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Characterization Of Heat-shock And Chemical-stress Induced Changes In The Gene Expression Of Mammalian Lymphocytes

David Ivan Rodenhiser

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CHARACTERIZATION OF HEAT-SHOCK AND CHEMICAL-STRESS INDUCED CHANGES IN THE GENE EXPRESSION OF MAMMALIAN LYMPHOCYTES

by

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of the requirements for the degree of
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ABSTRACT

To investigate the effect of heat-shock (HS) on the gene expression of mammalian blood cells, human, mouse and rabbit blood cells were cultured and incubated at elevated temperatures or in the presence of ethanol or sodium arsenite, and the patterns of polypeptides synthesized by these cells analyzed by one- and two-dimensional polyacrylamide gel electrophoresis (PAGE). The *in vitro* exposure of human lymphocytes or human myeloma cells to short term increases in incubation temperature (41°- 43°C) results in the enhanced synthesis of heat-shock proteins (HSPs) of 110, 100, 90, 70 and 65 kilodaltons, and the depressed synthesis of many polypeptides normally synthesized at a control (37°C) temperature. This response is dependent on the duration and severity of the HS. Heat-shocked mouse spleen cells, and mouse and rabbit peripheral blood lymphocytes, synthesize HSPs with molecular masses and isoelectric points similar to those synthesized by human lymphocytes. HSP synthesis by mouse spleen cells is transcription dependent, and is a transient event, since a gradual recovery of normal patterns of protein synthesis is observed following HS. Spleen cells from mice exposed to whole-body thermal stress also synthesize HSPs comparable to those synthesized by similar cells heat-shocked *in vitro*.

Cultured mouse spleen cells treated with arsenite or ethanol exhibit new and/or enhanced synthesis of most of the HSPs, but do not exhibit enhanced synthesis of the 100 kDa HSP. Short-term concurrent exposure of mouse lymphocytes to HS and a level of ethanol, which individually do not induce detectable HSP synthesis,

results in the pronounced synthesis of HSPs similar to those seen following exposure to higher levels of either stress applied separately. Thus, hyperthermia and ethanol stress can act synergistically to dramatically change the gene expression of mouse spleen cells. Quantification of the IgG constitutively synthesized and secreted *in vitro* by control and heat-shocked mouse spleen cells and splenic B lymphocytes revealed no differences between control and heat-shocked cells. These results demonstrate that synthesis and secretion of IgGs is not affected by thermal stresses sufficient to induce HSP synthesis and depress the synthesis of other polypeptides normally synthesized at a control temperature.

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For Susan and Kate, with love.

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LIST OF ABBREVIATIONS

1-D.....one-dimensional

2-D.....two-dimensional

BSA.....bovine serum albumin

CHO.....Chinese hamster ovary

DMSO.....dimethyl sulfoxide

EGTA.....ethylene-bis (oxyethylene nitrile) tetraacetic acid

ELISA.....enzyme linked immunoabsorbent assay

GAM HRP....Goat anti-mouse immunoglobulin G conjugated to Horseradish peroxidase

GAM IgG....Goat anti-mouse immunoglobulin G

GRP.....glucose-regulated protein

HBSS.....Hank's buffered saline solution

HRP.....Horseradish peroxidase

HS.....heat-shock

HSE.....heat-shock regulatory element

HSP.....heat-shock protein

HSTF.....heat-shock transcription factor

IEF.....isoelectric focussing

IgG.....immunoglobulin G

kDa.....kilodalton

LMM.....low molecular mass

MEM.....Minimum Essential Medium

mRNA.....messenger RNA

M_r.....relative molecular mass

PAGE.....Polyacrylamide gel electrophoresis

PBS.....phosphate buffered saline

pI.....isoelectric point

PMSF.....Phenylmethylsulfonylfluoride

PPO.....2,5-diphenyloxazole

SDS.....sodium dodecyl sulfate

INTRODUCTION

"Long ago it became evident that the key to every biological problem must finally be sought in the cell, for every living organism is, or at some time has been, a cell."

- Edmund B. Wilson, 1925.

1.1 General Introduction.

If one feature is shared by all organisms, be they plant or animal, protozoan or prokaryote, it is their continual interaction with a changing physical environment. In the short term, rapid and intense alterations in the environment can be of critical consequence; without a quick and effective response, the survival of an organism can be compromised. Over the long term, environmental change is selective, with those organisms surviving being a reflection of the environment itself.

Understanding the processes involved in coping with environmental stress has led to the study of the cellular response to a changing environment and, in particular, how environmental stimuli affect gene expression. One rapidly expanding area of study is the "heat-shock" (HS) or stress response, succinctly defined in Nover (1984) as "an instantaneous, complex, but transient reprogramming of cellular activities to ensure survival during the stress period, to protect essential cell components against heat damage and to allow a rapid and complete resumption of normal cellular activities in the recovery period".

The first accounts of the heat-shock response were reported in Drosophila by Ritossa (1962). Heat-shock alters the puff patterns of salivary gland polytene chromosomes resulting in the appearance

of new puffs in specific regions of the chromosomes, and the regression of pre-existing puffs (Ritossa 1962). Tissieres et al. (1974) reported changes in the pattern of protein synthesis in various types of heat-shocked Drosophila tissue. The rapid appearance of a set of six polypeptides (referred to as heat-shock proteins; HSPs) occurred within minutes of the HS exposure, and accounted for 30% of the label incorporated into protein by these cells. Other work coordinated altered puffing pattern with the cessation of RNA synthesis at preexisting puffs and the initiation of RNA synthesis at the active heat-shock induced sites (Ritossa 1964; Bonner and Pardue 1976).

These studies set the pattern for subsequent investigations of the HS response, by describing (1) alterations of gene expression at both the level of transcription and translation, (2) the enhanced synthesis of large quantities of a particular set of polypeptides (the HSPs), (3) the depressed synthesis of normally occurring polypeptides and (4), the re-establishment of normal patterns of protein synthesis following the heat-shock episode.

Interest in the heat-shock response has accelerated to the point that today, the heat-shock response has been identified in all manner of cells and organisms, including bacteria (Neidhardt et al. 1984), protozoa (Guttman et al. 1980), slime mould (Loomis and Wheeler 1980; Rosen et al. 1985), plants (Nover and Scharf 1984; Baszczynski et al. 1985; Key et al. 1985), sea urchins (Giudice 1985), insects (Vincent and Tanguay 1979; Dean and Atkinson 1983; and in particular Drosophila: Ashburner and Bonner 1979; Lindquist and DiDomenico 1985), as well as fish (Heikilla et al. 1982),

amphibians (Ketola-Pirie and Atkinson 1983), birds (Atkinson and Dean 1985; Schlesinger 1985) and mammals (Brown et al. 1982; Li and Werb 1982).

With only a few exceptions (to be discussed later), all cells examined display some alterations in protein synthesis following short heat-shock episodes. Furthermore, a wide variety of stresses besides heat-shock have been shown to alter gene expression in a myriad of cell types, doing so in a manner similar to, but not always identical with that seen following heat-shock. These stress agents include transition metals (zinc and cadmium; Levinson et al. 1980; Heikilla et al. 1982; Somerville 1984), oxidizing agents (hydrogen peroxide; Ashburner and Bonner 1979), steroid hormones (ecdysterone; Ireland and Berger 1982), amino acid analogues (canavanine; Li and Laszlo 1985), ionophores (A23187; Welch et al. 1983), ethanol (Li et al. 1982; Li and Laszlo 1985), and arsenite (Li et al. 1982; Bensaude and Morange 1984). Certain traumatic environmental changes also result in HSP synthesis, including glucose deprivation (Welch et al. 1983), viral infection (Collins and Hightower 1982; Kao and Nevins 1983), recovery from anoxia (Ashburner and Bonner 1979; Li and Werb 1982), and wounding (Currie and White 1981; Hightower and White 1982). An extensive list of agents, other than heat-shock, capable of inducing HSP synthesis is presented in Nover (1984).

The number of HSPs expressed by stressed cells, their molecular weights, and their location within the cytoplasm and nucleus vary greatly among cell types, and create a difficult picture to interpret. In a recent review of the stress protein response of

mammalian cells, Subjeck and Shyy (1986) have clarified this complex situation by categorizing the stress proteins into four groups:

(1) The major HSPs: widely recognized stress proteins occurring at 110, 89, 70 and 68 kDa.

(2) polypeptides of 76 and 97 kDa, primarily considered as glucose-regulated proteins.

(3) The low-molecular-weight HSP group (18-25 kDa), best documented in Drosophila and plant tissues.

(4) Minor HSPs of 46 and 56 kDa which are inducible by heat treatment and glucose deprivation.

The multitude of stressors capable of inducing the synthesis of a common and relatively small set of polypeptides in a wide variety of cell types points to a unified and conserved response to environmental stress. While reports in the literature often describe the polypeptides induced by a particular stress as being specific to that stress (i.e. glucose regulated proteins, Welch et al. 1983; trauma-induced proteins, Hightower and White 1982; as well as heat-shock proteins), it appears that each stressor represents part of an all-encompassing cellular "stress response" (Subjeck and Shyy 1986). Therefore, to maintain a consistent terminology in describing the stress response throughout this thesis, the term "heat-shock proteins" (HSPs) will be used as a general term to describe the polypeptides induced by exposure to environmental stresses. In those experiments where a specific type of chemical stress has been used (such as ethanol or arsenite), the polypeptides will be identified as either being "chemical-stress" induced, or induced by the specific stress.

1.2 Characteristics of the Heat-Shock Response.

Considerable evidence establishes the HS response as a universal and fundamental mechanism for cell protection during periods of diverse environmental stress. This evidence includes the wide variety of stresses capable of inducing HSP synthesis (Subjeck and Shyy 1986), the conservation through evolution of HSP genes and the consensus sequences required for their transcription (Craig 1985; Pelham 1985), the protection that mild heat treatment or chemical-stress (thermotolerance) offer to cells (Li and Laszlo 1985) and animals (Dean and Atkinson 1983) subsequently exposed to lethal temperatures, and the correlation of HSP synthesis resulting from mild heat pretreatment with protection against phenocopy induction (Mitchell et al. 1979). The constitutive synthesis of at least some of the HSPs at control temperatures, and the subsequent enhancement in their synthesis following heat-shock or chemical-stress suggests that some of these polypeptides function in cells during non-stress conditions and that their importance is amplified during the stress episode.

Heat-shock induces rapid alterations in the pattern of polypeptides synthesized by heat-treated cells, as a result of a reprogramming of gene expression. In most cell systems, [illegible] synthesis is primarily the result of new and/or [illegible] transcription of the HSP genes, although exceptions to [illegible] characteristic include: (a) Xenopus oocytes, where high constitutive levels of mRNA for the 70 kDa HSP are present under normal conditions, but remain untranslated until a temperature shift occurs (Bienz and Gurdon 1982), and (b) early cleavage stage

embryos where HSPs and HSP mRNAs are not synthesized during the early stages of embryogenesis (Heikkila et al. 1985). Regulation of gene expression at the level of transcription involves the repression of pre-existing transcription patterns and the preferential transcription of the heat-shock genes (Ashburner and Bonner 1979; Lindquist and DiDomenico 1985).

Transcriptional activation of the heat-shock genes appears to be dependent on a widely conserved promotor sequence or "heat-shock regulatory element" (HSE), upstream of the so-called "TATA" box (Bienz 1985; Pelham 1985). Single or multiple copies of the HSE possessing the homologous core consensus sequence "C-GAA-TTC-G" have been shown by cloning and sequencing analysis to exist with striking homology in HSP genes of Drosophila (encoding the 22-27, 68, 70 and 83 kDa HSPs), Xenopus (30 and 70 kDa HSPs), soybean (17 kDa HSP) and Dictyostelium (Pelham 1985).

In Drosophila, transcription of the 70 kDa HSP gene has been shown to be controlled by HSE regions activated by a heat-shock transcription factor (HSTF; Parker and Topol 1984). This HSTF is inactive in control cells but, when activated by heat-shock, binds to the HSE and initiates transcription of the HSP genes. The efficiency of transcription is dependent on the number and precise sequence of the HSEs possessed by the HSP genes, the proximity of the HSE to the TATA box, the accessibility of the HSTF to the HSE and the binding ability of the HSTF (Bienz 1985; Pelham 1985). Constitutive synthesis of HSPs at control temperatures, and the non-coordinate and developmental regulation of heat-shock genes can be accounted for by this regulatory model. Increased affinity

between the HSE and HSTF may account for the low level synthesis of certain HSPs (i.e. the mammalian 90 kDa HSP or [redacted]sophila 83 kDa HSP) at normal growth temperatures (Bienz [redacted] Pelham 1985). Tissue-specific, non-coordinate, and developmental regulation of HSP synthesis during embryogenesis (Moran et al. 1984; Heikkila et al. 1985) may be due to low levels of activatable HSTF during particular periods of differentiation or result from alterations in chromatin organization during development which could limit accessability of transcription factors to the HSE (Bienz 1985).

Some degree of post-transcriptional control of HSP gene expression does occur. This involves (1) the preferential selection and translation of HSP mRNAs, (2) a decrease in synthesis of most "control" polypeptides (Ashburner and Bonner 1979), (3) the improper processing of pre-existing mRNA precursors (Ashburner and Bonner 1979; Thomas et al. 1982), and (4) the preservation of pre-existing mRNAs in an untranslated form until the heat-shock period has ended and their translation is again required (Mirault et al. 1978; Lindquist 1980; Petersen and Mitchell 1981). During the recovery period following the HS episode, HSP synthesis decreases and the normal pattern of protein synthesis returns as the sequestered, pre-existing mRNAs are again recruited for translation (Craig 1985; Lindquist and DiDomenico 1985).

Evidence that HS elicits a conserved cellular response to environmental stress exists at several levels. As already discussed, heat-shocking eukaryotic cells results in the enhanced synthesis of a highly conserved set of polypeptides, most of which

fall in molecular mass ranges of 80-90 kDa, 68-74 kDa and 18-30 kDa (Craig 1985). While the molecular masses for particular HSPs from various species may vary by several thousand daltons, DNA sequences of HS genes from various species show remarkable homology. For example, the gene for Drosophila 83 kDa HSP shows a 60% sequence homology with the yeast 90 kDa HSP gene and a 45% homology with an E. coli HSP gene (Craig 1985).

The genes for the 70 kDa HSPs are probably the most highly conserved across the species, with the Drosophila 70 kDa HSP gene possessing a 60 - 80% degree of homology with similar genes from yeast, E. coli, maize, amphibians, and birds (Craig 1985; Pelham 1985). As well, a significant degree of homology is observed in Drosophila between the genes for the 70 kDa HSP and the 68 kDa HSP (Holmgren et al. 1979). The mechanism for transcriptional activation of the HS70 genes appears highly conserved, since cloned Drosophila HS70 genes introduced into rat cells (Burke and Ish-Horowicz 1982), mouse L cells (Lowe and Moran 1984), monkey COS cells (Mirault et al. 1982), or Xenopus oocytes (Pelham and Bienz 1982) will be transcribed when the recipient cells are heat-stressed (Pelham 1985).

Low molecular mass (LMM) HSPs are not widely expressed by heat-shocked mammalian cells, but are among the most prominent HSPs expressed at high levels by a variety of heat-shocked plant (Baszczynski et al. 1985) and Drosophila tissues (Lindquist 1980). A high degree of homology exists among the various Drosophila LMM HSP genes, and between these genes and genes for HSPs of similar molecular mass in Xenopus (Bienz 1984) and Caenorhabditis (Russnak

et al. 1983). However, perhaps the most interesting characteristic of these HSPs from both a functional and phylogenetic viewpoint, is the high degree of homology between the LMM HSP genes and the genes for the mammalian lens protein alpha-crystallin (Ingolia and Craig 1982; Southgate et al. 1985; Wistow 1985). The hydrophobicity and high-order aggregation of this lens polypeptide, and the similar characteristics reported for the Drosophila LMM HSPs suggest a role for these HSPs in maintaining the structural integrity of the cell during HS (Ingolia and Craig 1982; Tanguay 1985).

No single chemical property or biochemical mechanism, which could be considered a primary target for stress-induced changes in gene expression, appears to be shared by the variety of stressors capable of inducing HSP synthesis. To date, a number of general mechanisms have been proposed which attempt to present a common pathway for the induction of HSP synthesis. Of these, the two most often suggested are (1), oxidative-stress and (2), the presence of abnormal intracellular proteins. Most of the stressors that induce HSP synthesis can also cause protein denaturation (Ananthan et al. 1986), and several recent articles in the literature have presented data which appear to directly link HSP synthesis with increased intracellular levels of denatured protein (Goff and Goldberg 1985; Pelham 1985; Ananthan et al. 1986). A number of the stresses which induce HSP synthesis, fall into the category of an oxidative stress, including release from anoxia (Li and Werb 1982), ethanol (Li et al. 1982), and hydrogen peroxide (Ashburner and Bonner 1979). Finding the mechanism(s) directly responsible for induction

of the heat-shock response remains one of the major questions concerning heat-shock still to be answered.

1.3 Localization and Function of the HSPs.

Neither the precise role of the HS response in protecting cells from rapid changes in environmental conditions nor the specific function for any of the HSPs has been clarified completely. A number of studies have attempted to assign functions to particular HSPs by determining their subcellular location prior to and following heat-shock. These studies have utilized biochemical fractionation and/or immunocytochemical localization to assign HSPs to specific subcellular areas within the cytoplasm and nucleus.

The 68-74 kDa HSPs are major components of the heat-shock response in Drosophila and in mammals, and are a complex group of HSPs with a number of family members (Lindquist and DiDomenico 1985; Subjeck and Shyy 1986). Immunocytochemical localization detects low levels of the 70 kDa HSPs in the cytoplasm and nuclei of cells maintained at normal temperatures (Craig et al. 1983). Following heat-shock, the presence of these HSPs in both the cytoplasm and nucleus is increased. The specific association of the 70 kDa HSPs with the cytoskeleton (Kelley and Schlesinger 1982; Schlesinger et al. 1982) and nuclear matrix (Pouchelet et al, 1983; Atkinson and Pollock 1982; Welch and Feramisco 1984) have been described, as well as the specific localization of these HSPs within the nucleolar regions of heat-shocked cells (Arrigo et al. 1980; Welch and Feramisco 1984; Pelham 1984; Welch and Feramisco 1985). Interestingly, the nucleolar association of these HSPs is

not evident when cells are stressed with sodium arsenite or amino acid analogues (Welch and Feramisco 1984).

The 70 kDa HSP also has been shown to possess an RNA binding ability (DiDomenico et al 1982; Kloetzel and Bautz 1983). Velazquez and Lindquist (1984) have suggested that the 70 kDa HSP is transported to the nucleus during heat-shock to protect nuclear structure and function, and to re-establish the translation of normal cellular mRNA after heat-shock (Lindquist and DiDomenico 1985). In Drosophila, polypeptides of similar molecular mass as the 70 kDa HSPs are also synthesized at normal temperatures. These "heat-shock cognate" polypeptides are transcribed from genes which possess a high degree of homology with the HSP genes, but while transcribed at normal temperatures, these genes are not heat-inducible (Craig et al. 1983; Craig 1985).

Several major HSPs appear to share a nucleolar locale following their induction by heat-shock. The 68 kDa HSP is located in the nucleolus of heat-shocked mammalian cells (Welch and Feramisco 1984), but appears to lose this specific association and possess a more general nuclear presence during recovery from the heat-shock treatment. A similar nucleolar association has been presented for the 68 kDa HSP in Drosophila (Tanguay 1985). The 110 kDa HSP, like the 68 kDa HSP, is also located in the nucleolus, apparently having an affinity for RNA (Subjeck et al. 1983; Subjeck 1986).

The mammalian 90 kDa HSP (and Drosophila 83 kDa HSP) differs from the other HSPs in being a soluble polypeptide and strictly cytoplasmic in location (Welch et al. 1982; Kelley and Schlesinger 1982; Tanguay 1985). In Drosophila (Duband et al. 1983; Tanguay

1985), this HSP appears closely associated with the plasma membrane soon after heat-shock and, later, is present throughout the cytoplasm.

The subcellular locale of these HSPs can only be suggestive of possible functions for these polypeptides. To date, evidence of specific roles for any of the HSPs is, at best, fragmented. Recent studies have shown the 90 kDa HSP to be complexed with steroid hormone receptors (Sanchez et al. 1985; Catelli et al. 1985) and with the $pp60^{src}$ transforming protein of Rous sarcoma virus (Schuh 1985). The 90 kDa HSP binds to $pp60^{src}$ and a 50 kDa phosphoprotein, and remains complexed to these proteins while the $pp60^{src}$ is transported to, and becomes associated with, the plasma membrane (Welch and Feramisco 1982; Brugge et al. 1983). The association of the 90 kDa HSP with steroid hormone receptors from chick oviduct (Sanchez et al. 1985; Catelli et al. 1985) and mouse fibroblasts (Housley et al. 1985), implicates this HSP in the processing of steroid hormone receptors. With regard to the other HSPs, one member of the HSP 70 family having ATPase activity is responsible for the release of clathrin from coated membrane vesicles (Ungewickell 1985; Chappell et al. 1986). A low molecular weight HSP - ubiquitin - is responsible for targeting proteins destined for degradation (Bond and Schlesinger 1985), and the similarities between the low molecular weight HSPs and the lens protein 'alpha-crystallin' suggest a role for these HSPs in maintaining cell integrity during the stress period (Ingolia and Craig 1982).

1.4 Physiological Relevance of the Heat-Shock Response.

While it is not yet possible to present an integrated scenario for HSP function, various characteristics of the response bear witness to a beneficial protective function for the HSPs in maintaining cell integrity during short term periods of environmental stress. The induction of HSP synthesis has been correlated with the phenomenon of thermotolerance - the acquisition of a transient resistance to subsequently severe, normally lethal, heat-shock episodes (Li and Laszlo 1985). For instance, CHO cells exposed to a normally lethal temperature of 45°C (for 45 min) will survive this stress if they are pretreated with a mild HS episode (15 min at 45°C or 60 min at 41°C), followed by a several hour recovery period at the normal culture temperature. Acquistion of thermotolerance can be correlated with the enhanced synthesis of HSPs (Subjeck et al. 1982; Landry et al. 1982; Dean and Atkinson 1983; Li and Laszlo 1985), suggesting that the HSPs have a role in the development of heat resistance. Furthermore, other HSP-inducing stresses, such as ethanol (Li and Hahn 1978), sodium arsenite (Johnston et al. 1980; Li 1983), cadmium (Hahn and Li 1982) and chronic hypoxia (Li and Werb 1982), can confer upon mammalian cells a tolerance to subsequent heat treatments.

The physiological relevance of the HS response has been strengthened by studies demonstrating the induction of HSP synthesis in tissues of intact insects (Dean and Atkinson 1983), quail (Dean and Atkinson 1985), and mammals (Brown et al. 1982; Rodenhiser et al. 1985; Rodenhiser et al. 1986), subjected to hyperthermic stress (Atkinson and Dean 1985) or chemical agents

such as LSD (Brown 1985). As well, thermotolerance can be conferred to intact organisms, as shown by Dean and Atkinson (1983) in experiments correlating HSP synthesis with thermotolerance in larvae of the butterfly (Calpodes ethlius), and by Li et al. (1983), who showed that intact mice gained protection from a lethal whole body heat-dose of 42°C (for 35 min) when pretreated with a heat-dose of 41°C for 40 min.

Similarly, the occurrence of developmental defects caused by severe heat treatments (termed phenocopies; Goldschimdt 1935) can be prevented by a mild heat pretreatment prior to the lethal stress (Mitchell et al. 1979). This "prevention of phenocopy induction" and increased survival allowed by a mild heat pretreatment correlates with the induction of HSP synthesis, with the best prevention occurring when pretreatments induce HSP synthesis but do not depress normal protein synthesis (Mitchell et al. 1979; Mitchell and Petersen 1981; Subjeck and Shyy 1986).

Stress-induced changes in gene expression (both in vitro and in vivo) have important implications in understanding how a naturally occurring environmental stressor, either alone or in concert with other adverse environmental agents, may be involved in a variety of pathogenetic syndromes. The effect that specific stressors, such as heat and ethanol, have on depressing normal gene expression, has profound implications with respect to the appropriate functioning of cells. Indeed, the stress-induced decreased synthesis of normally occurring polypeptides may interfere with the proper timing of developmental decisions during embryonic growth and differentiation, or may affect the normal physiological function of

cells such that they are no longer functioning in an appropriate manner.

Hyperthermia and ethanol have each been shown to have adverse effects on embryonic and fetal development. Chicken embryos subjected to temperatures 3-4°C above normal body temperature (approximately 38°C) exhibit abnormalities of the developing cardiovascular system (Nilsen 1984). In mammals, female rodents exposed to short term hyperthermic episodes during pregnancy have young with an increased incidence of neural tube defects, which can be correlated with the timing of the hyperthermic episode (Finnell et al. 1986; Germain et al. 1985; Edwards 1967). A mild temperature elevation of 2.0 to 2.5°C above normal temperature (for at least 1 - 2 h) applied during a critical period of development causes birth defects in rats (Germain et al. 1985) and guinea pigs (Edwards 1969). Acute, short term temperature increases (43°C for 10 - 15 min) result in malformed rat (Germain et al. 1985; Mirkes 1985) and mouse (Finnell et al. 1985) embryos.

Indirect evidence suggests that hyperthermia (or ethanol) exposure may alter human fetal development in a manner similar to that seen with animal studies. Epidemiological studies of pregnant women suggest an association of maternal hyperthermia during pregnancy (within a range of temperatures as low as 38.9°C) with neural tube defects and facial abnormalities in their offspring (Smith et al. 1978; Layde et al. 1980; Smith et al. 1982). Such periods of elevated body temperature occur infrequently in humans, but can involve increases of 3°C or higher, especially during periods of fever (Smith et al. 1982). Certain physical activities

can also raise body temperature; during extensive exercise such as marathon running, increases in core temperature of 4.6°C have been reported (to approximately 42°C or 108°F; Maron et al. 1977), while individuals using saunas and hot tubs have been shown to have elevated core temperatures of 3.0°C (Smith et al. 1978; Harvey et al. 1981).

Ethanol is an inducer of HSP synthesis, which directly or through its metabolites, is also a teratogen (Chernoff 1982), altering carbohydrate and lipid metabolism and inhibiting protein synthesis (Mezey 1985). Chronic maternal ethanol exposure during pregnancy in humans and animals results in the Fetal Alcohol Syndrome (FAS) - an altered pattern of fetal development which involves fetal growth retardation, facial malformations, various neural anomalies and increased fetal mortality (Nitowsky 1980; Webster et al. 1980; Sulik et al. 1981; Priscott 1982; Abel 1984).

While numerous studies have documented the teratogenic effects of individual stressors, such as heat and ethanol, several studies have shown that a combination of teratogenic agents can act synergistically to produce deformities in developing embryos. In guinea pigs, hyperthermia and lead act synergistically to retard prenatal brain development (Edwards and Beatson 1984), while concurrent hyperthermia and arsenic treatment of female hamsters increases the rate of fetal malformations to higher levels than when either agent in present separately (Ferm and Kilham 1977).

Upon first consideration, the ability of environmental stressors such as heat or ethanol to alter the sequence of development appears to contradict the proposed protective function for the

heat-shock response. The short term benefit allowed by the HS response may be the ability of cells to survive the stress episode, but heat-shock induced depression in the expression of genes normally synthesized by cells at critical times during the process of differentiation may have profound effects on the development of the organism following the stress event. As German (1984) suggested with his 'embryonic stress hypothesis of teratogenesis', the HS response may exist as:

> "the basis for a proportion of human developmental errors ... (due to) the failure of essential loci to be transcribed into mRNA and translated into protein at some critical moment during embryonic life as a result of premption of the transcriptional and translational machinery of the cell at that time by heat-shock protein induction ... (This) would interrupt some of the myriad intricate and carefully programmed cell-cell interactions, cell migrations and cell death that occur before and during normal organogenesis."

Depending on the timing and duration of the stress, the consequences of a stress episode which interrupts the normal sequence of developmental decisions may be quite deleterious to the fate of differentiating cells in embryonic tissue. If this is true, the HS response could be considered a common pathway to account for the teratological effects of various types of environmental stresses, such as heat, ethanol or a combination of the two.

Alterations in the synthesis of polypeptides normally expressed by differentiated cells and required for a specific intra- or extra-cellular function may also be of important consequence for an organism as a whole, especially during periods of naturally occurring, and physiologically relevant, stress. Fever is a naturally occurring type of hyperthermia and a common response to infection in humans and many animal species (Roberts 1979).

Generally, fever is considered to be beneficial to the immune defense mechanisms, as various studies investigating alterations in cellular immunity following hyperthermia have demonstrated an enhanced response to mitogenic stimulation (Ashman and Nahmais 1977), the enhanced response to, and production of the leucocyte migration inhibition factor (LIF; Roberts and Sandburg 1979), an increased recruitment of cytotoxic T-lymphocytes (Smith et al. 1979) and interleukin-1 induced T-lymphocyte proliferation (Duff and Atkins 1982), and increases in the number of antibody- forming cells in mouse spleen cell cultures (Saririan and Nickerson 1982). Fever has also been shown to have adverse effects on immune function in vitro, including the depressed functioning of polymorphonuclear leukocytes (Peterson et al. 1976), and cytotoxic T-lymphocytes (Harris 1976; MacDonald 1977).

1.5 Summary and Thesis Objectives:

As discussed in this introduction, the heat-shock response is a highly conserved, rapid, and reversible cellular response to a wide variety of environmental stressors, which results in transient alterations in the patterns of polypeptides synthesized by the challenged cells. The enhanced synthesis in vitro of a select set of polypeptides - the HSPs - has been shown in nearly all cell types studied (Nover 1984; Atkinson and Walden 1985), and by a number of organisms heat-shocked in vivo (Dean and Atkinson 1983; Baszczynski et al. 1985; Dean and Atkinson 1985; Rodenhiser et al. 1985).

Surprisingly, up until 1982 when this research project was under-

taken, few reports had investigated the HS response of human cells, other than with transformed cell lines of human origin. Anderson et al. (1982) reported that HS induced the synthesis of 65 kDa HSPs (which they named HShock:1,2, and 3) and 80-100 kDa HSPs (HShock:4 and 5) in human lymphoblastoid cells, while heat-shocked human fibroblasts and peripheral blood lymphocytes only synthesized the 65kDa HSP (HShock:1). Tsekuda et al. (1981) showed that heat-shocked lung carcinoma cells synthesized 70, 90 and 100 kDa HSPs, while normal lung cells only synthesized the 70 kDa HSP. Atkinson and Pollock (1982) reported the synthesis of a 64 kDa HSP in heat-shocked human epidermoid carcinoma cells, and a number of groups described the HS response of HeLa cells, reporting the synthesis of HSPs with molecular masses of 100, 80, 70-74 and 27-37 kDa (McCormick and Penman 1969; Goldstein et al 1974; Slater et al. 1981; Hickey and Weber 1982; and Burdon et al. 1982). The paucity of data concerning the effect of heat-shock and chemical-stress on the gene expression of cultured non-transformed human cells initiated the research described herein.

The main goal of this thesis was to investigate and characterize the effect(s) of heat-shock on gene expression in mammalian blood cells. Blood seemed an appropriate source of cells with which to study the effect(s) of periods of thermal- and chemical- stress on gene expression, since cells of the circulatory system are exposed often, in their normal millieu, to elevated temperatures (i.e. periods of fever and hyperthermia), and to a variety of absorbed environmental pollutants and drugs (i.e. arsenic, heavy metals, ethanol). The use of blood cells from mammals (humans and non-

human) allowed a comparison of the heat-shock responses among cells of similar function from several species, and the use of cells isolated from mammals other than humans (i.e. mice) permitted considerable flexibility with regard to the types of experiments which could be performed. As well, the possibility of any genetic variability within the heat-shock response which might occur among human beings was eliminated by the use of inbred mouse strains.

In the first part of this thesis, human lymphocytes isolated from peripheral whole blood were cultured and incubated at control (37°C) or elevated temperatures or at 37°C in the presence of ethanol and sodium arsenite, and the patterns of polypeptides synthesized by these cells analyzed by one- and two-dimensional polyacrylamide gel electrophoresis (PAGE). The polypeptides synthesized by these lymphocytes were compared to those synthesized by control (37°C) and heat-shocked cells from a human transformed cell line.

The heat-shock response at the level of protein synthesis was further examined in cultured mouse and rabbit lymphocytes from peripheral whole blood, and in cultured mouse spleen cells and B lymphocytes. The pattern of polypeptides synthesized by these cells at control and heat-shock temperatures was compared to the pattern of polypeptides synthesized by similarly treated human lymphocytes and cells from a mouse transformed cell line, and to the pattern of polypeptides synthesized <u>in situ</u> by spleen cells isolated from mice at normal body temperature or exposed to whole-body thermal-stress.

The effect of chemical stresses (sodium arsenite or ethanol), on

the pattern of polypeptides synthesized by mouse spleen cells was examined and compared to the changes observed following heat-shock. Given the similar manner in which these various stressors were found to induce HSP synthesis, experiments were performed in which mouse spleen cells were concurrently exposed to low levels of several stressors known to induce HSP synthesis. Finally, the markedly altered gene expression - new and/or enhanced synthesis of HSPs and depressed synthesis of normally occurring polypeptides - found in experiments with stressed mammalian lymphocytes, prompted an investigation of the effect of heat-stress on the synthesis and secretion of IgGs - a major protein product of these cells. Mouse spleen cells and B lymphocytes were cultured at control and heat-shock temperatures and the quantity and specific activity of the IgGs secreted was detected by an enzyme-linked immunosorbent assay (ELISA).

In summary, the questions raised in this thesis are as follows:

(1) Do cultured human lymphocytes exposed to short term increases in incubation temperature synthesize heat-shock proteins?
(2) Are the levels of a constitutively synthesized proteins affected by heat-shock?
(3) Is this pattern of HSP synthesis similar to that observed with human transformed cell lines heat-shocked in culture?
(4) Is the pattern of HSP synthesis by human lymphocytes similar to that synthesized by cultured blood cells from other mammals?
(5) Do blood cells in thermally-stressed mice synthesize heat-shock

proteins *in situ*?

(6) Do cultured mammalian blood cells exposed to chemical stresses such as ethanol or arsenite, synthesize heat-shock proteins?

(7) Can several different, concurrently applied stressors act synergistically on cultured blood cells to cause alterations in gene expression and the synthesis of heat-shock protein synthesis?

(8) Are the levels of a constitutively synthesized and secreted protein of mammalian lymphocytes (i.e. IgG) affected by heat-shock?

MATERIALS AND METHODS

2.1 Chemicals

All chemicals used in this study were purchased from J.T. Baker Chemical Co.. (Phillipsburg, N.J.) or Fisher Scientific (Toronto) unless otherwise indicated.

Minimum Essential Medium (MEM), Medium 199, Hanks Buffered Saline Solution, Dulbecco's Modified Eagles Medium, Fetal Calf Serum, glutamine, and Non-Essential Amino Acids were purchased from Grand Island Biological Co. (GIBCO, Grand Island, N.Y.).

