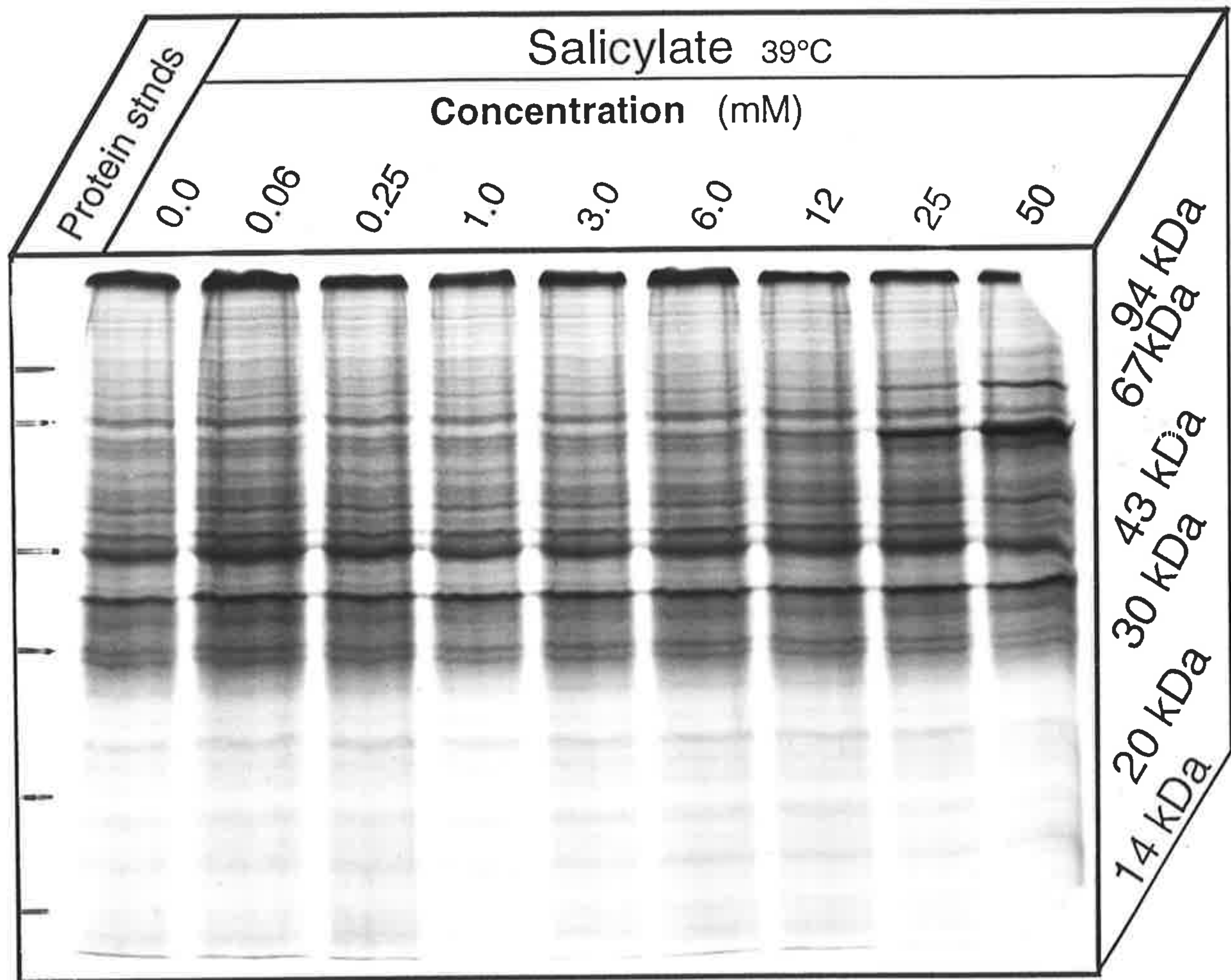


Figure 4.1 shows the autoradiograph of MNC proteins, after the cells were exposed to different salicylate concentrations for 30min at 37°C followed by incubation at 39°C for a further 15min.

Figure 4.1

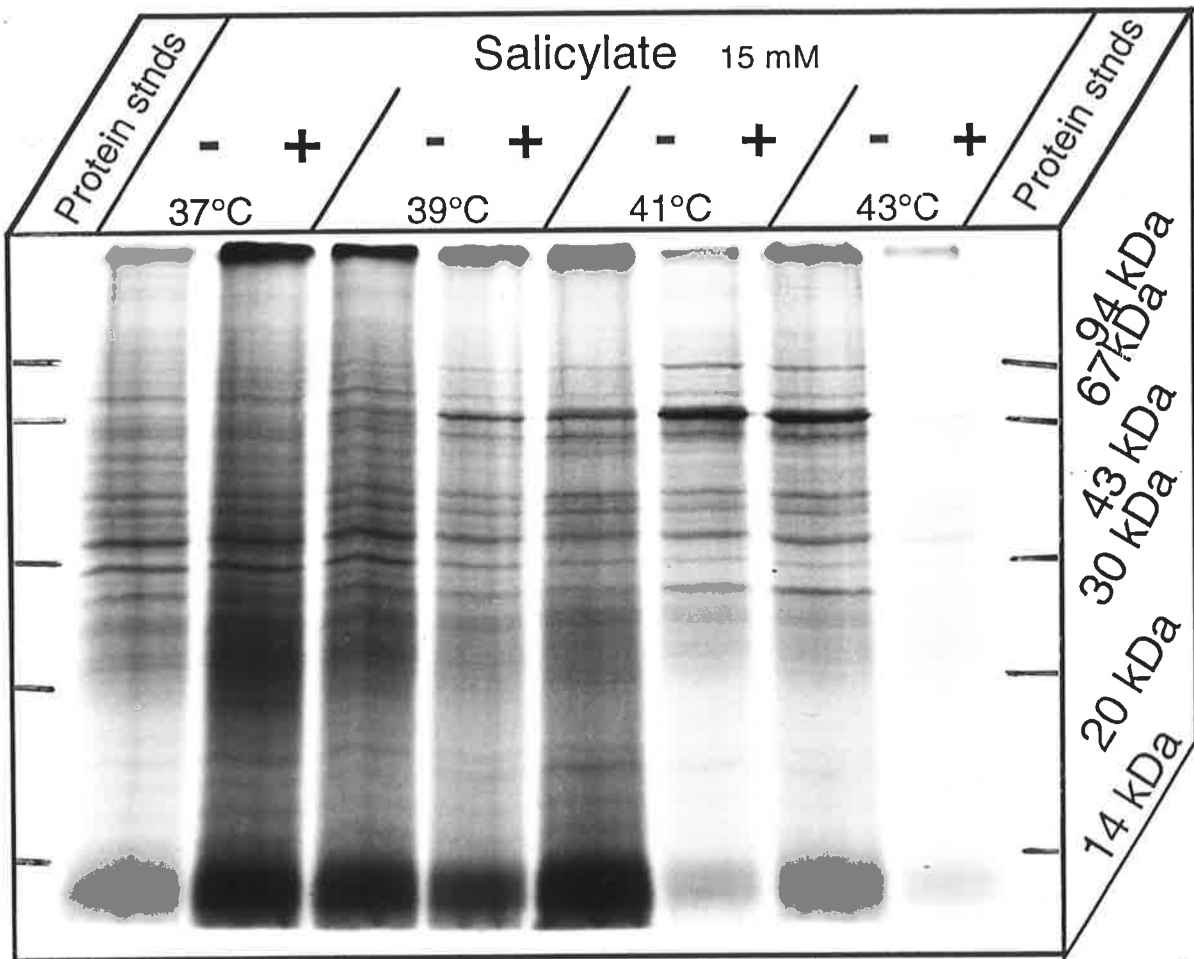


Figure 4.2 shows the autoradiograph of MNC proteins separated by SDS PAGE after MNC were exposed to a combination of salicylate and HS. MNC were incubated either without or, with 15mM salicylate for 10min at 37°C before exposure to a HS of 39°C, 41°C or 43°C for 15min. HSP synthesis was induced, after HS at 39°C, in salicylate-treated MNC, by the appearance of a hsp70 band, when compared to untreated MNC, also exposed to 39°C, which do not show any detectable HSP synthesis. After MNC were exposed to a HS of 41°C both the untreated and treated MNC synthesise HSP, however HSP synthesis in salicylate-treated MNC is considerably higher than that of the untreated cells, which show the induction of hsp70 only. After MNC were incubated at 43°C the untreated cells demonstrate as much HSP synthesis as salicylate treated MNC after exposure to 41°C, but the salicylate treated cells after a HS of 43°C demonstrate severe inhibition of all protein synthesis including HSP synthesis.

Figure 4.2

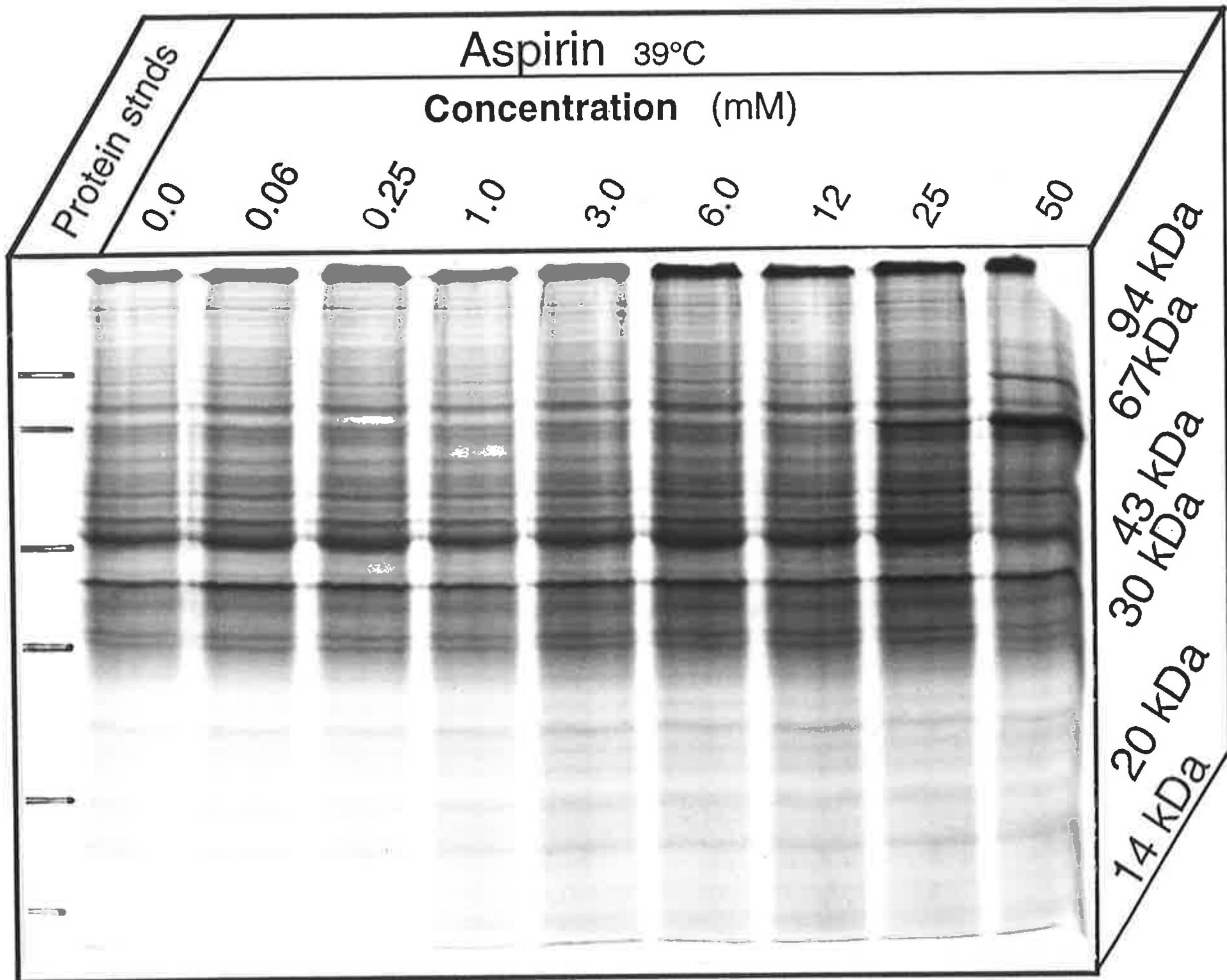


Figure 4.3 shows the autoradiograph of MNC proteins, after the cells were exposed to different aspirin concentrations for 30min at 37°C followed by incubation at 39°C for a further 15min.

Figure 4.3

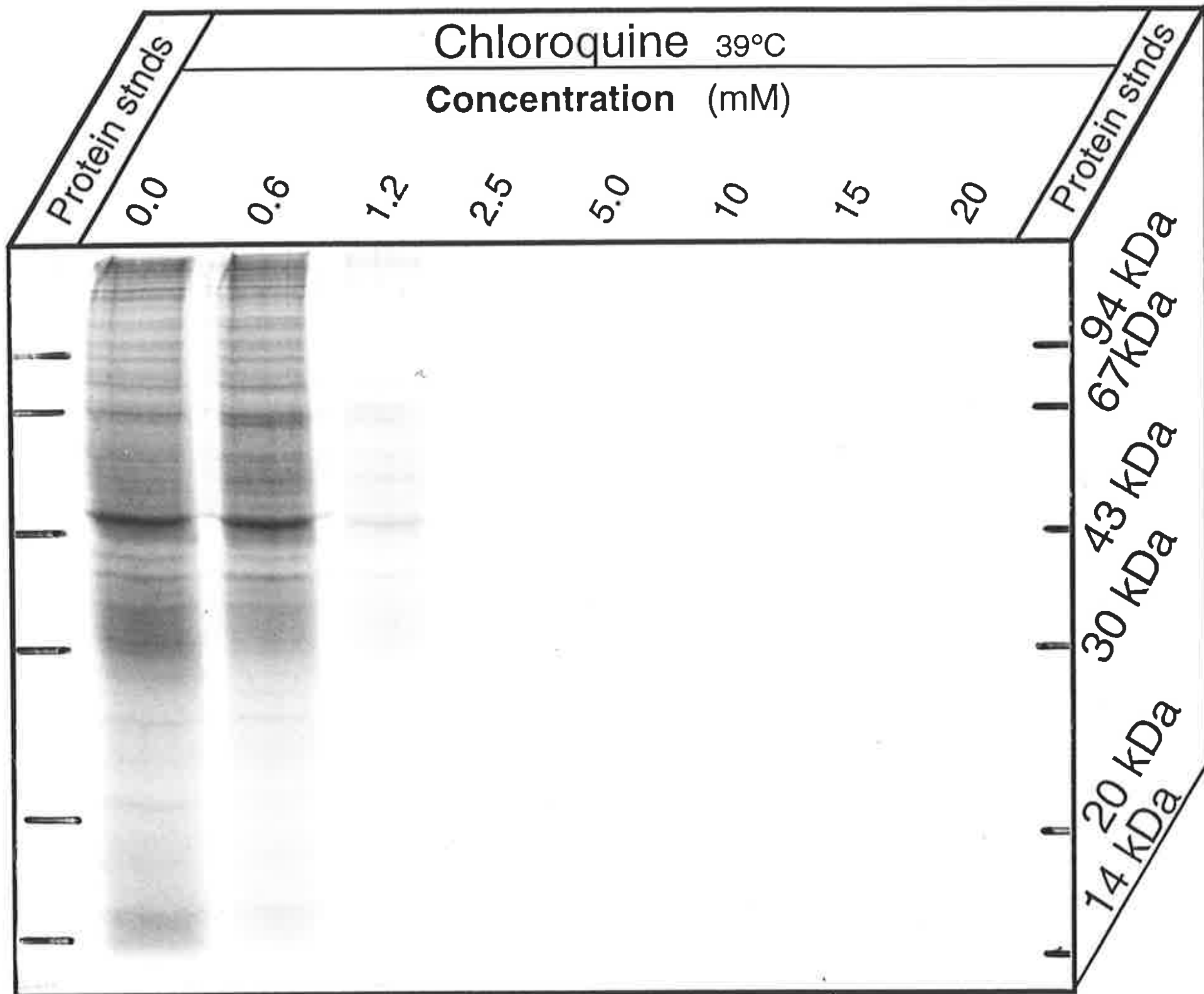


Figure 4.4 shows the autoradiograph of MNC proteins, after the cells were exposed to different chloroquine concentrations for 30min at 37°C followed by incubation at 39°C for a further 15min.

Figure 4.4

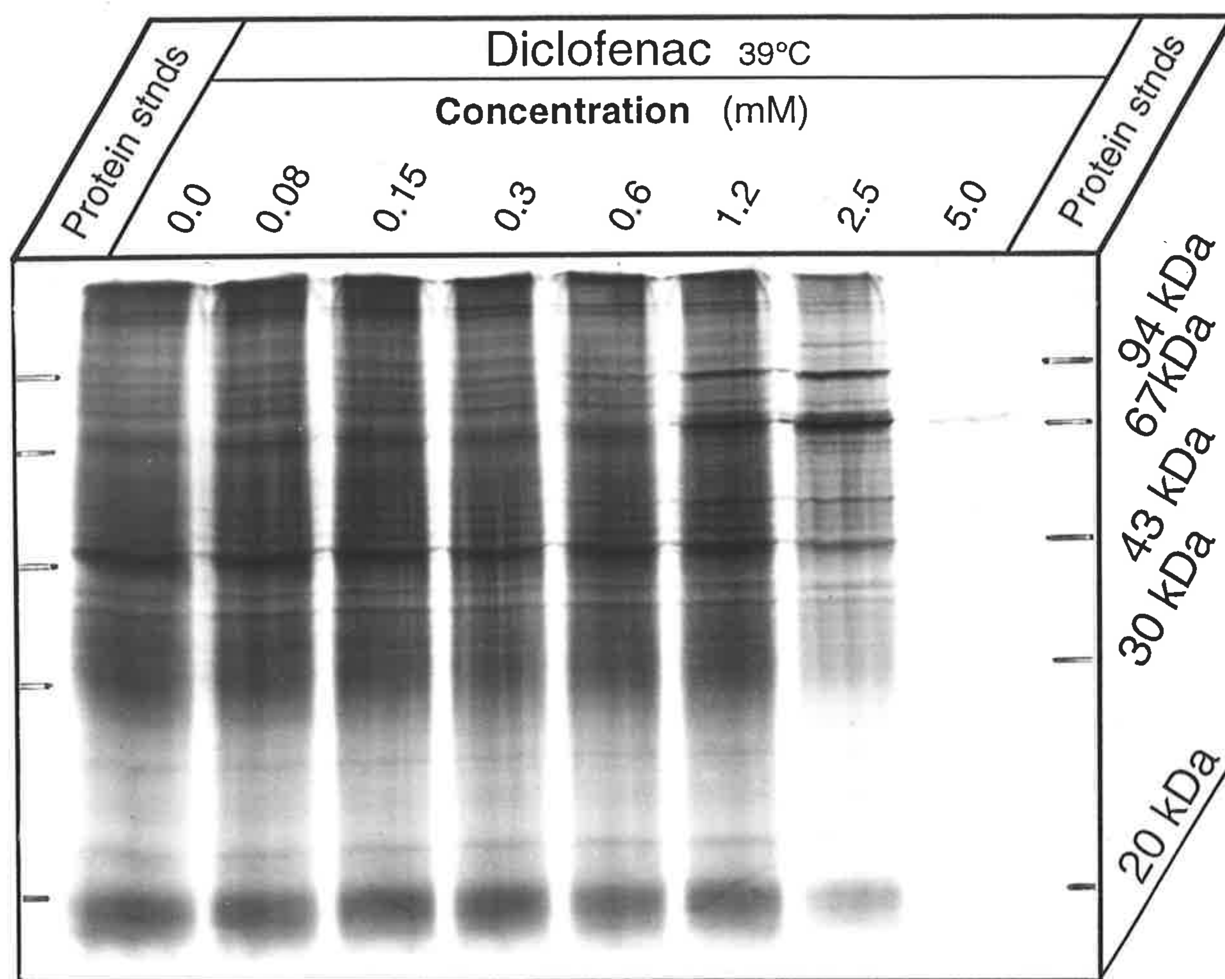


Figure 4.5 shows the autoradiograph of MNC proteins, after the cells were exposed to different diclofenac concentrations for 30min at 37°C followed by incubation at 39°C for a further 15min.

Figure 4.5

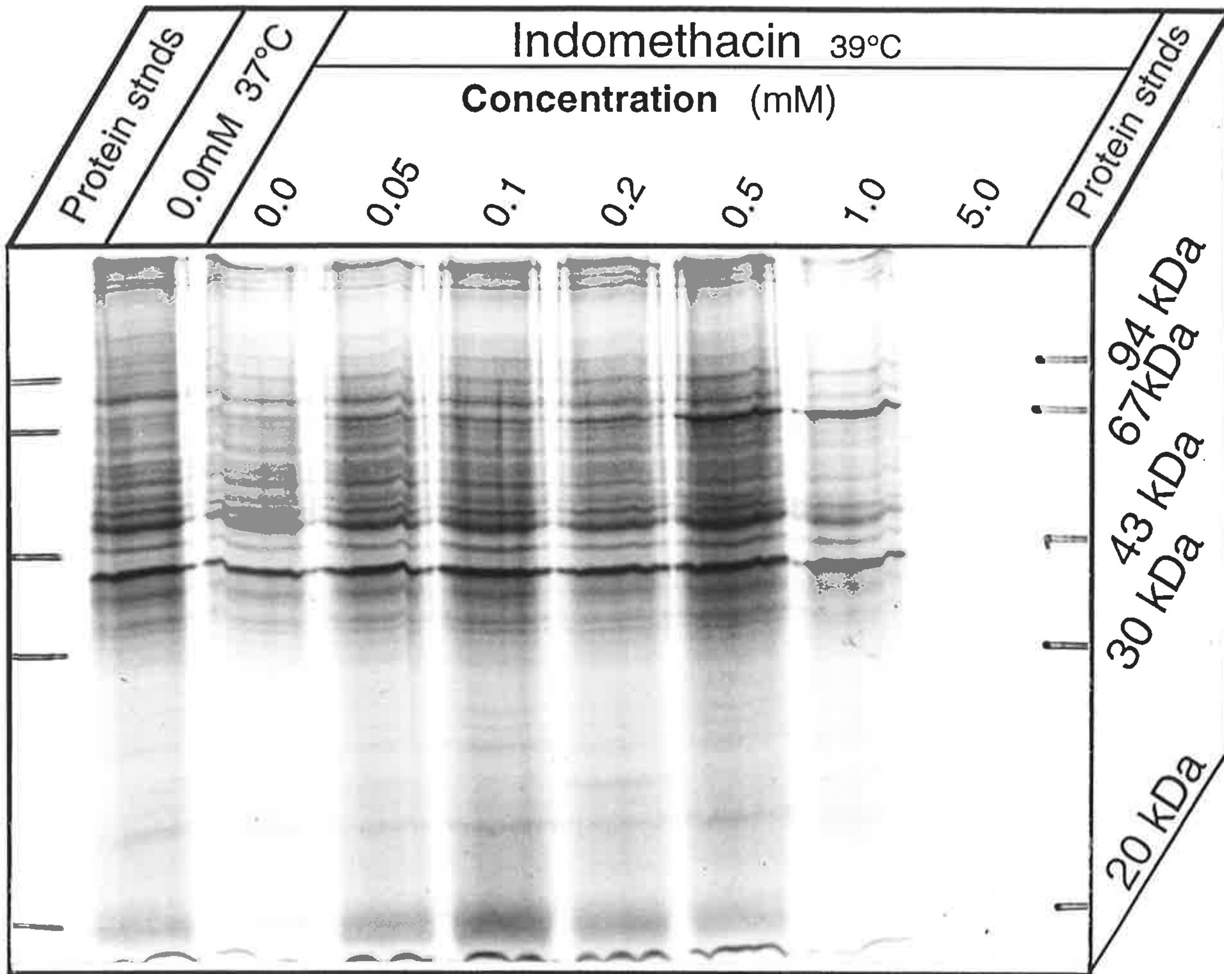
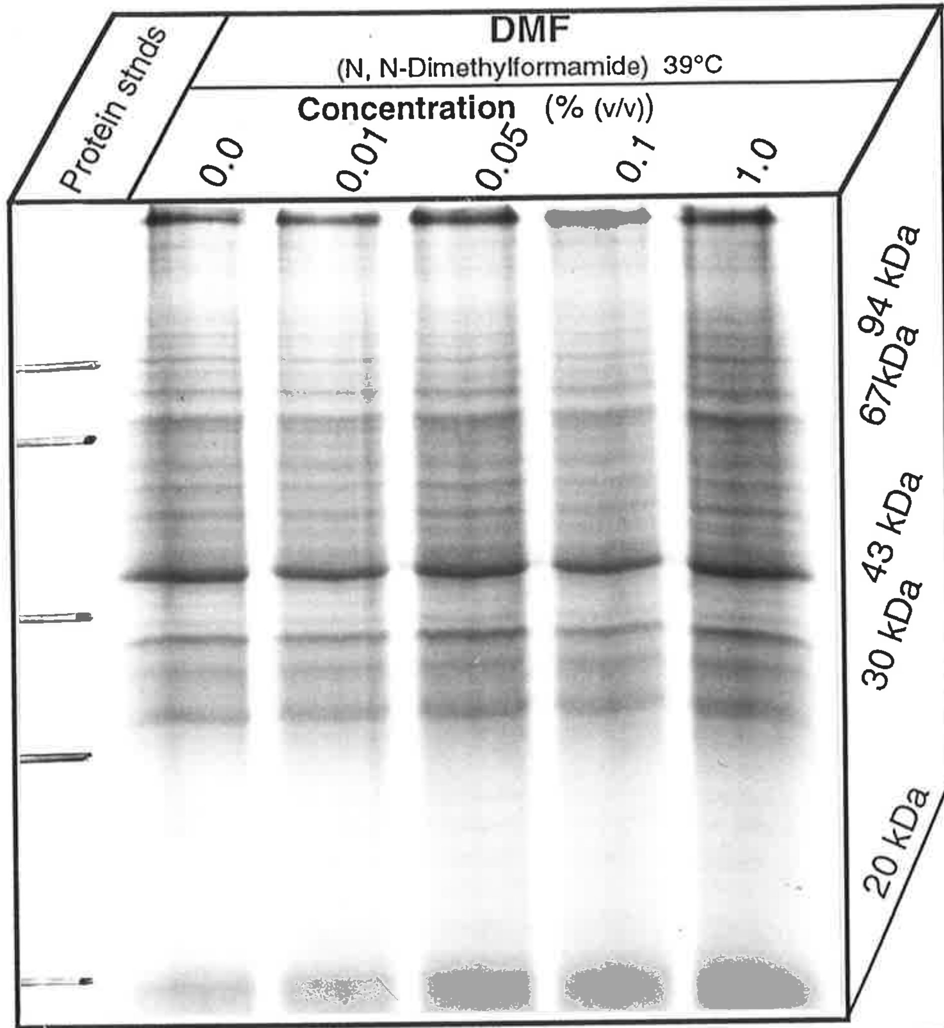
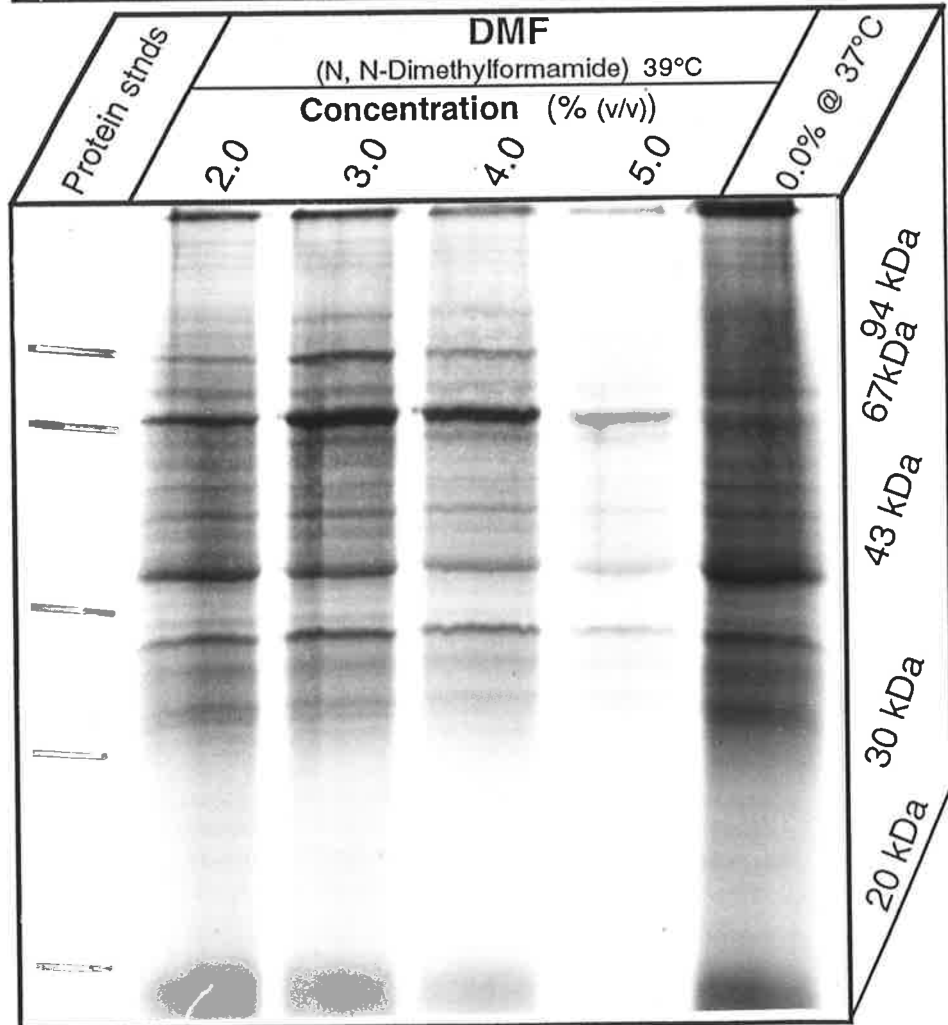


Figure 4.6 shows the autoradiograph of MNC proteins, after the cells were exposed to different indomethacin concentrations for 10min at 37°C followed by incubation at 39°C for a further 15min in the presence of indomethacin. As described in Section 2.1.3, DMF (0.2%v/v) was present in the final preparation of indomethacin supplemented culture medium. On the opposite page are the autoradiographs of MNC proteins, after cells were treated with different DMF concentrations.

Figure 4.6



Protein stnds
DMF
(N, N-Dimethylformamide) 39°C
Concentration (% (v/v))
0.0
0.01
0.05
0.1
1.0
94 kDa
67kDa
43 kDa
30 kDa
20 kDa



Protein stnds
DMF
(N, N-Dimethylformamide) 39°C
Concentration (% (v/v))
2.0
3.0
4.0
5.0
0.0% @ 37°C
94 kDa
67kDa
43 kDa
30 kDa
20 kDa

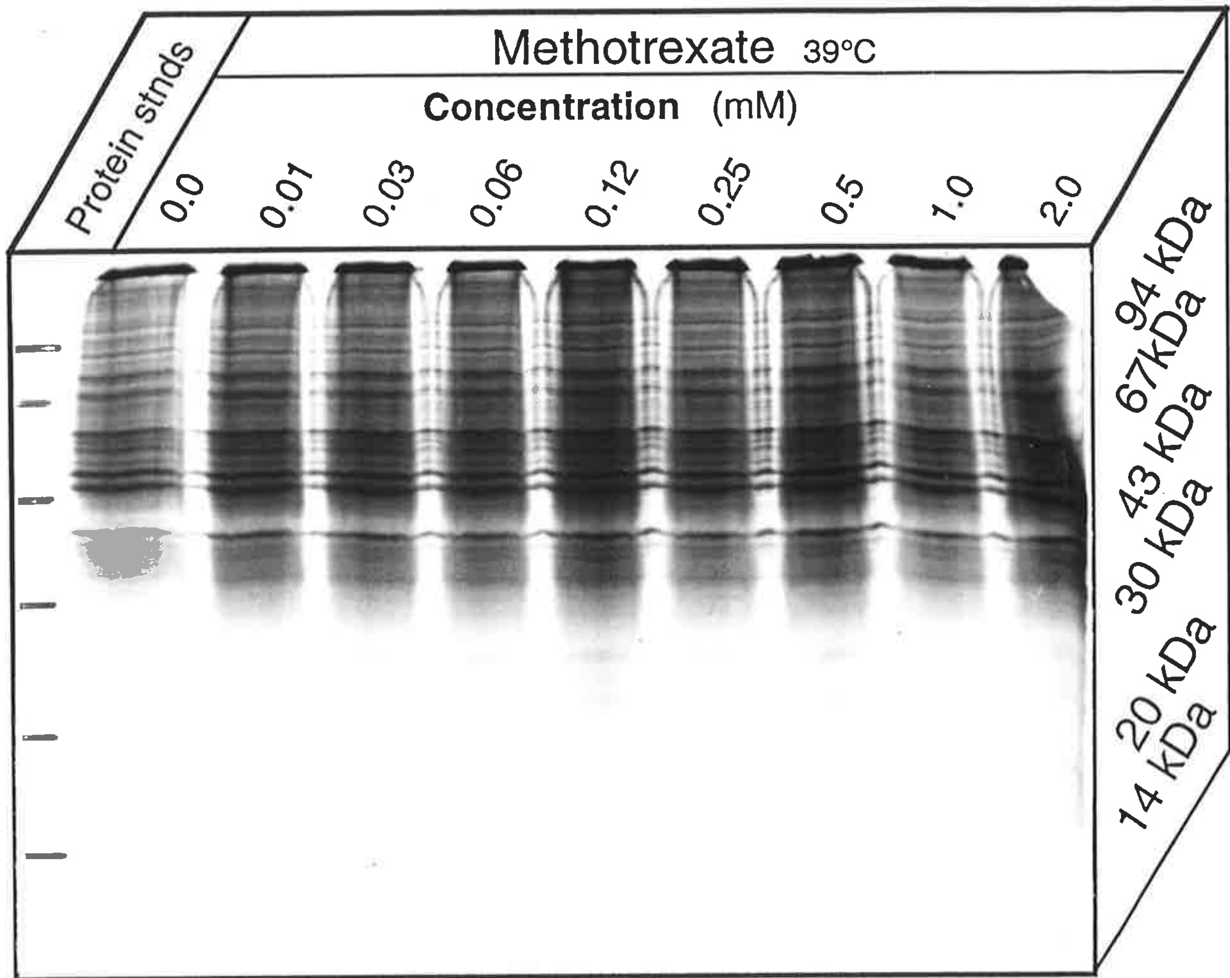


Figure 4.7 shows the autoradiograph of MNC proteins, after the cells were exposed to different methotrexate concentrations for 30min at 37°C followed by incubation at 39°C for a further 15min.

