

Heat Shock Protein 70 as an *In Vitro* Measure of Toxicity.

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Declaration. I declare that all material contained in this thesis is my own unaided work.

Abstract

The aim of this study was to assess the potential of the heat shock protein 70 as an *in vitro* measure of toxicity. The heat shock response was analysed by one-dimensional electrophoresis and a range of immunological methods.

The two cell lines used, mouse connective tissue (L929) and normal rat kidney (NRK), were found to be extremely sensitive responding to a classic heat shock treatment. One-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) combined with silver staining revealed the induction of a 70kDa protein with only a 3°C increase in temperature. Western blotting identified this as the inducible isoform of heat shock protein 70 (hsp70). Exposure to three metals cadmium, mercury and copper showed induction of hsp70 coupled with a repression of normal protein synthesis. At the median lethal concentration (LC₅₀), measurement of hsp70 revealed a more sensitive response than that of cell mortality as determined by the neutral red assay. Hsp70 induction was significant at the no effect level (NEL) determined by the neutral red assay as the concentration at which no cell mortality could be measured. Hsp70 induction was also shown at the lowest observed effective concentration (LOEC). Measurement of hsp70 revealed a more sensitive response for mixtures of the three metals than that of cell mortality. In addition, hsp70 induction revealed toxin interaction that resulted in an additive response.

The use of ELISA in conjunction with hsp70 induction to measure *in vitro* toxicity revealed similar results to those found using one-dimensional SDS-PAGE. Below the

NEL hsp70 induction was slightly higher for several of the metals suggesting that the ELISA assay was more sensitive. The use of immunocytochemistry in conjunction with hsp70 induction to measure *in vitro* toxicity also revealed similar results to those found using one-dimensional SDS-PAGE. The use of immunocytochemistry allows for qualitative and quantitative assessment of hsp70 induction. This assay appears more suited to low level toxicity as above the LC₅₀ cell mortality becomes the greater response. Using this assay a heterogeneous response was observed at intermediate toxin concentrations. To assess the potential of hsp70 induction as an *in vitro* measure of toxicity a novel compound 2-Isobutyl piperidine was tested. Measuring the induction of hsp70 revealed a more sensitive response than cell mortality. Significant hsp70 induction was shown at the NEL and LOEC.

Hsp70 induction showed a more sensitive response than the neutral red assay, which is considered a very sensitive measure of cell mortality. Induction of hsp70 at the NEL demonstrates the assays ability to identify sub lethal toxicity. The high level of induction at the LOEC demonstrates the sensitivity of the assay for measuring sub lethal toxicity. The repression of normal protein synthesis aids this assay by removing potentially interfering cellular proteins. Identifying toxin interaction suggests the assay could be applied to true environmental situations. The ability of hsp70 induction to be used in conjunction with ELISA and immunocytochemistry methods to measure *in vitro* toxicity provides the opportunity to develop a routine automated assay. The use of hsp70 induction to measure the toxicity of 2-Isobutyl piperidine demonstrates the potential to assess the toxicity of newly synthesised compounds.

Abbreviations

ATP	Adenosine Triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
GRP	Glucose regulated protein
HSE	Heat shock element
HSF	Heat shock factor
HSP	Heat shock protein
HTD	Highest tolerated dose
LC ₅₀	Median lethal concentration
LD ₅₀	Median lethal dose
LDH	Lactate dehydrogenase
LMW	Low molecular weight
LOEC	Lowest observed effective concentration
MIT	Metabolic inhibition test
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NBT	Nitro blue tetrazolium
NEL	No effect level
PBSA	Phosphate buffered saline A
PVDF	Polyvinylidene difluoride
Rf	Relative mobility
RNA	Ribonucleic acid
SARS	Structure activity relationships
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide electrophoresis
TEMED	NNNN'-tetramethylethylenediamine

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1 Introduction.

1.1 Animal experimentation.

The lone voice of Rachel Carson (1962) warning of impending environmental disaster has been joined over the last 38 years by a ground swell of concern about the effects of mans activities on our planet and such concern appears justified. Among, the 70,000 different chemicals in everyday use, only about 10% have been fully tested for toxicity (Rhodes, 1989). Although sensitive and accurate analyses exist for such chemicals, they cannot provide information on the ability of these substances, especially when present in complex mixtures, to enter and affect living organisms. Traditionally animals have been the only biological models available to assess these chemicals (Table 1, Appendix 1). The use of animals in experimentation is becoming increasingly unacceptable due to the expense, time constraints and questionable scientific merit of results. Estimates of the number of animals used annually in U.S. laboratories range from 17 to 70 million (Orlans, 1994). In the European Union the number of animals used in experiments in 1996 was estimated to be over 12 million (European Commission, 1999) (Table 2, Appendix 2). The U.S. Department of Defence, the Veterans Administration and the National Institutes of Health use an estimated 1.6 million animals (Office of Technology Assessment, 1986). These figures do not include experiments that were contracted out to non-governmental laboratories thus the actual total of animals is probably much higher. Some 2,000 chimpanzees are maintained in U.S. laboratories (Cantor, 1993) and approximately 100 chimps are born each year to captive mothers (Prince *et al*, 1988). Every year, 5.7 million animals are used in secondary and college science classes for dissections (National Anti-Vivisection Society, 1994) and about five

million animals die in toxicity tests performed in the United States (Humane Society of the United States, 1984). The Office of Technology estimates that between 3.4 and 3.7 million rats are killed annually in research laboratories, and estimates from other sources range as high as 23.6 million every year (Office of Technology Assessment, 1986).

Out of 800 chemicals tested on rats and mice, 520 (65%) caused cancer in the animals but not in humans and different results between rats and mice were reported (Lave *et al*, 1988). Metabolic differences in rats make them unsuitable for extrapolating results from studies of heart disease, cancer and stroke the top three killers in the U.S.A. (Luginbuhl, 1966). There are many physiologic and anatomical differences between animals and humans. Data obtained on chimpanzees cannot be extrapolated safely to humans. Chimps injected with the HIV virus (Nara *et al*, 1989) or hepatitis B virus (Ponzetto *et al*, 1988) do not show clinical symptoms.

The U.S. National Institute of Health (NIH) is the world's largest funder of animal experiments. It dispenses seven billion tax dollars in grants annually, of which about \$5 billion goes toward studies involving animals (Stoller, 1990). In addition, the U.S. Department of Defence spent about \$180 million on experiments using 553,000 animals in 1993. This figure represents a 36% increase in the number of animals used over the past decade (Office of Technology Assessment, 1986). It costs about \$2 million to test a single chemical on rats and mice (Brinkley, 1993). In addition, estimates for the cost of lifetime care for one chimp range as high as \$250,000 (Rowan and Moore, 1990). In cancer studies, animal tests of a single substance may take four to eight years and cost

\$400,000 or more, whereas short-term non-animal studies cost as little as \$200-\$4,000 and can be completed in just days (Colwell, 1996). In this context, there is increasing interest in the use of *in vitro* alternative toxicity testing for the rapid and low cost toxicological screening of chemicals. The positive social effects of *in vitro* toxicity testing in reducing the use of animals are recognised by pharmaceutical, chemical and cosmetic industries (Rhodes, 1989; Balls *et al*, 1990). Corrositex is an *in vitro* test approved by the U.S. Department of Transportation as a substitute for the traditional rabbit skin test. The method gives results in just a few hours for as little as \$100 per test (Roush, 1996). Tissue culture is currently the most promising alternative overcoming many of the cost, ethical and statistical problems associated with animal experimentation.

1.2 Tissue culture:

Tissue culture has been in existence since the beginning of the 20th century. Early experiments in tissue culture used undisaggregated fragments of frog tissue to study the behaviour of cells free of systemic variation (Harrison, 1907; Carrel, 1912). Frog tissue was used as no incubation was required and tissue regeneration is more common in lower vertebrates. The term tissue culture is used as a generic name to include both organ and cell culture. Organ culture refers to the three dimensional culture of undisaggregated tissue whereas cell culture refers to cultures derived from dispersed cells (Freshney, 1983). The culture of cells from primary explants of undisaggregated tissue dominated for over fifty years, however since the 1950's there has been a huge expansion in dispersed cultures.

Further development resulted from the cultivation of tissue of warm-blooded animals. Embryonated hen's eggs were a favourite choice giving accessibility to many different tissues that grew well in culture. The development of experimental animal husbandry, particularly with genetically pure strains of rodents brought mammals to the forefront as a preferred material. Chick embryo and rodent tissue provided a diversity of cell types in primary culture and had the advantage of producing continuous cell lines (Earle *et al*, 1943). Medical science developed tissue culture as a clinical tool when it was demonstrated that human tumours could give rise to continuous cell lines most notably the HeLa cell line (Gey *et al*, 1952). The use of mammalian tissue allowed more reliable extrapolation to humans, as normal and pathological development is similar for both although tissue cultures lack systemic systems. The commercial supply of media, sera, antibiotics, cell lines and clean air equipment have made tissue culture accessible to a wide range of scientific interests resulting in further development and expansion.

1.3 Cytotoxicity Testing:

Cell culture cytotoxicity testing is currently the most promising alternative to animal experiments and overcomes many of the ethical, cost and statistical problems associated with animal experimentation. The use of multi-well plates allows replicate testing with numerous chemical concentrations. Cytotoxicity tests are statistically more valid than animal tests due to the numbers of cells used, which can be as high as 10^5 - 10^6 cells per ml. The uniformity of cell lines between replicate samples also adds to the statistical reliability of cytotoxicity tests. The use of electronically controlled incubators, which regulate temperature, humidity and carbon dioxide levels, and the use of defined media and serum allow for strict control of environmental conditions. In addition, cells are

exposed directly to the reagent at lower defined concentrations with direct access to the target cell. In contrast, over 90% of the chemicals used in whole animal tests are excreted from or distributed to tissues not under study (Freshney, 1983). Although cell culture cytotoxicity tests have many advantages over animal tests, their use as an alternative will never be accepted without validation and standardisation. The lack of systemic systems in the cell cultures and the difficulty in extrapolating results from cells to higher organisms appear to be the main concerns with respect to cytotoxicity tests.

1.3.1 Systemic systems:

The two cell lines used in this study were the mouse connective tissue cell line (L929) and the normal rat kidney cell line (NRK). Both cell lines are established cultures growing as monolayers attached to a flask surface. One of the main concerns regarding the use of cell culture for toxicity testing is the fact that the growth of cells *in vitro* does not represent their growth *in vivo*. The disassociation of the three-dimensional tissue structure onto a two dimensional substrate, results in the loss of cell interactions characteristic of the tissue. As the cells proliferate, their differentiation is further reduced. The lack of systemic systems in cell cultures as found in whole animals permits only the assessment of basal toxicity unless specialised cultures and conditions are employed. The use of cell cultures to assess toxicity would therefore appear to have limited potential with regard to the extrapolation of results for humans and animals. However, this appears to be of less importance than initially thought. Systemic toxicity has been shown to have an 80% correlation with basal toxicity suggesting that systemic toxicity is merely a reflection of basal toxicity to specific cells (Ekwall and Ekwall,

1988). Thus, simple cytotoxicity assays are very often predictive of acute systemic toxicity, provided allowance is made for the toxico-kinetics of the chemicals (Ekwall *et al*, 1991).

1.3.2 Extrapolation of cytotoxicity testing:

The extrapolation of cytotoxicity test results to human and animal toxicity has been questioned. Such concern would appear reasonable, as the growth of cells *in vitro* does not represent their growth *in vivo*. In addition, it is difficult to correlate *in vitro* and *in vivo* results due to different routes of administration, modes of administration and different test species (Babich and Borenfreund, 1990). Conversely, it has been found that animal toxicity is often not well related to human toxicity (Bernsen *et al*, 1987). Rats exposed to 0.03-1.2mg/l formaldehyde develop nasal cancer, however at concentrations of 1mg/l human workers developed no nasal cancer. Conversely, arsenic and benzene are known human carcinogens but do not produce cancers in test animals (Pugh, 1985). Therefore, any attempt to correlate *in vitro* cytotoxicity, animal toxicity and human toxicity directly is unwarranted. The normal rat kidney cell line used in this study has an epithelial morphology and thus is characteristic of many of the cells exposed to contaminants. Cadmium has been found in epithelial cells of the liver, kidney, skin, stomach, intestines and mucosa of the lungs (Marion and Denizeau, 1983). However, it would appear that the use of cells derived from the target tissues of toxicants is unnecessary and provides no better extrapolation than if non-specific cells are used. Babich and Borenfreund (1990) observed that fresh corneal cells provided no more information than established cell lines for comparisons with the Draize ocular

irritancy test. The two cell lines used in this study were chosen to act simply as biological models to assess toxicity. The most appropriate method to allow extrapolation is to establish a rank order of toxicities using cell and animal systems, which is then related to human toxicity (Clothier *et al*, 1989).

1.3.3 Sensitivity of the cell lines:

Another concern regarding the use of cell cultures in toxicity testing revolves around the varying sensitivities of the different cell lines to toxins. Several investigators have reported that the toxicity of a given chemical varies depending on the sensitivity of the particular cell line employed. Kfir and Prozeskey (1981) observed that HeLa, BGM (buffalo green monkey) and ML (mouse lymphoma) cells were found to have different sensitivities to chemicals. HeLa cells were found to be the least sensitive and BGM cells the most sensitive. Other investigators have also found cell lines of varying sensitivities including PLHC-1 cells, flounder kidney cells (Ryan and Hightower, 1994), human epithelial cells, RTG-2 cells (Marion and Denizeau, 1983), Fathead minnow cells and Bluegill sunfish cells (Babich and Borenfreund, 1987). Such variations in sensitivities have been partly attributed to different incubation temperatures and serum requirements (Marion and Denizeau, 1983; Ryan and Hightower, 1994). Median lethal concentrations (LC₅₀) (Trevan, 1927) have been found to vary from 1.3mg/l (Garza-Ocanas *et al*, 1990) to 55mg/l (Mazziotti *et al*, 1990) for mercuric chloride an over 40 fold difference, from 0.2mg/l (Clothier *et al*, 1988) to 76mg/l (Brandao *et al*, 1992) for cadmium chloride nearly a 400 fold difference and from 5mg/l (Clothier *et al*, 1988) to 800mg/l (Brandao *et al*, 1992) for copper sulphate a 160 fold difference. However, investigators using

different cell lines and chemicals have observed that the sensitivity of the cell line appears to be of less importance in toxicity testing than originally thought (Knox *et al*, 1986; Garza-Ocanas *et al*, 1990; Mazziotti *et al*, 1990). Babich and Borenfreund (1987) observed that although Fathead minnow cells (FHM) were more sensitive than bluegill sunfish cells both cell lines ranked toxins similarly with a correlation coefficient of 0.988. Using the median lethal concentration (LC₅₀) to rank toxins rather than comparing absolute toxin concentrations overcomes the variability associated with differing cell line sensitivities (Clothier *et al*, 1988).

1.4 Methods of investigation:

A number of different assays have been employed in cell culture toxicity tests, cloning efficiency (Kfir and Prozeskey, 1981; Hunt *et al*, 1987), total protein (Marion and Denizeau, 1983; Dykes *et al*, 1984; Shopsis and Eng, 1985; Stark *et al*, 1986; Riddell *et al*, 1986; Knox *et al*, 1986; Babich and Borenfreund, 1987; Hunt *et al*, 1987; Clothier *et al*, 1988), Deoxyribonucleic acid (DNA), Ribonucleic acid (RNA) synthesis (Marion and Denizeau, 1983), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Mosmann, 1983; Benford and Good, 1987; Babich and Borenfreund, 1990), neutral red (Borenfreund and Puerner, 1985; Riddell *et al*, 1986; Stark *et al*, 1986; Hunt *et al*, 1987; Benford and Good, 1987; Babich and Borenfreund, 1987), morphology (Borenfreund and Puerner, 1985; Riddell *et al*, 1986), acid phosphatase (Connolly *et al*, 1986; Mazziotti *et al*, 1990), uridine uptake (Shopsis and Sathe, 1984; Stark *et al*, 1986), metabolic inhibition test (MIT) (Hunt *et al*, 1987), Adenosine triphosphate (ATP) (Benford and Good, 1987) and cell detachment assays (Babich and Borenfreund, 1987).

Several studies have been conducted which compare these assays (Borenfreund and Puerner, 1985; Shopsis and Eng, 1985; Riddell *et al*, 1986; Babich and Borenfreund, 1987; Mazziotti *et al*, 1990). The total protein assays were found to give an earlier indication of toxicity than measuring DNA or RNA synthesis (Marion and Denizeau, 1983) and were more reliable than the lactate dehydrogenase (LDH) assay (Mazziotti *et al*, 1990). Riddell *et al*, (1986) observed that the highest tolerated dose (HTD) showed morphological alterations for some toxins that were not identified by the neutral red or kenacid blue assay. However, as the HTD is a subjective endpoint it is not considered viable for routine use. In a comparative test, Hunt *et al*, (1987) noted that the cloning efficiency assay was the most technically demanding and expensive in terms of equipment and time. Benford and Good (1987) concluded that the ATP assay was lengthy, complicated and expensive. In addition, they observed that the neutral red assay produced no false positives but overestimated the toxicity of several irritants. It was noted that this is more desirable than underestimates especially in a screening program, where further analysis would identify false positives. Babich and Borenfreund (1990) observed that protein determination may greatly underestimate the toxicity of the test agent as dead cells and cellular fragments adhere to the substratum, they also observed the neutral red absorbance was about twice as high as that of MTT allowing less cells to be used in the assay. Shopsis and Eng (1985) noted that when using a total protein assay, protein inhibitors would give false positive results whereas DNA damaging agents that allowed protein synthesis to continue would give false negative results.

Good correlation has been shown for the total protein assay, versus neutral red ($r=0.94$) (Stark *et al*, 1986; Babich and Borenfreund, 1987) uridine uptake ($r=0.87$) (Shopsis and Sathe, 1984; Stark *et al*, 1986), and cell detachment ($r=0.988$) (Babich and Borenfreund, 1987). The neutral red assay also correlated with HTD and uridine uptake (Borenfreund and Puerner, 1985; Shopsis and Eng, 1985; Stark *et al*, 1986). Comparison of neutral red, MTT and ATP showed a correlation coefficient of 0.93 (Benford and Good, 1987). The choice of endpoint depends more on personal preference and ease of use than absolute sensitivity. Hunt *et al*, (1987) concluded that when choosing an endpoint it is important to consider comparability, reproducibility, ease of use and sensitivity. The neutral red assay was used in this study to establish toxicity data as it is cheap, rapid, reproducible, sensitive and requires limited technical dexterity having the potential for automation. In addition, other investigators have used the neutral red assay extensively allowing comparison.

1.4.1 The neutral red assay:

The neutral red assay was developed initially to serve as a potential alternative to the Draize rabbit ocular irritancy test (Borenfreund and Borrero, 1984; Borenfreund and Puerner, 1985). In addition to providing information on ocular and skin irritancy the assay was a good predictor of *in vivo* acute toxicity. The neutral red assay has been used to evaluate the relative potencies of a spectrum of test agents including, inorganic metals, organometals, environmental pollutants, pharmaceuticals including chemotherapeutics and over the counter drugs, surfactants, industrial chemicals, natural

toxins, complex mixtures and biomaterials. Furthermore, this assay was applied to investigations of:

1. Metabolism mediated cytotoxicity
2. Structure activity relationships (SARS) for a series of related chemicals
3. Phototoxicity
4. Temperature toxicity interactions
5. Synergistic and antagonistic interactions between combinations of test agents
6. Growth promoters and stimulators

(Babich and Borenfreund, 1990)

1.4.1.1 Principle of the assay:

Neutral red (3-amino-7-dimethyl-2-methylphenazine hydrochloride) is a water soluble, weakly basic supravital dye that accumulates in lysosomes of viable cells. The neutral red assay is an *in vitro* cell viability test that was developed and extensively studied for *in vitro* cytotoxicity determinations (Babich and Borenfreund, 1990). Earlier *in vitro* tissue culture studies using the neutral red dye were developed for assessments of viral cytopathogenicity (Finter, 1969) and for immunotoxicity assays (Mulbacher *et al*, 1984). The neutral red assay is based on the incorporation of neutral red into the lysosomes of viable cells after their incubation with test agents. Cellular uptake of dye is accomplished by passive transport across the plasma membrane. Accumulation of neutral red within the lysosomes occurs either, from the binding of neutral red to fixed acidic charges, such as those of polysaccharides within the lysosomal matrix, or from

the trapping of the protonated form of neutral red within the acid milieu of the cytoplasmic vacuoles. The plasma membrane does not act as a barrier to retain the neutral red within the cells (Bulychev *et al*, 1978). The neutral red assay was specifically designed to meet the needs of industrial, pharmaceutical, environmental and other testing laboratories concerned with acute toxicity testing. The numerous advantages of the neutral red assay, such as its simplicity, speed, economy, and sensitivity are apparent. Good reproducibility of neutral red cytotoxicity data among different laboratories has been demonstrated (Babich and Borenfreund, 1990).

1.4.2 Correlation with animal tests:

Although cell cultures do not possess the systemic systems found in whole animals, results from several studies show high correlation between the results of cell culture and animal tests. The ranking of chemicals based on their toxicity and not their definitive concentration appears to facilitate such good correlation. The neutral red assay has shown strong correlations with:

1. The *in vivo* Draize test for the ranking of surfactants (Borenfreund and Puerner, 1985; Shopsis and Eng, 1985).
2. Rat oral median lethal dose values (LD_{50}) ($r=0.77$) for six divalent metals (Benford and Good, 1987).
3. *In vitro* and *in vivo* data for the Bluegill sunfish ($r=0.988$) and fathead minnow ($r=0.835$) (Babich and Borenfreund, 1987).
4. Golden Orfe 48 hr LC_{50} data ($r=0.89$) (Brandao *et al*, 1992).
5. Rat and *Daphnia magna* data for butylated tins (Babich and Borenfreund, 1988).
6. Mud crab and *Daphnia magna* studies for the ranking of diorganotins (Babich and Borenfreund, 1988).
7. Corneal opacity scores ($r=0.84$) (Dierickx and Gordon, 1990).

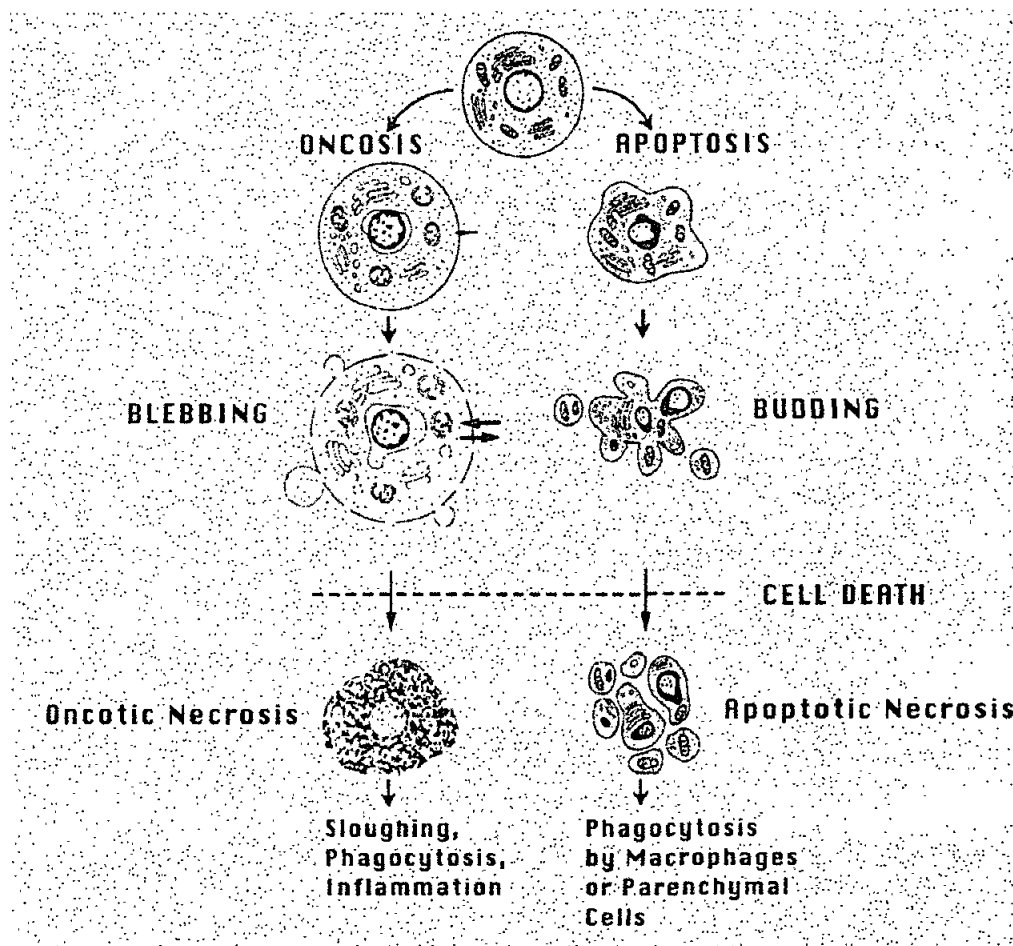
In vitro toxicity testing uses biochemical, cellular, and physiological biomarkers as diagnostic screening tools in environmental monitoring. Ideally, it should be possible for such biomarkers to be used across a broad range of organisms exposed to a variety of stress conditions, to correlate with decreased physiological function or survival and to be easily and economically measured. Traditionally, changes at higher levels of

biological complexity (metabolism, physiology, morphology, histology and immunology) have been used as biomarkers. However, such biomarkers by their nature are time consuming assays. Changes at the lower molecular and cellular levels of organisation in DNA and proteins can register exposures to and effects of environmental pollutants more efficiently and at an earlier time point. However, studies in toxicology have been hindered by a lack of mechanistic understanding of physiology at the cellular level. One obstacle to this understanding has been the difficulty in distinguishing primary and secondary cellular responses to pollutants. Primary responses are more likely to indicate environmentally induced damage and to correlate with sub-lethal toxicity. Secondary responses are more likely to be symptomatic of cellular damage (Sanders, 1993). The endpoints used in cytotoxicity testing typically measure secondary responses that involve cell death and are thus incapable of assessing sub-lethal toxicity. A primary protective response by the cell, the heat shock response, may be the solution.

1.5 Reactions of cells to injury:

Following injury, cells alter their structure and function in processes known as apoptosis or oncosis (Figure 1). These two types of change differ remarkably in structure and function. The predominant early response to death by oncosis is cell swelling (Majno and Joris, 1995). Oncosis often follows a variety of injuries, such as toxins and ischemia applied *in vivo* and *in vitro*, because many of these interfere with ATP synthesis and thus destroy control of the interior environment of the cell by destroying control at the plasma membrane. The lack of ATP and/or loss of the cell membrane integrity mean that control of cellular ion content, normally resulting from a balance between Na⁺ entry

Figure 1: Pathways of cell death.



Legend:

The two pathways of cell death, namely, oncosis and apoptosis, which lead a normal cell (top) to necrosis. The left side of the diagram depicts schematically a cell entering and passing through oncosis while the right side that of apoptosis. Note the differences in morphology, e.g., cell swelling with blebbing and increased permeability of the plasma membrane (arrows) in oncosis, while in apoptosis there is cell shrinkage with budding. Note also the marked nuclear chromatin clumping with near-normal cell organelles in apoptosis. Both pathways pass through the "point of no return" or cell death and onto necrosis. In oncototic necrosis, changes include sloughing, phagocytosis, and inflammation, while in apoptotic necrosis, cells break up into clusters of apoptotic bodies with phagocytosis by macrophages or parenchymal cells. Reproduced with permission from Trump and Berezsky (1998).

and active Na^+ extrusion, is lost. Apoptosis involves cell shrinkage and fragmentation (Kerr *et al*, 1972). Apoptosis is the type of change observed during embryologic cell death. However, apoptosis like oncosis can readily be induced by external stressors, including environmental toxins and therapeutic agents. Cells have apparently developed complex systems to distinguish between types of injurious stimuli. Although each cell has a built in program to effect death, these mechanisms are differentially activated by different stimuli. The difference seems to involve whether or not the injury interferes with ATP synthesis and/or cellular Na^+ regulation. In the case of oncosis there is typically no source of ATP, cellular Na^+ increases, and the cells swell. Necrosis refers to the changes that occur following the death of the cell. They principally involve degradative responses that result in the decomposition of organelles and other macromolecular systems. Rapid induction of heat shock proteins (hsp), such as heat shock protein 70 (hsp70), also accompanies the early stages of oncosis following injury from a variety of agents. This induction is Ca^{2+} dependent and is due to phosphorylation of the transcription factor. Experiments with ATP synthesis in Ehrlich ascites tumour cells have indicated that the resistance to cell killing in stationary as opposed to exponentially growing cells is correlated with expression of hsp68, hsp27 and elevated hsp90 (Trump and Berezsky, 1998). Kabakov and Gabai (1995) also showed that prior induction of hsp70 by heat treatment considerably suppressed the growth induced actin aggregation and rate of necrosis.

1.6 The Heat Shock Response:

1.6.1 Origin:

Ritossa (1962) observed temperature shock induced, well-defined variations in the puffing patterns in the chromosomes of *Drosophila busckii*. The variations always involved the same bands and specific metabolic activities. Temperature shock of 30°C for 30 minutes resulted in the appearance of new puffs and the regression of normal puffs. Removal of the temperature shock resulted in the induced puffs receding and the reappearance of the normal puffs. Induction depended on the rapid attainment of a given temperature threshold and not merely the increase of 5-6°C. Isolation of *Drosophila* salivary glands showed the phenomenon to occur at the cellular level and did not require organ interaction. Puffs corresponded to changes in the synthetic activity of the chromosome bands concerned. The use of a radioactive tracer showed the puffs were sites of RNA synthesis. After heat shock RNA synthesis was absent at the site of normal regressed puffs. These findings support the idea that puffs are sites of active genes. The results suggest the function of genes is reversible and dependent on environmental conditions.

These findings in 1962 were to pioneer research into the heat shock response phenomenon. Ironically, it was not for a further ten years that significant developments were made with regard to the heat shock response. Tissieres *et al*, (1974) observed heat shock also resulted in the induction of protein bands, which appeared to have a direct relationship to the induced puffs. Six protein bands were induced which accounted for 30% of the total protein synthesis, one band accounting for 15%. This was accompanied

by a reduction in the synthesis of other protein bands. Tissieres observation sparked a renewed interest in the heat shock response that continues to date. After four decades of research the mechanism of action, regulation and the function of the heat shock proteins are better understood.

1.6.2 Nomenclature:

For largely historical reasons, the heat shock proteins (hsp's) from different organisms are referred to by a variety of different names (Table 3). Unfortunately, this often leads to some confusion when discussing the structure and function of a particular member of the heat shock protein family. Following the nomenclature first used in *Drosophila*, the various hsp's in animal cells are referred to on the basis of their mode of induction, and apparent molecular mass on sodium dodecyl sulphate (SDS) one dimensional gels. Hence, their designation as heat shock protein 70 (hsp70) refers to heat-inducible proteins of 70kDa. In bacteria, the nomenclature used, to refer to the hsp's, is based on earlier genetic studies examining bacterial host functions that were essential for bacteriophage growth. The DnaK protein is the prokaryotic homologue of hsp70, while the groEL/groES proteins are the prokaryotic homologues of hsp60 and hsp10. Finally, in *Saccharomyces cerevisiae*, the hsp's are referred to by genetic nomenclature or sometimes by their apparent size. Another class of stress-induced proteins, distinct from those induced by heat, was first observed to exhibit an increased expression in eukaryotic cells starved of glucose, and therefore were called the glucose-regulated proteins (grp).

Table 3: Nomenclature of heat shock proteins.

Family	Name	Location	Size (kDa)
Bacteria	DnaK	Cytosol	70
	groEL	Cytosol	60
	groES	Cytosol	10
Yeast	Ssa	Cytosol	70
	Ssb	Cytosol	70
	Ssc	Mitochondria	70
	Kar2	Endoplasmic reticulum	70
Eukaryotes	Hsp90	Cytosol	90
	Hsc70	Cytosol/nucleus	70
	Hsp70	Cytosol/nucleus	70
	Hsp60	Mitochondria	60
	Rubisco	Chloroplasts	60
	TCP-1	Cytosol	60
	Grp75	Mitochondria	75
	Grp78 (BiP)	Endoplasmic reticulum	78

1.6.3 Conservation of the heat shock response:

One of the most promising aspects of the heat shock response as a biomarker of environmental pollution is its high degree of conservation throughout evolution. The heat shock response has been observed in all taxonomic classes from bacteria to man (Schlesinger *et al*, 1982). Evidence suggests the hsp's are related to each other and serve less function than originally thought (Craig *et al*, 1982). The small hsp's 27, 26, 23, and 22kDa are 40% homologous, possibly evolved from a single gene and are similar to mammalian alpha-crystallin (Craig *et al*, 1982).

In *Drosophila* hsp70 is the most abundant hsp and is encoded at two cytological locations 87a and 87c (Craig *et al*, 1982). Molecular weights vary from 70-75kDa depending on gel concentration, markers and species (Mirault *et al*, 1978). One and two-dimensional electrophoretic analysis showed hsp70 has several isoforms. Tryptic fingerprinting showed these were similar, possibly resulting from post-transcriptional modification of the parent polypeptide (Mirault *et al*, 1978). However, evidence suggests that as hsp70 is encoded by the 87a and 87c heat shock puff complex and that there are multiple copies of the coding sequence at these sites, it may be that each of these proteins is a product of a particular gene copy (Ashburner and Bonner, 1979). In *S. cerevisiae*, nine genes related to hsp70 of higher eukaryotes have been identified (Craig, 1985; Lindquist and Craig, 1988). Neidhardt *et al*, (1982) identified a regulon (genes/proteins sharing a common regulatory element) of 13 proteins induced by heat shock in *Escherichia coli*. Amongst these were groEL, groES, DnaK & lysU. At the amino acid residue level hsp70 in *E. coli* is 45-50% homologous with *Drosophila* and yeast heat shock genes, *D. melanogaster* shows >70% homology with human hsp70

(Craig *et al*, 1982). Homology between a prokaryotic high temperature responsive gene and the major heat shock genes of *Drosophila* and yeast establishes the conservation of these hsp genes in evolution.

The glucose regulated protein grp78 shares 60% homology with hsp70 (Munro and Pelham, 1986) and is identical to the immunoglobulin heavy chain binding protein (Bip). Hsp70 is related to normal developmental proteins, suggesting a small number of genes code for hsp's, and that different functions are carried out by the differentially regulated members of the multigene family (Craig *et al*, 1982). Antibodies raised against hsp70 identified two proteins 68 & 70kDa, the latter found to be more abundant in stressed cells (Schlesinger *et al*, 1982), hsp70 and hsp68 are 73% homologous. Using these antibodies with Western blotting Schlesinger *et al*, (1982) found cross reactivity with numerous taxonomic classes. This high degree of conservation makes the heat shock response an ideal biomarker allowing the response to be measured across a broad range of taxonomic classes.

However, Sanders *et al*, (1994) using various antibodies found the major hsp60 and hsp70 heat shock protein families are less highly conserved in invertebrates and fish than in mammals and appear to vary between tissues. They suggested care should be taken to characterise completely the cross-reactivity of an antibody whenever a new species is studied.

1.6.4 Ubiquity of the heat shock response:

The heat shock response is induced by a variety of agents besides heat shock (Table 4). However, an equally wide range of agents does not induce the response. The ability of such a diverse range of agents to cause the heat shock response has perplexed researchers. Is the cellular damage that causes the induction of hsp's of some specific type, is it perhaps by a common pathway, independent or converging pathways? An initial theory suggested that induction centres around a sulfhydryl containing target molecule, because arsenic, zinc, cadmium, copper and mercury bind to sulfhydryl groups (Kelley and Schlesinger, 1978). Chelating drugs, transition series metals, sulfhydryl reagents, heat shock and amino acid analogues also appear to induce similar if not identical proteins in cells. Kelley & Schlesinger (1978) showed the induction of three proteins by the amino acid analogue canavanine. The incorporation of the amino acid analogue appeared to trigger induction of hsp's. They suggested that the amino acid analogue incorporates into a protein, which acts as a repressor of hsp induction thus altering its function and preventing it repressing hsp induction.

Hightower (1980) also induced typical hsp's with amino acid analogues. He found that the addition of actinomycin, an inhibitor of mRNA synthesis, blocked further increases in hsp's and prevented the rapid decline of the hsp's after removal of the treatment. He deduced that the changes in the rates of accumulation were caused by alterations in mRNA synthesis, thus the induction of the heat shock response was under the control of gene expression. To confirm the inducible gene expression theory Hightower showed the cellular response was not caused by the direct stimulation of protein accumulation by a specific amino acid analogue or its metabolites, because a variety of structurally

Table 4: Agents that induce the heat shock response:

Inducer	Reference
Anoxia	Lewis <i>et al</i> , 1975
Amino acid analogues	Kelley and Schlesinger, 1978
Sulphydryl agents	Levinson <i>et al</i> , 1980
LSD	Brown <i>et al</i> , 1982
Teratogenic drugs	Buzin and Bournias-Vardiabasis, 1982
Water stress/ Wounding	Bonham-Smith <i>et al</i> , 1988
Heavy metals	Anderson and Ronne, 1990
Chemotherapeutics	Low-Friedrich <i>et al</i> , 1991
Salinity	Gonzalez and Bradley, 1994
Pesticides	Stringham and Candido, 1994
H ₂ O ₂	Marshall and Kind, 1994
River extract	Muller <i>et al</i> , 1995
Alcohols	Delmas <i>et al</i> , 1995
Lipopolysaccharide	Opanashuk and Finhelstein 1995
Polychlorinated Biphenyls Polyaromatic Hydrocarbons	Grosvik and Goksoyr, 1996

and catabolically distinct analogues were effective. In addition, he ruled out the absence of an essential amino acid by its addition after treatment, or the absence of newly synthesised biologically active proteins as shown by protein synthesis inhibition by cycloheximide. He found that the incorporation of puromycin and amino acid analogues resulted in the formation of aberrant polypeptides and that these aberrant polypeptides were rapidly degraded in the cell. Therefore, he suggested that the induced proteins were either changes due to (a) the presence of abnormal proteins or (b) catabolism of abnormal proteins. Hightower suggested that the induction of proteins was an organised, adaptive response to alterations in cellular metabolism caused by abnormal proteins, i.e. hsp synthesis in response to the degradation of large amounts of aberrant protein. They possibly function as structural components of a degradative organelle, as protease's or as enzymes.

Anderson *et al*, (1982) found high concentrations of hsp in murine testis. The peculiar requirement of the testis to maintain a high rate of protein catabolism may mimic the heat shock response. This concurs with the idea that the heat shock effect is a general response to the need for increased protein catabolism within the cell. All inducers are capable of reacting with or causing denaturation of proteins. Evidence for this was also found after heat shock on various fish cell lines. It was not merely a fixed increase in temperature above optimum that induced the response rather, the ascertainment of a critical temperature, which coincidentally results in protein denaturation (Hightower and Renfro, 1988).