Methionine-free Minimum Essential Medium, and Leucine-free Minimum Essential Medium, were purchased from Flow Labs (McLean, Va.). Phenylmethylsulfonylfluoride, 2-mercaptoethanol, acrylamide, dithiothrietol, sodium dodecyl sulfate, Coomassie [redacted]R, ammonium persulfate, ethylene-bis (oxyethylene nitrile) te[redacted]cetic acid (EGTA), and N,N' methylene bis-acrylamide, were from Sigma Chemical Co., St. Louis, Mo..

Triton-X 100 was from New England Nuclear, Boston, Ma., Ficoll-paque was from Pharmacia Fine Chemicals (Pharmacia Inc., Piscataway N.J.), ampholines were from LKB Instruments Inc., Rockville Md., and Nonidet p-40 was from BDH Chemicals, Poole, England. Nulcear Chicago Solubilizer (NCS) was from Amersham Corp., Arlington Heights, Il.. Tween-20 and 4-chloro-1-napthol were from Biorad Canada, Mississauga, Ont., O-phenylene diamine was from Aldrich Chemicals, Milwaukee, Wis., and sodium arsenite was from Matheson, Coleman and Bell, Cincinnati, Oh..

2.2 Collection, Heat-Shock and Radiolabelling of Human Lymphocytes.

Whole blood samples were obtained by venipuncture from ostensibly normal individuals. Samples were collected in heparinized Vacutainer tubes (Becton Dickinson Canada, Mississauga, Ont.), added to an equal volume of methionine-free Minimum Essential Medium (methionine-free MEM; Flow Labs, Mclean, Va.) and these diluted samples placed over 4 mL of Ficoll-Paque (Pharmacia Fine Chemicals, Pharmacia Inc., Piscataway, N.J.) and centrifuged at 400 x g for 35 min in a clinical centrifuge. The lymphocyte fraction was collected from the sample-Ficoll interface and washed by resuspending the cells in Minimum Essential Medium (MEM; GIBCO, Grand Island, N.Y.) and centrifuging for 10 min at 2000 rpm. These cells were then resuspended in MEM, and incubated for various times (see results for details) at control (37°C) and heat-shock (40, 41, 42, and 43°C) temperatures, in a humidified atmosphere of 5% CO_2 in air. Following this incubation, cells were pelleted by centrifugation, and resuspended in an equal volume of methionine-free MEM or leucine-free MEM (Flow Labs, Mclean, Va). The samples were labelled with either 10 uCi/mL of L-[^{35}S]-methionine (approximately 1000 Ci/mmol) or 3.3 uCi/mL of L-[^{14}C(U)]-leucine (342 mCi/mL) (New England Nuclear, Boston, Ma.; 1Ci = 37 GBq), and incubated for 2 h or more at control or heat-shock temperatures (see results for details).

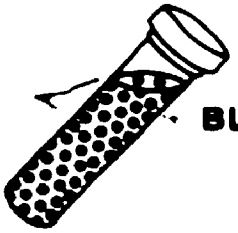
Radioactive labelling was terminated by placing samples on ice for 5 min. The cells were collected by centrifugation, and lysed in 5% 2-mercaptoethanol, 9 M urea, 1 mM phenylmethylsulfonylfluoride

(PMSF), and sonicated on a Kontes Micro-Ultrasonic cell disrupter (Kontes Scientific, Vineland, N.J.). Cell lysates were clarified by centrifugation in a Beckman Microfuge (Beckman Instruments, Spinco Division, Palo Alto, Ca.) and either used directly or stored at -70°C prior to electrophoretic analysis. An outline of this treatment procedure is presented in Figure 1.

In some experiments, whole blood samples were aliquoted directly into plastic culture dishes or siliconized glass tubes for heat-shock treatments. Following the incubation period, the samples were centrifuged and the blood plasma removed with a Pasteur pipet and replaced with an equal volume of methionine-free MEM containing 10 μCi/mL of L-[^{35}S]-methionine, and incubated for 2 h at their control or heat-shock temperature. Radioactive labelling was terminated by diluting with an equal volume of cold Hanks Buffered Saline Solution (HBSS; GIBCO), and the diluted samples were placed over Ficoll-Paque and centrifuged as described above. The lymphocyte fraction was collected, lysed, and stored, as previously described.

In a number of experiments, blood samples from newborn and premature infants were made available to us as aliquots of samples taken for normal diagnostic procedures. These samples were obtained from the Regional Medical Genetics Center at the Children's Hospital of Western Ontario. The small volumes of these blood samples (500-1000 μL) were manipulated in a manner similar to that previously described. Samples were collected and aliquoted into Venipuncture tubes, incubated for 2 h at control or heat-shock temperatures, and labelled with 10 μCi/mL of ^{35}S-methionine for 2 h at 37°C. Radioactive labelling was terminated by diluting with

Figure 1. A schematic diagram outlining the procedure for the isolation, heat-shock (or chemical-stress), and radiolabelling of human lymphocytes. A detailed description of these procedures is given in sections 2.2 and 2.6.



BLOOD SAMPLES

COLLECTED

LYMPHOCYTES COLLECTED FROM FICOLL GRADIENT

CELLS WASHED AND ALIQUOTED

CONTROL

HEAT SHOCK

37°

40°–43°

CHEMICAL STRESS

37°+ ETOH

37°+As

15–120 min INCUBATION IN MEM

SPIN

RESUSPEND

LABEL

60–120 min MEM–met + ^{35}S–met

SOLUBILIZE UREA/PMSF/2–me

1–D and 2–D PAGE ANALYSIS

an equal volume of cold HBSS, and the diluted samples (200-500 µL) were placed over 200 µL of Ficoll-Paque in siliconized 1000 µL glass test tubes, and centrifuged, washed, lysed and stored, as previously described.

2.3 Isolation, Heat-Shock and Radiolabelling of Mouse Peripheral Blood and Spleen Lymphocytes.

2.3.1 Animals

Male and female 6-12 week old mice from five inbred strains were used in this study. BALB/c and Swiss-Webster strains were obtained from Canada Breeding Farms, St. Constant, Charles River, Que.. C3H/HeSnJ and 129/ReJ strains were obtained from Jackson Labs, Bar Harbour, Me., and CsB - an acatalassemic line with C3H background - was originally obtained from Dr. T.W. Clarkson, University of Rochester, N.Y. All mice were maintained on a 14h/10h light/dark cycle in polycarbonate cages with sawdust bedding, fed Purina Mouse Chow and water ad libitum, and kept in the Department of Zoology, University of Western Ontario, London, Canada.

2.3.2 Mouse Peripheral Blood Lymphocytes.

BALB/c mice were killed by cervical dislocation and whole blood collected by cutting the heart and pipetting the blood pooled in the thoracic cavity in heparinized glass test tubes. The whole blood was incubated for 2 h, at control or heat-shock temperatures, and radiolabelled with 10 µCi/mL L-[^{35}S]-methionine. Radioactive labelling was terminated by addition of an equal volume of cold HBSS, and the diluted samples placed over Ficoll-Paque and

centrifuged at 400 x g for 25 min in a clinical centrifuge. The lymphocyte fraction was washed in HBSS, and collected by centrifugation (10 min at 2000 rpm) in a clinical centrifuge. The pelleted cells were lysed in 5% 2-mercaptoethanol, 9M urea and 1 mM PMSF, and the cell lysates sonicated, clarified, and stored at -70°C as described previously for human lymphocytes.

2.3.3 Mouse Spleen Lymphocytes: in vitro Heat-Shock Experiments.

Mice were killed by cervical dislocation, and their spleens removed and placed in 60 mm plastic petri dishes. Spleens were cut into two pieces and spleen cells (referred to hereafter as lymphocytes) were collected by repeated gentle flushing of the spleen with methionine-free MEM from a tuberculin syringe fitted with a 26 gauge needle, which was inserted beneath the connective tissue capsule. The lymphocytes were aliquoted into 15 mL plastic centrifuge tubes (Corning Glass Works, Corning N.Y.), brought up to 2 mL with methionine-free MEM, and incubated for various times (see results for details) at control (37°C) and heat-shock (41,43°C) temperatures, in a humidified atmosphere of 5% CO_2 in air. Following this incubation the samples were labelled with 10 uCi/mL of L-[^{35}S]-methionine and incubated for 1-2 h at control or heat-shock temperatures.

Radioactive labelling was terminated by placing the samples on ice, then pelleting the cells by centrifugation for 10 min at 2000 rpm in a clinical centrifuge. The pelleted cells were lysed in 5% 2-mercaptoethanol, 9 M urea, and 1 mM PMSF, and the cell lysates sonicated, clarified and stored at -70°C as described for human

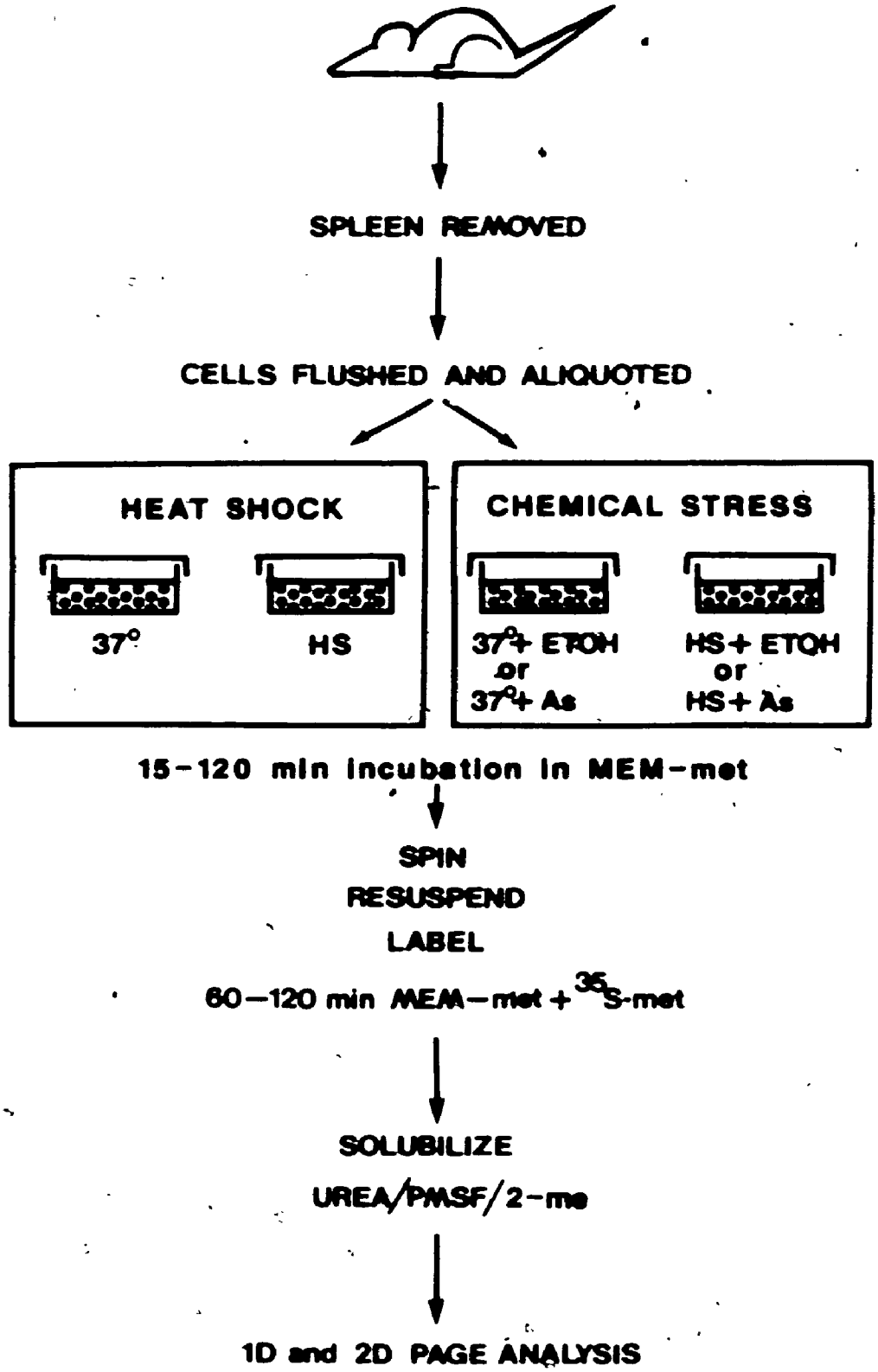
lymphocytes. An outline of the mouse spleen cell heat-shock procedure is presented in Figure 2.

2.3.4 Mouse Spleen Lymphocytes: in vivo Heat-Shock Experiments

In some experiments, the polypeptide synthesis patterns of lymphocytes heat-shocked in vivo were examined. Male BALB/c mice were anaesthetized with Avertin (0.2 mL/10 gm body weight of a 1:80 dilution of 4 gm 2,2,2-tribromoethanol in 2.5 mL tertiary amyl alcohol; Jones and Krohn 1960) and placed in a ventilated 50 mL plastic centrifuge tube. Each restrained mouse was placed on an electric heating pad, and a thermistor attached to a YSI-47 Scanning Tele-thermometer (Yellow Springs, Oh.) inserted into its rectum such that core temperature was monitored continuously. The heating pad was not activated for the control (non heat-stressed) animals used in this study. At specified times (see results) mice were killed by cervical dislocation, and lymphocytes were collected from their spleens, as previously described (section 2.3.3). The cells were resuspended in methionine-free MEM, radiolabelled with 10 µCi/mL [^{35}S]-methionine, and incubated for 2 h at 37°C. Following this labelling period, cells were collected by centrifugation, lysed in 5% 2-mercaptoethanol, 9 M urea and 1 mM PMSF, and the cell lysates sonicated, clarified and stored, as previously described.

In some cases, heat-stressed mice were allowed to recover from the anaesthetic and heat treatment, and following 30 min at room temperature, an intraperitoneal injection of ^{35}S-methionine (30 µCi/mouse) was administered. After 2 h, the mice were killed by

Figure 2. A schematic diagram outlining the procedure for the isolation, heat-shock (or chemical-stress), and radiolabelling of mouse spleen cells. A detailed description of these procedures is given in sections 2.3.3 and 2.6.


SPLEEN REMOVED
CELLS FLUSHED AND ALIQUOTED
HEAT SHOCK
37°
HS
CHEMICAL STRESS
37°+ ETOH
or
37°+ As
HS+ ETOH
or
HS+ As
15-120 min incubation in MEM-met
SPIN
RESUSPEND
LABEL
60-120 min MEM-met + 35S-met
SOLUBILIZE
UREA/PMSF/2-me
1D and 2D PAGE ANALYSIS

cervical dislocation, and their spleen cells collected, lysed and prepared for electrophoretic analysis, as previously described.

2.3.5 Isolation and Heat-Shock of Mouse Splenic B Cells.

Mouse B cells were purified from mouse spleen cell suspensions, using an Affi-gel (anti-mouse) Cell Sorting System (Biorad). This is an affinity chromatographic technique which employs rabbit anti-mouse IgG bound to cross-linked acrylic beads (diameter 250±50 μm) to select B cells (possessing surface IgG) from a mixed cell population.

Spleen cells were collected from BALB/c mice by repeated gentle flushing of spleens with methionine-free or Medium 199 (as described in section 2.3.3). Cells were placed into 15 mL plastic centrifuge tubes and incubated for 30 min at 37°C to remove cytophilic IgG. The cells were then centrifuged, resuspended in 1-2 mL of methionine-free MEM and applied directly to the 10 cm Affi-gel column, where they were incubated for 30 min at 4°C to allow B cell binding to the Affi-gel beads.

Following this incubation, non-binding cells were eluted from the column with 4-8 bed volumes (1 bed volume = 5 mL) of PBS (0.15 M NaCl, 0.01 M KH_2PO_4, and 0.1% NaN_3; pH 7.25). To elute the bound B cells, one bed volume of methionine-free MEM containing non-specific mouse IgG was added to the column and incubated for 30 min at room temperature. The cells were then released from the beads by vigourously agitating the beads with a polyethylene pipet, and were collected by elution with 8 bed volumes of PBS. B cell and non-binding cell fractions were collected from the elution buffer

by centrifugation and were washed twice with methionine-free MEM, and counted with a Coulter Counter Model ZB1 (Coulter Electronics, Hialeah, Fl.).

The cells were aliquoted in 2 mL volumes (1-2 X10^6 cells/mL) into 15 mL plastic centrifuge tubes, labelled with 10 µCi/mL ^{35}S-methionine and incubated for 4 h at control (37°C) or heat-shock (41 or 43°C) temperatures. Radiolabelling was terminated by placing the samples on ice and pelleting the cells by centrifugation. The pelleted cells were lysed in 5% 2-mercaptoethanol, 9M urea, and 1 mM PMSF, and the cell lysates sonicated, clarified, and stored at -70°C as described in section 2.3.3.

2.4 Isolation, Heat-Shock and Radiolabelling of Rabbit Peripheral Blood Lymphocytes.

2.4.1 Animals

Female 10-month old New Zealand white rabbits used in this study were obtained from a local breeder and were maintained in the Department of Zoology, University of Western Ontario. Rabbits were fed Purina Rabbit Chow and water ad libitum.

2.4.2 Rabbit Peripheral Blood Lymphocytes.

Whole blood was collected in heparinized tubes from ear veins, and the lymphocyte rich fraction collected over Ficoll-Paque as described for mouse lymphocytes (section 2.3.2). The cells were washed, suspended in methionine-free MEM for 1-2 h, at control or heat-shock temperatures, then radiolabelled with ^{35}S-methionine (10 µCi/mL) for 2 h at 37°C or 43°C (see results for details).

Radioactive labelling was terminated by placing the cells on ice. Cells were collected by centrifugation, and the pelleted cells lysed in 5% 2-mercaptoethanol, 9M urea amd 1 mM PMSF. The cell lysates were prepared for electrophoretic analysis, as described previously for mouse spleen cells.

2.5 Heat-Shock and Radiolabelling of Mouse and Human Myeloma Cell Lines.

Mouse (Sp 2/0-Ag 14) and human (LICR-LON-HMy2) myeloma cells were maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% Fetal Calf Serum (GIBCO), 2 mM glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids (GIBCO), and antibiotics (penicillin, streptomycin and fungizone) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells used for experimentation were pelleted by centrifugation, resuspended in methionine-free MEM at concentrations of 1-2 x 10^6 cells/mL, and incubated for 1-2 h at control (37°C) or heat-shock temperatures (see results for details). Cells were subsequently radiolabelled with ^{35}S-methionine (10 μCi/mL) for 1-2 h at 37°C or 43°C. Radioactive labelling was terminated by placing the cells on ice. Cells were collected by centrifugation, and the pelleted cells lysed in 5% 2-mercaptoethanol, 9 M urea and 1 mM PMSF. The cell lysates were prepared for electrophoretic analysis, as previously described.

2.6 Chemical-Stressing of Human Lymphocytes and Mouse Spleen Lymphocytes.

2.6.1 Chemical-Stressing of Human Lymphocytes.

The lymphocyte fractions from human whole blood samples were collected from Ficoll-Paque (as described in section 2.2) and re-suspended in methionine-free MEM. Lymphocytes were chemically-stressed with either ethanol or sodium arsenite ($NaAsO_2$). For ethanol-stress treatments, 95% ethanol was added to cell suspensions to give final v/v concentrations of 1 to 6% ethanol, and cells were then incubated for 1-2 h at 37°C in a humidified atmosphere of 5% CO_2 in air. For arsenite stress treatments, sodium arsenite (obtained from Matheson, Coleman and Bell, Cincinnati, Oh.; from an 0.01 M stock solution in water) was added to the cell suspensions to give final concentrations of 1-100 μM, and the cells incubated for 2 h at 37°C in a humidified atmosphere of 5% CO_2 in air.

Following chemical-stress, cells were pelleted by centrifugation, resuspended in methionine-free MEM, radiolabelled with 10 uCi/mL ^{35}S-methionine, and incubated for 1-2 h at 37°C. Radiolabelling was terminated by placing samples on ice, then the cells were pelleted by centrifugation, lysed, and the cell lysates prepared for electrophoretic analysis (see also Figure 1).

2.6.2 Ethanol-Stressing of Mouse Spleen Lymphocytes.

Mice were killed and their spleen cells collected, suspended in methionine-free MEM and aliquoted, as previously described in section 2.3.3. Ethanol (95%) was added to the cell suspensions to give final v/v concentrations of 1 to 6% ethanol. Cells were incubated for 15, 30, 60, 90, or 120 min at 37°C in a humidified

atmosphere of 5% CO_2 in air. In some experiments, cells were exposed to concurrent heat-shock and ethanol-stress. Ethanol was added to cell suspensions to give final v/v concentrations of 1 to 6 % ethanol, and the cells were then incubated for 15, 30, 60 or 90 min at 41°C, in a humidified atmosphere of 5% CO_2 in air.

Following these various treatments, the cells were collected by centrifugation (10 min at 2000 rpm) in a clinical centrifuge, resuspended in methionine-free MEM (without ethanol), radiolabelled with 10 μCi/mL ^{35}S-methionine and incubated for 1-2 h at 37°C. Radiolabelling was terminated by placing the samples on ice, then the cells were collected by centrifugation, lysed, and the cell lysates prepared for electrophoretic analysis (see also the outline of this protocol in Figure 2).

2.6.3 Arsenite-Stressing of Mouse Spleen Lymphocytes.

Mice were killed, and their spleen cells collected, suspended in methionine-free MEM and aliquoted, as previously described in section 2.3.3. Sodium arsenite was added to the cell suspensions to give a final concentrations of 1 to 100 μM, and the cells incubated for 1-2 h at 37°C in a humidified atmosphere of 5% CO_2 in air. In some experiments, cells were exposed to concurrent heat-shock and arsenite-stress. In these cases, sodium arsenite was added to the cell suspensions to give final concentrations of 1 to 100 μM and the cells were incubated for 1 h at 41°C in a humidified atmosphere of 5% CO_2 in air.

Following these various treatments, the cells were collected by centrifugation (10 min at 2000 rpm) in a clinical centrifuge,

resuspended in methionine-free MEM (without arsenite) and radiolabelled with 10 μCi/mL ^{35}S-methionine for 1-2 h at 37°C. Radiolabelling was terminated by placing the samples on ice, then the cells were collected by centrifugation, lysed, and the cell lysates prepared for electrophoretic analysis.

2.7 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis SDS-PAGE.

2.7.1. Protein and Radioactivity Determination.

The protein concentration of cell lysates was determined using the method of Lowry et al. (1951). The protein concentrations of those cell lysates containing 2-mercaptoethanol were determined using a turbidimetric assay modified from the method of Comings and Tack (1972). In all cases, bovine serum albumin (BSA) was used as a protein standard.

Samples for radioactivity determination were prepared by precipitation in 10% trichloroacetic acid (45 min), followed by an ether wash and then air dried. These air dried samples were then resuspended in 5% 2-mercaptoethanol, 9 M urea and 1 mM PMSF, and 50 μL aliquots were added to a Triton-X 100/toluene/Omnifluor (New England Nuclear) cocktail. Radioactivity of ^{14}C and ^{35}S in the sample/cocktail mixture was measured in a Beckman LS 255 liquid scintillation counter.

2.7.2 One dimensional (1-D) PAGE.

One dimensional (1-D) polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed accord-

ing to the procedure of Laemmli (1970), with the modifications described by Merrifield (1979).

Acrylamide gradient separating gels (5-15% or 7.5-17.5%) were cast in glass molds to yield gels of 10 x 15 x 0.15 cm. A 3% acrylamide stacking gel was poured on top of the polymerized separating gel. Sample wells were formed in the stacking gel by inserting a Teflon "comb" into the unpolymerized stacking gel between the bottom of the wells and the separating gel.

Polymerized gels were affixed to plexiglass tanks, and the tanks filled with running buffer (pH 8.3; 25 mM Tris-HCl, 193 mM glycine, and 0.1 mM SDS). Samples were prepared for electrophoresis by addition of 10 uL of a solution of 2% SDS, 5% 2-mercaptoethanol, 20% glycerol, 80mM Tris-HCl (pH 6.8), and 5-10 uL of a tracking dye (bromophenol blue in 1% SDS). Molecular weight standards were added to at least one lane of each gel to allow the characterization of the relative molecular mass (M_r) of the electrophoretically-separated polypeptides. Two sets of molecular weight standards were used. Unstained standards from Pharmacia Fine Chemicals (Uppsala, Sweden) contained a mixture of: phosphorylase-b, 94 000; albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; trypsin inhibitor, 20 100; a-lactalbumin, 14 400. Prestained molecular weight standards (Bethesda Research Labs, GIBCO) precluded the necessity to stain gels to visualize standards, thus allowing more rapid processing of gels. These standards contained a mixture of: myosin (H chain), 200 000; phosphorylase-b, 97 400; bovine serum albumin, 68 000; ovalbumin 43 000; a-chymotrypsin 25 700; B-lactoglobulin, 16 800; and cytochrome-C, 12 300.

Gels were run by applying 5 mA current to each gel overnight, or until the samples entered the separating gels. The current was then increased to 15-20 mA/gel until 30 min after the dye front had run off the bottom of the gel. After the run was completed, gels were stained for 2-4 h with 0.2% Coomassie Blue-R (Sigma) in 50% methanol and 10% acetic acid. Destaining was performed with several changes of 50% methanol and 10% acetic acid, followed by 20% methanol and 10% acetic acid. Final destaining was performed using 10% methanol and 10% acetic acid, and these destained gels were stored in 7% acetic acid. In those cases where Coomassie Blue-R staining was omitted, gels were fixed in 50% methanol and 10% acetic acid, then processed following the same destaining procedure, as described above.

2.7.3 Two-dimensional (2-D) PAGE.

Two-dimensional (2-D) analysis of proteins in cell lysates followed, with some modifications, the methods described by O'Farrell (1975).

Isoelectric focussing gels containing 3.5% acrylamide, 1.8% Nonidet P-40, 9 M urea and 2% ampholines were cast in glass tubes as tube gels approximately 10.5 x 0.4 cm in diameter. A mixture of ampholines was used to create a pH gradient from 3.5 to 8.5 and consisted of 40% 3.5-10 ampholines and 60% 5-8 ampholines (LKB Instruments Inc., Rockville, Md.). Once poured, the gels were allowed to polymerize overnight at room temperature.

The following day, the bottom ends of the tubes were covered with a 2 cm^2 piece of dialysis membrane and mounted in a Biorad model

150A electrophoresis tank. The apparatus was filled with upper (0.2 M NaOH) and lower (0.018 M H_3PO_4) buffers, and the gels were pre-run (prior to the samples being loaded) according to the following sequence: 200V for 15 min, 300V for 30 min, 400V for 30 min. Following this period, the upper tank buffer was replaced with fresh buffer, and the samples (prepared to a final 90 µL volume with 5% Nonidet P-40, 5% 2-mercaptoethanol, 5% ampholines and 9 M urea) were loaded unto the tops of the gels. One or two gels of each set were designated for pH determination; therefore samples were not added to these particular gels. All gels were electrophoresed for 16-20 h at 400V, and then at 800V for 2 h, for a total of 8000-9600 volt-hours.

At the completion of this run, gels were extruded from the glass tubes with a water-filled syringe. Those gels designated for pH determination were treated as follows: (1) the gels were refrigerated at 4°C for 1-2 h, (2) the gel was then sliced in 0.5 cm slices and (3) each slice was placed in a test tube with 2 mL of freshly boiled and cooled double distilled water. After incubation overnight, the pH of the water was measured and recorded as the mean pH of the gel slice (Saleem and Atkinson 1976). Those gels intended for 2-D gel electrophoresis were extruded from the glass tubes (as described above) and equilibrated for 15 min in 10 mL of 80 mM Tris-HCl (pH 6.8), 5% 2-mercaptoethanol, 2% SDS, and 20% glycerol.

Acrylamide gradient (3-15% or 7.5-17.5%) separating gels were cast in glass molds and covered with a 3% stacking gel as described previously. An equilibrated IEF gel was affixed to each polymerized stacking gel with a layer of melted agar (1% agar in 1% SDS). When

the agar had set, the 2-D gel was run, stained and destained, as previously described, and prepared for fluorography (see below).

2.7.4 Fluorography.

Stained and unstained acrylamide slab gels were prepared for fluorography using the method of Bonner and Laskey (1974). Gels were dehydrated by three 30 min washes in dimethyl sulfoxide (DMSO) and then soaked for 3 h in 100 mL of 22% 2,5-diphenyloxazole (PPO) in DMSO, to give a final concentration of 17.5% PPO in the gel. Following this period, the PPO was precipitated in the gels by immersing the gels in tap water for at least 3 h. The PPO impregnated gels were dried down on Whatmann 3mm filter paper using a Biorad model 224 gel dryer. The dried gels were mounted on cardboard, apposed to Kodak RP-Royal X-Omat film (XP-1), preflashed to an optical density of 0.15 (Laskey and Mills, 1975) and stored at -70°C for the appropriate exposure times. Fluorograms were developed in Kodak GBX developer and fixed in Kodafix.

2.7.5 Determination of Radioactivity Incorporated into Individual Gel slices.

In some experiments, the relative amounts of radioactivity incorporated into individual polypeptides separated by 1-D and 2-D gel electrophoresis were determined. Fluorograms of the particular gels to be used were carefully aligned over the dried gel, and regions of the gel which corresponded to bands or spots on the fluorogram were excised with a razor blade, and hydrated overnight in 1-2 mL of distilled water. Gel slices were dried of excess water

and incubated in 90% NCS (Amersham Corp., Arlington Heights, Il.) for 3 h at 55°C, to allow solubilization of protein present within the gel slices. Radioactivity was determined by scintillation counting of the solubilized samples in a toluene based fluor (Turner 1968) on a Beckmann LS 255 scintillation counter. Similar sized slices from blank areas of the gels were used to determine background and efficiency.

2.8 Enzyme Linked Immunosorbent Assay (ELISA) of Mouse Spleen Cell Immunoglobulin G.

2.8.1 Establishment of a Standard Curve for IgG Detection and Quantification.

A series of experiments was performed to determine if mouse spleen cells maintained at 37°C or heat-shocked at 41 or 43°C differ in the amount of immunoglobulin G (IgG) they secrete into their growth medium. To accomplish this, a protocol involving an enzyme linked immunosorbent assay (ELISA) coupled with an indirect immunoperoxidase staining procedure was established with mouse IgG (purchased from Sigma) and subsequently used to quantify the IgGs secreted into the growth media of control and heat-shocked cultured spleen cells and B lymphocytes.

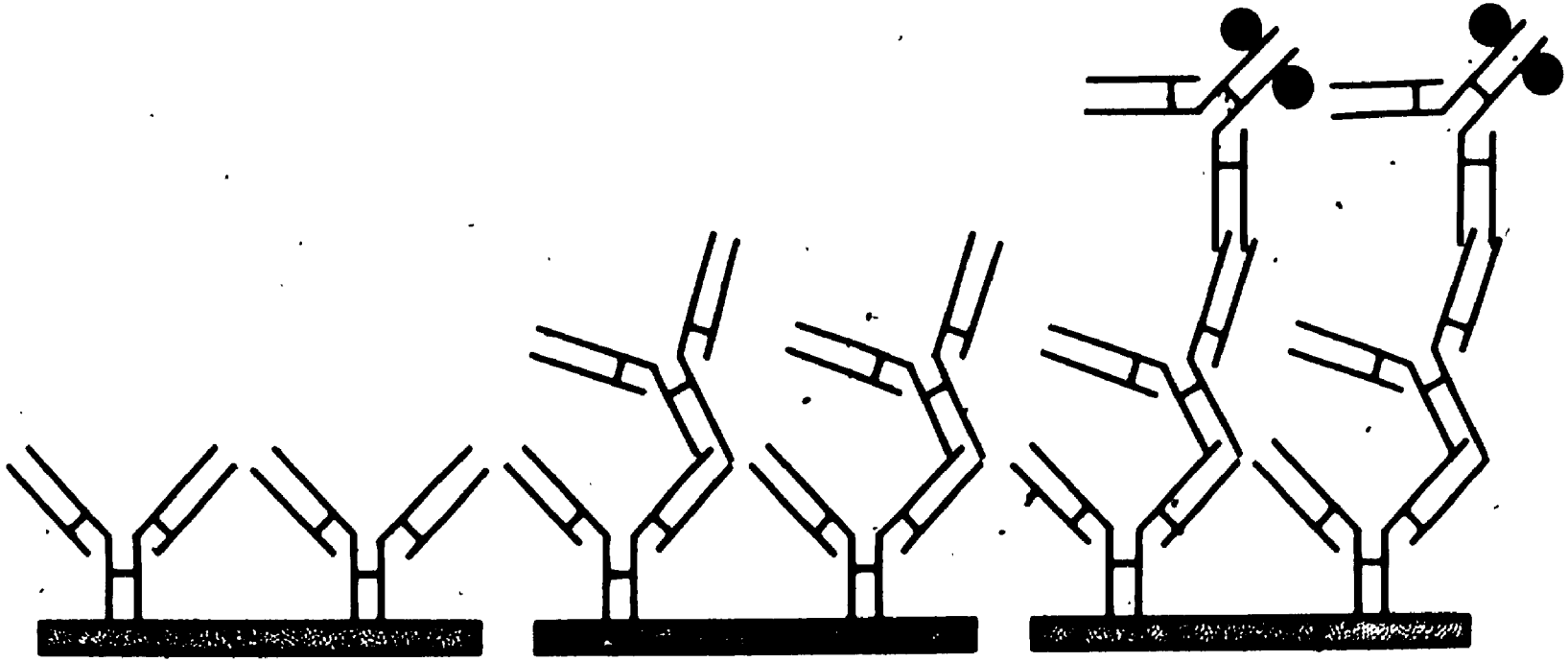
The ELISA/immunoperoxidase procedure, modified from the protocol of Weetman et al. (1982) was standardized with purchased IgG (Sigma), and can be briefly described as follows (see also Figure 3). First, a multi-well assay plate was coated with antibody raised against the test substance. Since the test substance in these experiments was mouse IgG, the multi-well plates were coated with

Figure 3. A schematic diagram outlining the ELISA/immunoperoxidase procedure described in section 2.8.

GAM IgG : Goat anti-mouse IgG (Fab'_2 fragment)

Mouse IgG : Mouse Immunoglobulin G

GAM IgG + HRP : Goat anti-mouse IgG conjugated to Horseradish peroxidase



STEP 1

PLATES COATED WITH GAM IgG

STEP 2

ADDITION OF MOUSE IgG

STEP 3

ADDITION OF GAM IgG + HRP

antibodies made in goats against mouse IgG (Goat anti-mouse IgG: GAM IgG). Second, the test substance (mouse IgG) was added to the multi-well plate and allowed to bind to the GAM IgG attached to the plate. Third, Goat anti-mouse IgG antibodies bound to the enzyme horseradish peroxidase (HRP) were added to the plate and bound to the mouse IgG. The subsequent addition of hydrogen peroxide to the multi-well plate resulted in the formation of a brown-coloured HRP reaction product. Colourimetric analysis of the amount of reaction product present allowed quantitation of the test substance present in the growth medium. A detailed description of this experimental procedure is provided in section 2.8.1.