Figure 4.7

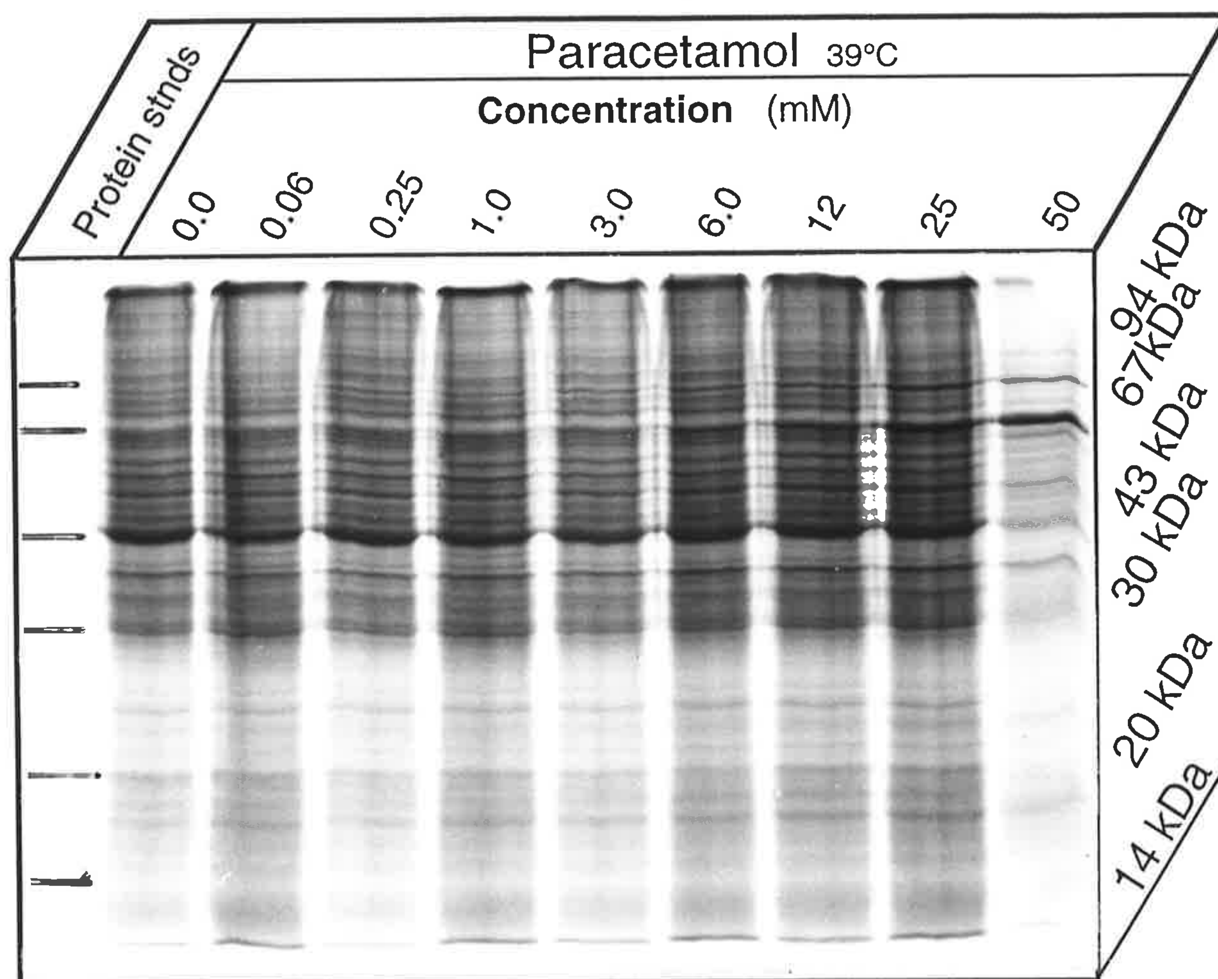


Figure 4.8 shows the autoradiograph of MNC proteins, after the cells were exposed to different paracetamol concentrations for 60min at 37°C followed by incubation at 39°C for a further 15min.

Figure 4.8

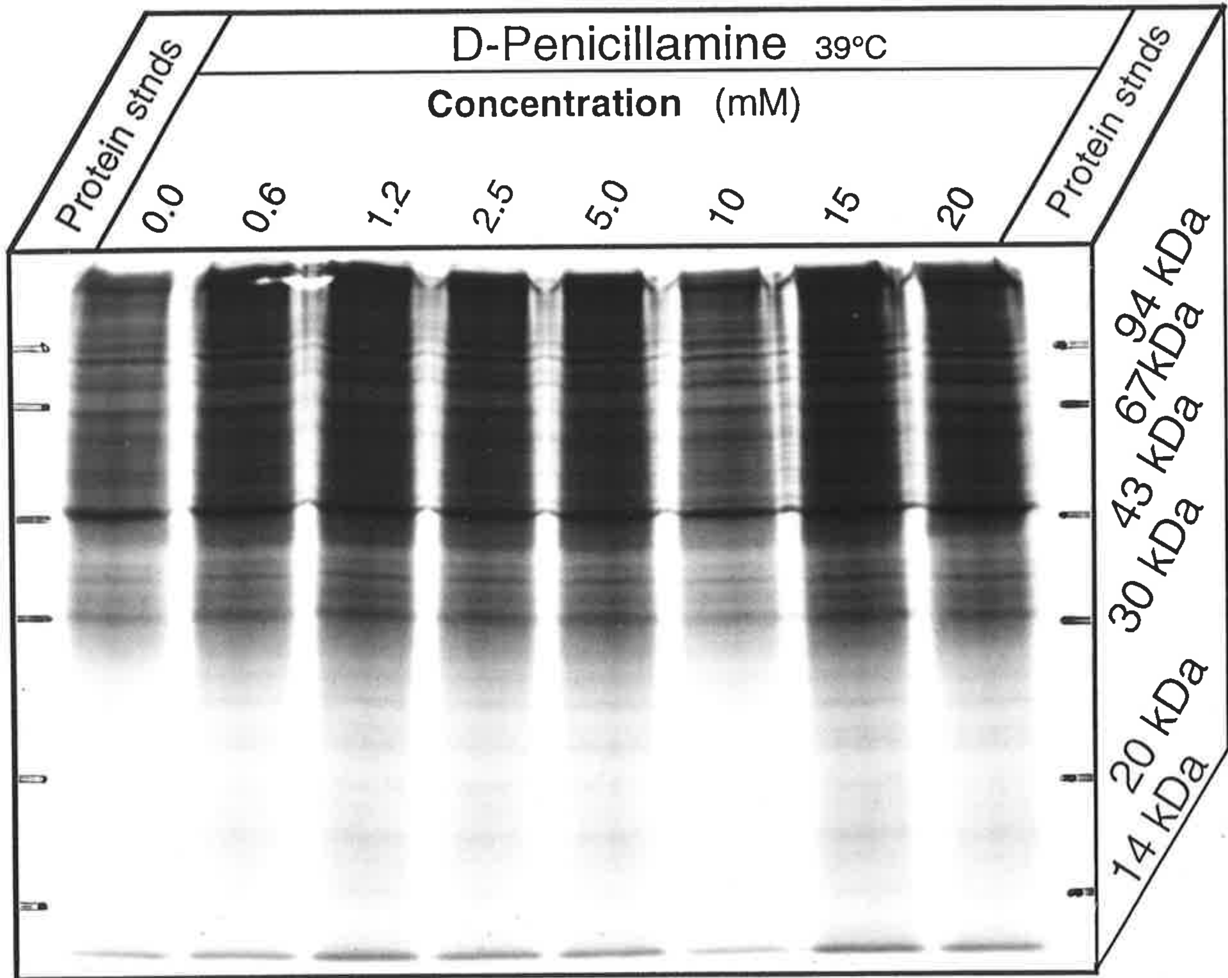


Figure 4.9 shows the autoradiograph of MNC proteins, after the cells were exposed to different D-penicillamine concentrations for 30min at 37°C followed by incubation at 39°C for a further 15min.

Figure 4.9

CHAPTER FIVE

The Effects of Anti-Inflammatory Agents on HSP Expression at Different Temperatures

5.1 INTRODUCTION

In the previous chapter, MNC were exposed to anti-inflammatory/rheumatic agents followed by exposure to mild hyperthermia. From these experiments it was possible to determine the minimum and optimum concentrations of each agent required to induce HSP in *in vitro* cultured MNC. In this chapter, these or similar concentrations will be used to investigate the effect each anti-inflammatory/rheumatic agent has on HSP induction in MNC, over a range of temperatures. The experimental design in this chapter will compare HSP responses between MNC incubated with an anti-inflammatory/rheumatic agent to MNC incubated without any agent and subsequently exposed to different HS temperatures. MNC were exposed to 37°C or to a HS temperature of 39°C, 41°C or 43°C, for 15min. As demonstrated in Section 3.2, the optimum HS conditions for MNC were 42°C for approximately 30 to 60min, which induced high levels of synthesis as well as a wide range of different HSP. In this chapter, the severity of HS was decreased by reducing the duration of HS to 15min. The HS temperatures of 39°C and 41°C were selected to determine the effect anti-inflammatory/rheumatic agents have on HSP synthesis when MNC are exposed to suboptimal HS conditions, which also, more closely reflect *in vivo* fever temperatures. HS at 43°C was selected as a positive control because of its ability to induce HSP in untreated MNC. But also, a HS of 43°C for 15min, will identify how anti-inflammatory/-rheumatic agents influence HSP synthesis in MNC, i.e. do they further enhance or do they suppress HSP synthesis.

5.2 HSP INDUCTION BY A COMBINATION OF HYPERTHERMIA AND ANTI-INFLAMMATORY AGENTS

5.2.1 Aspirin

The results for aspirin in Section 4.3.1 showed that HSP synthesis was significantly induced by 25mM aspirin. Therefore in these experiments similar concentrations were used. In the first experiment, presented in Fig. 5.1, aspirin, which was one of four agents investigated, was used at 17mM. The other agents, i.e. paracetamol, indomethacin and DMF, are discussed in later Sections of this Chapter. MNC were pretreated with 17mM aspirin for

30min before being exposed to 37°C, 39°C, 41°C or 43°C for a further 15min. Control MNC were treated in the same way except aspirin was omitted from the media. All MNC cultures were radiolabelled and processed using SDS PAGE as described in Sections 2.2.5 to 2.2.8.

Normal protein synthesis in untreated and aspirin-treated MNC remained unaffected at all the incubation temperatures. Hsp70 synthesis was faintly induced in untreated MNC incubated at 41°C, but at 43°C was significantly increased along with other HSP. At 43°C there was not only an increase in HSP synthesis compared to 41°C, but also induction of HSP not expressed at 41°C. The effect of aspirin on HSP expression was similar to that of untreated MNC, except that at 41°C the expression of hsp70 was further increased compared to untreated MNC and at 43°C, the expression of all HSP was higher.

In further experiments, pretreatment of MNC with aspirin was increased from 30min to 1.5hrs before exposure to HS. The results appear in the autoradiograph given in Fig. 5.2. Again no difference in normal protein synthesis was observed between the untreated and aspirin-treated MNC at any of the incubation temperatures. In the untreated MNC, HSP expression did not occur until the cells were exposed to 43°C. In aspirin-treated MNC, HSP were clearly expressed at 41°C, and at 43°C, HSP expression was significantly higher than the levels reached in the untreated cells.

These results suggest that aspirin, like its metabolite salicylate, facilitates HSP expression at moderate temperatures, while at higher temperatures, which already induce HSP expression, aspirin further increased HSP expression. However, there are some significant differences between the two compounds. For example, salicylate-treated MNC express hsp70 at 39°C, and expression of HSP synthesis at 41°C, was much greater than in aspirin-treated MNC, in both amount and number of HSP. However, at 43°C, both normal protein and HSP synthesis was almost totally inhibited in salicylate-treated MNC.

5.2.2 Chloroquine

MNC were preincubated with 0.8mM chloroquine for 30min before being exposed to 37°C, 39°C, 41°C or 43°C for a further 15min. MNC were processed as described in Sections 2.2.5 to 2.2.8 and the corresponding autoradiograph of the SDS PAGE analysis appears in Fig. 5.3.

Normal protein synthesis in untreated MNC remained unaffected at all the incubation temperatures. Although the untreated MNC, in Fig. 5.3, exposed to 41°C, showed a higher level of radiolabelled normal proteins, this was attributed to the presence of aggregated MNC in this culture. This was a common problem, and although care was taken to fully resuspend the MNC after centrifugation, an aggregate aliquoted into one of the cultures significantly increased the cell number in that particular culture which in turn, resulted in more radiolabelled protein in that lane after SDS PAGE. Through the use of densitometry and computer analysis, a correction factor of 0.76 was calculated for the untreated MNC incubated at 41°C in Fig. 5.3. The corrected values, when compared to the density values obtained for untreated MNC incubated at 37°C and 39°C, showed no significant induction of HSP at 41°C. Therefore, untreated MNC first demonstrated HSP expression at 43°C.

At all temperatures, chloroquine-treated MNC demonstrated lower levels of normal protein synthesis. This was consistent with most other experiments where MNC were treated with chloroquine. However, inhibition of normal protein synthesis increased at 41°C and was almost completely inhibited at 43°C. In contrast, HSP synthesis was first seen at 41°C, with hsp70 expression being the most noticeable: however at 43°C, although still induced, hsp70 expression was considerably reduced while other HSP were (or almost) totally inhibited.

Although chloroquine-treated MNC demonstrated HSP expression at the lower temperature of 41°C, while the untreated MNC demonstrated HSP expression at 43°C, these results must be interpreted with caution as chloroquine had an inhibitory effect on total protein synthesis (normal protein and HSP synthesis) at all temperatures. The possibility that chloroquine may be cytotoxic to MNC must not be ignored, as cytotoxic agents do, in general, cause HSP induction (Edwards *et al.* 1990). The effects of chloroquine on MNC viability and protein synthesis are further investigated in Section 6.2.2.

5.2.3 Diclofenac

MNC were pretreated with 1.0mM diclofenac for 30min before being exposed to 37°C, 39°C, 41°C or 43°C for a further 15min. All MNC cultures were radiolabelled and processed

using SDS PAGE as described in Sections 2.2.5 to 2.2.8 and the corresponding autoradiograph appears in Fig 5.4.

Normal protein synthesis in untreated MNC remained unaffected at all the incubation temperatures, whereas induction of HSP synthesis occurred at 43°C with a strong expression of hsp70. Diclofenac-treated MNC incubated at 37°C and 39°C demonstrated elevated levels of normal protein synthesis. This was consistently observed in some experiments after MNC were treated with 1.0mM diclofenac. Normal protein synthesis returned to control levels in diclofenac-treated MNC exposed to 41°C and dropped below control levels at 43°C. In diclofenac-treated MNC, HSP synthesis was strongly induced after HS at 39°C and continued strongly at 41°C. However, at 43°C hsp70 expression diminished significantly and expression of other HSP was almost entirely inhibited. Therefore, these results suggest that diclofenac, like salicylate, strongly facilitates the expression of HSP at moderate temperatures, while at higher temperatures, which already induce HSP expression, diclofenac further increased HSP expression. However, at 43°C, although both salicylate and diclofenac inhibited normal protein synthesis, hsp70 synthesis in diclofenac-treated MNC continued to survive.

5.2.4 Indomethacin

MNC were pretreated with 0.6mM indomethacin for 30min before being exposed to 37°C, 39°C, 41°C or 43°C for a further 15min. All MNC cultures were radiolabelled and processed using SDS PAGE as described in Sections 2.2.5 to 2.2.8 and the corresponding autoradiograph appears in Fig 5.1.

Normal protein synthesis in untreated MNC remained unaffected at all the incubation temperatures. Hsp70 synthesis was faintly induced in MNC incubated at 41°C, but at 43°C was significantly increased along with other HSP. At 43°C there was not only an increase in HSP synthesis compared to 41°C, but also induction of HSP not expressed at 41°C. As indomethacin was first dissolved in DMF before dilution in aqueous culture media, (resulting in a final DMF concentration of 0.2% (v/v)), MNC were also incubated with DMF alone. Both normal protein and HSP synthesis was unaffected by DMF (Fig. 5.1). In indomethacin-treated MNC there, once again, was no change in normal protein synthesis in MNC exposed to 37°C and 39°C, but at 41°C normal protein synthesis was inhibited and

at 43°C all protein synthesis (normal protein synthesis and HSP) was completely inhibited. HSP expression in indomethacin-treated MNC first appeared at 39°C with a strong induction of hsp70 expression along with significant induction of other HSP. However, at 41°C all HSP had decreased by a similar amount and were completely inhibited at 43°C. These results suggest that indomethacin, like salicylate and diclofenac, strongly facilitates the expression of HSP at moderate temperatures. Unlike salicylate- and diclofenac-pretreated MNC, where normal protein synthesis began to show the first signs of inhibition while the induced HSP synthesis continued unaffected until exposure at a higher temperature, indomethacin-pretreated MNC demonstrated a proportional decrease in both HSP synthesis and normal protein synthesis at all temperatures. However, at 43°C, indomethacin, like salicylate, inhibited all protein synthesis.

5.2.5 Methotrexate

MNC were pretreated with 10mM methotrexate for 30min before being exposed to 37°C, 39°C, 41°C or 43°C for a further 15min. All MNC cultures were radiolabelled and processed using SDS PAGE as described in Sections 2.2.5 to 2.2.8 and the corresponding autoradiograph appears in Fig. 5.5.

Normal protein synthesis in untreated MNC remained unaffected at all the incubation temperatures. HSP expression first occurred in untreated MNC when incubated at 43°C with the induction of hsp70 and several other HSP. Normal protein synthesis was essentially unaffected in methotrexate-treated MNC. At 41°C expression of hsp70 was faintly induced. However, at 43°C the effect of methotrexate on HSP expression was similar to that of untreated MNC. These results suggest that although methotrexate had a weak facilitating effect on HSP expression, at higher temperatures (43°C), which already induce HSP expression, the effect of methotrexate was indistinguishable from the untreated MNC.

5.2.6 Paracetamol

MNC were pretreated with 20mM paracetamol for 30min before being exposed to 37°C, 39°C, 41°C or 43°C for a further 15min. All MNC cultures were radiolabelled and

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processed using SDS PAGE as described in Sections 2.2.5 to 2.2.8 and the corresponding autoradiograph appears in Fig. 5.1.

Normal protein synthesis in untreated MNC remained unaffected at all the incubation temperatures. Hsp70 synthesis was faintly induced in MNC incubated at 41°C, but at 43°C was significantly increased along with other HSP. In paracetamol-treated MNC there was no change in normal protein synthesis over the different incubation temperatures, except at 43°C when normal protein synthesis appeared to decrease marginally. HSP expression, in paracetamol-treated MNC, began at 41°C with a strong expression of a range of different HSP. However, at 43°C, HSP expression decreased along with normal protein synthesis. These results suggest that paracetamol facilitates HSP expression by increasing the intensity as well as the number of HSP. However, unlike salicylate, diclofenac and indomethacin, paracetamol does not significantly change the temperature at which HSP expression first appears.

5.2.7 D-Penicillamine

MNC were incubated with 20mM D-Penicillamine for 40min at 37°C before being exposed to 37°C, 39°C, 41°C or 43°C for a further 15min. All MNC cultures were then processed as described in Sections 2.2.5 to 2.2.8 and the corresponding autoradiograph of the SDS PAGE appears in Fig. 5.6.

Normal protein synthesis in untreated MNC remained unaffected at all the incubation temperatures, while HSP expression first appeared in MNC incubated at 43°C. In D-penicillamine-treated MNC there was no change in normal protein synthesis over the different incubation temperatures. At 41°C hsp70 expression was faintly induced in D-penicillamine-treated MNC, while at 43°C, HSP expression was similar to that of untreated MNC, except that all HSP were expressed at significantly higher levels. These results suggest that D-penicillamine has a mild effect on HSP expression by gently facilitating HSP expression at moderate temperatures which in the absence of agent may not necessarily induce HSP expression. At higher temperatures, which already induce HSP synthesis, D-penicillamine further increased HSP expression.

5.3 COMPARSON OF HSP70 INDUCED BY HEAT SHOCK AND THE ANTI-INFLAMMATORY AGENTS

In Section 3.2.5 an anti-hsc/hsp70 monoclonal antibody was used to identify the HS induced hsp70 on Western blots. However, in Section 3.2.5, the location of hsp70, induced by a combination of anti-inflammatory/ rheumatic agent and hyperthermia, on SDS PAGE was not characterised. In this section, a combination of anti-inflammatory/rheumatic agents and HS, or HS, or prostaglandin A_1 (PGA_1) were used to induce HSP synthesis. *(PGA_1 was chosen for a control for HSP70 induction as it activates HSF and induces HSP70 synthesis in mammalian cells (Santoro 1989; Amici and Santoro 1991; Amici et al. 1992, 1993)).* MNC were subsequently analysed by SDS PAGE and autoradiography and hsp70 induced by a combination of anti-inflammatory/rheumatic agents and HS was compared to the hsp70 induced by HS or by PGA_1 as a means of demonstrating the uniformity of HSP induction by the different inducers/facilitators.

In addition, a direct comparison of coomassie blue staining of proteins was made to autoradiography when detecting *de novo* HSP synthesis: clearly demonstrated the differences in sensitivity between the two techniques.

5.3.1 Results

MNC were prepared as described in Sections 2.2.2 and 2.2.3 and exposed to HS or PGA_1 or pretreated with one of the anti-inflammatory/rheumatic agents and subsequently exposed to HS. The pretreatment and HS conditions are summarised in Table 5.3.

Table 5.3

Treatment	Agent Concentration (60min Pretreatment)	Heat Shock (42°C/15min)	HSP Induction	Normal Protein Synthesis
37°C		No	Nil	Normal
42°C		Yes	++	Normal
44°C		44°C/15min	+++++	Normal
Prostaglandin A_1	4μg/ml	No	+	Normal
Aspirin	22mM	Yes	++++	Normal
Salicylate	17mM	Yes	++++	Normal
Paracetamol	20mM	Yes	+++++	Normal
Indomethacin	0.6mM	Yes	+++	Inhibited
Chloroquine	0.8mM	Yes	+++	Inhibited
Methotrexate	10mM	Yes	+++	Normal
D-Penicillamine	20mM	Yes	++++	Normal
Diclofenac	1.0mM	Yes	+++++	Normal

After pretreatment and exposure to HS the MNC cultures were processed as described in Sections 2.2.5 to 2.2.8 and the autoradiographs appear in Fig. 5.7 while the coomassie blue stained gels appear in Fig.5.8.

The control cells, incubated at 37°C and without an agent, are shown in Fig. 5.7 lane 1. MNC exposed to 42°C (lane 2) showed a mild induction of HSP expression whereas MNC exposed to 44°C (lane 3) showed strongly induced hsp70 and other HSP. While PGA_1 (lane 4) had the smallest effect on HSP expression by inducing hsp70 expression faintly. MNC pretreated with aspirin, salicylate, paracetamol, indomethacin, chloroquine, methotrexate, penicillamine or diclofenac .and subsequently exposed to a HS all demonstrated the induction of hsp70 and several other HSP. While inhibition of normal protein synthesis occurred in indomethacin (lane 8) and chloroquine (lane 9) treated MNC. As can be seen from Fig. 5.7, hsp70 and the other induced HSP do not differ between cultures pretreated by different agents. Therefore, these results suggest that the HSP induced by the anti-inflammatory/ rheumatic agents and HS are the same proteins.

Comparison of the coomassie blue stained gels in Fig. 5.8 with the autoradiographs of these gels in Fig. 5.7 demonstrates that autoradiography is the more sensitive technique for detecting induced HSP expression. In particular, when comparing hsp70 in MNC incubated at 44°C (lanes 3 in Figs. 5.7 and 5.8) the autoradiograph showed a strongly induced protein while the stained gel demonstrated no increased staining of hsp70 or any of the other HSP. Likewise, the inhibition of normal protein synthesis by indomethacin and chloroquine is clearly observed on the autoradiograph (Fig. 5.7 lanes 8 & 9) while the corre-

sponding lanes on the stained gel (Fig. 5.8 lanes 8 & 9) demonstrate no sign of normal protein inhibition.

5.4 DISCUSSION

In chapter three, HSP expression was induced in MNC by hyperthermia alone. HSP induction was found to be temperature dependent, with new and different HSP becoming induced while existing HSP expression was increased to even greater levels as the severity of HS increased. When the severity of hyperthermia approached higher levels, normal protein synthesis started to decline while HSP expression persisted unaffected until the severity of hyperthermia became too great and then HSP expression also declined.

Under the experimental conditions used in this chapter, none of the selected agents induced HSP synthesis in MNC incubated at 37°C. However, most anti-inflammatory/ rheumatic agents had a positive effect on HSP induction when MNC were subsequently exposed to a HS, with changes in HSP expression ranging from mild to dramatic. MNC pretreated with 1.0mM diclofenac, 0.6mM indomethacin or 15mM salicylate (Section 4.2.2), produced the most dramatic increases in HSP induction. These agents decreased the temperature at which HSP expression was first induced as well as increasing the amount of HSP expression at temperatures which already induced HSP in the untreated MNC. These agents also lowered the temperature at which normal protein synthesis became significantly inhibited.

Paracetamol also facilitated the induction of HSP but the effects were not as dramatic as those produced by diclofenac, indomethacin or salicylate. Paracetamol considerably enhanced HSP synthesis in MNC at temperatures which already induced HSP synthesis in untreated MNC, but did not lower the induction temperature. Paracetamol did not inhibit normal protein synthesis at higher temperatures to the same degree as diclofenac, indomethacin or salicylate.

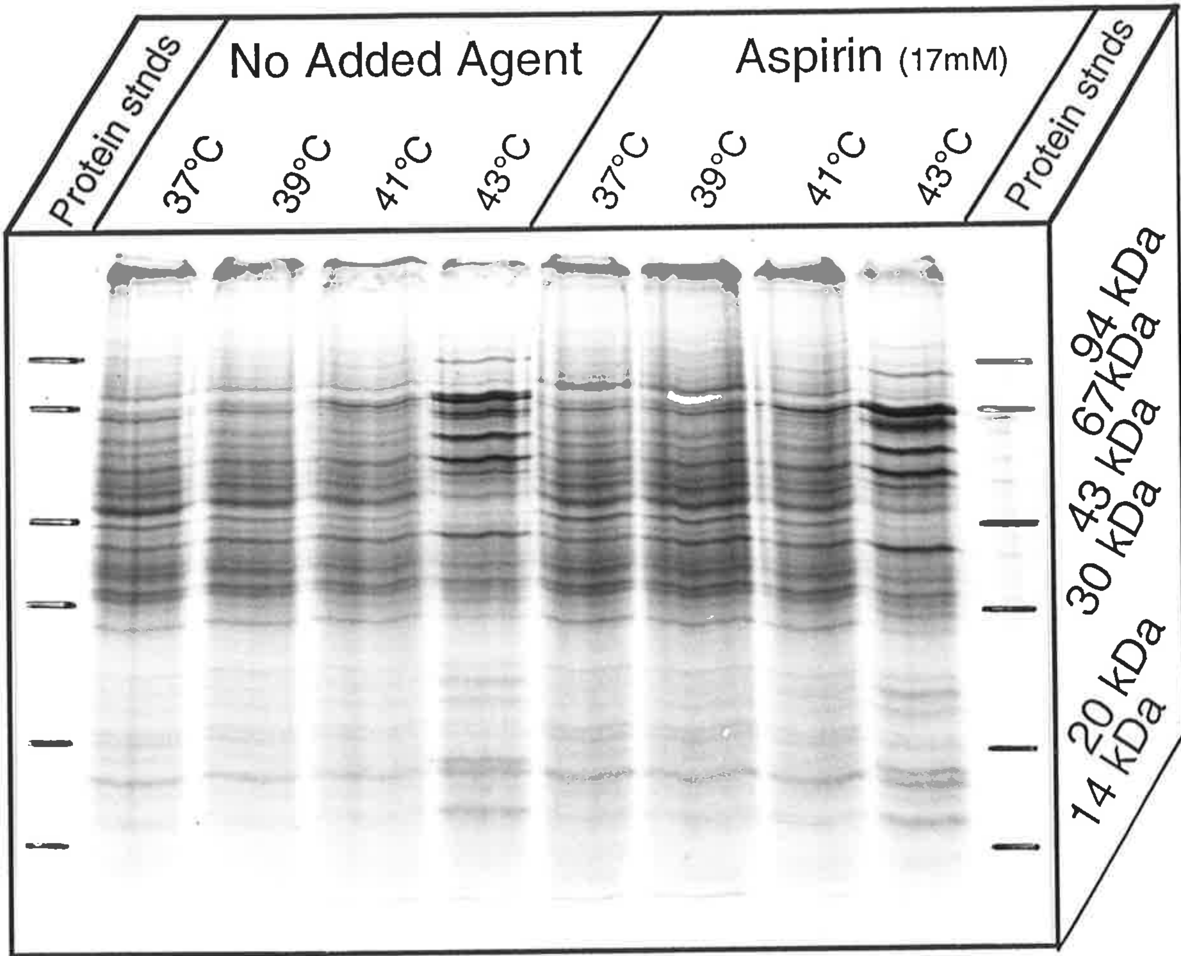
Aspirin and D-penicillamine had similar effects on HSP synthesis. They both enhanced the induction of HSP expression to a much lesser extent than paracetamol but, had no apparent effect on normal protein synthesis. Chloroquine, however, had an unusual effect on MNC protein synthesis, which was not characteristic of changes in cellular protein synthesis during HSP induction. Normally in untreated MNC, HSP synthesis was

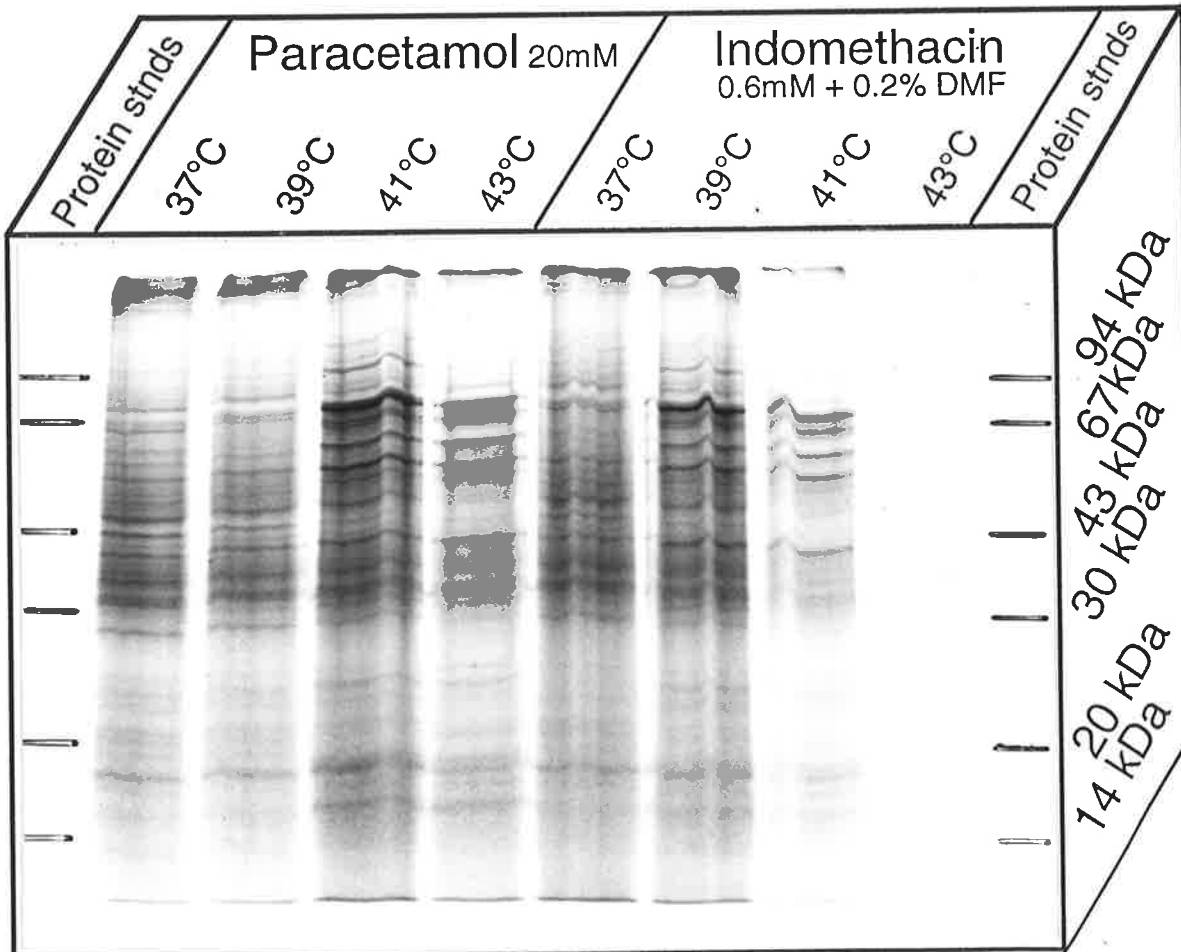
induced by mild hyperthermia (41°C) while normal protein synthesis was unaffected but after severe HS (45°C) non-HSP synthesis was also inhibited. However, in chloroquine-treated MNC normal protein synthesis was inhibited at all temperatures with inhibition increasing as the HS become more severe. HSP synthesis was observed after exposure to 41°C, but normal protein synthesis was already considerably suppressed. These data suggest that chloroquine may have an adverse effect on cellular protein synthesis well before HSP induction occurs. Methotrexate, on the other hand, had no effect on both normal protein synthesis and HSP expression was only marginally increased at 41°C. At temperatures which already induced HSP expression in untreated MNC, methotrexate had no apparent additional effect.