1.6.5 Induction of Transcription:

The speed at which hsp's are synthesised suggests a highly regulated mechanism exists for the proficient induction of hsp mRNA transcription. At "puff level" induction is apparent after 1' minute, in addition to the time required to synthesise its mRNA, it would approximately take 3-4 minutes to synthesise the polypeptide. Thus since hsp's can be detected 8-10 minutes after shock (Mirault *et al*, 1978), the processing of the primary transcript must also be very rapid. This rate of induction suggests that the heat shock genes although inactive in normal cells are maintained in an "alert" state ready for immediate transcription (Kothary *et al*, 1984). The rapid induction of heat shock puffs must result from a regulatory molecule whose function is the induction of transcription at the puff sites (Bonner, 1982). Isolation of cytoplasmic extracts showed that the puff inducing molecule is heat stable, not present in normal cells and production of the active inducer is a cytoplasmic event (Bonner, 1982). Bonner concluded that the molecule was a protein or several proteins due to its characteristics. The failure of inhibitors of protein synthesis to affect heat shock puff induction indicates that if the agents that signal the genome are in fact proteins, these agents must exist in the cell prior to the application of the inducing agent (Ashburner and Bonner, 1979). Perhaps inducers bring about a change in the conformation or sub-cellular compartmentalisation of these signal molecules. In support of Bonners findings, Yamamori *et al*, (1982) isolated a gene "*hin*", which codes for a positive regulatory protein. This protein called the heat shock transcription factor (HSF) quantitatively activates transcription of several hsp genes at high temperatures in *E. coli*. Walsh and Crabb (1989) studied the heat shock transcription factor in *Drosophila*. They observed that under normal conditions it does not bind to the heat shock element (HSE). However, under stressful conditions this

factor binds to the heat shock element. The HSF-HSE complex promotes the transcription of the heat shock gene. Further studies showed that the transcription of hsp70 is mediated by one or more of a family of transcription factors (HSF) that interact with a specific regulatory element, the heat shock element (HSE) present in the promoter of the hsp70 as well as other hsp genes (Sorger, 1991; Morimoto, 1993). In most mammalian cells, HSF is present constitutively, but in an inactive form. Stress results in the activation of HSF to a form that binds the HSE. The activation process requires the oligomerisation of the HSF monomers to a trimeric state and is associated with hyperphosphorylation of the transcription factor (Baler *et al*, 1993; Sistonen *et al*, 1994). Binding of the HSF trimers to the HSE is necessary for transcription of heat shock promoters. Baler *et al*, (1992) demonstrated that the heat shock transcription factor was auto-regulated by hsp70 levels within the cell.

1.7 Control of the heat shock response:

1.7.1 Transcriptional control:

Gene expression in the heat shock response appears to be controlled at both the transcriptional and the translational levels. The initial reaction of a cell to a stressful treatment such as heat shock is the rapid breakdown of almost the entire population of polysomes (McKenzie *et al*, 1975). Approximately ten minutes after the treatment new polysomes appear which contain new RNA transcribed from the induced heat shock genes. Actinomycin D addition prior to treatment results in polysome depletion but no reappearance, suggesting that new polysomes are formed on mRNA synthesised only at the higher temperature. The abrupt halt in normal protein synthesis observed and

induction of specific proteins may be ascribed to the disappearance of pre existing polysomes and the build-up of new polysomes (McKenzie *et al*, 1975). At the transcriptional level the heat shock response exercises its control through the preferential synthesis of hsp mRNA at high rates.

1.7.2 Translational control:

At the translational level, the heat shock response exercises its control by stopping the translation of pre existing mRNAs. In spite of translational changes the messages encoding the normal proteins are still found in the cytoplasm (Mirault *et al*, 1978). Thus, the synthesis of hsp's represents a specific recognition and selective translation of hsp mRNAs, from a pool that includes pre-existing cytoplasmic messages (Ballinger and Pardue, 1982). At intermediate temperature the cells appear unable to distinguish between normal and hsp mRNAs, indicating that the mechanism by which hsp mRNA is distinguished from normal mRNA is not a property of the mRNA itself but induced by growth at high temperature. Several theories exist to account for this preferential translation. Studying sub-cellular compartmentalisation in control and heat shocked cells, it appears normal mRNAs at high temperature have a specific block of elongation (Ballinger and Pardue, 1983). If elongation of normal mRNA is not completely blocked in heat shocked cells there must also be a factor decreasing the rate of initiation (Ballinger and Pardue, 1983). Hickey and Weber (1982) suggest heat shock mRNAs possess structural features that confer on them a high intrinsic translational efficiency. Thus, the reduction in mRNA translation by heat shock effects hsp translation to a much lesser extent. Several authors investigated the role of phosphorylation and

dephosphorylation of mRNA as a regulatory control of hsp synthesis and normal protein repression (Ernst *et al*, 1982; Glover, 1982; Sanders *et al*, 1982). Thomas and Mathews (1982) found normal mRNA co-sedimented in an inactive form which deproteinisation reactivated. Alternatively, there is the possibility that newly synthesised normal protein is specifically degraded in heat shock cells (Ballinger and Pardue, 1983). It is interesting that the induction of hsp genes and repression of most other genes suggests that the transcription of most genes in the cell is subject to heat shock control either stimulatory or inhibitory (Morimoto *et al*, 1982).

1.8 Regulation of the heat shock response:

1.8.1 Transcriptional Regulation:

Induction patterns, although depending on the severity of the shock, are highly reproducible both in terms of quantity of hsp's produced and kinetics of their repression (Lindquist *et al*, 1982). The reproducible character of induction and repression indicates that the intensity of the response is tightly regulated. Possibly an independent regulator measures the stress and induces the appropriate amount of hsp's. On the other hand, the response may be self-regulated with hsp's acting to repress their own synthesis when they have accumulated in sufficient levels to handle the stress (Lindquist *et al*, 1982).

If an independent regulator of the heat shock response is involved then the same level of induction should be achieved whether or not functional hsp's are produced (DiDomenico *et al*, 1982). However, if the cells mechanism is to assess the adequacy of its response then the absence of functional hsp's should result in over-induction. To

discover if the heat shock response was independently or self regulated DiDomenico *et al*, (1982) incorporated amino acid analogues that alter the function of the proteins, without affecting transcription and translation processes. Incorporation resulted in a normal stress induction, however in recovery normal protein synthesis did not occur and hsp's were over induced, as though, the cell did not recognise the hsp-amino acid incorporated protein. To eliminate the possibility that the analogue was causing this affect an amino acid that does not incorporate with protein was used, here a typical heat shock response was observed. In addition, DiDomenico *et al*, (1982) allowed the cell to produce functional hsp's after incorporation and showed it was not the presence of incorporated protein but a lack of hsp that kept the heat shock response going. From this they showed that a specific quantity of hsp was required to initiate recovery, and the same quantity of hsp was required to initiate restoration of normal protein synthesis and repression of hsp synthesis. Addition of cycloheximide showed the same effect. DiDomenico *et al*, (1982) concluded that an independent regulator was not involved in the heat shock response. The heat shock response appears to be auto-regulated with functional hsp's repressing their own transcription. Localisation of hsp's in the nucleus after shock appears to support their involvement in transcriptional regulation (Lin *et al*, 1982).

1.8.2 Post-transcriptional regulation:

Recovery from heat shock shows a rapid decrease in hsp mRNA. However, the addition of cycloheximide pre heat shock results in continued mRNA synthesis. The addition post-heat shock when sufficient hsp has been synthesised results in normal kinetics

(Lindquist *et al*, 1982). This suggests that the heat shock response is regulated by hsp's at the post-transcriptional level. To identify post-transcriptional regulation, Lindquist *et al*, (1982) used actinomycin to fix mRNA concentrations at various times during heat shock. The addition of actinomycin reduced the amount of mRNA synthesis and resulted in a more prolonged synthesis of hsp's before repression. If hsp mRNA has a fixed decay rate hsp synthesis should decline in proportion to the amount of mRNA. If it is self regulated then hsp synthesis should decline more rapidly for those that have more mRNA. Lindquist *et al* found that initiation of hsp repression varied depending on the length of actinomycin fixing with hsp synthesis declining more rapidly for those that had more mRNA. Repression always started after a certain level of hsp's was attained. Restoration of normal mRNA synthesis was delayed proportionately to the delay in initiation of hsp repression. Pre incubation with actinomycin blocked hsp synthesis completely and translation of normal mRNA was never restored. Thus, Lindquist *et al*, (1982) concluded that the heat shock response was post transcriptionally self-regulated with hsp accumulation inhibiting further transcription of hsp genes and destabilising pre existing hsp transcripts in the cytoplasm. Evidence for this is shown with gradual and rapid heat shocks that produce different recovery kinetics demonstrating post-transcriptional regulation. The protein patterns are the same, however the gradual heat shock initiates recovery more rapidly. This suggests that rapid heat shock requires time for cells to produce mRNAs and synthesise hsp's before a threshold level is attained which triggers recovery.

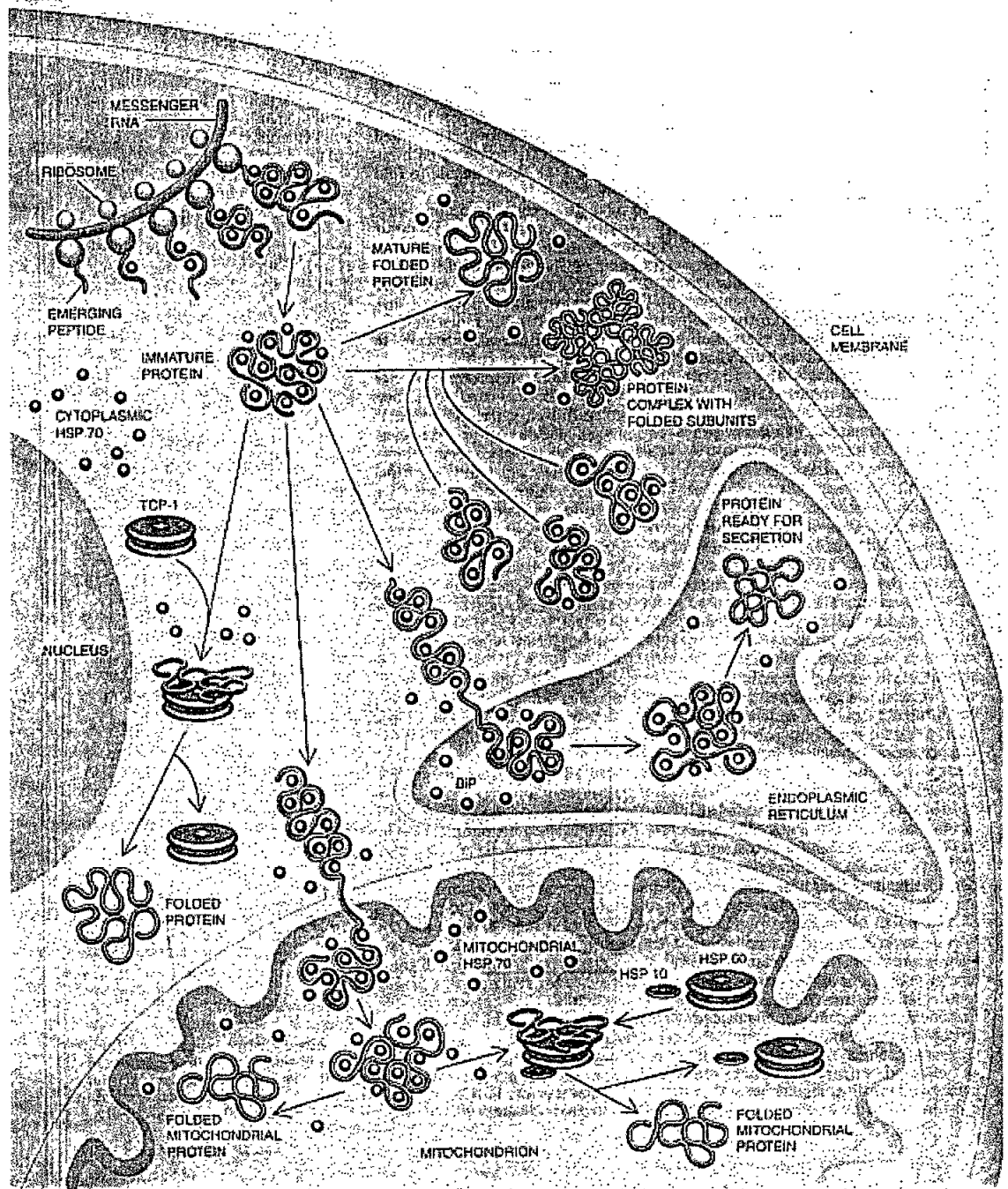
DiDomenico *et al*, (1982) believe hsp70 is involved in the regulation of the heat shock response. Evidence for this arises from experiments with cycloheximide and amino acid

analogues. These indicate that the regulator is a protein and that the protein is produced at high temperature as tests were conducted at maximum hsp70 induction levels where few other proteins are present. Finally, hsp70 induction was repressed when a specific amount of hsp70 had accumulated. This theory is supported by others, Baler *et al*, (1992) suggests that hsp70 is involved in the regulation of the heat shock response. This was supported by experiments that they carried out on the heat shock transcription factor. Self regulation is an important feature of any defence mechanism allowing for a rapid response without the overproduction of a protein.

1.9 Functions of heat shock proteins:

The heat shock proteins encompass a group of related proteins that were originally characterised based on their induction after exposure to increasing temperature. Several classes of heat shock proteins have been identified and are grouped according to their apparent molecular weight on one-dimensional sodium dodecyl sulphate polyacrylamide electrophoretic gels (SDS-PAGE). The majority of heat shock proteins are constitutively found functioning in the homeostasis of the cell. Under normal conditions, they have a chaperonein function in protein assembly, disassembly and transport (Figure 2). Under adverse conditions, the cell ceases the synthesis of normal proteins and concentrates solely on producing heat shock proteins. Under such conditions the heat shock proteins normal function takes on a new defensive role protecting proteins from denaturation and facilitating the removal of aggregated proteins.

Figure 2: Functions of the heat shock proteins.



Legend:

The functions of heat shock proteins in protein assembly, disassembly and transport.

Reproduced with permission from Welch (1993).

1.9.1 Hsp90:

Hsp90 has been found to associate with a number of cellular proteins, including steroid receptors (Catelli *et al*, 1985; Ziemiecki *et al*, 1986), several kinases (Rose *et al*, 1989) and a number of retrovirus encoded oncogene proteins many of which are tyrosine specific protein kinases (Brugge, 1986; Welch, 1990). The unifying functional theme in each of these cases appears to be that the binding of hsp90 to these cellular components regulates their activity by preventing them from carrying out their normal functions.

Hsp90 binds to some kinases immediately after synthesis, forming an inactive complex, which upon disassociation is phosphorylated and inserted into the membrane as an active kinase (Brugge *et al*, 1981, 1983). Hsp90 forms a stable complex with several members of the nuclear receptor super family (Sanchez *et al*, 1987; Perdew, 1988; Carson-Jurica *et al*, 1989; Chambraud *et al*, 1990). Hsp90 is believed to inhibit activation and translocation of the receptors into the nucleus in the absence of ligand (Baulieu and Catelli, 1989). There is also evidence to suggest that hsp90 must bind to these receptors for their subsequent activation (Baulieu and Catelli, 1989). Receptors form an inactive complex with hsp90 in the absence of hormone (Brunt *et al*, 1990; Nover, 1991). Hsp90 regulates receptor action by masking the DNA binding site of the receptor in the absence of hormone (Sanchez *et al*, 1985; DeMarzo *et al*, 1991). Upon binding of the receptor to the hormone, hsp90 disassociates and the hormone-receptor complex binds hormone-responsive elements on the DNA. Because the binding of hsp90 to the aporeceptor increases the subsequent affinity of the receptor for the hormone, it also plays a role in signal transduction (Picard *et al*, 1990). This heat shock protein may also be involved in the initial folding of DNA-binding proteins with which it does not form a complex (Shanknovich *et al*, 1992). Thus, the possibility that hsp90

may play a broader role in protein folding and activation of other proteins, particularly those that are transcription factors. Finally, hsp90 also binds non-covalently with actin and tubulin of the cytoskeleton under polymerising conditions (Koyasu *et al*, 1986, 1989) stabilising it during shock (Schlesinger, 1990). Upon exposure to environmentally stressful conditions, the synthesis of hsp90 increases and may redirect cellular metabolism to enhance tolerance. However, the specific mechanisms involved have not been elucidated.

1.9.2 Hsp70:

The ubiquitous hsp70 family is the most highly conserved and largest of all the heat shock protein families; it also has been the most extensively studied. Members of the hsp70 family are found in several sub-cellular compartments. They bind to target proteins to modulate protein assembly, disassembly and transport. Hsp70 functions as a molecular chaperone by binding to hydrophobic sites on unfolded segments of polypeptide chains (Pelham, 1988; Lindquist and Craig, 1988; Beckmann *et al*, 1990; Schlesinger, 1990). These unfolded segments may be presented as either nascent polypeptides emerging from the ribosome or from the lipid bilayer after membrane translocation, as sequences exposed by partial protein denaturation following an environmental stress, or as peptide loops extending from an otherwise native protein molecule (Gething and Sambrook, 1992). Hsp70 facilitates the correct folding of nascent peptides by stabilising the peptide chains in a loosely folded state until synthesis has been completed, and subsequently preventing the peptide from folding incorrectly (Beckmann *et al*, 1990; Gething and Sambrook, 1992). Once released the polypeptide

has the opportunity to complete its folding by forming intramolecular interactions, or to assemble into oligomeric structures with nearby polypeptide chains, or with another chaperone such as hsp60. If such interactions are not formed rapidly hsp70 proteins which are present at high concentration may rebind and again stabilise the unfolded protein. Under normal physiological conditions productive folding or rebinding to hsp70 molecules are more likely events than misfolding (Gething and Sambrook, 1992). In addition, hsp70 maintains some proteins in an unfolded intermediate configuration for targeting and translocation to cellular compartments, including the endoplasmic reticulum, chloroplast, and mitochondria (Marshall *et al*, 1990; Philips and Silhavy, 1990; Nover, 1991; Sanders *et al*, 1992). Hsp70 also facilitates the subsequent folding of peptides after import (Chirico *et al*, 1988; Craig, 1990; Hartl and Neupert, 1990; Watson, 1990). In all cases, ATP hydrolysis appears necessary for dissociation of hsp70 with the protein during the folding process (Pelham, 1988). In addition to these roles in protein homeostasis, hsp70 also may be involved in gene regulation through interactions with transcription factors (Wickner *et al*, 1991).

Under adverse environmental conditions, the synthesis of hsp70 increases and it takes on new but related roles to protect the cell from proteotoxicity. It is obvious that any increased probability of misfolding could be reversed by increasing the local concentration of hsp70. It is equally clear that the cell has developed mechanisms to sense increased amounts of nascent or unfolded proteins in different cellular compartments and to respond by inducing the transcription of the appropriate hsp70 gene. Cytoplasmic hsp70 migrates to the nucleus, where it binds to pre-ribosomes and other protein complexes to help protect them from denaturation (Lindquist, 1986; Gething and Sambrook, 1992). A major feature of stressed cells is the loss of integrity

of the nucleolus and the associated inhibition of rRNA synthesis and ribosomal activity (Welch, 1990). Under stress, hsp70 migrates to the nucleolus where it is speculated to resolubilise denatured pre ribosomal complexes and restore nucleolar function during recovery from stress (Welch and Feramisco, 1984). During recovery it migrates to the cytoplasm where it associates with ribosomes and polyribosomes where it is speculated that it may bind to denatured proteins and in an ATP dependent manner facilitating their resolubilisation (Welch and Feramisco, 1984; Pelham, 1988). It also prevents the formation of insoluble aggregates, which are particularly damaging to the cell (Ellis, 1990; Pelham, 1990). There is also evidence that hsp70 can break up existing aggregates and allow damaged proteins to refold and attain their original biological activity (Pelham, 1988; Ellis, 1990; Gaitanaris *et al*, 1990; Skowyra *et al*, 1990). It has been suggested that hsp70 assists correct folding not only through their anti-folding function but by disentangling malformed or aggregated proteins using the energy released during ATP hydrolysis. It is suggested that rather than actively unwinding the polypeptide chains in a catalytic process, hsp70 may bind to the peptide loops that are transiently exposed and in a mechanism that parallels its putative role during normal folding, stabilise the polypeptide chain in a state competent for subsequent refolding to the correct conformation. ATP hydrolysis would then be required to promote release of hsp70 to allow the polypeptide to continue the folding process. Finally, hsp70 vectors badly damaged proteins to the lysosomes for degradation (Chiang *et al*, 1989). Hsp70 expression is an excellent example of an evolutionary conserved cellular defence mechanism that protects cells against heat and other types of stress.

1.9.3 Hsp60:

The chaperonin family (hsp60) also binds target proteins to facilitate folding and assembly, however, chaperonin and hsp70 perform different folding functions. Hsp70 associates with imported polypeptides even before their translocation is complete, whereas hsp60 becomes involved at a later stage (Gething and Sambrook, 1992). They also appear to play a different role during the process of polypeptide formation. Hsp70 stabilises unfolded forms of their substrates, with folding occurring after hsp70 release. Hsp60 allows partial folding to take place on its surface. Under normal conditions, this complex binds incompletely folded proteins and directs the folding peptide to the correct conformation in a specific ATP-dependent fashion (Buchner *et al*, 1991). The complex also prevents aggregation of incompletely folded proteins until they are competent for oligomer assembly (Cheng *et al*, 1989; Ostermann *et al*, 1989; Martin *et al*, 1991). An important feature of chaperonins is that they do not form part of the final assembled protein. Their role in protein assembly and translocation, although essential, is a transient one (Watson, 1990). Under adverse environmental conditions that cause an increase in protein denaturation, the synthesis of hsp60 increases. It takes on an additional role during protein repair by binding to damaged proteins to help refold them to their native conformation. Increased levels of hsp60 also can protect against protein denaturation and aggregation (Martin *et al*, 1992). However, unlike hsp70, hsp60 is not able to break up existing aggregates (Buchner *et al*, 1991).

1.9.4 Low Molecular Weight heat shock Proteins:

A more diverse group of heat shock proteins, referred to as low molecular weight (LMW) heat shock proteins, is comprised of a large number of heat inducible proteins in the 20-kDa range. The LMW heat shock proteins are more species specific than the other major heat shock protein families and less highly conserved (Nover, 1991). However, all members of this heat shock protein family examined to date are partially homologous to alpha crystallin and share the ability to form higher order structures of approximately 500,000 kDa. Unlike hsp90, hsp70, and hsp60, these heat shock proteins are not synthesised under normal conditions. Their synthesis is induced under adverse environmental conditions and is developmentally regulated. This group of heat shock proteins has long been implicated in thermotolerance (Subject and Sciandra, 1982; Landry *et al*, 1982, 1989; Lindquist, 1986; Bosch *et al*, 1988). However, little is known regarding their specific cellular function or the mechanisms involved in their role in thermotolerance.

1.9.5 Other heat shock proteins:

Ubiquitin is a low molecular weight (7kDa) protein involved in the non lysosomal degradation of intracellular proteins (Schlesinger and Hershko, 1988) however, due to its size it is difficult to identify by SDS PAGE. A number of other heat shock proteins have been reported. However, little is known about their prevalence or function. Some of these, such as hsp104 and hsp110, appear to be induced by a variety of stressors in addition to heat shock. The hsp104 associates with the nucleolus upon heat shock and is required for thermotolerance in yeast (Sanchez and Lindquist, 1990). It has been

suggested that it is involved in rRNA synthesis and processing (Black and Subject, 1991). Hsp104 promotes the recovery of heat damaged splicing in the absence of other protein synthesis, it does not protect splicing from the initial disruption, suggesting hsp104 functions to repair splicing rather than prevent initial damage, hsp70 also has a role in this function (Vogel *et al*, 1995). Hsp110 associates with RNA or with a complex of proteins that bind RNA, it is speculated that hsp110 is induced to protect it (Subject *et al*, 1983). Using indirect immunofluorescence Lin *et al*, (1982) found hsp100 was localised in the golgi apparatus suggesting that it may be involved in the metabolic or catabolic processes of the golgi apparatus.

1.9.6 Haem oxygenase:

Although not a classic heat shock protein, haem oxygenase has been identified as a stressor specific protein. This 32kDa protein is an enzyme essential for haem catabolism that cleaves haem to form biliverdin, which is subsequently reduced to bilirubin (Keyse and Tyrrell, 1989). It is most highly induced by a variety of stressors that cause oxidative damage, such as UVA radiation, sodium arsenite and hydrogen peroxide (Keyse and Tyrrell, 1989). Cadmium, copper, zinc, lead, gold and sodium arsenite induce haem oxygenase (Keyse and Tyrrell, 1989).

1.9.7 Metallothionein:

Like haem oxygenase metallothionein is mentioned because of its role as a stressor specific protein. This 10kDa protein is a low molecular weight cysteine rich metal binding protein (Caltabiano *et al*, 1986). This metal binding ligand appears to be part of a cellular compartmentalisation or sequestration system which evolved to regulate the uptake and tissue distribution of essential trace metals such as the transition elements zinc and copper (Viarengo *et al*, 1985). Metallothionein synthesis is induced upon trace metal exposure (Caltabiano *et al*, 1986).

1.9.8 Thermotolerance:

Thermotolerance is the ability of a mild heat shock to confer a greater tolerance to a subsequent lethal heat shock. Hsp's have been implicated in thermotolerance as it was shown that protein synthesis was necessary during the intermediate heat shock (Craig, 1985). Other inducers besides heat produced the same effect and heat shock conferred resistance to other stressors. In support of hsp's role in thermotolerance, Yamamori *et al*, (1982) showed the synthesis of hsp's plays a critical role in supporting growth of *E. coli* at high temperatures and that the higher the temperature, the greater the amounts of hsp's that are required for normal cell growth. Hsp expression during thermotolerance varies depending on the treatment and severity (Li *et al*, 1982). Baler *et al*, (1992) investigating the regulation of the heat shock factor demonstrated the role of hsp70 in thermotolerance. They proposed that if inhibition of HSF activation were mediated by one of the hsp's, it should be more difficult to heat activate the HSF in cells that contained elevated levels of the hsp. Evidence for this was provided by a pre heat shock

that rendered the cells thermotolerant to a subsequent heat shock. Cycloheximide addition preventing hsp synthesis did not result in tolerance. Baler *et al*, (1992) also demonstrated hsp70 was involved in thermotolerance by the specific interaction of hsp70 and the HSF. Brown *et al*, (1982) studied hsp's within the intact body tissues of thermoregulating animals. Thermotolerance may find a role in cancer treatment, identifying sensitive tissues and allowing treatments to be timed when cells are most vulnerable (Li *et al*, 1982).

1.9.9 Developmental control:

Heat shock proteins also appear to function in embryonic development. Heat shock sufficient to turn off RNA and protein synthesis in embryos, results in abnormal phenotypes of *Drosophila* (Mitchell and Petersen, 1982). A preheat shock treatment which allows RNA and protein synthesis to recover more rapidly after heat shock prevents abnormal phenotypes. Drugs that inhibit differentiation in primary embryonic cultures stimulate the synthesis of three low weight hsp's (Buzin and Bournias-Vardiabasis, 1982) suggesting a possible protective role.

1.10 Decline of hsp70 expression during aging:

Changes in the heat shock response with age could seriously compromise the capacity of an organism to respond to changes in its environment. For example, Luce and Cristofalo (1992) found that late passage cells were more sensitive to heat shock than early passage cells. Because aging and senescence are characterised by a reduced ability to maintain homeostasis in response to stress, a number of investigators have compared

the abilities of young and old cells to express hsp70 (Richardson and Holbrook, 1996). The current data indicate that the induction of hsp70 expression by a variety of stresses generally declines with cellular aging both *in vivo* and *in vitro*. Hsp70 expression has been shown to decline with aging in heat-shocked cells, including lung and skin fibroblasts (Fargnoli *et al*, 1990), hepatocytes (Heydari *et al*, 1993) and splenocytes (Pahlavani *et al*, 1995). Although the magnitude of the age related decline varies somewhat the response is typically reduced by half. The only exception appears to be for *D. melanogaster* where an increase in hsp70 expression with aging was observed (Fleming *et al*, 1988). Decline of hsp70 expression during aging occurs with other stressors besides heat including heavy metals and amino acid analogues (Luce and Cristofalo, 1992). Hsp70 is not the only heat shock protein whose expression declines with aging. Liu *et al*, (1989) observed decreases in hsp's of molecular weights ranging from 90 to 25kDa. In addition, Heydari *et al*, (1994) observed glucose regulated proteins declined.

The age related decline in hsp70 expression appears to be due to reduced heat shock transcription factor (HSF) binding to the heat shock element (HSE). Liu *et al*, (1989) observed the hsp70 promoter was less active in late passage cells with a >90% decline in heat stress induced HSE binding activity, other investigators have also reported similar findings (Heydari *et al*, 1993; Fawcett *et al*, 1995; Pahlavani *et al*, 1995). The decreased binding activity could arise from a decrease in the expression of the HSF, however similar levels of HSF have been observed in old and young cells (Heydari *et al*, 1994; Fawcett *et al*, 1995). Thus, it appears that the age related decline in HSF binding activity is due to a decrease in its activation to a binding form. Current evidence

indicates that the transcription of hsp70 is under negative regulation (Clos *et al*, 1990; Lis and Wu, 1993; Morimoto, 1993). A regulatory factor, thought to be a protein and possibly hsp70 (Morimoto, 1993), has been proposed to stabilise HSF in its monomeric form and prevent oligomerisation. Stressors are believed to disrupt the binding of the regulatory molecule and allow oligomerisation. The mechanism responsible for the decrease in HSF oligomerisation with age possibly involves an increase in the level or activity of the negative regulatory protein. However, mixing young and old cell extracts Fawcett *et al*, (1995) observed the age related decline in HSF binding activity is not due to the accumulation of a negative regulatory protein. Another suggestion is an alteration in the HSF molecule resulting in decreased ability to oligomerise (Fawcett *et al*, 1995). The HSF has a relatively slow turn over rate and thus is potentially vulnerable to posttranslational modifications. It remains to be determined why HSF activity is reduced in aged cells. Is it due to a defect in the signal transduction processes or is it due to a defect or alteration in the HSF protein itself? Heydari *et al*, (1993) observed dietary restriction reduced the decline in hsp70 expression that occurs with aging and correlated this to the increase in HSF binding activity. Volloch *et al*, (1998) demonstrated that the diminished inducibility of hsp72 in aged cells results in increased rate of apoptotic cell death following heat shock. Forced induction of hsp72 in aged cells leads to a restoration of thermotolerance manifested in a lower rate of apoptosis.

The decrease in stress proteins with aging may serve as a useful biological marker of age related diseases. Schipper *et al*, (2000) have observed haem oxygenase levels are decreased in subjects with sporadic Alzheimer dementia and suggest its expression may aid the diagnosis of early sporadic Alzheimer dementia.

1.11 Kinetics of the heat shock response:

The kinetics of the heat shock response are very rapid, *in vivo* induction of puffs occurs within 1 minute of the temperature increase reaching a maximum about 1 hour later at which time hsp's account for 50% of total precursor incorporation (Ashburner and Bonner, 1979). The new proteins can be detected by electrophoresis after 10 minutes treatment (Lewis *et al*, 1975). After 3 hours RNA synthesis is half its maximum, with synthesis decreasing more rapidly if the heat shock is removed (Ashburner and Bonner, 1979). The continuation of hsp synthesis rules out the possibility that the decrease is due to cell death (Kothary *et al*, 1984). Return to normal temperature after 1 hour shows a decline in hsp synthesis until it ceases after 8 hours (Mirault *et al*, 1978; Kelly and Schlesinger, 1978). Continuous heat shock results in hsp accumulation due to their long half life's, by 6-8 hours hsp's represent about 10 % of the cells total protein (Moron *et al*, 1978). The rates of accumulation of most of the heat shock RNAs increase with an increase in the severity of the shock (Spradling *et al*, 1977). The severity of the shock is related to the delay before maximum synthesis is achieved. The greater the shock the slower the response, as would be expected if the response depends for its execution on some cellular system that itself may have been damaged (Anderson *et al*, 1982). Although synthesis of hsp's appears simultaneous, control of different hsp synthesis although similar is not necessarily co-ordinate. At 37°C, the 38kDa band forms first followed by the 67kDa, 70kDa, 26kDa and finally the 25kDa and 20kDa with respect to time (Lewis *et al*, 1975). The duration of hsp synthesis after shock has been removed is proportional to the severity of the shock. Initiation of normal synthesis is much more gradual than initial hsp synthesis. Pre existing mRNAs return to translation at approximately the same rates. Hsp mRNAs repression is asynchronous hsp70 always

first and hsp82 always last. Hsp70 repression is co-ordinate with reactivation of normal synthesis (Lindquist *et al*, 1982).

1.12 Application of the heat shock response:

1.12.1 Medicine:

Investigators are only beginning to explore the applications of the heat shock response, but already it shows huge potential in medicine and toxicology (Table 5, Appendix 3). Anderson *et al*, (1982) suggested the potential clinical importance of monitoring the induction of heat shock proteins could afford a diagnostic measurement of the severity of the stress. The potential of the heat shock response is attributed to the dramatic increase in the synthesis of heat shock proteins with appropriate damage rather than merely causing their release from the tissues into the blood. Thus, there is an opportunity to measure a responsive aspect of gene expression itself instead, of the degree of leakiness of affected cells. In cases of intentionally induced hyperthermia as used in certain cancer treatments, the measurement of the heat shock response would indicate the thermal sensitivity of target tissues (Anderson *et al*, 1982, Li *et al*, 1982). Kiang *et al*, (1997) noted that treatments that elicit a heat shock response could increase the resistance of tumour cells. However, Otova *et al*, (1999) demonstrated that repeated heat shock aids the therapeutic effect of some anti cancer treatments. Wu *et al*, (1998) observed that heat shock under strict conditions influenced the immunogenicity of tumour cells. Steels *et al*, (1992) investigated thermotolerance in human tumour cells suggesting the measurement of the heat shock response would permit optimum treatment times to be established. The small heat shock protein hsp27 has been

implicated in the tumorigenesis of breast cancer (Townson *et al*, 2000). Although, it appears unsuitable as a marker for breast cancer (Oesterreich *et al*, 1996), it may prove useful in monitoring treatment (Ciocca and Elledge, 2000).

Several investigators have examined the effects of pharmacological drugs used in the treatment of diseases. Levinson *et al*, (1980) examined the effect of drugs used in the treatment of chronic alcoholism, anti-tumour and anti-viral drugs. The cardiotoxicity of pharmaceuticals used in chemotherapy and in transplant medicine was investigated by Low-Friedrich *et al*, (1990, 1991). Teratogenic drugs were observed to induce specific heat shock proteins in embryonic cultures (Buzin and Bournias-Vardiabasis, 1982). In addition, Mitchell and Peterson (1982) investigated the role of heat shock proteins in developmental abnormalities. Levinson *et al*, (1980), and Anderson *et al*, (1982) suggested that measurement of the heat shock response could be useful in the evaluation or diagnosis of certain types of poisoning such as heavy metal or sulphhydryl poisons.

Induction of heat shock proteins in cardiac cells (Hightower and White, 1982) and brain tissue (White and Currie, 1982) suggested the possibility of using the heat shock response to diagnose diseases or injury to specific organs. Heat shock proteins improve cardiac recovery from subsequent ischemia/reperfusion (MacDonald and Stoodley, 1998). However, the beneficial effects of this response are compromised by initial tissue injury, which limits its clinical applicability (Gray *et al*, 1999). Several applications have been researched in an attempt to overcome such tissue injury including chronic mild hyperthermia (Su *et al*, 1999), localised heating (Gowda *et al*, 1998), mild pre ischemic treatment (Richard *et al*, 1996) and pre conditioning with heat (Gill *et al*,

1998). The ability of psychotropic drugs (Brown *et al*, 1982) and neurotoxins (Opanashuk and Finkelstein, 1995) to induce the heat shock response adds to its potential for assessing brain injuries. The presence of hsps in the synapses of the nervous system suggests the possibility of their use in treating neurodegenerative diseases (Ohtsuka and Suzuki, 2000). The increase in hsps after spinal injury suggests the possibility of using them to increase neuron survival (Gower *et al*, 1989).

Several investigators have examined the role of heat shock proteins in diseases. Zatloukal *et al*, (1988) investigated the heat shock response in chronically diseased livers. In a similar study, Walsh and Crabb (1989) investigated the heat shock response in relation to ethanol and its metabolites. Yonemoto *et al*, (1982) investigated the relationship between the activation of the *Rous Sarcoma* virus and heat shock proteins. Schafer and Williams (2000) investigated the role of hsp's in pancreatic disease. Matthews and Burnie (1996) observed hsp90 protected against *Candida* and suggested it had potential as a therapeutic agent. Watson (1990) reviewed the role of heat shock proteins as major immune response targets in a number of human pathogen and infectious diseases, including leprosy, malaria and tuberculosis.

The presence of hsps on the surface of tumours suggests the possibility of developing immunotoxins (Piselli *et al*, 1995). Thomas *et al*, (2000) observed that T cells that lyse tumour cells recognise hsps on the tumour surface. Rico *et al*, (1999) used hsps to stimulate T cell proliferation. Vanaja *et al*, (2000) prevented tumours using hsp induced from anti tumour compound. Gazit *et al*, (1995) used a Grp78 promoter that targets transcription in tumour cells to fight cancer.

However, to date no clinical assay has been developed that incorporates the heat shock response.

1.12.2 Toxicology:

The application of the heat shock response to toxicology has proceeded further than in medicine with several assays developed. This may be due to the characteristics of the heat shock response, which lend themselves to toxicity testing:

- The conservation of the heat shock response occurring across taxonomic classes.
- The ubiquity of the response been induced by a vast array of stimuli.
- The function of the heat shock proteins as a cellular defence mechanism.
- The speed of the response permitting rapid assessment.
- And the possibility of identifying stressor and/or tissue specific responses.