2.8.2 ELISA Protocol for Quantifying and Determining the Specific Activity of IgGs Secreted by Cultured Mouse Spleen Cells and B Lymphocytes.

Mouse spleen cells were collected (as described in section 2.3.3), counted using a Coulter Counter, and incubated for 30 min at 37°C to allow the removal of cytophilic IgG. The cells were then collected by centrifugation, resuspended in methionine-free MEM or Medium 199, and aliquoted at various cell concentrations (2×10^6, 1×10^6, 5×10^5, 1×10^5, or 5×10^4 cells/mL) into plastic centrifuge tubes. The cells were radiolabelled with 10 µCi/mL ^{35}S-methionine, and incubated for 4 h at control (37°C) or heat-shock (41 or 43°C) temperatures. Following this incubation period, the cells were collected by centrifugation; the growth medium was removed for later ELISA experiments to quantify IgG, and the cell pellets lysed and prepared for electrophoretic analysis.

In some experiments, mouse spleen cells were fractionated through the Affigel Cell Sorting Column and B lymphocytes (containing surface IgG) were collected. These cells were labelled, aliquoted and incubated at control and heat-shock temperatures as described above for the total spleen cell population, and the amount of IgG in their growth media was determined by the procedure described below.

MicroELISA 96 well plates (NUNC ImmunoPlate I (GIBCO, Burlinton, Ontario) or Cooke Microtiter plates (Dynatech Labs, Alexandria, Va.) were coated with 150-200 uL aliquots of goat anti-mouse IgG $F(ab')_2$ (Cappel Scientific, Malvern, Pa.) at a concentration of 1.6 µg/mL coating buffer (pH 9.6; 1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.2 g NaN_3; in 1 L), using a Titertek multichannel pipet (Flow Labs, Mclean, Va.). These plates were then refrigerated overnight at 4°C.

The following day, plates were washed 3 times (3 min between washes) with wash buffer (pH 7.4; 8.0 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 0.2 g KCl, 0.2 g NaN_3, 500 uL Tween-20; in 1 L) using a Dynatech Microwash apparatus (Dynatech Labs). Samples (media in which mouse spleen cells had been cultured) were added to the plates in 4-8 replicates each, and the plates incubated for 1-2 h at 37°C in 5% CO_2 in air. To determine non-specific binding of antibody to the multiwell plate, the initial step of this procedure, which involved coating the multiwell plate with goat anti-mouse IgG $F(ab')_2$ was omitted prior to the addition of 200 µL samples (at various concentrations) of mouse IgG (H and L chains; Sigma) to the plates.

Following this period, samples were aspirated from the plates,

which were washed 4 times with 200 uL wash buffer. Goat anti-mouse IgG conjugated with horseradish peroxidase (GAM+HRP, Biorad Labs, Mississauga, Ont.; 1:500 dilution in wash buffer) was added (150-200 uL/well) and the plates incubated for 1-2 h at 37°C. The plates were washed again, and 150-200 uL of substrate solution [pH 5.0; 24.3 mL 0.1 M citric acid, 25.7 mL 0.2 mL Na_2HPO_4, 50 mL distilled water, 40 mg O-phenylene diamine (Aldrich Chemicals, Milwaukee, Wis.), and 40 uL H_2O_2] was added to each well. After 15 min at room temperature, 50 uL stopping solution (2.5 M H_2SO_4) was added to stop the development reaction in the wells. Absorbances (at 492 nm) were determined using a Dynatech MR600 Microplate reader.

For statistical analysis of the absorbance data, Student's T tests or Analysis of Covariance (ANCOVA) were used (Snedecor and Cochran 1980). The ANCOVA was used to control for the effects of (log_{10}) cell concentration, and examine the effects of temperature on IgG concentration (i.e. 37°C versus heat-shock temperature) as measured by (log_{10}) absorbance units. I added 2 units to all absorbance values before transforming by log_{10} to account for some 0 and -1 absorbance values at some low cell concentrations.

Following some ELISA experiments, the secreted mouse IgG bound to the multiwell plate was released from the wells and the radioactivity determined. Subsequently, IgG was separated by 1-D PAGE, and visualized by fluorography. To accomplish this, the development and stopping solution present in the wells was removed, 200 uL of 10% SDS was added to each of the wells, and the multiwell plate was incubated at 37°C for 2-4 h. The SDS was then aspirated

from the plate, added to the previously removed development and stopping solution, and the protein present in this mixture was precipitated with trichloracetic acid, washed with ether, and air dried. The protein samples were resolubilized with 9 M urea, 5% 2-mercaptoethanol, and 1 mM PMSF, and the amount of acid-precipitable radioactivity present in the samples was determined. Equal amounts of acid-precipitable radioactivity from the samples were added to 5-15% 1-D polyacrylamide gels, the proteins electrophoretically separated and analyzed by fluorography (see Section 2.7.4).

RESULTS

3.1 Changes in Gene Expression of Human Lymphocytes in Response to Heat-Shock and Chemical-Stress.

In the following section of this thesis, I will present the results of experiments performed to determine if short term exposures to elevated temperatures (heat-shock) or chemical stress alter the patterns of gene expression in isolated human lymphocytes.

3.1.1 Heat-Shock of Human Lymphocytes.

To investigate the response of human lymphocytes to short term exposures to elevated temperatures, lymphocytes were isolated from peripheral whole blood by centrifugation through Ficoll-Paque, as described in the Materials and Methods section. Light microscope examination of lymphocyte preparations isolated from whole blood (Figure 4) reveals a homogeneous population of small lymphocytes which possess a central basophilic nucleus surrounded by a small rim of cytoplasm. Some platelet contamination of the lymphocyte preparation was observed. No differences in cell morphology were seen between control lymphocytes maintained at 37°C and lymphocytes heat-shocked at 43°C.

Human peripheral blood lymphocytes were incubated at elevated temperatures to determine if changes in gene expression occur in response to heat-shock. Lymphocytes were isolated from whole blood, incubated for 2 h at control (37°C) or heat-shock (40, 41, 42, or 43°C) temperatures, then labelled with ^{35}S-methionine for 2 h at

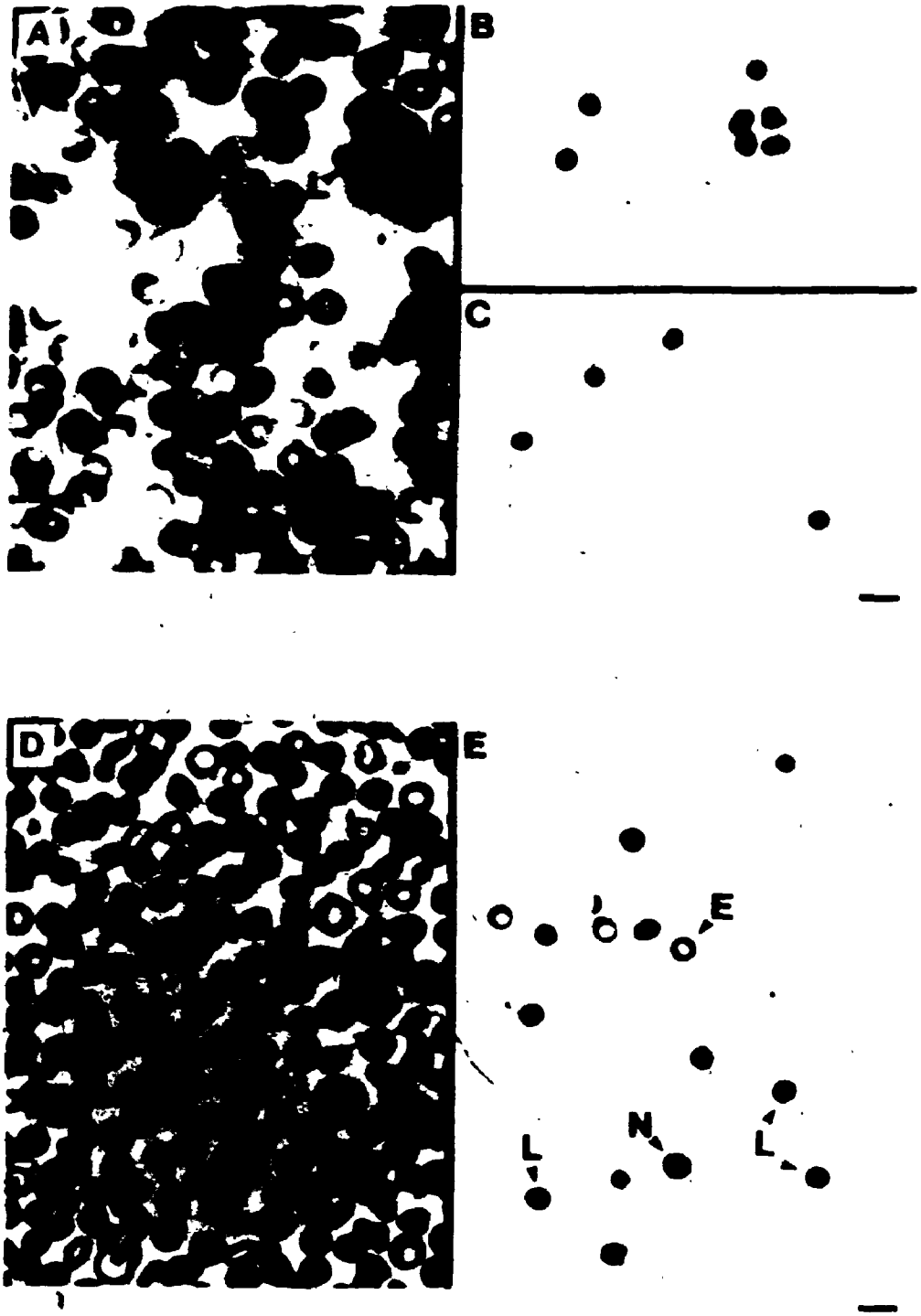
Figure 4. Light micrographs of the human (A,B,C) and mouse (D,E) cells used in this study. (A) depicts a human whole blood smear, stained with Giemsa; "L" indicates lymphocytes. (B) and (C) depict Giemsa-stained human lymphocytes isolated from whole blood by centrifugation following incubation at control (B) or heat-shock (C) temperatures (see results section for details). (D) depicts a mouse spleen cell suspension culture and (E) shows a Giemsa-stained preparation of simlar mouse spleen cells. Abbreviations stand for:

E : erythrocyte

N : neutrophil

L : lymphocyte

Magnification bar = approximately 10 micrometers.


A
B
C
D
E
O
O
O
E
N
L
L

37°C. The cells were then lysed, the amount of radioactivity incorporated into synthesized protein was determined, and equal amounts of acid-precipitable radioactivity from each cell lysate were electrophoretically-separated by one-dimensional (1-D) polyacrylamide gel electrophoresis (PAGE).

Figure 5A depicts the Coomassie-blue stained gel showing the staining pattern of polypeptides present in the cell lysates, and reveals that no qualitative differences exist among the polypeptide staining patterns as a result of the various heat treatments. The fluorogram of the same gel (Figure 5B) reveals the polypeptides synthesized by the cells during the labelling period. Incubation of these cells for a brief period (2 h) at temperatures 4-6° (41-43°C) above a control (37°C) temperature results in the enhanced and/or de novo synthesis (relative to the amounts of other polypeptides) of a select group of polypeptides (referred to hereafter as heat-shock proteins: HSPs). These HSPs have relative molecular masses (M_rs) of 110 000, 100 000, 90 000, 70 000 and 65 000 daltons. The fluorogram shown in Figure 5B indicates that exposure to a temperature of at least 41°C for at least 2 h is required to initiate synthesis of HSPs by human lymphocytes, and that synthesis of the HSPs increases with increasing temperature. To establish the minimum time required to induce HSP synthesis at a particular temperature, lymphocytes were isolated from whole blood, incubated for 15, 30, 60 and 90 min at 41 and 43°C and subsequently incubated for 2 h at 37°C in the presence of ^{35}S-methionine. The fluorograms shown in Figure 6 reveal that at least a 60 min incubation at 41°C is required to induce detectable levels of the HSPs, while a slight

Figure 5. The effects of incubation temperature on the types of polypeptides synthesized by human lymphocytes in culture. Lymphocytes were isolated from whole blood, incubated for 2 h at control (37°C) or heat-shock (40, 41, 42, or 43°C), then labelled with ^{35}S-methionine for 2 h at 37°C. The cells were lysed and equal amounts of acid-precipitable radioactivity from the lysates were added to each well of the 1-D SDS-polyacrylamide (3-15%) gel. Panel A depicts the Coomassie-blue stained gel showing the staining pattern of polypeptides present in the cell lysates. Panel B shows the fluorogram of the same gel, revealing the polypeptides newly synthesized by the cells during the labelling period. Protein standards of known molecular mass (M_rs) are denoted on the left side of the figure ("STDs"). The M_rs shown on the right side of this figure denote the relative molecular masses (X 10^{-3}) of the HSPs. The incubation temperature prior to labelling is shown at the bottom of the figure.

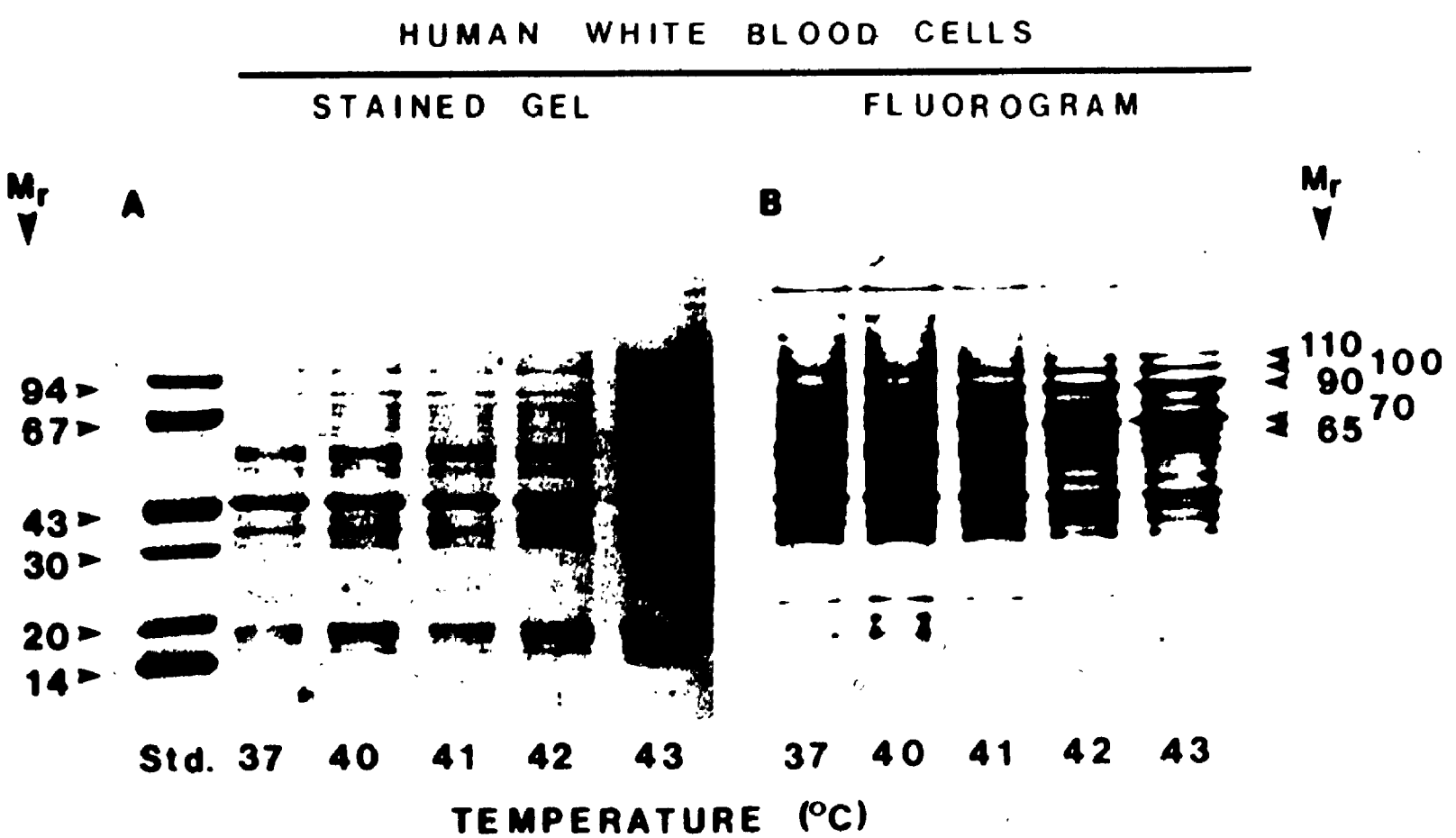
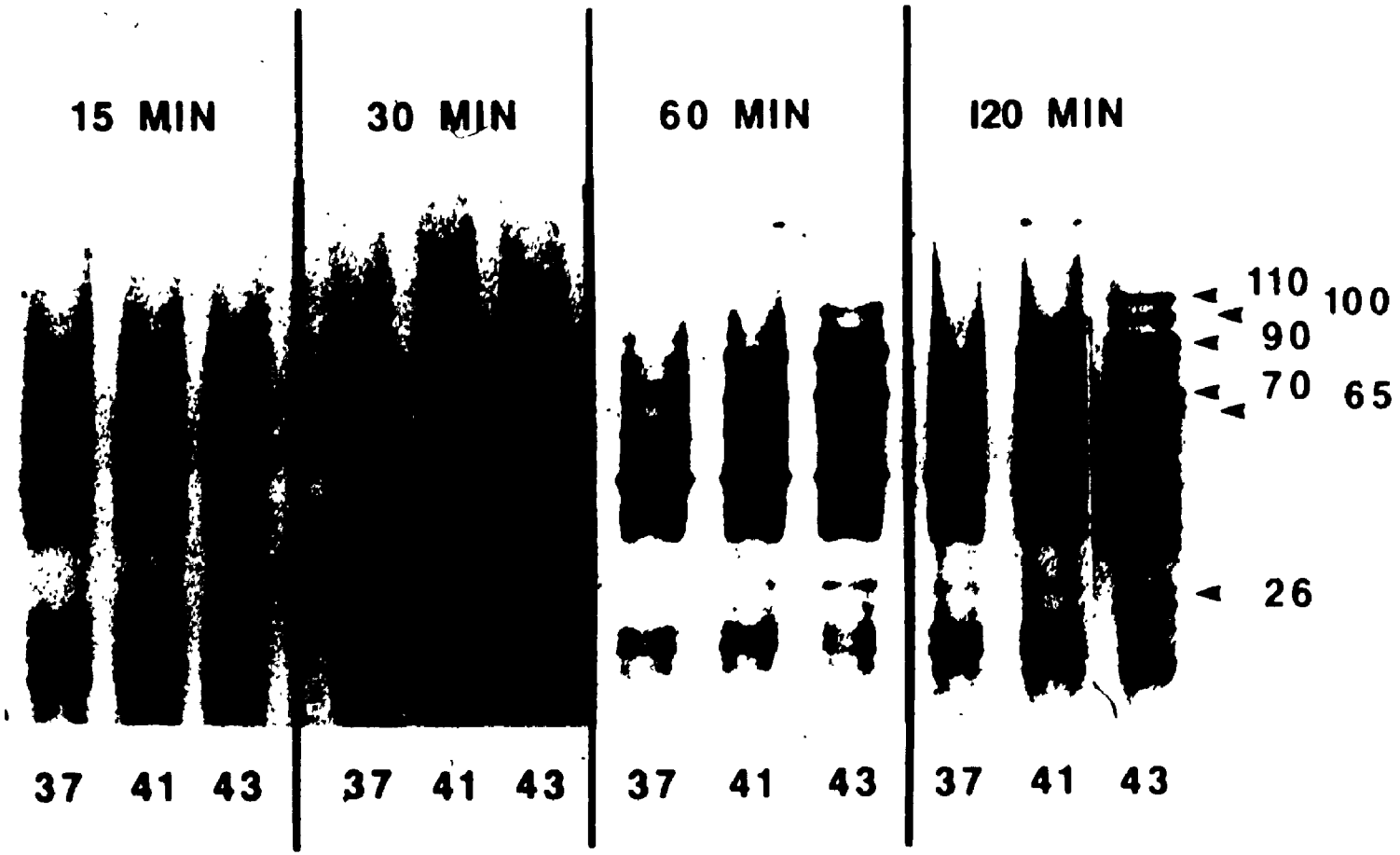

HUMAN WHITE BLOOD CELLS
STAINED GEL
FLUOROGRAM
Mr
A
B
Mr
94
67
43
30
20
14
110
100
90
70
65
Std. 37 40 41 42 43 37 40 41 42 43
TEMPERATURE (°C)

Figure 6. Fluorographic analysis of the time required at heat-shock temperatures (41°C and 43°C) to elicit a HS response from human lymphocytes. In each case, lymphocytes were collected, incubated for 15, 30, 60, or 120 min at 37°C (control) or 41°C or 43°C (heat-shock temperatures), then labelled with ^{35}S-methionine for 2 h at 37°C. Equal amounts of acid-precipitable radioactivity from the lysates were added to each well of a 1-D SDS-polyacrylamide (3-15% gradient) gel. The numbers shown on the right side denote the M_rs (X 10^{-3}) of the HSPs.


15 MIN
30 MIN
60 MIN
120 MIN
110
100
90
70
65
26
37 41 43
37 41 43
37 41 43
37 41 43
TEMPERATURE (°C)

enhancement in HSP synthesis is observed after as little as 30 min exposure at 43°C, and is much more enhanced after 60 min at 43°C.

To determine if synthesis of HSPs could be detected using a radioactive amino acid other than ^{35}S-methionine, human lymphocytes were maintained at 37, 41, or 43°C and then labelled at either 37°C or 43°C for 2 h with either ^{35}S-methionine or ^{14}C-leucine. In each case, equal amounts of acid-precipitable radioactivity from the lysates were electrophoretically-separated by 1-D PAGE. A comparison of the fluorographic results obtained from using ^{35}S-methionine or ^{14}C-leucine as the radioactive amino acid (Figure 7) reveals that HSPs with the same M_rs are detectable with either radioactive amino acid. Enhanced and/or de novo synthesis of the 26 kDa HSP is, however, more apparent when ^{14}C-leucine is incorporated.

To further establish that the synthesis of HSPs is elevated by increasing temperature, quantification of the radioactivity incorporated into specific electrophoretically-separated polypeptides from control and heat-shocked lymphocytes was determined. Lymphocytes were treated and labelled with ^{35}S-methionine or ^{14}C-leucine as described above for Figure 7. Cell lysates were separated by 1-D PAGE, and areas from the gels corresponding in M_r to the 90 000 and 70 000 daltons (90 and 70 kDa) HSPs and a 42 kDa non-HSP were excised from the gels. The radioactivity incorporated into these polypeptides was determined (see section 2.7.5), and the values presented as a percentage of the total acid-precipitable radioactivity present in the cell lysates. The results, as shown in Figure 8, indicate that synthesis of the 90 and 70 kDa HSPs and the 42 kDa non-HSP is temperature dependent. The 70 kDa HSP

Figure 7. Comparison of fluorographic results obtained from using ^{14}C-leucine or ^{35}S-methionine for radioactive labelling of polypeptides synthesized by control and heat-shocked human lymphocytes. Lymphocytes were maintained at:

(A) 37°C for 2 h and labelled at 37°C for 2 h

(B) 41°C for 2 h and labelled at 37°C for 2 h

(C) 43°C for 2 h and labelled at 37°C for 2 h

(D) 43°C for 2 h and labelled at 43°C for 2 h

Equal amounts of acid-precipitable radioactivity from the cell lysates were added to each well of the 1-D SDS-polyacrylamide (3-15% gradient) gel. Protein standards (STDs) of known molecular mass (X 10^{-3}) are denoted on the left side of the figure. The relative molecular masses (X 10^{-3}) shown on the right side denote the HSPs and a 42 kDa non-HSP.

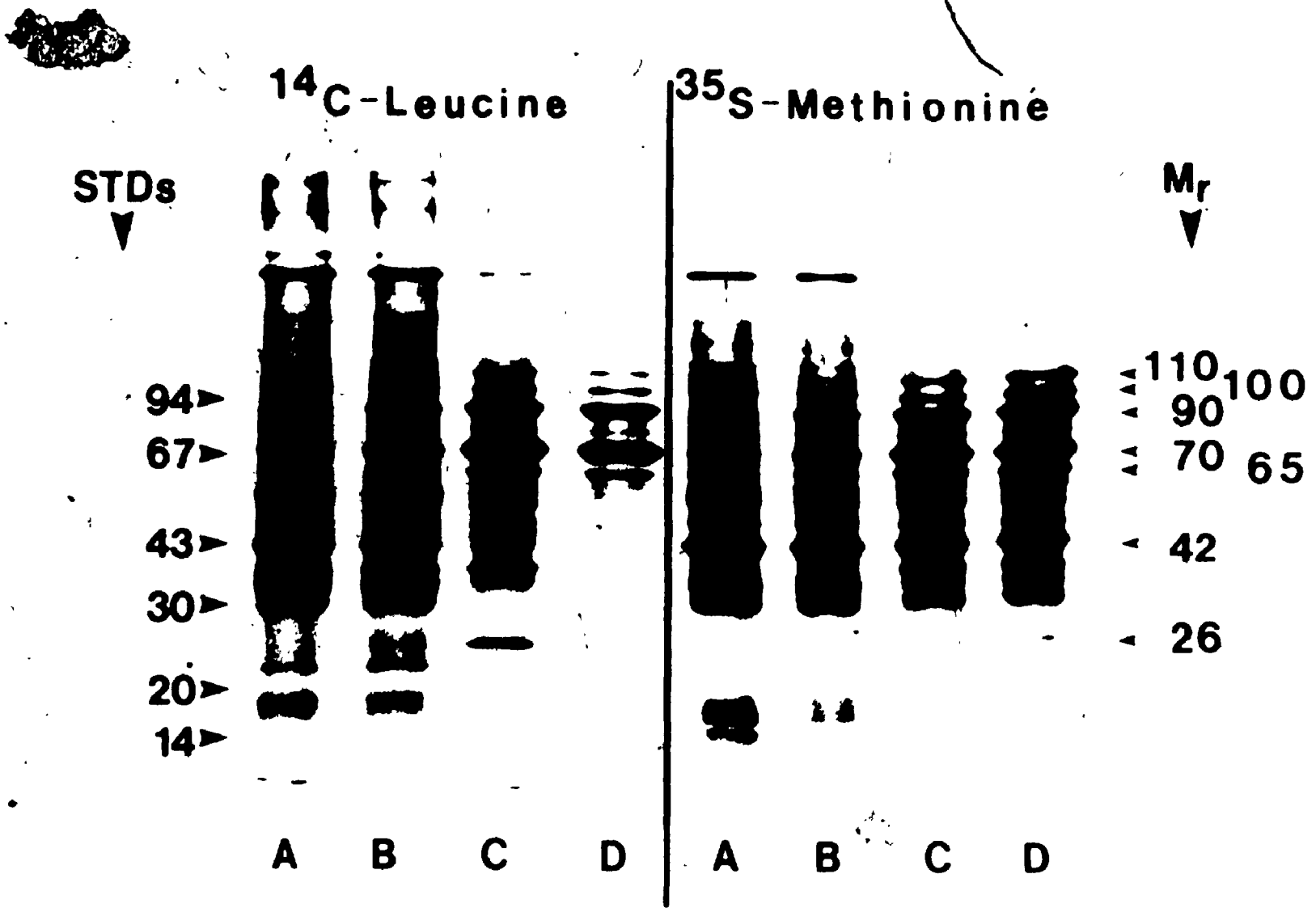

^{14}C-Leucine
^{35}S-Methioninė
STDs
M_r
94
67
43
30
20
14
110
100
90
70
65
42
26
A
B
C
D
A
B
C
D

Figure 8. Quantification of the radioactivity (^{14}C-leucine and ^{35}S-methionine) incorporated into specific, electrophoretically-separated polypeptides from the control and heat-shocked human lymphocytes shown in Figure 7. After 1-D PAGE separation of the polypeptides and fluorographic analysis of the gels, the amount of radioactivity in individual polypeptides (expressed as a percentage of the total acid-precipitable radioactivity) was determined as described in section 2.7.5.

The lymphocyte lysates used in this figure are from lymphocytes maintained at:

(A) 37°C for 2 h and labelled at 37°C for 2 h

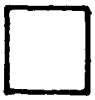
(B) 41°C for 2 h and labelled at 37°C for 2 h

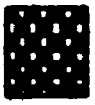
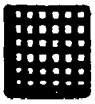
(C) 43°C for 2 h and labelled at 37°C for 2 h

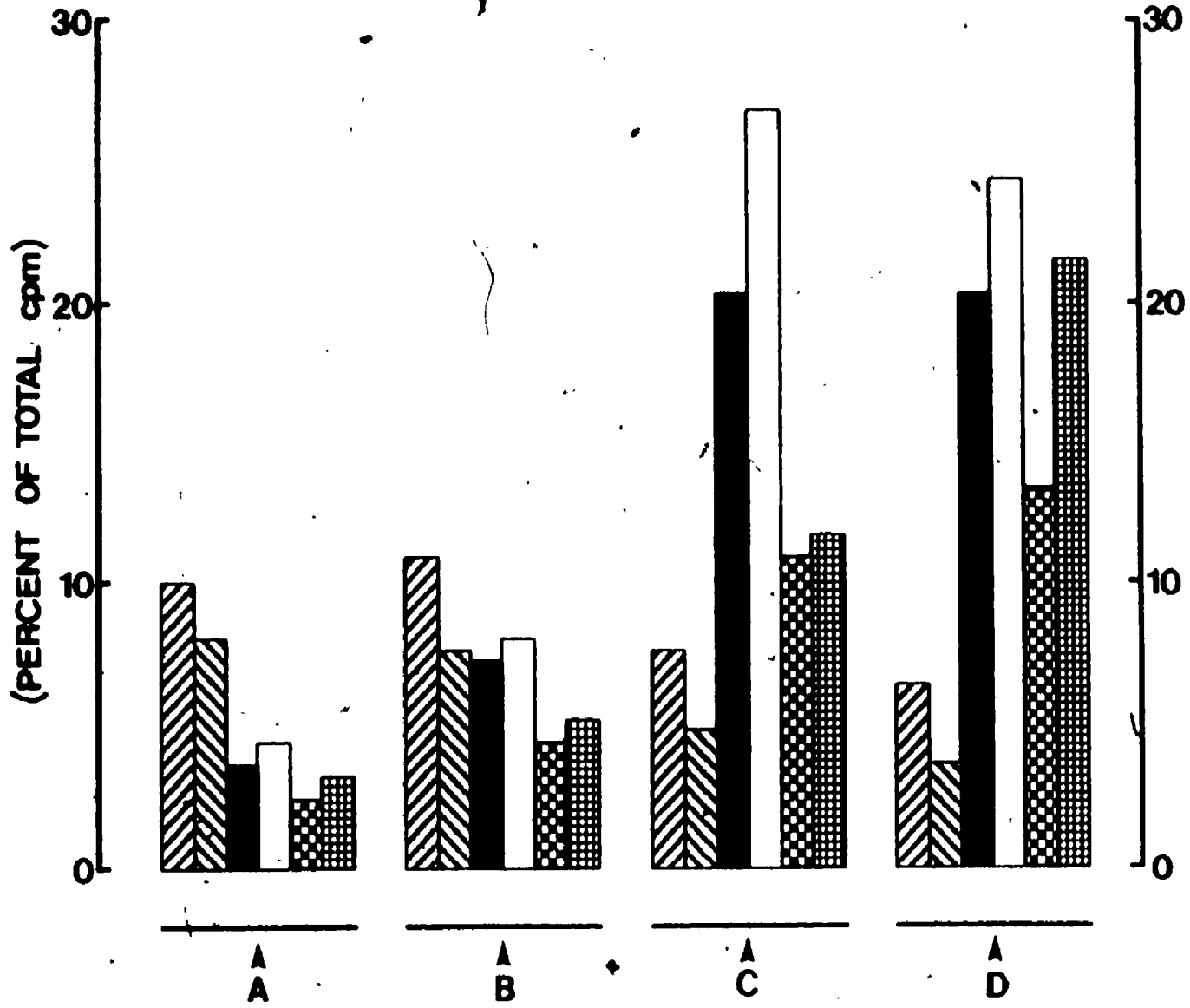
(D) 43°C for 2 h and labelled at 43°C for 2 h

The histogram bars indicating ^{14}C incorporation and ^{35}S incorporation are coded as follows:

42 kDa non-HSP:  ^{14}C;  ^{35}S.

70 kDa HSP:  ^{14}C;  ^{35}S.

90 kDa HSP:  ^{14}C;  ^{35}S.

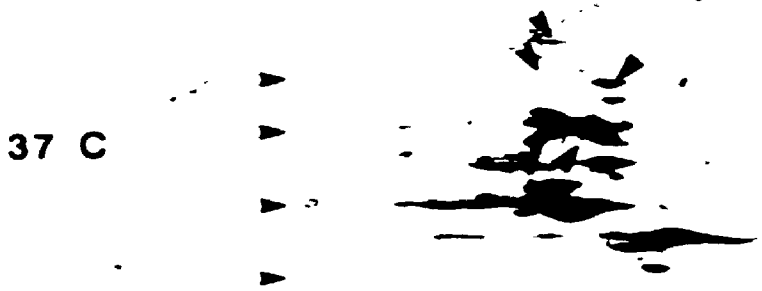

30
20
10
0
(PERCENT OF TOTAL cpm)
30
20
10
0
A
B
C
D

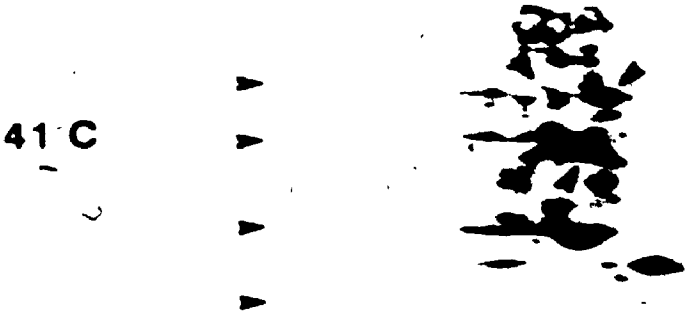
represents 4% of the radioactivity present in the 37°C cell lysate, 8% of the 41°C cell lysate, and between 20 - 30% of the 43°C cell lysate. The 90 kDa HSP represents 3% of the radioactivity present in the 37°C cell lysate, 5% of the 41°C cell lysate, and 10 - 20% of the 43% cell lysate. On the other hand, synthesis of the 42 kDa non-HSP decreases in relative amount in the cell lysate as the incubation temperature increases. This polypeptide represents 8 - 12% of the radioactivity present in the 37°C cell lysate, 7 - 12% of the 41°C cell lysate, and 4 - 8% of the 43°C cell lysate.