The facilitation of HSP expression by some anti-inflammatory agents is a relatively novel finding and therefore there is a lack of literature with respect to these effects. However, investigators working in other areas have reported findings which, from some aspects, are similar to the findings in this Chapter. For example Liu *et al.* (1994), demonstrated induced hsp70 expression after treating mouse fibroblasts with salicylate. However, the salicylate concentrations Liu *et al.* (1994), used were more than three times higher than those used in this thesis (up to 120mM salicylate). In the experiments of Liu *et al.* (1994), hsp70 expression was noticeable but only after first exposing cells to 60mM salicylate: even at 120mM salicylate, the increase in hsp70 expression was only small.(Liu *et al.* 1994) In a cardiotoxicity study, Low-Friedrich *et al.* (1990) demonstrated the induction of a 30kDa stress protein by methotrexate, while normal protein synthesis remained unaffected. In yet another study, Smith *et al.* (1983) used indomethacin and showed the abolition of increased protein synthesis, induced by arachidonic acid, in rabbit muscle cells.

Finally, the inhibition of normal protein synthesis by HS has been previously reported in *Drosophila* and in the nematode *Caenorhabditis elegans*, in experiments where HSP synthesis was induced. Investigations showed that the sudden induction of HSP synthesis was due to the appearance of HSP mRNA, however the suppression of normal protein synthesis was not due to the degradation or modification of normal protein mRNA but due to a change in ribosomal preference from normal and HSP mRNA to only HSP mRNA.(Snutch and Baillie 1983; Kruger and Benecke 1981; Didomenico *et al.* 1982; Storti *et al.* 1980) Although the phenomenon of normal protein synthesis inhibition, in MNC, by a

combination of HS and anti-inflammatory agent has similarities to these models, the mechanisms of how anti-inflammatory agent influence the inhibition of normal protein synthesis will still need further elucidation.


Protein stnds
No Added Agent
Aspirin (17mM)
Protein stnds
37°C
39°C
41°C
43°C
37°C
39°C
41°C
43°C
94 kDa
67kDa
43 kDa
30 kDa
20 kDa
14 kDa


Protein stnds
Paracetamol 20mM
Indomethacin
0.6mM + 0.2% DMF
Protein stnds
37°C
39°C
41°C
43°C
37°C
39°C
41°C
43°C
94 kDa
67kDa
43 kDa
30 kDa
20 kDa
14 kDa

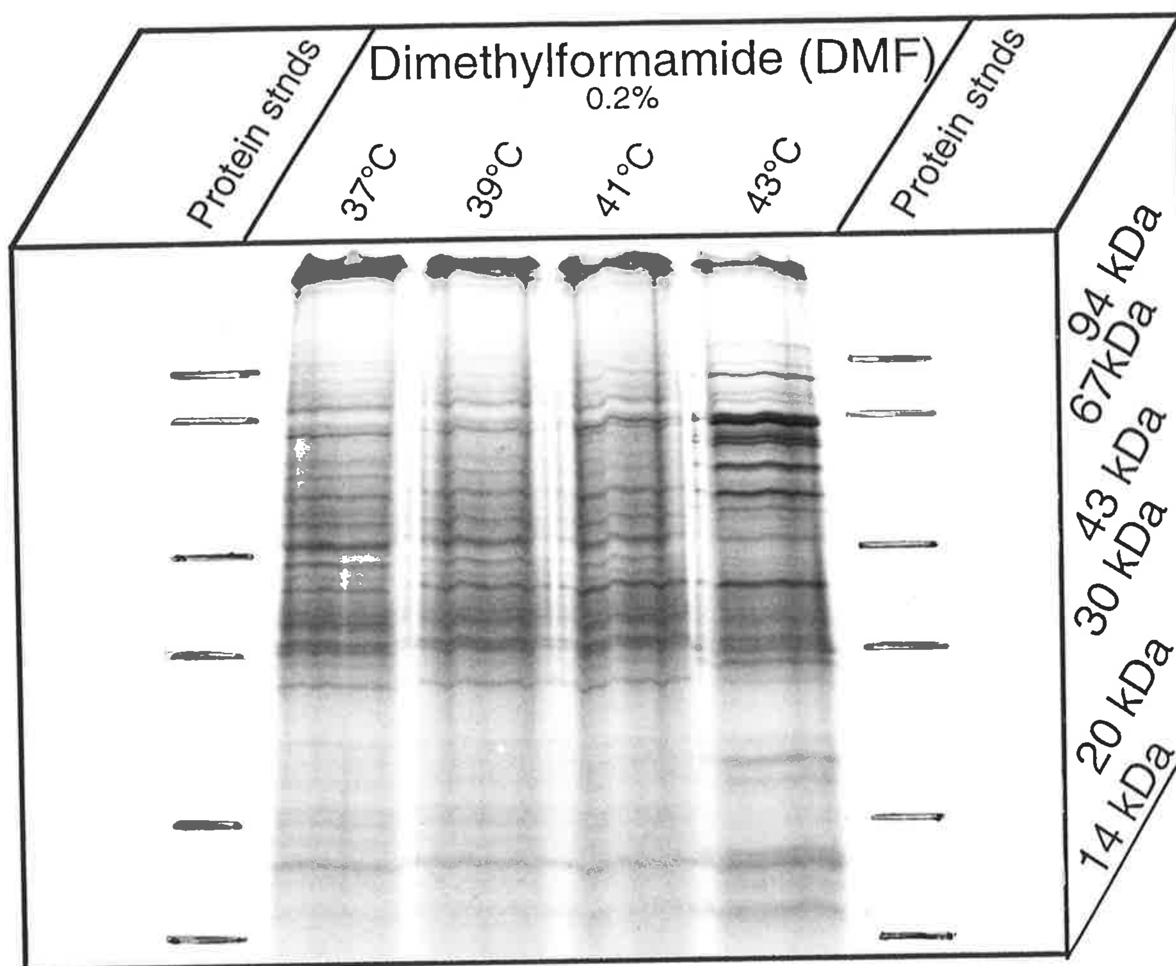


Figure 5.1 shows autoradiographs of MNC proteins separated by SDS PAGE from unstressed and HS-stressed MNC which were incubated with agent or incubated without agent. MNC were incubated with aspirin, paracetamol, indomethacin or DMF for 30min and then incubated at 37°C or HS at 39°C, 41°C or 43°C for 15min. MNC incubated without agent show HSP induction beginning at 41°C with very faint induction of the hsp70 band. At 43°C other HSP are induced and the bands are much darker. MNC incubated with DMF demonstrate an identical HSP induction as the untreated MNC and with identical band intensities. Once again, MNC incubated with aspirin also demonstrate an identical HSP profile after HS at 41°C and 43°C to that of untreated cells, except the hsp70 band induced at 41°C is slightly darker for aspirin-treated MNC. Densitometric analysis of this band confirms that it is darker, having a relative density of 0.68 for aspirin-treated MNC compared to 0.61 for untreated MNC. Paracetamol-treated MNC also show the first signs of HSP synthesis at 41°C, however a broad HSP profile is induced at this temperature. Indomethacin-treated MNC show HSP induction at the much lower temperature of 39°C and continues at 41°C but at 43°C a total protein synthesis inhibition is observed.

Figure 5.1

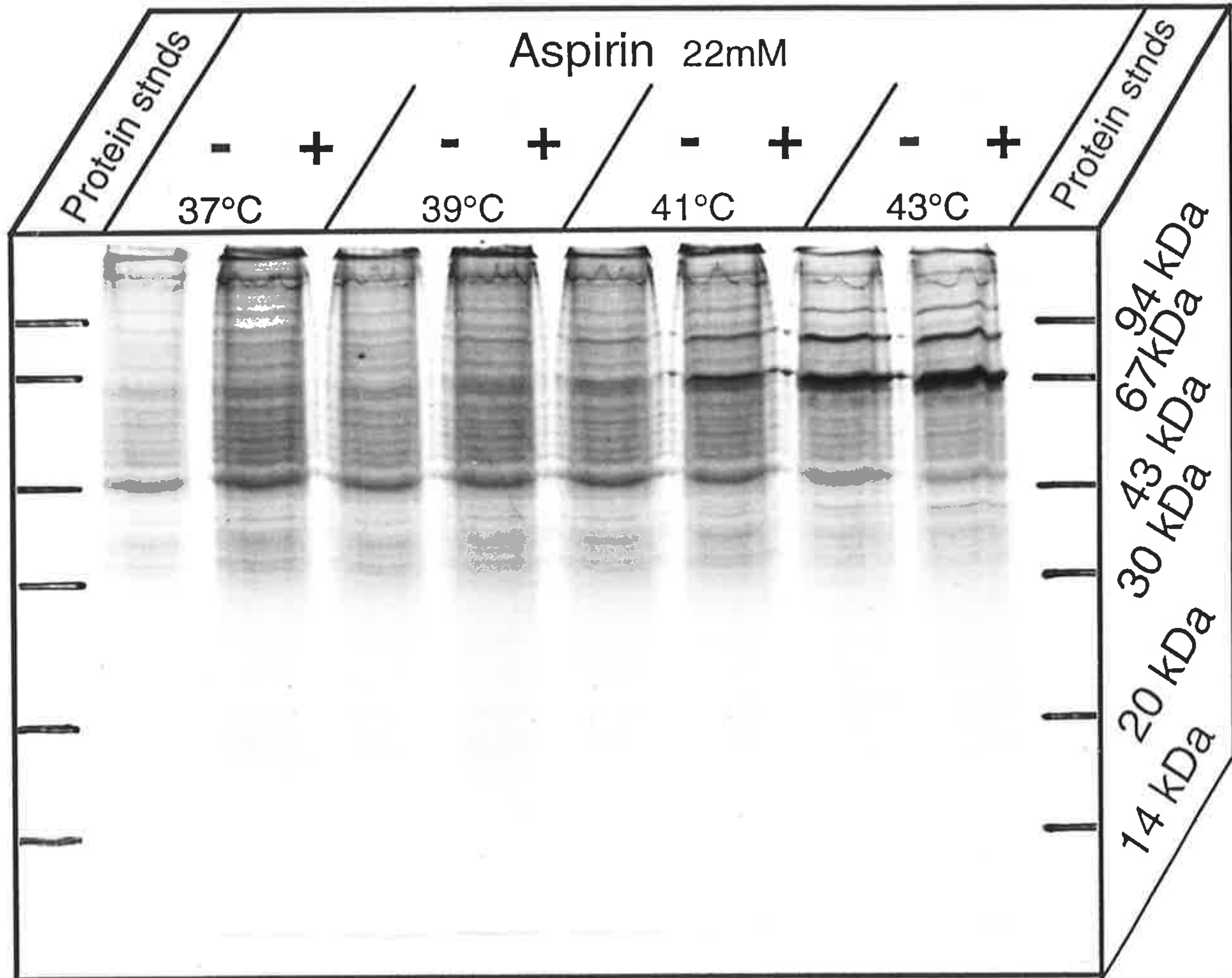


Figure 5.2 shows the autoradiograph of MNC proteins separated by SDS PAGE after MNC were exposed to a combination of aspirin and HS. MNC were incubated either without (-) or, with 22mM aspirin (+) for 1.5hrs at 37°C before exposure to a HS of 39°C, 41°C or 43°C for 15min. HSP synthesis is clearly induced, after HS at 41°C, in aspirin-treated MNC when compared to untreated MNC also exposed to 41°C. After MNC were exposed to 43°C both the untreated and treated cells strongly synthesize several different HSP, however hsp70 synthesis appears to be slightly greater in aspirin-treated MNC.

Figure 5.2

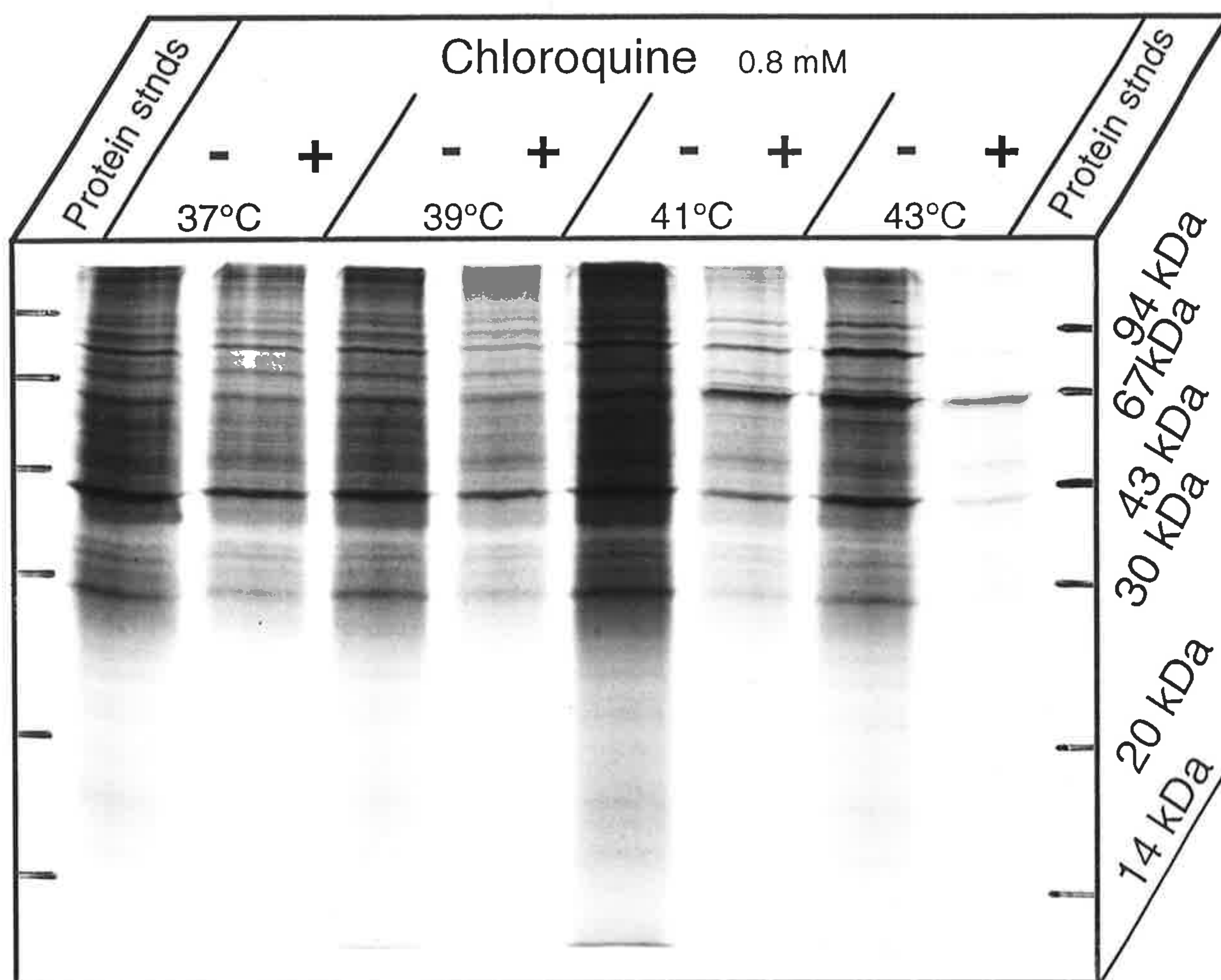


Figure 5.3 shows the autoradiograph of MNC proteins separated by SDS PAGE after MNC were exposed to a combination of chloroquine and HS. MNC were incubated either without (-) or, with 0.8mM chloroquine (+) for 30min at 37°C, before exposure to a HS of 39°C, 41°C or 43°C for 15min. MNC not exposed to chloroquine demonstrate a clear HSP synthesis at 43°C and normal protein synthesis was unaffected by HS when compared to MNC incubated at 37°C.

Figure 5.3

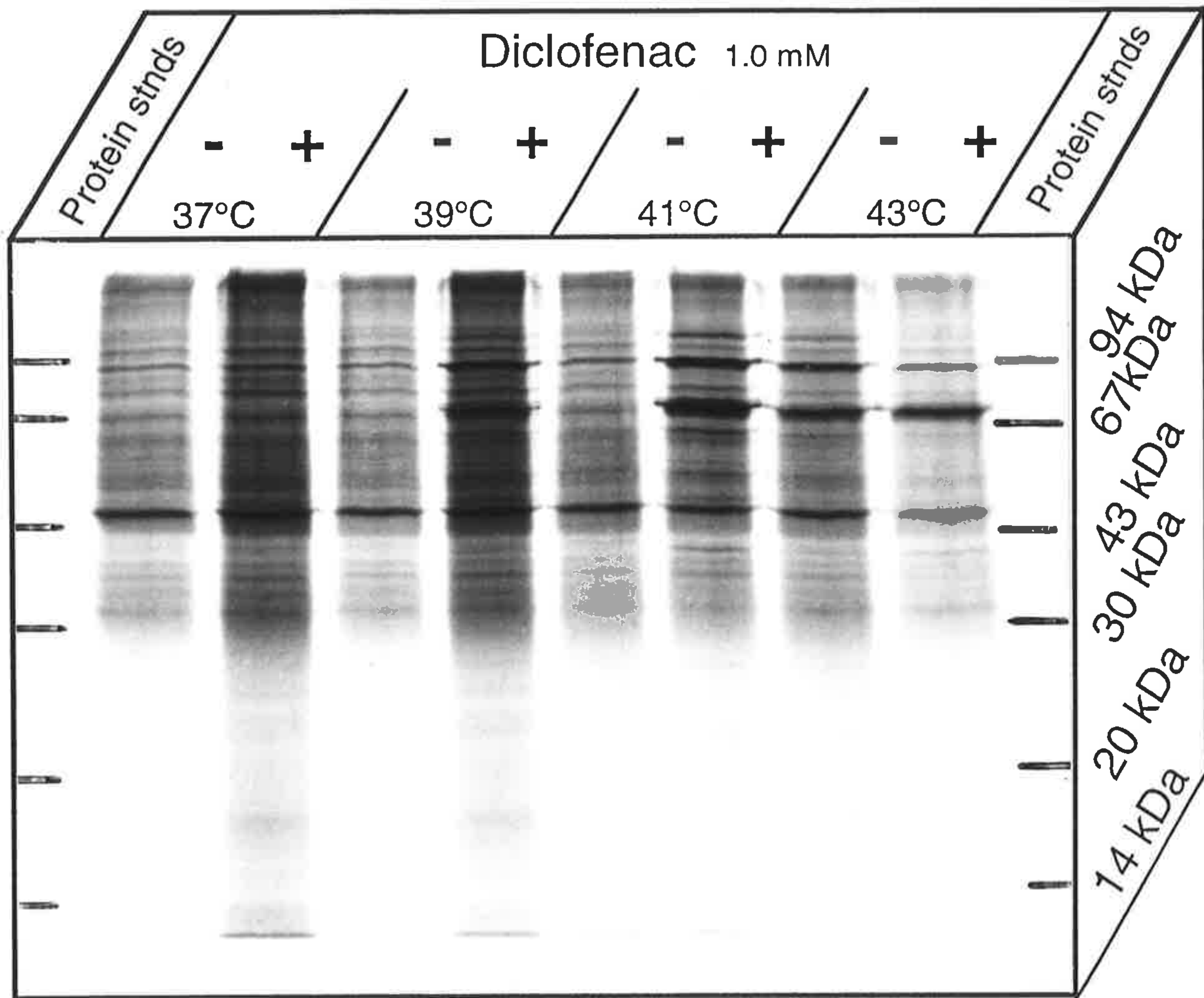


Figure 5.4 shows the autoradiograph of MNC proteins separated by SDS PAGE after MNC were exposed to a combination of diclofenac and HS. MNC were incubated either without (-) or, with 1.0mM diclofenac (+) for 30min at 37°C before exposure to a HS of 39°C, 41°C or 43°C for 15min.

Figure 5.4

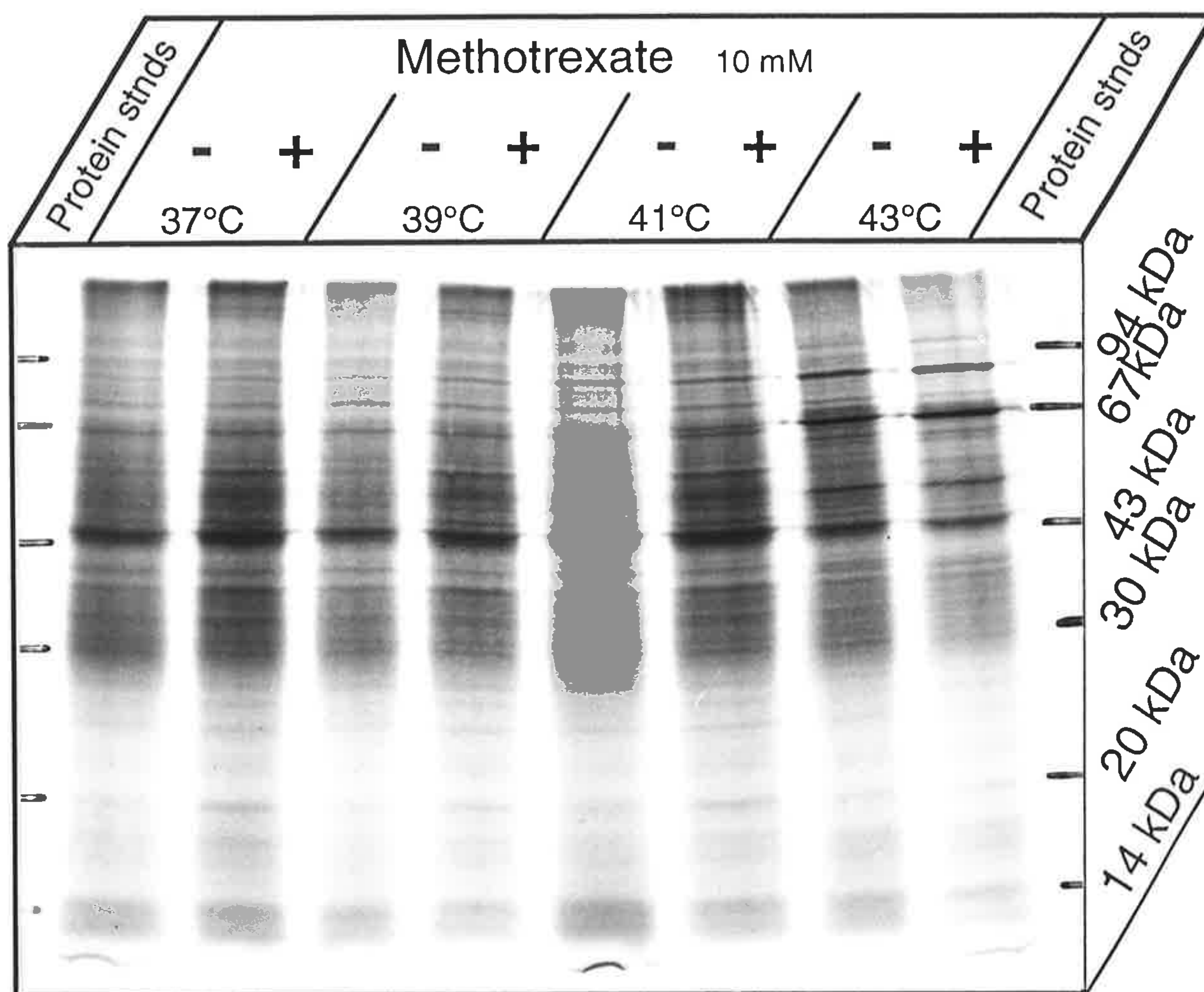


Figure 5.5 shows the autoradiograph of MNC proteins separated by SDS PAGE after MNC were exposed to a combination of methotrexate and HS. MNC were incubated either without (-) or, with 10mM methotrexate (+) for 40min at 37°C before exposure to a HS of 39°C, 41°C or 43°C for 15min.

Figure 5.5

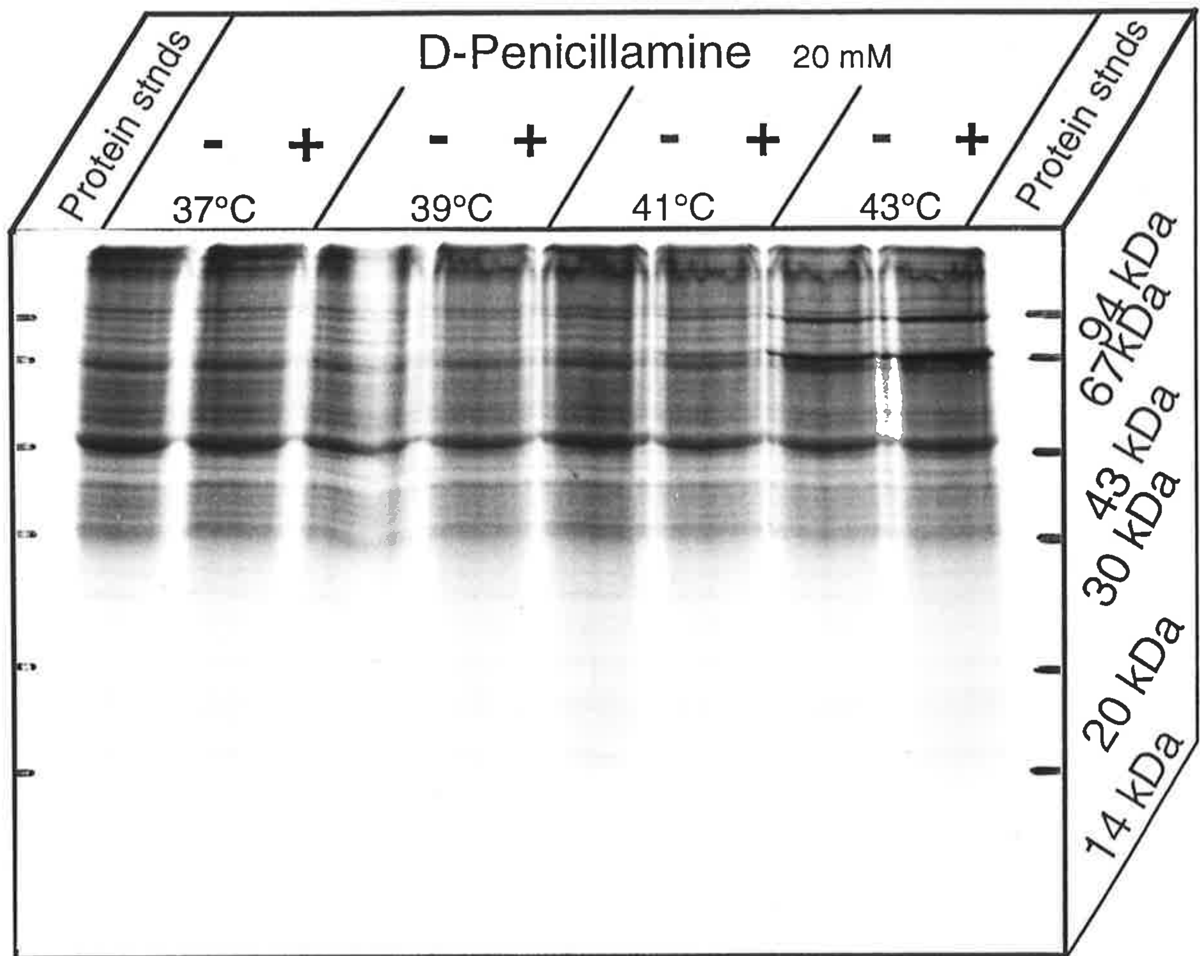


Figure 5.6 shows the autoradiograph of MNC proteins separated by SDS PAGE after MNC were exposed to a combination of D-penicillamine and HS. MNC were incubated either without (-) or, with 20mM D-penicillamine (+) for 40min at 37°C before exposure to a HS of 39°C, 41°C or 43°C for 15min.

Figure 5.6

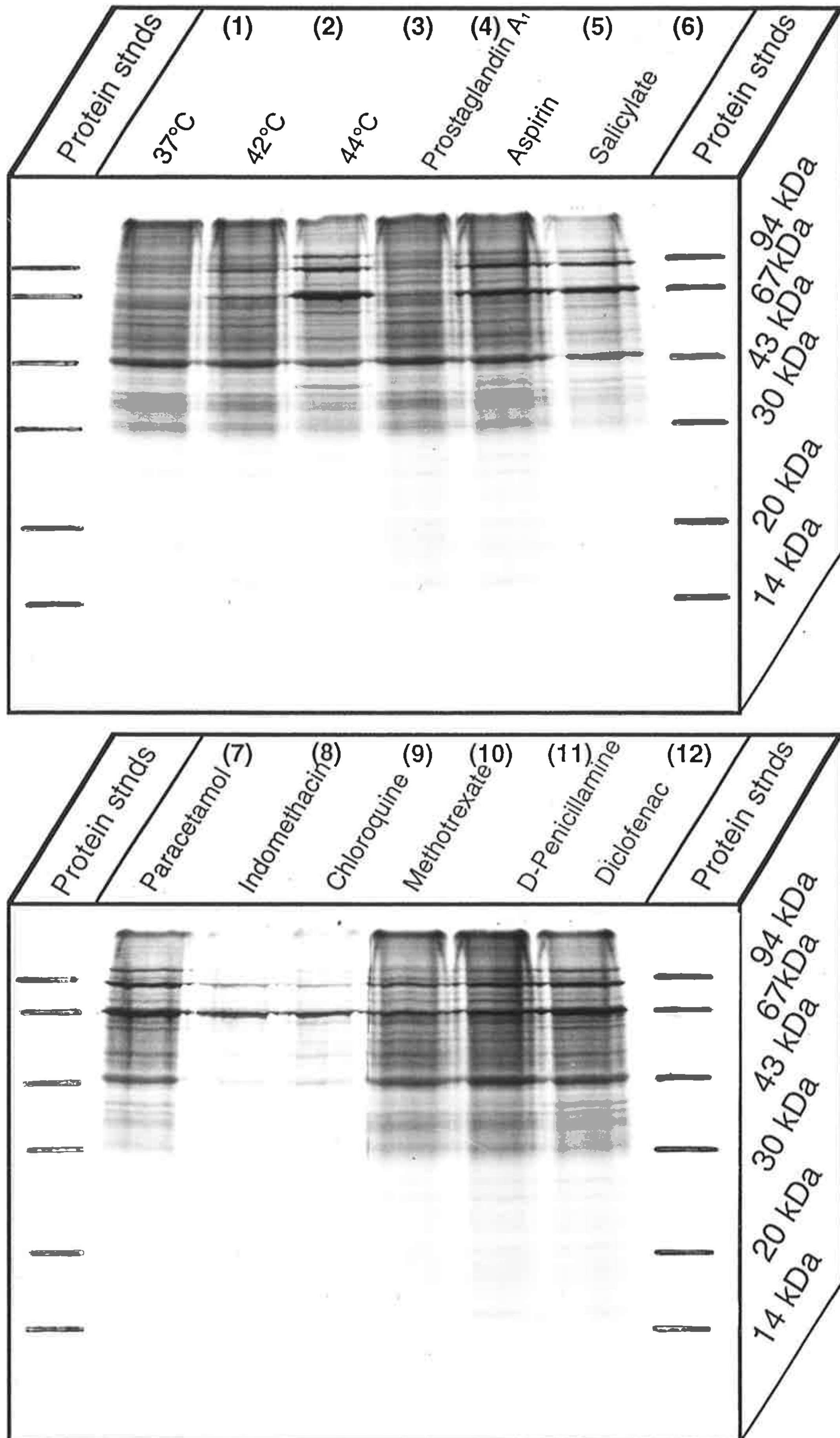


Figure 5.7 shows the autoradiograph of MNC proteins separated by SDS PAGE after MNC were exposed to a combination of anti-inflammatory/rheumatic agent and HS or HS alone. The exposure conditions are summarised in Table 5.3.