The heat shock response has been applied to various aspects of toxicology such as the adaptation of organisms to various types of environment (Brown *et al*, 1995), the possibility of differentiating between natural and anthropogenic stressors (Gonzalez and Bradley, 1994), determining the toxicity of dental materials (Oshima *et al*, 1997; Schmalz *et al*, 1997), assessing the sensitivity of cell lines (Wagner *et al*, 1999) and determining the toxicity of hepatotoxins (Salminen *et al*, 1996). The heat shock response to various distinct pollutants as a biomarker of toxicity has also been investigated (Grosvik and Goksoyr, 1996; Camatini *et al*, 1999; Kammenga *et al*, 1998). Several investigators have compared the heat shock response with existing

measures of toxicity. Vedel and Depledge (1995) examined the heat shock response as a biomarker for copper pollution in the gills of shore crabs and concluded that the heat shock response was less sensitive than measuring copper levels in the gills. In contrast, Delmas *et al*, (1995) investigated the heat shock response suggesting it would provide a sensitive, rapid and universal measure of environmental aggression. They observed heat shock protein induction was more sensitive than growth rate upon exposure to various alcohols. Sanders (1990) also found the heat shock response to be a more sensitive measure than scope for growth in *Mytilus edulis* exposed to copper.

The heat shock response has been used to assess toxicity in a number of distinct environments. Terrestrial pollutants and environmental conditions that affect plant growth and yields have been investigated (Kelley and Freeling, 1982; Bonham-Smith *et al*, 1988; Wollgiehn and Neumann, 1995; Eckwert and Kohler, 1997; Kohler and Eckwert, 1997). Werner and Nagel (1997) investigated the potential of the heat shock response to measure the toxicity of a variety of distinct pollutants in sediments. The heat shock response as a biomarker of aquatic pollution has also been investigated (Sanders, 1993; Siesko *et al*, 1997; Karouna-Renier and Zehr, 1999). Muller *et al*, (1995) examined the toxicity of river water using the heat shock response however only concentrated samples induced hsp70.

A number of different approaches have been adopted to apply the heat shock response to toxicity testing. Dilworth *et al*, (2000) used liver spheroids to assess toxicity avoiding the induction of hsps associated with establishing primary cultures (Dilworth and Timbrell, 1998). Witzmann *et al*, (1995) used two-dimensional electrophoresis to

establish the normal protein patterns and levels of heat shock proteins in kidney and liver providing a baseline to permit the detection of toxicity. Marshall and Kind (1994) developed a cytochemical assay to detect and identify localisation of hsp70 suggesting it as a useful biomarker of toxicity. Several investigators have placed genes under the control of the hsp's to quantify toxicity (Fischbach *et al*, 1993; Ait-Aissa *et al*, 2000). Using this approach Fischbach *et al*, (1993) found good correlation with *in vivo* and *in vitro* data for cancer and genetic abnormalities. Bartosiewicz *et al*, (2000) used hsp DNA bound to a solid substrate to identify gene expression on exposure to compounds. Stringham and Candido (1994) developed transgenic strains of nematodes as biological monitors of environmental stress and observed a tissue specific heat shock response suggesting the possibility of identifying tissue specific stressors. Ryan and Hightower (1994) used the heat shock response in conjunction with the neutral red assay to identify toxicity.

Several investigators have reported heat shock protein induction below a toxins median lethal concentration (LC₅₀) suggesting the potential to identify sub lethal toxicity (Ryan and Hightower, 1994; Stringham and Candido, 1994; Werner and Nagel, 1997). However, to date no studies have demonstrated heat shock protein induction at toxin concentrations that result in no cell mortality. Thus, there is a need to examine the potential of the heat shock response to identify toxicity at these sub lethal concentrations. In addition, for the heat shock response to have potential in toxicity testing there is a need to develop an assay suitable for routine application. The heat shock response assay needs to be rapid, reproducible, sensitive, economic and easy to use.

1.13 Aims of the present study

- To assess the potential of heat shock protein 70 as an *in vitro* measure of toxicity.
- To identify sub-lethal toxicity using heat shock protein 70 induction.
- To assess the potential of the heat shock response to be used in conjunction with Enzyme linked immunosorbent assay (ELISA) and immunocytochemistry techniques to assess toxicity.
- To investigate the toxicity of a novel compound using heat shock protein 70.

2 Materials & Methods:

2.1 Chemicals and Reagents:

Dulbecco's modified Eagles media, HEPES buffer, Sodium bicarbonate buffer, L-glutamine, Non-essential amino acids, Foetal bovine serum, Penicillin/Streptomycin, phosphate buffered saline A (PBSA) and trypsin were all cell culture grade and purchased from Biowhitaker, Inc. 8830 Biggs Ford Road, M16, Walkersville, MD 21793-0027. Monoclonal anti-hsp70 mouse IgG₁ clone C92F3A-5 was purchased from Stressgen Biotechnologies Ltd, Glanford Avenue, Victoria, Canada. Monoclonal anti-hsp70 mouse ascites fluid clone BRM-22, goat anti-mouse IgG₁ (whole molecule) alkaline phosphatase conjugate, BCIP/NBT tablets (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium), polyvinylidene difluoride membrane (PVDF), molecular weight markers high range (36-205 kDa), sodium dodecyl sulphate, Tris buffer, acrylamide (specially purified), N.N-methylenebisacrylamide ("Electran"), mercaptoethanol, ammonium persulphate, (Ultragrade), dithiothrietol, bovine serum albumin (BSA), NNNN'-tetramethylethylenediamine (Ultragrade), sodium azide and glycine were all of electrophoresis grade unless otherwise stated, Bromophenol blue, trichloroacetic acid, sulphosalicylic acid, silver methylamine, Hoechst 33258 stain, Whatman No1. filter paper, p-Nitrophenyl phosphate, Folin reagent, Kodak™ hypo clearing reagent, Tween-20, Triton X-100, diethanolamine and methylamine were purchased from Sigma Aldrich, Poole, Dorset, U.K. Cadmium chloride, mercury chloride, copper sulphate, magnesium chloride, acetone, crystal violet, neutral red, sulphorhodamine B, formaldehyde, acetic acid, calcium chloride, glycerol, ethanol,

methanol, sodium acetate, sodium hydroxide, sodium bicarbonate, sodium potassium tartrate, citric acid, sodium thiosulphate pentahydrate, ammonia, silver nitrate, hydrochloric acid and sodium chloride were purchased from BDH Chemicals, Poole, Dorset, U.K. All chemicals were of Analar grade unless otherwise stated. Tryptone Soya agar and Sabouraud dextrose agar were purchased from Oxoid Ltd. Wade Road, Basingstoke, Hampshire RG248PW, U.K.

2.2 Apparatus:

Gelaire BSB 4 Laminar flow cabinet from Flow Laboratories, Rickmansworth, Herts WD3 1PQ, England. Zeiss "Telaval 31" inverted microscope from Carl Zeiss Jena GmbH, Zeiss Gruppe, Geschäftsbereich Mikroskopie, Tatzendpromenade, D-07745 Jena, Germany. Olympus fluorescent microscope from Olympus Ltd. Great Western Industrial park, Dean Way, Southall, Middlesex UB24SB, U.K. Heraeus CO₂ incubator from Heraeus Instruments GmbH, Heraeusstraße 12-14, D-63450, Hanau. Eppendorf pipettes (10µl, 100µl, 1000µl and 8 tipped multichannel), sterile 1ml & 10ml pipettes, 0.22µm filters, sterile plastic universals, cryopreservation vials and Neubauer haemocytometer from Sigma Aldrich, Poole, Dorset, U.K. Multi-well plates (24 & 96) and 25cm² flasks from Corning Ltd. One the Valley Centre, Gordan Road, GB-Highwycombe, Bucks, HP136EQ, UK. "ATTO" vertical electrophoresis unit (ATTO AE-6220) and "ATTO" semi dry electroblotter from ATTO Corporation, 2-3, Hongo 7-chrome, Bunkyo-Ku, Tokyo 113, Japan. Consort E773 power supply (0-500 V range) from Consort nv, Parklann 36, B2300 Turnhout, Belgium. Anthos 2010 plate reading spectrophotometer from Anthos Labtec Instruments Ges.m.b.H. CREAM Densitometric

software from Kem-en-Tec, A/S, Haraldsgrade 68, Copenhagen DK-2100. Heidolph DSG 304/M4 plate shaker from Heidolph Instruments, Walpersdorfer Str, 12, 91126, Schwabach, Germany. IEC Centra MP4R centrifuge from IEC International Equipment Company, 300 Second Avenue, Needham Heights, Massachusetts, 02494, U.S.A. Degassing pump from Charles Austin Pumps Ltd. 100 Royston Road, Byfleet, Surrey, England.

2.3 Cell lines:

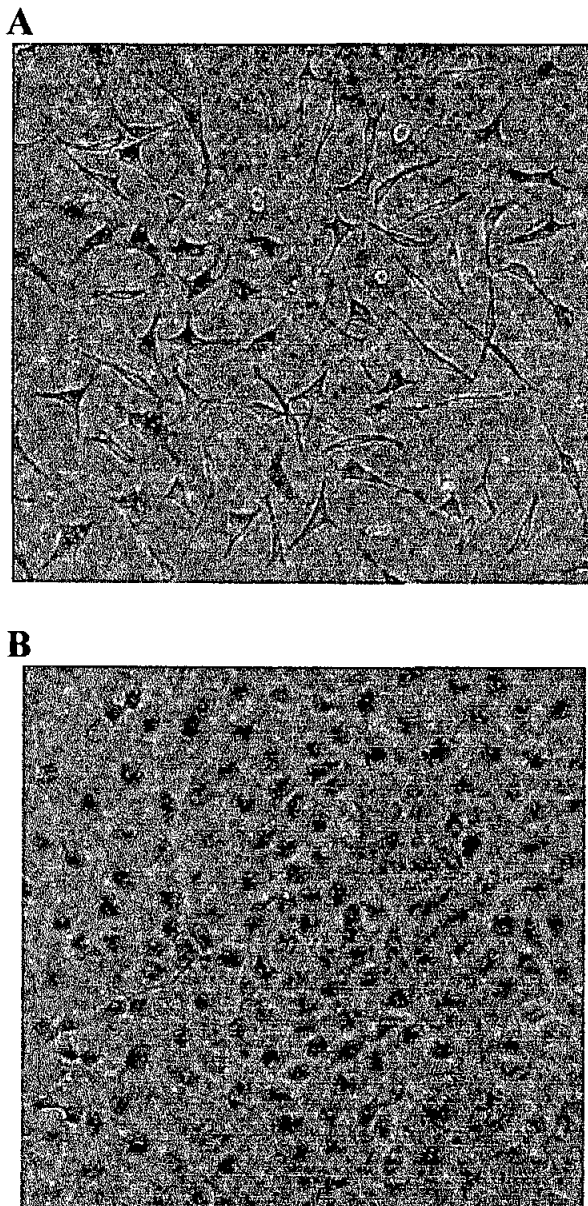
2.3.1 Mouse connective tissue cell line: (L929)

Mouse connective tissue cell line has a fibroblastic-like morphology (Figure 3A, Appendix 4). The cell line was chosen due to its ease of culture. It is a continuous cell line growing as monolayers attached to a flask surface. Subcultures are prepared by the trypsinisation method. Prior to testing the cells were thawed and grown in culture for 10 days to allow adaptation.

2.3.2 Normal rat kidney cell line: (NRK-52E)

Normal rat kidney cell line has an epithelial-like morphology (Figure 3B, Appendix 5). The cell line was chosen due to its ease of culture. It is a continuous cell line growing as monolayers attached to a flask surface. Subcultures are prepared by the trypsinisation method. Prior to testing the cells were thawed and grown in culture for 10 days to allow adaptation.

Figure 3A, 3B: Cell lines in culture.



Legend:

A. Mouse connective tissue cell line: (L929) **B.** Normal rat kidney cell line: (NRK).

Cells grow as a monolayer attached to the plate surface. L929 cells have a fibroblastic-like morphology. NRK cells have an epithelial-like morphology.

2.4 Hsp70 Antibodies:

2.4.1 Clone C92F3A-5 (Stressgen):

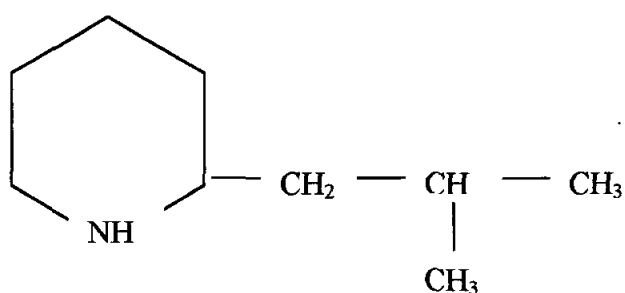
The antibody was developed against purified hsp70 from human HeLa cells (Welch and Suhan, 1986). Following three rounds of single cell cloning, the antibody was isolated and demonstrated to be specific for hsp70, inducible form. The antibody detects a 70kDa protein corresponding to the expected molecular mass of hsp70, in numerous mammalian cell lines including: human, monkey, rabbit, rat, mouse, hamster, guinea pig, bovine, sheep, dog, pig, Chinook salmon embryo and carp epithelioma cell lysates as well as beluga brain tissue extracts by Western blotting. The epitope is in the region of aa. 436-503 of human hsp70. There is no reactivity with the constitutive form of hsp70.

2.4.2 Clone BRM-22 (Sigma):

Monoclonal anti-hsp70 IgG₁ is derived from the BRM-22 hybridoma, produced by the fusion of mouse myeloma cells and splenocytes from BALB/c mice immunised with purified hsp70 isolated from bovine brain. The isotype was determined using Sigma immunoassay kit (Sigma ISO-1) and by double diffusion immunoassay using mouse monoclonal antibody isotyping reagents (Sigma ISO-2). The antibody reacts against hsp70 inducible and constitutive isoforms. The antibody recognises brain hsp70 of bovine, human, rat, chicken, and guinea pig. It also recognises hsp70 in *Drosophila* and human fibroblasts cell extract (Sigma-Aldrich, 1998).

2.5 Test solutions:

Cadmium (100mg/L $\text{CdCl}_2 \cdot 2\frac{1}{2}\text{H}_2\text{O}$), Mercury (100mg/L HgCl_2 anhydrous), and Copper (1000mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) stock solutions were prepared from Analar grade reagents and sterilised through 0.22 μm filters. All metal solutions were stored (up to six months) in sterile plastic containers to avoid metals leaching from glassware. Metal concentrations were verified by atomic absorption spectroscopy (APHA, 1998). Stock solutions were diluted into the culture medium at a 10% final concentration for testing. 2-Isobutyl piperidine was dissolved 1:4 in acetone (10%), sterilised through 0.22 μm filters and diluted in the cell culture medium (Werner and Nagel, 1997). 2-Isobutyl piperidine was synthesised and kindly provided by Professor Janus Szfrank at the University of Gdansk in Poland. It is similar in structure to a toxin found in the Colorado potato beetle and was therefore considered potentially toxic.



Chemical structure of 2-Isobutyl Piperidine.

2.6 Cell culture:

2.6.1 Maintenance media:

For routine maintenance the mouse connective tissue cell line (L929) and normal rat kidney cell line (NRK) were cultured in 25cm² flasks at 37°C. Both cell lines were propagated in Dulbecco's modified Eagles media (pH 7.3) containing 4.5g/l glucose amended with 10% (v/v) foetal bovine serum, L-glutamine (200mM), non-essential amino acids (100x) and HEPES buffer (20mM).

2.6.2 Trial media:

For toxicity and heat shock protein tests both cell lines were incubated at 37°C in a 5% CO₂ atmosphere with 95% humidity. The cells were propagated in Dulbecco's modified Eagles media (pH 7.3) containing 4.5g/l glucose amended with 10% (v/v) foetal bovine serum, L-glutamine (200mM), non-essential amino acids (100x) and sodium bicarbonate buffer (2g/l). No antibiotics were included in trial media as these are known to interfere in some cases with chemicals toxicity (Horner and Shah, 1984).

2.6.3 Sub culturing:

At confluence, the cells were sub cultured by trypsinisation (Freshney, 1983). The media was removed and the flask washed twice, using phosphate buffered saline (3ml). Trypsin (1ml) was added to the flask for 1 minute and decanted. The cells were re-incubated for

5-8 minutes to allow the residual trypsin to breakdown the proteins and mucoproteins attaching the cells to the flask. The flask was tapped gently to dislodge the cells and cell detachment confirmed by microscopic examination. The cells were resuspended in media (2ml) and the cell suspension drawn gently up and down a 1ml pipette in order to achieve a single cell suspension. An aliquot (100 μ l) was removed and the cell number counted using a haemocytometer. The split ratio was determined and appropriate aliquots of cell suspension distributed to fresh flasks containing maintenance media.

2.6.4 Cell preservation:

Cells from a near confluent 25cm² flask were detached by trypsinisation and counted using a haemocytometer. The cells were resuspended in media amended with cryostatic agent (10% glycerol). The optimum number of cells for preservation was achieved by dilution with media. Aliquots (1-2mls) were distributed into cryopreservation vials for freezing. A slow reduction from the optimum temperature to -170°C is required. The temperature decrease should be approximately 1-3 $^{\circ}\text{C}$ per minute. This was achieved by placing the cryopreservation vials on ice for 15 minutes, then dry ice at -70°C for 2 hours and finally into the liquid nitrogen at -170°C . The cells were recovered by rapid thawing at 37 $^{\circ}\text{C}$, seeded into 25cm² culture flasks and the cryostatic agent was removed.

2.6.5 Sterile Technique:

To ensure sterility, all cell culture reagents and consumables were either purchased sterile, filter sterilised or autoclaved. The laminar flow cabinet and related equipment

were sterilised using 70% ethanol. Reagents and cultures were routinely tested for microbial contamination using Tryptone Soya agar and Sabouraud dextrose agar. The presence of mycoplasmas was tested for using the Hoechst fluorescent staining technique (Chen, 1977). Briefly, cells were grown on coverslips in a Petri dish at 37°C in a humidified CO₂ incubator for 24 hours to achieve sub confluence. The cells were fixed to the coverslip by adding a 3:1 solution of 100% ethanol and glacial acetic acid to the medium. After 2 minutes the medium fixative solution was decanted. Fixative was added directly to the coverslips for 5 minutes and then this was repeated. The coverslips were air dried and stained with a solution of Hoechst 33258 stain (0.05µg/ml) for 10 minutes. The coverslips were washed twice with distilled water and examined by fluorescent microscopy (Chen, 1977).

2.7 Toxicity testing:

2.7.1 Plate seeding:

The production of a non-aggregated cell suspension is extremely important to ensure accurate seeding of the multi-well plate. The optimal seeding density of 4×10^4 cells/ml resulted in exponential growth after 24 hours. Cells from a near confluent 25cm² flask were trypsinised and counted by haemocytometer. The cell suspension was diluted in trial media to achieve the optimum seeding density. The cell suspension was mixed thoroughly by gentle inversion to ensure a homogeneous suspension and aliquots (100µl per well) were dispensed by multi-channel micropipette into eleven columns of the 96 well plate. The twelfth column provided the reagent blank. Between the seeding of

subsequent plates the suspension was mixed by drawing it up and down the multi-channel pipette. The plates were covered and incubated for 24 hours in a humidified, 5% CO₂ atmosphere.

2.7.2 Toxin dilutions:

The three heavy metals were diluted using a similar protocol. The stock solution was serially diluted to give standard solutions that were 10 times the required media concentration. The standard solutions were diluted 1:10 into trial media. In this way the volume of toxin remained constant (10%) for all concentrations tested. Sterile distilled water was diluted 1:10 into trial media to act as a control. The organic chemical, 2-Isobutyl piperidine was insoluble in water and was diluted 1:4 with acetone and filter sterilised (0.22µm). This was serially diluted into trial media as above. Acetone (10%) was diluted 1:10 into trial media to act as a solvent control.

2.7.3 Toxin application:

After 24 hours incubation when the cells had attached to the plate the media was removed by inverting the plate. Each end of the plate was “flicked” over a waste tray to remove the maximum amount of media from each half. This technique was the most effective. The cells were washed twice with sterile PBSA (3ml) before toxin application. The test solutions were dispensed sequentially into each column of the plate (100µl per well). The plate was covered, labelled and returned to the CO₂ incubator for the trial period (24 hours).

2.7.4 Neutral red assay:

The protocol used in this study was as described by Borenfreund and Puerner (1985). Briefly, the toxin amended media was removed from the wells by inversion and the cells re-incubated for 3 hours in a sterile solution of 40µg/ml neutral red, prepared in media (100µl), (the neutral red is incubated in media for 24 hours to allow crystal formation and the crystals removed by centrifugation 1,500g for 10 minutes). After three hours the media was decanted and the cells washed with a 1% formaldehyde/10% calcium chloride solution (100µl). This not only removes any remaining dye crystals but also fixes the cells to the plate. The neutral red dye is eluted from the cells using a 1% acetic acid/50% ethanol solution (100µl). To increase the efficiency of the elution process the plates were agitated on a plate shaker for 15 minutes. The optical density of each well was measured on a plate reading spectrophotometer at 492nm. The optical density of neutral red for each test concentration was expressed as a percentage of the control well values and reported as percentage cell mortality (Borenfreund and Puerner, 1984).

2.7.5 Crystal violet dye assay:

The Crystal violet assay was carried out according to the method of Dykes *et al*, (1984). Briefly, the washed cells were fixed to the plates using 10% formaldehyde for 10 minutes (100µl). The formaldehyde was removed and the cells washed twice with phosphate buffered saline. The cells were stained with 0.25% crystal violet dye for 30 minutes (100µl), (pre-filtered through Whatman No1. Filter paper). The stain was removed and the cells were washed five times with phosphate buffered saline. The plates were left to

dry and 33% acetic acid was added to elute the crystal violet within the cells (100µl). The plates were agitated on a plate shaker for 15 minutes. The optical density of each well was determined in a plate reader at 492nm. The optical density of crystal violet for each test concentration was expressed as a percentage of the control well values and reported as percentage cell mortality (Dykes *et al*, 1984).

2.7.6 Sulphorhodamine B assay:

The protocol used in this study was as described by Doyle *et al*, (1993). Briefly, the cells were fixed by adding 50µl of 50% trichloroacetic acid on to the toxin-amended media for 1 hour at 2°C. The cells were washed five times with distilled water and allowed to dry and stained with 200µl sulphorhodamine B (4% sulphorhodamine B in 1% acetic acid) for 30 minutes. Excess stain was removed by washing with 1% acetic acid (4 washes). The plates were allowed dry and 10mM Tris buffer, pH 10.5 added (200µl/well), followed by agitation on a plate shaker for 15 minutes and the optical density read at 492nm on a plate reading spectrophotometer. The optical density of sulphorhodamine B for each test concentration was expressed as a percentage of the control well values and reported as percentage cell mortality (Doyle *et al*, 1993).

2.7.7 Alkaline phosphatase assay:

The protocol used in this study was as described by Connolly *et al*, (1986). The toxin-amended media was removed from the wells by inversion and the cells washed twice with PBSA. Reaction buffer (0.1M sodium acetate pH 5.5, 0.1% Triton X-100 and 10mM p-

Nitrophenyl phosphate) was added to each well (100µl). The plates were incubated at 37°C in a 5% CO₂ humidified atmosphere for 2 hours. The reaction was stopped with the addition of 10µl of 1N NaOH. The optical density of each well was determined in a plate reader at 405nm. The optical density of alkaline phosphatase for each test concentration was expressed as a percentage of the control well values and reported as percentage cell mortality (Connolly *et al*, 1986).

2.7.8 Comparison of assays:

To establish the most accurate, reproducible and rapid assay for toxicity testing, 96 well plates were seeded with L929 cells as described above. Cadmium concentrations were added to each plate as previously described and incubated overnight. After 24 hours each assay was applied to a 96 well plate. Cell mortality and accuracy estimates were determined for each assay and the results compared.

2.7.9 Plate seeding accuracy:

To establish the accuracy of the plate seeding technique the cell lines were seeded into 96 well plates as described above. The plates were covered and incubated overnight in a humidified, 5% CO₂ atmosphere. Cell density was determined using the neutral red assay. The accuracy of the plate seeding technique was estimated using the coefficient of variation (%CV).

2.8 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

2.8.1 Protein determination:

The Lowry protein assay (Lowry *et al*, 1951) was used to ensure equal loads of sample protein application for separation by electrophoresis. After 24 hours the toxin amended media was removed from the 96 well plate and each well rinsed twice with 100µl of phosphate buffered saline. Cells were lysed in sample buffer, containing 2% w/v SDS, 20% v/v glycerol, 5% v/v mercaptoethanol, and 62.5mmol/l Tris-HCl; pH 6.8. Cell lysates from eight replicate wells were combined and protein determined by the Lowry assay.

However, using this method protein levels could not be determined as it was found that, the sample buffer interfered in the Lowry assay suppressing the colorimetric endpoint development. Sodium hydroxide has previously been used to lyse cells (Hightower, 1980) and therefore the lowest concentration that lysed the cells was determined. 1N NaOH was serially diluted and added to cells in a 96 well plate. Cell lysis was determined by microscopic examination. The lowest concentration that lysed cells was 0.05N NaOH (5µl/well). Lysates from eight replicate wells were combined.

Protein concentrations were determined by the Lowry method. An aliquot from the combined cell lysate (20µl) was mixed with 20µl of 2N NaOH and hydrolysed at 100°C for 10 minutes in a boiling water bath. After cooling to room temperature, freshly mixed complex forming reagent (2% w/v Na₂CO₃, 1% w/v CuSO₄.5H₂O and 2% w/v Sodium potassium tartrate; mixed 100:1:1 v/v/v respectively) was added for 10 minutes (200µl). Using a vortex mixer Folin reagent was added (20µl) and the mixture was let stand at

2.8.4 Gel preparation:

Polyacrylamide gradient electrophoresis gels (4-22.5% w/v) (10cmx10cmx2mm) were prepared as previously described (Marshall and Williams, 1986). Stock acrylamide was prepared by dissolving 29.2g acrylamide (T=30%) and 0.8g N-N-methylenebisacrylamide (C=5%) in 100ml distilled water for 1 hour (stored in the dark at 4°C for up to 1 month). The dense solution was prepared by mixing 16.9ml of stock acrylamide with 5.6ml gel buffer (1.5M Tris-HCl containing 0.4% w/v SDS and 0.01% w/v NaN₃). The light solution was prepared by mixing 16.9ml of distilled water with 5.6ml gel buffer. Both mixtures were degassed for 2 minutes in a Buchner flask using a vacuum pump (Charles Austin Pump). Without degassing sample wells did not polymerise completely. The light solution was added to the second column of the gradient maker and the compensating rod inserted, the valve was opened momentarily to remove any air bubbles. The dense solution was added to the first column and the valve opened to remove any air bubbles. The dense solution was continuously mixed with a stirring rod (Pharmacia, 1983). Polymerisation was initiated by adding 6.75µl of neat NNN'N'-tetramethylethylenediamine (TEMED) and 22.5µl of 40% ammonium persulphate (APS) and was complete within 1 hour.

2.8.5 SDS PAGE:

Sample wells were prepared using an acrylic comb template (ATTO Corporation) inserted into the top of the acrylamide immediately after pouring. Each well was filled with electrophoresis buffer containing bromophenol blue (250µl of aqueous 0.1%

BPB/10ml of buffer). The denatured sample was loaded through the BPB with a micropipette. The gels (two per electrophoresis tank) were carefully immersed in pre-cooled (4°C) electrophoresis buffer (25mmol/l Tris containing 200mmol/l glycine and 0.1% w/v SDS) (Gorg *et al*, 1981) and electrophoresed without a stacking gel, at 50mA constant current for approximately 3 hours to resolve individual bands, progress was monitored by viewing the bromophenol blue dye front (Marshall, 1984a). Molecular weight standards (36-200kDa) were treated identically to samples and co-migrated on each gel.

2.8.6 Silver staining:

The electrophoretic gels were fixed overnight in 10% trichloroacetic acid and 5% sulphosalicylic acid (200ml/gel). The proteins were detected by staining in alkaline silver nitrate using a modified procedure of Marshall (1984b) (Table 6).

2.9 Densitometer:

Silver stained SDS-PAGE gels were scanned using a black and white video camera. Protein patterns were examined using the CREAM densitometric software. Background correction, averaging and sensitivity were all optimal. The peak area defined as the Gaussian distribution below the peak of each protein band (Kem-en-Tec, 1990) for each test concentration was expressed as a percentage of the control peak area.

Table 6. Alkaline silver nitrate stain. (Marshall, 1984b).

1. Wash in two changes of water (10, 20 min) (a).
2. Incubate in aqueous 0.1% w/v formaldehyde (30 min).
3. Cool in water (20°C, 10 min).
4. Incubate in silver methylamine (b) (10 min).
5. Quickly rinse in water (2 changes) and developer (formaldehyde, 0.02% w/v containing 0.1% w/v citric acid). Change developer at 5 min intervals until gel blackens (approx. 30 min).
6. Rinse in three changes of water (10, 20, 30 min).
7. Incubate in destaining solution (c) until gel background is golden yellow (1-4 min).
8. Rinse in water, incubate in aqueous 2.5% w/v Kodak hypo clearing agent (d) (30 min) and wash in three changes of water (10, 20, 30 min).

Footnotes:

- a) Perform all steps with gentle shaking in a fume cupboard; steps 1, 2 at 60°C (with reagent volume of 200ml/gel) and steps 3-8 at room temperature (100ml/gel).
- b) For 100ml (1gel): mix commercial methylamine solution (30%) with 0.36% w/v sodium hydroxide (1:5, v/v), add (approx. 10ml) to 4ml of a stirring solution of 20% w/v silver nitrate till it just clears and dilute to 100ml with water.
- c) For 800ml (8 gels): Solution A, dissolve 11.1g sodium chloride and 11.1g cupric sulphate in 285ml water and add ammonium solution (25%) till the precipitate clears to a deep blue solution (final volume approx. 300ml). Solution B, dissolve 44g sodium thiosulphate pentahydrate in 85ml water (final volume 100ml). Mix solutions A and B (3:1, v/v) and dilute to 800ml with water.
- d) Gels may be photographed at this stage.

2.10 Western blotting:

Western blotting was carried out according to the method of Towbin *et al*, (1979). Following SDS-PAGE, proteins were transferred to PVDF membranes by electroblotting (Figure 4) for 30 minutes at 112mA using transfer buffer (25mM Tris, 192mM Glycine, 20% methanol. pH 8.3). The membranes were blocked using buffer containing 10mM Tris, 150mM NaCl and 5% BSA overnight and were subsequently probed with mouse anti-hsp70 antibody (clone BRM-22 or C29F3A-5) for 2 hours (1µg/ml in blocking buffer). The membrane was washed three times in washing buffer (10mM Tris, 150mM NaCl containing 0.1% Tween-20). The membrane was probed with goat anti-mouse alkaline phosphatase conjugate secondary antibody for 2 hours (0.5µg/ml in blocking buffer) and the proteins visualised after 30 minutes incubation with alkaline phosphatase substrate (NBT/BCIP) (Figure 5).

room temperature for 30-60 minutes. Absorbance was read at 750nm using a spectrophotometer.

2.8.2 Sample preparation:

Analytical SDS-PAGE was performed using a modified procedure of Laemmli (1970). The Lowry protein assay was used to ensure equal protein loadings were separated by electrophoresis. Double strength sample buffer, containing 4% w/v SDS, 40% v/v glycerol, 10% v/v mercaptoethanol, and 125mmol/l Tris-HCl; pH 6.8, was mixed with an equal aliquot of the combined cell lysate. Cell lysates were heated to 90°C for five minutes then allowed to cool (Marshall, 1984a).

The ability of Dithiothrietol to break up thiol groups as an alternative to mercaptoethanol was also assessed. Double strength sample buffer containing 4% w/v SDS, 40% v/v glycerol, 20mM dithiothrietol, and 125mmol/l Tris-HCl; pH 6.8, was mixed with an equal aliquot of the combined cell lysate. Cell lysates were heated to 90°C for five minutes then allowed to cool.

2.8.3 Heat shock assay:

Cells were seeded into 25cm² flasks at a density sufficient to ensure exponential growth after 24 hours incubation. The flasks were immersed in a water bath at the appropriate treatment temperature (37-52°C) for 30 minutes and re-incubated at normal temperature for 4 hours. The cells were washed twice with PBSA and prepared for electrophoresis as above.

Figure 4: Schematic diagram of semi dry blotter.

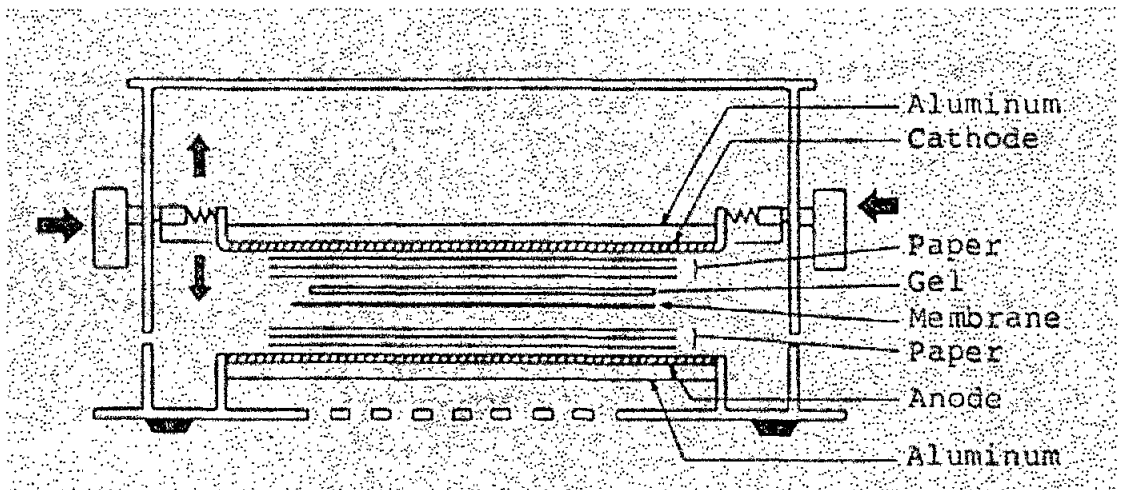
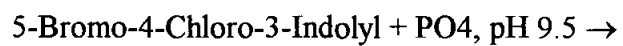
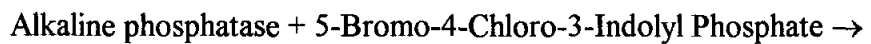


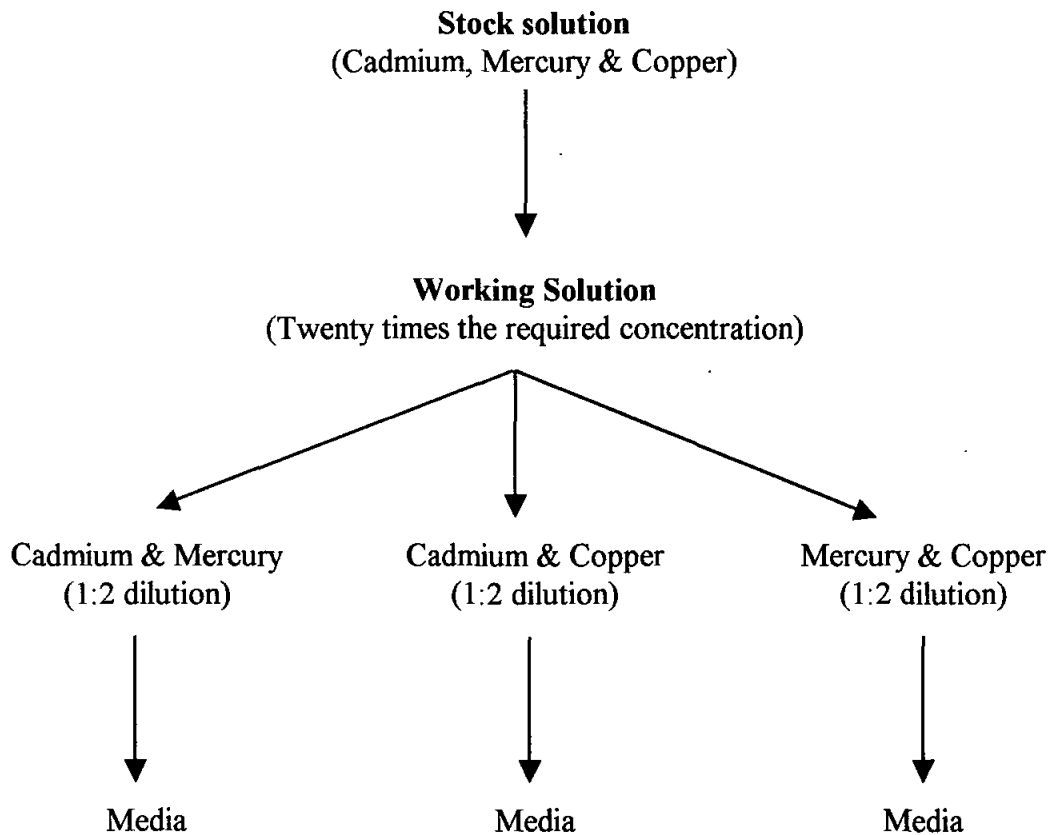
Figure 5: Reaction of Alkaline phosphatase with BCIP/NBT substrate.



2.11 Toxin mixtures:

Toxin mixtures to reflect possible environmental situations were assessed. The mixtures were of cadmium and mercury, cadmium and copper and mercury and copper. The three heavy metal mixtures were diluted using the same protocol (Figure 6). Stock solutions were diluted to give standard solutions that were 20 times the required concentration. The standard solutions were diluted 1:2 with each other to generate the mixtures. The mixtures were then diluted 1:10 in trial media. In this way the volume of toxin remained constant (10%) for all concentrations tested. Sterile distilled water was diluted 1:10 into trial media to act as a control. For each mixture, 96 well plates were seeded in duplicate as in section 2.7.1 and the toxins applied as in section 2.7.3. Cell mortality was assessed for one 96 well plate using the neutral red assay. The optical density of neutral red for the individual toxins and mixtures of toxins was expressed as a percentage of the control well values and reported as percentage cell mortality. Cell mortality for the toxin mixtures was compared to that of the individual toxins. Stress protein induction was assessed for the other 96 well plate as in section 2.8. The peak area of the stress protein bands for the individual toxins and mixtures of toxins was expressed as a percentage of the corresponding control peak area. Stress protein induction for the toxin mixtures was compared to that of the individual toxins and to the corresponding cell mortality estimates.

Figure 6: Flow diagram of dilutions used for mixtures in toxin interaction assay.



2.12 ELISA:

Equal protein loads of cell lysates were bound to the ELISA plate overnight at 37°C in a humidified incubator. ELISA plates were covered with cling film to reduce evaporation. The plates were washed three times with washing buffer (10mM Tris, 150mM NaCl containing 0.1% Tween-20). To prevent non-specific binding by the antibodies the plates were blocked using buffer containing 10mM Tris, 150mM NaCl, 5% BSA for 2 hours

and were subsequently probed with mouse anti-hsp70 antibody (Clone C95F3A-5) for 2 hours (1µg/ml in blocking buffer). The plates were washed three times in washing buffer (10mM Tris, 150mM NaCl containing 0.1% Tween-20). The plates were probed with the goat anti-mouse alkaline phosphatase conjugate secondary antibody for 2 hours (1µg/ml in blocking buffer). The absorbencies of individual wells were read using a plate reading spectrophotometer at 405nm after 30 minutes incubation with 10mg p-Nitrophenyl phosphate in 10ml of 10mM diethanolamine (pH 9.5) containing 0.5mM MgCl₂.