To further characterize the polypeptides induced in heat-shocked lymphocytes, lysates from control and heat-shocked cells were separated by 2-dimensional IEF-PAGE. Cells were maintained at a control (37°C) or heat-shock (41-43°C) temperature for 2 h, and labelled for 2 h at 37°C with ^{35}S-methionine. Equal amounts of acid-precipitable radioactivity from the cell lysates were electrophoretically-separated by 2-D IEF-PAGE. Figure 9 shows that heat-shocked human lymphocytes synthesize HSPs with M_rs of 110, 100, 90, 70 and 65 kDa. Small but detectable levels of the 110 kDa, 90 kDa, 70 kDa, and 65 kDa HSPs are synthesized by cells maintained at 37°C. Several of the HSPs consist of families of polypeptides with different isoelectric points (pIs). The 110 kDa HSP has a pI of 5.5 and the 100 kDa HSP has a pI of 5.7 - 5.8. The 90 kDa HSP family has members with pIs ranging from 4.5 to 5.4, the 70 kDa HSP family has members with pIs from 5.3 to 5.8, and the 65 kDa HSP family has members with pIs of 5.3 to 5.5. A 26 kDa HSP was not detected on fluorograms of these 2-D gels.

In the aforementioned experiments, human peripheral blood

Figure 9. Fluorographic analysis (2-D) of the heat-shock response in human lymphocytes. In each case, lymphocytes were collected, placed at a control temperature (37°C), or heat-shocked at 41 or 43°C for 2 h and subsequently transferred to 37°C and labelled with ^{35}S-methionine for 2 h (see Materials and Methods). The lymphocytes were lysed and equal amounts of acid-precipitable radioactivity from the lysates were added to each first dimensional (IEF) gel; the second dimension SDS gel consisted of a 5-15% polyacrylamide gradient. Protein standards of known molecular mass (M_rs) are denoted on the left side of the figure ("STDs"). The M_rs shown on the right side of this figure denote the HSPs and a 42 kDa non-HSP; arrowheads indicate the position of the HSPs.


37 C


41 C


STDs
94
67
43
30
43 C
Mr
110
100
90
70
65
42

lymphocytes were isolated from blood samples donated by individuals aged 20 - 40 years. To further establish a human heat-shock response across the age spectrum, lymphocytes were isolated from blood samples received from individuals of various ages. These individuals were classified into the following groups: premature infants (less than 38 weeks gestation at birth), full term infants, child, young adult (20 to 40 years old), and older adult.

Lymphocytes were isolated from whole blood samples on a discontinuous Ficoll gradient, incubated for 2 h at control (37°C) or heat-shock (41 or 43°C) temperatures, and then labelled with ^{35}S-methionine for 2 h at 37°C. Figure 10 depicts fluorograms of 1-D (3-15% gradient) SDS-polyacrylamide gels on which equal amounts of acid-precipitable radioactivity from the cell lysates were electrophoretically-separated. Fluorographic analysis shows that although cells from the different individuals express different patterns of protein synthesis at 37°C, cells from all individuals synthesize the 110, 100, 90, 70, and 65 kDa HSPs following incubation at 41 or 43°C.

3.1.2 Comparison of the Heat-Shock Response of Human Lymphocytes with Human Myeloma Cells.

To compare the heat-shock response between non-transformed and transformed cells, human lymphocytes isolated from whole blood and myeloma cells from a human breast carcinoma line (LOM-MLCR-HMy2) were maintained for 2 h at control (37°C) or heat-shock (43°C) temperatures, then labelled with ^{35}S-methionine for 2 h at 37°C. Figure 11 depicts 1-D and 2-D electrophoretic separations of

Figure 10. Fluorographic comparison (1-D) of the heat-shock response in human peripheral blood lymphocytes from individuals of various ages. Lymphocytes were isolated from whole blood samples (see Materials and Methods for details), incubated for 2 h at control (37°C) or heat-shock (41-43°C) temperatures, and then labelled with ^{35}S-methionine for 2 h at 37°C. The cells were lysed and equal amounts of acid-precipitable radioactivity from the lysates were added to each well of the 1-D SDS-polyacrylamide (3-15%) gels. The age group of the individual donating the blood sample is given above the respective fluorograms ("prem" refers to premature infant). The temperature of incubation prior to labelling is indicated below the gel lanes. The molecular masses (X 10^{-3}) indicated at the right of the figure denote the HSPs.

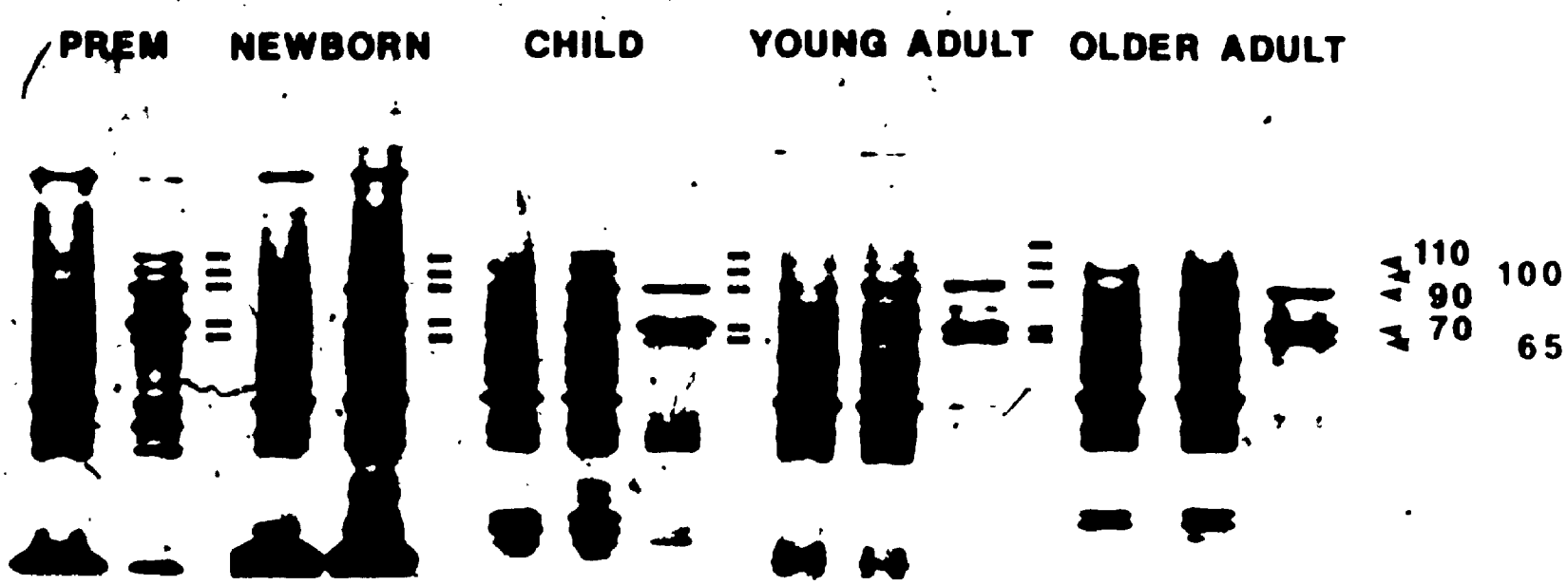
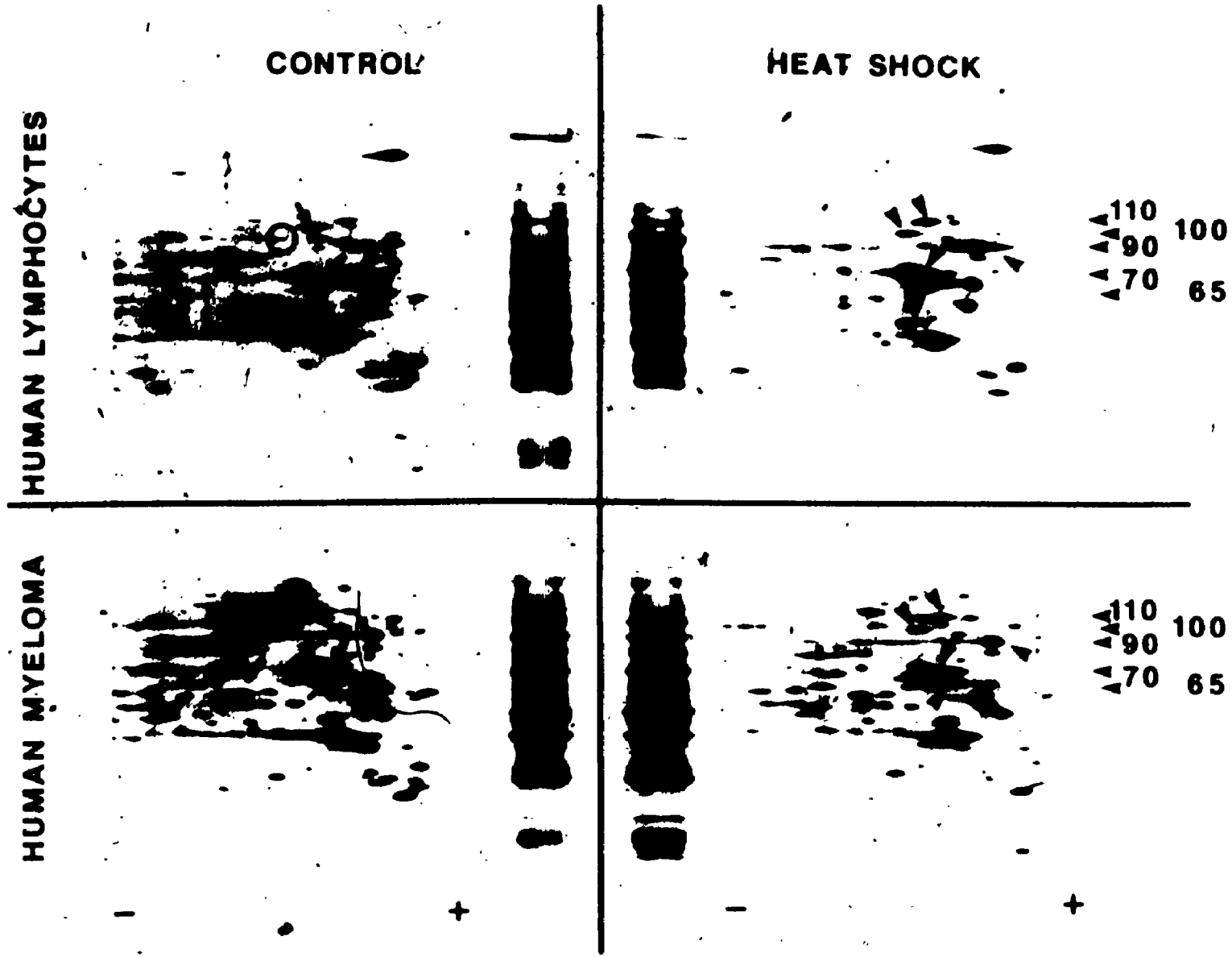

PREM
NEWBORN
CHILD
YOUNG ADULT
OLDER ADULT
110
90
70
100
65
37 43 37 43 37 41 43 37 41 43 37 41 43
TEMPERATURE (°C)

Figure 11. Fluorographic comparison (1-D and 2-D) of the heat-shock response in human lymphocytes and human myeloma cells. These cells were maintained for 2 h at control (37°C) or heat-shock (43°C) temperatures, then labelled for 2 h with ^{35}S-methionine at 37°C. Equal amounts of acid-precipitable radioactivity from the lysates were added to each well of the 1-D 5-15% SDS-polyacrylamide gels. For 2-D analysis equal amounts of acid-precipitable radioactivity were added to each first dimensional (IEF) gels and the second dimensional (SDS) gels consisted of a 7.5-17.5% polyacrylamide gradient. The panels on the right half of the figure indicate the heat-shock cells; on the left are cells maintained at 37°C prior to labelling. The molecular masses ($X\ 10^{-3}$) of the HSPs are given to the right of the figure; arrowheads indicate their position on the 2-D gels. HSPs synthesized constitutively in significant amounts at the control temperature are indicated by open arrows in the 2-D gels. Circled area (O) in the control gels corresponds to the area in which the 100 kDa HSP is located in the gels from lysates of heat-shocked cells.


CONTROL
HEAT SHOCK
HUMAN LYMPHOCYTES
HUMAN MYELOMA
110
90
70
100
65
110
90
70
100
65
−
+
−
+

lysates from these cells (equal amounts of acid-precipitable radioactivity from each lysate). A comparison of these fluorograms indicates that the heat-shocked human myeloma cells synthesize polypeptides of similar molecular masses and isoelectric points as the HSPs synthesized by heat-shocked human lymphocytes. Further inspection of these fluorograms suggests that transformed cells maintained at 37°C constitutively synthesize amounts of the 110, 90, 70 and 65 kDa HSPs approximating those found in their heat-shocked counterparts. To confirm this observation, areas of the 2-D gels corresponding to these HSPs were excised from the gels and the radioactivity incorporated into these spots was determined (not shown), as described in section 2.7.5. Quantification of radioactivity into these polypeptides showed that synthesis of the HSPs in heat-shocked myeloma cells increased only 0.2 - 0.5 times over levels found in myeloma cells maintained at a control temperature of 37°C. By comparison, heat-shocked lymphocytes exhibited a two to seven fold increase in HSP synthesis over levels found in lymphocytes maintained at 37°C.

3.1.3 Chemical Stress of Human Lymphocytes.

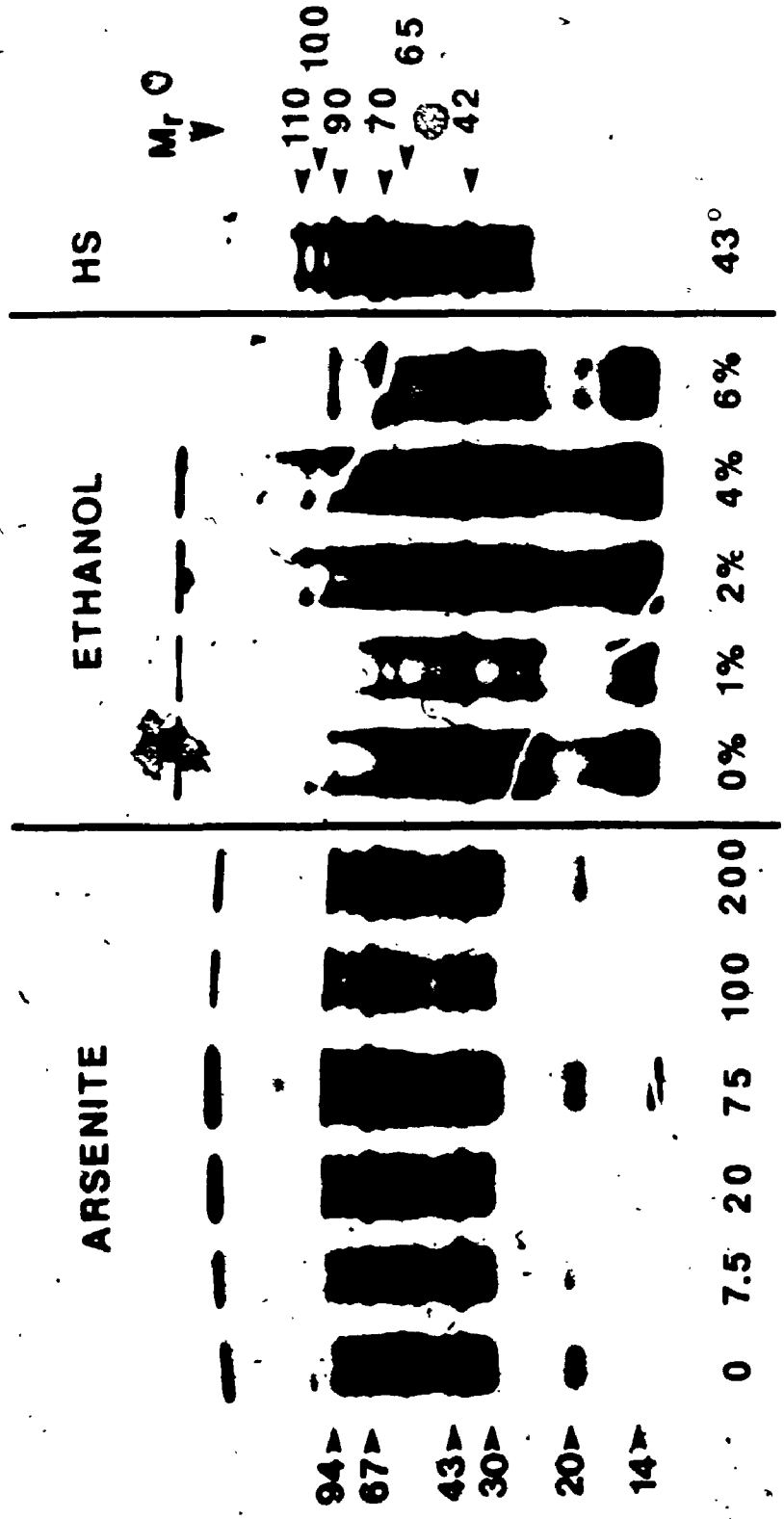
Having established that heat-shock alters gene expression in human lymphocytes, further experiments were performed to examine the response of these cells to chemical stresses such as ethanol or arsenite. Human lymphocytes were exposed to sodium arsenite, by incubating the cells for 1 h at 37°C with sodium arsenite at concentrations of 5, 7.5, 20, 75, 100, and 200 μM. The cells were then labelled with ^{35}S-methionine for 2 h at 37°C in the absence of

arsenite. Human lymphocytes were also exposed to another type of chemical stress, in the form of ethanol. Cells were incubated for 1 h at 37°C with 0, 1, 2, 4, or 6% ethanol, and then labelled with ^{35}S-methionine for 2 h at 37°C in the absence of ethanol.

Figure 12 depicts fluorograms of the 1-D gels on which lysates from arsenite-stressed and ethanol-stressed human lymphocytes were electrophoretically-separated. A short term arsenite-stress results in the enhanced or de novo synthesis (relative to amounts of other polypeptides) of a group of polypeptides with M_rs similar to the HSPs seen following heat-shock. These polypeptides have M_rs of 110, 90, 70, and 65 kDa. Enhanced synthesis of these polypeptides appears to be dependent on the concentration of arsenite present in the medium. No enhanced synthesis of HSP-like polypeptides is observed with arsenite levels less than 7.5 uM.

Lymphocytes treated with 6% ethanol display the enhanced or de novo synthesis of a set of polypeptides with M_rs of 110, 90, 70, and 65 kDa. No changes in the pattern of protein synthesis are seen with cells treated with ethanol concentrations less than 6% for the 1 h period at 37°C. These ethanol-stress induced polypeptides are similar in M_r to the HSPs synthesized by heat-shocked and arsenite-stressed lymphocytes. As with arsenite-stress, ethanol-stress of human lymphocytes does not result in the enhanced synthesis of a 100 kDa polypeptide similar to the 100 kDa HSP synthesized following heat-shock. These results show that short term exposure to chemical stresses such as arsenite or ethanol can induce changes in the pattern of protein synthesis in human lymphocytes. These changes involve the enhanced or de novo

Figure 12. Comparison of fluorographic results (1-D) obtained from arsenite-, ethanol-stressed and heat-shocked (HS) human lymphocytes. Cells were incubated for 1 h at 37°C in media containing various concentrations of sodium arsenite (in uM) or ethanol (at the concentrations given in the figure). Heat-shocked (HS) cells were incubated for 1 h at 43°C. Following these treatments, cells were radioactively labelled with ^{35}S-methionine at 37°C for 1 h in the absence of ethanol or arsenite. Equal amounts of acid-precipitable radioactivity from the lysates were added to each well of the 1-D SDS-polyacrylamide gels (3-15% gradient for arsenite-stressed cells; 5-15% gradient for ethanol-stressed cells). The M_rs (X 10^{-3}) of protein standards are denoted on the left of the figure. The M_rs (X 10^{-3}) shown on the right of the figure denote the HSPs (or HSP-like polypeptides induced by chemical-stress), and a 42 kDa non-HSP.


HS
Mr
0
110
90
70
65
42
100
43°
ETHANOL
0%
1%
2%
4%
6%
ARSENITE
0
7.5
20
75
100
200
94
67
43
30
20
14

synthesis of HSP-like polypeptides similar in M_r to most of the HSPs induced by heat-shock.

3.2 Changes in Gene Expression of Mouse Cells in Response to Heat-Shock and Chemical-Stress.

Following the establishment of a heat-shock and chemical-stress response in human lymphocytes, experiments were performed using mouse spleen cells and lymphocytes to further investigate the heat-shock and chemical-stress response in mammalian cells.

In the following section of this thesis, I will describe the response of mouse spleen cells to short term *in vitro* and *in vivo* heat-shock. I will present data outlining the response of mouse spleen cells to chemical-stress and to concurrent chemical-stress and heat-shock *in vitro*, and I will present results which compare the heat-shock response of mouse cells with human and rabbit lymphocytes.

3.2.1 Heat-Shock of Mouse Spleen Cells: in vitro Studies.

Mouse spleen cells are easily obtained and manipulated in culture, and consist of a differentiating population of cells which are active in protein synthesis. A light microscope examination of spleen cell preparations (Figure 4 D and E), reveals a mixed population of lymphocytes, erythrocytes and other blood elements, including eosinophils, basophils, and monocytes.

Mouse spleen cells from several inbred strains of mice were heat-shocked to determine if different strains express different

heat-shock polypeptides (HSPs) in response to thermal stress. Figure 13 depicts fluorograms of 1-D gels of electrophoretically-separated polypeptides from mouse spleen cells incubated at a control (C) temperature (37°C), or heat-shocked (HS) at 43°C for 2 h, and subsequently labelled with ^{35}S-methionine at 37°C for 2 h. Equal amounts of acid-precipitable radioactivity from the spleen cell lysates were applied to the gels. Fluorographic analysis of these 1-D gels shows that although spleen cells from different strains of mice express different patterns of protein synthesis at 37°C, spleen cells incubated at an elevated temperature (43°C) from all of the mouse strains examined exhibit a similar altered pattern of gene expression when compared to cells from the same strains which were maintained at 37°C. This involves the enhanced or de novo synthesis (relative to the amounts of other polypeptides) of a select group of polypeptides (HSPs), with relative molecular masses (M_rs) of 110 000, 100 000, 90 000, 70 000, and 65 000 daltons (Figure 13). Since no differences were found in the HSP response of spleen cells from these mouse strains, all subsequent experiments were performed with lymphocytes isolated from BALB/c mice.

A comparison of the fluorographic results obtained from using ^{35}S-methionine or ^{14}C-leucine as the radioactive label (Figure 14), reveals that HSPs with the same M_rs are detectable whether either amino acid is used. The 100 kDa HSP readily apparent by ^{35}S-methionine labelling is not as apparent with ^{14}C-leucine labelling. Enhanced and/or de novo synthesis of a 26 kDa HSP (evident in human lymphocytes labelled with ^{14}C-leucine) is not seen in mouse spleen cells labelled with either radioactive amino acid.

Figure 13. Fluorographic analysis of the heat-shock response in spleen cells obtained from various mouse strains. In each case, spleen cells were collected, placed at a control (C) temperature (37°C), or heat-shocked (HS) at 43°C for 2 h and subsequently transferred to 37°C and labelled with ^{35}S-methionine for 2 h (see Materials and Methods). The spleen cells were lysed and equal amounts of acid-precipitable radioactivity from the lysates were added to each well of the 1-D SDS-polyacrylamide (5-15% gradient) gel. The mouse strains are named at the bottom of the figure. Protein standards of known molecular mass (M_rs) are denoted on the left side of the figure ("STDs"). The M_rs (X 10^{-3}) shown on the right side of this figure denote the HSPs and a 42 kDa non-HSP.

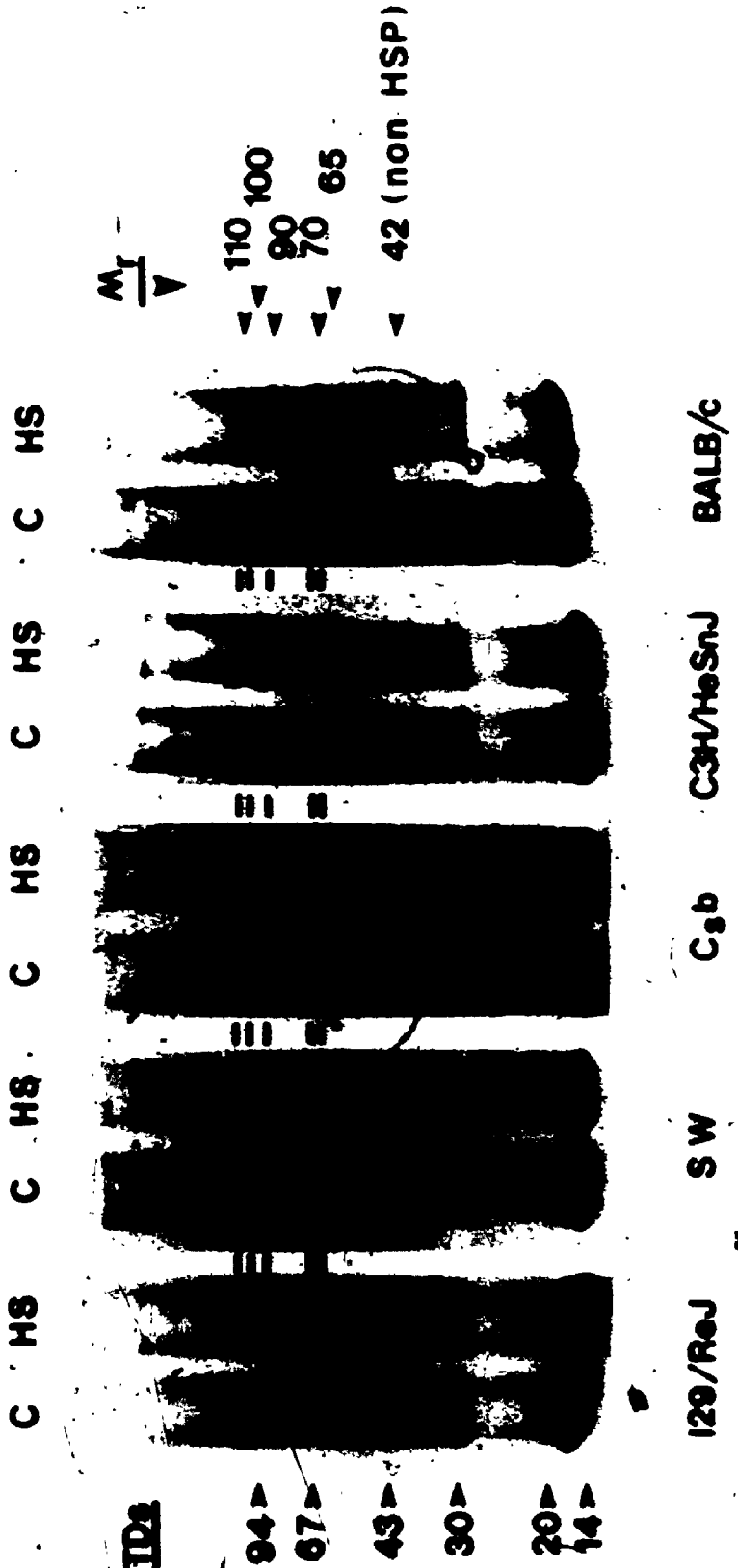
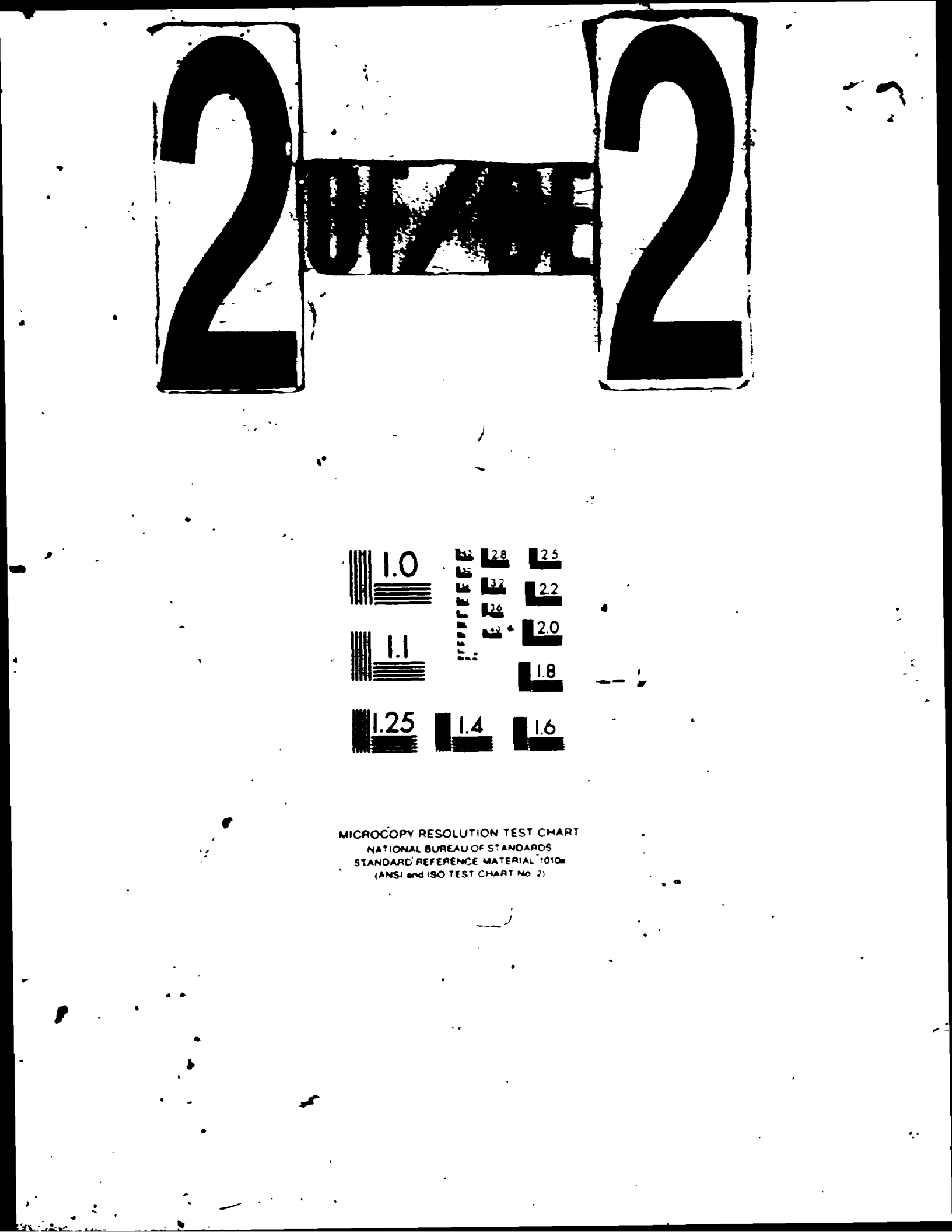
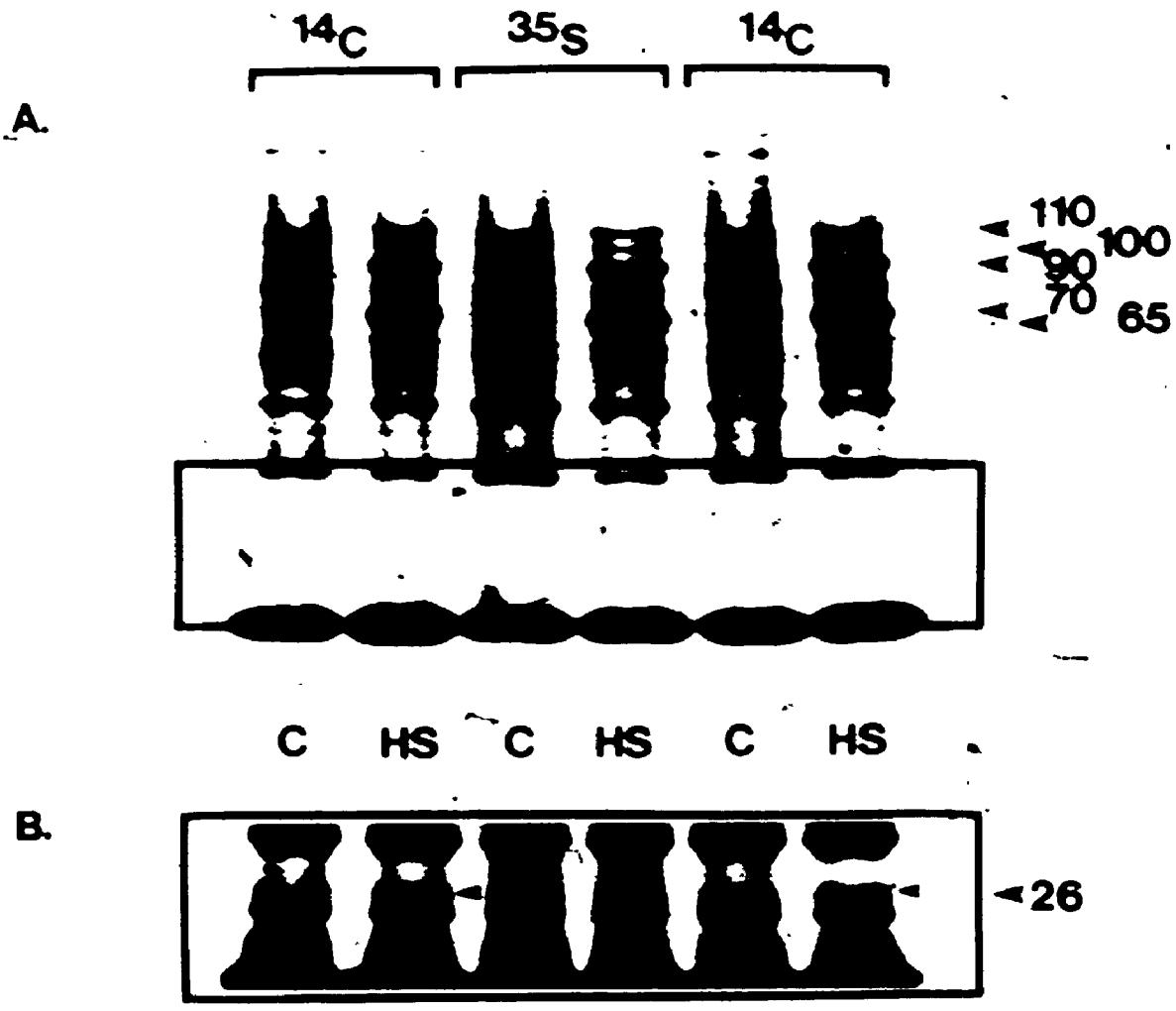

M_r
110
100
90
70
65
42 (non HSP)
C HS C HS C HS C HS C HS
129/RoJ SW C3b C3H/HeSnJ BALB/c
STDs
94
67
43
30
20
14

Figure 14. Comparison of fluorographic results obtained from using ^{14}C-leucine or ^{35}S-methionine for radioactive labelling of polypeptides synthesized by control (C) and heat-shocked (HS) mouse spleen cells. Cells were maintained at 37°C or 43°C for 2 h, prior to radiolabelling at 37°C for 2 h. Equal amounts of acid-precipitable radioactivity from the cell lysates were added to each well of the 1-D SDS-polyacrylamide (5-15% gradient) gel.