Figure 5.7

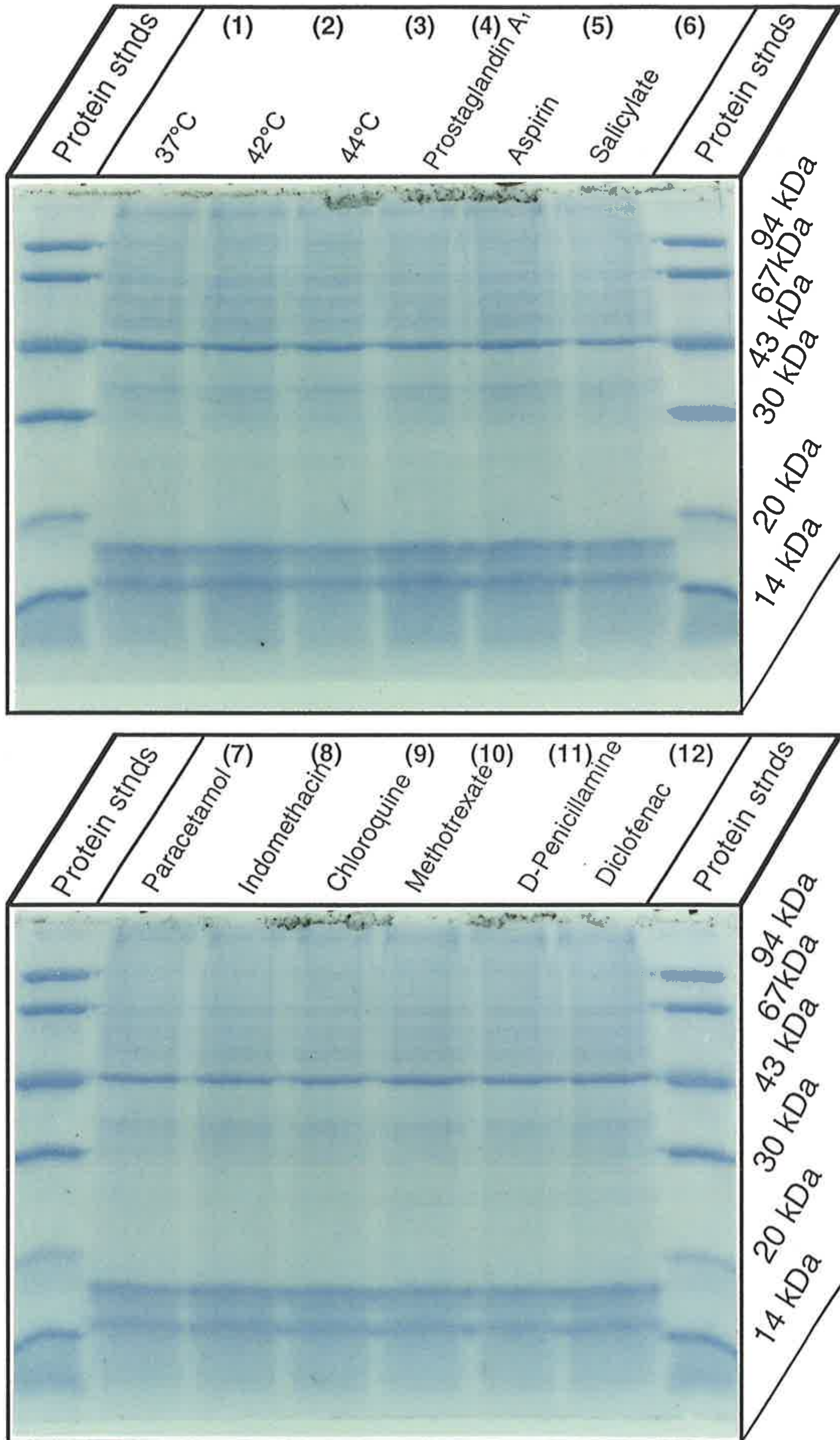


Figure 5.8 shows the coomassie blue stained gels which correspond to the autoradiographs shown in Fig. 5.7.

Figure 5.8

CHAPTER
SIX

Variations in the Induction of HSP Associated with Exposure to Anti-inflammatory Agents

6.1 INTRODUCTION

In chapters four and five the standard exposure of MNC to anti-inflammatory agents consisted of an initial incubation at 37°C for, usually, no longer than 30min plus an additional 15min of HS and thereafter the cells were removed from the agent. On analysis of total protein synthesis, two major effects were consistently observed. The first effect was the induction of HSP after a sufficient HS and sometimes after HS combined with an anti-inflammatory/rheumatic agent. The second effect was the inhibition of normal protein synthesis when MNC were exposed to a particular combination of HS and an anti-inflammatory agent. Inhibition of normal cellular protein synthesis may indicate one of two situations having occurred: (a) a possible redirection of the protein synthesis machinery and resources from normal cellular protein synthesis to HSP synthesis through the selective inhibition of normal protein synthesis (Lindquist 1986; Yost *et al.* 1990) or (b) disruption of normal cellular protein synthesis which may be mediated by the anti-inflammatory/rheumatic agents due to cytotoxic levels of agent acting on the cell.

This chapter investigates changes in MNC protein synthesis after MNC were incubated for 30min or longer with anti-inflammatory/rheumatic agents. Although MNC protein synthesis was not effected by anti-inflammatory/rheumatic agents, after short periods of exposure without a subsequent HS, longer exposures may have allowed more of the agent to diffuse across the cell membrane, before a toxic intracellular concentration accumulates, or may have allowed the agent more time to react with the cell to cause chemical or other changes which result in cytotoxicity. In this chapter, I establish whether or not each agent alone, used at a particular concentration, causes adverse changes to normal MNC protein. In the event of adverse effects occurring, I investigate whether or not these effects are likely to influence experiments using the standard incubation protocol. I also check cell survival, by the trypan blue dye exclusion method after the longer exposures to anti-inflammatory/rheumatic agents.

Also in previous chapters, where MNC were treated with both HS and anti-inflammatory/rheumatic agents, the HS was applied to MNC while the cells were still exposed to an anti-inflammatory/rheumatic agent. Therefore, the effects of temperature on normal protein synthesis were investigated, by comparing normal protein synthesis in

MNC exposed to HS in the absence or presence of an anti-inflammatory/rheumatic agent. over longer periods (e.g. up to 5.5hrs).

6.2 EFFECT OF EXPOSURE TIME TO ANTI-INFLAMMATORY AGENTS ON NORMAL PROTEIN SYNTHESIS

In this section MNC were incubated without or with one of the anti-inflammatory/ rheumatic agents for 30min, 1.5hrs, 3.5hrs or 5.5hrs at 37°C and subsequently incubated in the presence of the agent for an additional 15min at either 37°C or 41°C. The cells were then metabolically radiolabelled as described in Section 2.2.5. The MNC cultures were processed further as described in Sections 2.2.5 to 2.2.8. and the corresponding autoradiographs appear in Figs. 6.1a to 6.1c. The results shown in Figs. 6.1a to 6.1c are also summarised in Table 6.1.

6.2.1 Aspirin

MNC exposed to 22mM aspirin and incubated at 37°C demonstrated no significant change in normal protein synthesis after 3.5hrs (Fig. 6.1a). MNC incubated with aspirin and then exposed to HS also did not demonstrate a significant change in normal protein synthesis after 3.5hrs. There was no change in viability by trypan blue exclusion for MNC preincubated between 30min and up to 5.5hrs at 37°C regardless of whether or not aspirin was present in the media. This result suggests that the conditions used in previous chapters, where MNC were incubated for <3.5hrs with 22mM aspirin, did not severely affect normal cellular protein synthesis.

6.2.2 Chloroquine

MNC exposed to chloroquine for 30min and subsequently exposed to 41°C (Fig. 6.1a) demonstrated normal protein synthesis inhibition. MNC incubated with chloroquine for greater than 30min, whether exposed to a HS or not, demonstrated inhibition of normal protein synthesis with the MNC exposed to a HS showing a slightly greater level of inhibition. After MNC had been incubated with chloroquine for 3.5hrs, normal protein

synthesis inhibition was almost complete and after 5.5hrs was completely inhibited in both the MNC incubated at 37°C and exposed to HS. Although normal protein synthesis was severely affected, trypan blue staining of MNC did not increase.

6.2.3 Diclofenac

MNC exposed to diclofenac demonstrated normal protein synthesis inhibition after 1.5hrs in the cells that had been subsequently exposed to a HS at 41°C (Fig 6.1a). However, inhibition of normal protein synthesis was more pronounced in MNC treated with diclofenac for 3.5hrs and incubated at 37°C or exposed to a HS. At 5.5hrs, normal protein synthesis inhibition increased only slightly. HSP expression was first observed after MNC were treated with diclofenac for 30min and exposed to a HS. Interestingly, after 1.5hrs, HSP synthesis also occurred in diclofenac-treated MNC not exposed to a HS. HSP expression also occurred in diclofenac-treated MNC incubated at 37°C for 3.5hrs and 5.5hrs. Once again trypan blue cell viability analysis showed no increase in positive staining as inhibition of normal protein synthesis increased.

6.2.4 Indomethacin

In the experiment shown in Fig. 6.1b, indomethacin-treated MNC did not demonstrate any noticeable change in normal protein synthesis. However in Fig. 5.1, of the previous chapter, MNC exposed to indomethacin and a HS of 41°C for 15min demonstrated the beginnings of normal protein synthesis inhibition. Therefore, HS at 41°C is possibly on the limits of affecting normal protein synthesis in cells treated with indomethacin. However, in this section, as the incubation time was increased from 30min to 5.5hrs, there was no significant difference between the normal protein synthesis of indomethacin-treated and untreated MNC. This suggested that longer exposures to a fixed concentration of indomethacin did not affect normal cellular protein synthesis when MNC were subsequently exposed to HS but possibly, the combination of both indomethacin and even higher HS temperatures are what have the greatest effect on normal cellular protein synthesis. Trypan blue exclusion showed no change in MNC viability during the course of the experiment.

6.2.5 Methotrexate

MNC incubated with methotrexate for 30min did not demonstrate any significant change in normal cellular protein synthesis at 37°C and a decrease at 41°C (Fig 6.1b). But after 1.5hrs, 3.5hrs and 5.5hrs, normal cellular protein synthesis showed a consistent inhibition in both the methotrexate-treated MNC subsequently exposed to 37°C or a HS at 41°C. Trypan blue staining of the cell cultures demonstrated no change in cell viability for cultures preincubated for 30min to 3.5hrs. After 5.5hrs, staining increased significantly in methotrexate-treated MNC, but was unaffected in untreated MNC. Therefore, the experimental results obtained with 30min methotrexate preincubated MNC in Chapters 4 and 5 are not likely to be influenced by cell death, as cell death only increased in methotrexate-treated MNC at 5.5hrs.

6.2.6 Paracetamol

No change in normal protein synthesis occurred in MNC exposed to paracetamol for 30min or 1.5hrs (Fig. 6.1b). However, a significantly noticeable inhibition set in after MNC were incubated for 3.5hrs at 37°C and in MNC exposed to a HS. The inhibition of normal protein synthesis further increased, although only marginally, in MNC incubated with paracetamol for 5.5hrs. Frequency of trypan blue staining did not change throughout this experiment.

6.2.7 D-Penicillamine

D-Penicillamine demonstrated no inhibition of normal protein synthesis in MNC incubated at 37°C or 41°C for 30min up to 5.5hrs (Fig. 6.1c). Again, the frequency of trypan blue staining did not change throughout this experiment.

6.2.8 Salicylate

Salicylate-treated MNC demonstrated no change in normal protein synthesis for up to 1.5hrs of incubation (Fig. 6.1c). After 3.5hrs, MNC incubated with salicylate and exposed to a HS showed inhibition of normal protein synthesis when compared to

untreated MNC exposed to a HS or when compared to salicylate-treated or untreated MNC exposed to 37°C after 3.5hrs. After 5.5hrs, salicylate-treated MNC incubated at 37°C demonstrated a slight inhibition of normal protein synthesis. However, MNC subsequently exposed to a HS of 41°C demonstrated a stronger inhibition. Trypan blue staining showed no change with increased duration of MNC pretreatment with salicylate.

6.2.9 Summary

This section is primarily concerned with investigating the effects of extended exposures of MNC to anti-inflammatory/rheumatic agents on normal protein synthesis. Table 6.1 summarises the relative changes in normal protein synthesis in MNC exposed to different anti-inflammatory/rheumatic agents. Although the combined effects of some anti-inflammatory/rheumatic agents and HS includes some degree of normal MNC protein synthesis inhibition, which may be due to the rededication of the cellular protein synthesis resources and machinery from normal protein synthesis to HSP synthesis, reduced levels of normal protein synthesis occurring in agent-treated MNC after a 30min preincubation with the agent in the absence of HS, were interpreted in this chapter as potential cytotoxic effects. Trypan blue staining was also used to identify cultured MNC which had been killed by exposure to an anti-inflammatory/rheumatic agent.

Table 6.1 shows that only two (indomethacin and D-penicillamine) of the eight agents had no inhibitory effects on normal protein synthesis after 5.5hrs at 37°C. The remaining six agents all demonstrated, to some extent, inhibition of normal protein synthesis. While salicylate and aspirin only affected normal protein synthesis after 5.5hrs at 37°C, diclofenac and paracetamol caused inhibition after 3.5hrs and chloroquine and methotrexate after only 1.5hrs preincubation at 37°C. Because the inhibitory effects caused by all of these agents took affect at 1.5hrs or later they are not expected to have interfered with experiments which use the 30min preincubation period. This may not always have been the case with chloroquine as in Section 5.2.2, it caused a small but significant reduction in normal protein synthesis in MNC which had been incubated at 37°C for 30min without a subsequent HS.

Normal protein synthesis, in MNC treated with chloroquine, diclofenac, methotrexate and salicylate and subsequently exposed to 41°C, became inhibited after shorter incubation periods when compared to MNC incubated at 37°C. MNC treated with chloroquine and methotrexate demonstrated inhibition of normal protein synthesis after only 30min (Table 6.1), suggesting that these agents may be affecting cell viability at 41°C. However, in the case of chloroquine-treated MNC at 30min, there was strong hsp70 synthesis (Table 6.2) observed at this temperature.

Despite the inhibition of normal protein synthesis by most of these agents, only methotrexate increased trypan blue staining at 5.5hrs and this directly corresponded with the increase in the inhibition of normal protein synthesis. In comparison, the negative trypan blue staining of MNC treated with the other agents which caused protein synthesis inhibition, indicated that the decrease in protein synthesis was insufficient to cause cell death. For example, with chloroquine at 41°C, there was a marked decrease in normal protein synthesis but no cell death as indicated by negative trypan blue staining and elevated HSP synthesis (Table 6.2).

Table 6.1

The Comparison of Normal Protein Synthesis with Time in MNC Incubated with an Anti-Inflammatory Agent

Agent	Exposure Temperature & Time							
	37°C				41°C			
	0.5hr	1.5hr	3.5hr	5.5hr	0.5hr	1.5hr	3.5hr	5.5hr
Aspirin	0	0	0	- -	0	0	0	- -
Chloroquine	0	- -	- - - - -	- - - - -	- -	- - - -	- - - - -	- - - - -
Diclofenac	0	0	- - -	- - - -	0	- -	- - - -	- - - -
Indomethacin	0	0	0	0	0	0	0	0
Methotrexate	0	-	-	- - -	-	-	- -	- - -
Paracetamol	0	0	- - -	- - - -	0	0	- -	- - - -
D-Penicillamine	+	+	0	0	+	+	++	0
Salicylate	0	0	0	- -	0	0	- - -	- - - -

Normal protein synthesis was scored by comparing the agent-treated sample with its untreated control for each incubation period. Increases were scored with the positive symbol (+) and decreases were scored with the negative symbol (-), with "-" representing a slight decrease and "- - - - -" representing a total inhibition of normal protein synthesis. While "0" means no change was observed and a single "+" means a slight increase.

6.3 EFFECT OF EXPOSURE TIME TO ANTI-INFLAMMATORY AGENTS ON HSP SYNTHESIS

The previous section investigated the differences in normal protein synthesis in MNC preincubated with an anti-inflammatory/rheumatic agent for extended periods up to 5.5hrs. This section summarises the effect of extended exposures of MNC to anti-inflammatory/rheumatic agents on HSP expression and answers the following questions: (i) Do any of the anti-inflammatory/rheumatic agents cause the induction of HSP synthesis in MNC without a subsequent exposure to HS when the preincubation period is extended?, (ii) does exposing MNC to anti-inflammatory/rheumatic agents for longer durations result in an increase or decrease in HSP induction after a subsequent moderate HS?

Table 6.2 shows that of all the anti-inflammatory/rheumatic agents tested only diclofenac caused a clear and distinct induction of HSP in MNC without the need for a subsequent HS. This induction occurred only after the MNC had been exposed to 1.0mM diclofenac for 1.5hrs or longer.

As the length of MNC exposure to anti-inflammatory/rheumatic agents increased, HSP synthesis/inducibility increased in MNC treated with only two of the agents tested, aspirin and D-penicillamine. Both agents demonstrated clear increases of HSP synthesis, with longer preincubation times, but only when the treated MNC were exposed to HS. Aspirin demonstrated a significant increase in HSP synthesis/inducibility when the preincubation period was extended from 30min to 1.5hrs but a decrease with longer preincubation periods. D-penicillamine similarly demonstrated an increased HSP synthesis/inducibility but only after MNC had been preincubated for 3.5hrs or longer. Other agents, in conjunction with HS, caused a decrease in HSP synthesis with increased exposure times, with chloroquine showing a significant fall at 1.5hrs. Again, methotrexate did not facilitate HSP induction.

Table 6.2

The Comparison of hsp70 Expression with Time in MNC Incubated with an Anti-Inflammatory Agent

Agent	Exposure Temperature & Time							
	37°C				41°C			
	0.5hr	1.5hr	3.5hr	5.5hr	0.5hr	1.5hr	3.5hr	5.5hr
Aspirin	0	0	0	0	++	+++	+++	++
Chloroquine	0	0	0	0	+++	+	0	0
Diclofenac	0	+++	+++	++	+++++	+++	+++	++
Indomethacin	0	0	0	0	+++	++	++	+
Methotrexate	0	0	0	0	0	0	0	0
Paracetamol	0	0	0	0	++++	++++	+++	++
D-Penicillamine	0	0	0	0	0	0	++	++
Salicylate	0	0	0	0	+++++	+++++	+++	+

Hsp70 synthesis was scored by comparison of the agent-treated sample with its untreated control for each incubation period. Increases in HSP synthesis were scored with the positive symbol (+). With the single positive symbol "+" representing a slight increase and "+++++" representing a strong induction of HSP synthesis.

6.4 DIFFERENCES IN HSP EXPRESSION BY MONONUCLEAR CELLS EXPOSED TO HS IN THE PRESENCE OR IN THE ABSENCE OF THE ANTI-INFLAMMATORY AGENT

This section investigates the differences in protein synthesis in MNC preincubated with an anti-inflammatory/rheumatic agent and then subsequently exposed to HS in the absence or presence of the same agent. In the previous section, it was found that most of the agents had some effect on normal MNC protein synthesis when MNC had been incubated at 37°C for longer than 30min and then exposed to HS for a further 15min, all in the presence of an agent. Altogether, MNC were exposed to an agent for 45min. In this section MNC are exposed to an agent for 30min at 37°C and thereafter the cells are either, incubated with agent at 37°C for a further 15min or exposed to a HS of 42°C for 15min, in the absence or presence of the same agent. Control MNC were processed in the same way with the exception that agents were not included in the culture medium. For practical reasons the anti-inflammatory/rheumatic agents were divided into two groups and MNC were exposed to each group in separate experiments. This allowed for better control over MNC exposure times to agent. The resulting autoradiographs appear in Figs. 6.2a to 6.2c with separate controls for each experiment. Other than this, the experiments were performed as described in Sections 2.2.5 to 2.2.8. Densitometric analysis was also

performed on the autoradiographs appearing in Figs. 6.2a to 6.2c and the area under each of the peaks representing hsp70 was estimated and is given in arbitrary units. The data and the densitometric scans appear in Figs. 6.3a to 6.3e. A summary of the results is shown in Table 6.3.

Experiment One

The first experiment included MNC exposed to aspirin, salicylate, paracetamol or indomethacin and the results appear in Figs. 6.2a to 6.2b and Figs. 6.3a to 6.3c. The control MNC showed no difference in normal protein synthesis between MNC incubated at 37°C or MNC exposed to a HS of 42°C. Hsp70 in the control MNC exposed to a HS (lane 2) and processed in the same way (e.g. inclusion of an extra washing step) as MNC pretreated with an agent before exposure to HS was estimated to be 0.278 units (12%) greater than in the untreated control MNC (lane 3) processed like MNC exposed to a HS in the presence of an agent (e.g. no wash step). This suggested that the additional processing of MNC cultures, that is removal of the anti-inflammatory/rheumatic agent and resuspension in fresh medium, resulted in an increased hsp70 synthesis after HS.

6.4.1 Aspirin

MNC exposed to aspirin (lanes 4 to 6) demonstrated no variation in normal protein synthesis between the MNC exposed to 37°C and MNC exposed to HS in the absence or the presence of aspirin. Hsp70 levels were estimated at 0.112 units (5%) greater in MNC exposed to HS in the presence of aspirin (lane 6) compared to MNC exposed to HS in the absence of aspirin (lane 5).

6.4.2 Salicylate

Normal protein synthesis in salicylate-treated MNC (lanes 7 to 9) was noticeably lower when compared to the control MNC (lanes 1 to 3), and decreased slightly further in MNC exposed to a HS in the presence of salicylate (lane 9) compared to MNC exposed to a HS in the absence of salicylate (lanes 8). However, hsp70 was higher by 0.632 units (46%)

in MNC exposed to HS in the presence of salicylate as compared to MNC exposed to HS in the absence of salicylate.

6.4.3 Paracetamol

Paracetamol-treated MNC demonstrated a slight decrease in normal protein synthesis when exposed to a heat shock in the presence of paracetamol (lane 12) when compared to MNC exposed to HS in the absence of paracetamol (lane 11). Hsp70 synthesis was estimated to be 0.562 units (24%) higher in the MNC exposed to HS in the presence of paracetamol compared to MNC exposed to HS in the absence of HS.

6.4.4 Indomethacin

Normal protein synthesis in indomethacin-treated MNC exposed to a HS in the presence of indomethacin (lane 15) was significantly reduced when compared to MNC incubated at 37°C (lane 13) or MNC exposed to HS in the absence of indomethacin (lane 14). Hsp70 synthesis in MNC exposed to a HS in the presence of indomethacin was 0.304 units (12%) higher than in the cells exposed to HS in the absence of indomethacin.

Experiment Two

The second experiment included MNC exposed to chloroquine, methotrexate, penicillamine, or diclofenac with the results appearing in Figs. 6.2b to 6.2c and Figs. 6.3c to 6.3e. Control MNC (lanes 16 to 18) demonstrated virtually no difference in total protein synthesis, however before this conclusion was arrived at, allowances needed to be made in calculations for the fact that the proteins in lane 18 had diffused laterally resulting in a wider lane on the autoradiograph appearing lighter in density. Once again there was a difference in hsp70 levels in the two MNC cultures exposed to a HS. Hsp70 synthesis in the control MNC which were processed in the same way as cultures normally containing agents and exposed to HS in the absence of the agent (lane 17) was estimated to be 0.170 units (7%) greater than in the control MNC processed in the same way as MNC exposed to HS in the presence of an agent (lane 18). This was consistent with the first experiment where a similar trend (12%) was observed.

6.4.5 Chloroquine

Normal protein synthesis in chloroquine-treated MNC (lanes 19 and 20) was noticeably lower compared to the levels displayed in control MNC and considerably lower when MNC were exposed to a HS in the presence of chloroquine (lane 21). Hsp70 levels in MNC exposed to a HS in the presence of chloroquine (lane 21) was estimated to be 1.44 units (78%) lower than in MNC exposed to HS in the absence of chloroquine (lane 20). The decrease in hsp70 levels seems to be part of an overall effect on all protein synthesis caused by chloroquine when combined with a HS at 42°C for 15min rather than a selective depression of HSP synthesis.

6.4.6 Methotrexate

Normal protein synthesis in methotrexate-treated MNC (lanes 22 and 23) was noticeably lower than in the untreated control cells, with normal protein synthesis in MNC exposed to a HS in the presence of methotrexate (lane 24) was even lower when compared to lanes 22 and 23. The level of hsp70 synthesised in MNC exposed to a HS in the presence of methotrexate (lane 24) was 1.373 units (68%) lower when compared to the methotrexate-treated MNC exposed to HS in the absence of methotrexate (lane 23). Once again, as for chloroquine-treated MNC, the lower levels of hsp70 seem to be due to an overall effect on protein synthesis caused by methotrexate when combined with a HS at 42°C for 15min rather than a selective depression of HSP synthesis.

6.4.7 D-Penicillamine

D-Penicillamine-treated MNC (lanes 25 to 27) demonstrated no significant difference in normal protein synthesis between MNC incubated at 37°C and MNC exposed to HS in the absence or presence of D-penicillamine. Hsp70 levels in MNC exposed to HS in the presence of D-penicillamine (lane 27) were estimated to be 0.256 units (12%) higher than in MNC exposed to a HS in the absence of D-penicillamine (lane 26).

6.4.8 Diclofenac

Normal protein synthesis in diclofenac-treated MNC remained high in MNC incubated at 37°C (lane 28) and in MNC exposed to HS in the absence of diclofenac (lane 29). However, normal protein synthesis was considerably reduced in MNC exposed to a HS in the presence of diclofenac (lane 30). Hsp70 levels in the MNC exposed to a HS in the presence of diclofenac (lane 30) were estimated to be 0.827 units (37%) lower than in MNC exposed to a HS in the absence of diclofenac (lane 29). Once again, as for chloroquine- and methotrexate-treated MNC, the lower levels of hsp70 seem to be due to the overall effect on protein synthesis caused by diclofenac (lane 30) when combined with a HS at 42°C for 15min rather than a selective depression of HSP synthesis.

6.4.9 Summary

In this section, HSP induction and synthesis was investigated in MNC treated with an anti-inflammatory/rheumatic agent and then exposed to a HS in the absence or the presence of the agent. The results are summarised in Table 6.3. Aspirin, indomethacin, paracetamol, D-penicillamine and salicylate all demonstrated a stronger induction of hsp70 synthesis when HS was applied while the anti-inflammatory/rheumatic agent was present in the culture medium. There are several possible explanations for this:

(i) By removing the agent prior to the HS, the exposure period of MNC to agent was limited to the 30min preincubation period, while MNC exposed to HS in the presence of the agent, experienced the agent for an additional 15min making the total exposure period 45min.

(ii) The higher temperature of HS increased the rate of agent diffusion across the membrane therefore a higher intracellular concentration of agent accumulates and therefore exerts a greater effect on HSP inducibility.

(iii) The removal of the agent from the medium may have allowed the agent to diffuse out of the cells before and during the HS hence reducing the intracellular concentration of the agent.

Although chloroquine, methotrexate, and diclofenac all decreased hsp70 synthesis in MNC exposed to HS in the presence of one of these agents, the decrease has to be interpreted in context with the inhibition of normal protein synthesis. Because, as the severity of inhibition increased, not only was normal protein synthesis inhibited but HSP synthesis was also inhibited. Therefore, an agent like diclofenac, having demonstrated considerable HSP synthesis enhancement after HS (Section 5.2.3 at 41°C), showed the opposite effect because of the severe inhibitory effect on all cellular protein synthesis after HS at this temperature (42°C).

Table 6.3

Hsp70 Expression in MNC Exposed to HS in the Absence or Presence of an Anti-Inflammatory Agent

Agent	Change in hsp70	Normal Protein Synthesis
Nil (Expt 1)	– 12%	Control
Aspirin	5%	Unchanged *(lanes 5 & 6)*
Salicylate	46%	Unchanged *(lanes 8 & 9)*
Paracetamol	24%	Unchanged *(lanes 11 & 12)*
Indomethacin	12%	Inhibited *(lanes 14 & 15)*
Nil (Expt 2)	– 7%	Control
Chloroquine	– 78%	Inhibited *(lanes 20 & 21)*
Diclofenac	– 37%	Inhibited *(lanes 29 & 30)*
Methotrexate	– 68%	Inhibited *(lanes 23 & 24)*
D-Penicillamine	12%	Unchanged *(lanes 26 & 27)*

Table 6.3 summarises the difference in hsp70 expression between MNC exposed to a 42°C HS in the absence of an anti-inflammatory/rheumatic agent and MNC exposed to 42°C in the presence of an anti-inflammatory/rheumatic agent. The lanes being compared are given in italics.

FIGURE 6.1:

Preincubation Time Course

MNC were preincubated with (+) or without (-) an anti-inflammatory/ rheumatic agent for either 30min, 1.5, 3.5 or 5.5hrs before exposing to a HS of 41°C for 15min or incubating at 37°C for the same length of time. Thereafter, the culture media was immediately changed to media containing radiolabelled methionine and no anti-inflammatory/rheumatic agents and the MNC were incubated at 37°C for 2hrs before a small aliquot of the MNC suspension was removed and used for trypan blue staining. MNC protein synthesis was then analysed by SDS PAGE and autoradiography. Photographs of the autoradiographs appear on the following three pages of this figure.