2.13 Immunocytochemistry:

L929 cells were grown in 24 well plates at 37°C in a 5% CO₂ humidified incubator at a cell density of 4 x 10⁴ cells per ml. The last well received no cells and was used as a reagent blank. After 24 hours attachment cells were exposed to increasing cadmium concentrations as described in section 2.7.3. The toxin-amended medium was removed and the cells washed twice with PBSA. The cells were fixed using a solution of ethanol and acetone (1:1 v/v) at 4°C for 10 minutes (Marshall and Kind, 1994). The cells were washed three times with PBSA and were subsequently probed with mouse anti-hsp70 antibody (clone C29F3A-5) for 2 hours (5µg/ml in PBSA). The cells were washed three times in PBSA. The cells were probed with goat anti-mouse alkaline phosphatase conjugate secondary antibody for 2 hours (5µg/ml in PBSA). Heat shock protein 70 was either qualitatively assessed by microscopic examination after 30 minutes incubation using an alkaline phosphatase substrate (NBT/BCIP) that precipitates at the site of hsp70, or quantitatively assessed by spectrophotometer at 405nm after 30 minutes

incubation using an alkaline phosphatase substrate 10mg p-Nitrophenyl phosphate in 10ml of 10mM diethanolamine (pH 9.5) containing 0.5mM MgCl₂ that produces a soluble coloured end product. To ensure alkaline phosphatase leakage that occurs due to the toxin increasing the permeabilisation of the cell membrane does not interfere with the assay a second control plate was assessed. The control plate was prepared in an identical manner to the test plate. However, instead of assessing hsp70 induction the alkaline phosphatase endpoint was used as in section 2.7.7 to assess the degree of alkaline phosphatase leakage.

2.14 Data handling:

To ensure standardisation between replicate tests cell density was kept constant at 4×10^4 cells per ml. For both toxicity and stress protein tests the control and complete toxin concentration range were assayed on the same 96 well plate. For toxicity tests a reagent blank was included in each plate to account for the absorption of dye to the well surface. The absorbance of the reagent blank was subtracted from all test absorbances. The absorbances of replicate tests (n=31) were averaged and standard deviations calculated. Variability was estimated by calculating the coefficient of variation (%CV). The optical density of the dye for each test concentration was expressed as a percentage of the control well values and reported as percentage cell mortality (Borenfreund and Puerner, 1984). Percentage cell mortalities for replicate tests were averaged and plotted against toxin concentrations to generate dose response curves. The coefficient of variation (%CV) was plotted as Y-axis error bars. The median lethal concentration (LC₅₀) was taken as the concentration that resulted in 50% inhibition of cell viability (Trevan, 1927).

The median lethal concentration was estimated by regression of the linear portion of the dose response curve (Shopsis and Sathe, 1984). The no effect level (N.E.L.) was taken as the concentration whose cell mortality did not differ significantly from the control (Rand and Petrocelli, 1985). To account for variation in the technique the no effect level was determined by the Student T-test ($p=0.05$).

To ensure standardisation between replicate tests the control and complete toxin concentration range were separated on the same one-dimensional gel. Eight molecular weight markers were included in each gel to allow accurate measurement of the proteins molecular weight. The CREAM densitometer calculated the molecular weights of the unknown sample proteins based on the relative mobility (Rf) of the molecular weight markers (Kem-en-Tec, 1990). Changes in the level of individual stress protein bands were assessed using the protein bands peak area. The peak area of individual stress proteins for replicate separations ($n=8$) were averaged and standard deviations calculated. Variability was estimated by calculating the coefficient of variation (%CV). Stress protein band peak areas for test concentrations were measured relative to those of the control and the difference expressed as percentage induction. Percentage induction of individual stress proteins for replicate tests were averaged and plotted against toxin concentrations to generate stress response curves. The coefficient of variation (%CV) was plotted as Y-axis error bars. Stress protein inductions were compared to the median lethal concentration, the no effect concentration and the lowest observed effective concentration (L.O.E.C.). To account for variation in the technique the lowest observed effective concentration was determined by the Student T-test ($p=0.05$).

3 Results:

3.1 Choice of cytotoxicity assay:

A number of assays were examined to establish which was the most suitable for this study. Four assays were considered, the crystal violet assay, the sulphorhodamine B assay, the alkaline phosphatase assay and the neutral red assay. The crystal violet assay was not chosen as it was found to underestimate the degree of cell mortality. The sulphorhodamine B assay showed a more representative estimate of cell mortality however, it was not chosen along with the alkaline phosphatase assay due to the high variability found for cell mortality estimates. The neutral red assay gives a very good estimate of cell mortality, as only viable cells are capable of absorbing the dye. In addition, variability between replicates was low with coefficients of variation (%CV) of less than 3%. The neutral red assay was chosen to establish the toxicity data in this study.

3.2 Toxicity data:

Dose response curves were constructed using cell mortality versus toxin concentration. Data points represent the arithmetic mean of 31 replicate tests. Variation between replicate tests was estimated by the coefficient of variation (%CV) and was plotted as Y-axis error bars. From these dose response curves the traditional measures of toxicity the median lethal concentration value and the no effect level were estimated. The

median lethal concentration is defined as the concentration of test compound that results in the death of 50% of the test population within a defined period (Trevan, 1927). The LC_{50} was calculated by regression of the linear portion of the dose response curve. Lethal concentrations at 20% (LC_{20}) and 80% (LC_{80}) cell mortality were also calculated to allow the slope of the line to be estimated. The No Effect Level is defined as the concentration of test compound that results in no measurable effect to the test population within a defined period. To account for variability between replicates and spectrophotometry only concentrations that showed no significant difference (using the Students T-test) in the optical density of neutral red to the controls were taken to have no effect ($p=0.05$).

3.2.1 Median Lethal Concentration (LC_{50}) for the L929 cell line:

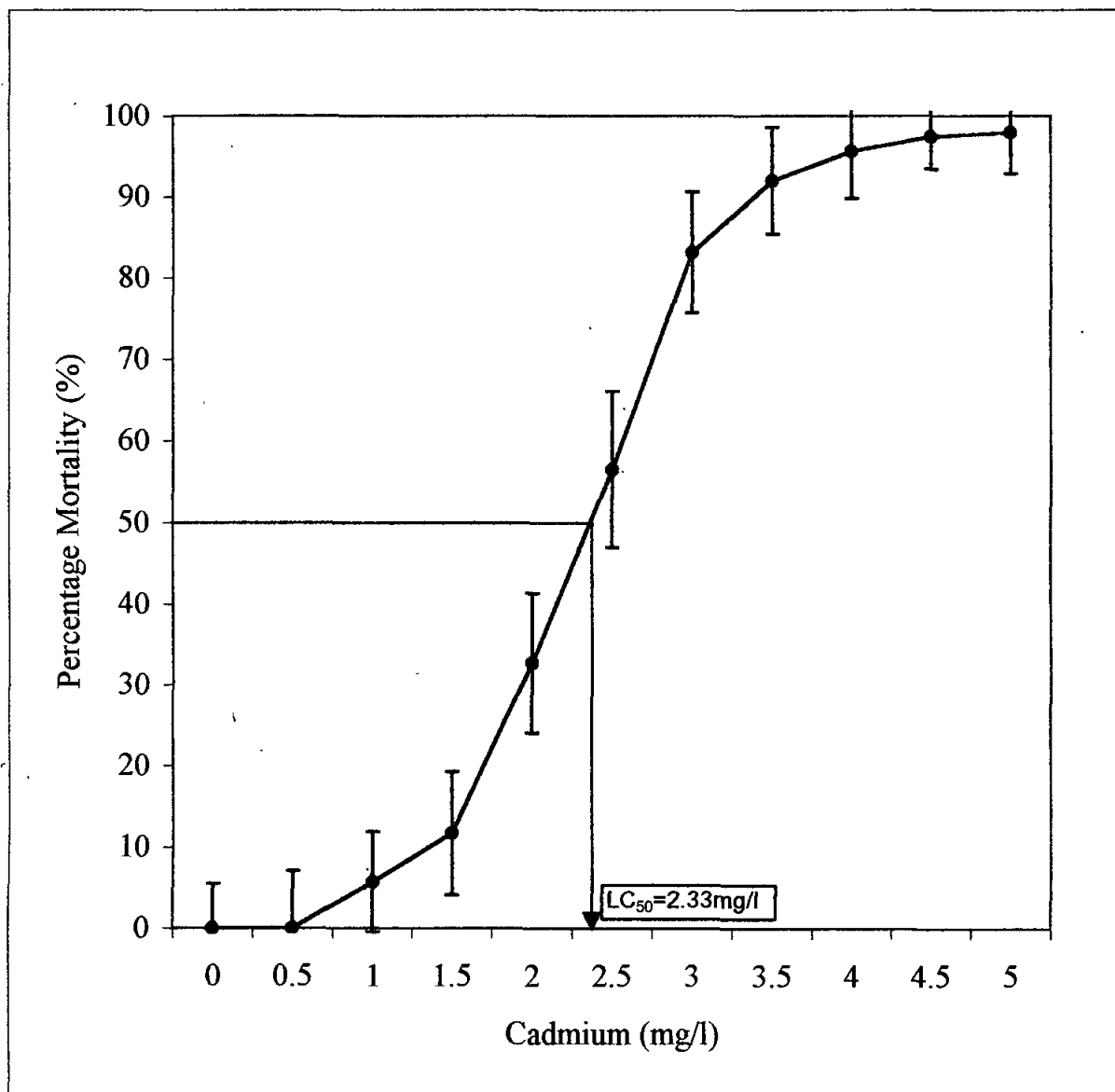
The L929 cell line dose response curves for cadmium, mercury and copper showed a typical sigmoidal shape with toxicity increasing in a dose dependent fashion (Figures 7-9). The median lethal concentration (LC_{50}) as well as the lethal concentrations at 20% (LC_{20}) and 80% (LC_{80}) cell mortality are presented in Table 7. For the L929 cell line at the LC_{50} value cadmium was found to be over 1 fold more toxic than mercury and over 17 fold more toxic than copper. Coefficients of variation (%CV) ranged from 3.89-9.55% (average C.V.=6.64%) for cadmium, 2.35-10.30% (average C.V.=5.18%) for mercury and 2.99-4.14% (average C.V.=3.47%) for copper. The slopes of the dose response curves for the three metals indicate the rate of increase in toxicity with concentration (Table 7).

Table 7. Lethal concentration values for the L929 cell line.

Toxin	LC_{20}	LC_{50}	LC_{80}	Slope
Cadmium	1.7 mg/l (C.V.=8%)	2.33 mg/l (C.V.=9%)	2.96 mg/l (C.V.=7.5%)	0.021
Mercury	2.8 mg/l (C.V.=5.9%)	3.13 mg/l (C.V.=5.65%)	3.46 mg/l (C.V.=5.36%)	0.011
Copper	32.64 mg/l (C.V.=3.2%)	39.95 mg/l (C.V.=3.42%)	47.27 mg/l (C.V.=3.5%)	0.244

Figure 7:

Dose response curve for the mouse connective tissue cell line (L929) to Cadmium.

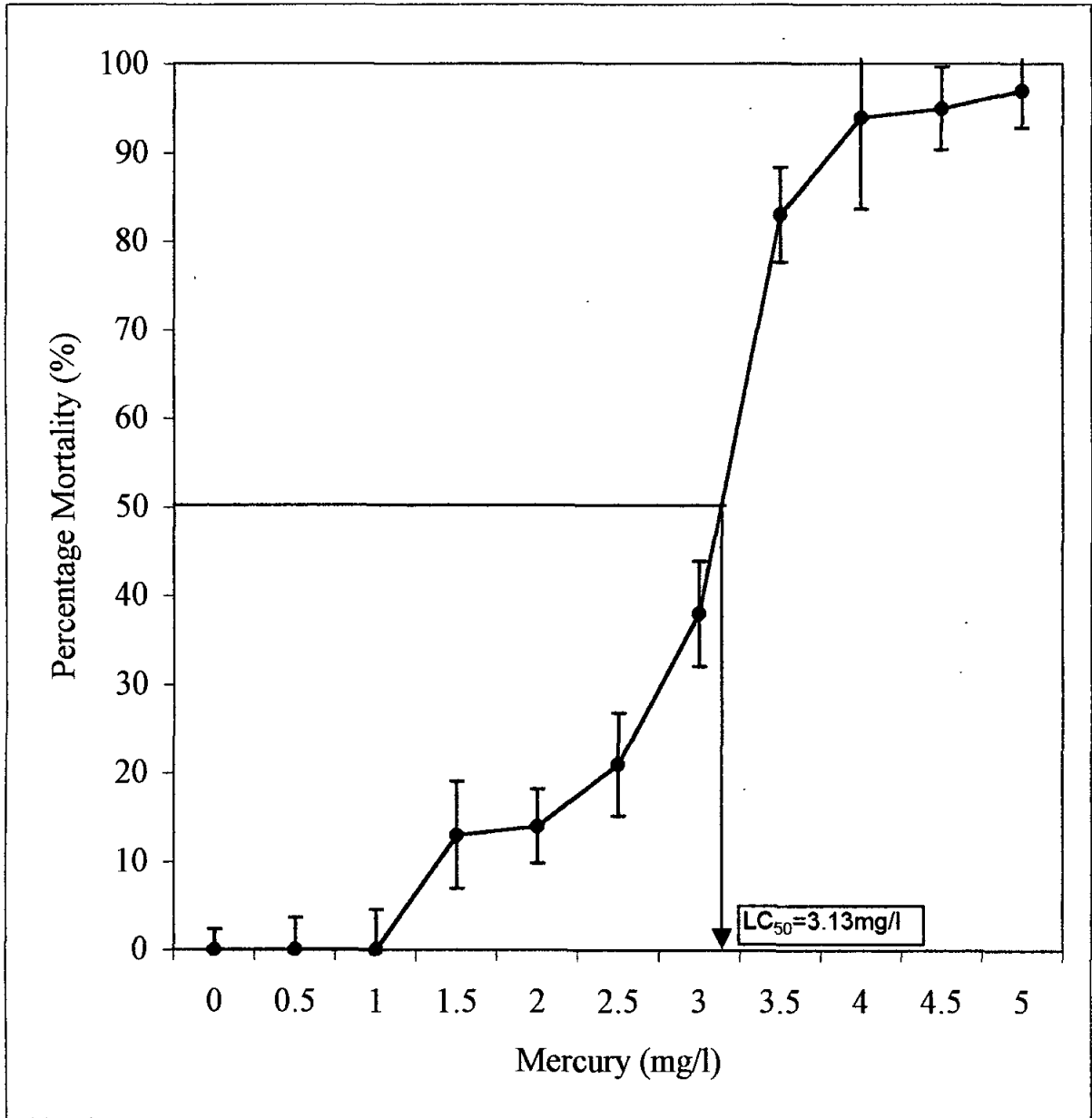


Dose response curve for the mouse connective tissue cell line (L929) to cadmium.

96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C . The attached cells were incubated with 100 μl s per well of cadmium amended medium (0-5mg/l Cd) for 24 hours at 37°C . Cadmium concentrations never exceeded 10% of the media volume. Cell mortality (●) was estimated by the neutral red assay. Neutral red optical density readings were expressed as a percentage of the untreated control. Data points represent the arithmetic mean of 31 replicates. The dose response curve for cadmium shows a typical sigmoidal shape with toxicity increasing in a dose dependent fashion. The median lethal concentration was estimated by regression of the linear portion of the curve to be 2.33 mg/l. Using the Students T-test ($p=0.05$) to determine significant changes in cell mortality the no effect level was estimated to be 1.5 mg/l. Coefficients of variation (%CV) are presented as Y-axis error bars, % CV's ranged from 3.89-9.55%.

Figure 8:

Dose response curve for the mouse connective tissue cell line (L929) to Mercury.

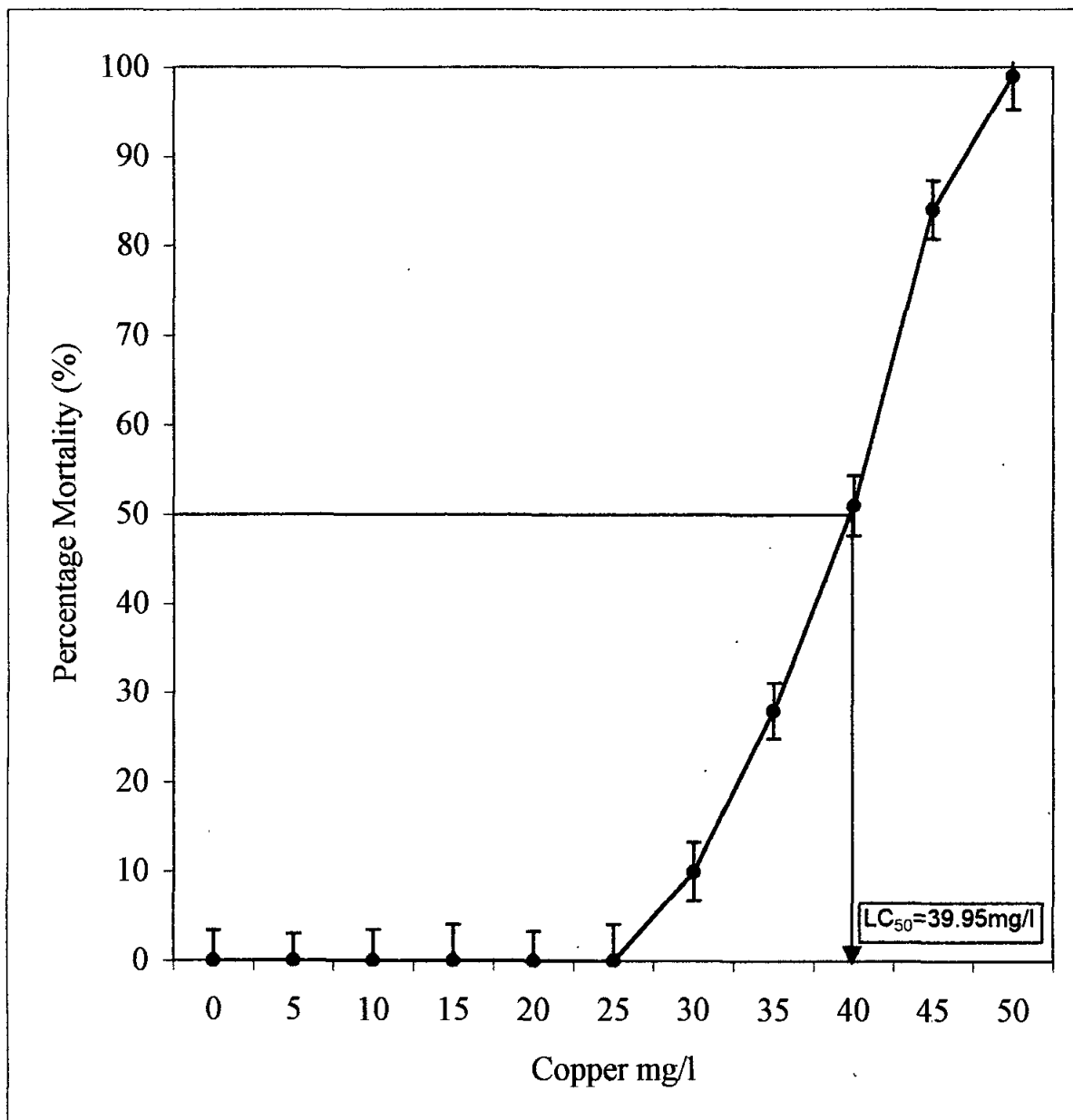


Dose response curve for the mouse connective tissue cell line (L929) to mercury.

96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C . The attached cells were incubated with $100\mu\text{l}$ s per well of mercury amended medium (0-5mg/l Hg) for 24 hours at 37°C . Mercury concentrations never exceeded 10% of the media volume. Cell mortality (●) was estimated by the neutral red assay. Neutral red optical density readings were expressed as a percentage of the untreated control. Data points represent the arithmetic mean of 31 replicates. The dose response curve for mercury shows a typical sigmoidal shape with toxicity increasing in a dose dependent fashion. The median lethal concentration (LC_{50}) was estimated by regression of the linear portion of the curve to be 3.13 mg/l. Using the Students T-test ($p=0.05$) to determine significant changes in cell mortality the no effect level was estimated to be 1.0 mg/l. Coefficients of variation (%CV) are presented as Y-axis error bars, % CV's ranged from 2.35-10.30%.

Figure 9:

Dose response curve for the mouse connective tissue cell line (L929) to Copper.



Dose response curve for the mouse connective tissue cell line (L929) to copper.

96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100µl per well of copper amended medium (0-50mg/l Cu) for 24 hours at 37°C. Copper concentrations never exceeded 10% of the media volume. Cell mortality (●) was estimated by the neutral red assay. Neutral red optical density readings were expressed as a percentage of the untreated control. Data points represent the arithmetic mean of 31 replicates. The dose response curve for copper shows a typical sigmoidal shape with toxicity increasing in a dose dependent fashion. The median lethal concentration (LC₅₀) was estimated by regression of the linear portion of the curve to be 39.95mg/l. Using the Students T-test ($p=0.05$) to determine significant changes in cell mortality the no effect level was estimated to be 25mg/l. Coefficients of variation (%CV) are presented as Y-axis error bars, % CV's ranged from 2.99-4.14%.

3.2.2 Median lethal concentration (LC₅₀) for the NRK cell line:

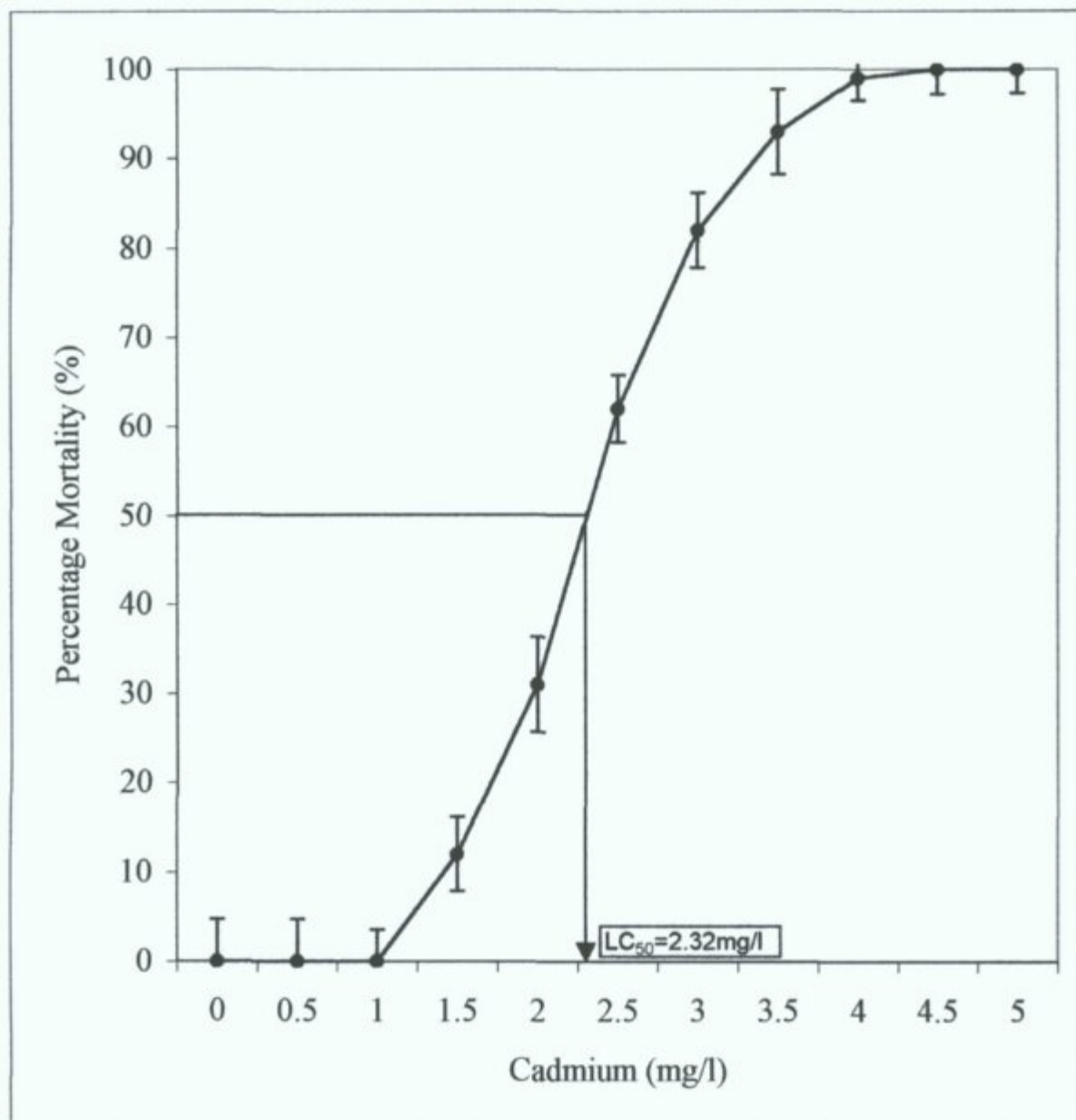
The NRK cell line dose response curves for cadmium, mercury and copper showed a typical sigmoidal shape with toxicity increasing in a dose dependent fashion (Figures 10-12). The median lethal concentration (LC₅₀) as well as the lethal concentrations at 20% (LC₂₀) and 80% (LC₈₀) cell mortality are presented in Table 8. Cadmium was found to be over one fold more toxic than mercury and nearly 8 fold more toxic than copper. Coefficients of variation (%CV) ranged from 2.65-5.31% (average C.V.=3.91%) for cadmium, 2.98-8.53% (average C.V.=5.56%) for mercury and 2.46-8.44% (average C.V.=4.73%) for copper. The slopes of the dose response curves for the three metals indicate the rate of increase in toxicity with concentration (Table 8).

Table 8. Lethal concentration values for the NRK cell line.

Toxin	LC₂₀	LC₅₀	LC₈₀	Slope
Cadmium	1.69 mg/l (C.V.=4.7%)	2.32 mg/l (C.V.=4.55%)	2.93 mg/l (C.V.=4.2%)	0.021
Mercury	2.83 mg/l (C.V.=6.7%)	3.16 mg/l (C.V.=7.24%)	3.5 mg/l (C.V.=7.4%)	0.011
Copper	15.26 mg/l (C.V.=3.9%)	18.48 mg/l (C.V.=4.50%)	21.71 mg/l (C.V.=5.1%)	0.108

Figure 10:

Dose response curve for the normal rat kidney cell line (NRK) to Cadmium.

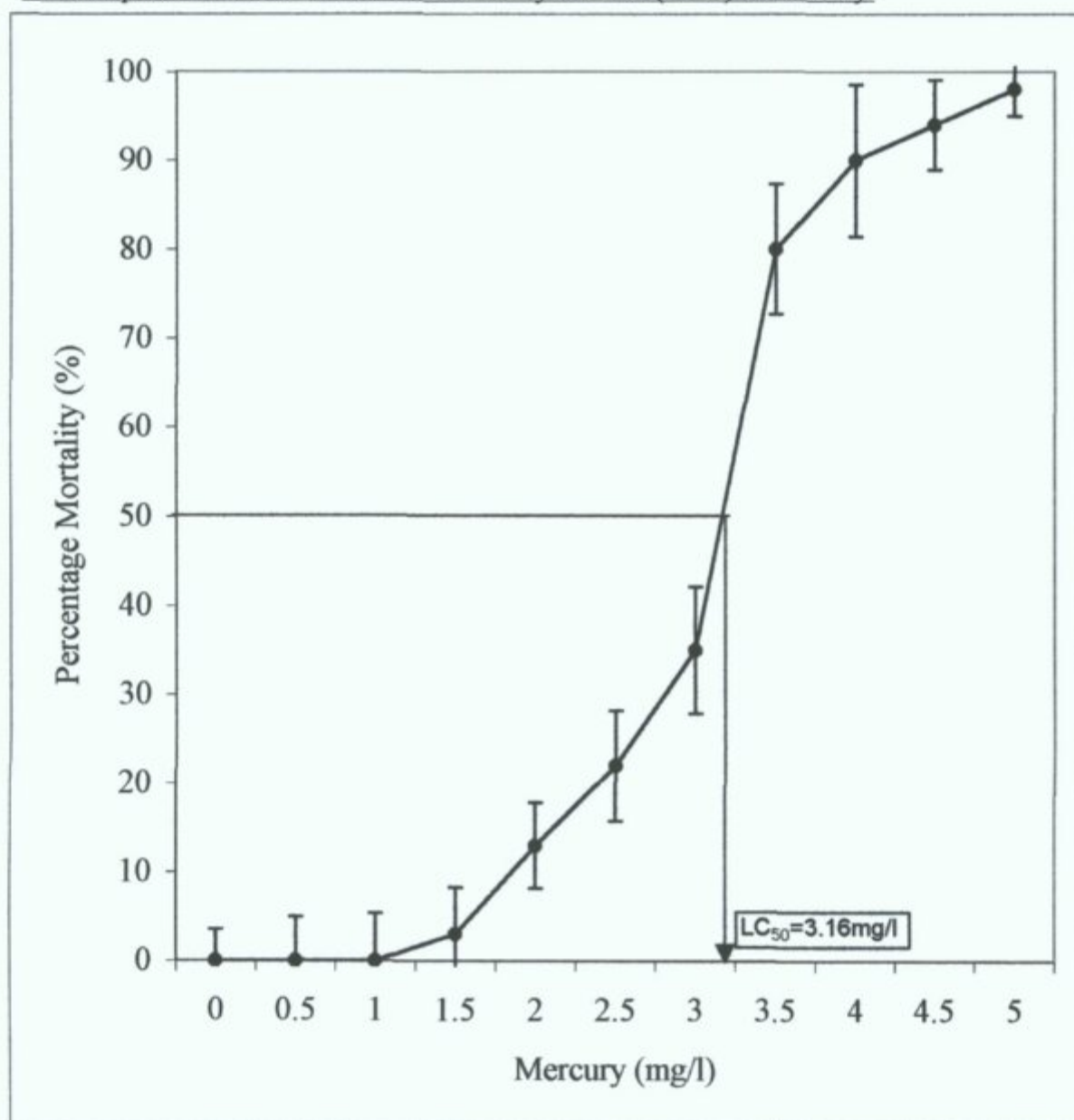


Dose response curve for the normal rat kidney cell line (NRK) to cadmium.

96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100µl per well of cadmium amended medium (0-5mg/l Cd) for 24 hours at 37°C. Cadmium concentrations never exceeded 10% of the media volume. Cell mortality (●) was estimated by the neutral red assay. Neutral red optical density readings were expressed as a percentage of the untreated control. Data points represent the arithmetic mean of 31 replicates. The dose response curve for cadmium shows a typical sigmoidal shape with toxicity increasing in a dose dependent fashion. The median lethal concentration (LC₅₀) was estimated by regression of the linear portion of the curve to be 2.32 mg/l. Using the Students T-test (p=0.05) to determine significant changes in cell mortality the no effect level was estimated to be 1.5 mg/l. Coefficients of variation (%CV) are presented as Y-axis error bars, % CV's ranged from 2.65-5.31%.

Figure 11:

Dose response curve for the normal rat kidney cell line (NRK) to Mercury.

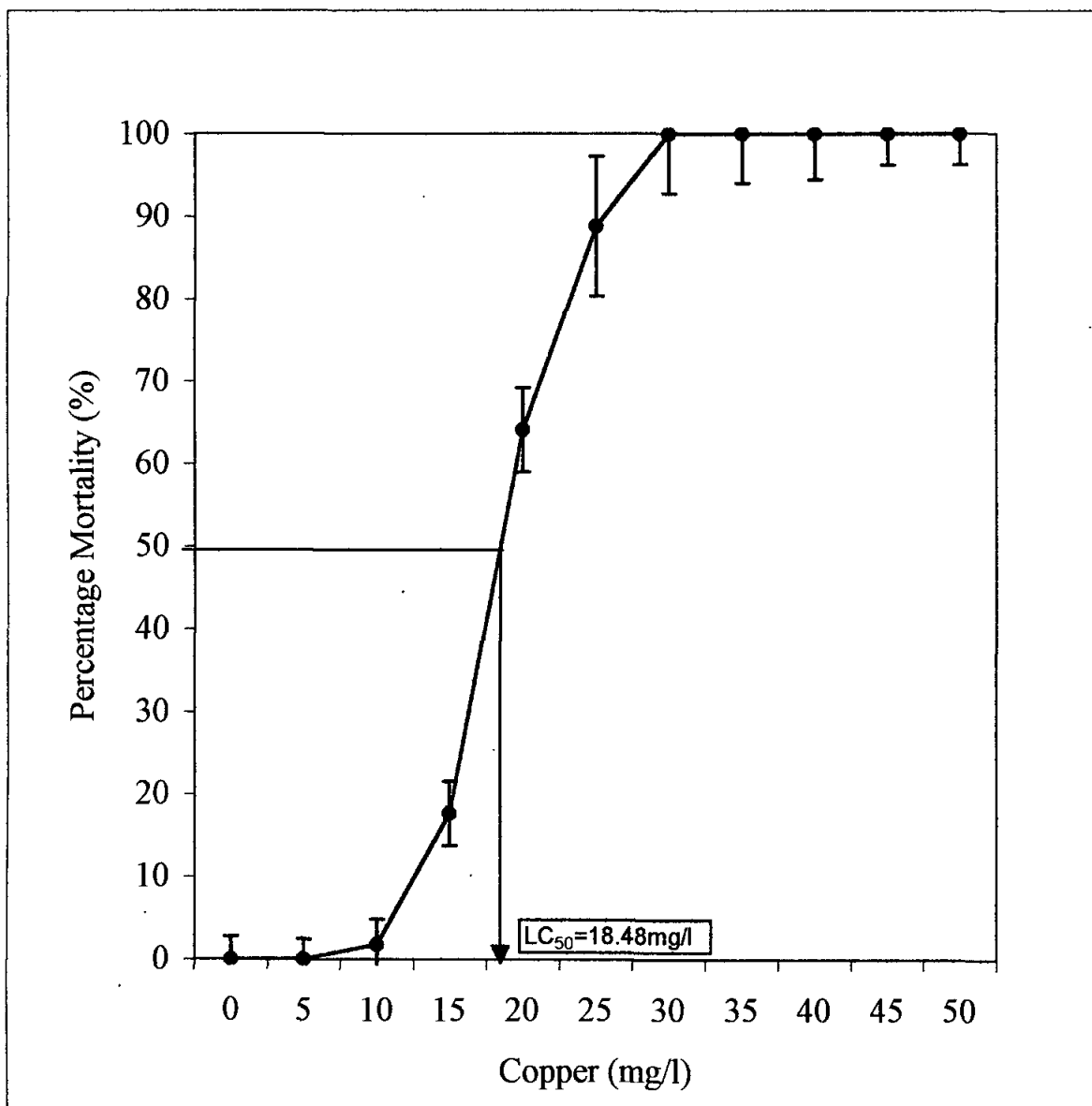


Dose response curve for the normal rat kidney cell line (NRK) to mercury.

96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100µl per well of mercury amended medium (0-5mg/l Hg) for 24 hours at 37°C. Mercury concentrations never exceeded 10% of the media volume. Cell mortality (●) was estimated by the neutral red assay. Neutral red optical density readings were expressed as a percentage of the untreated control. Data points represent the arithmetic mean of 31 replicates. The dose response curve for mercury shows a typical sigmoidal shape with toxicity increasing in a dose dependent fashion. The median lethal concentration (LC₅₀) was estimated by regression of the linear portion of the curve to be 3.16 mg/l. Using the Students T-test ($p=0.05$) to determine significant changes in cell mortality the no effect level was estimated to be 1.5 mg/l. Coefficients of variation (%CV) are presented as Y-axis error bars, % CV's ranged from 2.98-8.53%.

Figure 12:

Dose response curve for the normal rat kidney cell line (NRK) to Copper.



Dose response curve for the normal rat kidney cell line (NRK) to copper.

96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100µl per well of copper amended medium (0-50mg/l Cu) for 24 hours at 37°C. Copper concentrations never exceeded 10% of the media volume. Cell mortality (●) was estimated by the neutral red assay. Neutral red optical density readings were expressed as a percentage of the untreated control. Data points represent the arithmetic mean of 31 replicates. The dose response curve for copper shows a typical sigmoidal shape with toxicity increasing in a dose dependent fashion. The median lethal concentration (LC₅₀) was estimated by regression of the linear portion of the curve to be 18.48mg/l. Using the Students T-test ($p=0.05$) to determine significant changes in cell mortality the no effect level was estimated to be 10mg/l. Coefficients of variation (%CV) are presented as Y-axis error bars, % CV's ranged from 2.46-8.44%.

3.2.3 No Effect Level: (N.E.L)

The no effect levels (Table 9) show that the two cell lines have very similar sensitivities to cadmium and mercury. The L929 and NRK cell lines have differing sensitivities to copper, NRK cells were 2½ times more sensitive to copper than the L929 cells. Cadmium and mercury had the same no effect level for the NRK cell line, however for the L929 cell line mercury had a slightly lower no effect level than cadmium. For the L929 cell line mercury was marginally more toxic than cadmium and over 25 fold more toxic than copper. For the NRK cell line cadmium and mercury had equal toxicities and were over 6 fold more toxic than copper.

Table 9: No effect level for L929 and NRK cell lines

Toxin	L929	NRK
Cadmium	1.5 mg/l (C.V.=7%)	1.5 mg/l (C.V.=4.14%)
Mercury	1.0 mg/l (C.V.=4.57%)	1.5 mg/l (C.V.=5.25%)
Copper	25 mg/l (C.V.=4.14%)	10 mg/l (C.V.=3.04%)

3.3 Optimisation of the Heat shock response assay:

Once toxicity data for the three metals was established it was possible to assess the potential of the heat shock response. Under identical conditions the two cell lines were exposed to the three toxins. If the heat shock response were to have potential as a biomarker of toxicity it would have to provide more information than the neutral red

assay. That is, it would have to induce a more pronounced response allowing easier measurement or be capable of detecting stress induced toxicity below the no effect level.