(A) depicts the fluorogram of the 1-D gel. In (B), inspection of an overexposed fluorogram of the region enclosed by the box in (A) reveals the slightly enhanced synthesis of a 26 kDa HSP (arrowheads) by heat-shocked cells labelled with ^{14}C-leucine. The relative molecular masses (X 10^{-3}) shown on the right side of the Figure denote the HSPs.


2 OF 2
1.0
1.1
1.25
1.4
1.6
2.8
3.2
3.6
2.5
2.2
2.0
1.8
MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)


14C
35S
14C
A.
110
100
90
70
65
C
HS
C
HS
C
HS
B.
26

To determine the minimum time required to induce HSP synthesis at a particular temperature, spleen cells from BALB/c mice were incubated for 15, 30, 60, and 90 min at control (37°C) or heat-shock (41°C or 43°C) temperatures, and subsequently incubated for 2 h at 37°C in the presence of ^{35}S-methionine. Figures 15 and 16 depict fluorograms of the 1-D and 2-D electrophoretically-separated polypeptides of lysates from these cells. These fluorograms demonstrate that synthesis of the HSPs is non-coordinate, with the 2-D separations affording better resolution of the polypeptides synthesized. Fluorographic analysis of the 1-D gels shows that synthesis of the 90 kDa HSP is noticeably enhanced after 60 min at 41°C, while enhanced synthesis of the 110, 100, 70 and 65 kDa HSPs requires at least 90 min at this temperature. Fluorograms of the 2-D gels reveals that enhanced synthesis of the 100 kDa HSP can be detected easily following a 60 min incubation at 41°C (circled areas in Figure 16).

Analysis of proteins from lysates of spleen cells incubated at 43°C further demonstrates the noncoordinate synthesis of these HSPs, and also shows that enhanced HSP synthesis at a higher heat-shock temperature (43°C versus 41°C) occurs following a shorter time period at that higher temperature. One-dimensional analysis (Figure 15) reveals that the synthesis of 110, 90, 70, and 65 kDa HSPs is markedly enhanced after 30 min at 43°C (compared with 60 to 90 min at 41°C) while new and/or enhanced synthesis of the 100 kDa HSP would appear to require at least 60 min at 43°C (compared with 90 min at 41°C). Again, the resolving power of the 2-D gels detects the presence of the 100 kDa HSP in earlier samples

Figure 15. Fluorographic analysis (1-D) of the time required at heat-shock temperatures (41°C and 43°C) to elicit a HS response from mouse spleen cells. In each case, spleen cells were collected, incubated for 15, 30, 60, or 90 min at 37°C or the heat-shock temperatures indicated at the top of the figure, then labelled with ^{35}S-methionine for 2 h at 37°C. Equal amounts of acid-precipitable radioactivity from the lysates were added to each well of an 1-D SDS-polyacrylamide (5-15% gradient) gel. Protein standards of known molecular mass (M_rs given in kilodaltons) are denoted on the left side of the figure. The M_rs ($\times 10^{-3}$) shown on the right side denote the HSPs and a 42 kDa non-HSP.

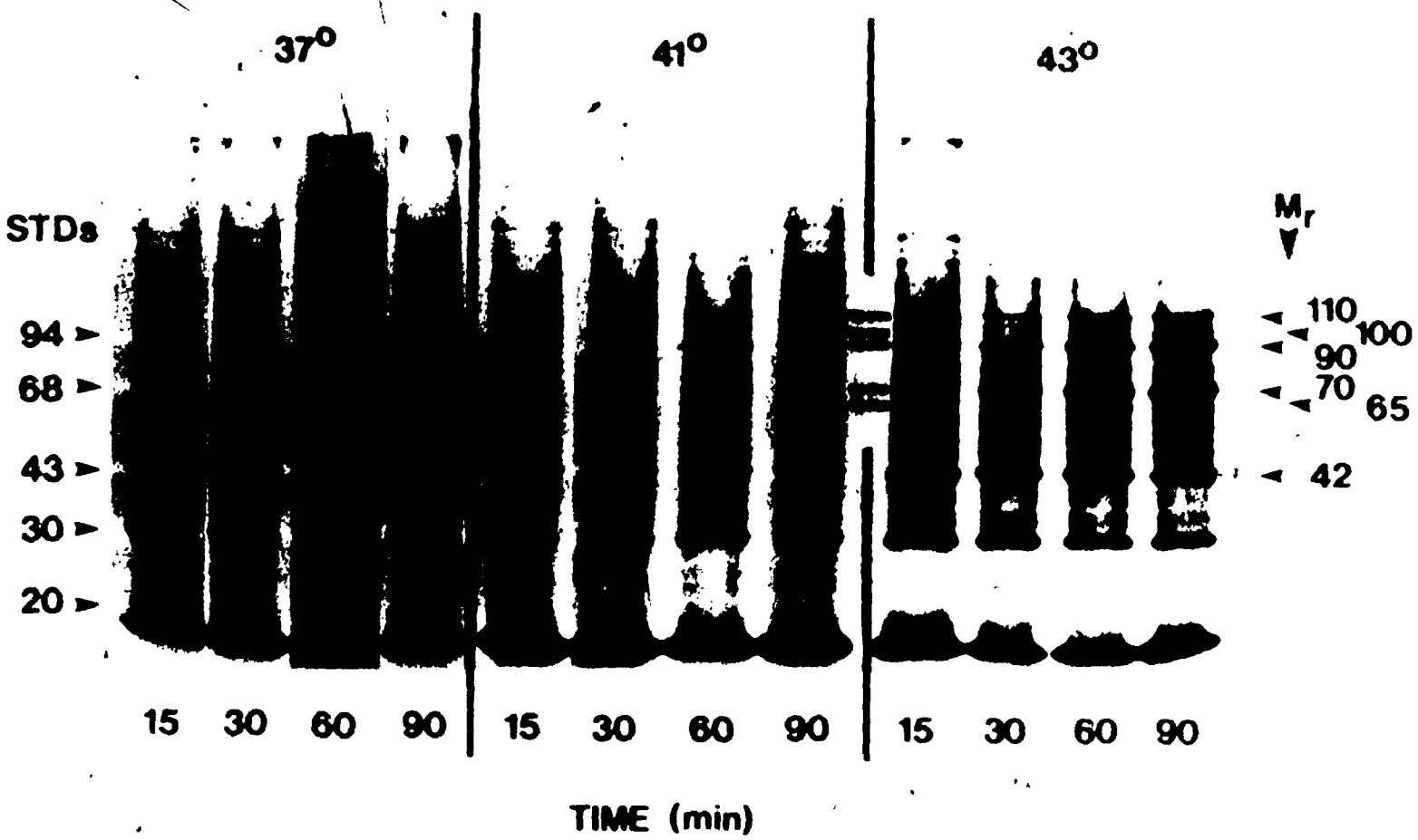
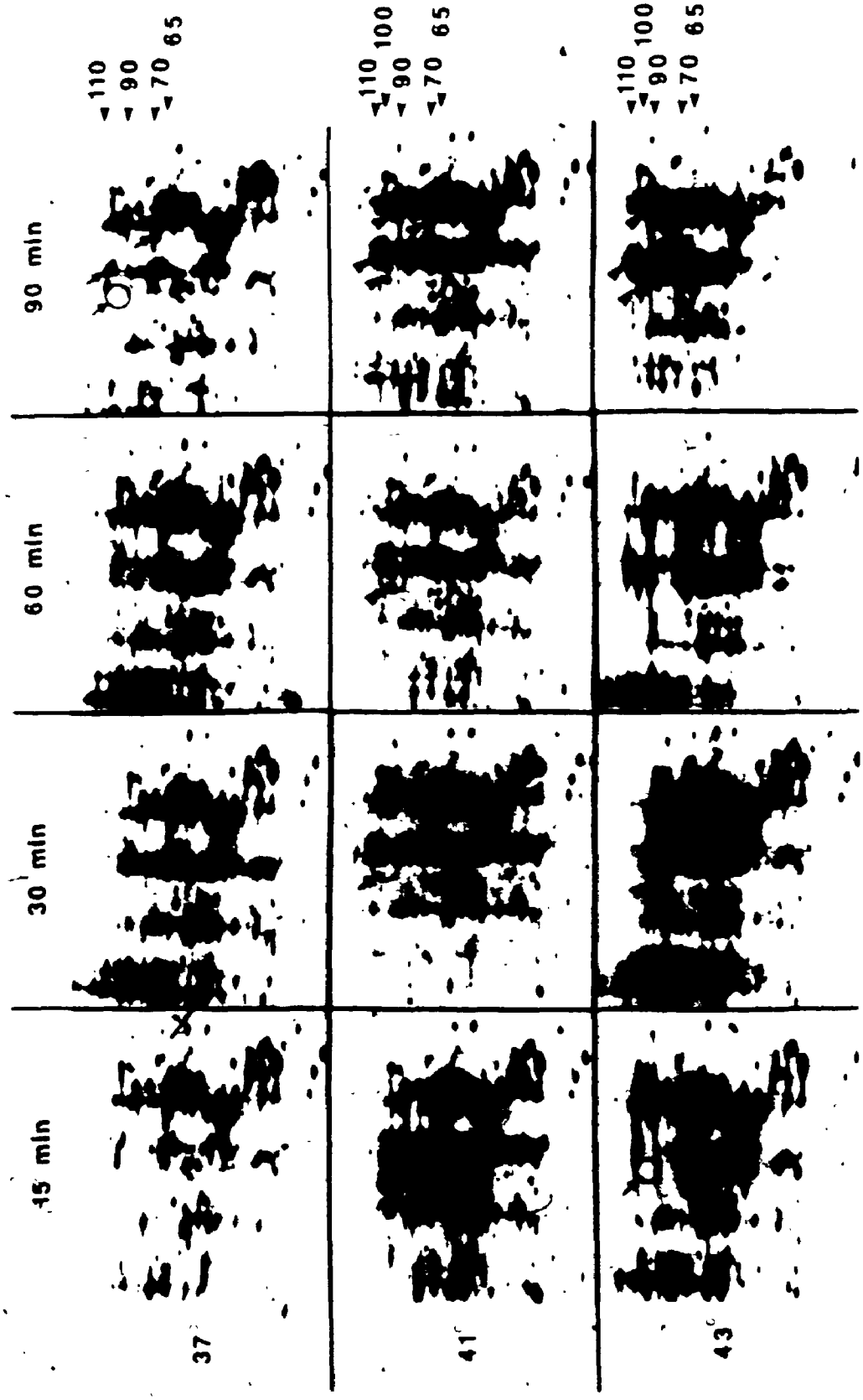

37°
41°
43°
STDs
94
68
43
30
20
Mr
110
100
90
70
65
42
15 30 60 90 15 30 60 90 15 30 60 90
TIME (min)

Figure 16. Fluorographic analysis (2-D) of the time required at heat-shock temperatures (41°C and 43°C) to elicit a HS response from mouse spleen cells. In each case, spleen cells were collected, incubated for 15, 30, 60, or 90 min at control (37°C) or heat-shock (41 or 43°C) temperatures, then labelled with ^{35}S-methionine for 2 h at 37°C. Equal amounts of acid-precipitable radioactivity from the lysates were added to each first dimensional (IEF) gel; the second dimension SDS gel consisted of a 7.5-17.5% polyacrylamide gradient. The M_rs (X 10^{-3}) shown on the right side denote the HSPs (arrowheads on figure). The open arrows indicate the positions of constitutively synthesized HSPs at 37°C; circles accompanied with an arrowhead indicate the 100 kDa HSP, while those circles accompanied with an open arrow indicate the position the 100 kDa HSP would occupy if it were present.


110
90
70
65
110
100
90
70
65
110
100
90
70
65
90 min
60 min
30 min
15 min
37°
41°
43°

than do the 1-D gels; it is evident in the 2-D gels after 30 min at 43°C.

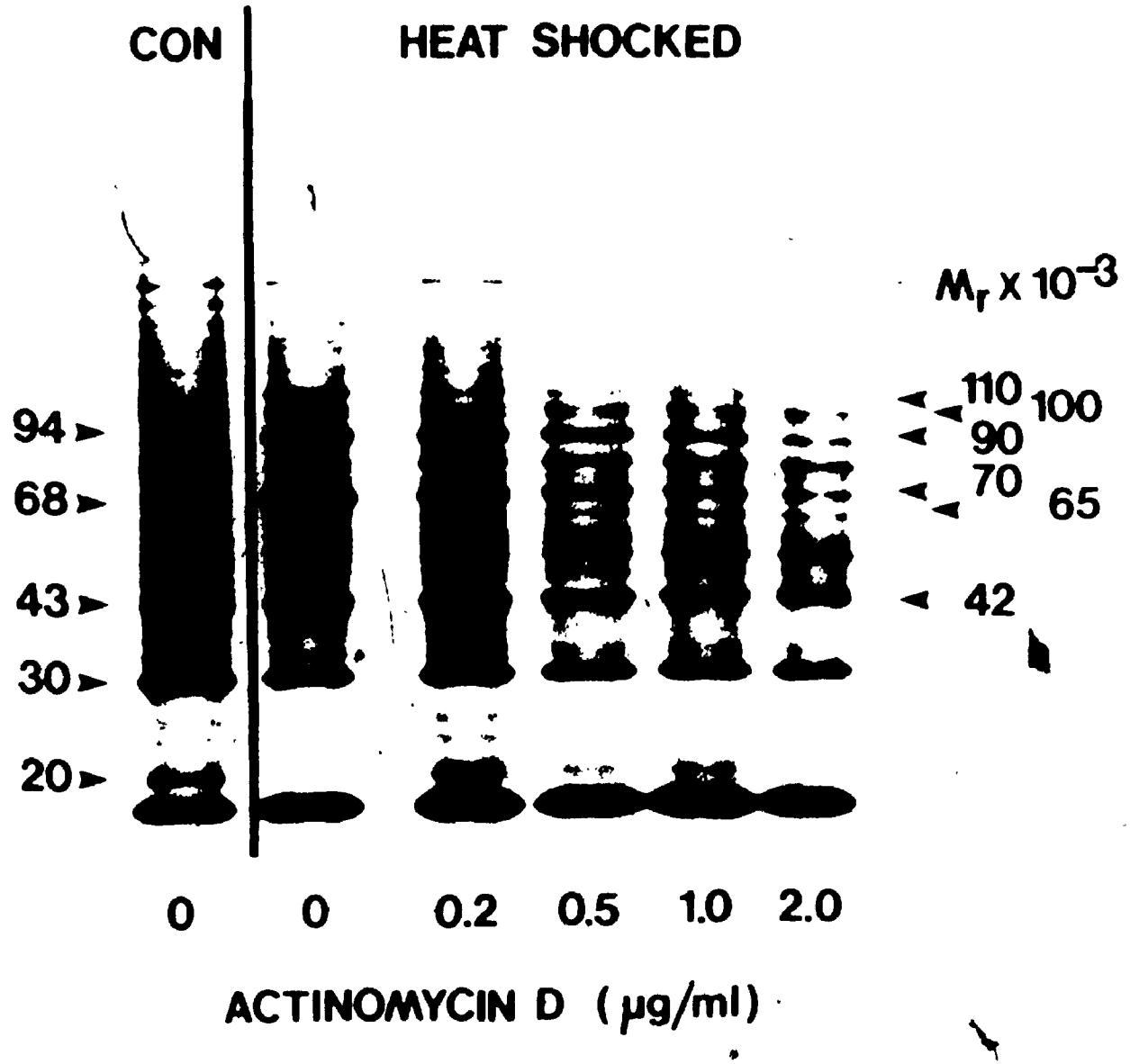
3.2.2 The Effect of Actinomycin-D Treatment on HSP Synthesis:

To determine if HSP synthesis is dependent on new transcription, spleen cells were heat-shocked for 1 h at 43°C, in the presence of the RNA polymerase inhibitor Actinomycin-D at concentrations of 0.2, 0.5, 1.0, and 2.0 uM. The cells were then labelled with ^{35}S-methionine for 1 h at 37°C. Figure 17 depicts the fluorogram of a 1-D gel on which equal amounts of acid-precipitable radioactivity from the cell lysates were electrophoretically-separated. Fluorographic analysis reveals that Actinomycin-D inhibits HSP synthesis in a dose-dependent manner. As the concentration of Actinomycin-D added to the cell suspensions increases, the relative amounts of the HSPs being synthesized by these cells decreases. Heat-shocked cells treated with 2.0 μM Actinomycin-D exhibit a marked decrease in HSP synthesis, in comparison with heat-shocked cells treated with lesser amounts of Actinomycin-D, and also possess a pattern of protein synthesis similar to control cells maintained at 37°C. These results suggest that new transcription is required for HSP synthesis.

3.2.3 Recovery from Heat-Shock in Mouse Spleen B Cells.

Experiments were performed to analyze the patterns of protein synthesis in mouse cells allowed to recover from heat-shock prior to labelling. B cells were purified from mouse spleen cell suspensions (see section 2.3.5) and placed for 1 h at a control

Figure 17. Fluorographic analysis (1-D) of heat-shocked mouse spleen cells treated with Actinomycin-D. Spleen cells were heat-shocked for 1 h at 43°C in the presence of Actinomycin-D (at the concentrations shown), then radioactively labelled for 1h at 37°C with ^{35}S-methionine, in the presence of Actinomycin-D. Equal amounts of acid-precipitable radioactivity from the lysates were added to each well of the SDS-polyacrylamide (5-15% gradient) gel. Lysates from control (CON) and heat-shocked cells which were not treated with Actinomycin-D are shown in the two lanes on the left of this figure (lanes marked "O"). Relative molecular masses (M_rs) of the HSPs are shown on the right side of this figure.


CON
HEAT SHOCKED
$M_r \times 10^{-3}$
94
68
43
30
20
110
100
90
70
65
42
0
0
0.2
0.5
1.0
2.0
ACTINOMYCIN D (μg/ml)

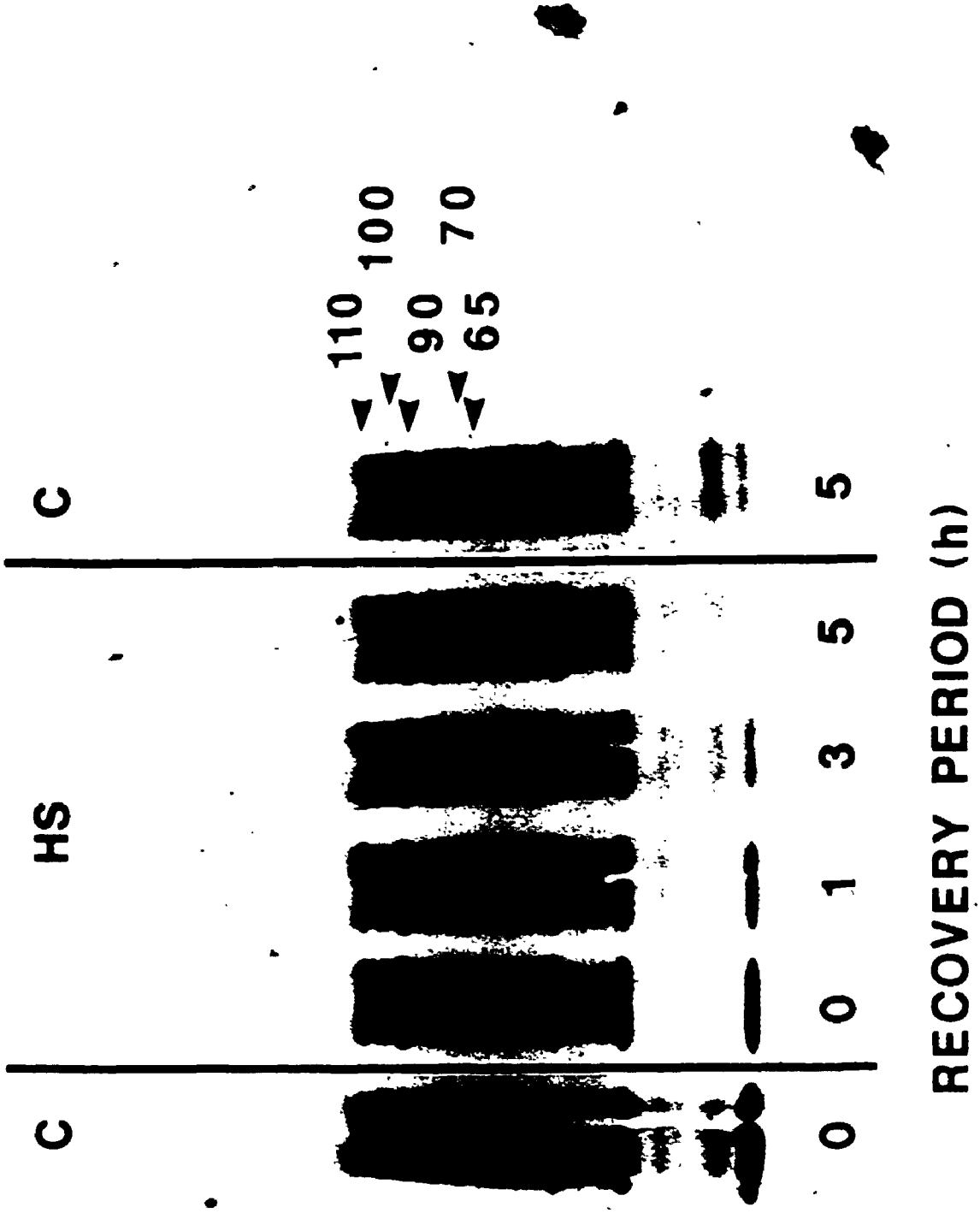
temperature (37°C), or heat-shocked at 43°C. The heat-shocked cells were then returned to 37°C for various time periods (0, 1, 3, and 5 h). Following this recovery period, the cells were collected by centrifugation, resuspended in methionine-free MEM and labelled with ^{35}S-methionine for 2 h at 37°C. Figure 18 depicts the fluorogram of a 1-D gel on which equal amounts of radioactivity from the cell lysates were electrophoretically-separated.

Fluorographic analysis reveals that mouse B cells do recover from heat-shock and that recovered cells possess a pattern of protein synthesis which resembles the pattern seen in control cells. Following a 1 h heat-shock (HS; lane "0"), mouse B cells exhibit the enhanced synthesis of HSPs with M_rs of 110, 100, 90, 70, and 65 kDa. Heat-shocked cells allowed to recover at 37°C for 1 h prior to labelling (HS; lane "1") still synthesize HSPs at enhanced levels. However, cells removed from heat-shock and allowed to recover at 37°C for 3 h or 5 h prior to labelling (HS; lanes "3" and "5") no longer exhibit the enhanced synthesis of HSPs, but possess a pattern of protein synthesis similar to that seen in control cells maintained for 5 h at 37°C (C; lane "5") prior to labelling. These results suggest that mouse cells can recover from heat-shock, that the rapid enhanced or de novo synthesis of the HSPs is transient, and that these cells can re-establish a "normal" pattern of protein synthesis following heat-shock.

3.2.4 Heat-Shock of Mouse Spleen Cells: in vivo studies.

Following the completion of the previously described experiments, which established that mouse spleen cells heat-shocked in

Figure 18. Fluorographic analysis of the protein synthesis during recovery from heat-shock. B cells were isolated from the spleen cells of mice, and were maintained at the control temperature (37°C; lanes "C"), or heat-shocked (lanes "HS") for 1 h at 43°C. Heat-shocked cells were then returned to 37°C for various time periods (0-5 h), prior to labelling with ^{35}S-methionine for 2 h at 37°C. Control cells were either labelled immediately after the 1 h incubation at 37°C (C, lane 0), or kept an additional 5 h at 37°C prior to labelling (C, lane 5). The cells were then lysed and equal amounts of acid-precipitable radioactivity from the lysates were added to each well of the 1-D SDS-polyacrylamide (5-15% gradient) gel. The M_rs (X 10^{-3}) shown on the right side of this figure denote the HSPs.

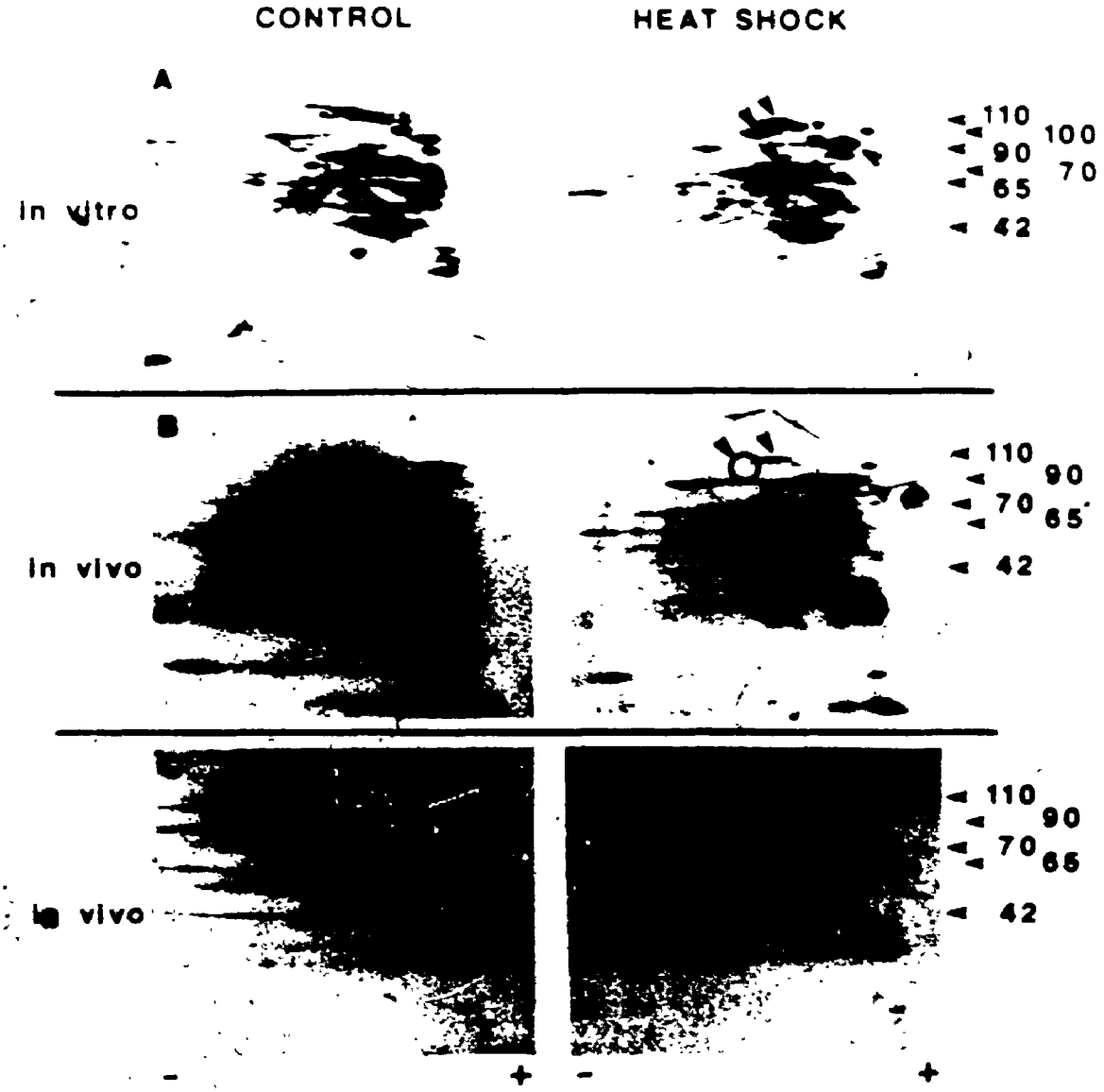

110
100
90
70
65
C
HS
C
0
1
3
5
0
5
RECOVERY PERIOD (h)

vitro exhibit enhanced or *de novo* HSP synthesis, experiments were performed to determine if spleen cells from mice subjected to brief periods of thermal stress exhibit changes in gene expression *in situ* similar to those seen with spleen cells heat-shocked *in vitro*.

Figure 19 shows fluorograms depicting (A) the polypeptides synthesized by cultured mouse spleen cells, and (B and C) the polytides synthesized by spleen cells from control mice (maintained at 37°C), or mice which were thermally stressed to raise their core temperature to 41°C for a period of 20 min (B and C). Cultured mouse cells shown in (A) were maintained at 37°C or heat-shocked for 1 h at 43°C, then labelled with ^{35}S-methionine for 2 h at 37°C. The spleen cells shown in (B) were isolated from control and hyperthermic mice and labelled for 2 h in culture at 37°C. In (C), spleen cells from control and hyperthermic mice were labelled *in vivo*; that is, following the 20 min period of induced hyperthermia, the mice were injected with 30 uCi of ^{35}S-methionine and labelled for 2 h. Following this time, the mice were sacrificed and their spleen cells collected and lysed.

Spleen cells from hyperthermic mice (B and C) exhibit enhanced or *de novo* synthesis of polypeptides with molecular masses and isoelectric points similar to the HSPs synthesized by spleen cells heat-shocked and labelled *in vitro* (A). These include the 110 kDa, 90 kDa, 70 kDa, and 65 kDa HSPs. Synthesis of a 100 kDa HSP was not observed in cells from hyperthermic mice. No differences in the patterns of HSP synthesis are seen between spleen cells from hyperthermic mice labelled in culture (B:heat-shock), and labelled *in situ* (C:heat-shock). Except for the application of heat, mice used

Figure 19. Fluorographic comparisons (2-D) of the polypeptides synthesized by cultured mouse spleen cells maintained at 37°C or heat-shocked for 1 h at 43°C (A), with the polypeptides synthesized by spleen cells from mice which were maintained at 37°C or which were thermally stressed to raise their core temperature to 41°C for a period of 20-30 minutes (B and C). Cultured mouse cells (A) were maintained at 37 or 43°C for 1 h and then labelled with ^{35}S-methionine for 2 h at 37°C. In (B), spleen cells isolated from control and hyperthermic mice were labelled in culture for 2 h at 37°C. In (C), spleen cells from control and hyperthermic were labelled in situ; that is, following the 20 min period of induced hyperthermia, the mice were injected with ^{35}S-methionine, labelled for 2 h, then sacrificed and their spleen cells collected and lysed. Equal amounts of acid-precipitable radioactivity from the lysates were added to each first dimensional (IEF) gel; the second dimension SDS gel consisted of a 5-15% polyacrylamide gradient. The numbers shown on the right side of this figure denote the M_rs (X 10^{-3}) of the HSPs and a 42 kDa non-HSP. The position the 100 kDa HSP would occupy (if present) is indicated by the circle, and the arrowheads denote the HSPs.


CONTROL
HEAT SHOCK
A
In vitro
110
90
65
42
100
70
B
In vivo
110
90
70
65
42
In vivo
110
90
70
65
42
-
+
-
+

as controls in these experiments were treated in the same manner as the thermally-stressed mice. The differences observed in gene expression between cells from control and thermally-stressed mice can therefore be attributed to the effects of thermal stress on the intact mouse.

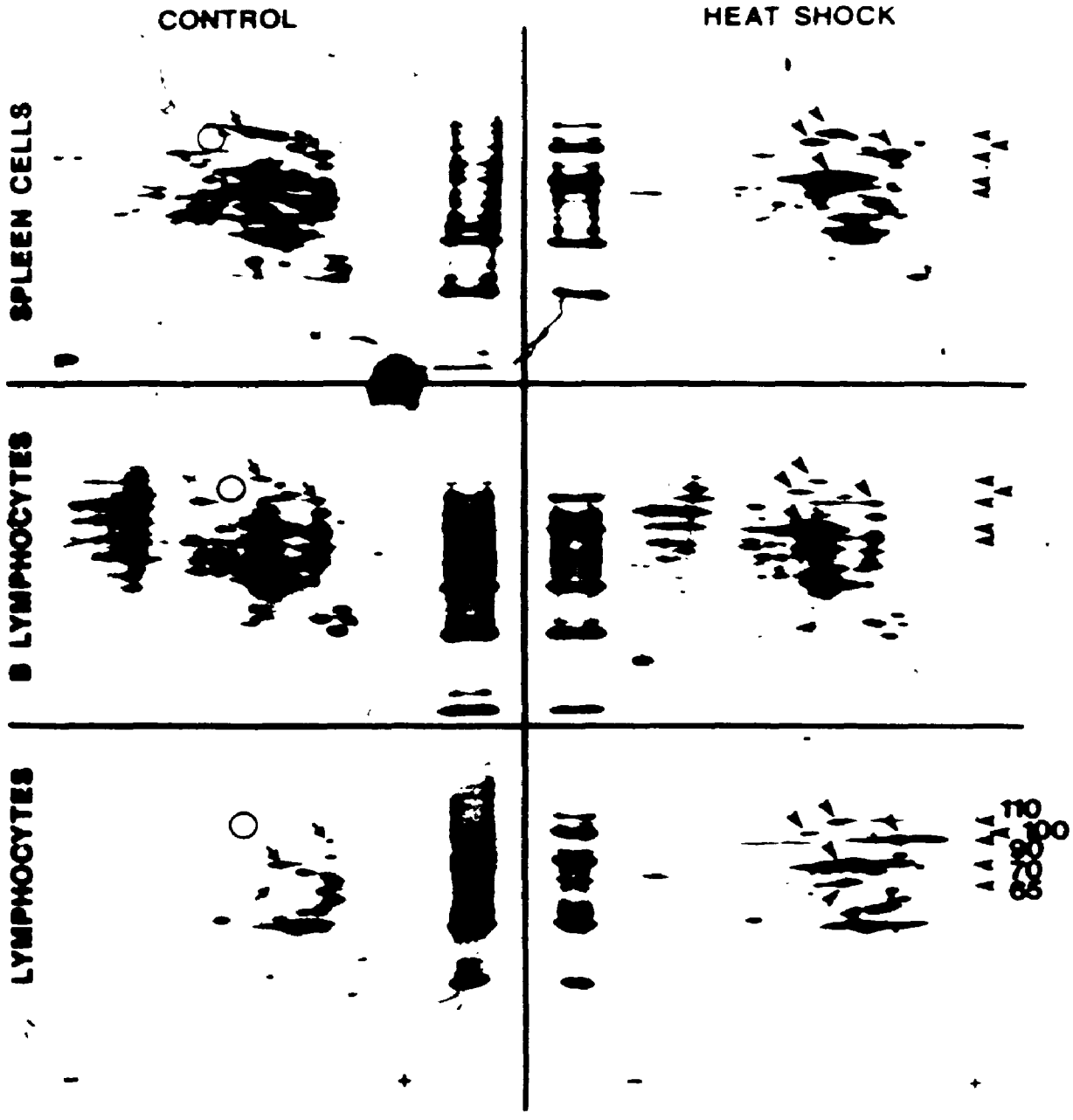
3.2.5 Comparison of the Heat-Shock Response in Mouse, Rabbit and Human Lymphocytes and with Mouse and Human Myeloma Cells.

To assess the generality of the heat-shock response in mammalian lymphocytes, cells from several mammalian sources were heat-shocked and their polypeptide synthesis patterns compared. As well, to compare the heat-shock response of non-transformed cells with transformed cell types, mouse spleen cells were heat-shocked, and their patterns of protein synthesis compared with heat-shocked mouse myeloma cells.