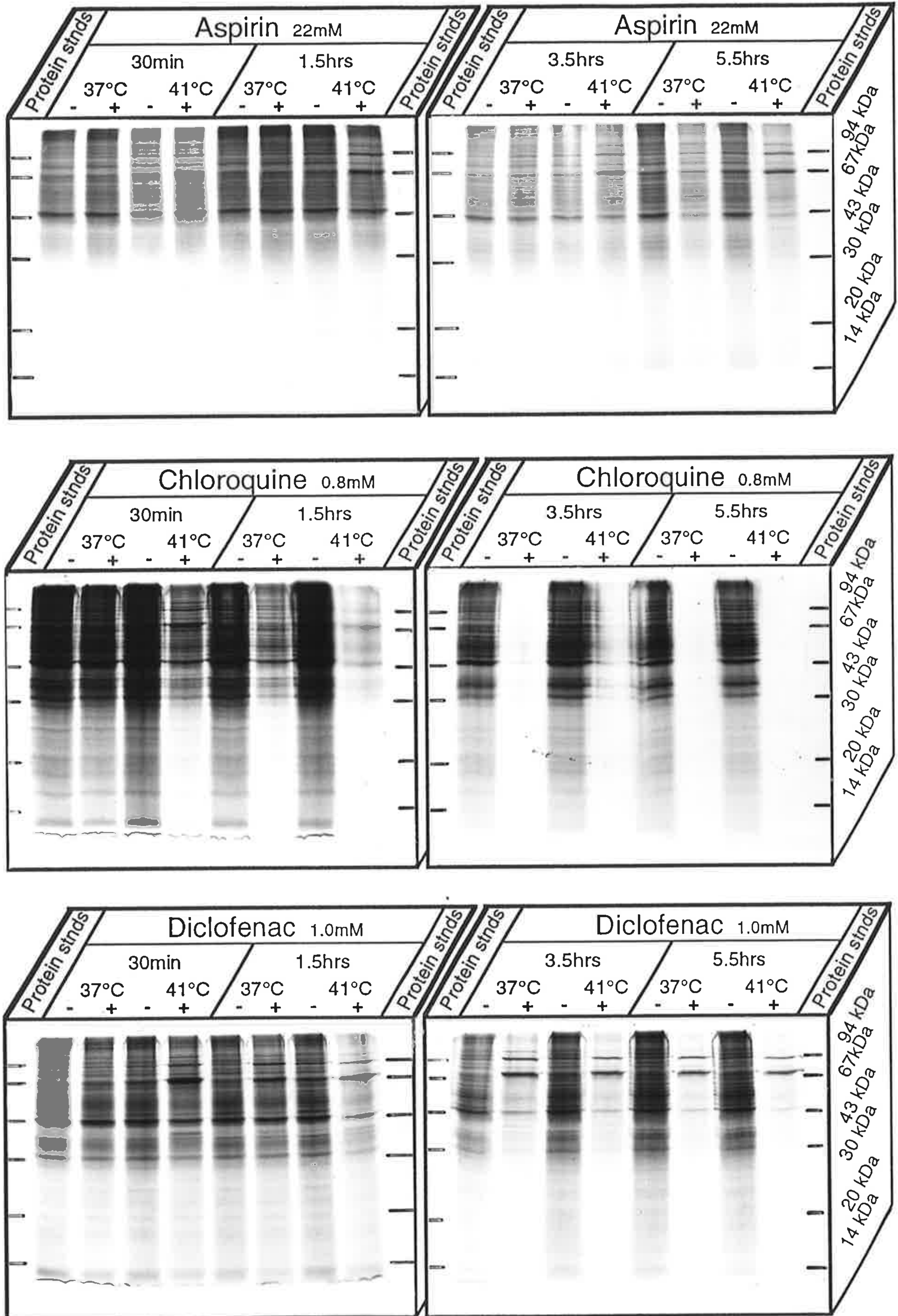
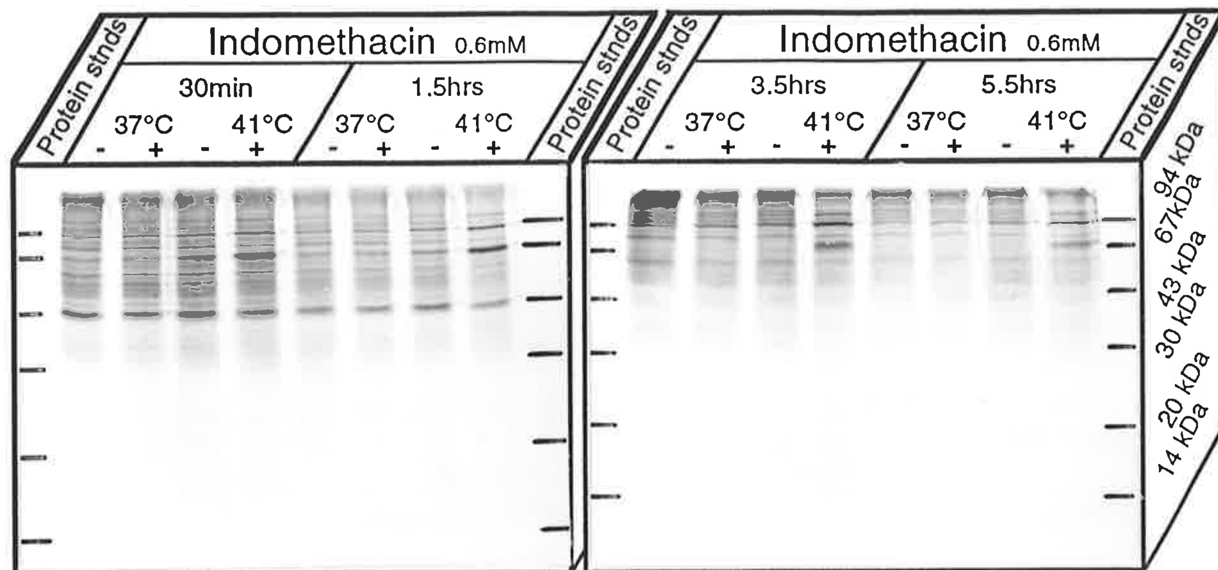
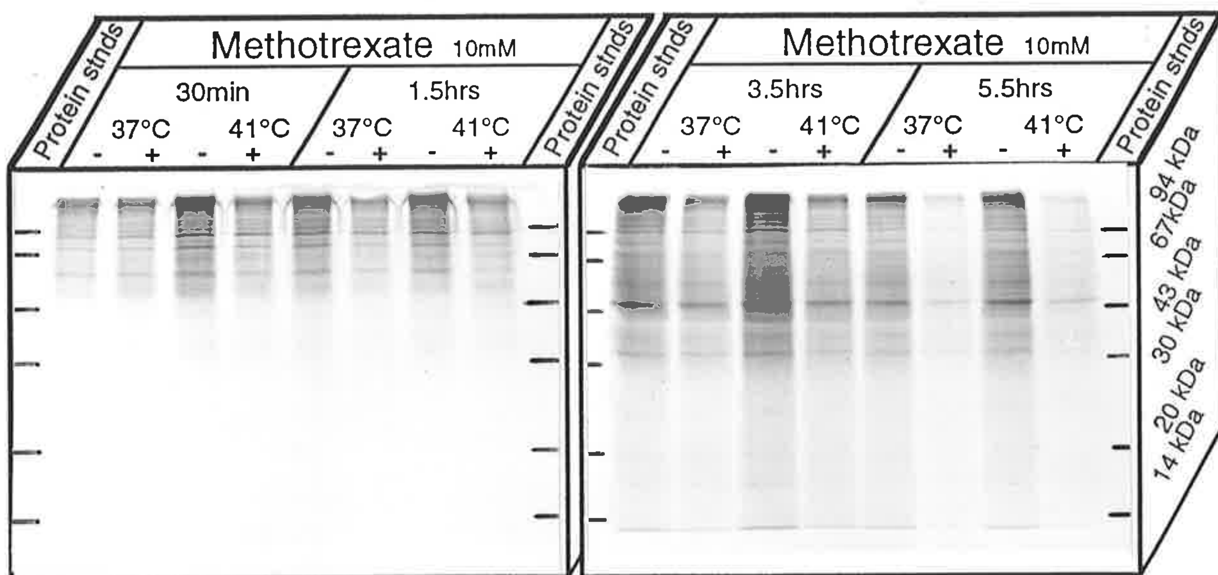


Figure 6.1a





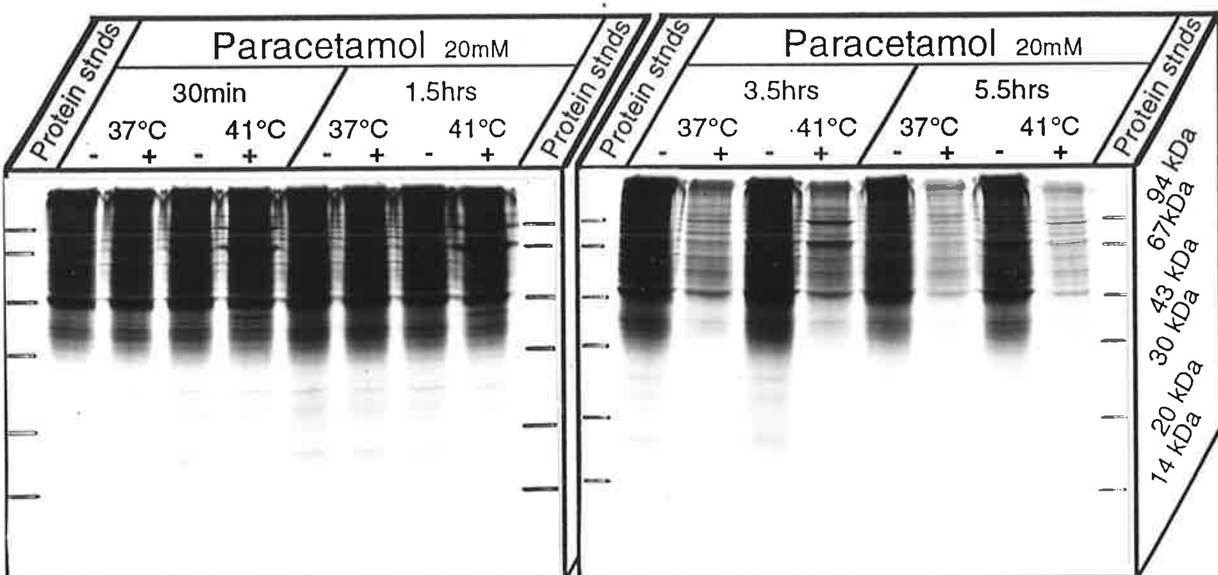
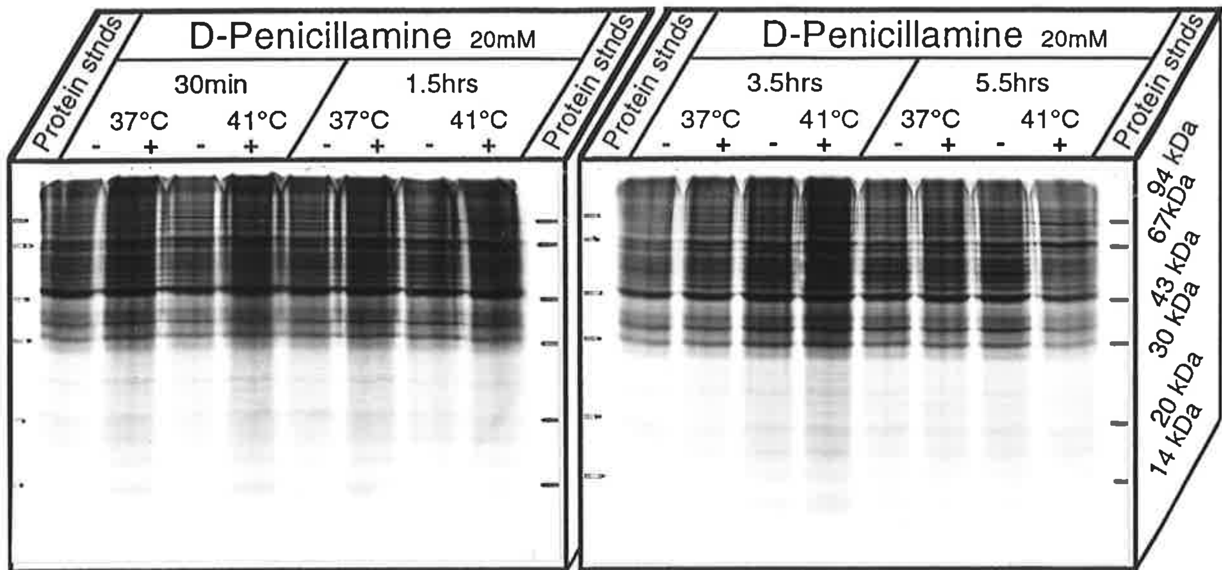


Figure 6.1b



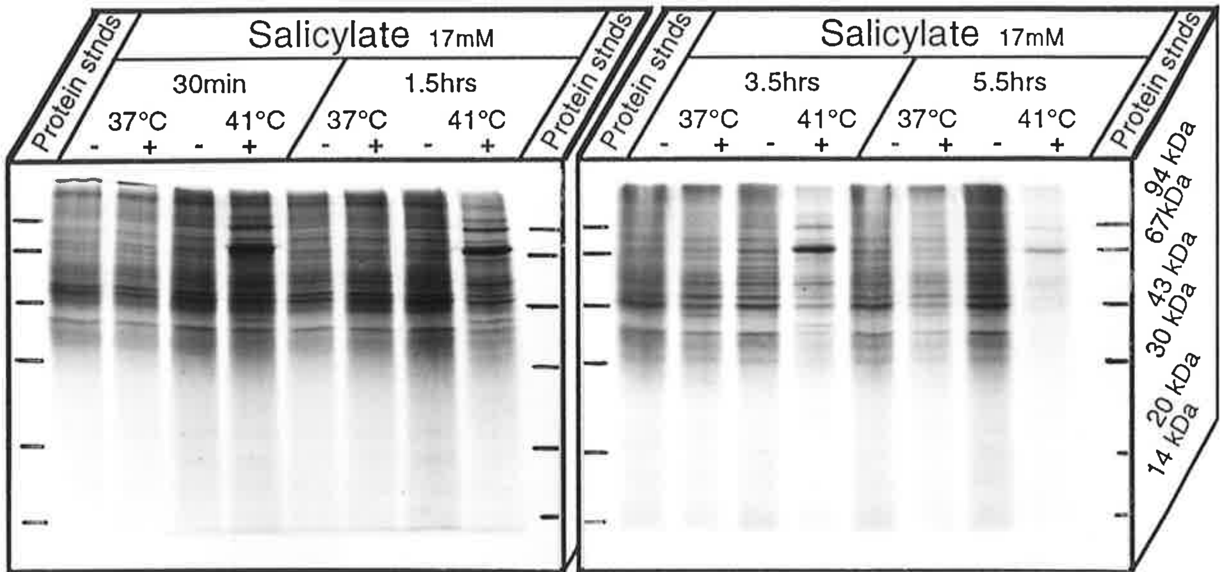
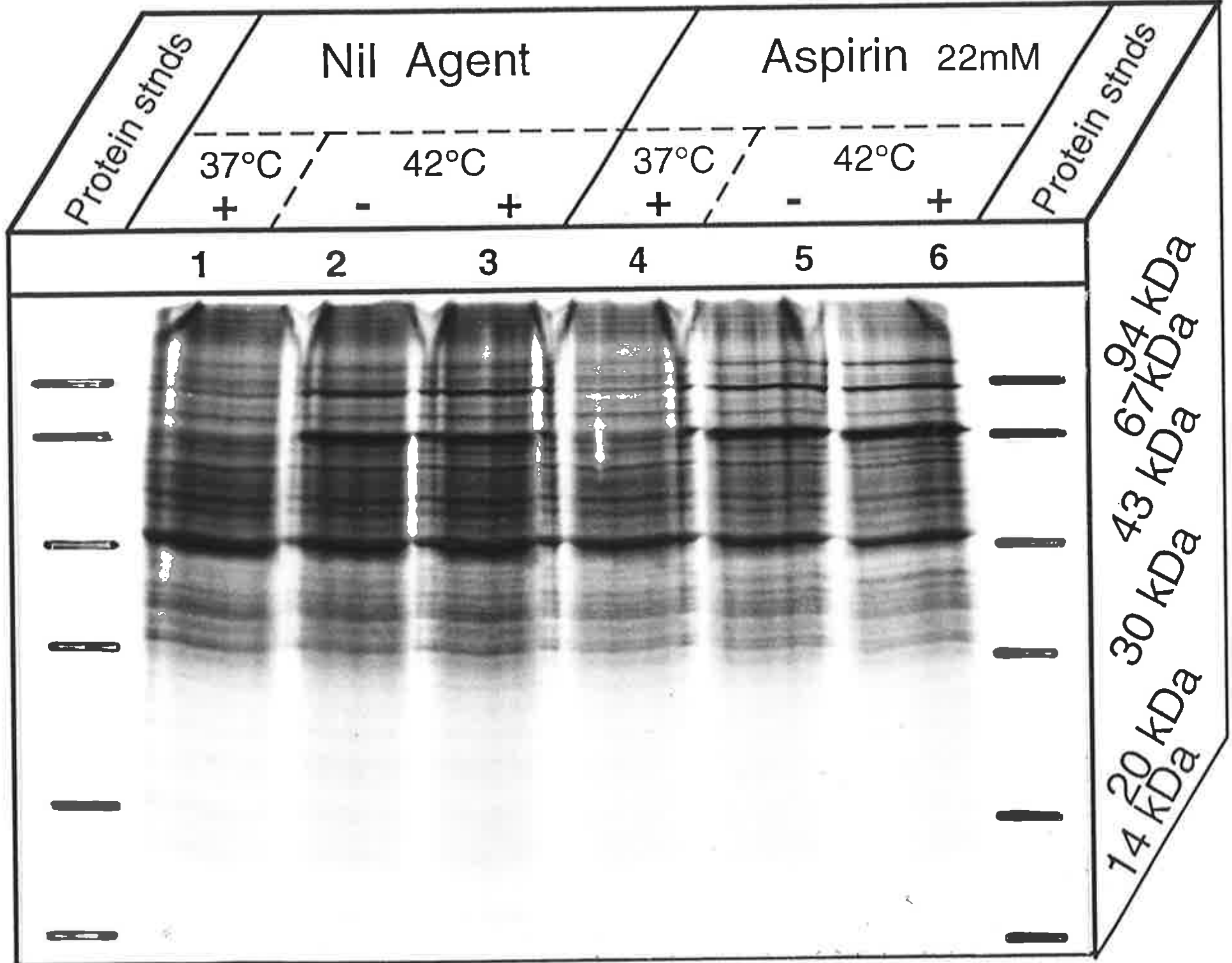


Figure 6.1c

FIGURES 6.2 - 6.3:

MNC Exposed to HS in the Absence or Presence of Agent

Three MNC cultures were preincubated with the same anti-inflammatory/ rheumatic agent for 30min at 37°C before the culture media in one of the cultures was replaced with media not containing an agent. The other two MNC cultures (+) were then either, further incubated for 15min at 37°C or exposed to a HS of 42°C for 15min. The MNC culture where the agent had been removed was also exposed to a HS of 42°C for 15min and represented the MNC culture exposed to a HS in the absence of an agent and appears in the following autoradiographs labelled with a minus sign (-). After the HS treatment MNC were radiolabelled and analysed by SDS PAGE and visulised by autoradiography (Figs. 6.2a to 6.2c. The autoradiographs were analysed by densitometry and the area under the hsp70 peak were estimated and are given in arbitrary units on the densitometric traces appearing in figures 6.3a to 6.3e.



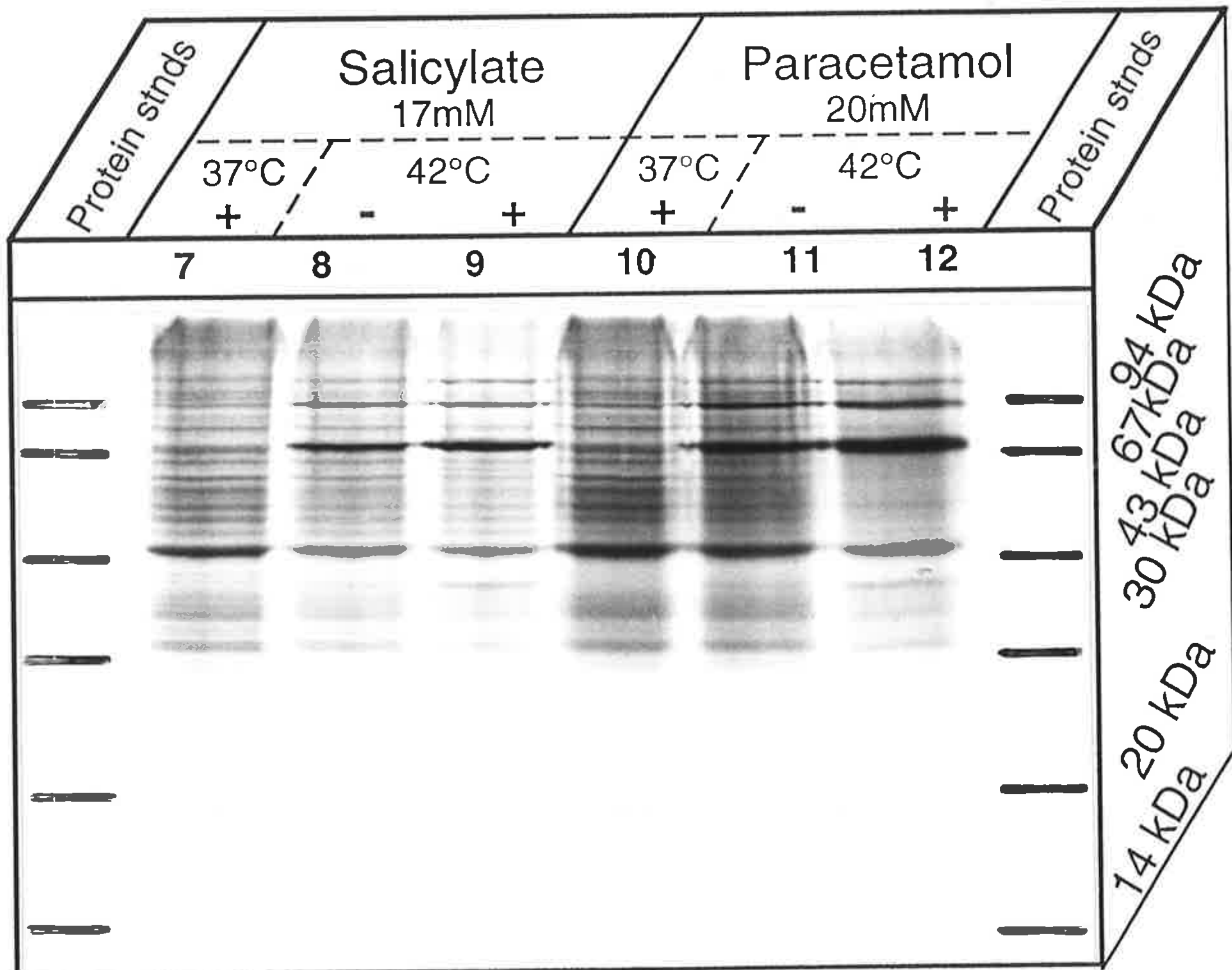
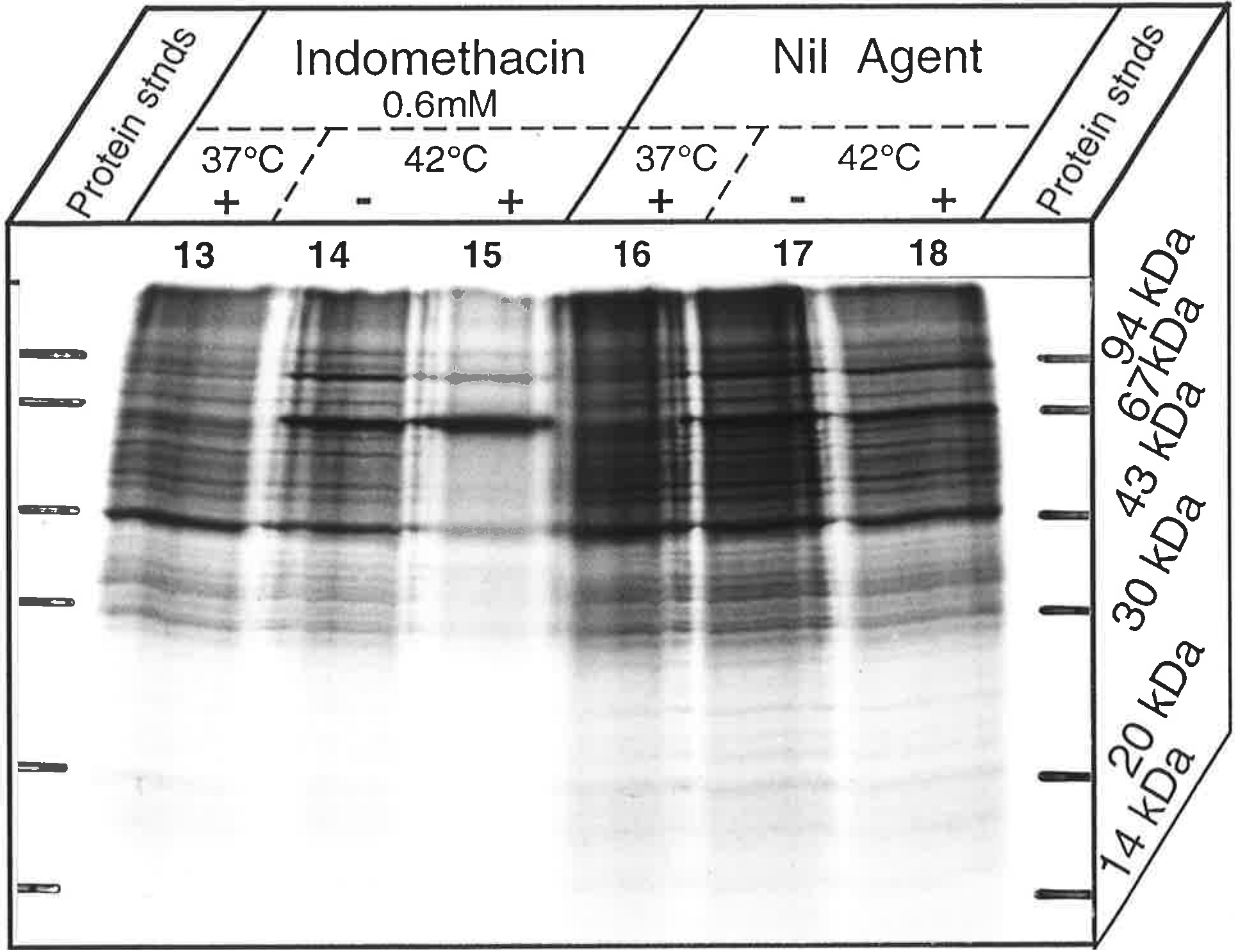