3.3.1 Normal Protein Pattern:

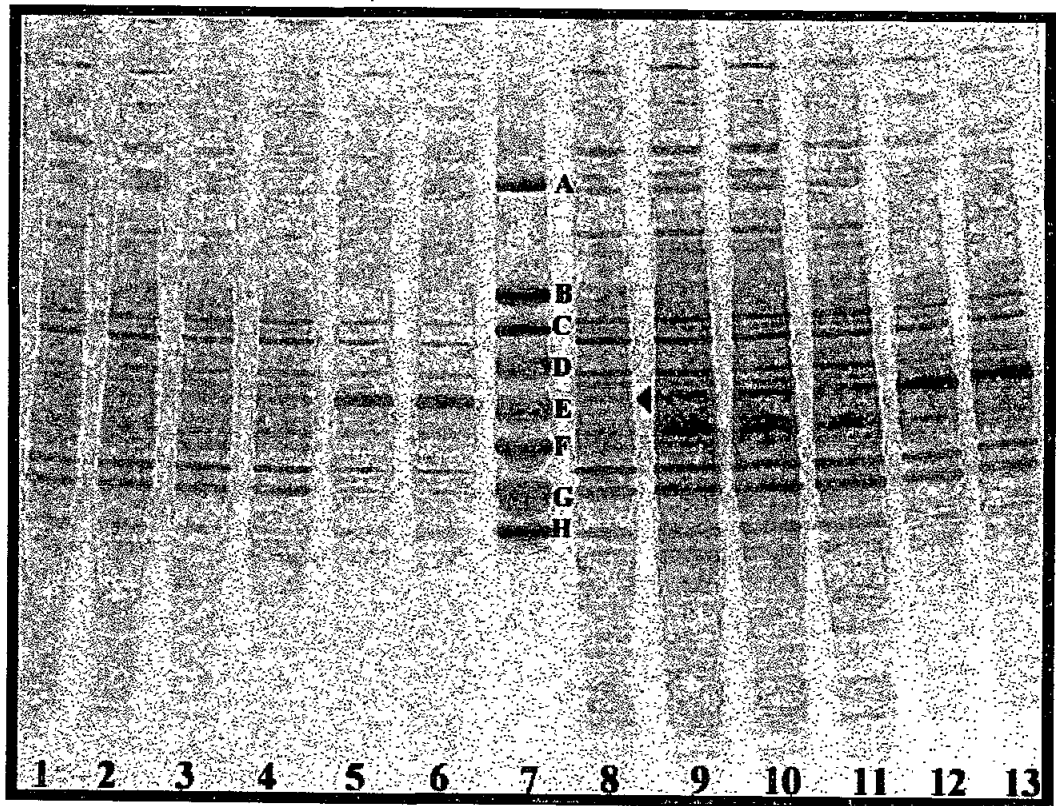
In order to measure changes in the amounts of heat shock proteins it was necessary to establish the normal protein patterns for the cell lines. L929 and NRK cells were separated by 1-D SDS-PAGE. The gel gradient is generated to obtain the optimal separation at approximately 50-100kDa, without obscuring the separation of other, potentially interesting, molecular weight proteins. Approximately, 25-30 different proteins could be resolved. To standardise each separation a control was included in every gel. The coefficient of variation (%CV) for control samples separated on replicate gels was 5% based on the peak area of hsp70 as determined by densitometry.

3.3.2 Heat shock response:

Initially to assess if the mouse connective tissue cell line L929 and normal rat kidney cell lines were capable of inducing heat shock proteins, a classic heat shock test was conducted. Cells were exposed to increasing heat shock (37-52°C) for 30 minutes and re-incubated at normal temperatures to permit heat shock protein synthesis. The cell proteins were separated by 1-D SDS-PAGE. Silver staining the electrophoretically separated proteins of the L929 and NRK cell lines after heat shock revealed induction of a 70kDa protein (Figure 13). This protein was positively identified as hsp70 by Western blot using a monoclonal mouse IgG anti-hsp70 antibody.

Figure 13:

One-dimensional SDS-PAGE separation of mouse connective tissue cells (L929) and normal rat kidney cells (NRK) exposed to increasing temperatures.



Legend:

One-dimensional SDS-PAGE separation of mouse connective tissue cells (L929) and normal rat kidney cells (NRK) exposed to increasing temperatures. 25cm² flasks were seeded with 4 x 10⁴ cells per ml for 24 hours at 37°C. The attached cells were immersed in a waterbath for 30 minutes at temperatures ranging from 37°C to 52°C and subsequently re-incubated at 37°C for 4 hours to permit stress protein induction. Cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Lane 1 represents the separation of L929 cells exposed to normal temperatures of 37°C. Lanes 2-6 represent separations of L929 cells exposed to increasing temperatures (40°C, 43°C, 46°C, 49°C & 52°C). Lane 7 represents separation of molecular weight markers (A=200 kDa, B=116 kDa, C=97 kDa, D=84 kDa, E=66 kDa, F=55 kDa, G=45 kDa & H=36 kDa). Lane 8 represents the separation of NRK cells exposed to normal temperatures of 37°C. Lanes 9-13 represent separations of NRK cells exposed to increasing temperatures (40°C, 43°C, 46°C, 49°C & 52°C). Arrowhead (◄) indicates the relative position of hsp70. Hsp70 induction showed a dose dependent increase. Repression of normal protein synthesis occurred with increasing temperature:

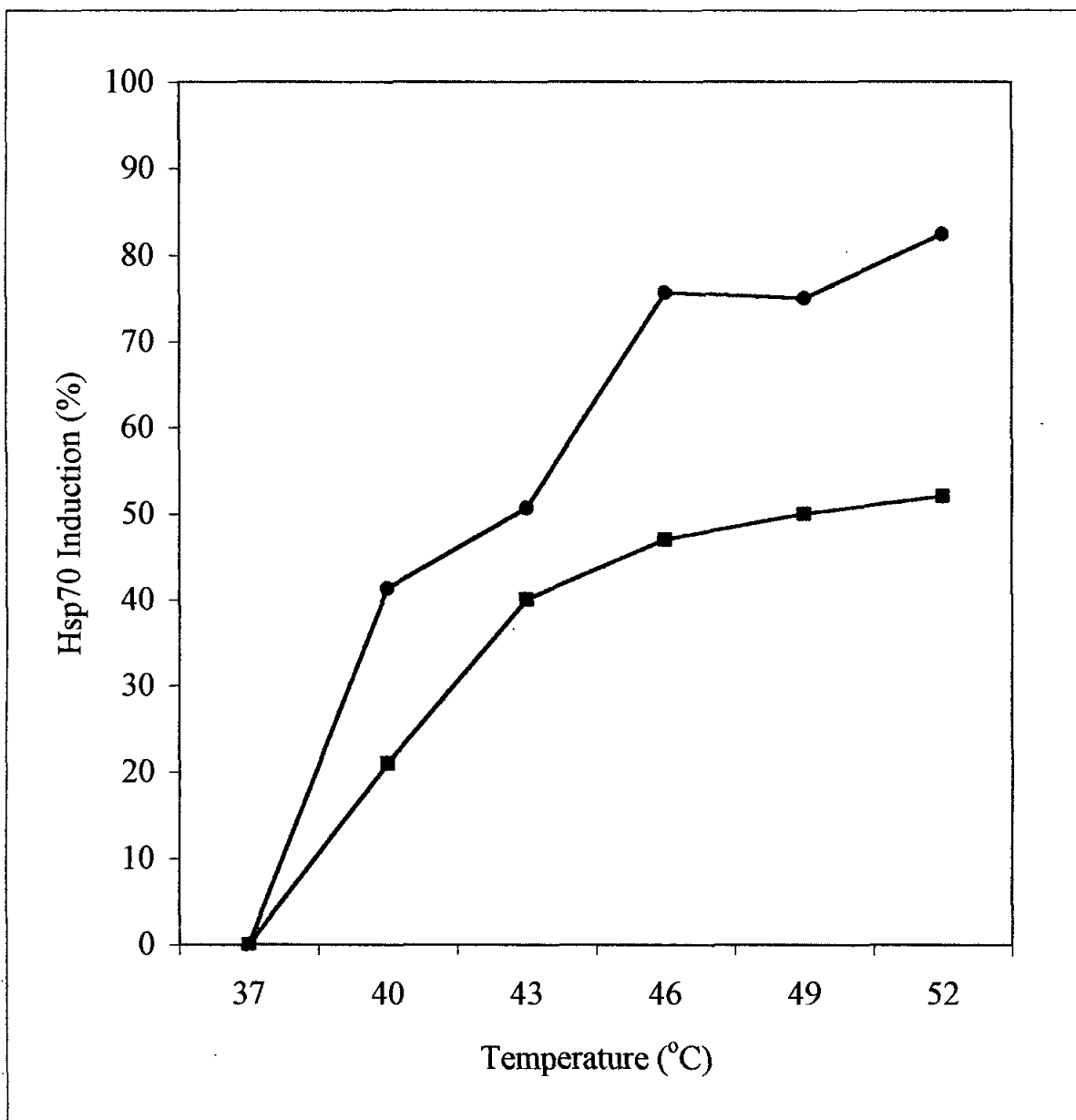
The cell lines were very sensitive to temperature shock demonstrating hsp70 induction with only a 3°C increase (Figures 14). NRK cells were found to have a 40% increase in hsp70 levels at 40°C over that found at 37°C. Above 46°C hsp70 levels began to plateau with hsp70 induction at 46-52°C ranging from 75-80%. The L929 cell line showed a lower induction of hsp70 at 40°C of only 20%. Above 46°C hsp70 levels began to plateau with hsp70 induction at 46-52°C ranging from 45-50%.

3.3.3 Heat shock protein induction by Heavy Metals:

Under identical test conditions, as the neutral red assay, cells were exposed to metallic stresses at concentrations ranging from sub-lethal to lethal. The cell lysates were separated by 1-D electrophoresis and proteins detected by silver staining. For both cell lines using the three toxins similar protein patterns were found. Hsp70 levels increased in a dose dependent fashion, above the LC₅₀ this increase began to plateau. Even with a reduced rate of induction hsp70 becomes the dominant protein above the LC₅₀ concentration. For the L929 cell line at the LC₅₀ value hsp70 represented 13%, 9% and 19% of the total cellular protein content for cadmium, mercury and copper respectively. Lower levels were found for the NRK cell line at the LC₅₀ value with hsp70 representing 6%, 6% and 4% of the total cellular protein content for cadmium, mercury and copper respectively. Repression of normal protein synthesis was also observed for the two cell lines using the three toxins. Normal protein synthesis was repressed in a dose dependent manner, below the LC₅₀ repression was less severe becoming more pronounced with increasing toxin concentration.

Figure 14:

Heat shock response curve for the normal rat kidney cell line (NRK) and mouse connective tissue cell line (L929) to temperature shock.



Heat shock response curve for the normal rat kidney cell line (●) and mouse connective tissue cell line (■) to temperature shock. 25cm² flasks were seeded with 4 x 10⁴ cells per ml for 24 hours at 37°C. The attached cells were immersed in a waterbath for 30 minutes at temperatures ranging from 37°C to 52°C and subsequently re-incubated at 37°C for 4 hours to permit stress protein induction. Cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp70 induction was expressed as a percentage of the untreated control level. The heat shock response curve shows a temperature dependent increase in hsp70 induction. Above 46°C hsp70 induction begins to plateau.

3.3.4 Mouse connective tissue cell line (L929):

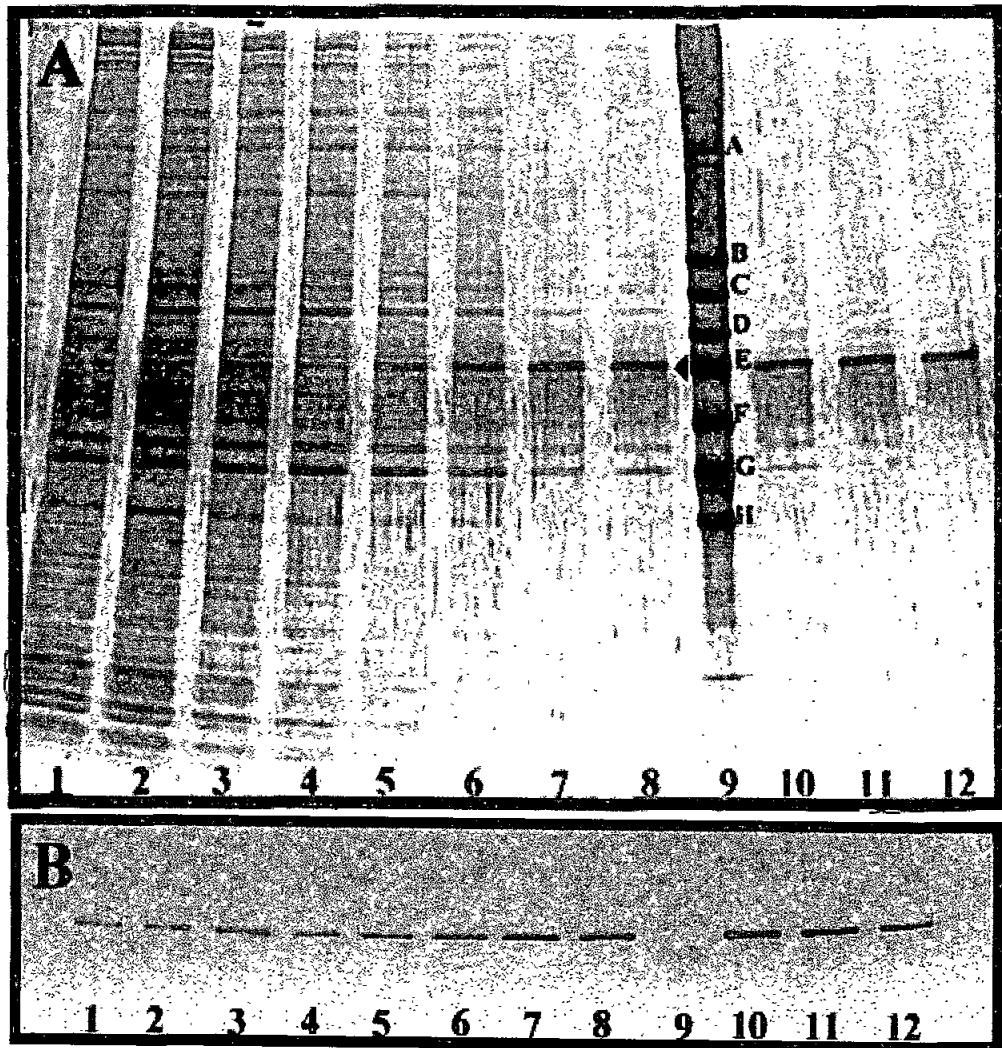
To allow comparison between the neutral red assay and heat shock protein assay the LC_{50} was used as a gauge to assess sensitivity. Heat shock protein induction at the LC_{50} was calculated by regression of the linear portion of the heat shock response curve. For the L929 cell line the heat shock response curve for cadmium, mercury and copper showed a dose dependent increase in hsp70 induction (Figures 15-20). Heat shock induction for the three metals at the LC_{50} is reported in Table 10. For the L929 cell line measurement of hsp70 induction showed a 25%, 5% and 25% greater response than found with measuring cell mortality using the neutral red assay for cadmium, mercury and copper. Coefficients of variation (%CV) ranged from 3-9% (average %C.V.=5%) for cadmium, 4-9% (average %C.V.=6%) for mercury and 3-9% (average %C.V.=6%) for copper.

Table 10: Hsp70 induction at the LC_{50} value for the L929 cell line

Toxin	Hsp70 induction at the LC_{50}
Cadmium	67% (C.V.=4.5%)
Mercury	53% (C.V.=5%)
Copper	67% (C.V.=7%)

Figure 15:

One-dimensional SDS-PAGE separation of mouse connective tissue cell line (L929) exposed to increasing concentrations of Cadmium.



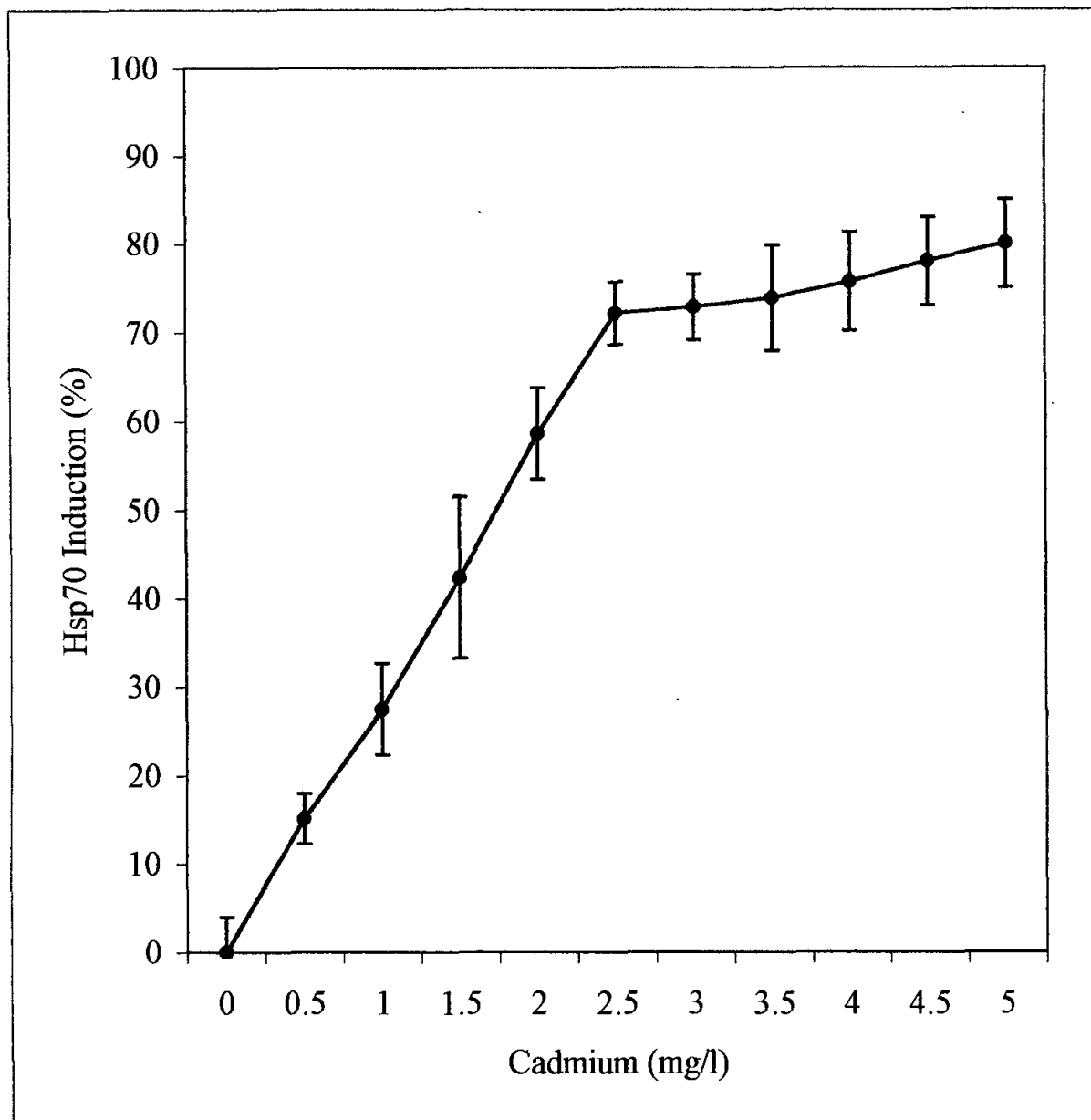
Legend:

A One-dimensional SDS-PAGE separation of the mouse connective tissue cell line (L929) to increasing concentrations of cadmium. 96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100 μ l per well of cadmium amended medium (0-5mg/l Cd) for 24 hours at 37°C. Cadmium concentrations never exceeded 10% of the media volume. Cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Lane 1 represents the separation of L929 cells exposed to unamended media. Lanes 2-8 & 10-12 represent separations of L929 cells exposed to increasing concentrations of cadmium (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 & 5.0 mg/l Cd). Lane 9 represents separation of molecular weight markers (A=200 kDa, B=116 kDa, C=97 kDa, D=84 kDa, E=66 kDa, F=55 kDa, G=45 kDa & H=36 kDa). Arrowhead (\blacktriangleleft) indicates the relative position of hsp70. Hsp70 induction showed a dose dependent increase. Repression of normal protein synthesis occurred with increasing cadmium concentration.

B One-dimensional SDS-PAGE separations were Western blotted on to PVDF membranes. Blots were probed with a mouse anti-hsp70 monoclonal antibody (clone C29F3A-5) and hsp70 detected by an alkaline phosphatase secondary antibody conjugate. A single band of hsp70 was identified with no cross reactivity with other proteins.

Figure 16:

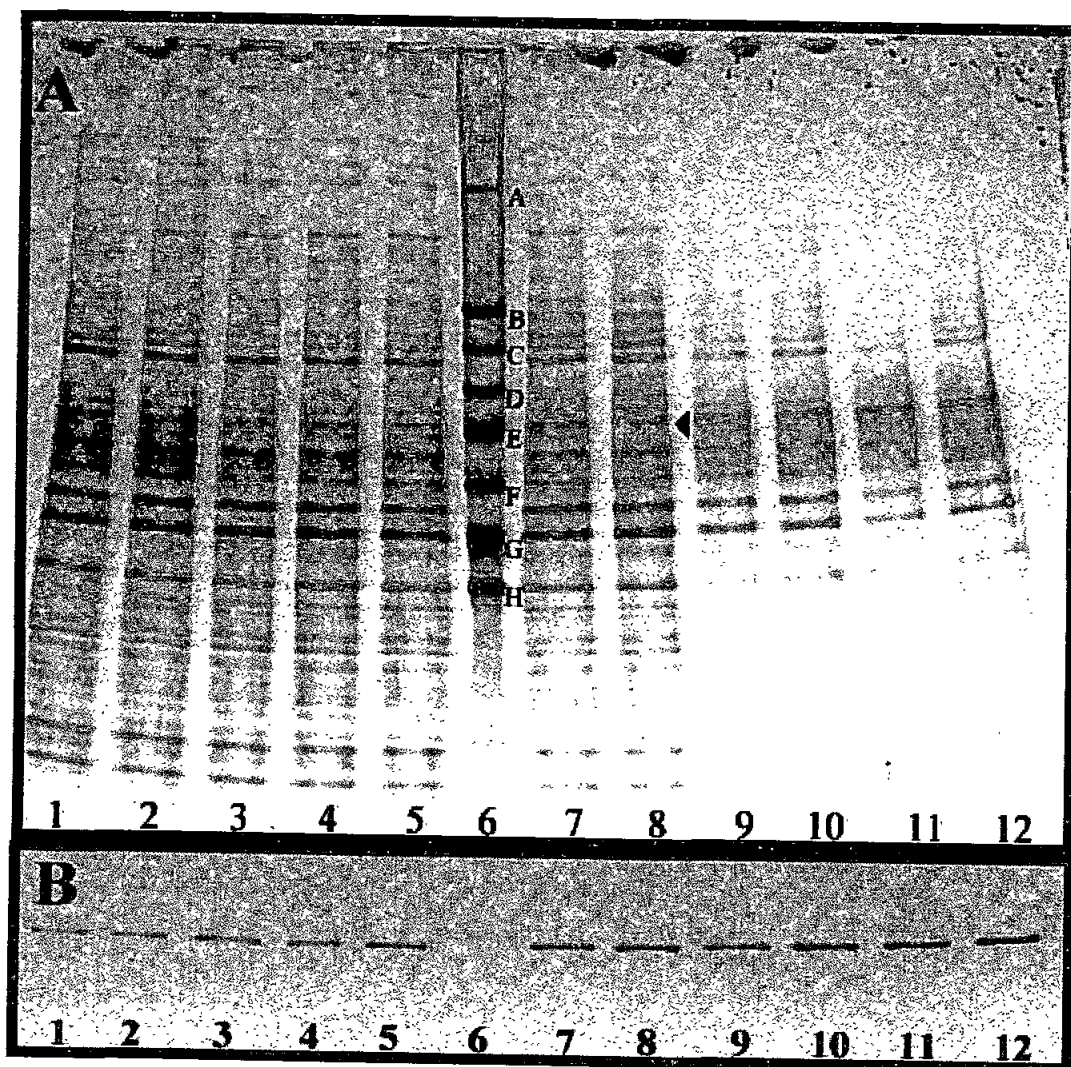
Heat shock response curve for the mouse connective tissue cell line (L929) to cadmium.



Heat shock response curve for the mouse connective tissue cell line (L929) to cadmium. 96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100µl per well of cadmium amended medium (0-5mg/l Cd) for 24 hours at 37°C. Cadmium concentrations never exceeded 10% of the media volume. Cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp70 induction (●) was expressed as a percentage of the untreated control level. Data points represent the arithmetic mean of 8 replicates. The stress response curve shows a dose dependent increase in hsp70 induction. Measurement of hsp70 induction at the median lethal concentration showed a 25% greater response than found with measuring cell mortality using the neutral red assay. Coefficients of variation (%CV) are presented as Y-axis error bars, % CV's ranged from 3-9%.

Figure 17:

One-dimensional SDS-PAGE separation of mouse connective tissue cell line (L929) exposed to increasing concentrations of Mercury.



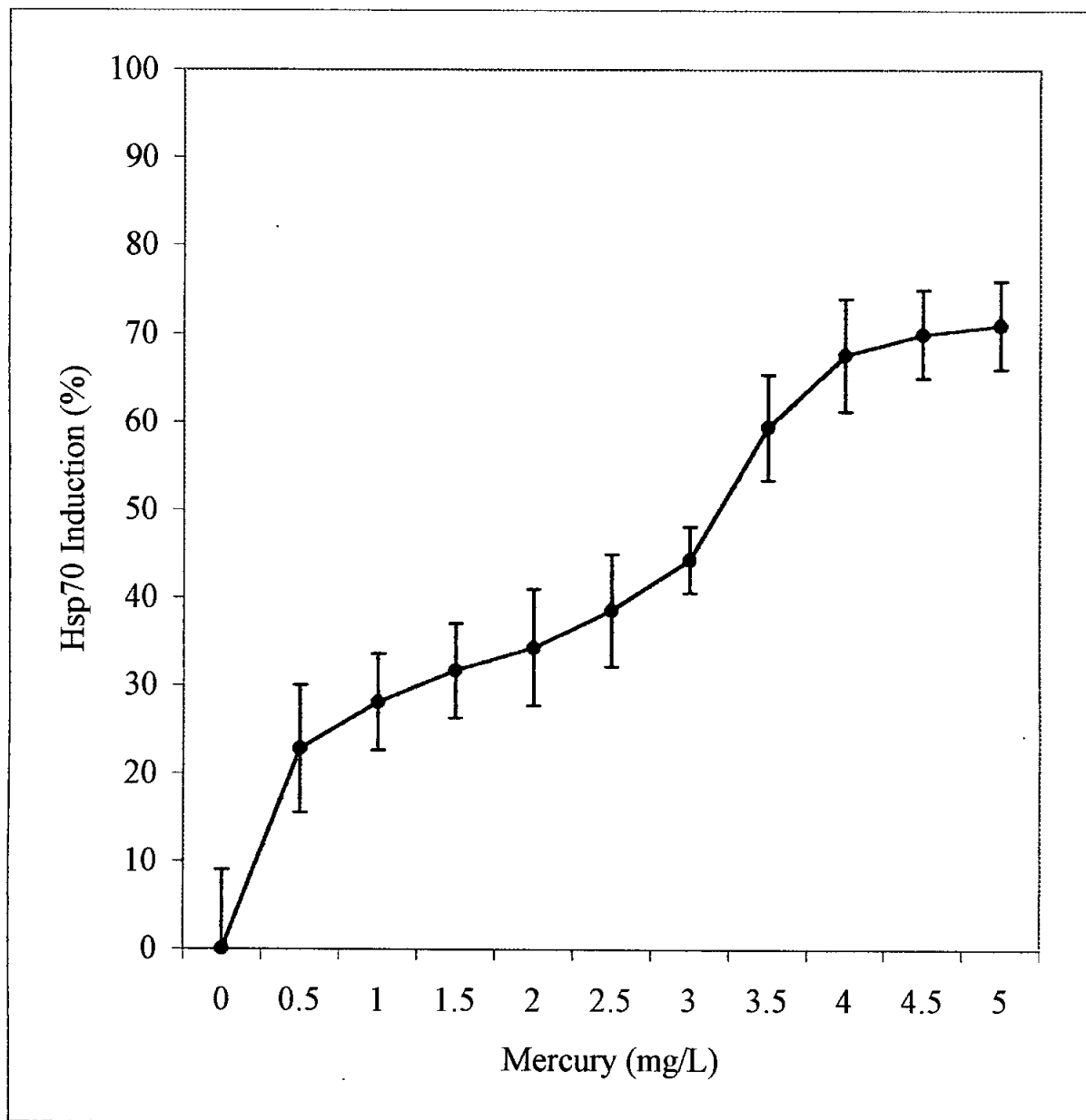
Legend:

A One-dimensional SDS-PAGE separation of the mouse connective tissue cell line (L929) to increasing concentrations of mercury. 96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100µl per well of mercury amended medium (0-5mg/l Hg) for 24 hours at 37°C. Mercury concentrations never exceeded 10% of the media volume. Cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Lane 1 represents the separation of L929 cells exposed to unamended media. Lanes 2-5 & 7-12 represent separations of L929 cells exposed to increasing concentrations of mercury (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 & 5.0 mg/l Hg). Lane 6 represents separation of molecular weight markers (A=200 kDa, B=116 kDa, C=97 kDa, D=84 kDa, E=66 kDa, F=55 kDa, G=45 kDa & H=36 kDa). Arrowhead (◄) indicates the relative position of hsp70. Hsp70 induction showed a dose dependent increase. Repression of normal protein synthesis occurred with increasing copper concentration.

B One-dimensional SDS-PAGE separations were Western blotted on to PVDF membranes. Blots were probed with a mouse anti-hsp70 monoclonal antibody (clone C29F3A-5) and hsp70 detected by an alkaline phosphatase secondary antibody conjugate. A single band of hsp70 was identified with no cross reactivity with other proteins.

Figure 18:

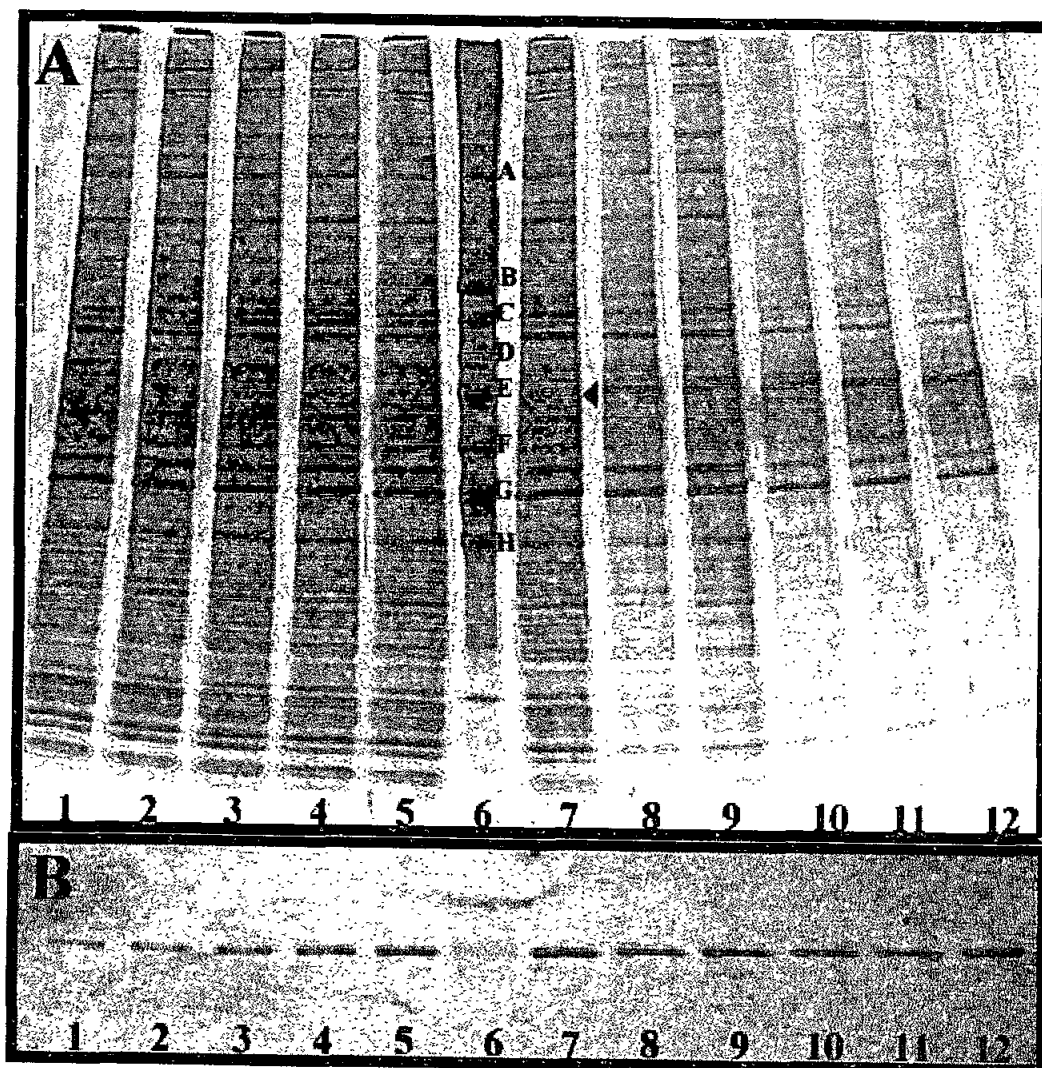
Heat shock response curve for the mouse connective tissue cell line (L929) to mercury.



Heat shock response curve for the mouse connective tissue cell line (L929) to mercury. 96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100µl per well of mercury amended medium (0-5mg/l Hg) for 24 hours at 37°C. Mercury concentrations never exceeded 10% of the media volume. Cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp70 induction (●) was expressed as a percentage of the untreated control level. Data points represent the arithmetic mean of 8 replicates. The heat shock response curve shows a dose dependent increase in hsp70 induction. Measurement of hsp70 induction at the median lethal concentration showed a 5% greater response than found with measuring cell mortality using the neutral red assay. Coefficients of variation (%CV) are presented as Y-axis error bars, % CV's ranged from 4-9%.

Figure 19:

One-dimensional SDS-PAGE separation of mouse connective tissue cell line (L929) exposed to increasing concentrations of Copper.



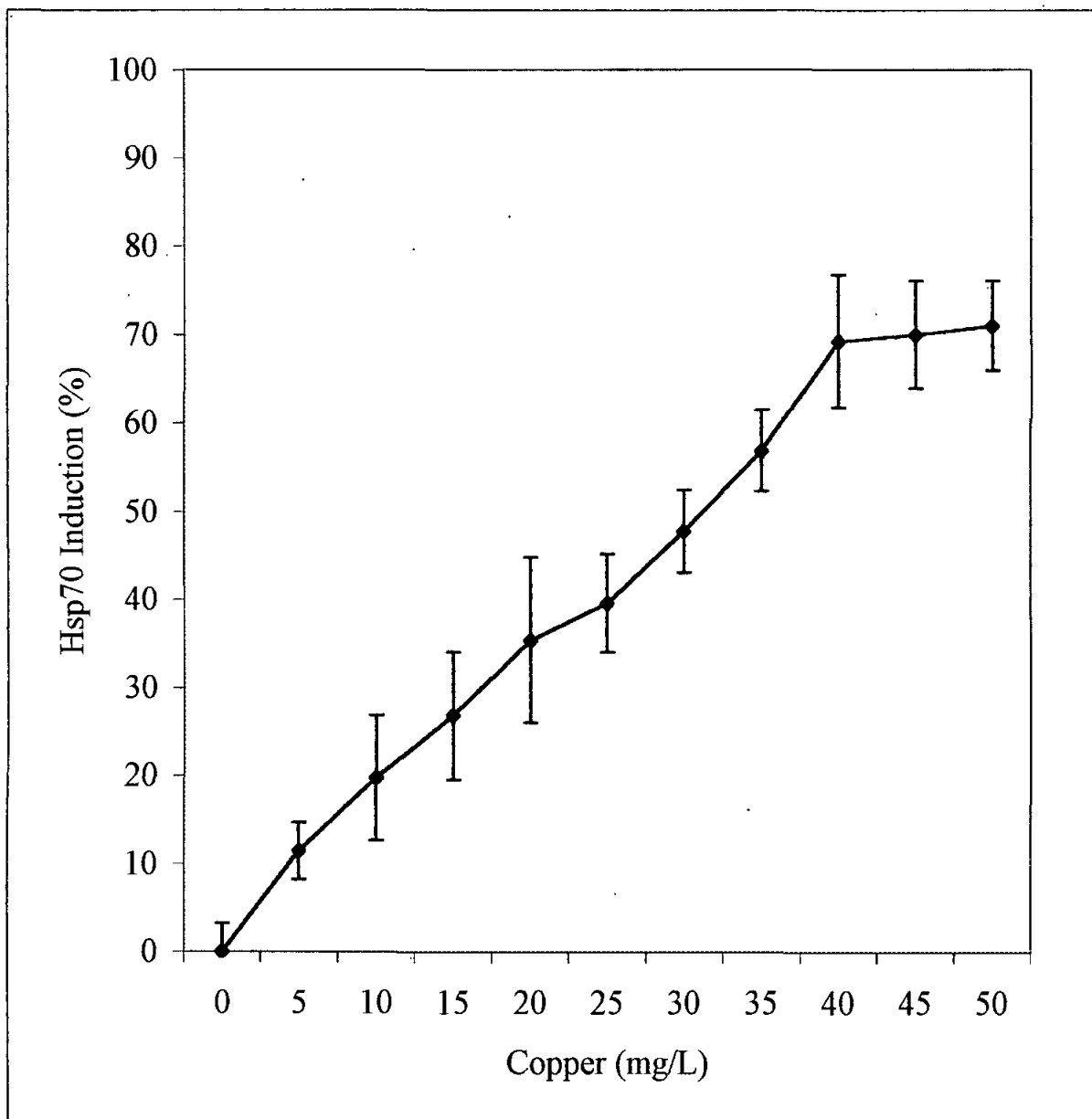
Legend:

A One-dimensional SDS-PAGE separation of the mouse connective tissue cell line (L929) to increasing concentrations of copper. 96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100 μ l per well of copper amended medium (0-50mg/l Cu) for 24 hours at 37°C. Copper concentrations never exceeded 10% of the media volume. Cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Lane 1 represents the separation of L929 cells exposed to unamended media. Lanes 2-5 & 7-12 represent separations of L929 cells exposed to increasing concentrations of copper (5, 10, 15, 20, 25, 30, 35, 40, 45 & 50 mg/l Cu). Lane 6 represents separation of molecular weight markers (A=200 kDa, B=116 kDa, C=97 kDa, D=84 kDa, E=66 kDa, F=55 kDa, G=45 kDa & H=36 kDa). Arrowhead (\blacktriangleleft) indicates the relative position of hsp70. Hsp70 induction showed a dose dependent increase. Repression of normal protein synthesis occurred with increasing copper concentration.