3.2.5.1 Comparison of the Heat-shock Response in several Mouse Cell Types.

A comparison of the HSPs synthesized by mouse spleen cells, mouse whole blood lymphocytes, and B lymphocytes isolated from mouse spleen are depicted in the fluorograms depicted in Figure 20. In each case, cells were incubated for 2 h at control (37°C) or heat-shock (43°C) temperatures and then labelled with ^{35}S-methionine for 2 h at 37°C. Equal amounts of acid-precipitable radioactivity from all lysates were applied to 1-D gels. Fluorographic analysis reveals that while each cell type expresses different patterns of protein synthesis at 37°C, all cell types

Figure 20. Fluorographic comparison of the HSPs synthesized by mouse spleen cells, mouse whole blood lymphocytes, and B lymphocytes isolated from mouse spleen. In each case, the cells were collected, placed at a control (37°C) or heat-shock (43°C) temperature for 2 h and subsequently transferred to 37°C and labelled with ^{35}S-methionine for 2 h. Equal amounts of acid-precipitable radioactivity from the lysates were added to each well of the 1-D 5-15% SDS-polyacrylamide gel. For 2-D analysis, equal amounts of acid-precipitable radioactivity were added to each first dimensional (IEF) gel and the second dimensional (SDS) gels consisted of a 7.5-17.5% polyacrylamide gradient. The panels on the right half of the figure indicate the heat-shock cells; on the left are cells maintained at 37°C prior to labelling. The molecular masses (X 10^{-3}) of the HSPs are given to the right of the figure; arrowheads indicate their position on the 2-D gels. The circle indicates the position the 100 kDa HSP would occupy if it were present.


CONTROL
HEAT SHOCK
SPLEEN CELLS
B LYMPHOCYTES
LYMPHOCYTES

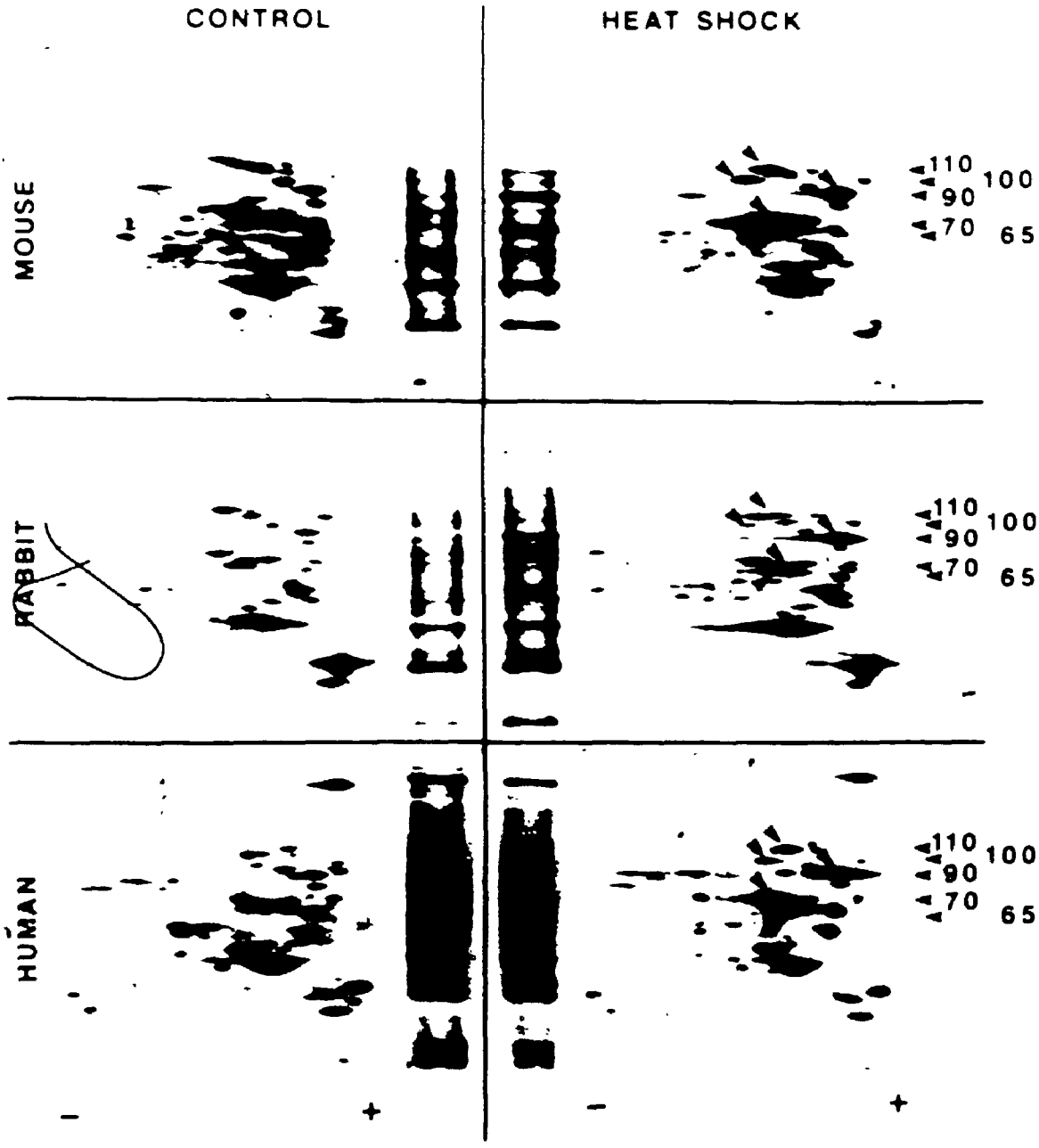
examined exhibit a similar altered pattern of gene expression in response to heat-shock: the enhanced synthesis of HSPs with molecular masses and isoelectric points of 110 kDa (pI 5.5), 100 kDa (pI 5.8), 90 kDa (pI 4.9 - 5.2), 70 kDa (pI 5.3 - 5.8), and 65 kDa (pI 5.3 - 5.5).

3.2.5.2 Comparison of the Heat-Shock Response in Mouse, Rabbit, and Human Lymphocytes.

The heat-shock response observed in mouse cells was also compared with that seen in lymphocytes isolated from rabbit and human whole blood. These cells were maintained in culture for 2 h at control (37°C) or heat-shock (43°C) temperatures, and were labelled with ^{35}S-methionine for 2 h at 37 or 43°C. Equal amounts of acid-precipitable radioactivity from the cell lysates were loaded on 1-D and 2-D gels. Fluorograms of these gels of control and heat-shocked mouse spleen cells, and rabbit and human lymphocytes are shown in Figure 21.

In general, similar patterns of HSP synthesis are observed in lymphocytes from each of the three species, although some differences do occur. Heat-shocking rabbit and mouse lymphocytes does stimulate enhanced or de novo synthesis of polypeptides with molecular masses and isoelectric points similar, if not identical, to the HSPs synthesized by the human cells. Neither mouse cells (radiolabelled with ^{35}S-methionine or ^{14}C-leucine) nor rabbit cells (radiolabelled with ^{35}S-methionine) appear to synthesize a 26 kDa HSP similar to that seen with human cells. A list of mammalian lymphocyte HSPs can be summarized (see also Table 1) as consisting

Figure 21. Fluorographic comparison (1-D and 2-D) of the heat-shock response in mouse spleen cells, rabbit lymphocytes, and human lymphocytes. These cells were maintained for 2 h at control (37°C) or heat-shock (43°C) temperatures, then labelled for 2 h with ^{35}S-methionine at 37 or 43°C. Equal amounts of acid-precipitable radioactivity from the lysates were added to each well of the 1-D 5-15% SDS-polyacrylamide gels. For 2-D analysis equal amounts of acid-precipitable radioactivity were added to each first dimensional (IEF) gel and the second dimensional (SDS) gels consisted of a 7.5-17.5% polyacrylamide gradient. The panels on the right half of the figure indicate the heat-shock cells; on the left are cells maintained at 37°C prior to labelling. The molecular masses ($\times 10^{-3}$) of the HSPs are given to the right of the figure; arrowheads indicate their position on the 2-D gels.


CONTROL
HEAT SHOCK
MOUSE
RABBIT
HUMAN
110
90
70
100
65
110
90
70
100
65
110
90
70
100
65
−
+
−
+

of 110 kDa (pI 5.5), 100 kDa (pI 5.7), 90 kDa (pI 4.8-5.4), 70 kDa (pI 5.3-5.8), 65 kDa (pI 5.3-5.5) and 26 kDa (human only) HSPs.

3.2.5.3 Comparison of the Heat-Shock Response in Mouse Spleen Cells and Mouse Myeloma Cells.

Mouse spleen cells and mouse myeloma cells (Sp 2/0-Ag 14) were maintained for 2 h at control (37°C) or heat-shock (43°C) temperatures, then labelled with ^{35}S-methionine for 2 h at 37°C. Figure 22 depicts 1-D and 2-D electrophoretic separations of lysates from these cells. A comparison of these fluorograms indicates that the heat-shocked mouse myeloma cells synthesize polypeptides of similar molecular masses and isoelectric points as the HSPs synthesized by heat-shocked mouse spleen cells. The fluorograms in Figure 22 also suggest that transformed cells of murine origin maintained at 37°C constitutively synthesize the 90, 70, and 65 kDa HSPs at levels approximating those found in their heat-shocked counterparts. Results similar to these were presented in Figure 11 with the comparison of the heat-shock response in human lymphocytes and human myeloma cells. In these experiments as well, human myeloma cells maintained at control temperatures constitutively synthesized HSPs at levels approximating those found in human myeloma cells which had been heat-shocked.

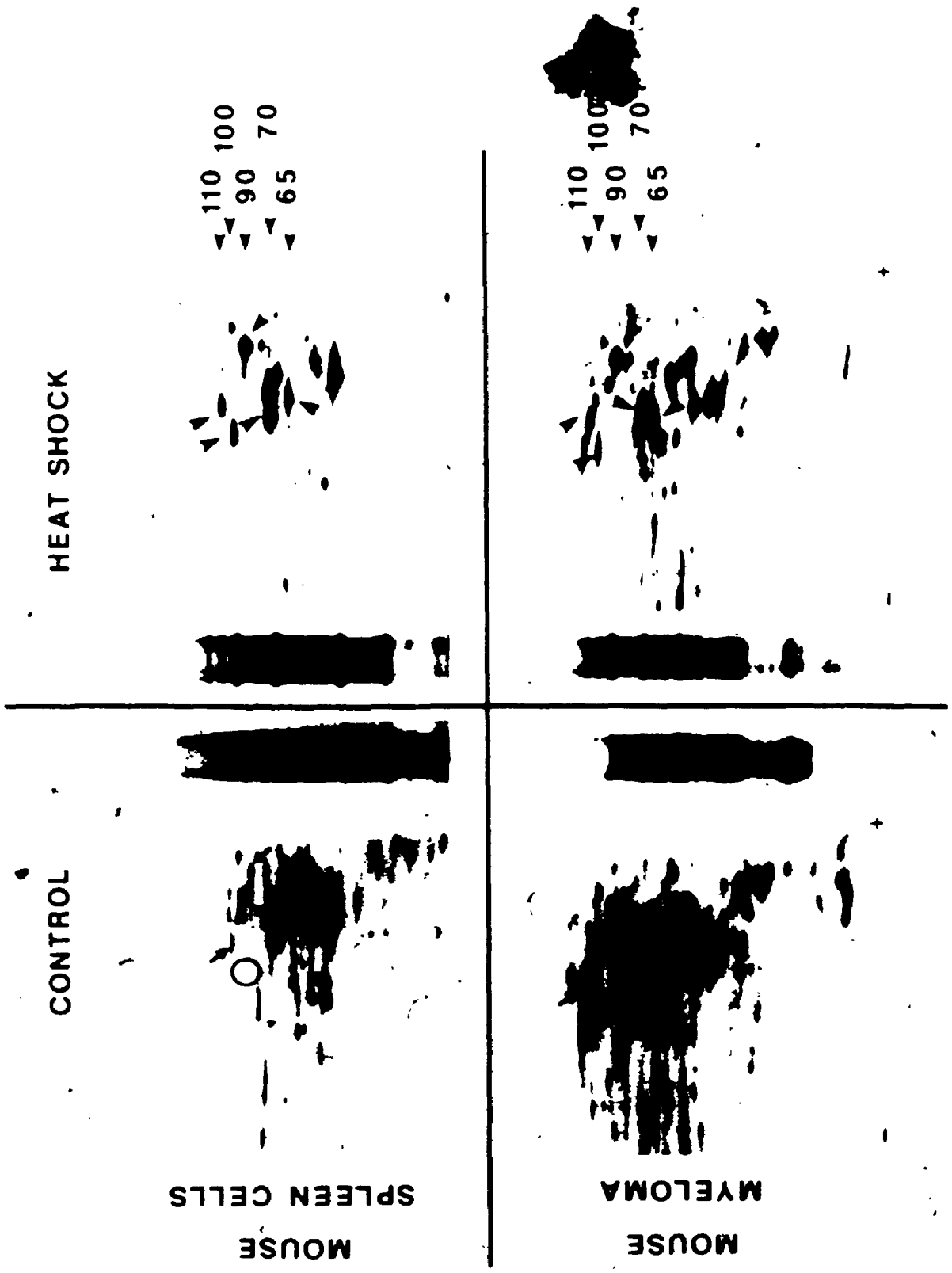
3.2.6 Chemical Stressing of Mouse Spleen Cells.

Having established that heat-shock alters gene expression in mouse spleen cells, further experiments were performed to examine the response of these cells to chemical agents known to alter gene

Table 1. Summary of the Molecular Masses and Isoelectric Points of the Heat-Shock Proteins (HSPs) Synthesized by Human Lymphocytes, Mouse Spleen Cells, and Rabbit Lymphocytes Radiolabelled with ^{35}S-methionine.

MOLECULAR MASS	ISOELECTRIC POINTS		
	HUMAN	MOUSE	RABBIT
110 kDa HSP	5.5	5.5	5.5 - 5.7
100 kDa HSP	5.7	5.8	5.7 - 5.8
90 kDa HSP	4.5 - 5.4	4.9 - 5.2	5.3
70 kDa HSP	5.4 - 5.7	5.3 - 5.8	5.4 - 5.7
65 kDa HSP	5.5	5.3 - 5.5	5.5 - 5.6

Figure 22. Fluorographic comparison (1-D and 2-D) of the heat-shock response in mouse spleen cells and mouse myeloma cells. These cells were maintained for 2 h at control (37°C) or heat-shock (43°C) temperatures, then labelled for 2 h with ^{35}S-methionine at 37°C. Equal amounts of acid-precipitable radioactivity from the lysates were added to each well of the 1-D 5-15% SDS-polyacrylamide gels. For 2-D analysis equal amounts of acid-precipitable radioactivity were added to each first dimensional (IEF)-gel and the second dimensional (SDS) gels consisted of a 7.5-17.5% polyacrylamide gradient. The panels on the right half of the figure indicate the heat-shock cells; on the left are cells maintained at 37°C prior to labelling. The molecular masses ($X \cdot 10^{-3}$) of the HSPs are given to the right of the figure; arrowheads indicate their position on the 2-D gels. HSPs synthesized constitutively in significant amounts at the control temperature at indicated by open arrows on the 2-D gels; the circled area in the control samples indicates the position the 100 kDa HSP would occupy if it were present.


110
100
90
70
65
HEAT SHOCK
CONTROL
MOUSE
SPLEEN CELLS
MOUSE
MYELOMA

expression in a manner similar to heat-shock. In this study, sodium arsenite and ethanol were chosen as chemical-stresses for mouse spleen cells. In some experiments, chemical-stress and heat-shock were applied concurrently to the cells, to determine how low levels of two different stresses (i.e. ethanol and heat) might act to alter gene expression in mouse spleen cells.

3.2.6.1 Ethanol-Stressing of Mouse Spleen Cells:

Mouse (BALB/c) spleen cells were exposed to varying concentrations of ethanol, to determine if changes in gene expression occur in response to chemical stress. The cells were incubated for 1 h at 37°C with 0, 1, 2, 4, or 6% ethanol, then labelled for 2 h at 37°C in the absence of ethanol. The cells were lysed, and equal amounts of acid-precipitable radioactivity from the cell lysates were electrophoretically-separated on 1-D gels. Fluorograms from the 1-D gels (Figure 23) and 2-D gels (Figure 24) of the control and ethanol-stressed cells show that a 1 h incubation at 37°C with 6% ethanol results in the relative enhanced synthesis of the 110, 90, 70 and 65 kDa HSPs. No enhancement of HSP synthesis is seen in cells incubated at 37°C for 1 h in ethanol concentrations of less than 6%; ethanol treatment does not result in the enhanced or de novo synthesis of a 100 kDa HSP synthesized following heat-shock.

To analyze further the polypeptides induced in heat-shocked and ethanol-stressed spleen cells, additional 2-dimensional PAGE separations were performed. Figure 25 shows fluorograms from 2-D separations of lysates from control (A), heat-shocked (B), and ethanol-stressed (C) cells, as well as a coelectrophoresed mixed

Figure 23. Comparison of fluorographic results (1-D) obtained from control (C), heat-shocked (HS), ethanol-, and arsenite-stressed mouse spleen cells. Cells were incubated for 1 h at 37°C in media containing ethanol or sodium arsenite at the concentrations given in the figure. Control and heat-shocked cells were incubated for 1 h at 37°C or 43°C respectively. Following these treatments, cells were radioactively labelled with ^{35}S-methionine at 37°C for 1 h in the absence of ethanol or arsenite. Equal amounts of acid-precipitable radioactivity from the lysates were added to each well of a 1-D SDS-polyacrylamide (5-15% gradient) gel. The relative molecular masses (X 10^{-3}) of the protein standards are denoted on the left of the figure. The M_rs (X 10^{-3}) shown on the right of the figure denote the HSPs (or HSP-like polypeptides induced by chemical-stress), and a 42 kDa non-HSP.

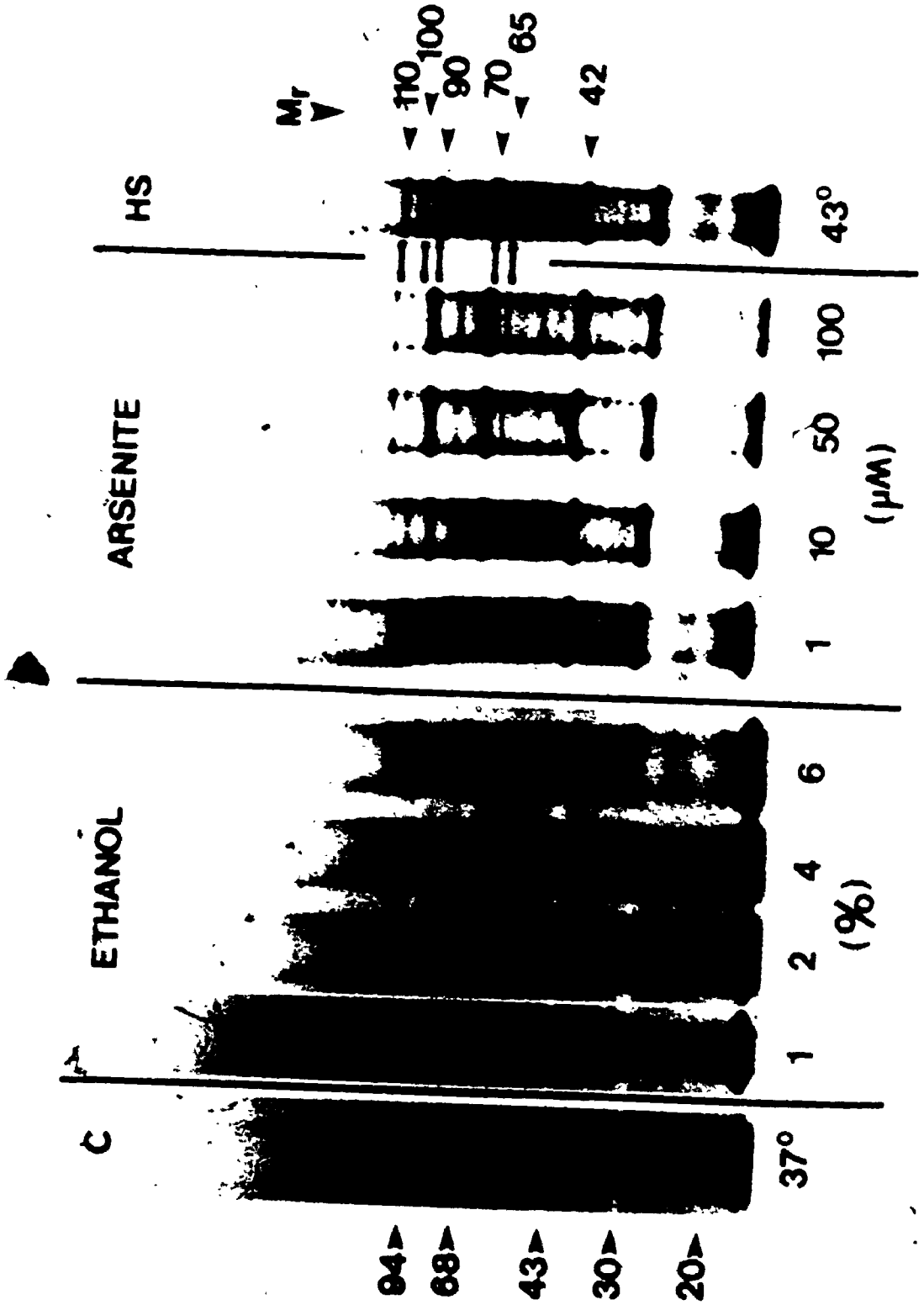
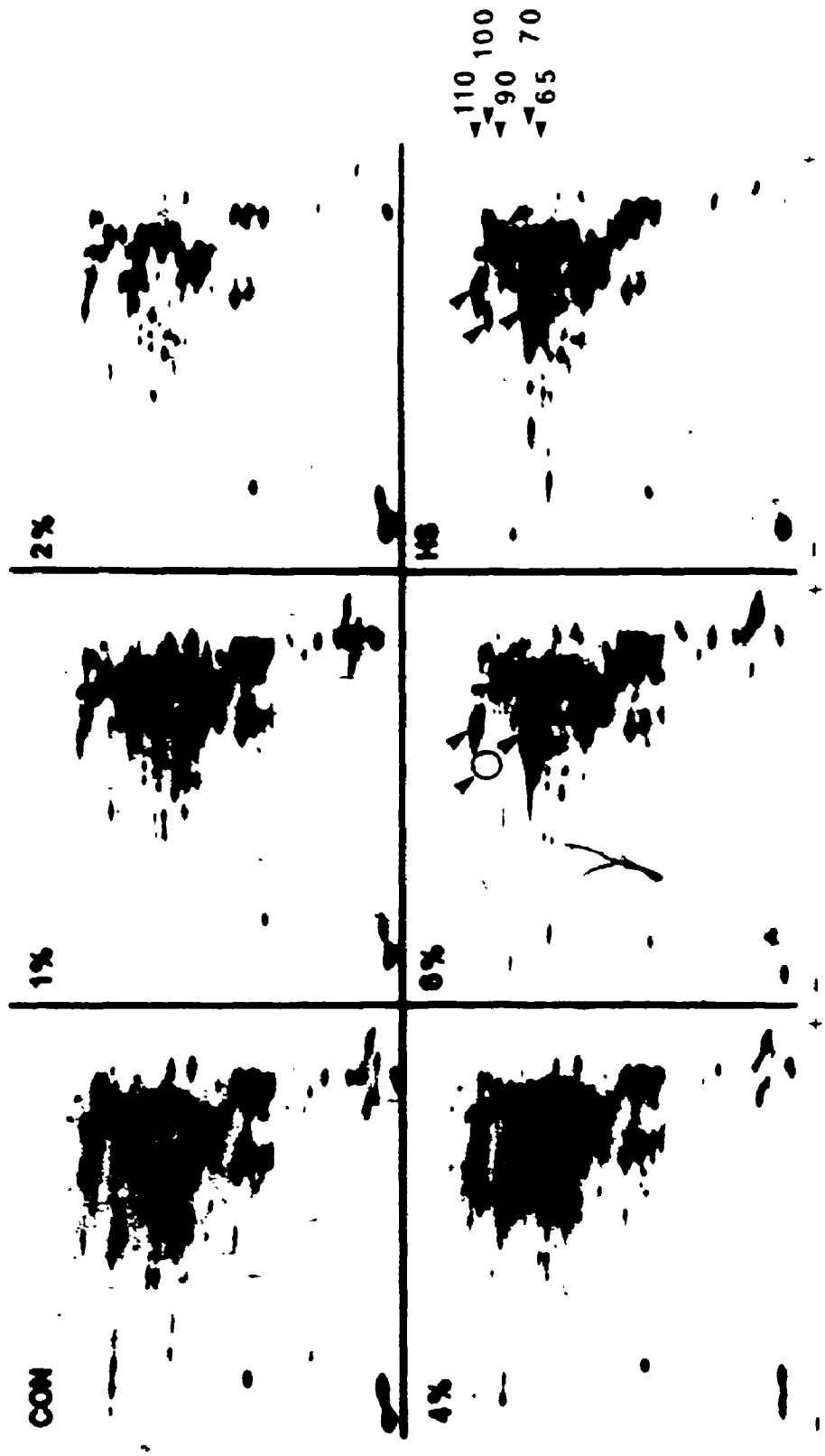

Mr
110
100
90
70
65
42
HS
43°
ARSENITE
100
50
10
1
(μM)
ETHANOL
6
4
2
1
(%)
C
37°
94
68
43
30
20

Figure 24. Fluorographic comparison (2-D) of the polypeptides synthesized by ethanol-stressed mouse spleen cells. Cells were incubated for 2 h at 37°C in media containing ethanol at the concentrations given in the figure. Control (CON) and heat-shocked (HS) cells were incubated for 1 h at 37°C or 43°C respectively. Following these treatments, cells were radioactively labelled with ^{35}S-methionine at 37°C for 2 h in the absence of ethanol. Equal amounts of acid-precipitable radioactivity from the lysates were added to each first dimensional (IEF) gel and the second dimensional (SDS) gels consisted of a 7.5-17.5% polyacrylamide gradient. The M_rs (X 10^{-3}) shown on the right of the figure denote the HSPs (or HSP-like polypeptides induced by ethanol-stress), and a 42 kDa non-HSP. The circled area indicates the position the 100 kDa HSP would occupy if it were present.

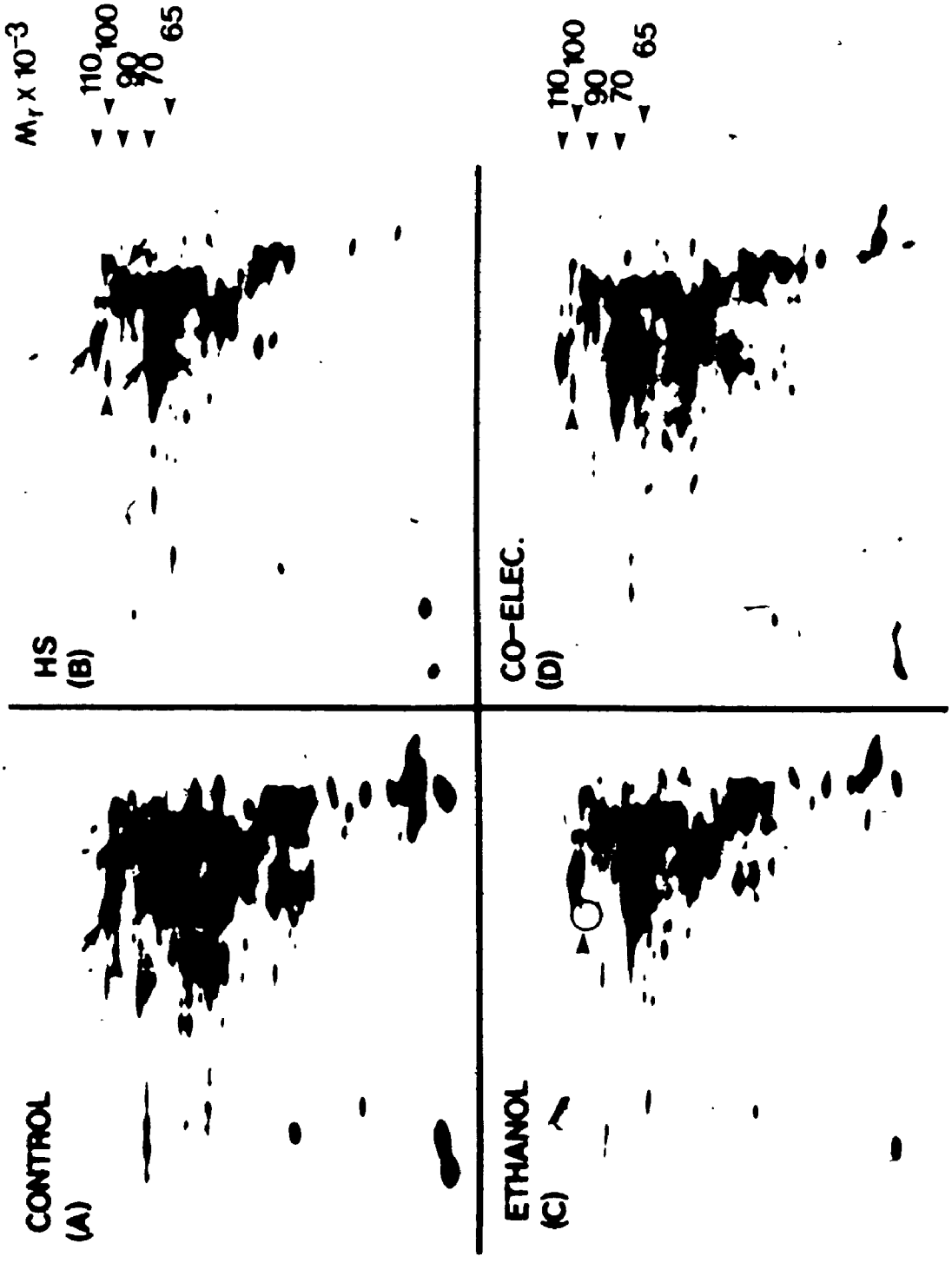

110
100
90
70
65
2%
MB
1%
0%
CON
4%

lysates (D) from heat-shocked and ethanol-stressed cells. Analysis of these fluorograms reveals that the HSPs (see arrows in B) exist as families of polypeptides with different isoelectric points, some of which are synthesized at low levels by spleen cells maintained at 37°C (compare the HSPs indicated by arrows in B to their counterparts in A). Heat-shock and ethanol-induced polypeptides appear to coelectrophorese (see D in Figure 25) and to be identical with respect to M_r and pI, except that the 100 kDa polypeptide induced by heat-shock (see arrowheads) is absent in the ethanol-stressed cells (see circle in C).

3.2.6.2 Concurrent Ethanol-Stress and Heat-Shock of Mouse Spleen Cells:

Having established the pattern of gene expression of spleen cells exposed to a single stressor (heat or ethanol), the response of spleen cells simultaneously exposed to both stressors was investigated. Mouse spleen cells were incubated for 30 min at 37°C (A,B) or 41°C (C,D) in the absence (A,C) or presence (B,D) of 4% ethanol, then labelled with ^{35}S-methionine for 2 h at 37°C, in the absence of ethanol. The cells were lysed, and equal amounts of acid-precipitable radioactivity from the cell lysates were loaded on 1-D and 2-D gels. Fluorographic analysis of these gels (Figures 26 and 27) show no differences in the polypeptides synthesized by control cells (Figures 26A and 27A) and cells exposed for only a limited period (30 min) to 4% ethanol at 37°C (Figures 26B and 27B), or to 41°C alone (Figures 26C and 27C). However, cells subjected concurrently to a limited period (30 min) of heat (41°C)

Figure 25. Fluorographic comparisons (2-D) of polypeptides synthesized by control (A), heat-shocked (B;HS), and ethanol-stressed (C) mouse spleen cells. Cells were incubated for 1h at 37°C or 43°C, or at 37°C with 6% ethanol, then labelled for 2h at 37°C with ^{35}S-methionine. Equal amounts of acid-precipitable radioactivity were added to each first dimensional (IEF) gel; the second dimensional (SDS) gel consisted of a 7.5-17.5% polyacrylamide gradient. A fourth panel (D;CO-ELEC) represents a mixture of lysates from heat-shocked and ethanol-stressed spleen cells which was electrophoresed on the same gel. Molecular masses (X 10^{-3}) of the HSPs are indicated on the right of the figure. Arrows indicate the HSPs; arrowheads indicate the 100kDa HSP which is absent in the ethanol stressed cells (see circled area in C).


$M_r \times 10^{-3}$
110
100
90
70
65
HS
(B)
CO-ELEC.
(D)
CONTROL
(A)
ETHANOL
(C)

and 4% ethanol (Figures 26D and 27D) display a dramatic synergism in inducing HSP synthesis - one comparable to cells exposed to a more severe single stress (e.g. 1 h exposure to 43°C or 6% ethanol; see Figure 27E,F). This involves the enhanced synthesis of HSPs with M_rs of 110, 90, 70, and 65 kDa. Synthesis of the 100 kDa HSP induced by heat-shock but absent following ethanol-stress (an exposure to 6% ethanol for 1 h at 37°C; Figure 27F and Figure 23), is not induced or enhanced following concurrent heat and ethanol stress (Figures 26D and 27D, circled area). As well, synthesis of several members of the 70 kDa HSP family (pIs of 5.3 - 5.4) which are present following separate heat-shock or ethanol-stress are absent following concurrent heat-shock and ethanol-stress (compare Figures 24 and 27B,C).

To quantify the increased synthesis of HSPs following concurrent heat and ethanol stress, areas of the gels from Figure 27 corresponding in M_r to the 110, 90, 70, and 65 kDa HSPs, and a 42 kDa non-HSP (as identified by fluorography), were removed and the radioactivity determined (see Materials and Methods). The results (Figure 28) indicate that concurrent exposure to limited periods of heat and ethanol increases the relative synthesis of the 110, 90, 70, and 65 kDa HSPs, but does not affect the synthesis of the 42 kDa non-HSP. In particular, cells stressed for 30 min at 41°C with 4% ethanol exhibit a 10-fold increase in the synthesis of the 70 kDa HSP, when compared to cells treated with either a limited period of heat (30 min at 41°C) or 4% ethanol.

To investigate further the response of spleen cells to concurrent heat-shock and ethanol stress, cells were incubated for varying

Figure 26. Fluorographic comparison (1-D) of the polypeptides synthesized by mouse spleen cells stressed by a single stressor (heat or ethanol) and a concurrent multiple stress (heat and ethanol). Spleen cells were incubated for 30 min at 37°C (A,B) or 41°C (C,D), in the absence (A,C) or presence (B,D) of 4% ethanol, then labelled with ^{35}S-methionine for 2 h at 37°C in the absence of ethanol. Equal amounts of acid-precipitable radioactivity from lysates were added to each well of the SDS-polyacrylamide (5-15% gradient) gel. The two lanes on the right (E,F) show the polypeptides synthesized by spleen cells heat-shocked for 1 h at 43°C prior to labelling (E), and spleen cells stressed with 6% ethanol for 1 h at 37°C (F). The molecular masses (X 10^{-3}) of protein standards (STDs) are indicated of the left of the figure; on the right are the molecular masses (X 10^{-3}) of the HSPs, or HSP-like polypeptides.