Figure 6.2a



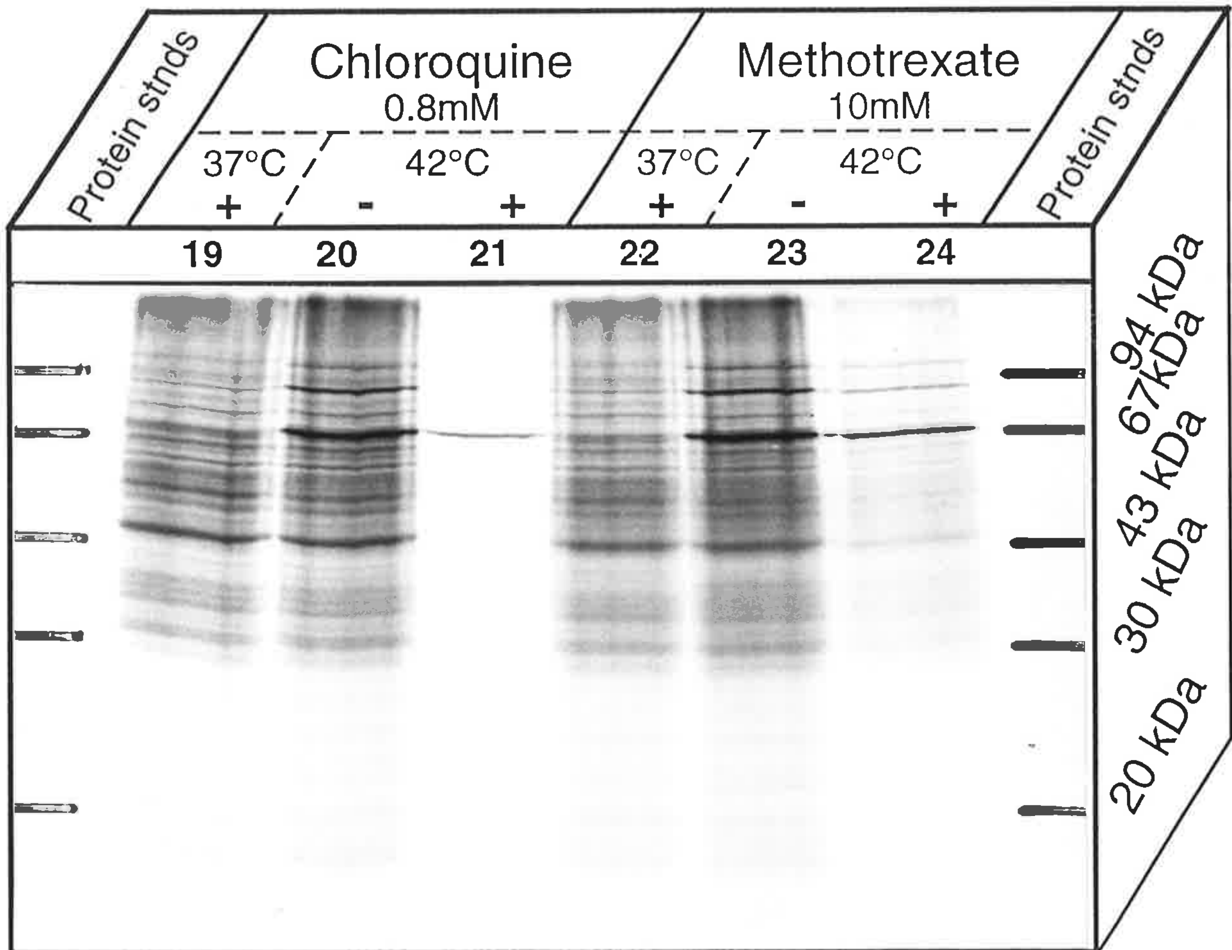


Figure 6.2b

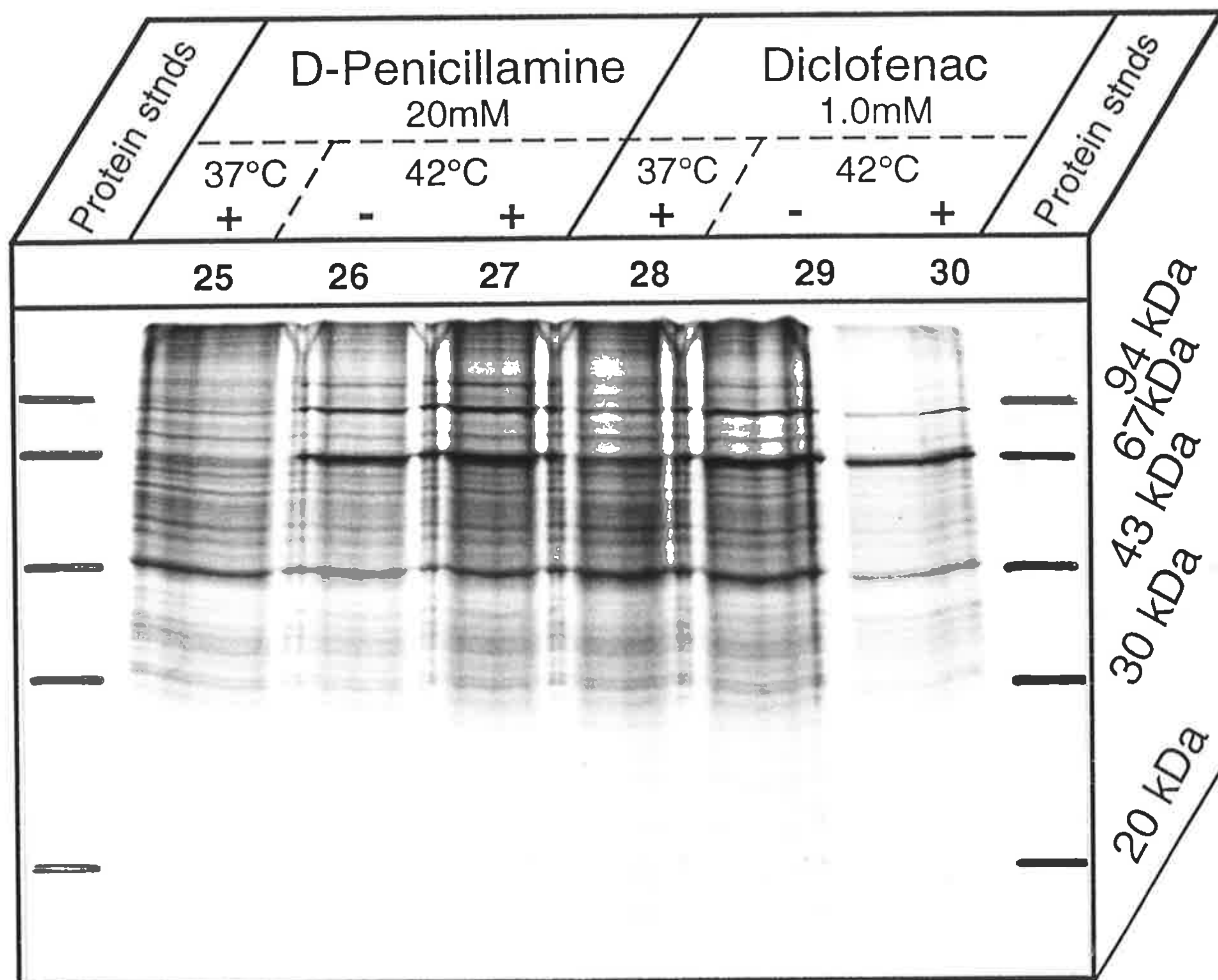


Figure 6.2c

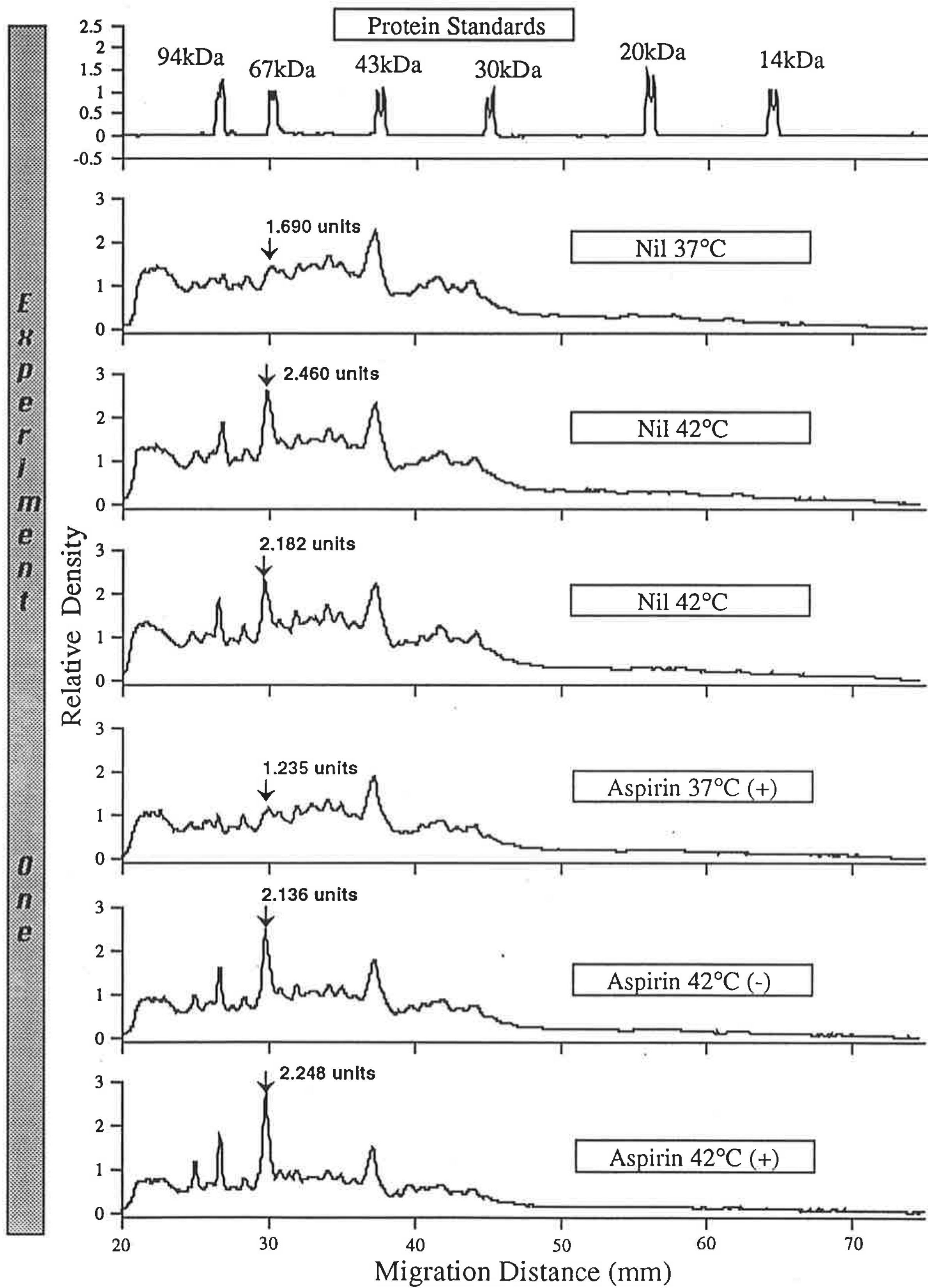


Figure 6.3a

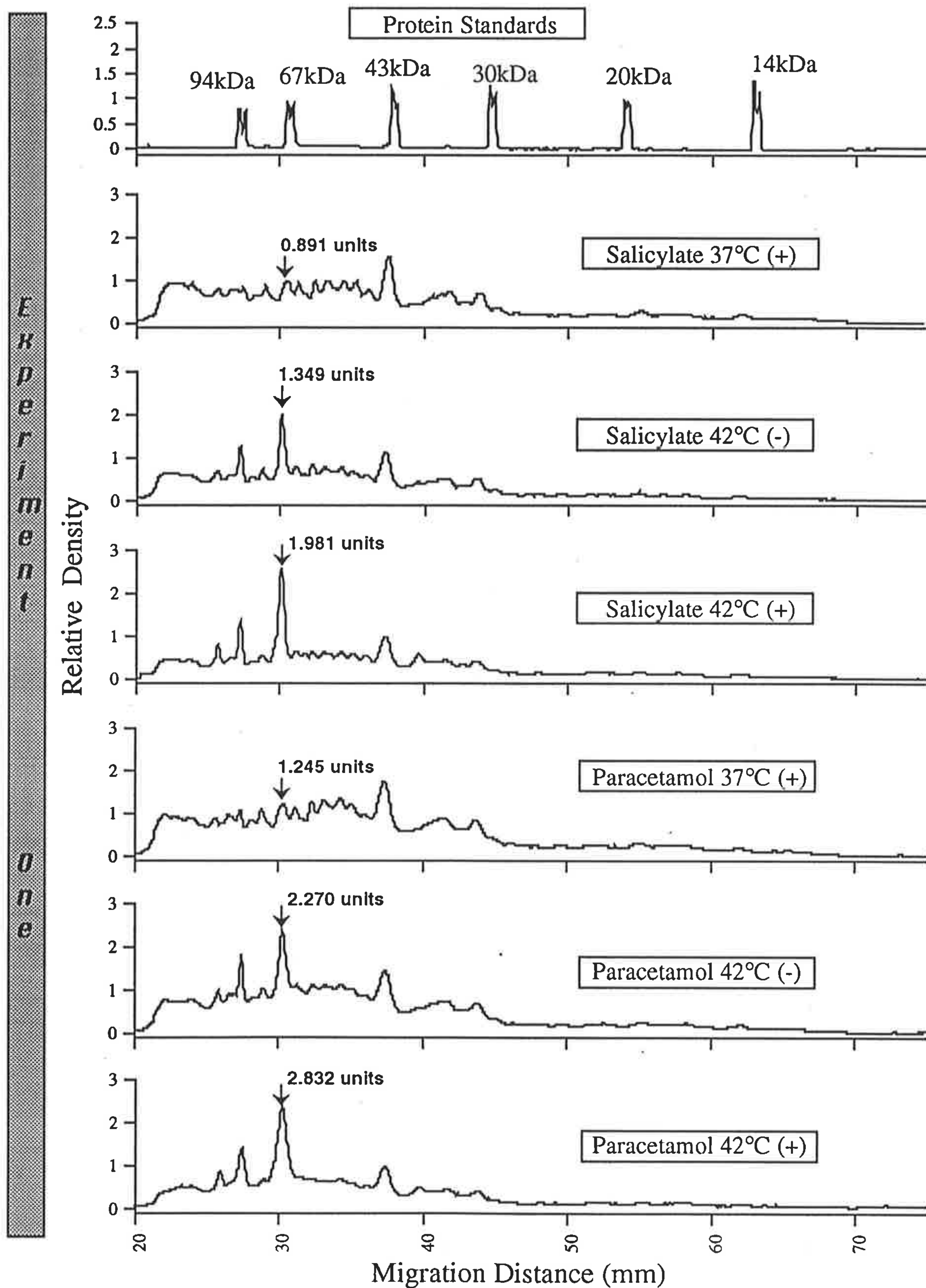


Figure 6.3b

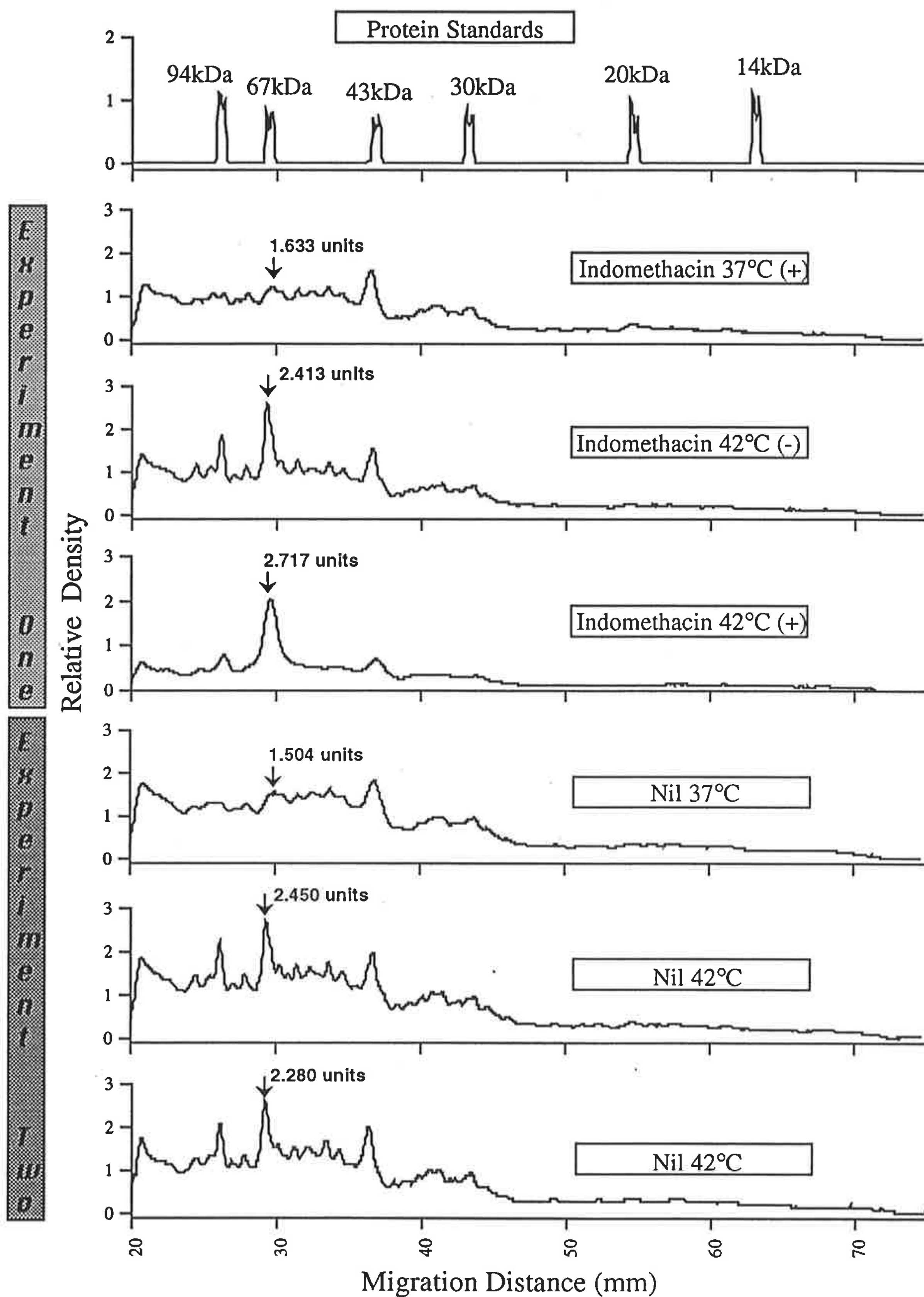


Figure 6.3c

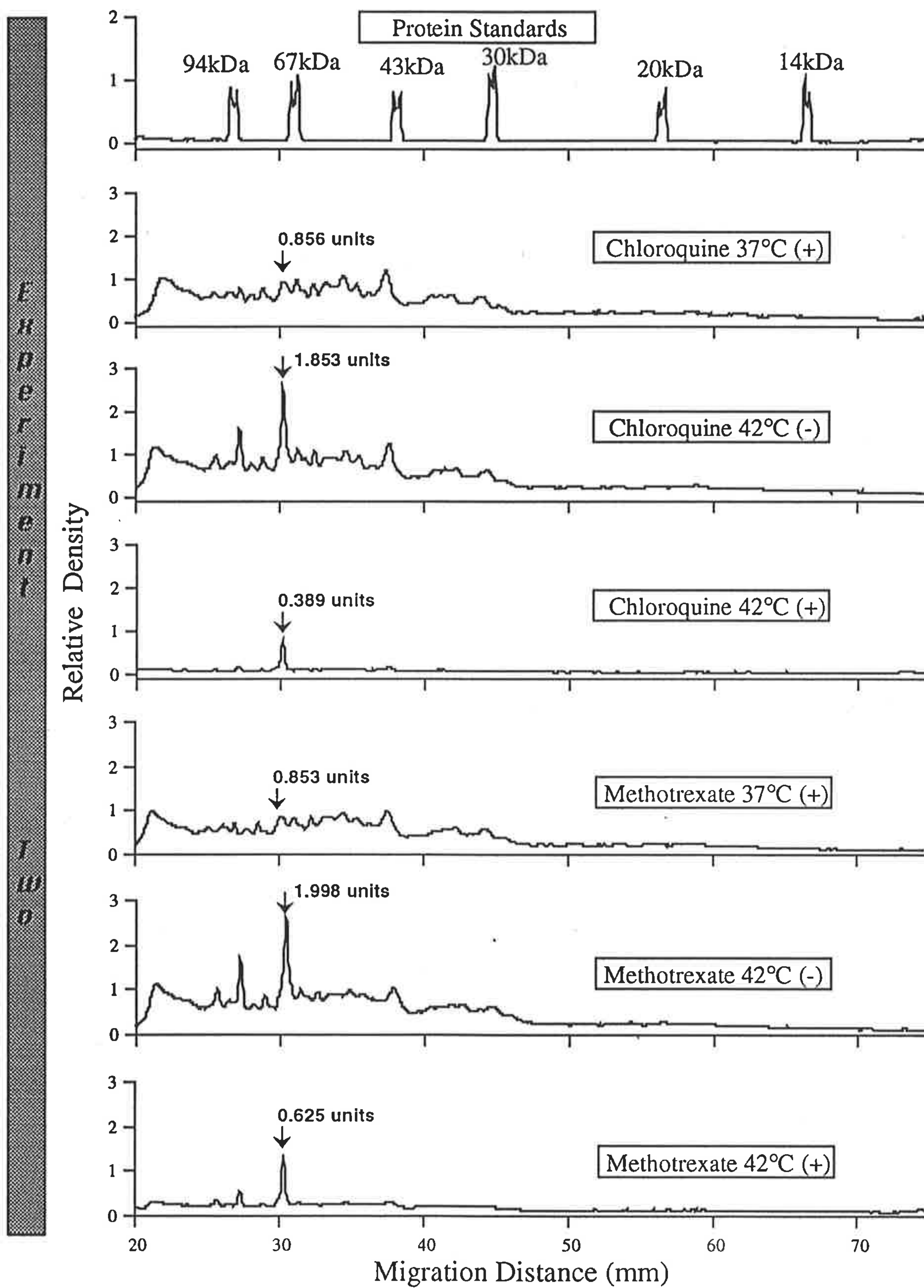


Figure 6.3d

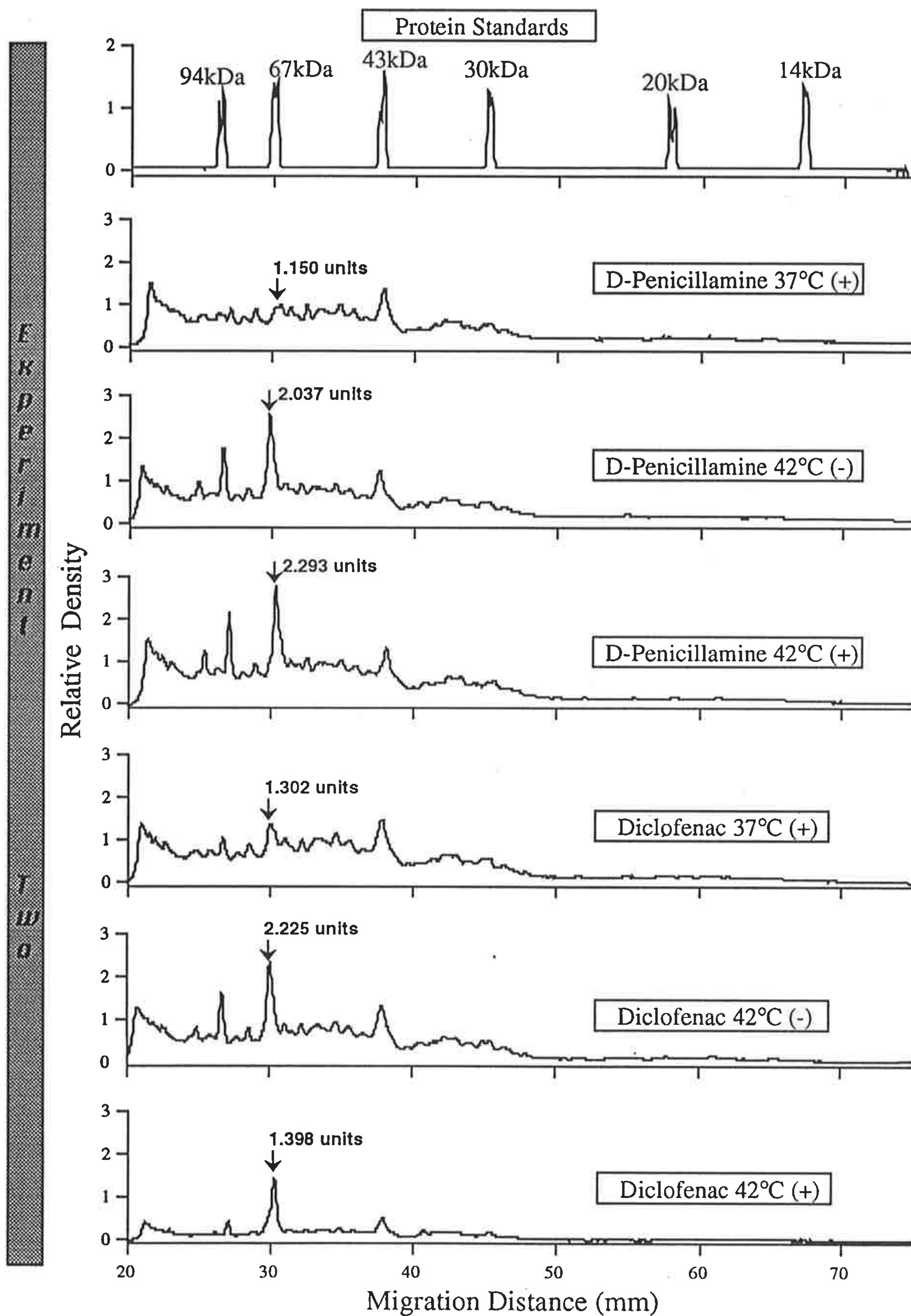


Figure 6.3e

CHAPTER SEVEN

A Combination of Anti-Inflammatory Agent and Mild Hyperthermia Protects Mononuclear Cells Against Hyperthermia

7.1 INTRODUCTION

In chapters four and five, anti-inflammatory/rheumatic agents demonstrated an ability to facilitate HSP expression in MNC exposed to a mild HS. As discussed in Section 1.1.1, HSP induced after a mild stress have been demonstrated to protect cells from harmful and/or lethal effects of more severe stresses (Ciavarra and Simeone 1990a; Mosser and Martin 1992). The severe stress may not necessarily kill MNC, but reduce their ability to function properly. Therefore HSP induced in MNC, by a combination of HS and anti-inflammatory or anti-rheumatic agents also have the potential to protect MNC from exposure to severe stress.

This chapter investigates the ability of MNC, exposed to a combination of HS and an anti-inflammatory/rheumatic agent, to maintain normal metabolic function, in particular protein synthesis, on exposure to a subsequent and more severe stress. Initially, conditions which protect normal MNC protein synthesis were established using a mild HS to induce HSP synthesis, followed by a recovery period allowing for HSP synthesis. A more severe HS was then used to challenge the cells and the amount of radiolabel incorporated into *de novo* proteins was used as a measure of protein synthesis. After establishing the protective pre-HS conditions, the severity of this pre-HS was further reduced until it alone did not have the ability to induce protection. Under these conditions I then tested whether or not the addition of anti-inflammatory/rheumatic agents, again protected normal protein synthesis. This would be expected since, as shown in Chapter Six, some combinations of mild HS and anti-inflammatory/rheumatic agent increased HSP induction.

7.2 HUMAN MONONUCLEAR CELLS EXPOSED TO HYPERTHERMIA DEMONSTRATE PROTECTION AGAINST SEVERE HYPERTHERMIA

This section establishes the pre-HS conditions required for a sufficient induction and accumulation of HSP in MNC which will protect normal protein synthesis from the inhibition caused by a severe stress.

MNC were prepared as described in Sections 2.2.2 and 2.2.3 and then the MNC were aliquotted into four groups. Three of the groups were exposed to a 45°C pre-HS for either

10, 20 or 30min, while a control group was exposed to 37°C for 30min. *(In the groups exposed to the 45°C pre-HS for10min, the MNC were exposed to 45°C for 10min and then transferred to 37°C for the remaining 20min to give a total preincubation time of 30min. Similarly MNC pre-HS for 20min were incubated for the remaining 10min at 37°C).* The MNC were then allowed to recover at 37°C for 3hrs. Each group was then further divided into four sub-groups. Each sub-group was exposed to a secondary HS of 45°C for either, 0min, 30, 40 or 50min *(When MNC were exposed to a 45°C secondary HS for 30min they were incubated at 45°C for 30min and then transferred to 37°C for the remaining 20min to give a total incubation of 50min. Similarly, MNC exposed to HS for 40min were incubated for the remaining 10min at 37°C).* The MNC cultures were then processed as described in Sections 2.2.5 to 2.2.8 and the resulting autoradiographs appear in Fig. 7.1.

Fig. 7.1 demonstrates that normal protein synthesis of control MNC (lanes 1 to 4, not exposed to a pre-HS) was increasingly inhibited as exposure to the secondary HS increased from 0 up to 50min. However, MNC exposed to a pre-HS of 45°C for 10min (lanes 5 to 8) demonstrated considerably higher levels of normal protein synthesis at all exposures to the secondary HS, with the greatest improvement after 50min. Likewise, MNC exposed to a 20min pre-HS (lanes 9 to 12) demonstrated similar levels of normal protein synthesis to those exposed to a 10min pre-HS. These results suggest that the amount of HSP, induced by a pre-HS of 10 or 20min and accumulated over 3hrs, prevented the inhibition of normal protein synthesis. MNC exposed to a pre-HS of 30min (lanes 13 to 16) demonstrated reduced amounts of normal protein synthesis at all exposures to the secondary HS, suggesting that the 30min pre-HS may have caused a considerable disruption of normal protein synthesis which did not recover even after 3hrs. Therefore, maximum protection of normal MNC protein synthesis, when induced by HS alone, was achieved after MNC were exposed to a pre-HS of 45°C for 10 to 20min.

7.3 HUMAN MONONUCLEAR CELLS EXPOSED TO A COMBINATION OF HYPERTHERMIA AND ANTI-INFLAMMATORY AGENTS DEMONSTRATE PROTECTION AGAINST SEVERE HYPERTHERMIA

Once again, as in Section 6.3, for practical reasons, the eight anti-inflammatory/ rheumatic agents used in this thesis, were divided into two equal groups and MNC were exposed to these agents in two separate experiments. Experiments consisted of MNC divided into groups, one group was used as the control, while the others were incubated with 4 different anti-inflammatory/rheumatic agents for 30min at 37°C. After this preincubation period, each of these groups were further divided into two groups and exposed to either 37°C or 42°C for 20min. The culture media with the anti-inflammatory/rheumatic agent was then removed and replaced (with media not supplemented with an agent) and then the MNC were incubated at 37°C for 5hrs. Finally, each of the ten cultures were then exposed to 37°C or a secondary HS of 45°C for either 30 or 50min. The MNC cultures were then processed as described in Sections 2.2.5 to 2.2.8 and the resulting autoradiographs appear in Figs. 7.2a to 7.2f. Aspirin, salicylate, paracetamol and indomethacin were used in the first experiment, while chloroquine, methotrexate, D-penicillamine and diclofenac were used in the second experiment.

Experiment One

Both the control MNC (Fig 7.2a lanes 1 and 4), whether preexposed to 37°C or 42°C, demonstrated equal amounts of normal protein synthesis. MNC exposed to 30min of secondary HS (lanes 2 and 5) demonstrated a considerable reduction in normal protein synthesis, and there was still no difference between the pretreated cells at 37°C and 42°C. After exposure to the secondary HS for 50min (lanes 3 and 6), normal protein synthesis in MNC pretreated at 37°C and 42°C, was almost completely inhibited. These results, suggest there was no protection of normal protein synthesis in MNC, which had been preexposed to 42°C for 20min. Note this was in contrast to the considerable protection obtained by pretreating MNC at 45°C for 20min (Section 7.2).

7.3.1 Aspirin

As for the untreated MNC, the aspirin-treated control MNC, preexposed at 37°C and 42°C (Fig. 7.2a lanes 7 and 10), demonstrated no difference in normal protein synthesis. Once again, as for the untreated MNC, normal protein synthesis was considerably reduced in aspirin treated MNC after 30min of secondary HS (lanes 8 and 11) and, was almost totally inhibited after 50min of secondary HS (lanes 9 and 12). These results suggest that pretreatment of MNC with aspirin, and a pre-HS, does not protect normal protein synthesis in MNC.

7.3.2 Salicylate

As for the untreated MNC, the salicylate-treated MNC, preexposed at 37°C and 42°C (Fig. 7.2b lanes 13 and 16), demonstrated no difference in normal protein synthesis. Once again, as for the untreated MNC, normal protein synthesis was considerably reduced in salicylate-treated MNC, preexposed to 37°C, after 30min of secondary HS (lane 14). However, MNC preexposed to 42°C (lane 17), demonstrated sustained levels of normal protein synthesis and expressed HSP strongly. After exposure to a 50min secondary HS, salicylate treated MNC preexposed to 37°C (lane 15) demonstrated almost total inhibition of normal protein synthesis, whereas MNC preexposed to 42°C (lane 18) demonstrated sustained levels of normal protein synthesis and a mild HSP expression. These results suggest that the combined effect of pretreating MNC with salicylate and preexposure to 42°C, protects normal protein synthesis from inhibition by a second severe HS.

7.3.3 Paracetamol

As for the untreated MNC (Fig. 7.2a, lanes 1 and 4), the paracetamol-treated MNC, preexposed at 37°C and 42°C (Fig. 7.2b lanes 19 and 22), demonstrated no difference in normal protein synthesis. Similarly, after 30min of secondary HS (lane 20), normal protein synthesis was considerably reduced in paracetamol-treated MNC, preexposed to 37°C, while MNC preexposed to 42°C (lane 23), demonstrated a highly sustained level of normal protein synthesis and a high amount of HSP expression. After exposure to a 50min secondary HS, paracetamol treated MNC preexposed to 37°C (lane 21) demonstrated almost total

inhibition of normal protein synthesis, whereas MNC preexposed to 42°C (lane 24) demonstrated a slightly sustained amount of normal protein synthesis as well as a small amount of HSP expression. These results suggest that the combined effect of pretreating MNC with paracetamol and preexposure to 42°C, protects normal protein synthesis from inhibition by a severe HS.

7.3.4 Indomethacin

Indomethacin-treated MNC, preexposed at 37°C (Fig. 7.2c lane 25), demonstrated no decrease in normal protein synthesis and no HSP expression whereas, the treated MNC preexposed to 42°C (lane 28) demonstrated a moderate decrease in normal protein synthesis as well as HSP expression. Once again, as for the untreated MNC, normal protein synthesis was equally reduced in indomethacin-treated MNC, preexposed to 37°C and 42°C, after 30min of secondary HS (lanes 26 and 29 respectively). However, MNC preexposed to 42°C (lane 29), demonstrated a higher level of HSP expression. After a 50min exposure to the secondary HS, indomethacin treated MNC preexposed to 37°C (lane 27) demonstrated almost total inhibition of normal protein synthesis: however, MNC preexposed to 42°C (lane 30) not only demonstrated HSP expression but also a slightly sustained level of normal protein synthesis. Therefore, although the indomethacin-pretreated control MNC, preexposed to 42°C, demonstrated an initial decrease in normal protein synthesis, indomethacin protected normal protein synthesis from inhibition when MNC were exposed to a 50min secondary HS.

Experiment Two

In experiment two, normal protein synthesis and HSP responses of untreated MNC were similar to those of the untreated MNC in experiment one. The untreated control MNC (Fig 7.2d lanes 31 and 34), whether preexposed to 37°C or to 42°C, demonstrated equal amounts of normal protein synthesis. MNC exposed to a 30min secondary HS (lanes 32 and 35) demonstrated a considerable reduction in normal protein synthesis, and no difference between the MNC pretreated at 37°C and 42°C. After exposure to a secondary HS for 50min (lanes 33 and 36), normal protein synthesis was almost completely inhibited. These

results, as in experiment one, suggest that there was no protection of normal protein synthesis in untreated MNC, preexposed to 42°C, from a severe HS.

7.3.5 Chloroquine

Unlike the untreated MNC, chloroquine-treated MNC, preexposed at 37°C (Fig. 7.2d, lane 37), demonstrated a marginal decrease in normal protein synthesis and MNC exposed to 42°C (lane 40) demonstrated a significant decrease in normal protein synthesis as well as an induced HSP expression. After a 30min secondary HS (lanes 38 and 41), normal protein synthesis, in chloroquine-treated MNC, was reduced below the levels of the untreated MNC exposed to a 30min secondary HS (lanes 32 and 35), except that chloroquine-treated MNC demonstrated a decreased HSP expression. Chloroquine-treated MNC exposed to a 50min secondary HS (lanes 39 and 42) demonstrated complete inhibition of normal protein synthesis and HSP expression, except for the MNC pretreated at 42°C (lane 42), which demonstrated only a faint HSP expression. Although HSP expression, in MNC preexposed to 42°C, survived after a 50min exposure to the secondary HS, normal protein synthesis was totally inhibited. Therefore, these results suggest that chloroquine does not appear to protect normal protein synthesis from a severe HS, in MNC preexposed to 42°C or to 37°C.

7.3.6 Methotrexate

Methotrexate treated MNC (Fig. 7.2e, lane 43) demonstrated a slight decrease in normal protein synthesis, while the MNC preexposed to 42°C (lane 46) demonstrated a significant decrease in normal protein synthesis. After a 30min exposure to the secondary HS (lanes 44 and 47) the decrease in normal protein synthesis and HSP expression in treated MNC was greater than the decrease in untreated MNC (Fig. 7.2d, lanes 32 and 35). After a 50min exposure to the secondary HS (lanes 45 and 48) methotrexate-treated MNC whether preexposed to 37°C or 42°C, demonstrated total protein synthesis inhibition. These results, therefore suggest, that methotrexate does not protect normal protein synthesis from inhibition by a severe HS of 45°C for 50min, in methotrexate-treated MNC preexposed to 37°C or to 42°C.

7.3.7 D-Penicillamine

Once again, as for the untreated MNC (Fig. 7.2d, lanes 31 and 34), the D-penicillamine-treated control MNC, preexposed at 37°C and 42°C (Fig. 7.2e lanes 49 and 54), demonstrated no difference in normal protein synthesis. Similarly, after 30min of secondary HS (lanes 50 and 53), normal protein synthesis was considerably reduced in D-penicillamine-treated MNC, preexposed to 37°C and 42°C. After exposure to a 50min secondary HS, D-penicillamine-treated MNC preexposed to 37°C (lane 51) demonstrated almost total inhibition of normal protein synthesis, whereas MNC preexposed to 42°C (lane 54) demonstrated a slightly higher sustained amount of normal protein synthesis as well as a small amount of HSP expression, in particular hsp70. These results suggest that the combined effect of pretreating MNC with D-penicillamine and preexposing to 42°C, protects normal protein synthesis from complete inhibition by a severe HS.

7.3.8 Diclofenac

MNC treated with diclofenac (Fig. 7.2f, lane 55) demonstrated a slight decrease in normal protein synthesis while MNC preexposed to 42°C (lane 58) demonstrated a severe decrease in normal protein synthesis and a clearly induced expression of HSP. MNC exposed to a 30min secondary HS (lanes 56 and 59) demonstrated a considerably greater reduction in normal protein synthesis, and also in HSP expression, than untreated MNC. After a 50min exposure to the secondary HS, diclofenac treated MNC (lane 57) demonstrated a complete inhibition of normal protein synthesis whereas, MNC exposed to 42°C (lane 60), although also demonstrating complete inhibition of normal protein synthesis, continued to express a small amount of HSP.

Because diclofenac treated MNC were not able to sustain normal protein synthesis above that of untreated MNC, these results therefore suggest that under the experimental conditions, diclofenac did not protect normal protein synthesis from inhibition by a severe HS.