B One-dimensional SDS-PAGE separations were Western blotted on to PVDF membranes. Blots were probed with a mouse anti-hsp70 monoclonal antibody (clone C29F3A-5) and hsp70 detected by an alkaline phosphatase secondary antibody conjugate. A single band of hsp70 was identified with no cross reactivity with other proteins.

Figure 20:

Heat shock response curve for the mouse connective tissue cell line (L929) to copper.



Heat shock response curve for the mouse connective tissue cell line (L929) to copper.

96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100µl per well of copper amended medium (0-50mg/l Cu) for 24 hours at 37°C. Copper concentrations never exceeded 10% of the media volume. Cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp70 induction (●) was expressed as a percentage of the untreated control level. Data points represent the arithmetic mean of 8 replicates. The heat shock response curve shows a dose dependent increase in hsp70 induction. Measurement of hsp70 induction at the median lethal concentration showed a 25% greater response than found with measuring cell mortality using the neutral red assay. Coefficients of variation (%CV) are presented as Y-axis error bars, % CV's ranged from 3-9%.

3.3.5 Normal Rat Kidney cell line (NRK):

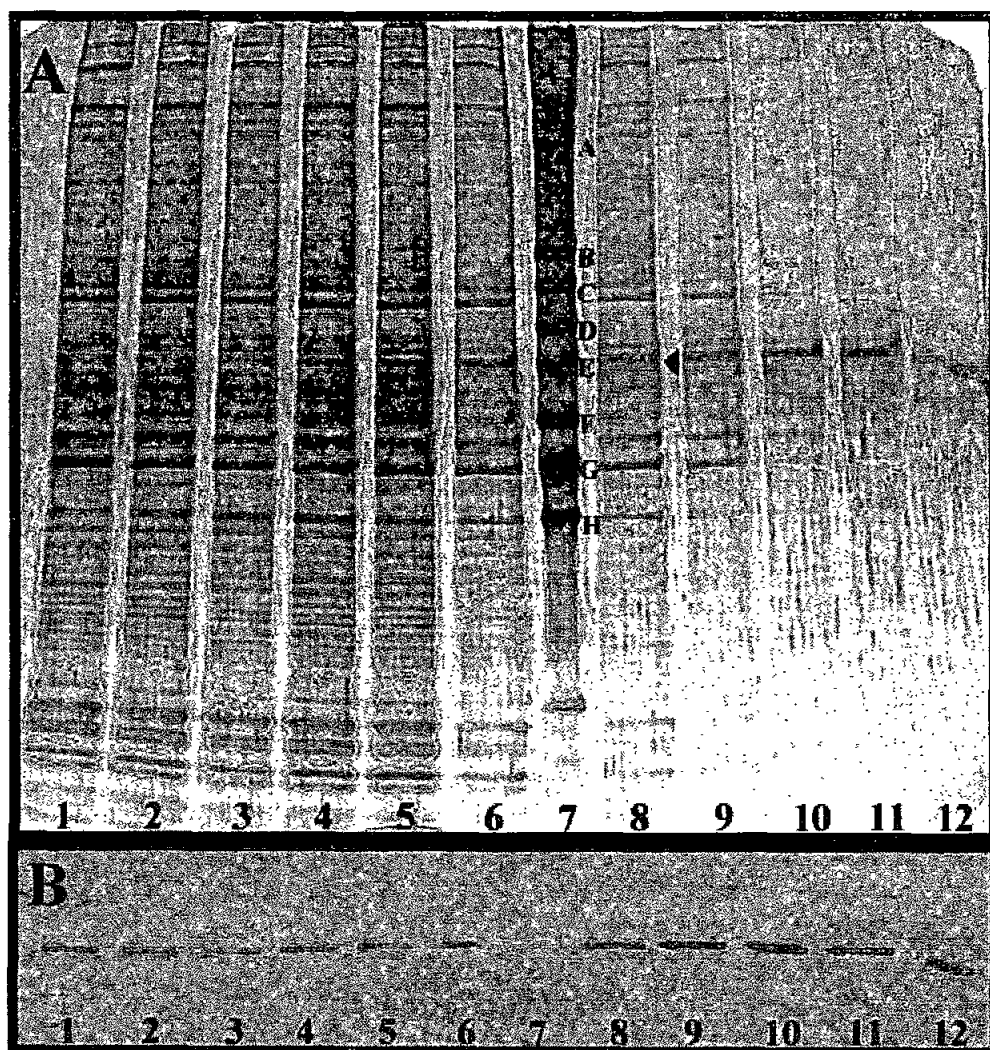
For the NRK cell line the dose response curves for cadmium, mercury and copper showed a dose dependent increase in hsp70 induction (Figures 21-26). Heat shock induction at the LC₅₀ was calculated by regression of the linear portion of the heat shock response curve. Heat shock protein induction for the three metals at the LC₅₀ is reported in Table 11. For the NRK cell line measurement of hsp70 induction showed a 25% greater response than that found with measuring cell mortality using the neutral red assay for cadmium and mercury. For the NRK cell line measurement of hsp70 induction showed a 16% lower response than that found with measuring cell mortality for copper. Coefficients of variation (%CV) ranged from 3-8% (average C.V.=6%) for cadmium, 3-11% (average C.V.=5%) for mercury and 3-8% (average C.V.=6%) for copper.

Table 11: Hsp70 induction at the LC₅₀ value for the NRK cell line

Toxin	Hsp70 induction at the LC₅₀
Cadmium	67% (C.V.=6%)
Mercury	66% (C.V.=3%)
Copper	42% (C.V.=6%)

Figure 21:

One-dimensional SDS-PAGE separation of normal rat kidney cell line (NRK) exposed to increasing concentrations of Cadmium.



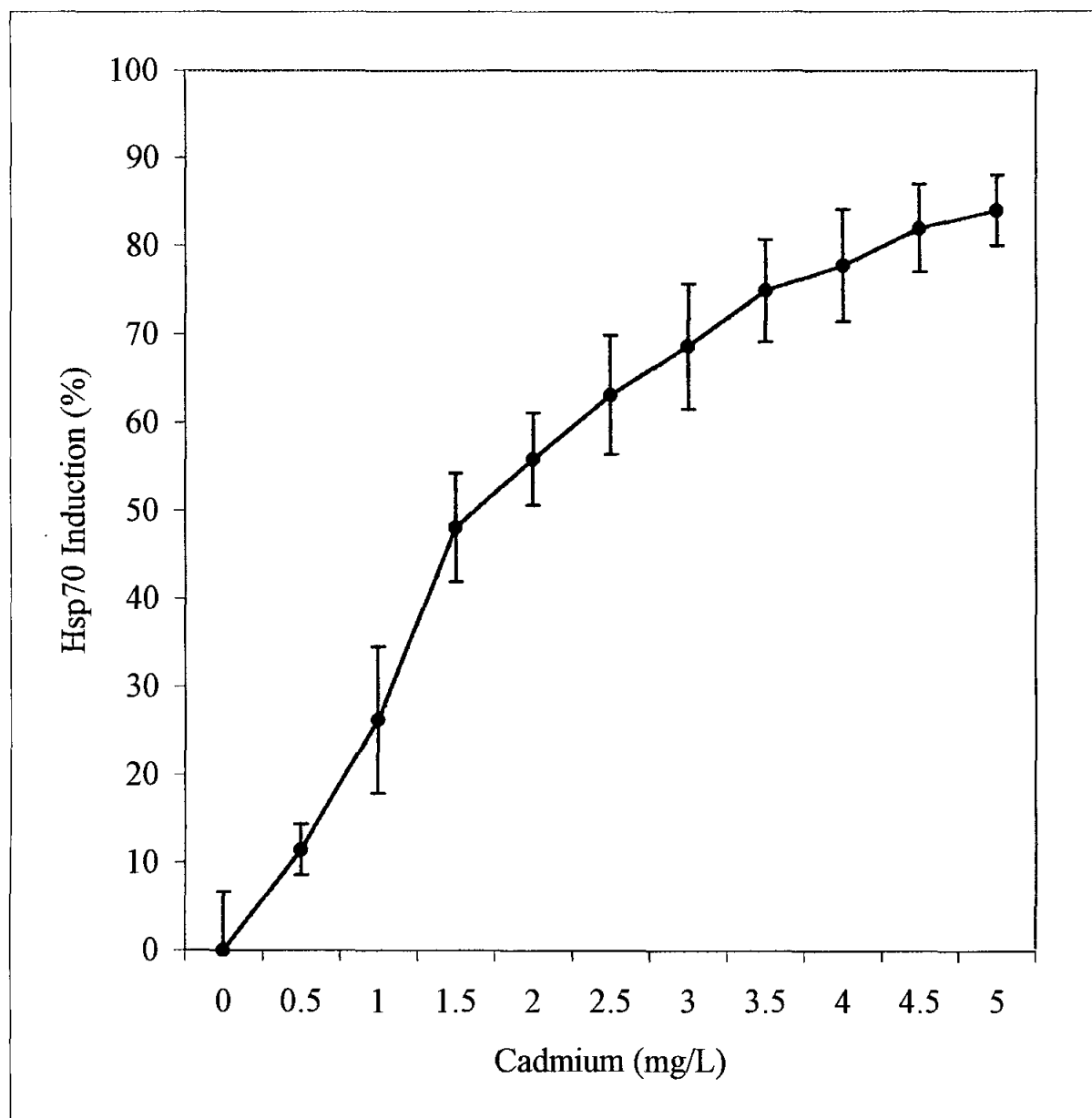
Legend:

A One-dimensional SDS-PAGE separation of the normal rat kidney cell line (NRK) to increasing concentrations of cadmium. 96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100µl per well of cadmium amended medium (0-5mg/l Cd) for 24 hours at 37°C. Cadmium concentrations never exceeded 10% of the media volume. Cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Lane 1 represents the separation of NRK cells exposed to unamended media. Lanes 2-6 & 8-12 represent separations of NRK cells exposed to increasing concentrations of cadmium (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 & 5.0 mg/l Cd). Lane 7 represents separation of molecular weight markers (A=200 kDa, B=116 kDa, C=97 kDa, D=84 kDa, E=66 kDa, F=55 kDa, G=45 kDa & H=36 kDa). Arrowhead (◄) indicates the relative position of hsp70. Hsp70 induction showed a dose dependent increase. Repression of normal protein synthesis occurred with increasing cadmium concentration.

B One-dimensional SDS-PAGE separations were Western blotted on to PVDF membranes. Blots were probed with a mouse anti-hsp70 monoclonal antibody (clone C29F3A-5) and hsp70 detected by an alkaline phosphatase secondary antibody conjugate. A single band of hsp70 was identified with no cross reactivity with other proteins.

Figure 22:

Heat shock response curve for the normal rat kidney cell line (NRK) to cadmium.

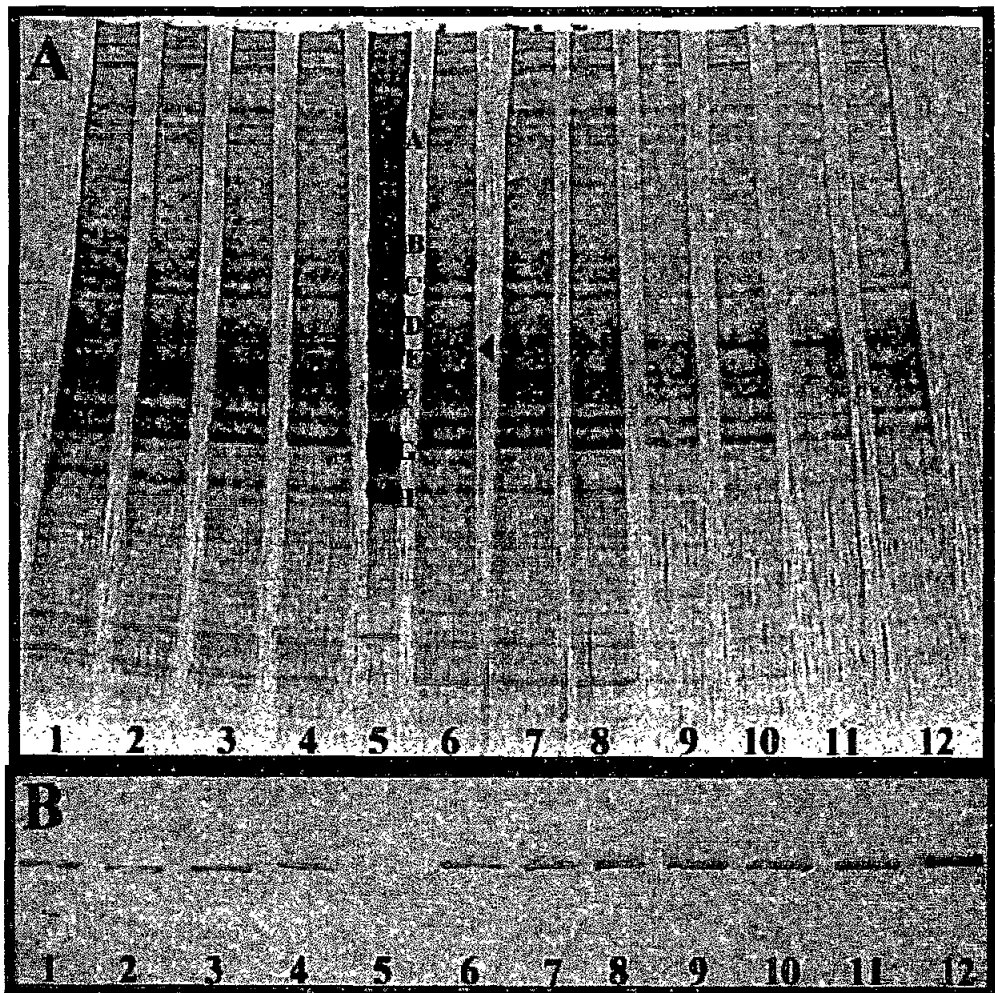


Heat shock response curve for the normal rat kidney cell line (NRK) to cadmium.

96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100µl per well of cadmium amended medium (0-5mg/l Cd) for 24 hours at 37°C. Cadmium concentrations never exceeded 10% of the media volume. Cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp70 induction (●) was expressed as a percentage of the untreated control level. Data points represent the arithmetic mean of 8 replicates. The heat shock response curve shows a dose dependent increase in hsp70 induction. Measurement of hsp70 induction at the median lethal concentration showed a 25% greater response than found with measuring cell mortality using the neutral red assay. Coefficients of variation (%CV) are presented as Y-axis error bars, % CV's ranged from 3-8%.

Figure 23:

One-dimensional SDS-PAGE separation of normal rat kidney cell line (NRK) exposed to increasing concentrations of Mercury.



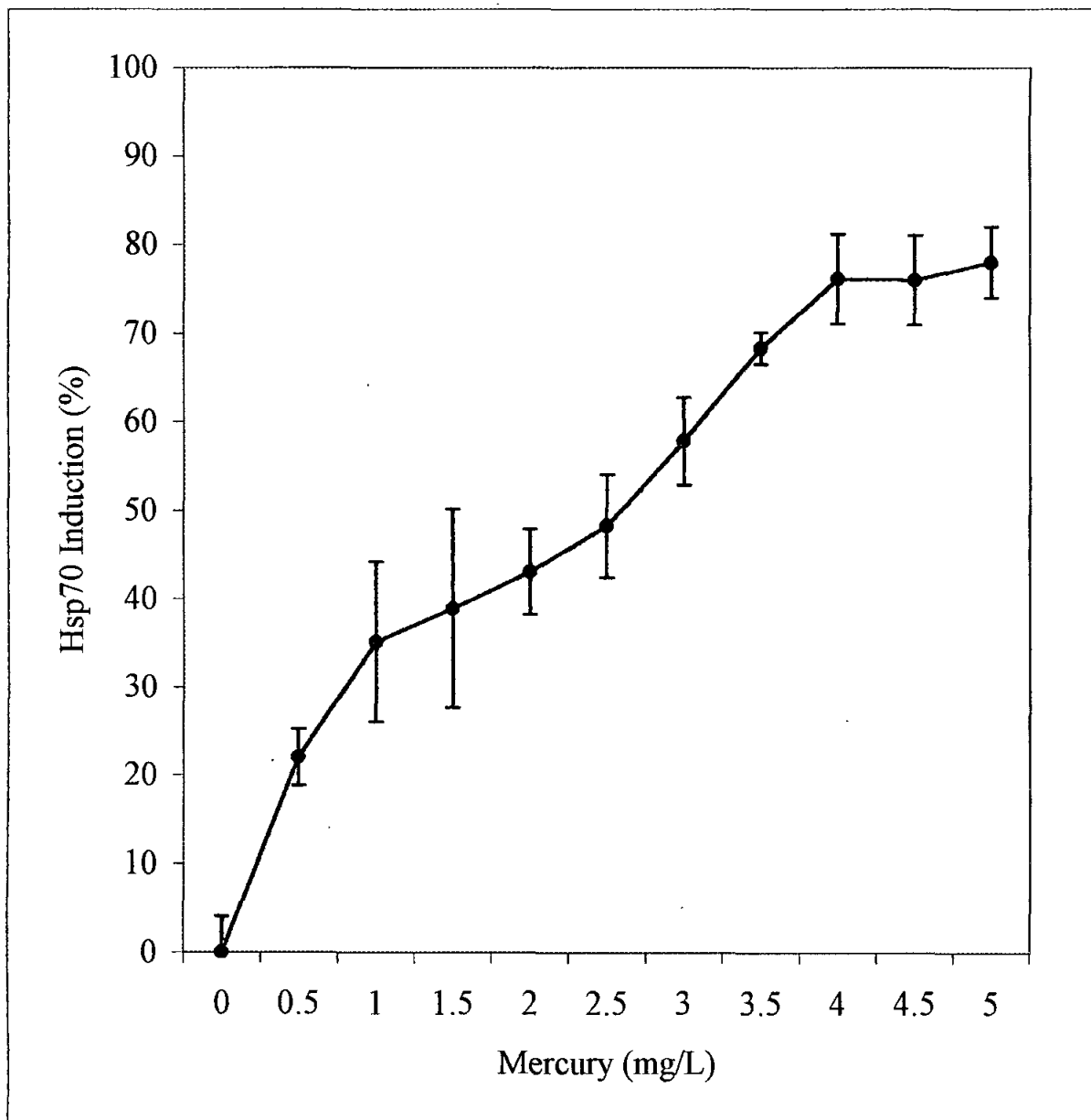
Legend:

A One-dimensional SDS-PAGE separation of the normal rat kidney cell line (NRK) to increasing concentrations of mercury. 96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C . The attached cells were incubated with $100\mu\text{l}$ per well of mercury amended medium (0-5mg/l Hg) for 24 hours at 37°C . Mercury concentrations never exceeded 10% of the media volume. Cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Lane 1 represents the separation of NRK cells exposed to unamended media. Lanes 2-4 & 6-12 represent separations of NRK cells exposed to increasing concentrations of mercury (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 & 5.0 mg/l Hg). Lane 5 represents separation of molecular weight markers (A=200 kDa, B=116 kDa, C=97 kDa, D=84 kDa, E=66 kDa, F=55 kDa, G=45 kDa & H=36 kDa). Arrowhead (A) indicates the relative position of hsp70. Hsp70 induction showed a dose dependent increase. Repression of normal protein synthesis occurred with increasing copper concentration.

B One-dimensional SDS-PAGE separations were Western blotted on to PVDF membranes. Blots were probed with a mouse anti-hsp70 monoclonal antibody (clone C29F3A-5) and hsp70 detected by an alkaline phosphatase secondary antibody conjugate. A single band of hsp70 was identified with no cross reactivity with other proteins.

Figure 24:

Heat shock response curve for the normal rat kidney cell line (NRK) to mercury.

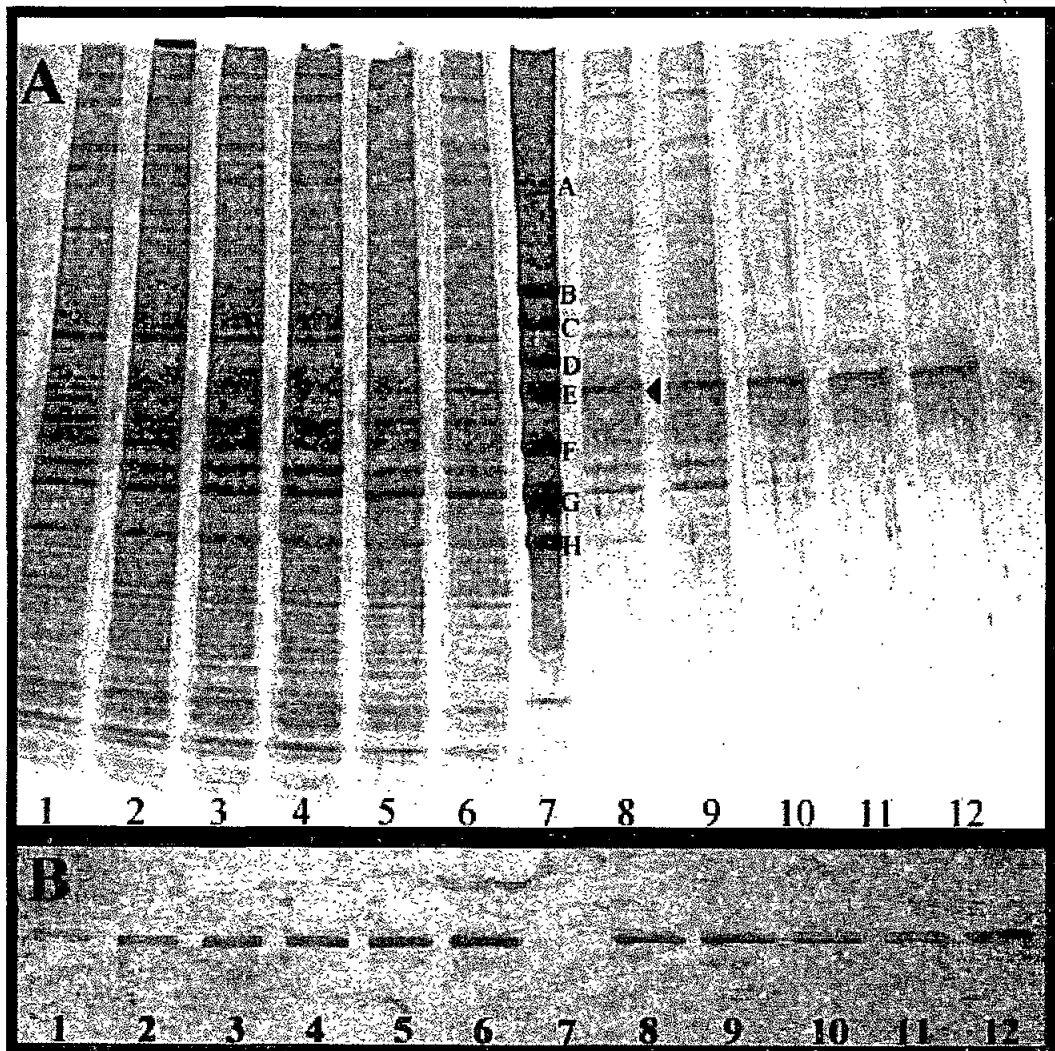


Heat shock response curve for the normal rat kidney cell line (NRK) to mercury.

96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100µl per well of mercury amended medium (0-5mg/l Hg) for 24 hours at 37°C. Mercury concentrations never exceeded 10% of the media volume. Cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp70 induction (●) was expressed as a percentage of the untreated control level. Data points represent the arithmetic mean of 8 replicates. The heat shock response curve shows a dose dependent increase in hsp70 induction. Measurement of hsp70 induction at the median lethal concentration showed a 25% greater response than found with measuring cell mortality using the neutral red assay. Coefficients of variation (%CV) are presented as Y-axis error bars, % CV's ranged from 3-11%.

Figure 25:

One-dimensional SDS-PAGE separation of normal rat kidney cell line (NRK) exposed to increasing concentrations of Copper.



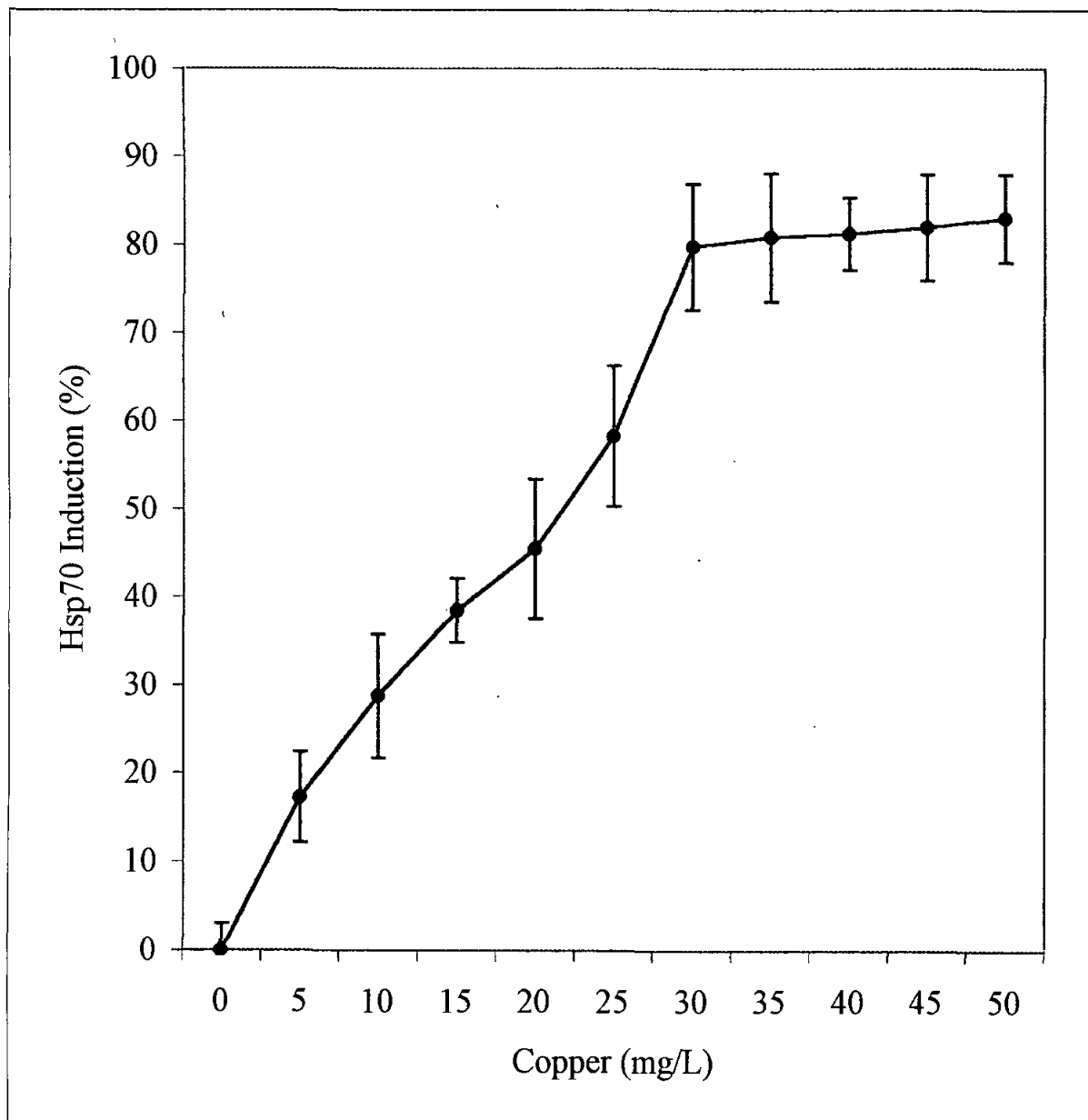
Legend:

A One-dimensional SDS-PAGE separation of the normal rat kidney cell line (NRK) to increasing concentrations of copper. 96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100 μ l per well of copper amended medium (0-50mg/l Cu) for 24 hours at 37°C. Copper concentrations never exceeded 10% of the media volume. Cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Lane 1 represents the separation of NRK cells exposed to unamended media. Lanes 2-6 & 8-12 represent separations of NRK cells exposed to increasing concentrations of copper (5, 10, 15, 20, 25, 30, 35, 40, 45 & 50 mg/l Cu). Lane 7 represents separation of molecular weight markers: (A=200 kDa, B=116 kDa, C=97 kDa, D=84 kDa, E=66 kDa, F=55 kDa, G=45 kDa & H=36 kDa). Arrowhead (\blacktriangleleft) indicates the relative position of hsp70. Hsp70 induction showed a dose dependent increase. Repression of normal protein synthesis occurred with increasing copper concentration.

B One-dimensional SDS-PAGE separations were Western blotted on to PVDF membranes. Blots were probed with a mouse anti-hsp70 monoclonal antibody (clone C29F3A-5) and hsp70 detected by an alkaline phosphatase secondary antibody conjugate. A single band of hsp70 was identified with no cross reactivity with other proteins.

Figure 26:

Heat shock response curve for the normal rat kidney cell line (NRK) to copper.



Heat shock response curve for the normal rat kidney cell line (NRK) to copper.

96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100µl per well of copper amended medium (0-50mg/l Cu) for 24 hours at 37°C. Copper concentrations never exceeded 10% of the media volume. Cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp70 induction (●) was expressed as a percentage of the untreated control level. Data points represent the arithmetic mean of 8 replicates. The heat shock response curve shows a dose dependent increase in hsp70 induction. Measurement of hsp70 induction at the median lethal concentration showed a 16% lower response than found with measuring cell mortality using the neutral red assay. Coefficients of variation (%CV) are presented as Y-axis error bars, % CV's ranged from 3-8%.

3.3.6 Sub-lethal toxicity:

In order to assess the potential of the heat shock response to measure sub-lethal toxicity concentrations below the no effect level were examined. Any measurable increase in hsp70 levels would identify sub-lethal toxicity. To allow direct comparison hsp70 induction at the no effect level was measured. For both cell lines using the three toxins considerable hsp70 induction was observed at the no effect levels established using the neutral red assay. Hsp70 induction at the no effect level for both the L929 and NRK cell lines is presented in Table 12. The heat shock response assay had a 42%, 28% and 40% greater response than the neutral red assay for the L929 cell line, identifying hsp70 induction at concentrations that were 1.5, 1.6 and 3 times lower than the median lethal concentration for mercury, cadmium and copper respectively. The heat shock response assay had a 48%, 39% and 29% greater response than the neutral red assay for the NRK cell line, identifying hsp70 induction at concentrations that were 1.5, 1.7 and 2 times lower than the median lethal concentration for cadmium, mercury and copper respectively.

Table 12: Hsp70 induction at the no effect level for L929 and NRK cell lines

Toxin	L929	NRK
Cadmium	42% (C.V.=9%)	48% (C.V.=6%)
Mercury	28% (C.V.=5%)	39% (C.V.=11%)
Copper	40% (C.V.=6%)	29% (C.V.=7%)

3.3.7 Lowest observed effective concentration:

As the heat shock response had shown a greater sensitivity than the neutral red assay measuring hsp70 induction at the no effect level, hsp70 induction at the lowest observed effective concentration was examined. Hsp70 induction at the lowest observed effective concentration for both the L929 and NRK cell lines using the three metals are presented in Table 13. The heat shock response assay identified hsp70 induction for the L929 cell line at concentrations that were 2, 3 and 5 times lower than the no effect level for mercury, cadmium and copper respectively. The heat shock response assay identified hsp70 induction for the NRK cell line at concentrations that were 2 times lower than the no effect level for copper and 3 times lower for cadmium and mercury.

Table 13: Hsp70 induction at the lowest observed effective concentration for the L929 and NRK cell lines

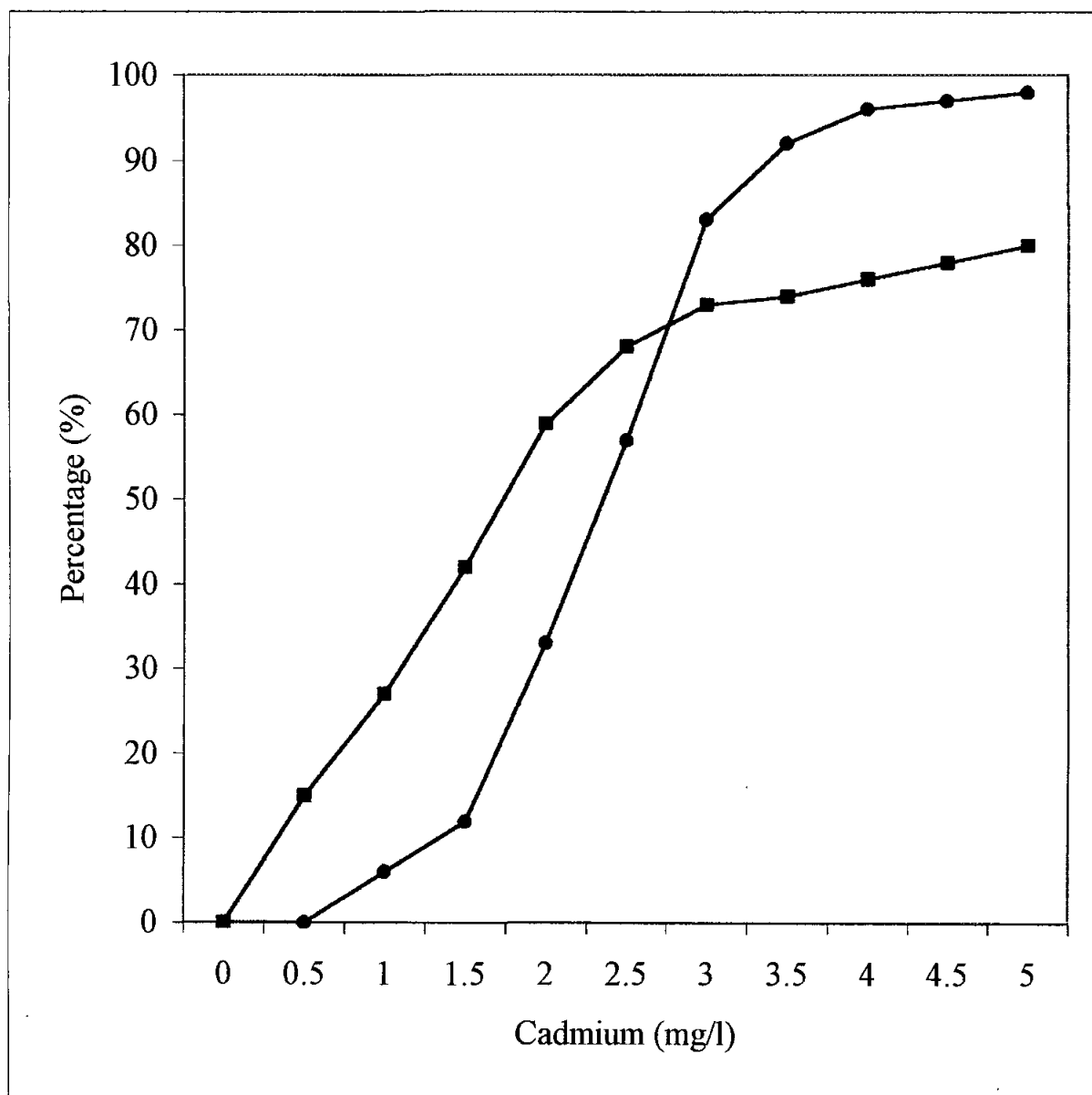
Toxin	L929	NRK
Cadmium (0.5mg/l)	15% (C.V.=3%)	11% (C.V.=3%)
Mercury (0.5mg/l)	23% (C.V.=7%)	22% (C.V.=3%)
Copper (5.0mg/l)	11% (C.V.=3%)	17% (C.V.=5%)

3.4 Heat shock protein induction verses toxicity data:

Comparing the toxicity data established using the neutral red assay and the heat shock response assay it is evident that the heat shock response assay is more sensitive (Figures 27-32). At the LC_{50} values measuring hsp70 induction the heat shock response assay identified a greater response than the neutral red assay. For several of the toxins this response was 25% greater than observed when measuring cell mortality. However, as hsp70 induction begins to plateau around the LC_{50} value hsp70's increase is less pronounced. Substantial hsp70 induction was observed at the no effect level and lowest observed effective concentration, at concentrations where the neutral red assay was unable to measure any cell mortality.

Figure 27:

Comparison of cell mortality and hsp70 induction for the mouse connective tissue cell line (L929) to cadmium.

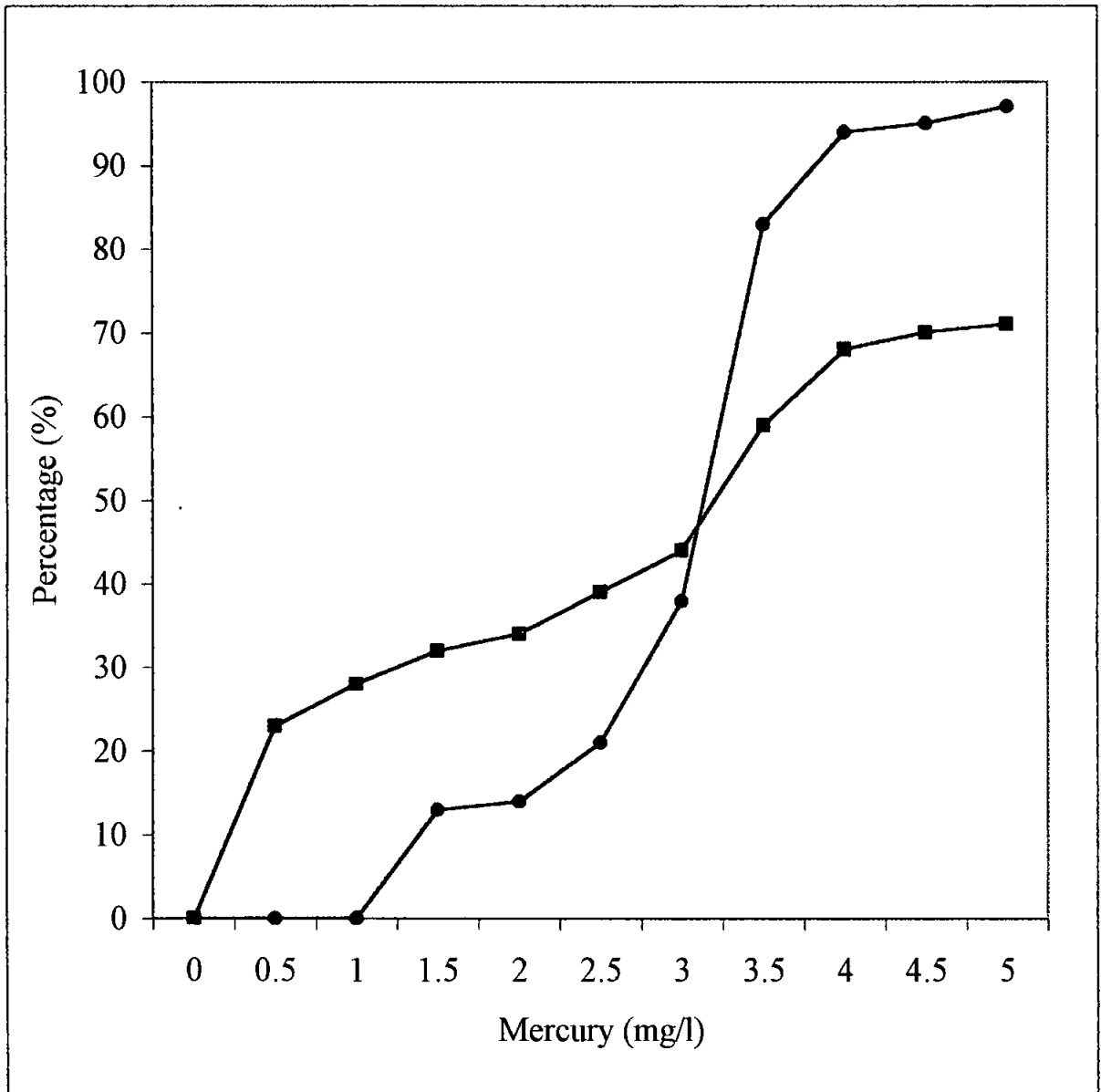


Comparison of cell mortality and hsp70 induction for the mouse connective tissue cell line (L929) to cadmium. Cell mortality (●) was estimated by the neutral red assay. The median lethal concentration was estimated by regression of the linear portion of the curve. Using the Students T-test ($p=0.05$) to determine significant changes in cell mortality the no effect level was estimated.