STDs
94
67
43
30
20
14
A
B
C
D
E
F
110
100
90
70
65
TEMPERATURE
37°
37°
41°
41°
43°
37°
ETHANOL
0
4%
0
4%
0
6%

Figure 27. Fluorographic comparison (2-D) of the polypeptides synthesized by mouse spleen cells stressed by a single stressor (heat or ethanol) and a concurrent multiple stress (heat and ethanol). Spleen cells were incubated for 30 min at 37°C (A,B) or 41°C (C,D), in the absence (A,C) or presence (B,D) of 4% ethanol, then labelled with ^{35}S-methionine for 2 h at 37°C in the absence of ethanol. Equal amounts of acid-precipitable radioactivity were added to each first dimensional (IEF) gel and the second dimensional (SDS) gel consisted of a 7.5-17.5% polyacrylamide gradient. Arrowheads indicate the HSPs; the circled area in D marks the position the absent 100 kDa HSP would occupy if it were present. The relative molecular masses (M_rs) of the HSPs are shown on the right side of this figure.

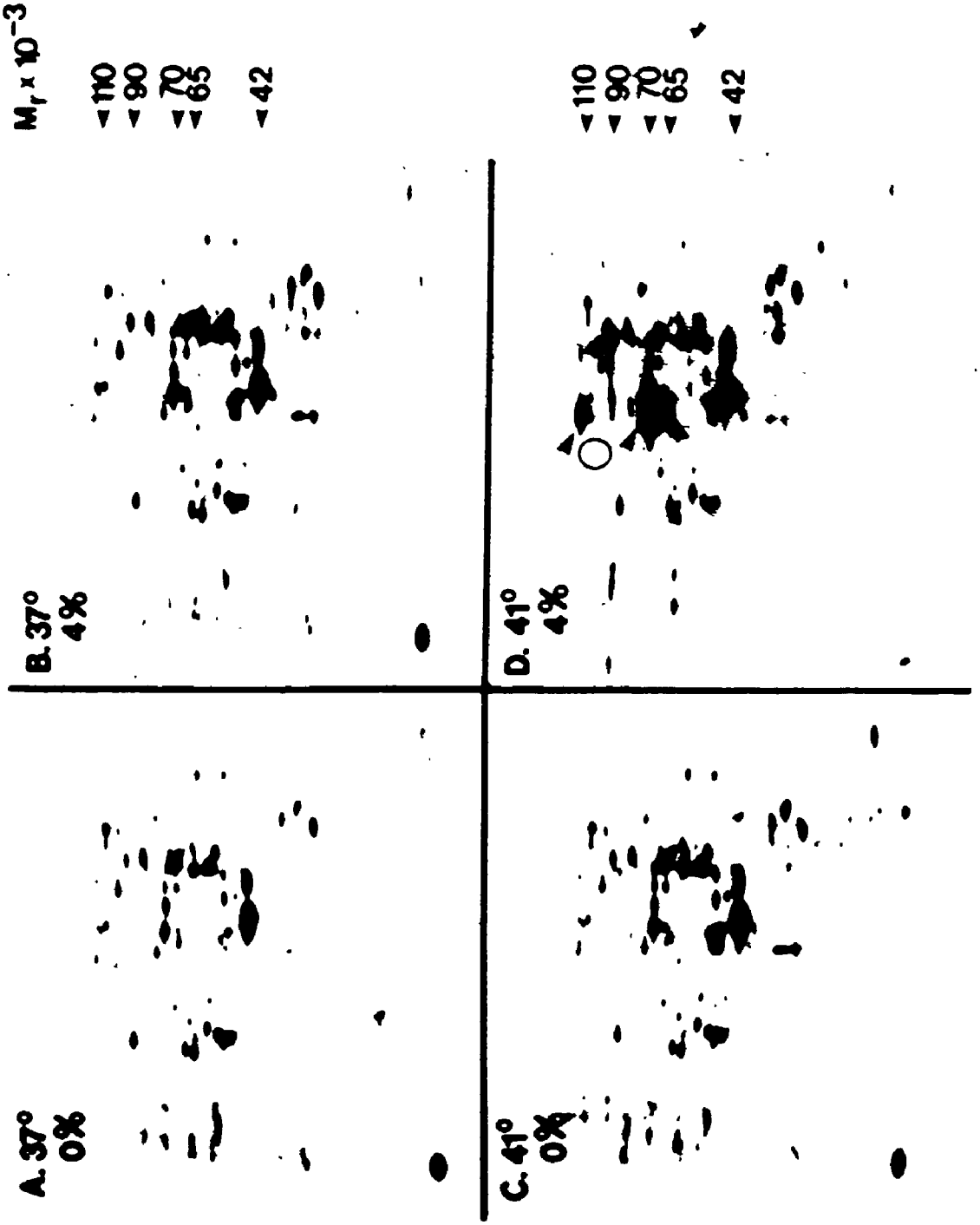

$M_r \times 10^{-3}$
110
90
70
65
42
110
90
70
65
42
B. 37°
4%
D. 41°
4%
A. 37°
0%
C. 41°
0%

Figure 28. Quantification of the radioactivity incorporated into specific, electrophoretically-separated polypeptides by mouse spleen cells stressed by a single stressor (heat or ethanol) or a concurrent multiple stress (heat and ethanol), as shown in Figure 27. The lysates used in this figure are from spleen cells incubated for 30 min at:

(A) 37°C without ethanol

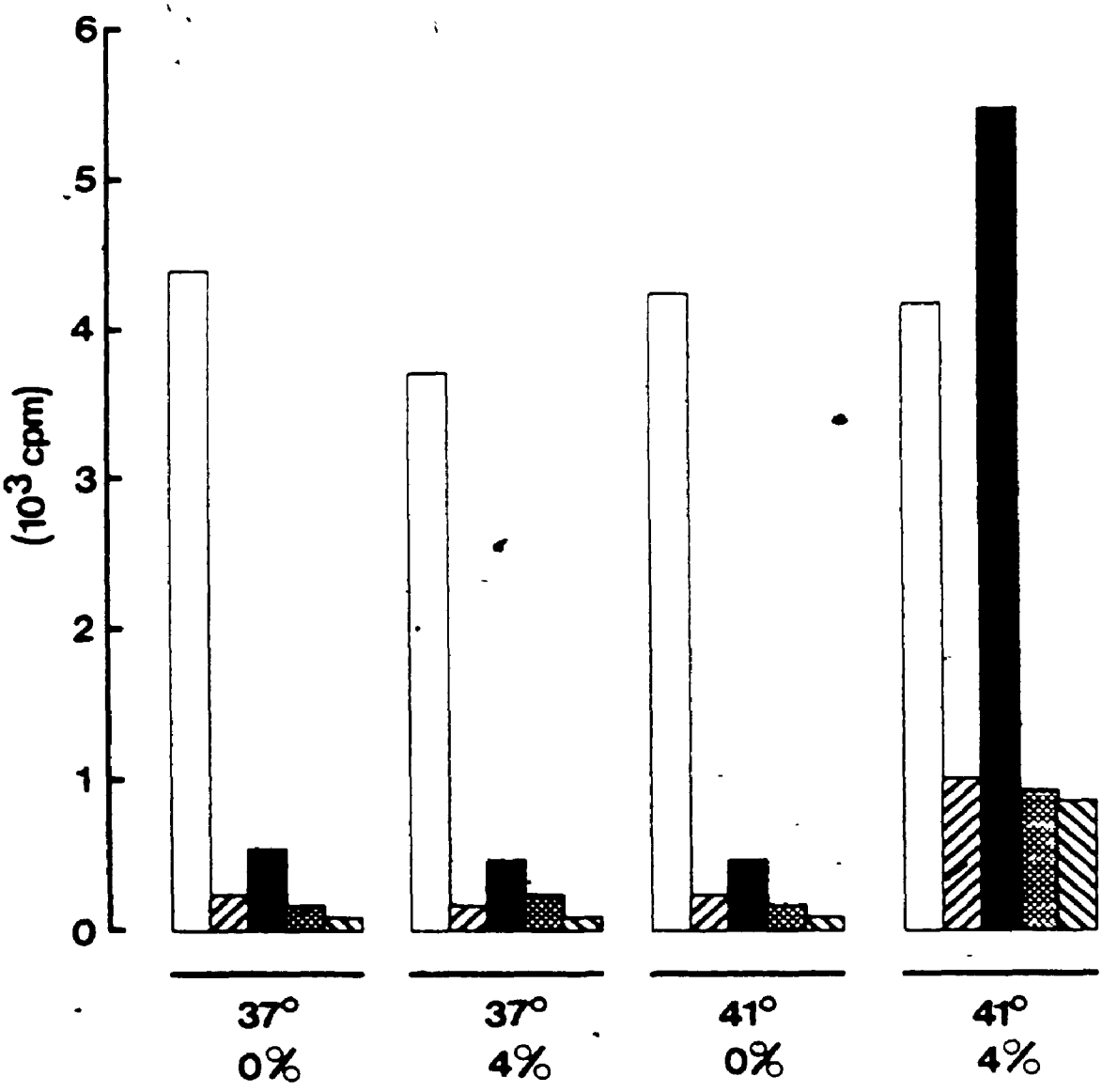
(B) 37°C with 4% ethanol

(C) 41°C without ethanol

(D) 41°C with 4% ethanol

Following these treatments, the cells were labelled for 2 h at 37°C in the absence of ethanol. After 1-D PAGE separation of the polypeptides and fluorographic analysis of the gels, the amount of radioactivity in individual polypeptides (expressed as counts per minute) was determined as described in section 7.7.5. The following histogram bars were used to represent the various polypeptides:

- 110 kDa HSP
- 90 kDa HSP
- 70 kDa HSP
- 65 kDa HSP
- 42 kDa non-HSP

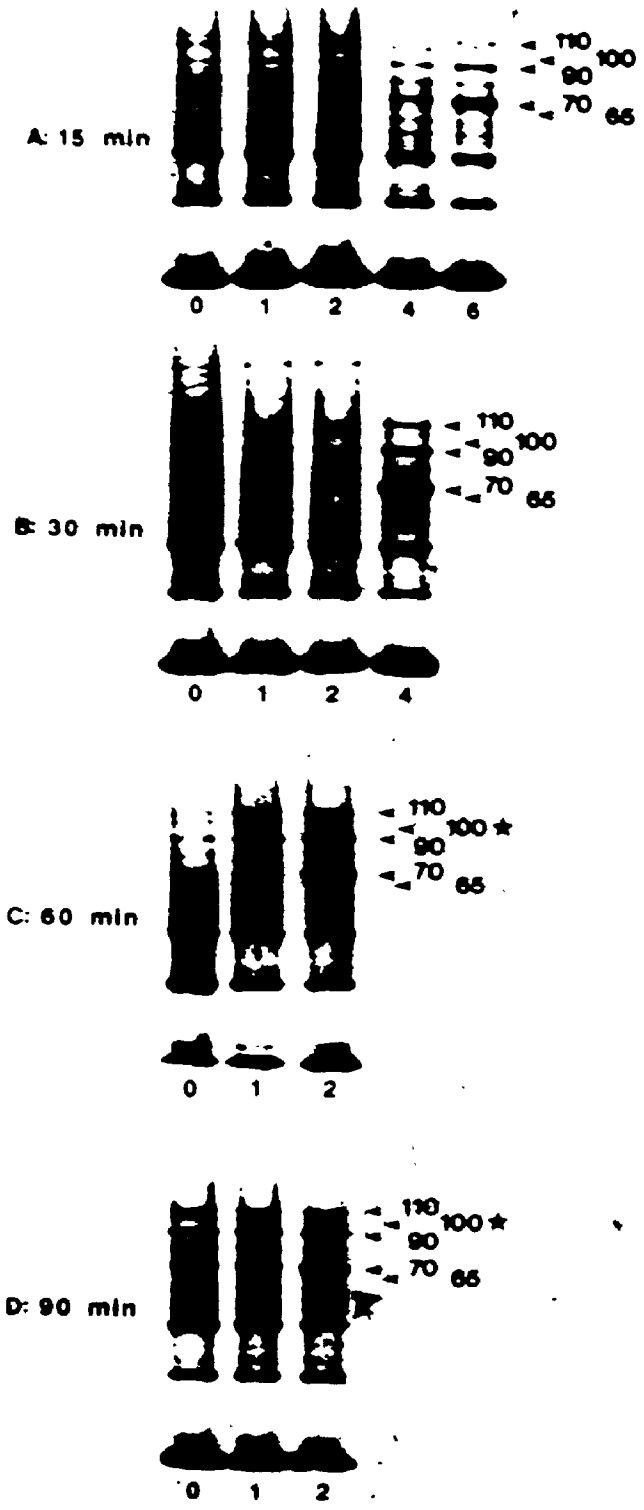

6
5
4
3
2
1
0
(10³ cpm)
37°
0%
37°
4%
41°
0%
41°
4%

time periods (15, 30, 60 or 90 min) at 41°C in the presence (or absence) of ethanol at v/v concentrations of 1, 2, 4, or 6%. Following these treatments, spleen cells were labelled with ^{35}S-methionine for 2 h at 37°C in the absence of ethanol, lysed, and equal amounts of acid-precipitable radioactivity for each lysate were separated by 1-D PAGE.

Fluorographic analysis of the 1-D gels (Figure 29) shows that changes in gene expression due to concurrent heat-shock and ethanol-stress are dependent on the duration of exposure to the stressors and the levels of stressors present. As the duration of heat-shock increases from 15 to 90 min, less ethanol is required to elicit a stress response. Spleen cells incubated for 15 min at 41°C (Figure 29A) require a concurrent exposure to 6% ethanol to elicit a "complete" chemical-stress response (i.e. enhanced synthesis of the 110, 90, 70, and 65 kDa HSPs), and do not express enhanced or de novo HSP synthesis at lower ethanol concentrations during the 15 min incubation at 41°C (Figure 29A). Spleen cells incubated for 30 min at 41°C (Figures 27 and 29B) require 4% ethanol to elicit a "complete" chemical-stress response. Exposure to 6% ethanol at 41°C for a period of 30 min is lethal to the cells.

As previously shown, spleen cells incubated for 60 min at 41°C express enhanced synthesis of the 90 and 100 kDa HSPs in the absence of ethanol, with a 90 min period at this temperature required for synthesis of the complete HSP set (Figure 16). The presence of 2% ethanol during this 60 min 41°C HS period induces the synthesis of the complete set of HSPs (Figure 29C). Exposure to higher ethanol levels than this for the 60 min period at 41°C is

Figure 29. Fluorographic comparison (1-D) of the polypeptides synthesized by mouse spleen cells stressed by concurrent heat-shock and ethanol-stress for various time periods. Spleen cells were incubated for 15, 30, 60 or 90 min at 41°C in the absence (lanes marked "0") or presence of ethanol (at the concentrations listed: 1, 2, 4 or 6%). Following the stress period, the cells were labelled with ^{35}S-methionine for 2 h at 37°C in the absence of ethanol. Equal amounts of acid-precipitable radioactivity from the lysate were added to each well of the SDS-polyacrylamide (5-15% gradient) gels. The molecular masses (X 10^{-3}) of the HSPs, or HSP-like polypeptides are indicated on the right side of the figure. No acid-precipitable radioactivity was recovered from lysates of cells treated with 6% ethanol for 30 min (B), or 4% and 6% ethanol for 60 min (C) and 90 min (D). The asterix beside the 100 kDa HSP (C,D) indicates the presence of this HSP in these cell lysates.


A: 15 min
110
100
90
70
66
0 1 2 4 6
B: 30 min
110
100
90
70
66
0 1 2 4
C: 60 min
110
100 ★
90
70
66
0 1 2
D: 90 min
110
100 ★
90
70
66
0 1 2

lethal to the cells. A 90 min HS period at 41°C (without any concurrent stressor) is sufficient to induce synthesis of the complete set of HSPs in mouse spleen cells, as previously seen in Figure 16. As would be expected, HSPs are synthesized when low amounts of ethanol (1 - 2%) are present during this 90 min HS. However exposure to higher levels of ethanol than this proves to be lethal to the cells. Interestingly, the 100 kDa HSP induced by heat-shock, but not by ethanol-stress (see Figure 23), is only synthesized by those cells which have been concurrently stressed for 90 min at 41°C. This is a time and temperature previously shown to enhance synthesis of this particular HSP in the absence of ethanol (see Figure 29D).

3.2.6.3 Arsenite Stressing of Mouse Spleen Cells.

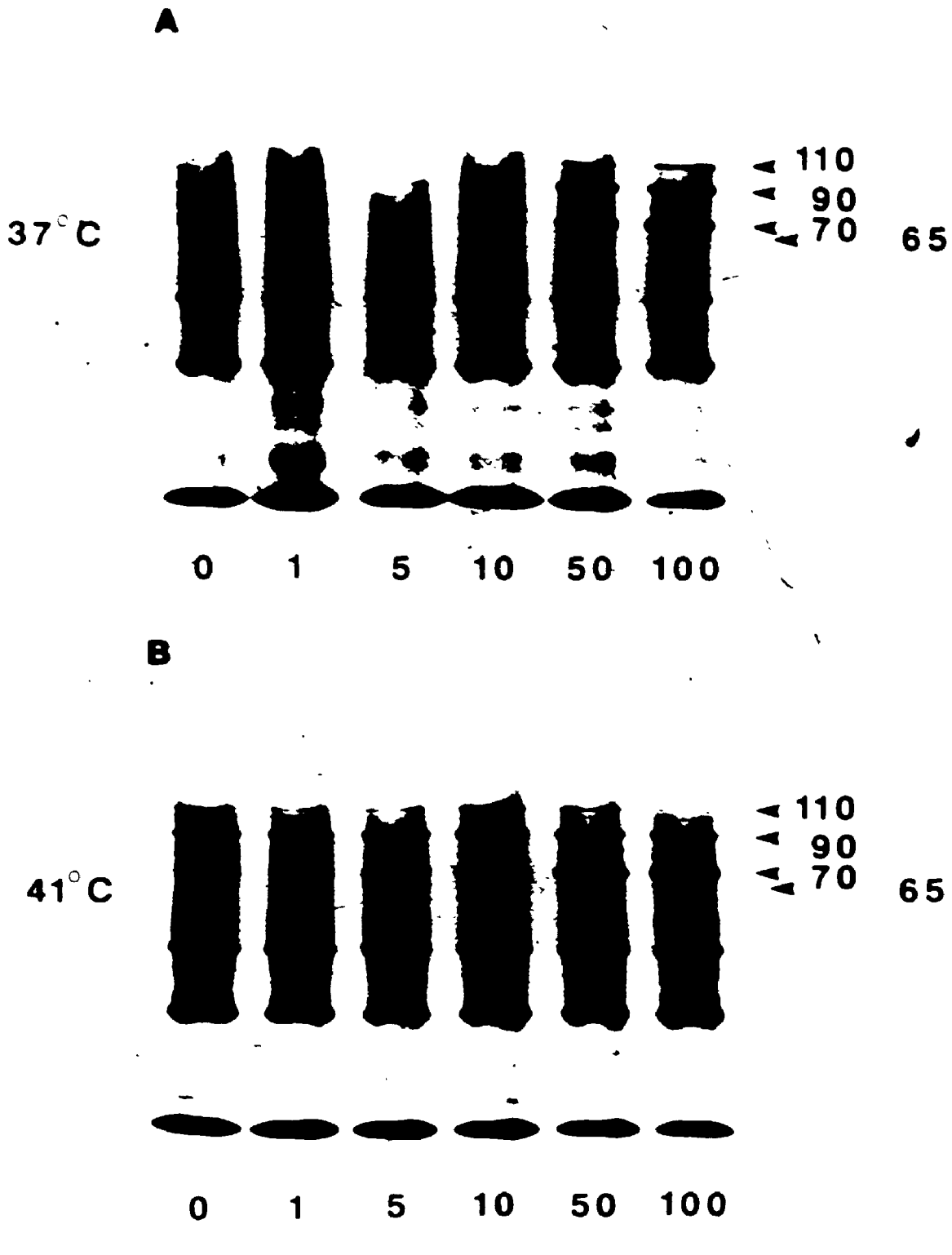
Mouse spleen cells were exposed to varying concentrations of sodium arsenite, to determine if changes in gene expression occur in response to this type of chemical stress. Spleen cells were incubated for 1 h at 37°C in the presence of 1, 10, 50, or 100 uM arsenite, labelled for 2 h at 37°C in the absence of arsenite, and then lysed. Fluorograms from 1-D gels of control and arsenite-stressed cells (Figure 23) show that a 1 h incubation at 37°C with 10, 50 or 100 μM arsenite results in the enhanced synthesis of the 110, 90, 70, and 65 kDa HSPs. Neither enhanced nor de novo synthesis of the 100 kDa HSP results following these arsenite treatments.

3.2.6.4 Concurrent Arsenite-Stress and Heat-Shock of Mouse Spleen Cells.

Experiments were performed to determine if concurrent arsenite-stress and heat-shock alter mouse spleen cell gene expression in a manner similar to concurrent ethanol-stress and heat-shock. Figure 30 depicts fluorograms of electrophoretically-separated polypeptides from mouse spleen cells incubated for 1 h at 37°C (Figure 30A) or 41°C (Figure 30B) in the presence of 0, 1, 5, 10, 50 or 100 μM sodium arsenite. Following these treatments, cells were labelled with ^{35}S-methionine for 2 h at 37°C in the absence of arsenite, then lysed, and equal amounts of acid-precipitable radioactivity from all lysates were separated on 1-D gels.

Fluorographic analysis of these 1-D gels shows that arsenite-stressed cells (Figure 30A) first exhibit enhanced or de novo synthesis of HSPs following treatment with 10 μM arsenite, and that synthesis of the "complete" set of chemical-stress induced HSPs is seen following the 50 μM arsenite treatment. As was previously shown in Figure 16 and is again seen in Figure 30B, cells incubated for 1 h at 41°C (in the absence of other stresses) express the enhanced synthesis of the 90 kDa HSP. Cells exposed to concurrent arsenite-stress and heat-shock exhibit enhanced or de novo synthesis of the HSPs at much lower arsenite concentrations than seen with arsenite alone. Enhanced or de novo synthesis of the "complete" set of HSPs occurs in these cells at 41°C when arsenite levels as low as 5 μM are present - a level considerably less than the 50 μM of arsenite required for HSP synthesis when cells were incubated at 37°C. These results suggest that concurrent arsenite-stress and heat-shock can act synergistically to enhance or induce HSP synthesis in mouse spleen cells.

Figure 30. Fluorographic comparison (1-D) of the polypeptides synthesized by mouse spleen cells stressed by a single stressor (heat or arsenite) and a concurrent multiple stress (heat and arsenite). Spleen cells were incubated for 30 min at 37°C (A), or 41°C (B), in the absence (lanes marked "0") or presence of sodium arsenite (at the concentrations listed: 1, 5, 10, 50, 100 uM). The cells were then labelled with ^{35}S-methionine for 2 h at 37°C in the absence of arsenite. Equal amounts of acid-precipitable radioactivity from lysates were added to each well of the SDS-polyacrylamide (5-15% gradient) gels. The molecular masses (X 10^{-3}) of the HSPs, or HSP-like polypeptides are indicated on the right side of the figure.


A
37°C
110
90
70
65
0
1
5
10
50
100
B
41°C
110
90
70
65
0
1
5
10
50
100

3.3 Immunodetection of IgG by ELISA.

Experiments were performed to estimate the levels of IgG secreted by mouse spleen cells maintained in short term culture at 37°C, or heat-shocked at 41 or 43°C. To accomplish this, the presence of IgG in the cell growth media was assayed using an Enzyme-linked Immunosorbent Assay (ELISA) coupled with an indirect immunoperoxidase staining procedure.

To establish the sensitivity of the ELISA technique, mouse immunoglobulin G (H and L chains, Sigma) at concentrations ranging from 1-1000 ng/mL was used as the test substance in a series of experiments. Figure 31 depicts the absorbance results and a photograph of an ELISA multiwell plate from one such experiment. The wells in lanes 3 to 11 of the multiwell plate were coated with goat anti-mouse IgG (Fab'_2 fragment), and mouse IgG was added (in replicates of 7) at the concentrations given in the figure legend. Goat anti-mouse IgG coupled to horseradish peroxidase was then added to the multiwell plate and the color reaction developed. As seen in Figure 31, the immunoperoxidase reaction was most intense at the highest IgG concentrations (lanes 3,4,5; 1000, 500, 250 ng/mL) and diminished as the mouse IgG concentration decreased from 100 ng/mL to 1 ng/mL (lanes 4 - 11). No significant differences among absorbance readings were seen at mouse IgG concentrations greater than 250 ng/mL, indicating that optimal binding of mouse IgG to the GAM-IgG had occurred under these experimental conditions.

In each experiment, tests were performed to account for non-specific binding of the test substance or the GAM-HRP to the

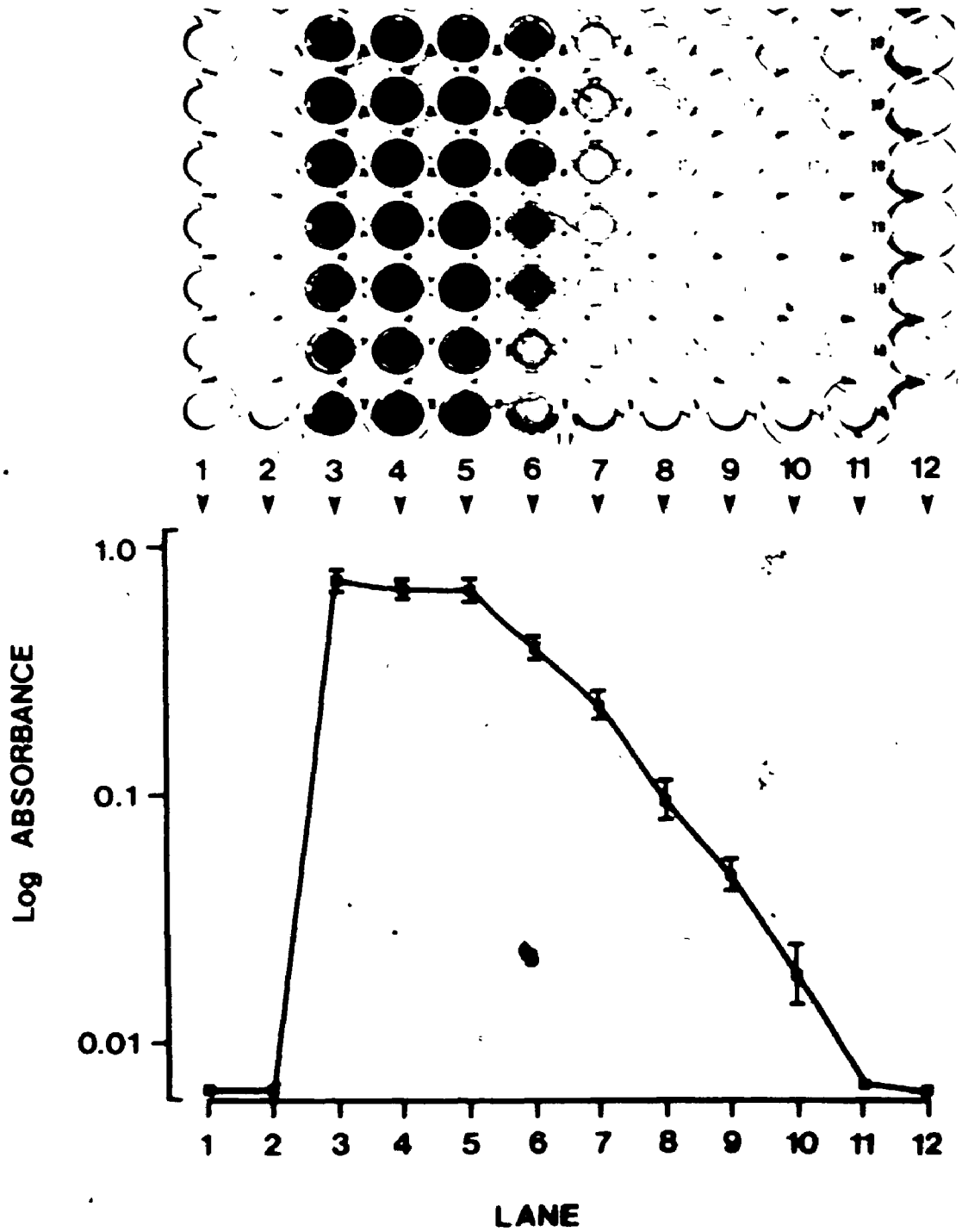
multiwell plates. To determine the amount of non-specific binding of the mouse IgG to the multiwell plate, wells in lane 2 were not coated with GAM-IgG (Fab'_2 fragment) prior to the addition of mouse IgG (1000 ng/mL) and the later addition of GAM-HRP. As seen in Figure 31, non-specific binding of the mouse IgG to the plate in negligible even at this high IgG concentration (compare lane 2 with lane 3, where wells were coated with GAM-IgG prior to the addition of the mouse IgG). To determine the level of non-specific binding of GAM-HRP to the microwell plate, the wells in lane 1 and 12 were not coated with GAM-IgG (Fab'_2 fragment), nor was mouse IgG added to these wells prior to addition of GAM-HRP. Figure 31 shows that only negligible levels of peroxidase reaction were seen in these wells in lanes 1 and 12. Similar very low levels of non-specific binding were observed when GAM-HRP was added to wells coated with GAM-IgG (Fab'_2 fragment in the absence of mouse IgG (data not shown in Figure 31).

Mouse spleen cells or B cells isolated from a total spleen cell population were maintained in short-term culture at 37°C or at heat-shock temperatures of 41°C or 43°C. The media these cells were grown in was collected, and assayed for IgG using the ELISA technique.

Figures 32 and 33 depict the results of representative experiments performed to determine the effect of incubation temperature and cell concentration on the secretion of IgG by mouse spleen cells, as measured by changes in absorbance at 492 nm. Growth medium was collected from cells maintained in short term (4 h) culture at 37°C, or at heat-shock temperatures of 41°C (Figure

Figure 31. Results from an experiment designed to establish the sensitivity of the ELISA technique, using mouse Immunoglobulin G (H and L chains, Sigma) as the test substance. The upper half of this Figure shows the microELISA multiwell plate, with the mean (log_{10}) Absorbance values for each lane of the multiwell plate shown in the bottom part of this Figure. The wells in each lane of this plate were treated as follows:

	GAM IgG	mouse IgG (ng/mL)	GAM+HRP
lane 1.	-	-	+
lane 2.	-	1000	+
lane 3.	+	1000	+
lane 4.	+	500	+
lane 5.	+	250	+
lane 6.	+	100	+
lane 7.	+	50	+
lane 8.	+	20	+
lane 9.	+	10	+
lane 10.	+	5	+
lane 11.	+	1	+
lane 12.	-	-	+


1 2 3 4 5 6 7 8 9 10 11 12
1.0
0.1
0.01
Log ABSORBANCE
1 2 3 4 5 6 7 8 9 10 11 12
LANE

32) or 43°C (Figure 33). The cells were incubated at the cell concentrations shown in the Figure legends. Absorbance data were analyzed by Analysis of Covariance (ANCOVA; as described in the Materials and Methods), to establish whether the levels of IgG found in the media from cells grown at 37°C differed from those levels found in media from cells maintained at 41 or 43°C.

The statistical analysis of these absorbance values reveals that at any given cell concentration, the amount of IgG detected in the media from cells incubated at 37°C was not significantly different from that seen in media from cells maintained at the heat-shock temperatures of 41°C (Figure 32) or 43°C (Figure 33). The absorbance values of any given cell concentration are significantly different than those for any other cell concentration, indicating that as the number of cells present in the media increases, the amount of IgG present in that media increases as well.

In some experiments, the IgG complexes present in the wells of the multiwell plates were stripped from the wells by SDS-solubilization, and electrophoretically-separated on 1-D PAGE gels. Fluorographic analysis reveals (Figure 34, C and D) that a similar pattern on polypeptides was isolated from the media of control and heat-shocked cells assayed by the ELISA technique. In each case, three polypeptides having M_rs of 77, 53, and 23 kDa were resolved on fluorograms of the 1-D polyacrylamide gels. These correspond to the molecular masses of mouse light chain IgG (23 kDa), and mouse heavy chain IgG (53 kDa) previously reported under reduced conditions (Eisen 1980). The 77 kDa band may correspond to a non-reduced light and heavy chain configuration, as the M_rs of

Figure 32. Analysis of the amount of IgG in media from cells maintained for 4 h at control (37°C) or heat-shock (41°C) temperatures, as detected by the ELISA-immunoperoxidase technique. This graph depicts the effect of cell concentration and incubation temperature (■-37°C, or □-41°C) on the $\log_{10}$ Absorbance values. Data is given as Mean ± S.E..