7.4 DISCUSSION

In Chapter Five, diclofenac, indomethacin, paracetamol and salicylate were highly effective in facilitating HSP expression in MNC, whereas aspirin and D-penicillamine

produced only a mild facilitating effect. The effects of methotrexate and chloroquine were very weak or obscured by a decrease in normal protein synthesis. Therefore, the ability of these agents to protect normal protein synthesis from a severe HS, should be related to their ability to facilitate HSP expression in MNC which have been pretreated with the agents and then preexposed to a mild hyperthermia. This hypothesis was tested in Section 7.3. When MNC were preexposed to HS conditions alone, there was no improvement in the ability of normal protein synthesis to survive the most severe secondary HS (50min at 45°C). MNC pretreated with either paracetamol, salicylate, indomethacin or D-penicillamine, clearly demonstrated sustained levels of normal protein synthesis after a severe HS (50min of 45°C). The strongest protection was afforded by paracetamol and salicylate. Although indomethacin demonstrated an initial decrease in normal protein synthesis, when MNC were preexposed to the mild hyperthermia, it did sustain normal protein synthesis through the most severe HS. Whereas D-penicillamine had the smallest protective effect on normal protein synthesis, it had no significant influence on normal protein synthesis until after MNC were exposed to 50min of the secondary HS, when normal protein synthesis was sustained by only a small amount.

The results with diclofenac were unexpected as diclofenac had shown earlier (Section 5.2.3) to strongly facilitate HSP synthesis, yet in this section failed to protect normal protein synthesis. This may be explained by the inhibitory effect diclofenac has on normal protein synthesis during a more severe HS. As in Fig. 7.2f, diclofenac-treated MNC preexposed to 42°C (lane 58) and allowed to recover at 37°C for 5hrs, still demonstrated very low levels of normal protein synthesis before exposure to the secondary HS. The subsequent exposure to the secondary HS further inhibited the surviving normal protein synthesis giving the impression that diclofenac-facilitated HSP was insufficient to protect normal protein synthesis. To ascertain if diclofenac could still protect against a severe HS, milder hyperthermia conditions (e.g. 39°C or 40°C), which do not inhibit normal protein synthesis, to the same extent, may be more appropriate. Such conditions would be less inhibitory not only to normal protein synthesis but also to HSP expression therefore allowing more HSP to accumulate and then protection would be more likely to occur. For example, indomethacin like diclofenac, inhibited normal protein synthesis, but although normal protein synthesis was initially decreased, after preexposure to 42°C (Fig. 7.2c, lane

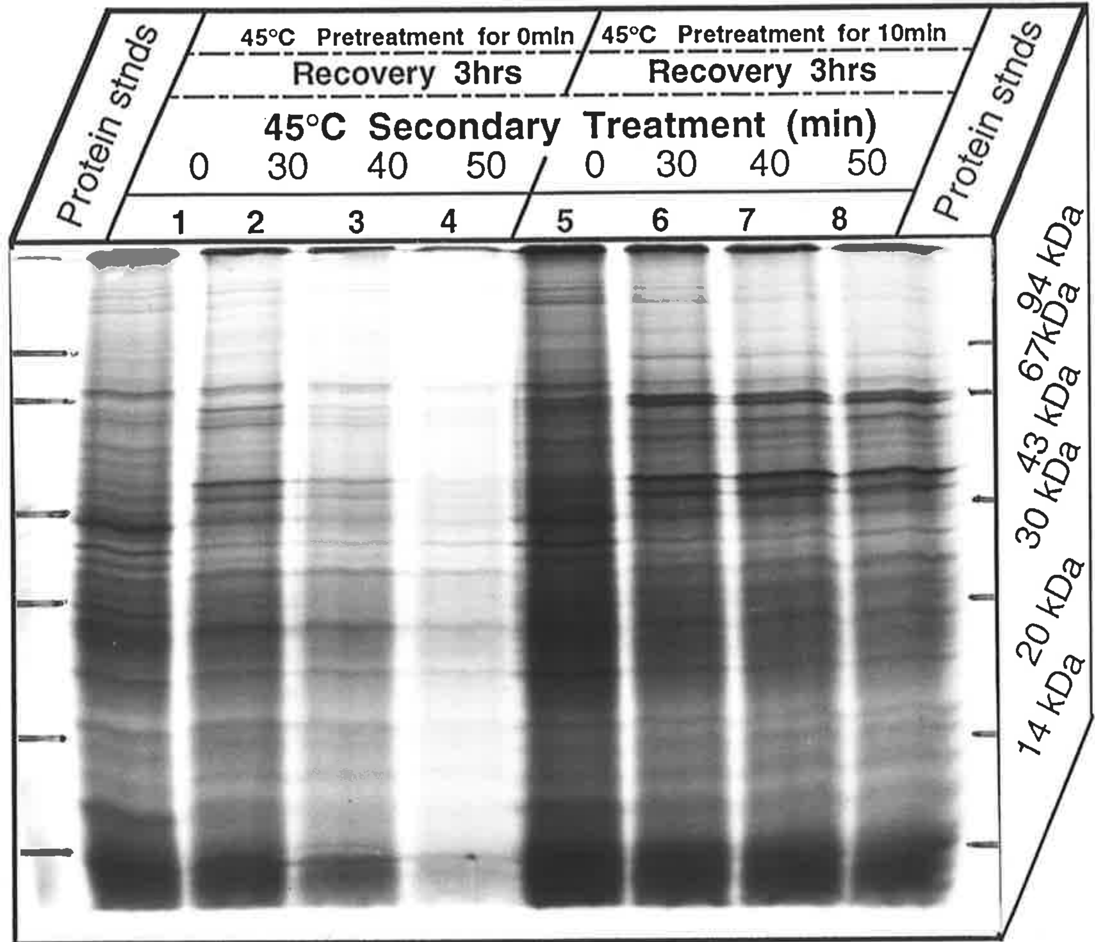
28), the inhibition was not as severe and therefore the MNC were in a better metabolic state to synthesise HSP and recover after 5hrs.

To summarise, the anti-inflammatory/rheumatic agents: paracetamol, salicylate, indomethacin and, D-penicillamine which also facilitated HSP expression in Chapter Five, protected normal protein synthesis when the MNC were first pretreated with an agent and then exposed to a pre-HS. Although diclofenac and aspirin were earlier shown to facilitate HSP expression (Chapter Five), they did not protect normal protein synthesis when MNC were pretreated with one of these agents for 30min before being exposing to 42°C for 20min.

FIGURE 7.1:

Protection of Normal Protein Synthesis by Pre-exposure to a Mild Heat Shock

MNC were preincubated at 37°C for 30min or exposed to 45°C for 10min and 37°C for 20min or, 45°C for 20min and 37°C for 10min or 45°C for 30min. The MNC were then incubated at 37°C for 3hrs before being incubated at 37°C for 50min or, 45°C for 30min and 37°C for 20min or, 45°C for 40min and 37°C for 10min or, 45°C for 50min. *De novo* MNC protein synthesis was then radiolabelled and analysed using SDS PAGE and autoradiography. Photographs of the autoradiographs appear on the following page.



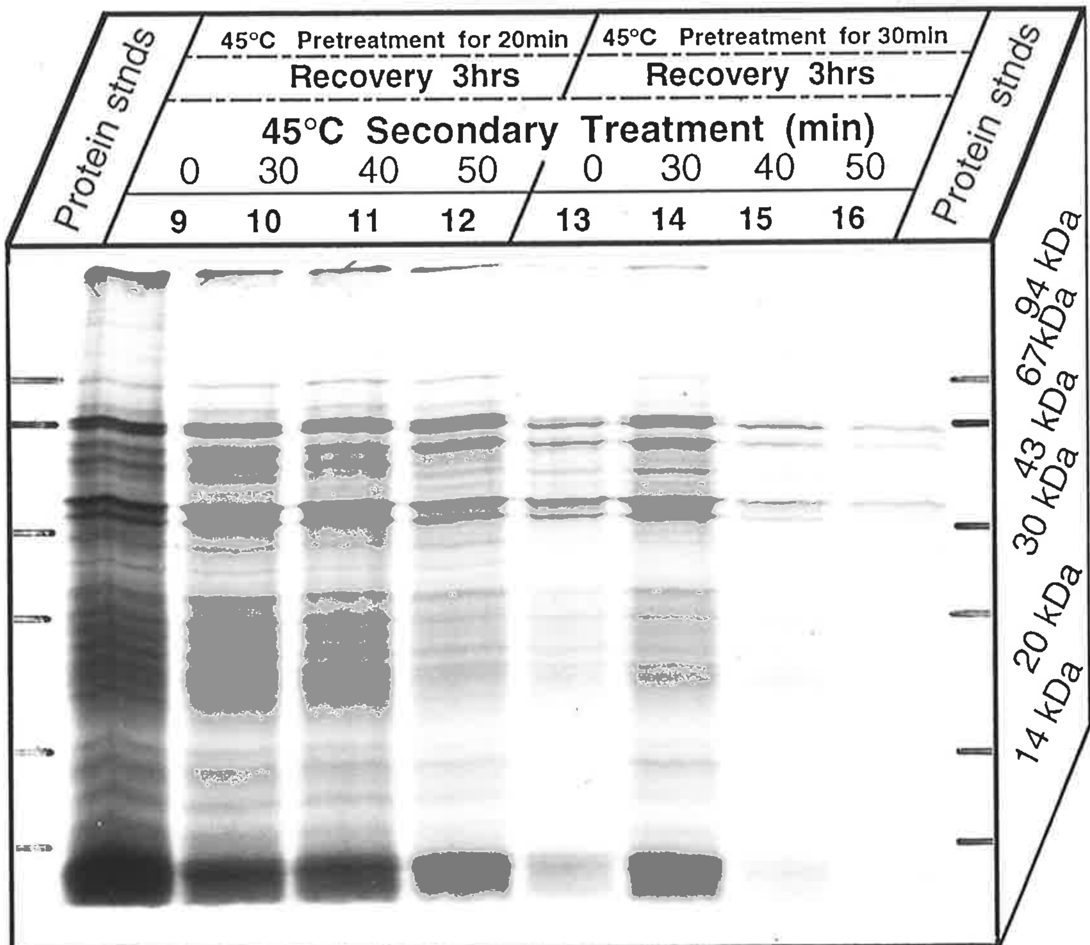
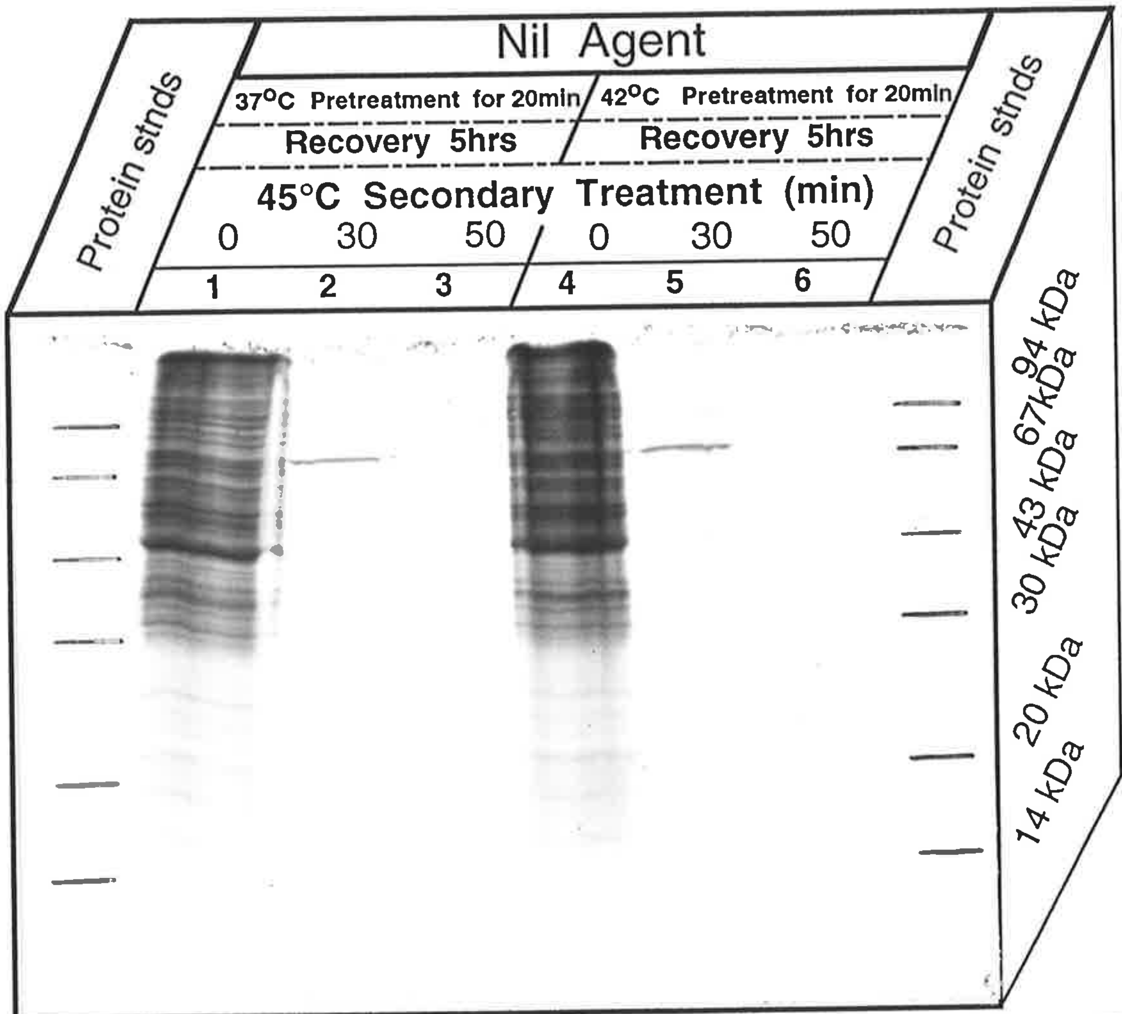


Figure 7.1

FIGURE 7.2:

Protection of Normal Protein Synthesis by Anti-Inflammatory/Rheumatic Agents

MNC were preincubated with or without an anti-inflammatory/ rheumatic agent for 30min, before being preexposed to 42°C for 20min or incubating at 37°C for the same length of time. All cultures were incubated at 37°C for 5hrs before being exposed to a more severe second HS at 45°C for 50min, 30min at 45°C and 37°C for 20min or incubated at 37°C for 50min. *De novo* MNC protein synthesis was radiolabelled and then analysed by SDS PAGE and autoradiography. Photographs of the autoradiographs appear on the following six pages of this figure.



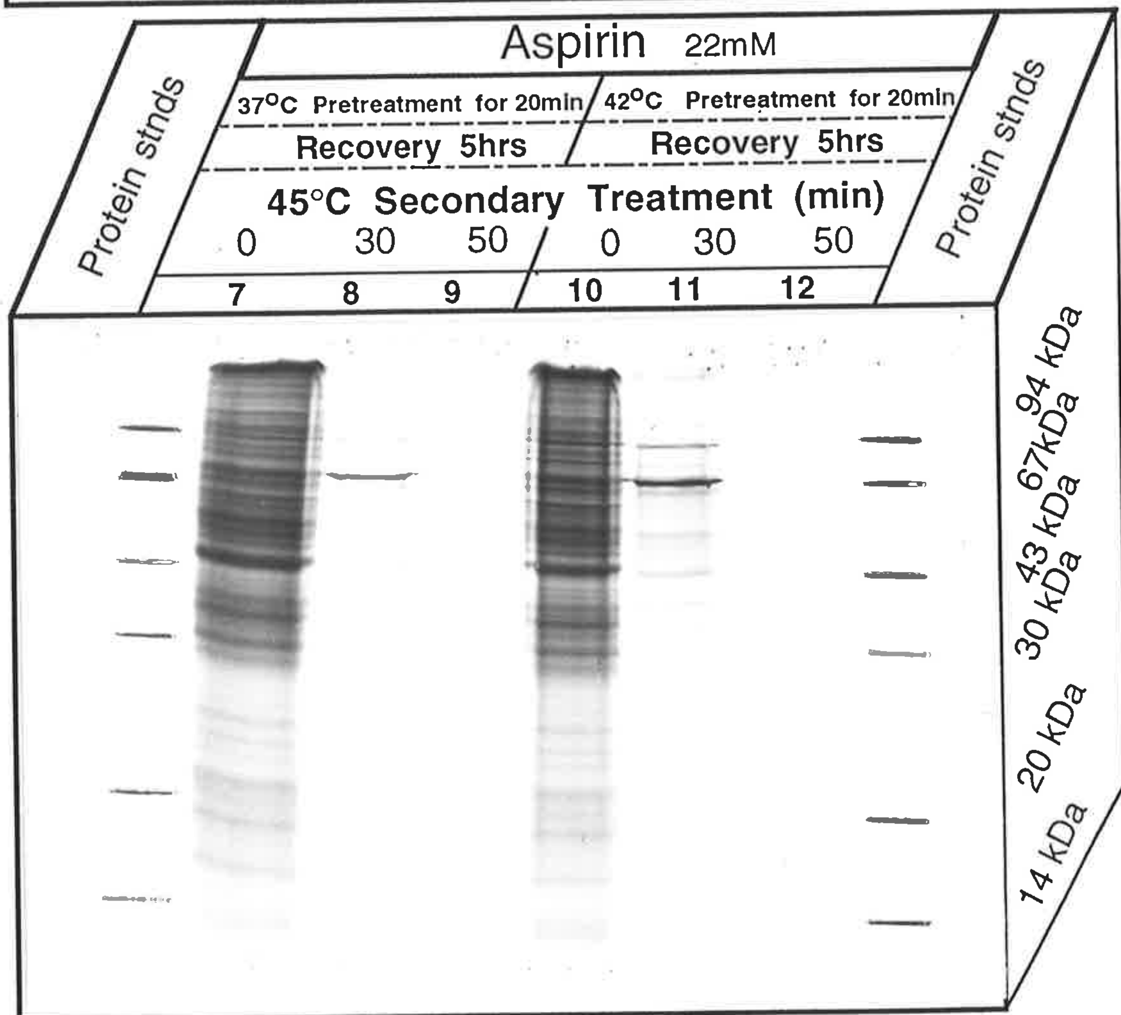
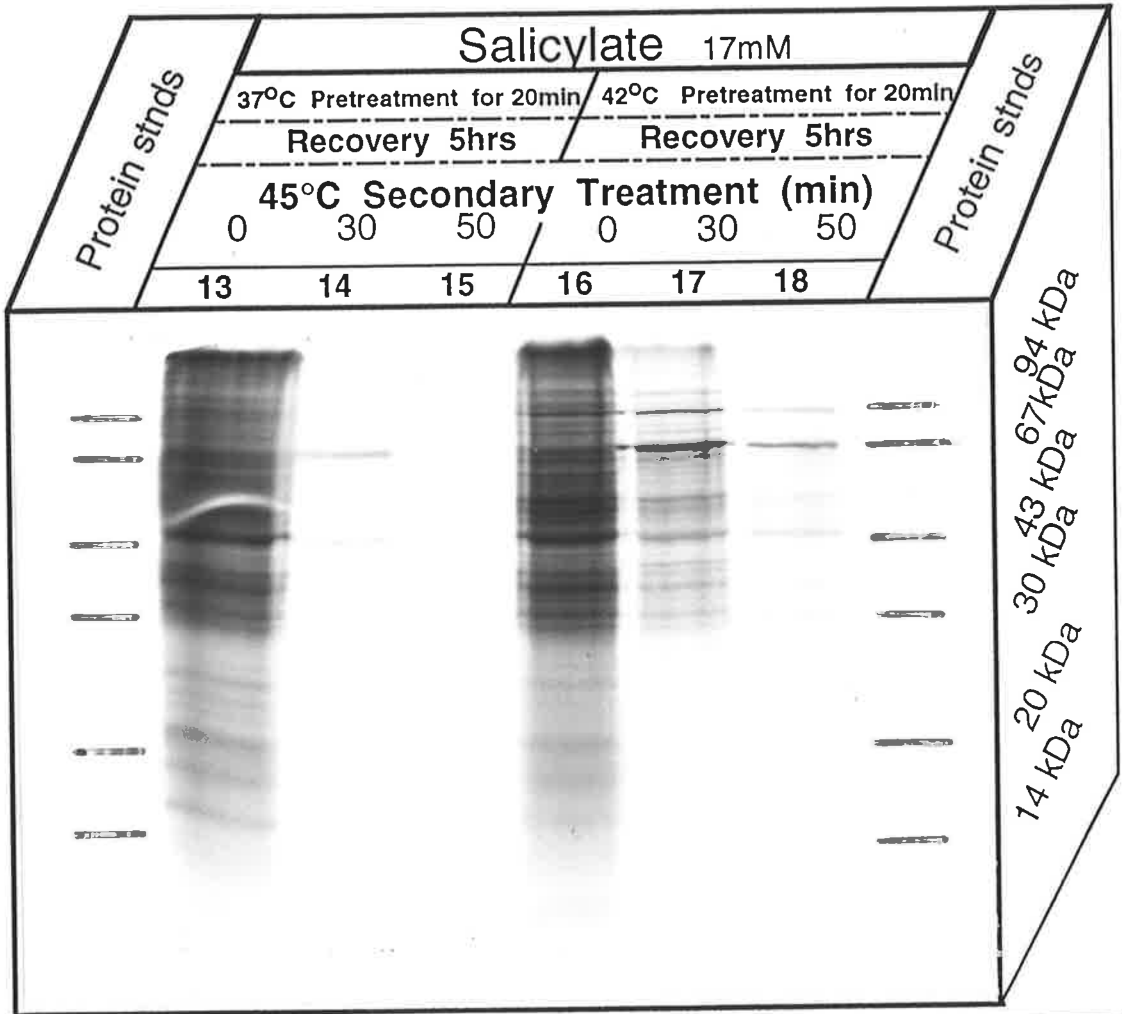


Figure 7.2a



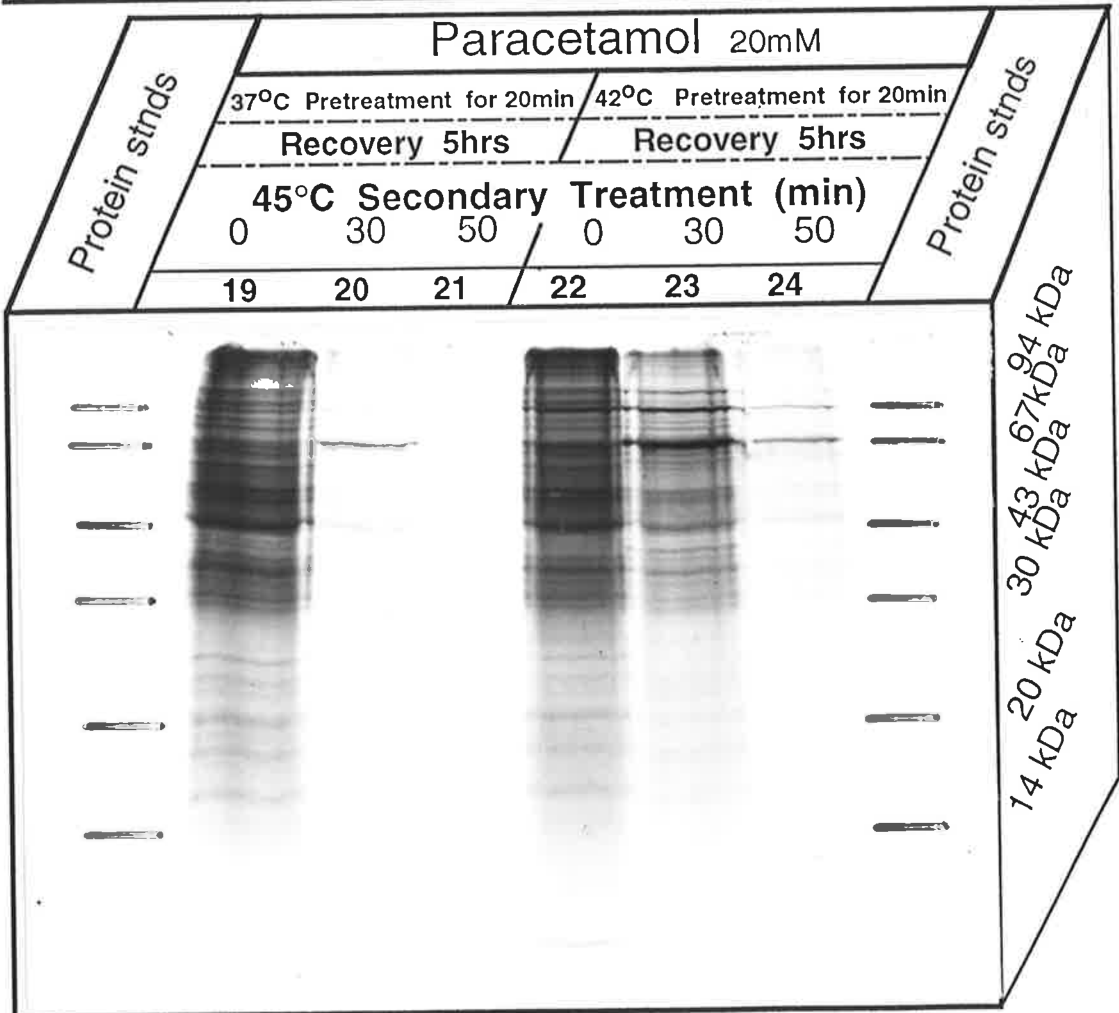


Figure 7.2b

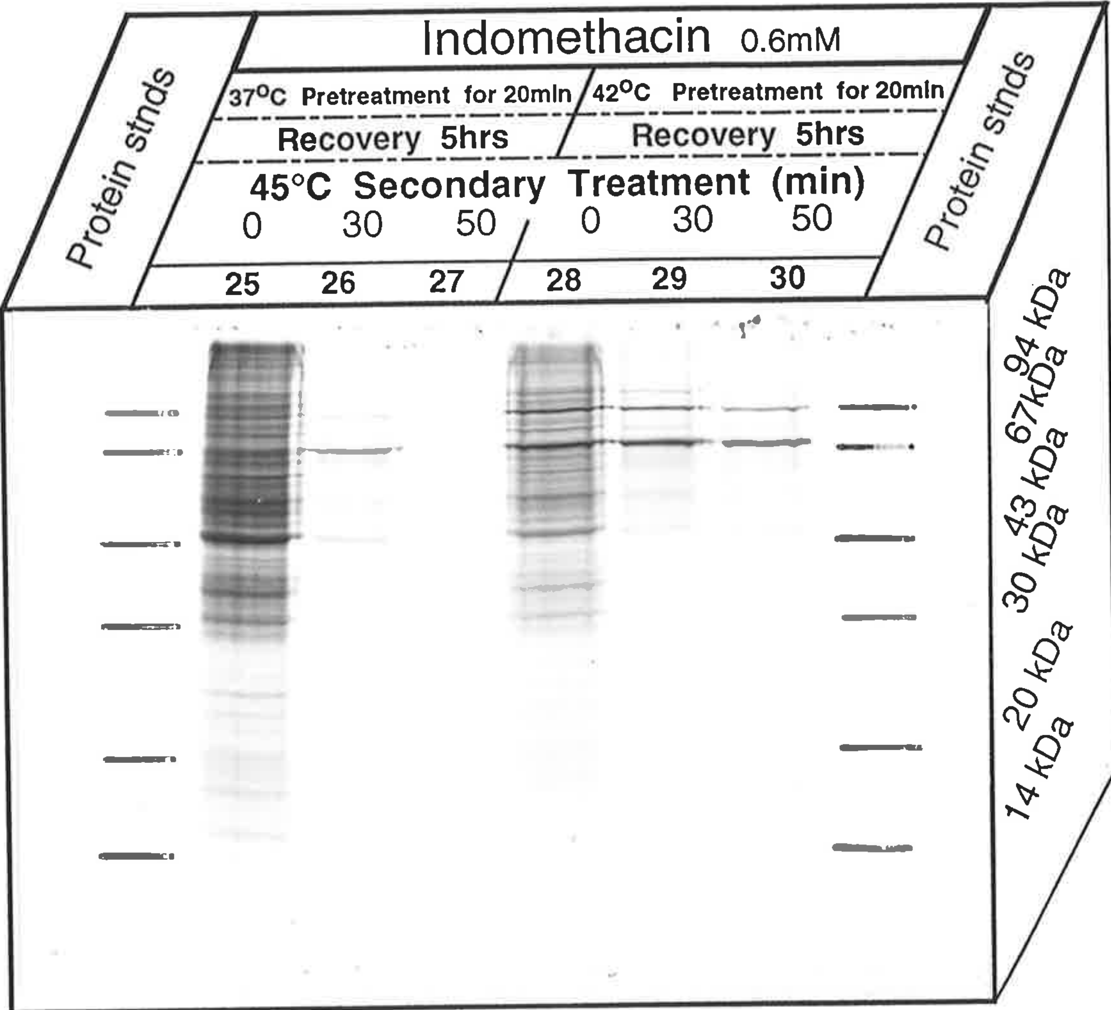
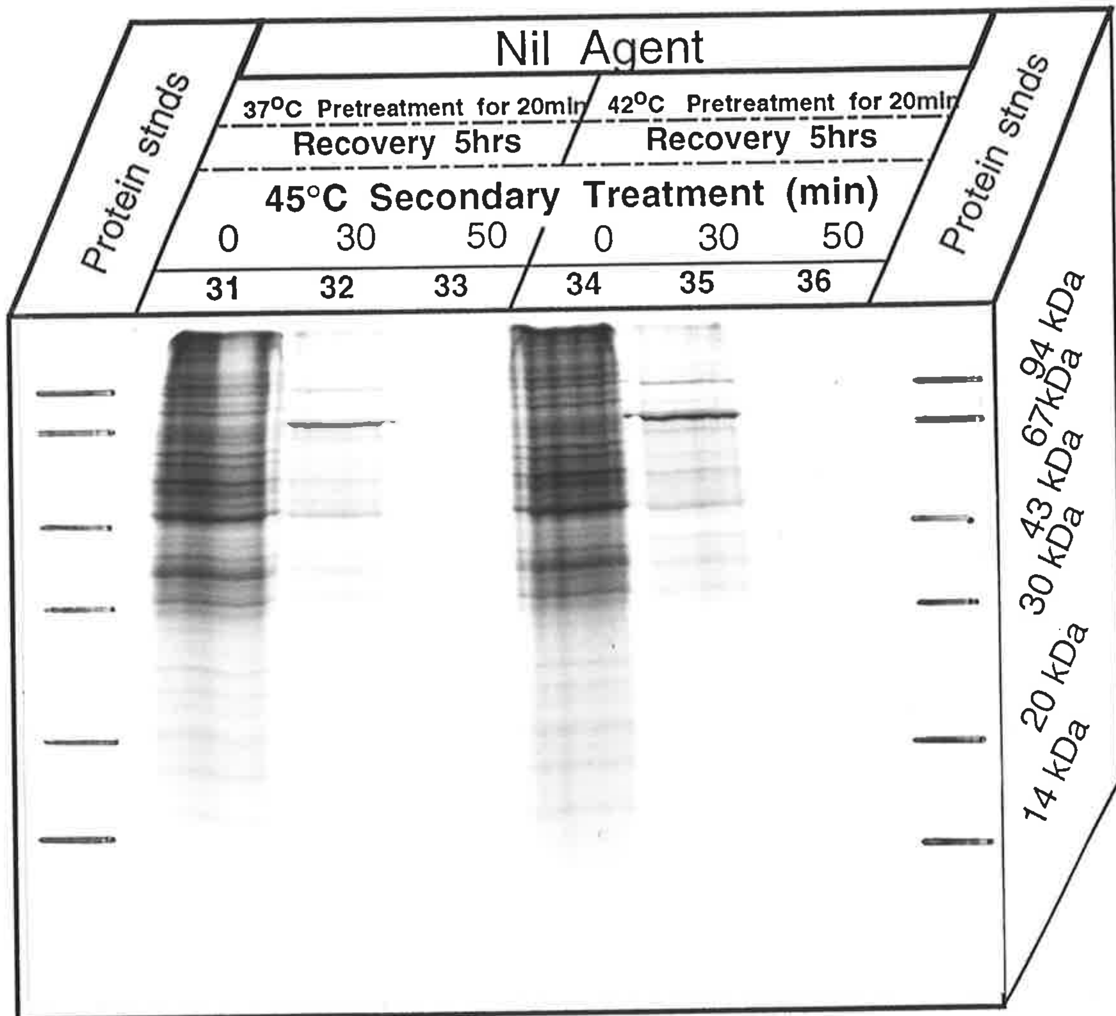