For the heat shock response assay cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp70 induction (■) was expressed as a percentage of the untreated control level. Measurement of hsp70 induction at the median lethal concentration, the no effect level and at the lowest concentration tested showed a greater response than found with measuring cell mortality using the neutral red assay.

Figure 28:

Comparison of cell mortality and hsp70 induction for the mouse connective tissue cell line (L929) to mercury.

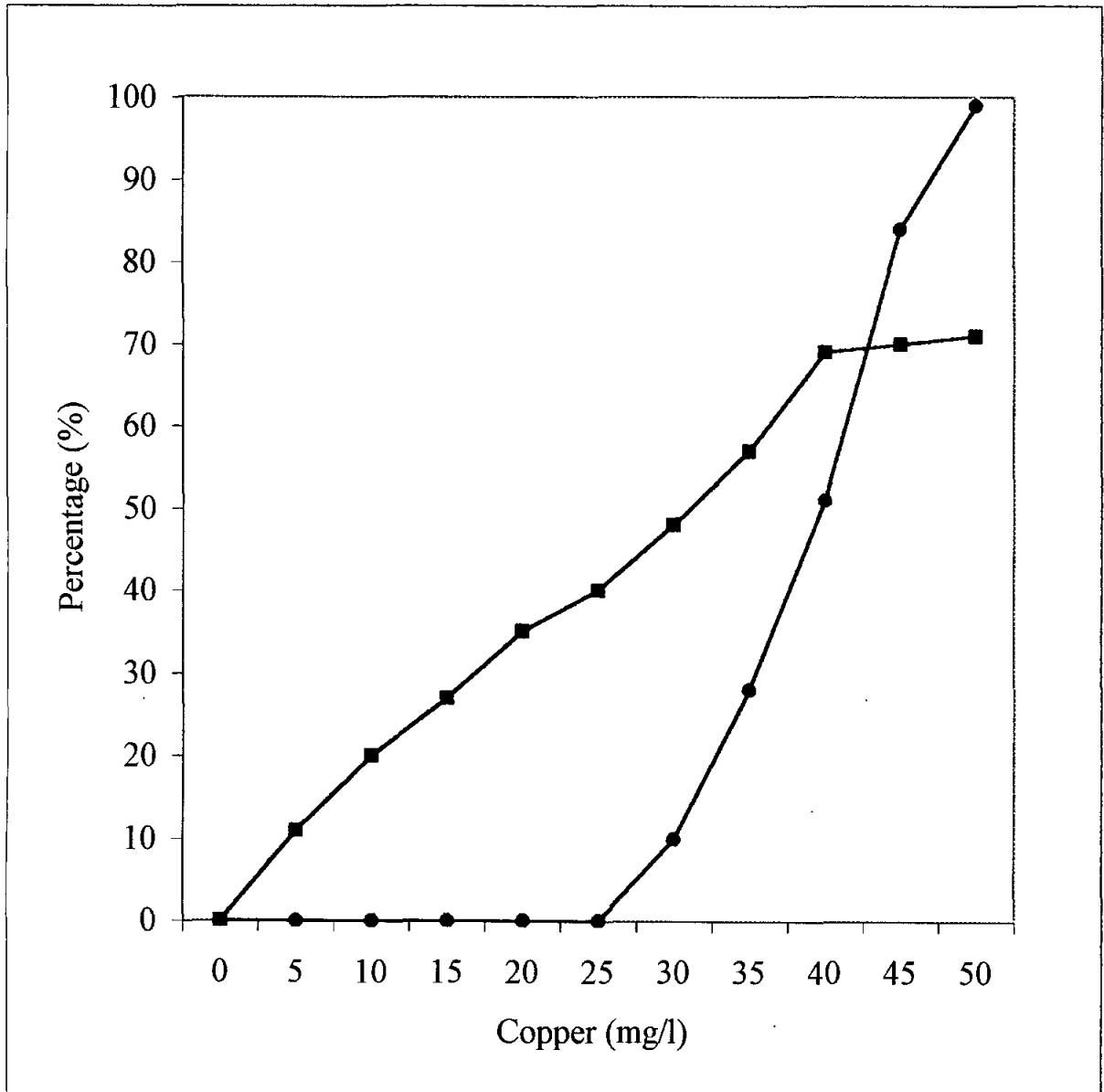


Comparison of cell mortality and hsp70 induction for the mouse connective tissue cell line (L929) to mercury. Cell mortality (●) was estimated by the neutral red assay. The median lethal concentration was estimated by regression of the linear portion of the curve. Using the Students T-test ($p=0.05$) to determine significant changes in cell mortality the no effect level was estimated.

For the heat shock response assay cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp70 induction (■) was expressed as a percentage of the untreated control level. Measurement of hsp70 induction at the median lethal concentration, the no effect level and at the lowest concentration tested showed a greater response than found with measuring cell mortality using the neutral red assay.

Figure 29:

Comparison of cell mortality and hsp70 induction for the mouse connective tissue cell line (L929) to copper.

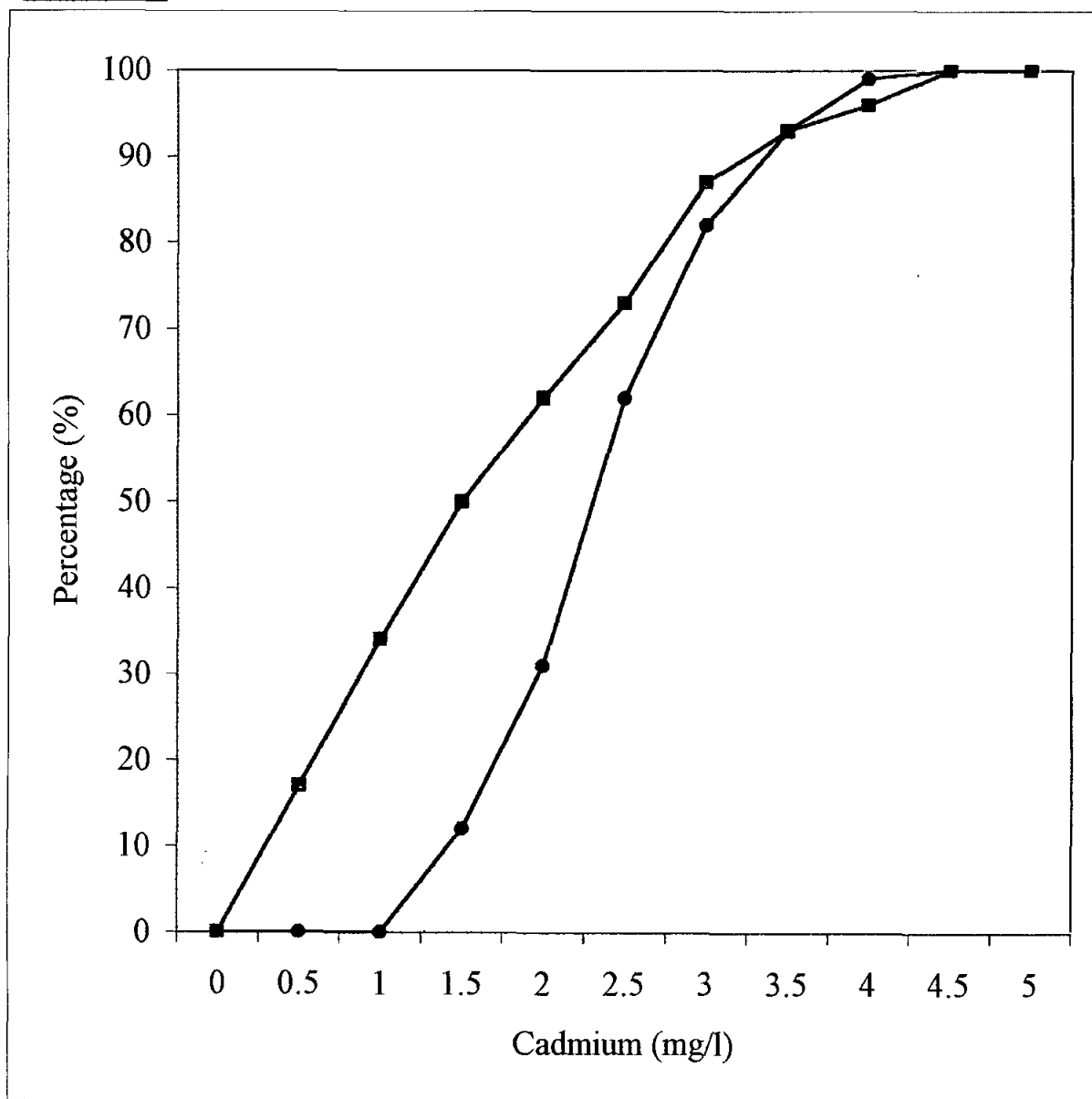


Comparison of cell mortality and hsp70 induction for the mouse connective tissue cell line (L929) to copper. Cell mortality (●) was estimated by the neutral red assay. The median lethal concentration was estimated by regression of the linear portion of the curve. Using the Students T-test ($p=0.05$) to determine significant changes in cell mortality the no effect level was estimated.

For the heat shock response assay cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp70 induction (■) was expressed as a percentage of the untreated control level. Measurement of hsp70 induction at the median lethal concentration, the no effect level and at the lowest concentration tested showed a greater response than found with measuring cell mortality using the neutral red assay.

Figure 30:

Comparison of cell mortality and hsp70 induction for the normal rat kidney cell line (NRK) to cadmium.

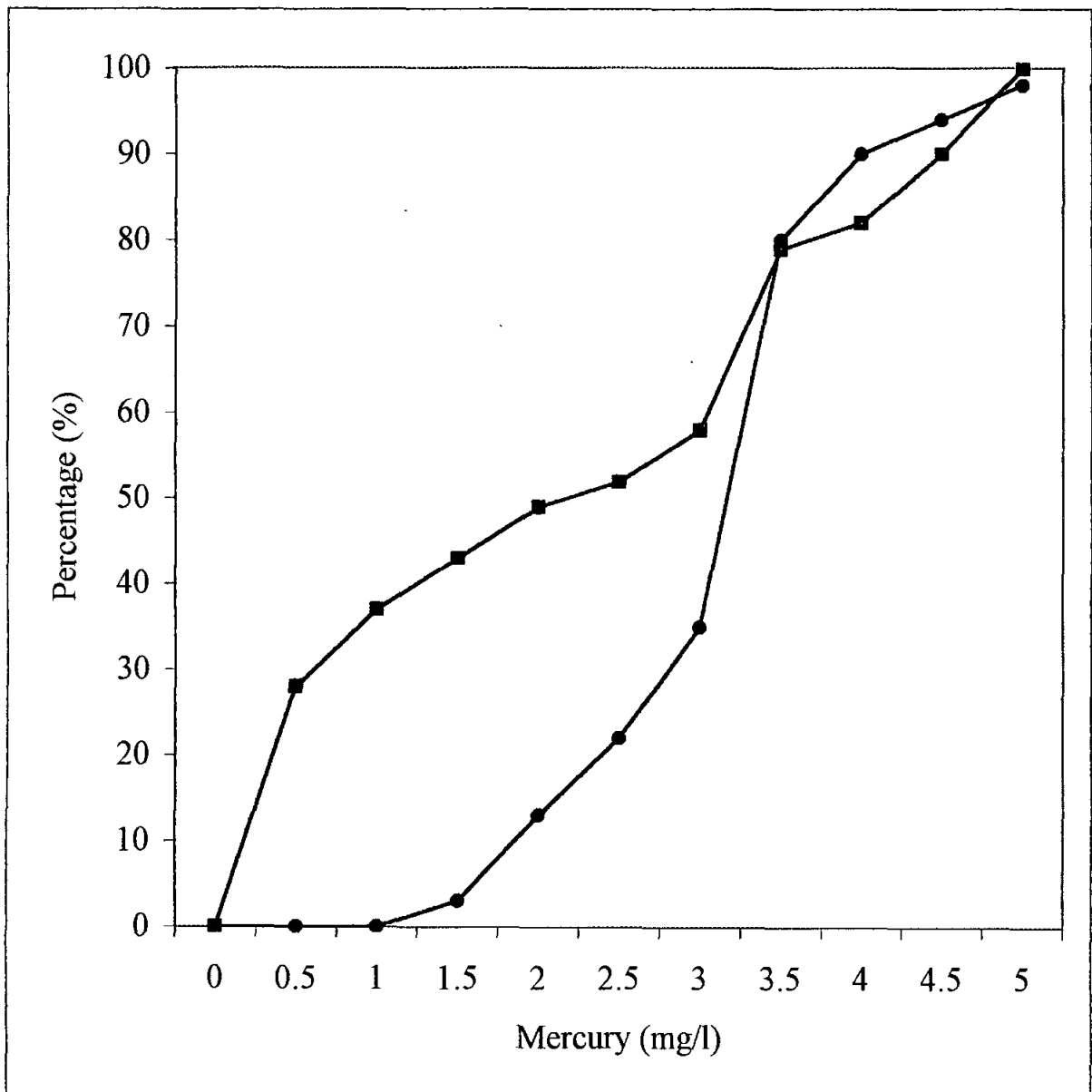


Comparison of cell mortality and hsp70 induction for the normal rat kidney cell line (NRK) to cadmium. Cell mortality (●) was estimated by the neutral red assay. The median lethal concentration was estimated by regression of the linear portion of the curve. Using the Students T-test ($p=0.05$) to determine significant changes in cell mortality the no effect level was estimated.

For the heat shock response assay cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp70 induction (■) was expressed as a percentage of the untreated control level. Measurement of hsp70 induction at the median lethal concentration, the no effect level and at the lowest concentration tested showed a greater response than found with measuring cell mortality using the neutral red assay.

Figure 31:

Comparison of cell mortality and hsp70 induction for the normal rat kidney cell line (NRK) to mercury.

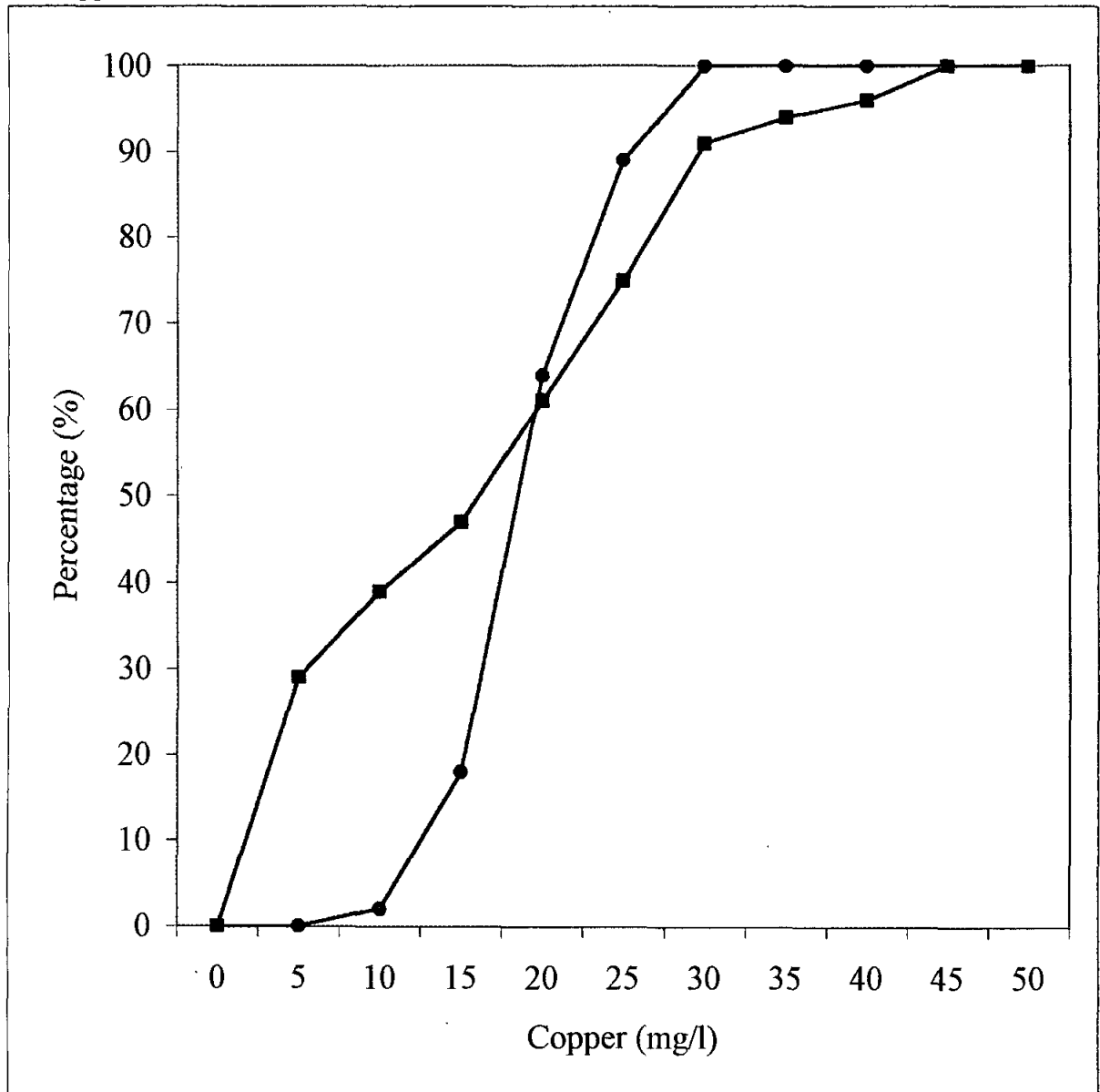


Comparison of cell mortality and hsp70 induction for the normal rat kidney cell line (NRK) to mercury. Cell mortality (●) was estimated by the neutral red assay. The median lethal concentration was estimated by regression of the linear portion of the curve. Using the Students T-test ($p=0.05$) to determine significant changes in cell mortality the no effect level was estimated.

For the heat shock response assay cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp70 induction (■) was expressed as a percentage of the untreated control level. Measurement of hsp70 induction at the median lethal concentration, the no effect level and at the lowest concentration tested showed a greater response than found with measuring cell mortality using the neutral red assay.

Figure 32:

Comparison of cell mortality and hsp70 induction for the normal rat kidney cell line (NRK) to copper.



Comparison of cell mortality and hsp70 induction for the normal rat kidney cell line (NRK) to copper. Cell mortality (●) was estimated by the neutral red assay. The median lethal concentration was estimated by regression of the linear portion of the curve. Using the Students T-test ($p=0.05$) to determine significant changes in cell mortality the no effect level was estimated.

For the heat shock response assay cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp70 induction (■) was expressed as a percentage of the untreated control level. Measurement of hsp70 induction at the median lethal concentration, the no effect level and at the lowest concentration tested showed a greater response than found with measuring cell mortality using the neutral red assay.

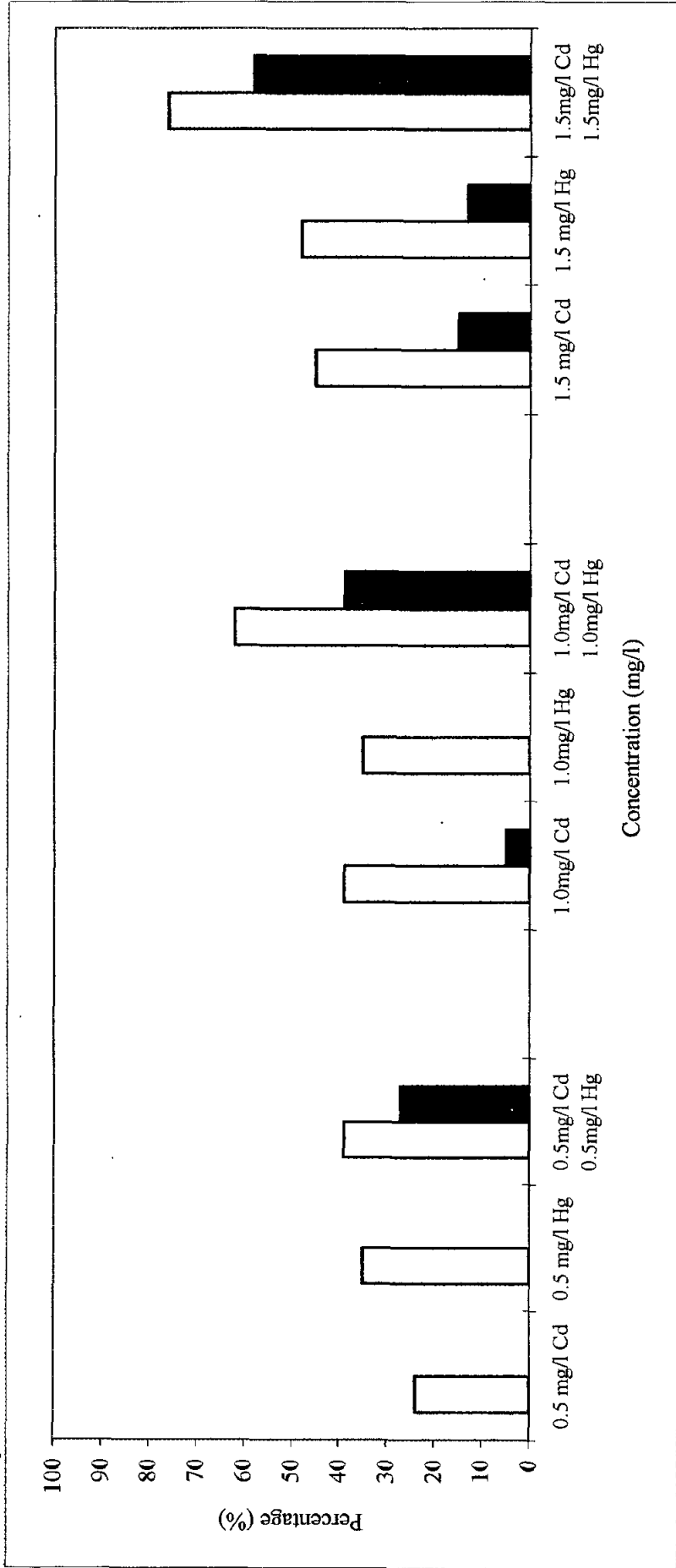
3.5 Western blotting:

In order to confirm that the 70kDa inducible protein observed in this study was hsp70 and thus allow comparison with other studies it was essential to identify hsp70 using specific antibodies. The initial antibody (clone BRM-22) used reacted with both the inducible and constitutive isoforms of hsp70. Using Western blotting techniques the antibody identified both hsp70 isoforms in the two cell lines. The inducible isoform was observed to increase significantly upon stress with the constitutive form decreasing. The second antibody (clone C29F3A-5) identified the inducible form of hsp70 alone. For each 1-D gel separation the corresponding Western blot demonstrates the induction of hsp70 (Figures 15, 17, 19, 21, 23 & 25).

3.6 Toxin interaction:

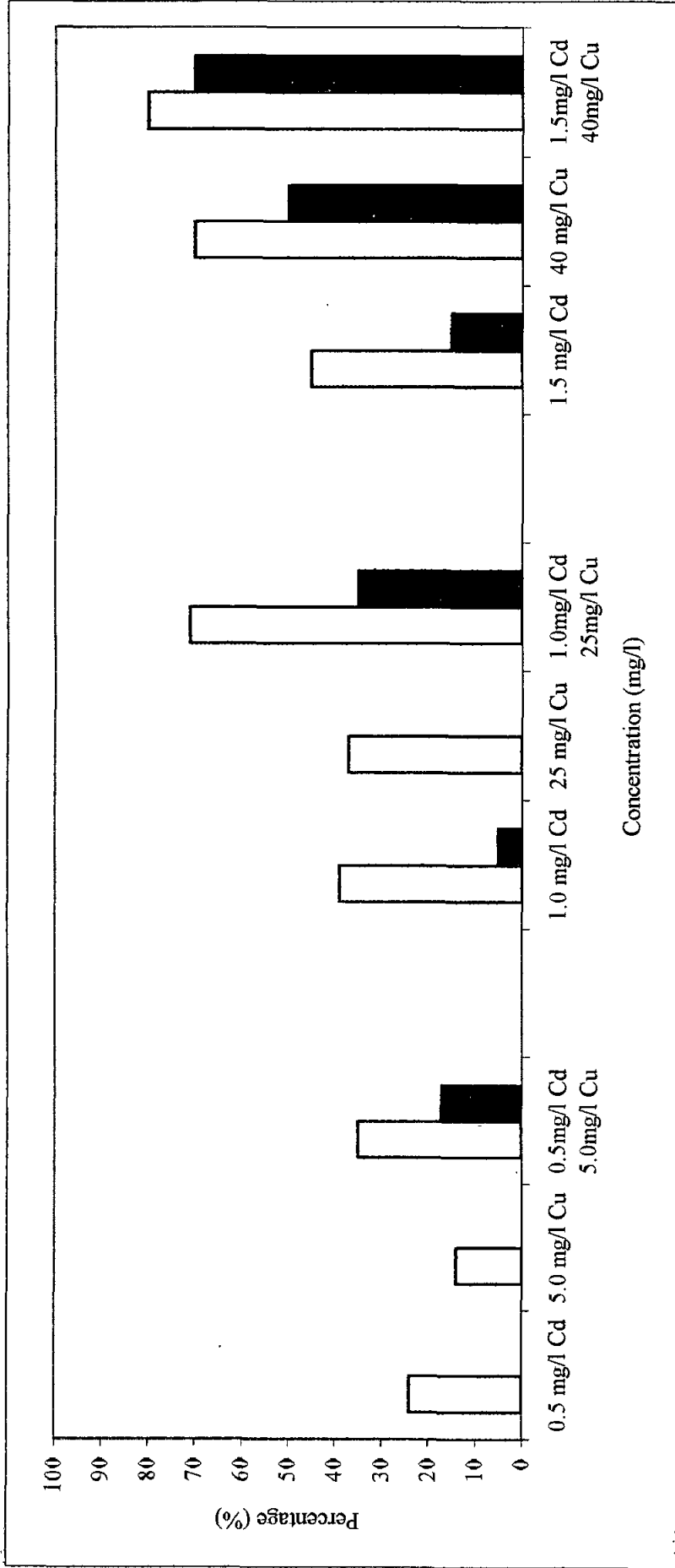
The ability of the heat shock response assay to measure hsp70 induction in mixtures as found in the real environment was also assessed. Initially toxicity data had to be established to identify the effects of mixing the toxins together. Using the neutral red assay cell mortality for the individual metals and for the metal mixtures was estimated. The mixtures were cadmium and mercury, cadmium and copper and mercury and copper. For the L929 and NRK cell lines the three mixtures showed a combined toxic effect (Figure 33 & 38). Tables 14-16 show the cell mortality for the metal mixtures for both the L929 and NRK cell lines. Under identical conditions the protein content of these mixtures were separated by 1-D electrophoresis. For the L929 and NRK cell line the three mixtures showed a combined toxic effect (Figures 33-38). Tables 17-19 show hsp70 induction for the metal mixtures for both cell lines.

Figure 33: Comparison of cell mortality and hsp70 induction for the mouse connective tissue cell line (L929) to a mixture of cadmium and mercury.



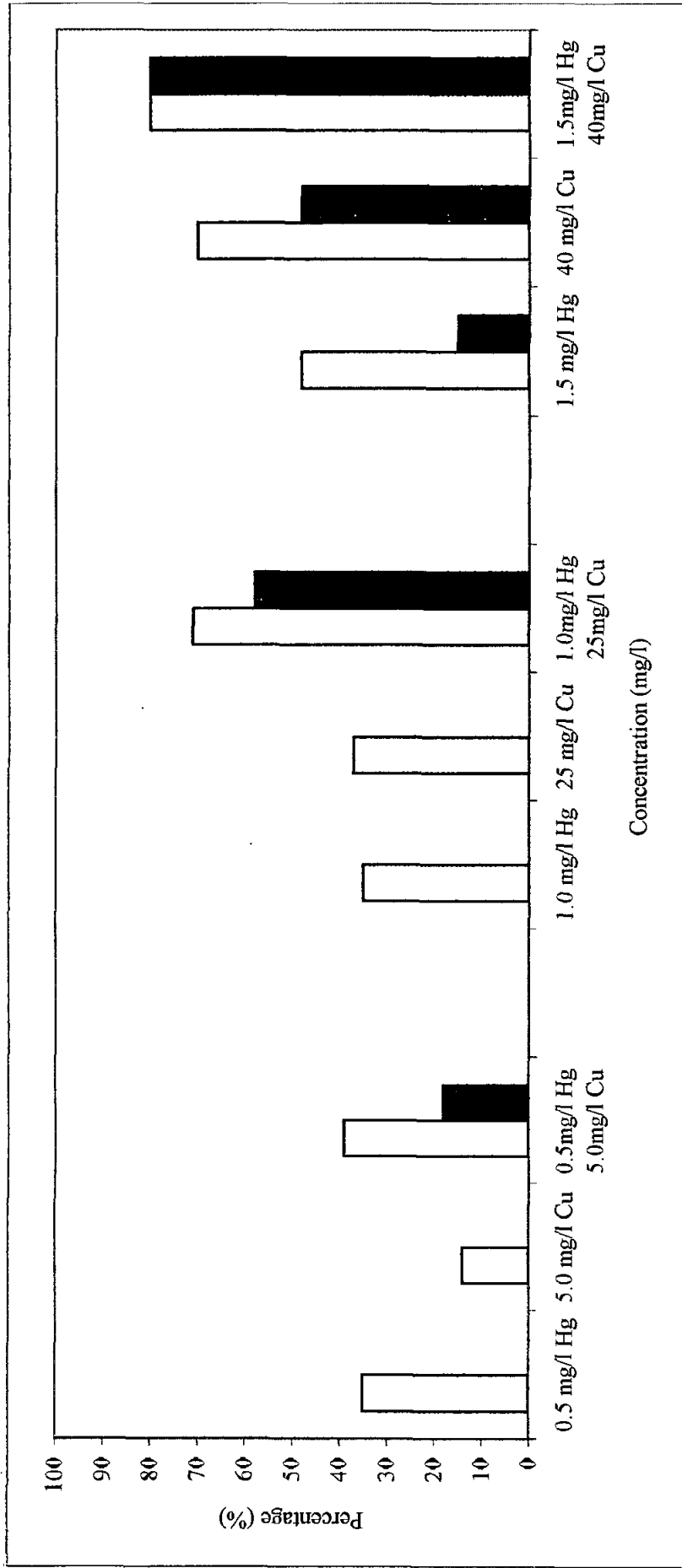
Comparison of cell mortality and hsp70 induction for the mouse connective tissue cell line (L929) to a mixture of cadmium and mercury. Cell mortality (■) was estimated by the neutral red assay. Data points represent the arithmetic mean of 4 replicates. For the heat shock response assay cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp 70 induction (□) was expressed as a percentage of the untreated control level. Data points represent the arithmetic mean of 4 replicates. Mixtures of cadmium and mercury showed a combined toxicity resulting in increased cell mortality. Measurement of hsp70 induction also identified the combined toxicity of the mixture. Measurement of hsp70 induction for both the individual metals and their mixtures showed a greater response than found with measuring cell mortality using the neutral red assay.

Figure 3.4: Comparison of cell mortality and hsp70 induction for the mouse connective tissue cell line (L929) to a mixture of cadmium and copper.



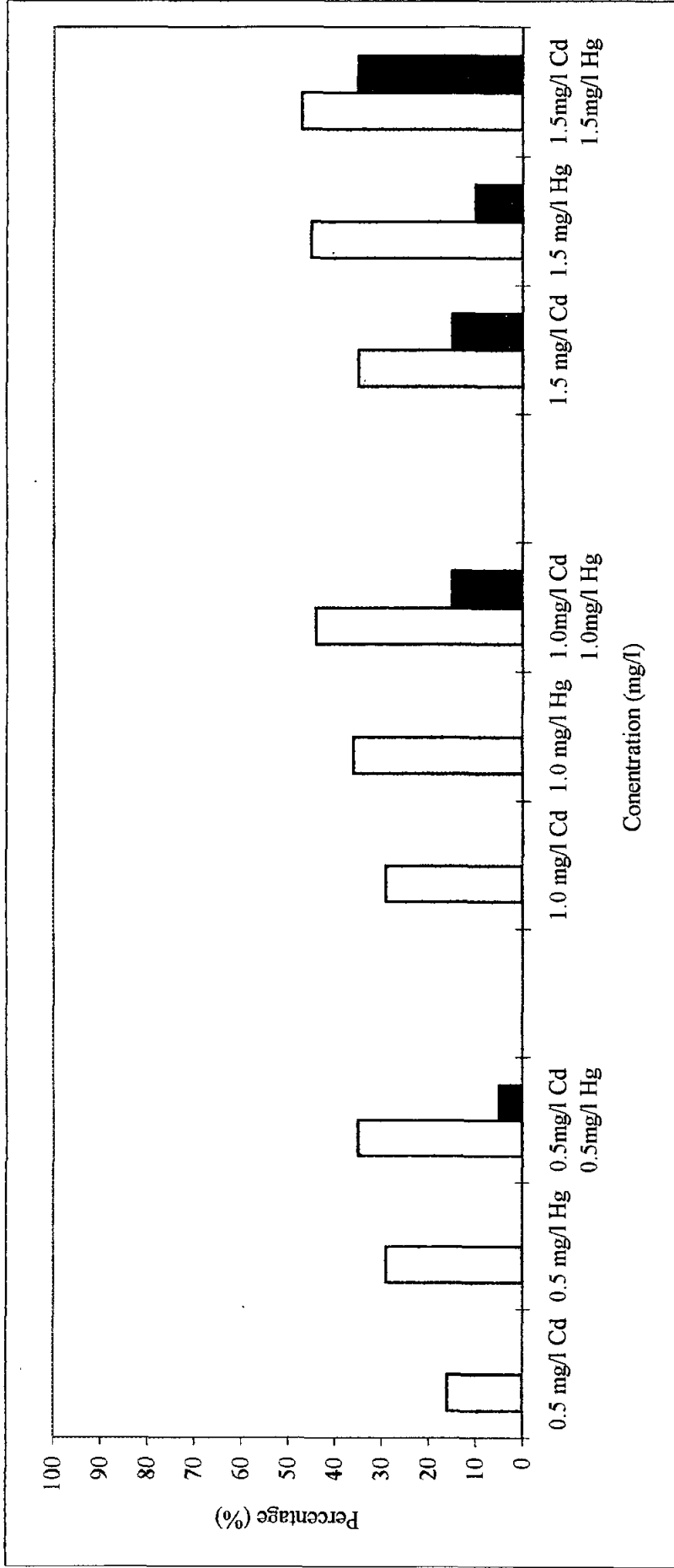
Comparison of cell mortality and hsp70 induction for the mouse connective tissue cell line (L929) to a mixture of cadmium and copper. Cell mortality (■) was estimated by the neutral red assay. Data points represent the arithmetic mean of 4 replicates. For the heat shock response assay cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp70 induction (□) was expressed as a percentage of the untreated control level. Data points represent the arithmetic mean of 4 replicates. Mixtures of cadmium and copper showed a combined toxicity resulting in increased cell mortality. Measurement of hsp70 induction also identified the combined toxicity of the mixture. Measurement of hsp70 induction for both the individual metals and their mixtures showed a greater response than found with measuring cell mortality using the neutral red assay.

Figure 35: Comparison of cell mortality and hsp70 induction for the mouse connective tissue cell line (L929) to a mixture of mercury and copper.



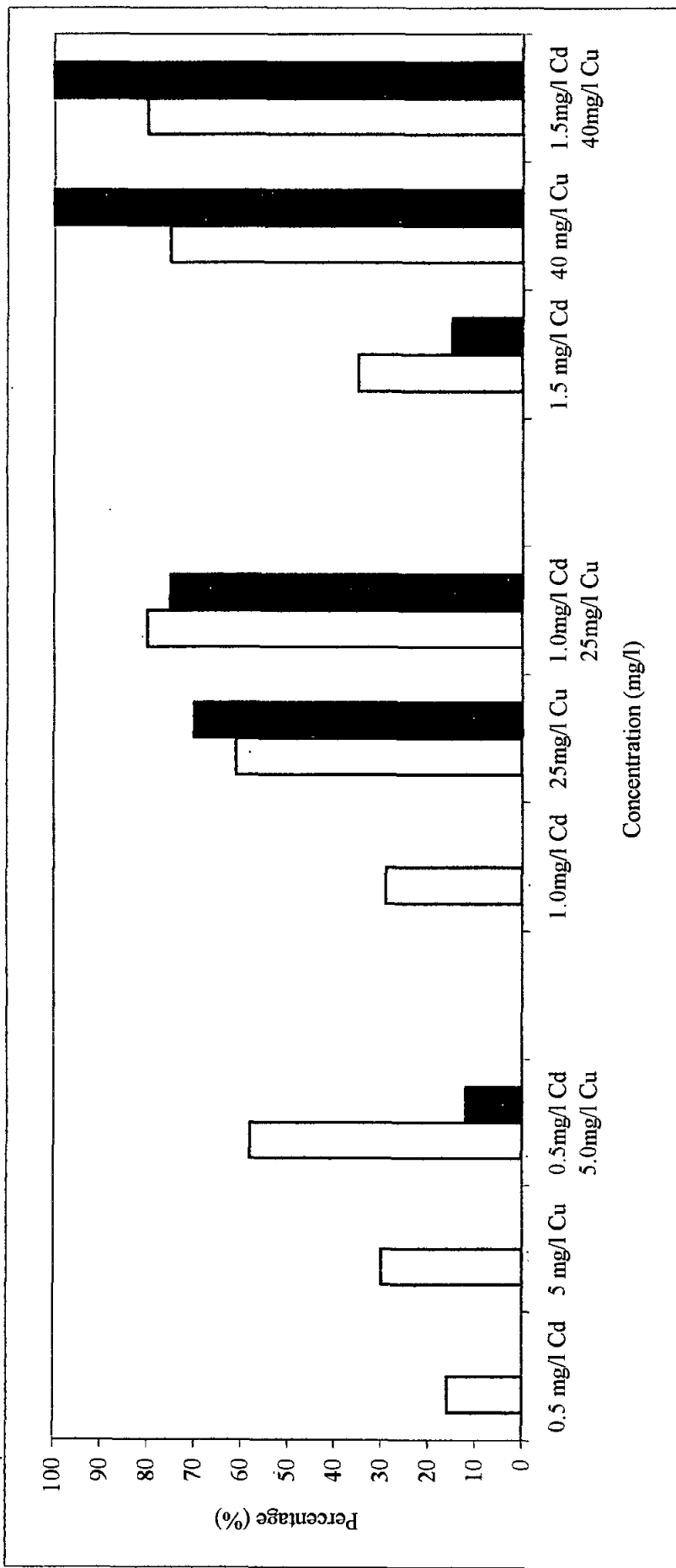
Comparison of cell mortality and hsp70 induction for the mouse connective tissue cell line (L929) to a mixture of mercury and copper. Cell mortality (■) was estimated by the neutral red assay. Data points represent the arithmetic mean of 4 replicates. For the heat shock response assay cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp70 induction (□) was expressed as a percentage of the untreated control level. Data points represent the arithmetic mean of 4 replicates. Mixtures of mercury and copper showed a combined toxicity resulting in increased cell mortality. Measurement of hsp70 induction also identified the combined toxicity of the mixture. Measurement of hsp70 induction for both the individual metals and their mixtures showed a greater response than found with measuring cell mortality using the neutral red assay.