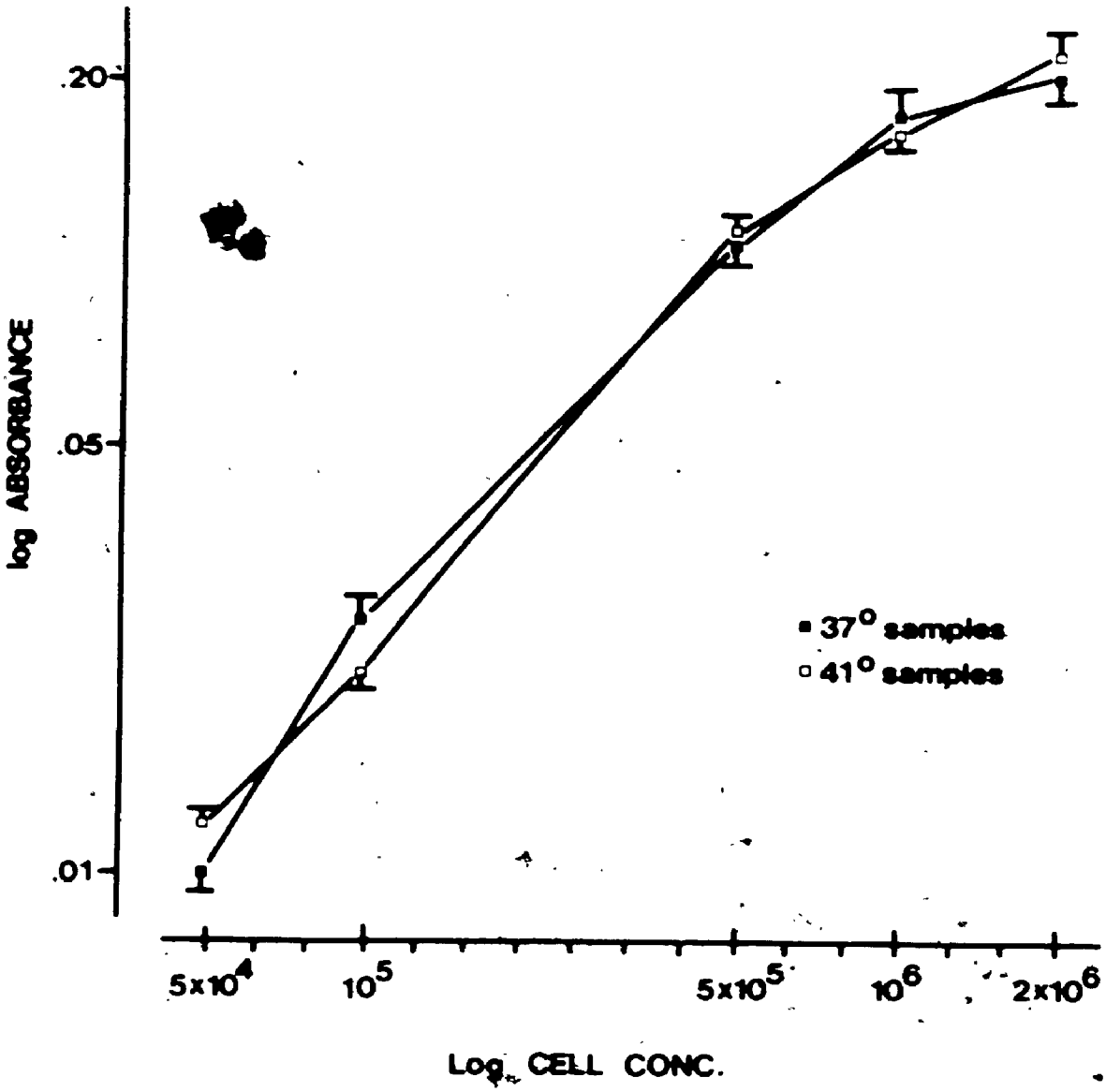
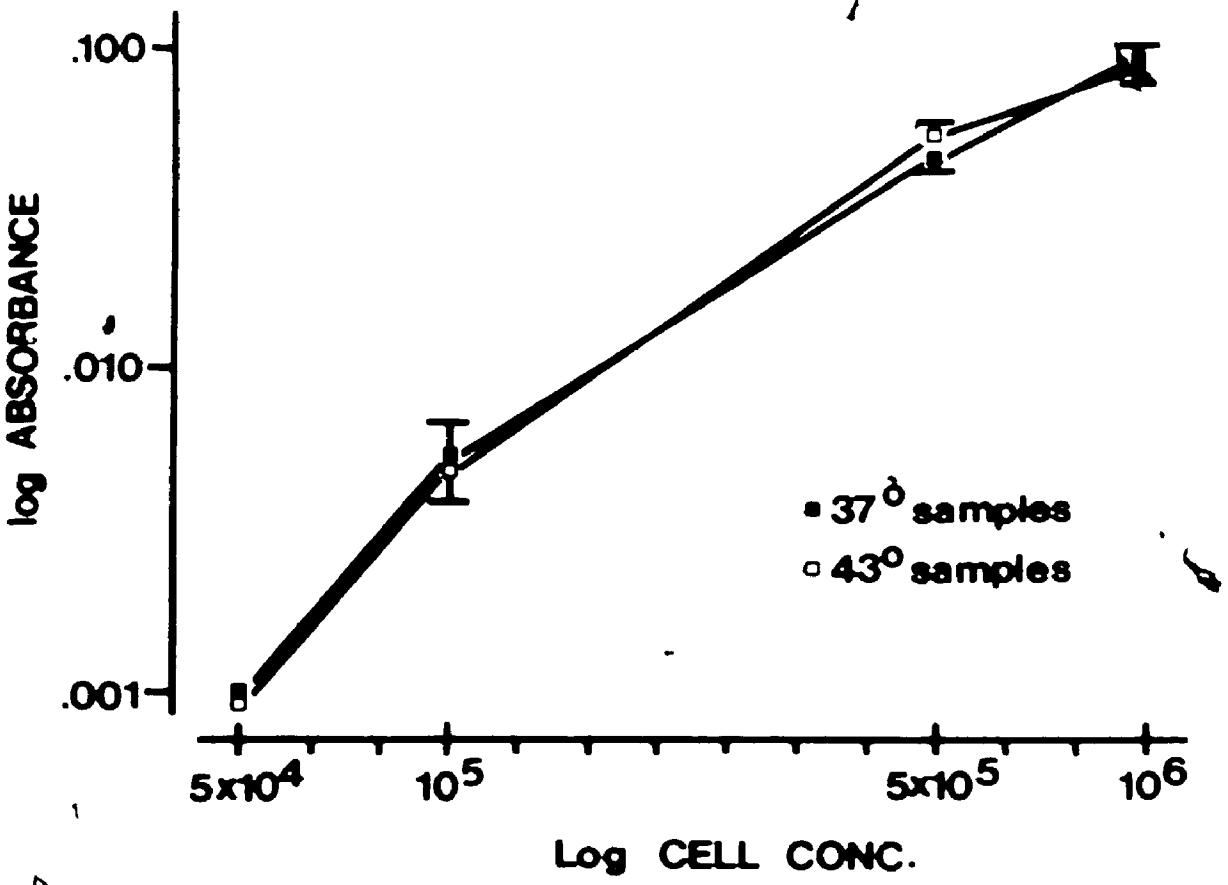

.20
.05
.01
log ABSORBANCE
▪ 37° samples
▫ 41° samples
5x10^4
10^5
5x10^5
10^6
2x10^6
Log CELL CONC.

Figure 33. Analysis of the amount of IgG in media from cells maintained for 4 h at control (37°C) or heat-shock (43°C) temperatures, as detected by the ELISA-immunoperoxidase technique. This graph depicts the effect of cell concentration and incubation temperature (■-37°C, or □-43°C) on the $\log_{10}$ Absorbance values. Data is given as Mean ± S.E..


.100
.010
.001
log ABSORBANCE
■ 37° samples
□ 43° samples
5x10⁴
10⁵
5x10⁵
10⁶
Log CELL CONC.

these two chains do add up to a similar molecular mass (76 kDa). This figure shows that the only radioactive polypeptides extracted from the ELISA plates, and presumably bound to the GAM-IgG, are the IgGs.

Levels of IgG were also determined in media in which B cells isolated from the total spleen cell population had been maintained at a control (37°C) temperature or at 41°C (Table 2; A and B), and with spleen cells maintained at 37° or 43°C. In some experiments, the concentration of the IgG detected in the multiwell plate (ng/mL) was determined by extrapolating the absorbance data from a mouse IgG standard curve similar to that presented in Figure 31. As seen in Table 2, no significant differences existed between the amount of IgG detected in the medium from cells maintained at control or heat-shock temperatures. Quantification of the specific activity of the IgG samples shown in Figure 34 (lanes C and D) and presented in Table 2 (C) shows that the specific activities (cpm/ng IgG) of IgG newly synthesized and secreted by cells maintained at control and heat-shock temperatures are comparable. These results suggest that synthesis and secretion of IgGs may not be affected by thermal stresses sufficient to induce HSP synthesis and depress the synthesis of other polypeptides normally synthesized at a control temperature.

Table 2. Detection of IgG by ELISA in the media of control and heat-shocked mouse spleen cells and splenic B cells.

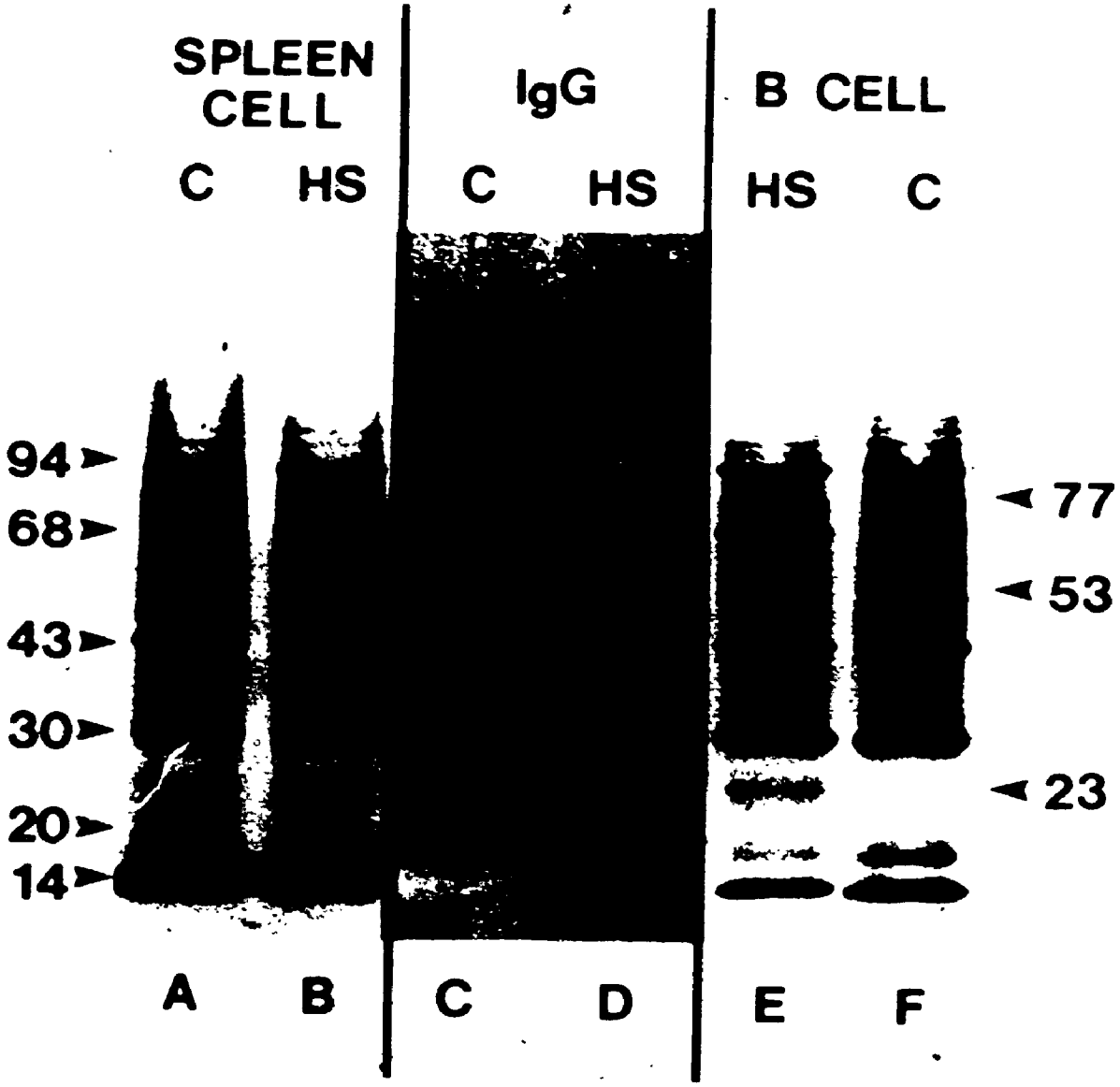
	INCUBATION TEMPERATURE (°C)	ABSORBANCE (± S.D.)	SIGNIFICANCE (p=0.05)	ng/mL IgG	SPECIFIC ACTIVITY (CPM/ng IgG)
EXPT A	37°	0.018 ± 0.004	N.S.	N.D.	N.D.
	41°	0.018 ± 0.002		N.D.	N.D.
EXPT B	37°	0.035 ± 0.008	N.S.	7.5 ± 1	N.D.
	41°	0.041 ± 0.011		8.0 ± 1	N.D.
EXPT C	37°	0.342 ± 0.033	N.S.	58 ± 6	179
	43°	0.321 ± 0.032		54 ± 6	224

(CPM/ng IgG) indicates counts per minute of radioactivity per nanogram of IgG.

N.S. indicates the compared values are not signicantly different.

N.D. indicates these values were not determined.

Figure 34. Fluorographic comparison of cell lysates from control and heat-shocked mouse spleen cells (A and B), mouse B cells (E and F), and IgG samples isolated from cell growth medium by ELISA, and subsequently released from the multiwell ELISA plate by SDS solubilization (C and D). Mouse spleen cells were incubated at 37°C or 43°C for 4 h, in the presence of ^{35}S-methionine; mouse B cells, isolated from the total spleen cell population, were incubated at 37°C or 41°C for 4 h in the presence of ^{35}S-methionine. The cells were lysed and equal amounts of acid-precipitable radioactivity from the lysates were added to each well of the 1-D SDS-polyacrylamide (5-15%) gradient gels. Media from control and heat-shocked spleen cells was assayed for the presence of IgG by the ELISA procedure, then the IgG complexes bound to the ELISA plate were released by solubilization with 10% SDS, and equal amounts of acid-precipitable radioactivity were separated by 1-D polyacrylamide gel electrophoresis. Protein standards of known molecular mass (M_rs given in kilodaltons) are denoted on the left side of the figure; the relative molecular masses of the IgG (lanes C and D) are given on the right side of the Figure.


SPLEEN CELL
IgG
B CELL
C
HS
C
HS
HS
C
94
68
43
30
20
14
77
53
23
A
B
C
D
E
F

DISCUSSION

In recent years, a considerable volume of work investigating the heat-shock phenomenon has been reported. Tissues and cells, from bacteria to mammals, have been challenged by all manner of environmental stress agents in attempts to characterize this ubiquitous cellular response to changes in the environment. When this thesis study was undertaken in 1982, the literature contained surprisingly few reports characterizing the HS response in human tissues. For the most part, those studies presenting human HS data described this response in HeLa cells (Slater et al. 1981; Thomas et al. 1982) or other transformed cell lines (Tsekuda et al. 1981; Anderson et al. 1982; Atkinson and Pollock 1982), with little attention given to examining the HS response in normal human tissues.

One objective of my study was to characterize the heat-shock response in normal human cells, and to correlate this response with that seen in similar, but non-human, mammalian cells. The data indicate that lymphocytes isolated from humans, mice and rabbits respond to a brief heat shock treatment by a similar rapid change in gene expression, as characterized by the new and/or enhanced synthesis of polypeptides with M_rs of 110, 100, 90, 70, 65 and 26 (with ^{14}C-leucine labelling only) kDa, and the depressed synthesis of proteins normally made at 37°C. With human lymphocytes, the depressed synthesis of polypeptides normally made at 37°C becomes more pronounced with increased temperature and/or with time spent at the elevated temperature (Figure 7).

The synthesis of HSPs by heat-shocked mammalian lymphocytes is dependent on transcription of new mRNA, as determined by experiments using Actinomycin-D, an inhibitor of RNA synthesis. When mouse lymphocytes were heat-shocked in the presence of Actinomycin-D synthesis of HSPs was not observed, although synthesis of control polypeptides from pre-existing cytoplasmic mRNAs did not appear to be affected (Figure 17). This suggests that new mRNA synthesis is required for induction of the HS response in mouse cells. Similar results have been reported for numerous systems, including heat-shocked avian erthryocytes (Atkinson and Dean 1985), and chicken embryo cells stressed by heat (Kelley and Schlesinger 1978) or arsenite (Johnston et al. 1980).

The new and/or enhanced synthesis of each HSP is dependent on the length of time the cells remain at a particular HSP-inducing temperature, and is not necessarily coordinated with the synthesis of the other HSPs. A temperature of at least 41°C (for greater than 1 h) is required to detect synthesis of the HSPs by human lymphocytes, with greater quantities of the HSPs being detectable as the incubation temperature increases to 43°C (see Figure 6). This finding suggests that the limited HS response reported by other studies with some human transformed cell lines may be a function of the short periods such cells were heat-shocked (10-30 min, Anderson et al. 1982). Similarly, with mouse lymphocytes (Figure 16), fluorographic analysis of the 2-D gels shows that detectable quantities of the 100 and 90 kDa HSPs are present after 60 min at 41°C, while at least 90 min at this temperature is required to detect noticeably enhanced synthesis of the 110, 70 and 65 kDa

HSPs. When the incubation temperature is increased to 43°C, HSP synthesis is markedly enhanced after only 30 min at this temperature. Similar evidence of non-coordinate HSP gene expression has been shown by others in Drosophila (Lindquist and DiDomenico, 1985) and in avian erythrocytes (Atkinson and Dean, 1985). This response of mammalian lymphocytes to heat-shock, which involves the rapid enhanced synthesis of the HSPs, is a reversible response. When heat-shocked cells are returned to a normal culture temperature, HSP synthesis gradually ceases and the cells recover their normal pattern of protein synthesis after several hours at the normal culture temperature (Figure 18).

The set of HSPs synthesized in culture by mammalian lymphocytes is similar, in whole or in part, to the HSPs synthesized by other mammalian cell types. HSPs of similar molecular mass and isoelectric point are synthesized by heat-shocked and arsenite-stressed human fetal tissue (German 1984), heat-shocked human lymphoblastoid cells (Anderson et al. 1982), heat-shocked and arsenite-stressed primary and secondary cultures of mouse embryonic fibroblasts and mouse teratocarcinoma cells (Bensaude and Morange 1983; Morange et al. 1984), ethanol- and arsenite-stressed CHO cells (Li and Werb 1982), and some, but not all heat-shocked HeLa cell lines (Thomas et al.; Welch et al. 1982), but are different, in one or more of these properties, from the HSPs synthesized by other similarly treated transformed lines of human or mouse cells (Imperiale et al. 1984; Morange et al. 1984). The most commonly reported HSPs synthesized in mammalian cells - the 90 and 70 kDa HSPs (Subjeck

and Shyy 1986) - are constitutively synthesized in low amounts by mammalian lymphocytes, but become major components of the protein synthetic pattern of these cells following heat-shock. In the absence of experimentally imposed stress, human lymphocytes synthesize low levels of the 90 and 70 kDa HSPs accounting for only 5 - 8% of the total radioactively- labelled amino acid incorporated into protein (Figure 8). Following heat-shock, 30 - 50% of the radioactively-labelled amino acid is present in these polypeptides.

While the constitutive synthesis of the 90 and 70 kDa HSPs in mammalian lymphocytes appears to be similar to the low levels of these polypeptides detected in primary cultures of mouse fibroblasts (Bensaude and Morange 1983), the myeloma cell lines tested synthesize considerable amounts of these polypeptides in the absence of heat-shock (Figures 11 and 22). While heat-shock of these cells does result in the enhanced synthesis of the 100 kDa HSP, only a moderate enhancement in synthesis of the 110, 90, 70 and 65 kDa HSPs is observed over the already elevated constitutive levels of these polypeptides. Similarly, other groups have shown that high levels of the 89 and 70 kDa HSPs are constitutively synthesized by some human and mouse cell lines (Morange et al. 1984; Imperiale et al. 1984). Alternatively, these polypeptides may be the products of genes that are homologous to the HSP genes, but which are transcribed at normal temperatures and are not heat inducible, and therefore have gene products indistinguishable from the HSPs on the basis of 2-D PAGE.

It was found that detection of a 26 kDa HSP is less apparent in human and mouse cells labelled with [^{35}S]-methionine than it is in

cells labelled with [^{14}C]-leucine. The difficulty in detecting this 26 kDa HSP in human lymphocytes labelled with radioactive methionine, and the lack of expression of a comparable low molecular-weight HSP in mouse and rabbit cells (also radiolabelled with ^{35}S-methionine) suggests that this polypeptide possesses little or no methionine, a finding in agreement with Hickey and Weber (1982), Kim et al. (1983) and Welch (1985).

The experiments which characterized the in vitro response of human and mouse lymphocytes to HS, suggest that this dramatic change in gene expression in vitro may represent a homeostatic response to stress which would also occur in cells enduring a thermal stress in vivo. To test this hypothesis, mice were exposed to brief periods of thermal stress, and the polypeptide synthetic patterns of lymphocytes collected from these mice were compared to those from polypeptides extracted from lymphocytes heat-shocked in vitro. It was found that lymphocytes obtained from, or in, mice subjected to a non-lethal, increase in body temperature (approx. 4°C) synthesize HSPs with pIs and M_rs identical to the full complement of HSPs synthesized by human, mouse and rabbit lymphocytes heat-shocked in culture, except for the absence of a 100 kDa HSP. These results, in terms of a more or less complete HSP response in situ, are similar to those reported for bone marrow from thermally-stressed adult mice, and for thermally-stressed 10- or 11- day old mouse embryos (German 1984), avian embryos (Atkinson et al. 1983) or the erythrocytes obtained from heat-stressed adult quail (Dean and Atkinson 1985; Atkinson and Dean 1985), but are

different from results obtained using tissue from intact organs of thermally stressed rats (Currie and White 1981; Hammond et al. 1981) and rabbits (Inasi and Brown 1982; Brown 1985). In these latter studies, LSD-induced hyperthermia resulted in the enhanced synthesis of only 71 and 74 kDa HSPs by tissues from intact organs (as well as a 95 kDa HSP in rabbit retinal cells), even though cultured cells from these mammals are known to respond to heat-shock by synthesizing a greater variety of HSPs (Landry et al. 1982; Welch 1985; Figure 21 of this thesis). Brown et al. (1982) suggest this limited HSP response by these tissues may be due to constraints on HSP induction in intact organs. However, our data and that of others which report the non-coordinate induction of the HSPs (Lindquist and Di Domenico 1985; Atkinson and Dean 1985) suggest that this limited response by tissues from intact organs of thermally-stressed mammals may either be the result of cells within these organs not receiving a stress of sufficient magnitude or duration to induce expression of the full complement of the HSPs, or that the method of hyperthermia induction (LSD-treatment) may be responsible for the limited HSP response.

The synthesis of HSPs by mammalian cells can be induced by exposure to a wide variety of stressors other than heat, such as chemical stress (i.e. ethanol or arsenite), anoxia, viral transformation or glucose deprivation (Nover 1984; Subjeck and Shyy 1986; see also section 1.1 of this thesis). It was found that human and mouse lymphocytes exposed to 10 - 100 μM arsenite or 6% ethanol (for 1 h at 37°C; see Figures 12 and 23 to 25), respond with the

new and/or enhanced synthesis of polypeptides with pIs and M_rs similar to most of the heat-induced HSPs (i.e. 110, 90, 70 and 65 kDa HSPs). These polypeptides have molecular masses similar to the major subset of mammalian HSPs categorized by Subjeck and Shyy (1986; M_rs of 110, 89, 70 and 68 kDa). As is the case with the heat-induced HSPs, synthesis of the chemical-stress-induced HSPs is dependent on new RNA synthesis.

Chemical stress (ethanol or arsenite) of human and mouse lymphocytes does not appear to induce the synthesis of a 100 kDa HSP. Previous reports have implicated this particular HSP as being a membrane-bound phosphoprotein involved in alterations of glucose metabolism (Welch et al. 1982; Lin and Queally 1982). Welch et al. (1983) have shown with rat-1 cells that heat-shock, calcium ionophore treatment and glucose depletion from the cell culture medium each result in the enhanced synthesis of a Golgi-associated 100 kDa HSP, which they refer to as a glucose-regulated protein (GRP). Other workers have also reported glucose-regulated proteins of similar molecular mass (95-105 kDa). Hightower and White (1982) report that synthesis of a 99 kDa glucose-regulated protein (GR99) is induced in glucose-deprived rat aorta cells, while a 95 kDa GRP has been reported in glucose-starved chicken embryo fibroblasts transformed with Rous sarcoma virus (Shiu et al. 1977) and with murine L cells cultured for prolonged periods in low-glucose medium (Kasambalides and Lanks 1981).

Rose and Khandjian (1985) have recently described a polypeptide (p105) which, like the 100 kDa GRP described by Welch et al.

(1983), is membrane associated, induced by glucose starvation, and primarily localized in the nucleus following heat-shock. These reports of a glucose-regulated 100 kDa polypeptide, along with my data showing only HS-specific induction of the 100 kDa HSP *in vitro*, and the absence of this polypeptide in cells heat-shocked *in vivo* (Figure 19) suggest that the 100 kDa HSP is a minor member of the HSP group whose synthesis is enhanced only in cultured cells following exposure to certain types of environmental stresses - such as heat-shock and glucose deprivation - but is not detectably induced by either ethanol- or arsenite- stress.

The data presented indicates that exposure of mammalian lymphocytes to such seemingly diverse environmental challenges as heat-shock or ethanol results in a similar pattern of enhanced HSP synthesis. As well, HSP synthesis is dependent on the level of stress and the length of time cells are exposed to that stress. For instance, a 60 min exposure to 41°C or 30 min exposure to 43°C each enhance HSP synthesis by mouse spleen cells, as does a 60 min exposure to 6% ethanol. However, no enhancement in HSP synthesis was observed with cells exposed to lesser levels of stress (i.e. 30 min at 41°C or 60 min with 4% ethanol), suggesting that a stress threshold must be reached before the HS response will be observed. These results raised the possibility that low levels (non HSP-inducing) of two or more stressors presented to cells concurrently might act synergistically to elicit a HS response.

To test this hypothesis, mouse splenic lymphocytes were concurrently exposed to sub-threshold levels (non HSP-inducing) of two

different stressors. Heat and ethanol were chosen as stressors, and lymphocytes were incubated for 30 min at 37°C or subjected to a "mild" heat shock (30 min at 41°C; a time period at a temperature which does not result in HSP synthesis), in the presence (or the absence) of 4% ethanol (a concentration which does not induce HSP synthesis in 30 min at 37°C). Fluorographic analysis of 1-D (Figure 26) and 2-D (Figure 27) electrophoretically-separated polypeptides and quantification of individual polypeptides synthesized by lymphocytes under these conditions (Figure 28) reveal no differences in the polypeptide synthetic patterns of lymphocytes maintained for 30 min at 37°C without ethanol, 37°C with 4% ethanol, or 41°C without ethanol. However, cells which were stressed for 30 min by a concurrent application of "mild" heat (41°C) and 4% ethanol synthesized striking amounts of all of the HSPs (except the 100 kDa HSP) synthesized by cells incubated for 1 h at 43°C or for 1 h at 37°C in the presence of 6% ethanol.

The synergistic action of two "mild" (non-HS inducing) stressors to elicit the HS response in mouse lymphocytes is dependent on the incubation temperature, the level of ethanol, and the duration of the stress period. As the length of the stress period (at 41°C) increases, less ethanol is required to elicit the HSP response. Cells stressed for 15 min at 41°C require a concurrent stress of 6% ethanol for HSP synthesis (Figure 29A), while cells stressed for 30 min at 41°C synthesize HSPs in the presence of 4% ethanol (Figures 26 and 29B). A similar HS response is observed with cells stressed for 60 min at 41°C in the presence of 2% ethanol (Figure 29C). Cells stressed for 90 min at 41°C (Figure 29D) synthesize HSPs in

the absence of ethanol, as was previously shown in Figure 15.

Further multiple stress experiments were performed, where mouse lymphocytes were exposed to concurrent subthreshold levels of heat and arsenite (Figure 30). Lymphocytes were incubated for 1 h at 37°C or subjected to a "mild" heat-shock (1 h at 41°C), in the presence (or absence) of arsenite (at concentrations ranging from 0 to 100 μM). The synergistic effect of heat and arsenite on gene expression is similar to that observed, herein, with heat and ethanol. Cells stressed with arsenite at 37°C exhibit the "full" HS response (enhanced synthesis of the 110, 90, 70 and 65 kDa HSPs) only when arsenite levels reach 50 μM. When the cells are incubated at 41°C in the presence of arsenite, this HS response is observed at arsenite concentrations as low as 5 μM. These results further demonstrate the synergistic effect multiple stressors at individual subthreshold levels have on gene expression.

Previous work in our lab (Somerville 1984) has shown that exposure to concurrent multiple stresses can enhance the HS response. Simultaneous heat (42°C) and chemical (arsenite, copper or zinc) treatments in quail myogenic cells greatly enhance synthesis of a 26 kDa HSP. Other workers have shown that synthesis of a canavanine-induced 22 kDa protein is considerably enhanced in chicken embryo fibroblasts when the incubation temperature is increased from 38-42°C (Kelley and Schlesinger 1978). Furthermore, various inducers of HSP synthesis can act cooperatively to confer thermotolerance - the acquisition of a transient resistance to lethal temperatures by cells previously exposed to a period of mild heat-shock or chemical-stress (Li and Laszlo 1985). Stressors other

than heat which induce HSP synthesis in mammalian cells, such as ethanol, chronic hypoxia and sodium arsenite, confer tolerance to subsequent heat treatment (Li 1983; Li and Werb 1982; Li and Hahn 1978), and can induce self-protection; for example, ethanol-pretreated cells become more resistant to subsequent ethanol treatment than control cells (Li et al. 1980). Collectively, these data suggest that heat-shock, and at least some types of chemical stresses affect a common "target" which directly or indirectly causes a rapid change in gene expression (Munro and Pelham 1985), resulting in the synthesis of heat-shock proteins.

Considerable evidence suggests that the HS response (i.e. synthesis of HSPs) may act as a universal and fundamental mechanism for cell protection during periods of diverse environmental stress. This evidence includes the wide variety of stresses capable of inducing HSP synthesis (Subjeck and Shyy 1986), the conservation through evolution of HSP genes and the consensus sequences required for their transcription (Craig 1986; Pelham 1985), the correlation of HSP synthesis with the protection that mild heat treatment or chemical-stress offer to cells (Li and Laszlo 1985) or animals (Dean and Atkinson 1983) subsequently exposed to lethal temperatures (thermotolerance), and the correlation of HSP synthesis resulting from mild heat pretreatment with protection against phenocopy induction (Mitchell et al. 1979). The constitutive synthesis of at least some of the HSPs at control temperatures and the subsequent enhancement in their synthesis following heat-shock or chemical-stress suggests that these polypeptides function in

cells during non-stress conditions, and that their importance is amplified during the stress episode.

Even though a large volume of data has characterized much of the heat-shock response, evidence for specific roles for any of the HSPs is at best fragmented, and it is not yet possible to establish an integrated scenario for HSP function. The 90 kDa HSP has been reported to associate both with steroid hormone receptors (Sanchez et al. 1985; Catelli et al. 1985) and the $pp60^{src}$ transforming protein of Rous sarcoma virus (Schuh 1985), one member of the 70 kDa HSP family releases clathrin from coated vesicles (Ungewickell 1985), the low-molecular weight HSPs possess striking sequence homology with the structural protein alpha-crystallin (Ingolia and Craig 1982) and ubiquitin is purported to be involved in protein degradation (Bond and Schlesinger 1985).

In this thesis, mammalian blood cells subjected to in vitro and in vivo heat-shock or in vitro chemical-stress were observed to have changes in gene expression at the level of protein synthesis which involved the enhanced synthesis of HSPs and the concomitant depression in synthesis of most normally synthesized polypeptides. While various characteristics of the HS response support the belief that the HSPs have a beneficial protective function, the depressed synthesis of many normally synthesized polypeptides - through the preferential transcription of the heat-shock genes (Ashburner and Bonner 1979; Lindquist and DiDomenico 1985), the preferential selection and translation of the HSP mRNAs and sequestering of pre-existing mRNAs translated prior to the heat-shock treatment

(Craig 1985; Lindquist and DiDomenico 1985) - could have adverse effects on cellular differentiation and function. If cells stressed by heat-shock or chemical-stress are involved with developmental processes such as the proper timing of developmental decisions during embryo growth and differentiation or are involved with physiological processes such as the continual secretion of physiologically required products, any alterations in normal protein synthesis could be of serious consequence. Indeed, evidence supporting the conjecture that stress-altered protein synthesis can lead to developmental defects is well established. Hyperthermia and ethanol have each been shown to have adverse effects on the embryonic and fetal development of rodents whose mothers were exposed to short periods of hyperthermia or ethanol-stress during pregnancy (Finnell et al. 1986; Germain et al. 1985; Nitowsky 1980; Webster et al. 1980; Edwards 1967). In humans, epidemiological studies of pregnant women suggest an association of maternal fever during pregnancy with neural tube defects in their offspring (Layde et al. 1980; Smith et al. 1978). Chronic maternal ethanol exposure during pregnancy in humans and animals results in an altered pattern of fetal development known as the Fetal Alcohol Syndrome (FAS, Abel 1984; Priscott 1982; Sulik et al. 1981).

Having established in earlier experiments that HS could induce alterations in the patterns of polypeptides synthesized by mammalian blood cells, it seemed appropriate to evaluate whether the adverse effects (i.e. the depressed synthesis of normal proteins) suggested for heat-shock on the gene expression of developing cells, might also affect differentiated cells involved

in the synthesis and secretion of protein products extremely relevant to the well-being of an organism. Blood cells, at least those which constitutively synthesize and secrete IgG, were deemed an appropriate and relevant model for this type of study, as it would seem to be advantageous for an organism to maintain an immune response (i.e. the synthesis and secretion of IgGs) during periods of hyperthermia, or feverous periods which often occur in the course of certain disease states (Roberts 1979). Thus, in this thesis, it was asked whether the amounts of constitutively synthesized and secreted immunoglobulin G detectable in the media from cell suspensions maintained at 37°C would be different from that found in the media of cells heat-shocked at 41°C or 43°C. These results demonstrate that the synthesis and secretion of IgG by mouse spleen cells or B lymphocytes does not appear to be affected by short exposure to heat-shock temperatures of 41° - 43°C. If these results from experiments with heat-shocked cultured cells are analogous to naturally occurring periods of hyperthermia, they would support the contention that the alterations in gene expression resulting from fever or hyperthermia are not detrimental and, in fact, may be beneficial to the mounting of an appropriate immune response during certain disease states.(Ashman and Nahmais 1977; Roberts and Sandburg 1979; Smith et al. 1979; Saririan and Nickerson 1982).

SUMMARY OF CONCLUSIONS:

(1) The *in vitro* exposure of human lymphocytes to short term increases in incubation temperature results in the synthesis of heat-shock proteins with relative molecular masses of 110 000, 100 000, 90 000, 70 000 and 65 000 daltons, and the depressed synthesis of many normally synthesized polypeptides. This response is dependent on the duration and severity of the heat-shock. Synthesis of a 26 000 dalton HSP is observed in cells radiolabelled with ^{14}C-leucine, but not in those cells radiolabelled with ^{35}S-methionine.

(2) The pattern of HSP synthesis observed in human lymphocytes is similar to that observed with human transformed cell lines heat-shocked *in vitro*. However, transformed cells constitutively synthesize higher levels of the HSPs than are synthesized by non-transformed lymphocytes.

(3) Lymphocytes from mice and rabbits, and mouse spleen cells and B lymphocytes synthesize HSPs with molecular masses and isoelectric points similar to those synthesized by human lymphocytes. HSP synthesis by mouse spleen cells is dependent on new transcription, and is a transient event, since heat-shocked cells returned to normal growth temperatures gradually recover normal patterns of protein synthesis.

(4) Spleen cells from mice exposed to whole-body thermal stress synthesize heat-shock proteins. The pattern of HSPs synthesized by these cells *in vivo* is similar to that observed by similar cells heat-shocked *in vitro*.

(5) Human lymphocytes and mouse spleen cells exposed *in vitro* to chemical stresses (ethanol or arsenite) synthesize heat-shock proteins with molecular masses and isoelectric points similar to the heat-induced HSPs.

(6) Low levels (non-HSP-inducing) of two different HSP-inducing stresses (heat and ethanol, or heat and arsenite) applied concurrently to cultured mouse spleen cells act synergistically to induce HSP synthesis in these cells.

(7) Quantification of the IgG constitutively synthesized and secreted *in vitro* by control and heat-shocked mouse spleen cells and splenic B lymphocytes revealed no differences between control and heat-shocked cells.

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