Figure 7.2c



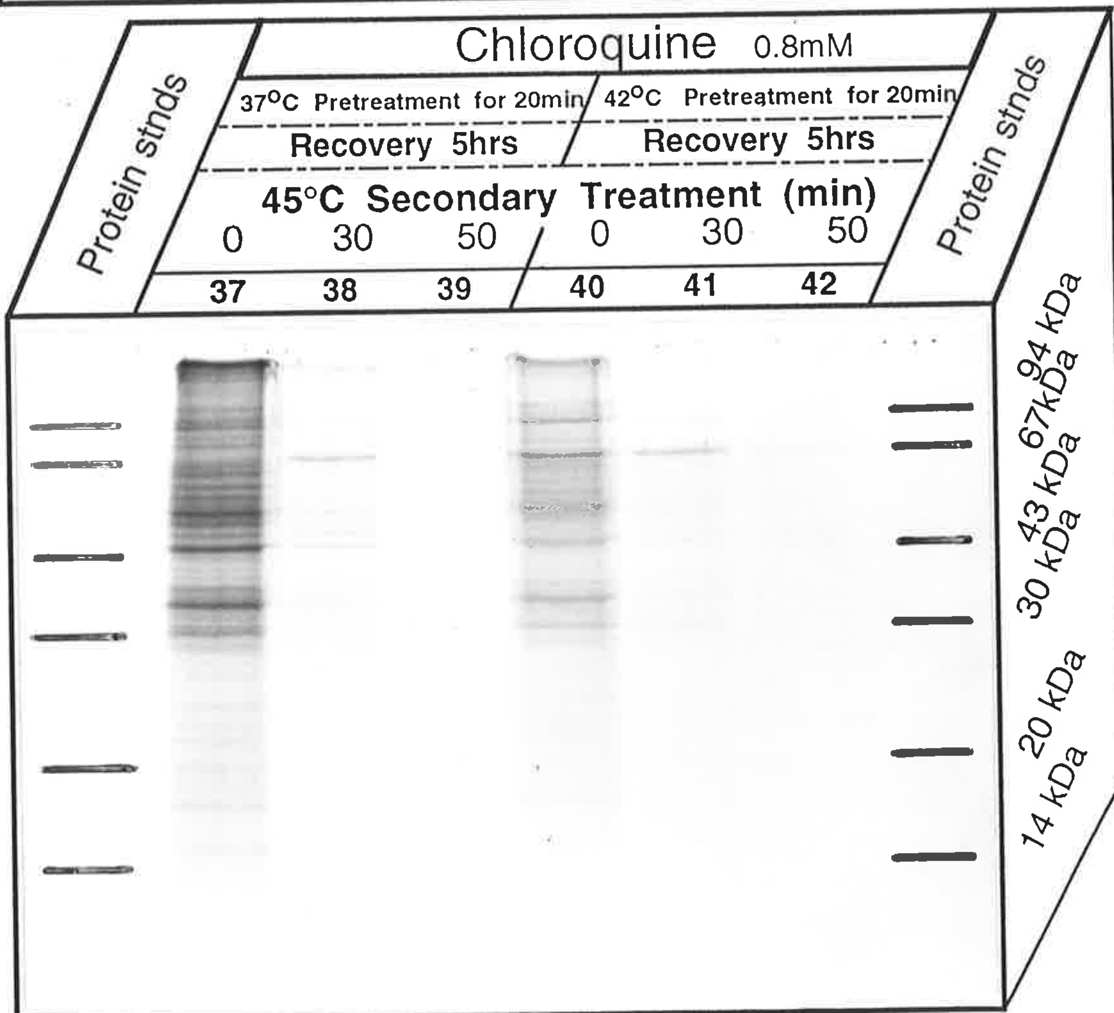
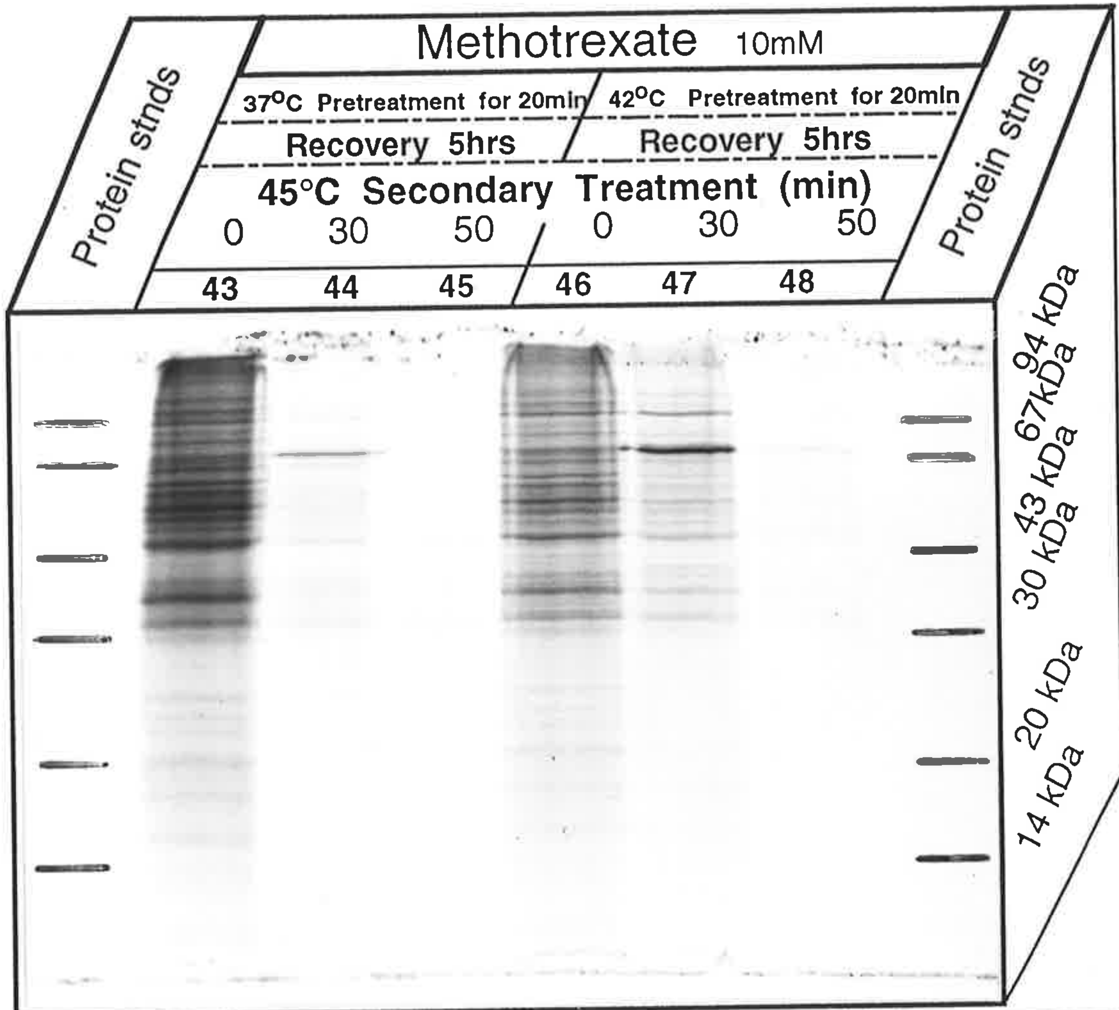


Figure 7.2d



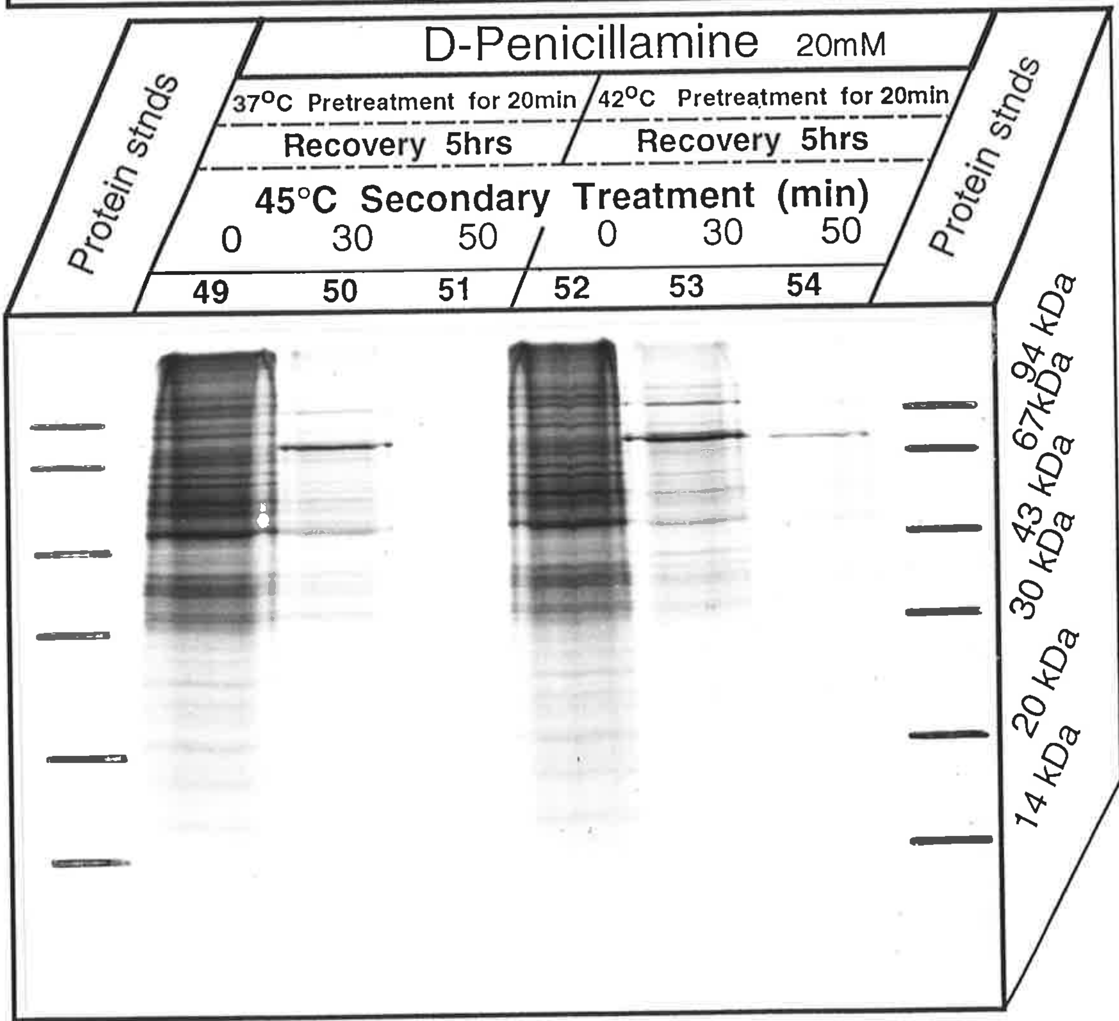


Figure 7.2e

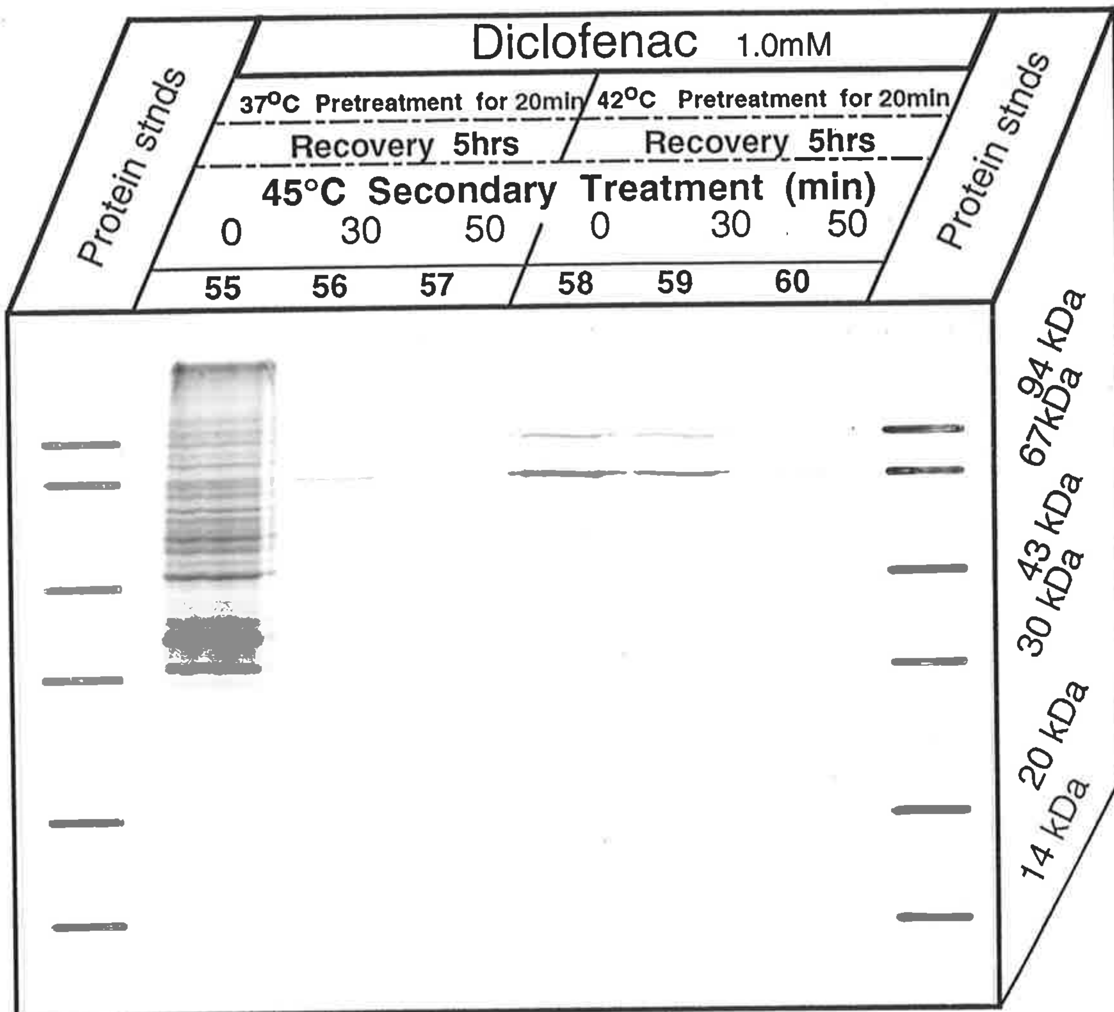


Figure 7.2f

CHAPTER EIGHT

Discussion of Results & Future Prospects

8.1 SUMMARY OF RESULTS

In Chapter Three, HSP expression was induced by hyperthermia and characterised in MNC. MNC demonstrated no induction of HSP expression at low temperatures (up to 40°C/30min), a strong induction at moderate temperatures (42°C to 43°C/30min) and reduced HSP expression at higher temperatures (>44°C/30min). Normal protein synthesis started to decrease when MNC were exposed to temperatures of 43°C/30min or higher.

HSP synthesis also increased with increasing HS duration. At the moderate temperature of 42°C, HSP synthesis continued to increase for up to an hour of exposure, while normal protein synthesis remained constant. Thereafter, HSP synthesis and normal protein synthesis progressively decreased.

Hsp70, which was the most prominent HSP, was identified on Western blots of whole MNC lysates separated by SDS PAGE, using an hsp70/hsc70 specific monoclonal antibody. The position of hsp70 on autoradiographs was identified relative to the other MNC proteins and the molecular weight protein standards.

In Chapter Four, salicylate was used to investigate its effects on HSP synthesis in MNC. Salicylate alone had no effect on HSP synthesis. However, when MNC were pretreated with salicylate and exposed to a mild HS, HSP synthesis occurred at temperatures which alone did not induce HSP expression. HSP expression was further increased at temperatures which already induced HSP but at more severe temperatures (43°C), salicylate inhibited normal protein synthesis as well as HSP synthesis. These results suggested that although salicylate alone did not induce HSP expression, it facilitated HSP expression by lowering the temperature threshold of HSP expression and also the temperature at which HS inhibits normal protein synthesis.

In light of the results with salicylate, other anti-inflammatory/rheumatic agents were then considered. The concentrations of aspirin, diclofenac, chloroquine, indomethacin, methotrexate, paracetamol and D-penicillamine were optimised for HSP induction in pretreated MNC subsequently exposed to a mild HS.

In Chapter Five the selected anti-inflammatory/rheumatic agents were investigated for their ability to facilitate HSP expression in pretreated MNC when exposed to different HS temperatures. Of the selected agents, diclofenac, indomethacin, salicylate and paracetamol demonstrated good facilitation of HSP synthesis.

In Chapter Six, the effects of the anti-inflammatory/rheumatic agents on normal protein synthesis were investigated to determine whether or not the concentrations of each agent were interfering with MNC metabolism by inhibiting normal protein synthesis. With all agents, except chloroquine and to a lesser extent methotrexate, there was no evidence that normal protein synthesis in MNC was affected by any of the other agents. Chloroquine-treated MNC demonstrated a significant reduction in normal protein synthesis when incubated at 37°C and inhibition increased further as the duration of exposure was increased or when the MNC were incubated at higher temperatures.

In Chapter Seven, MNC were pretreated with one of the anti-inflammatory/ rheumatic agents before exposure to a mild HS which alone did not induce HSP expression. These MNC were allowed to recover and synthesise HSP before being exposed to a severe HS which would normally inhibit normal protein synthesis in non-preheat shocked MNC. The combined pretreatment of MNC with an agent and subsequent exposure to HS, demonstrated protection of normal protein synthesis in MNC pretreated with either paracetamol, salicylate, indomethacin or D-penicillamine, but not by aspirin, chloroquine, methotrexate or diclofenac.

8.2 DISCUSSION OF INTERESTING OBSERVATIONS

In Section 3.2.1 an observation was made that one of the HSP induced by HS (Fig. 3.1a, protein band labelled 'd'), with an apparent molecular weight between 70 and 90kDa was absent from the results presented in Sections 3.2.2 and 3.2.3 under apparently similar conditions. As discussed in Section 3.3.2, Polla (1990) also reported a 84kDa stress protein induced by opsonised sheep red blood cells. However in this thesis, this protein was found to be one of the rarely induced HSP. The only other experiment to also demonstrate the clear induction of this protein, along with other more strongly induced HSP, is described in Section 5.2.4 and appears in Fig. 5.1. Why this protein was not more frequently induced after HS, or HS combined with an agent, was not investigated in this thesis. It can only be assumed that this protein was not always required after MNC had been exposed to apparently similar stress conditions. One possible explanation for this situation may involve other HSP, functionally substituting for some of, or all the functions of this protein, which would be a similar situation to that reported by Sanchez *et al.* (1993).

Sanchez *et al.* (1993) discovered that under stress conditions, thermotolerance in a *hsp70* mutant strain of *S. cerevisiae* was not reduced even though growth rates were effected. While strains mutant in *hsp104* showed a significant decrease in their ability to survive short exposures to extreme temperatures, but their growth rate was unaffected. Hence, in the absence of hsp104, hsp70 was very important for thermotolerance and Sanchez *et al.* (1993) concluded that a close functional relationship existed between the two HSP.

Similarly, other HSP induced by hyperthermia in Sections 3.2.1 to 3.2.3 were not always observed after every HS or after exposure to a combination of HS and anti-inflammatory/rheumatic agent. However, hsp70 acted as a good marker for HSP induction, as it was always the first HSP to be detected and the most abundantly synthesised. The variation in the number of different HSP induced in different experiments in subsequent chapters may be attributed to the reduced severity of HS, particularly when used in combination with anti-inflammatory/rheumatic agents .

In Chapters Four and Five (Sections 4.2 and 5.2.4) normal protein synthesis of MNC treated with the anti-inflammatory agents salicylate and indomethacin was inhibited at higher temperatures (41°C, 43°C). In comparison untreated MNC, need to be incubated at temperatures at least 2°C higher to achieve similar inhibitions of normal protein synthesis. This effect may be of significant importance with respect to Reye's Syndrome - a potentially lethal disorder which usually affects young children, suffering from fever, who have been treated with aspirin. This condition rarely occurs in adults, who tend not to attain the high temperatures seen in children with fever (Starko *et al.* 1980; Waldman *et al.* 1982; Halpin *et al.* 1982; Rennebohm *et al.* 1985). The pathogenesis of Reye's Syndrome includes damage to the liver as well as other parts of the body. This may be rationalised in the following manner:- Aspirin is metabolised by the liver producing salicylate, which in combination with fever acts to inhibit normal protein synthesis in liver cells. Inhibited protein synthesis may lead to cell death and liver damage if it continues for prolonged periods. However, there are no reported cases of Reye's Syndrome occurring in young children treated with indomethacin. This may be due to the limited use of indomethacin for treating young children. Furthermore, not all children treated with aspirin develop Reye's Syndrome. Further experiments, in animal models, will help to elucidate the

mechanism by which aspirin induces Reye's Syndrome and perhaps determine if indomethacin can also induce Reye's Syndrome.

Results with diclofenac-treated MNC often demonstrated increased levels of *de novo* normal protein synthesis (i.e. lanes containing diclofenac-treated samples were generally darker on autoradiographs). This effect was not observed with any of the other agents used in this thesis. As in Section 5.2.3 (Fig. 5.4), these effects were usually observed with diclofenac at 37°C and lower HS temperatures (39°C) but disappeared at higher temperatures (≥41°C). This situation did not seem to effect HSP induction. At 39°C diclofenac not only increased normal protein synthesis, but also facilitated HSP expression. This effect may be of benefit in rheumatoid arthritis (RA) as diclofenac can facilitate HSP expression to protect cells in the joint and at the same time increase the rate of protein synthesis to aid tissue regeneration and healing.

This thesis demonstrates two important points about anti-inflammatory/ rheumatic agents. One is that some agents can help facilitate HSP expression in combination with mild hyperthermia and the other, that normal protein synthesis in MNC can be protected from inhibition by a severe HS when MNC are pretreated with a combination of an agent and mild hyperthermia, but not when treated with either the agent or mild hyperthermia alone. Similarly, Ciavarra and Simeone (1990a) found that T cells preconditioned with a HS were capable of higher rates of protein synthesis, when subsequently challenged by a severe HS. Studies where HSP are induced by a combination of agents are rare. In a study by Fisher *et al.* (1986) hydrocortisone, a glucocorticoid used in the treatment of inflammation, was used in combination with hyperthermia and shown to facilitate hsp28 expression. However, hydrocortisone and other steroid hormones when used at physiological concentrations are able to induce HSP synthesis by themselves, whereas this thesis was using agents which alone did not demonstrate HSP induction. Only diclofenac showed an ability to induce HSP synthesis without a subsequent HS (Section 6.2.3, Fig. 6.1a), but only after an extended preincubation period of 1.5hrs.

8.3 ADVANTAGES AND LIMITATIONS OF THESE STUDIES

This thesis used *in vitro* cultured human peripheral blood MNC to study the effects of anti-inflammatory/rheumatic agents on HSP expression. *In vitro* cultures have certain advantages over *in vivo* MNC exposed to anti-inflammatory/rheumatic agents. *In vitro* cultures eliminate variations in HSP expression such as elevated body temperatures induced by exercise and changes in diet (e.g. vitamin, mineral, preservative, etc., supplemented foods). A better control over anti-inflammatory/rheumatic agent concentrations is also possible, as the culture medium does not include serum proteins which may bind to agents and reduce their effectiveness. In addition, variations between individuals, with respect to drug metabolism and clearance, can lead to variations in *in vivo* drug concentrations and exposure duration.

However, this *in vitro* system also has its limitations. For example, in some cases the metabolites rather than the parent drug possess therapeutic properties and *in vitro* cultures do not have the capacity to generate all, if any of the metabolites. It is also difficult to determine if therapeutic intracellular concentrations of the anti-inflammatory/rheumatic agent are achieved in these experiments. In most cases these are not even known. Furthermore, the intracellular concentrations depend on the physiochemical properties of an agent (lipophilic, lipophobic, water solubility, etc.) which in turn determine its diffusion rate across the cellular membranes. The diffusion rate of the selected anti-inflammatory/rheumatic agents may be an important factor in this thesis due to the short preincubation periods used. The final intracellular concentration achieved after the preincubation periods may have been lower than the extracellular concentration. French *et al.* (1987) from our laboratory, found this to be the case with the rate of chloroquine and hydroxychloroquine uptake into human peripheral MNC. The uptake of chloroquine and hydroxychloroquine was found to be time and dose dependent. Cells incubated with either 0.1mM chloroquine or hydroxychloroquine for 60min only accumulated intracellular levels comparable to those achieved during antirheumatic therapy. Therefore the effective intracellular concentrations of the agents used in this thesis may more closely resemble therapeutic levels. However, further studies are needed to investigate this possibility.

8.4 RELEVANCE OF THESE STUDIES TO THE INFLAMMATORY PROCESS AND OTHER MEDICAL CONDITIONS

Based on experimental studies showing mycobacterial hsp65-reactive T cells in the joints and the peripheral blood of RA sufferers, which have the potential to react with an autoantigen, some investigators have proposed that HSP may be the targets for these T cells. However, other studies do not fully support this theory. Hence the involvement of HSP in the pathogenesis of RA still remains unresolved. Generally these studies showed that healthy individuals also have cytotoxic T lymphocytes reactive towards BCG (bacillus Calmette-Guerin) and mycobacterial hsp65 as discussed in Section 1.4.2 (Ottenhoff *et al.* 1988; Kaleab *et al.* 1990a, 1990b; Munk *et al.* 1989; Kumararatne *et al.* 1990; Res *et al.* 1990). To further complicate the issue, the anti-rheumatic gold salts were also shown to induce HSP synthesis in human cells. Caltabiano *et al.* (1986; 1988) showed that auranofin and the other gold salts induced several HSP/SP after mammalian cells were incubated with these agents. *(Caltabiano et al. (1988) also tried other anti-rheumatic agents including indomethacin, penicillamine and chloroquine but these agents did not induce HSP in their experiments).* These findings therefore raise the question:- if HSP really contribute to the pathogenesis of RA then, why do some agents in the treatment of RA also induce HSP synthesis?

My work was based on the facts that HSP are essential components of, and beneficial to all cells, whether they are under stress or not. Therefore, an increased expression of HSP by the cells located in the RA joint, would help them survive and function better in the presence of the stresses associated with RA (as described in Sections 1.4.1 and 1.4.2).

Osteocytes, chondrocytes and synovial cells are important cell types in the joint. Osteocytes are responsible for the synthesis of organic bone matrix which subsequently is impregnated with calcium to become bone. Chondrocytes are responsible for synthesising the components of cartilage. Chondrocytes sometimes absorb cartilage by releasing proteolytic enzymes. Stressed osteocytes and chondrocytes result in reduced or abnormal bone and cartilage formation, which results in deformities and contributes to the excessive wear of the joint. Likewise, stress also effects the synovial cells that make up the synovial

membrane, which encapsulates the joint. Various stresses that develop during chronic inflammation result in abnormal growth of the synovial membrane and may contribute to the formation of pannus.

Furthermore, white blood cells (WBC) which enter RA-affected joints are also influenced by the stresses in the joint. Their ability to respond appropriately and resolve the initiating stimulus will be affected by those stresses. Therefore WBC primed for HSP induction by anti-inflammatory/rheumatic agents in the blood stream should be better prepared and express HSP sooner and in greater amounts than unprimed WBC.

The use of agents such as the anti-inflammatories/rheumatics to facilitate HSP synthesis would bolster the body's own systems responsible for preventing damage. Although anti-inflammatory/rheumatic agents also act in other ways to control the inflammatory process, an agent which also facilitates HSP synthesis, may also play a duel role by stemming the release of more pro-inflammatory compounds (e.g. cytokines, oxy radicals, etc.) by stressed cells as well as protecting these cells. Furthermore, developing new therapeutic agents, which significantly increase HSP expression, and which can be safely taken by patients along with the current anti-inflammatory/rheumatic agents, may provide a better method of treating RA. Also, the detrimental side effects of anti-inflammatory/rheumatic agents on the gastrointestinal system could be reduced by substituting some of the anti-inflammatory medications with the HSP inducers/ facilitators.

Therapeutic agents, specifically designed for facilitating HSP expression, may also have applications in other areas of medicine. For example, in the treatment of ischaemia of the central nervous system, or ischaemia and reperfusion injury to the heart after myocardial infarction or organ transplantation. Aoki *et al.* (1993) demonstrated that gerbil hippocampus cells preconditioned for a more rapid expression of hsp70 had a improved survival frequency after transient ischaemia compared to control cells. While Simon *et al.* (1993) demonstrated that preinduction of hsp70 in rat brains protects against ischaemia induced by an experimentally induced stroke. Donnelly *et al.* (1992) and Maulik *et al.* (1993) independently demonstrated that preinduced HSP synthesis in rat hearts protected them from damage by reperfusion after experimentally induced ischaemia. The difference being that Maulik *et al.* (1993) used an agent (IL-1) to induce HSP and superoxide dismu-

tase while Donnelly *et al.* (1992) used HS. In the case of ischaemia of the central nervous system or the heart, the use of drugs to facilitate protection by HSP would be of little use due to the instantaneous nature of the events, but in organ transplantation, would be very effective since most of the damage only occurs on reperfusion. Preinducing protective levels of HSP in an organ after excision but prior to transportation would increase organ survival and recovery, and possibly reduce the likelihood of rejection (Liu *et al.* 1992; Schumer *et al.* 1992).

Some findings of this thesis are also relevant to cancer therapy. The ability of anti-inflammatory agents, such as salicylate, to lower the temperature at which HS inhibits normal protein synthesis may be of value. By administering a therapeutic concentration of salicylate to cancer patients and using localised heat treatment, total protein synthesis may be inhibited more efficiently and for long enough to kill the tumour cells. This situation may be similar to Reye's Syndrome where aspirin combined with fever are associated with liver damage. Another possibility would be to use or develop HSP inhibitors, rather than HSP facilitators, to selectively inhibit the induction of HSP. Inhibitors of HSP synthesis, like the flavonoids, are already known. Hosokawa *et al.* (1990) reported flavonoids, such as quercetin, flavone, kaempferol and genistein, inhibit a range of HSP induced by sodium arsenite in human cell lines. Quercetin, in particular, was shown to inhibit hsp70 mRNA accumulation in stressed cells while in unstressed cells further reducing the already low levels of hsp70 mRNA without affecting non HSP mRNA (Hosokawa *et al.* 1990). In a later study, Hosokawa *et al.* (1992) showed that quercetin prevented hsp70 induction by preventing the activation of HSF binding to the HSE. Also using HSP inhibitors, Mitchell and Russo (1983) demonstrated the combined use of the thiol cysteine and heat treatment induced thermosensitisation rather than thermotolerance, making cells sensitive to damage by a subsequent HS. Thus, by reducing the ability of tumour cells to defend themselves against chemotherapeutic agents or heat treatment, HSP inhibitors may be a valuable tool in cancer therapy.

Although the mechanism is not known, studies have also shown that increasing HSP expression in tumour cells can make them more recognisable and therefore susceptible to destruction by the immune system. For example, Lukacs *et al.* (1993) demonstrated that tumour cells transfected with the mycobacterial hsp65 lost their ability to induce

tumours when implanted in mice. Similarly, Giardiello *et al.* (1993) demonstrated that sulindac (an anti-inflammatory agent) has the ability to reduce the size of colonic and rectal adenomas. Similarly, inducing or facilitating HSP expression in tumour cells (with anti-inflammatory agents) may provide another method for treating cancer.

8.5 FUTURE PROSPECTS

This thesis establishes the following: that HSP synthesis can be facilitated by some anti-inflammatory/rheumatic agents and, that the combined exposure of MNC to an anti-inflammatory/rheumatic agent and hyperthermia can protect MNC from a severe stress. The next stage will require an investigation of the effects of anti-inflammatory/rheumatic agents *in vivo*. The rat adjuvant arthritis (AA) model may provide an ideal system to study the agents which were shown to facilitate HSP synthesis *in vitro*. Several initial experiments would need to be performed using in vitro cultured rat MNC to determine if the same results can be achieved in rat MNC. From there, anti-inflammatory/rheumatic agent doses administered to rats would need to be optimised for the facilitation of HSP in healthy joints. This may involve administering an anti-inflammatory/rheumatic agent to the rat for a period of time, sacrificing the rat to remove the joint tissues or isolate MNC from the rat. These tissues or MNC could then be exposed to a mild hyperthermia (*ex vivo*) which normally would not induce HSP expression in tissues from untreated rats. The objective would be to demonstrate HSP facilitation in joint tissues and/or in MNC from agent treated rats but not untreated rats. After the facilitation of HSP expression by the combined treatment with an agent and hyperthermia has been demonstrated, the same doses of each anti-inflammatory/rheumatic agent could then be administered to rats pre and post AA induction. The facilitated expression of HSP should prevent or delay the development or, reduce the severity of AA in these rats. While rats with already established AA, would be expected to show a significant recovery of the inflamed joints after an anti-inflammatory/rheumatic agent is administered.

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