Figure 36: Comparison of cell mortality and hsp70 induction for the normal rat kidney cell line (NRK) to a mixture of cadmium and mercury.



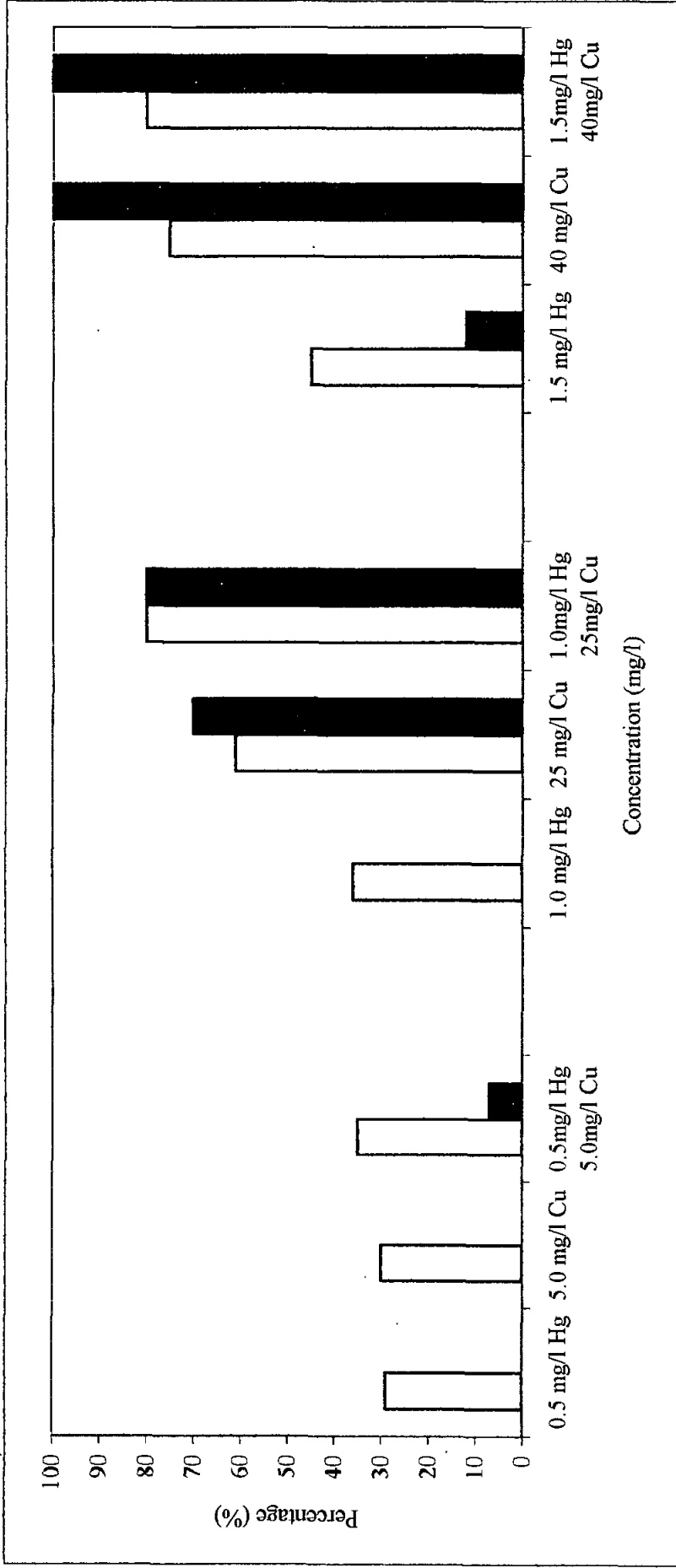
Comparison of cell mortality and hsp70 induction for the normal rat kidney cell line (NRK) to a mixture of cadmium and mercury. Cell mortality (■) was estimated by the neutral red assay. Data points represent the arithmetic mean of 4 replicates. For the heat shock response assay cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp70 induction (□) was expressed as a percentage of the untreated control level. Data points represent the arithmetic mean of 4 replicates. Mixtures of cadmium and mercury showed a combined toxicity resulting in increased cell mortality. Measurement of hsp70 induction also identified the combined toxicity of the mixture. Measurement of hsp70 induction for both the individual metals and their mixtures showed a greater response than found with measuring cell mortality using the neutral red assay.

Figure 37: Comparison of cell mortality and hsp70 induction for the normal rat kidney cell line (NRK) to a mixture of cadmium and copper.



Comparison of cell mortality and hsp70 induction for the normal rat kidney cell line (NRK) to a mixture of cadmium and copper. Cell mortality (■) was estimated by the neutral red assay. Data points represent the arithmetic mean of 4 replicates. For the heat shock response assay cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp70 induction (□) was expressed as a percentage of the untreated control level. Data points represent the arithmetic mean of 4 replicates. Mixtures of cadmium and copper showed a combined toxicity resulting in increased cell mortality. Measurement of hsp70 induction also identified the combined toxicity of the mixture. Measurement of hsp70 induction for both the individual metals and their mixtures showed a greater response than found with measuring cell mortality using the neutral red assay.

Figure 38: Comparison of cell mortality and hsp70 induction for the normal rat kidney cell line (NRK) to a mixture of mercury and copper.



Comparison of cell mortality and hsp70 induction for the normal rat kidney cell line (NRK) to a mixture of mercury and copper. Cell mortality (■) was estimated by the neutral red assay. Data points represent the arithmetic mean of 4 replicates. For the heat shock response assay cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp70 induction (□) was expressed as a percentage of the untreated control level. Data points represent the arithmetic mean of 4 replicates. Mixtures of mercury and copper showed a combined toxicity resulting in increased cell mortality. Measurement of hsp70 induction also identified the combined toxicity of the mixture. Measurement of hsp70 induction for both the individual metals and their mixtures showed a greater response than found with measuring cell mortality using the neutral red assay.

Table 14: Cell mortality as assessed by the neutral red assay for the L929 and NRK cell lines using mixtures of cadmium and mercury

Toxin Mixture	L929	NRK
0.5mg/l Cadmium & Mercury	27%	5%
1.0mg/l Cadmium & Mercury	39%	15%
1.5mg/l Cadmium & Mercury	58%	35%

Table 15: Cell mortality as assessed by the neutral red assay for the L929 and NRK cell lines using mixtures of cadmium and copper

Toxin Mixture	L929	NRK
0.5mg/l Cadmium & 5mg/l Copper	15%	12%
1mg/l Cadmium & 25mg/l Copper	37%	75%
1.5mg/l Cadmium & 40mg/l Copper	70%	100%

Table 16: Cell mortality as assessed by the neutral red assay for the L929 and NRK cell lines using mixtures of mercury and copper

Toxin Mixture	L929	NRK
0.5mg/l Mercury & 5mg/l Copper	18%	7%
1mg/l Mercury & 25mg/l Copper	58%	80%
1.5mg/l Mercury & 40mg/l Copper	80%	100%

Table 17: Hsp70 induction for the L929 and NRK cell lines using mixtures of cadmium and mercury.

Toxin Mixture	L929	NRK
0.5mg/l Cadmium & Mercury	39%	35%
1.0mg/l Cadmium & Mercury	62%	44%
1.5mg/l Cadmium & Mercury	76%	47%

Table 18: Hsp70 induction for the L929 and NRK cell lines using mixtures of cadmium and copper.

Toxin Mixture	L929	NRK
0.5mg/l Cadmium & 5mg/l Copper	35%	58%
1mg/l Cadmium & 25mg/l Copper	71%	80%
1.5mg/l Cadmium & 40mg/l Copper	80%	80%

Table 19: Hsp70 induction for the L929 and NRK cell lines using mixtures of mercury and copper.

Toxin Mixture	L929	NRK
0.5mg/l Mercury & 5mg/l Copper	39%	35%
1mg/l Mercury & 25mg/l Copper	71%	80%
1.5mg/l Mercury & 40mg/l Copper	80%	80%

3.7 ELISA:

For the two cell lines using the three toxins similar dose dependent increases in hsp70 induction were observed with the ELISA technique using the C92F3A-5 antibody as found when assayed by 1-D electrophoresis (Tables 20-22; Figures 39-44). At the median lethal concentration hsp70 induction had an increased response over that found using the neutral red assay of 25% for both cadmium and mercury and 18% for copper. At the no effect level the heat shock response assay identified hsp70 induction at concentrations that were 1.5, 1.6 and 3 times lower for the L929 cell line and 1.5, 1.7 and 2 times lower for the NRK cell line than the median lethal concentration for mercury, cadmium and copper respectively. At the lowest observed effective concentration the heat shock response assay identified hsp70 induction at concentrations that were 2, 3 and 5 times lower than the no effect level for mercury, cadmium and copper respectively. For the NRK cell line the heat shock response assay identified hsp70 induction at concentrations that were 2 times lower than the no effect level for copper and 3 times lower for cadmium and mercury.

Table 20: Hsp70 induction at the LC₅₀ value for the L929 and NRK cell lines as assayed by the ELISA technique.

Toxin	L929	NRK
Cadmium	67%	69%
Mercury	66%	50%
Copper	61%	59%

Table 21: Hsp70 induction at the no effect level for L929 and NRK cell lines as assayed by the ELISA technique.

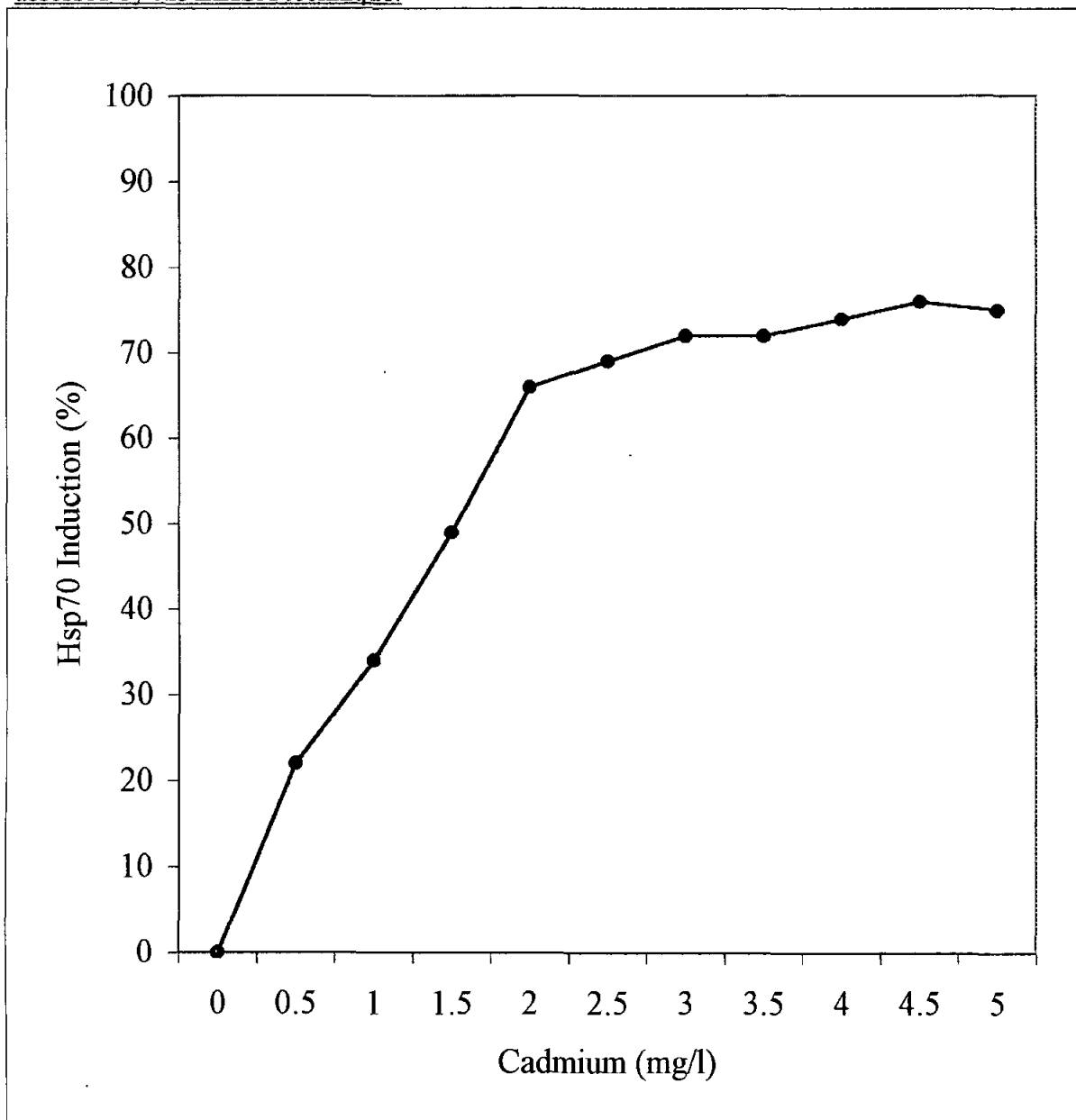
Toxin	L929	NRK
Cadmium	49%	35%
Mercury	28%	28%
Copper	35%	39%

Table 22: Hsp70 induction at the lowest observed effective concentration for the L929 and NRK cell lines as assayed by the ELISA technique.

Toxin	L929	NRK
Cadmium (0.5mg/l)	22%	10%
Mercury (0.5mg/l)	24%	15%
Copper (5.0mg/l)	15%	23%

Figure 39:

Heat shock response curve for the mouse connective tissue cell line (L929) to cadmium as assessed by the ELISA technique.

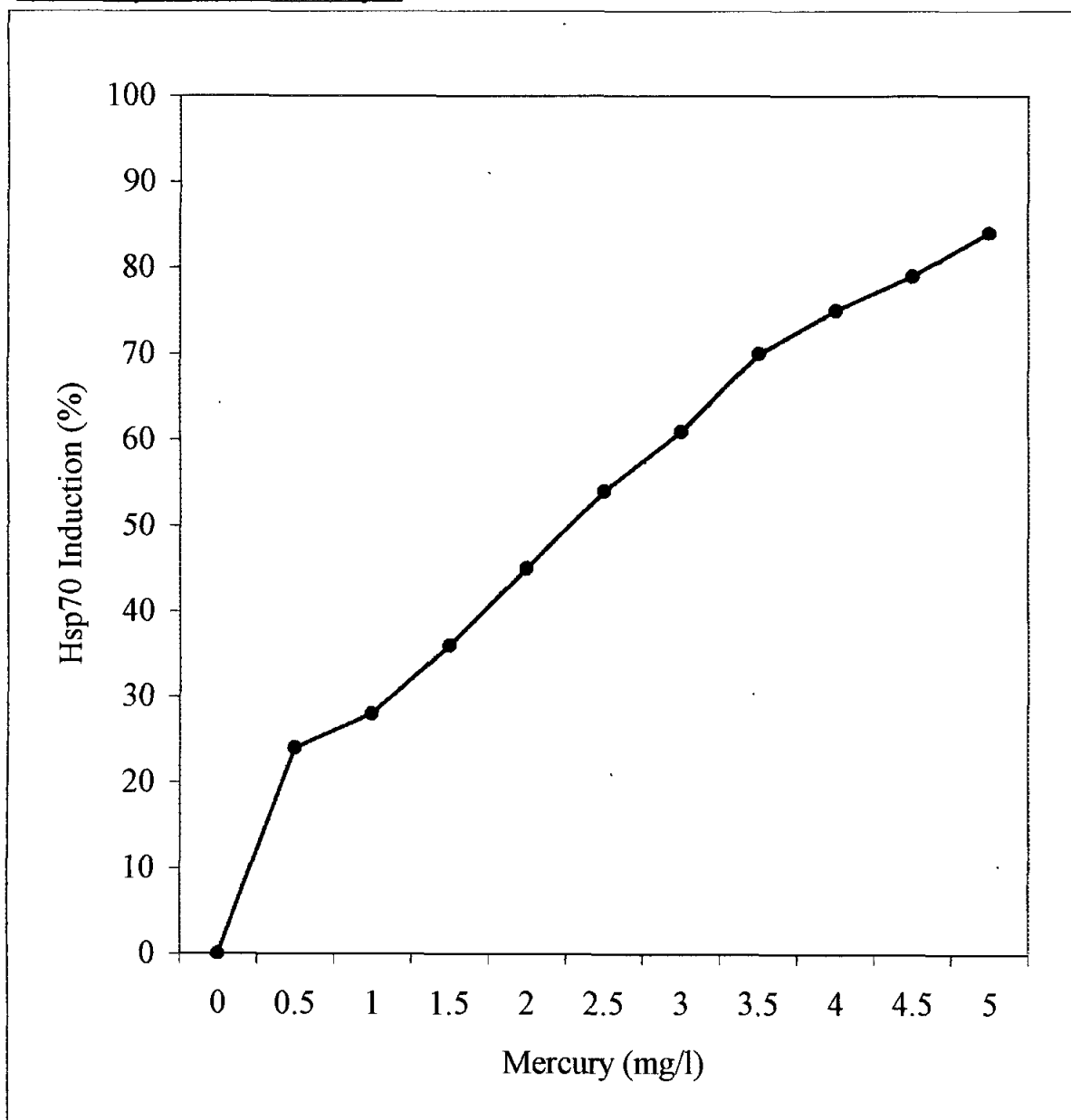


Heat shock response curve for the mouse connective tissue cell line (L929) to cadmium as assessed by the ELISA technique.

96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100µl per well of cadmium amended medium (0-5mg/l Cd) for 24 hours at 37°C. Cadmium concentrations never exceeded 10% of the media volume. Cell lysates were bound to 96 well ELISA plates. Cell lysates were probed with a mouse anti-hsp70 monoclonal antibody (clone C92F3A-5) and hsp70 detected by an alkaline phosphatase secondary antibody conjugate. Hsp70 induction (●) was expressed as a percentage of the untreated control level. The heat shock response curve shows a dose dependent increase in hsp70 induction. Hsp70 induction showed a similar response as found with 1-D electrophoresis. Measurement of hsp70 induction showed a greater response than found with measuring cell mortality using the neutral red assay.

Figure 40:

Heat shock response curve for the mouse connective tissue cell line (L929) to mercury as assessed by the ELISA technique.

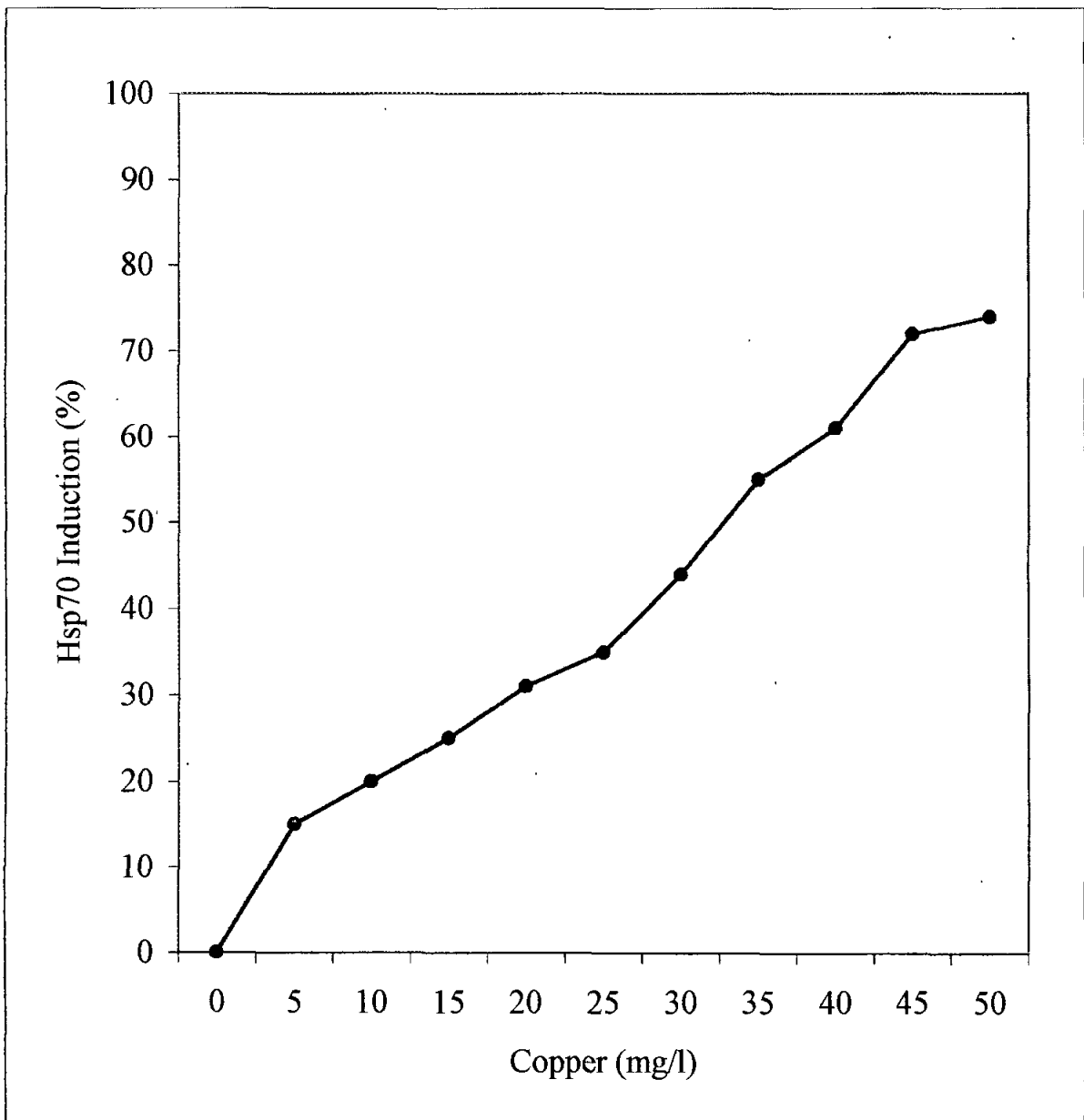


Heat shock response curve for the mouse connective tissue cell line (L929) to mercury as assessed by the ELISA technique.

96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100µl per well of mercury amended medium (0-5mg/l Hg) for 24 hours at 37°C. Mercury concentrations never exceeded 10% of the media volume. Cell lysates were bound to 96 well ELISA plates. Cell lysates were probed with a mouse anti-hsp70 monoclonal antibody (clone C92F3A-5) and hsp70 detected by an alkaline phosphatase secondary antibody conjugate. Hsp70 induction (●) was expressed as a percentage of the untreated control level. The heat shock response curve shows a dose dependent increase in hsp70 induction. Hsp70 induction showed a similar response as found with 1-D electrophoresis. Measurement of hsp70 induction showed a greater response than found with measuring cell mortality using the neutral red assay.

Figure 41:

Heat shock response curve for the mouse connective tissue cell line (L929) to copper as assessed by the ELISA technique.

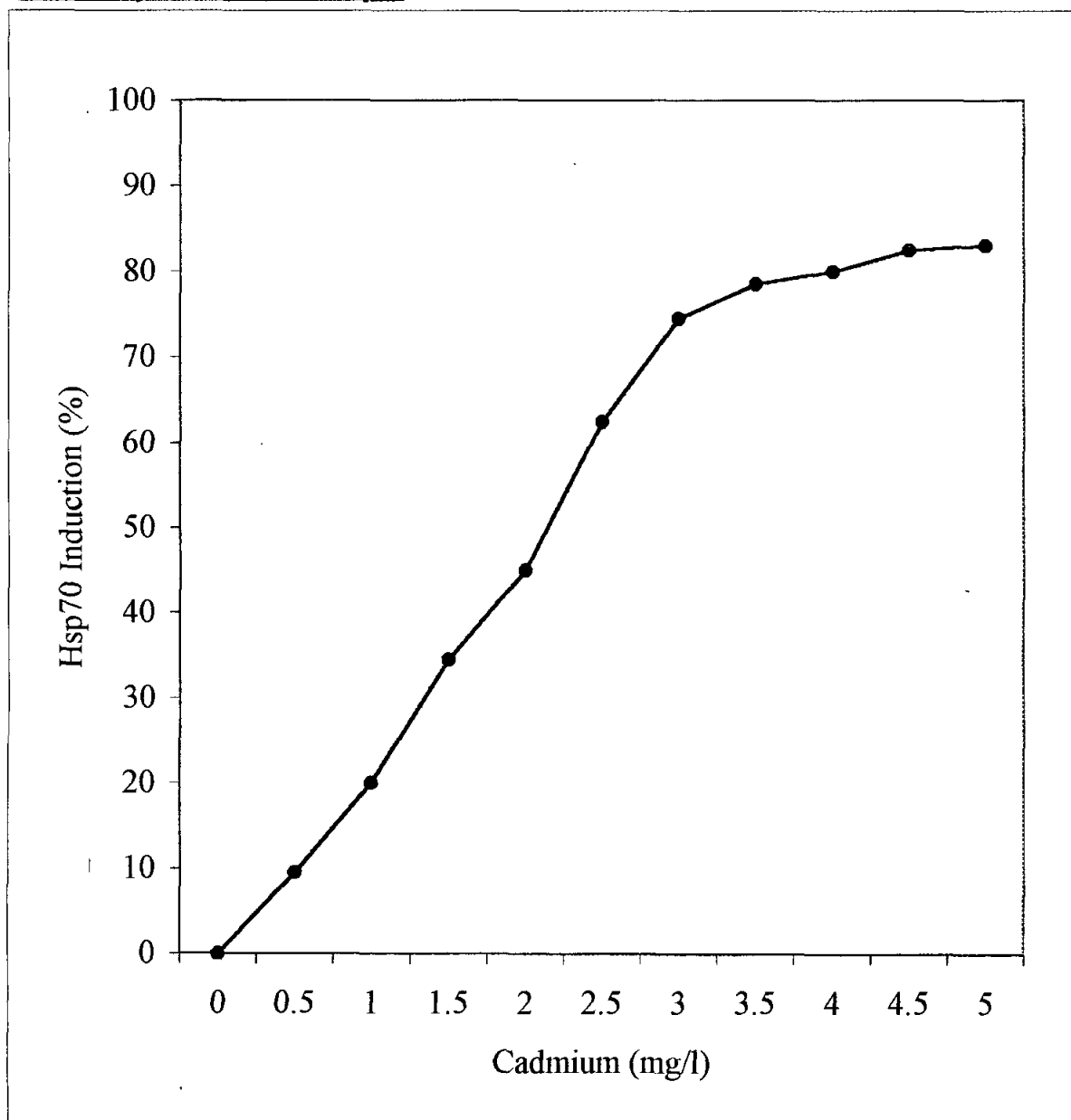


Heat shock response curve for the mouse connective tissue cell line (L929) to copper as assessed by the ELISA technique.

96 well plates were seeded with 4×10^4 cell's per ml for 24 hours at 37°C. The attached cells were incubated with 100µl's per well of copper ammended medium (0-50mg/l Cu) for 24 hours at 37°C. Copper concentrations never exceeded 10% of the media volume. Cell lysates were bound to 96 well ELISA plates. Cell lysates were probed with a mouse anti-hsp70 monoclonal antibody (clone C92F3A-5) and hsp70 detected by an alkaline phosphatase secondary antibody conjugate. Hsp70 induction (●) was expressed as a percentage of the untreated control level. The heat shock response curve shows a dose dependent increase in hsp70 induction. Hsp70 induction showed a similar response as found with 1-D electrophoresis. Measurement of hsp70 induction showed a greater response than found with measuring cell mortality using the neutral red assay.

Figure 42:

Heat shock response curve for the normal rat kidney cell line (NRK) to cadmium as assessed by the ELISA technique.

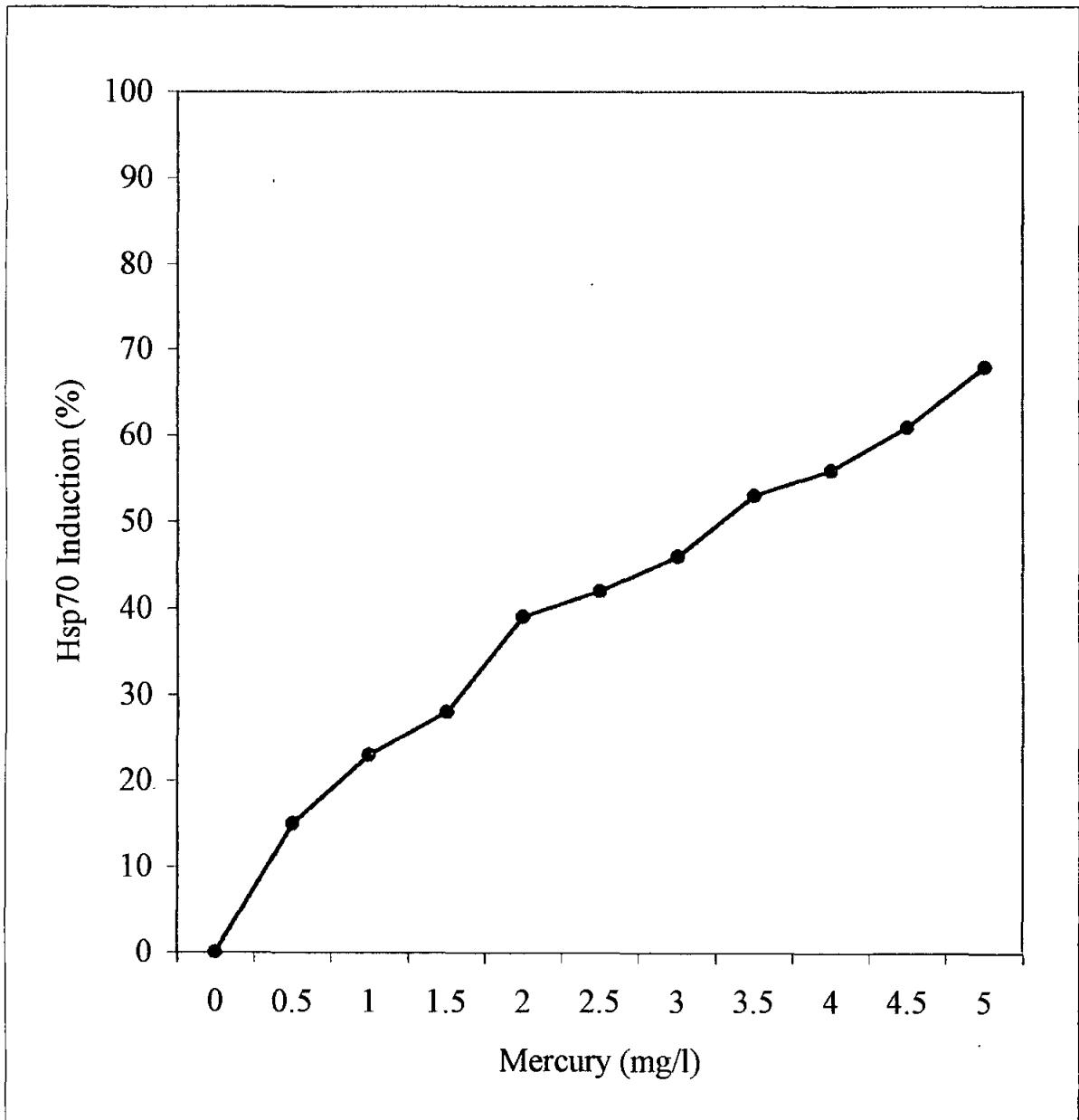


Heat shock response curve for the normal rat kidney cell line (NRK) to cadmium as assessed by the ELISA technique.

96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100µl per well of cadmium amended medium (0-5mg/l Cd) for 24 hours at 37°C. Cadmium concentrations never exceeded 10% of the media volume. Cell lysates were bound to 96 well ELISA plates. Cell lysates were probed with a mouse anti-hsp70 monoclonal antibody (clone C92F3A-5) and hsp70 detected by an alkaline phosphatase secondary antibody conjugate. Hsp70 induction (●) was expressed as a percentage of the untreated control level. The heat shock response curve shows a dose dependent increase in hsp70 induction. Hsp70 induction showed a similar response as found with 1-D electrophoresis. Measurement of hsp70 induction showed a greater response than found with measuring cell mortality using the neutral red assay.

Figure 43:

Heat shock response curve for the normal rat kidney cell line (NRK) to mercury as assessed by the ELISA technique.

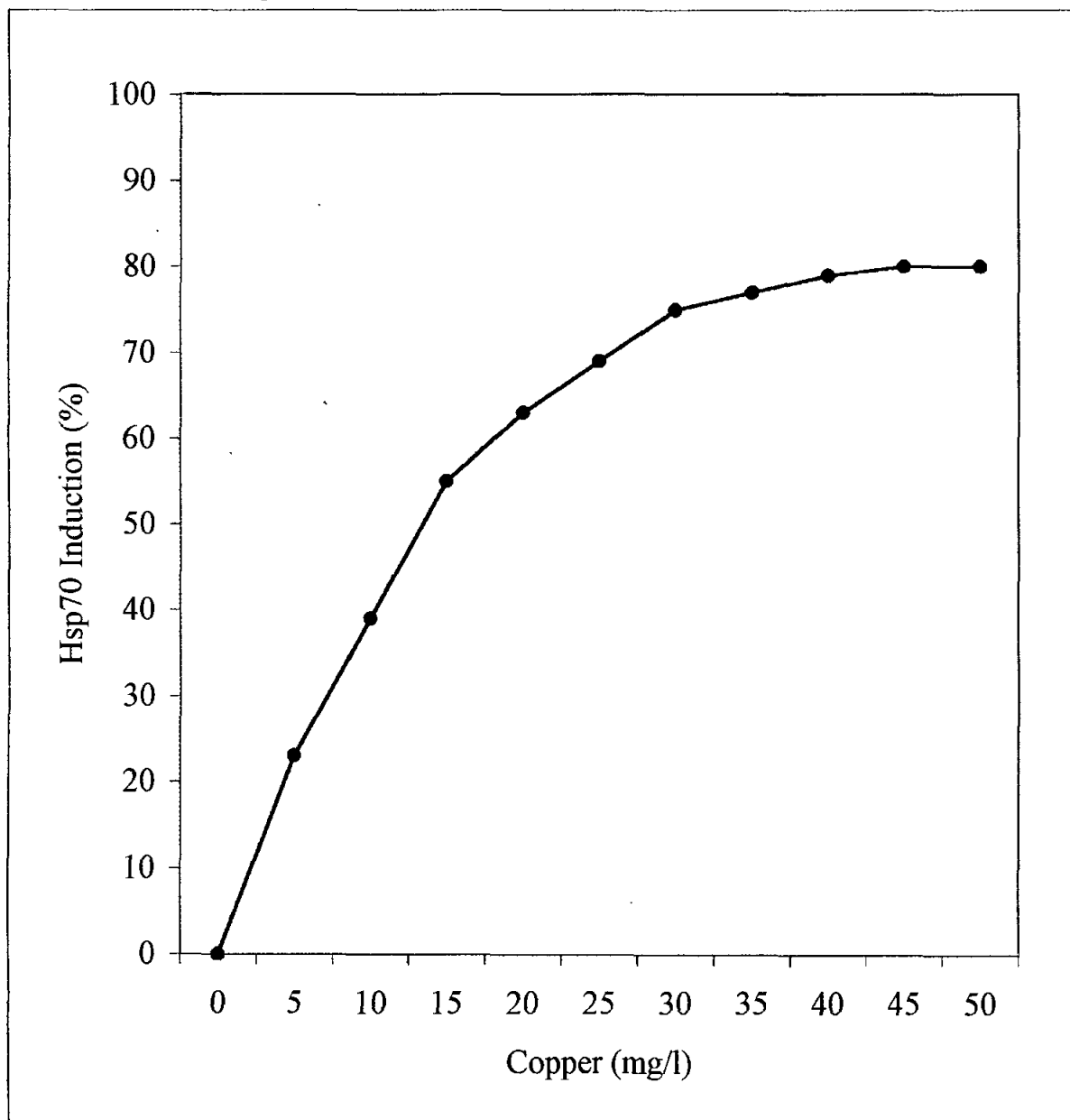


Heat shock response curve for the normal rat kidney cell line (NRK) to mercury as assessed by the ELISA technique.

96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100µl per well of mercury amended medium (0-5mg/l Hg) for 24 hours at 37°C. Mercury concentrations never exceeded 10% of the media volume. Cell lysates were bound to 96 well ELISA plates. Cell lysates were probed with a mouse anti-hsp70 monoclonal antibody (clone C92F3A-5) and hsp70 detected by an alkaline phosphatase secondary antibody conjugate. Hsp70 induction (●) was expressed as a percentage of the untreated control level. The heat shock response curve shows a dose dependent increase in hsp70 induction. Hsp70 induction showed a similar response as found with 1-D electrophoresis. Measurement of hsp70 induction showed a greater response than found with measuring cell mortality using the neutral red assay.

Figure 44:

Heat shock response curve for the normal rat kidney cell line (NRK) to copper as assessed by the ELISA technique.



Heat shock response curve for the normal rat kidney cell line (NRK) to copper as assessed by the ELISA technique.

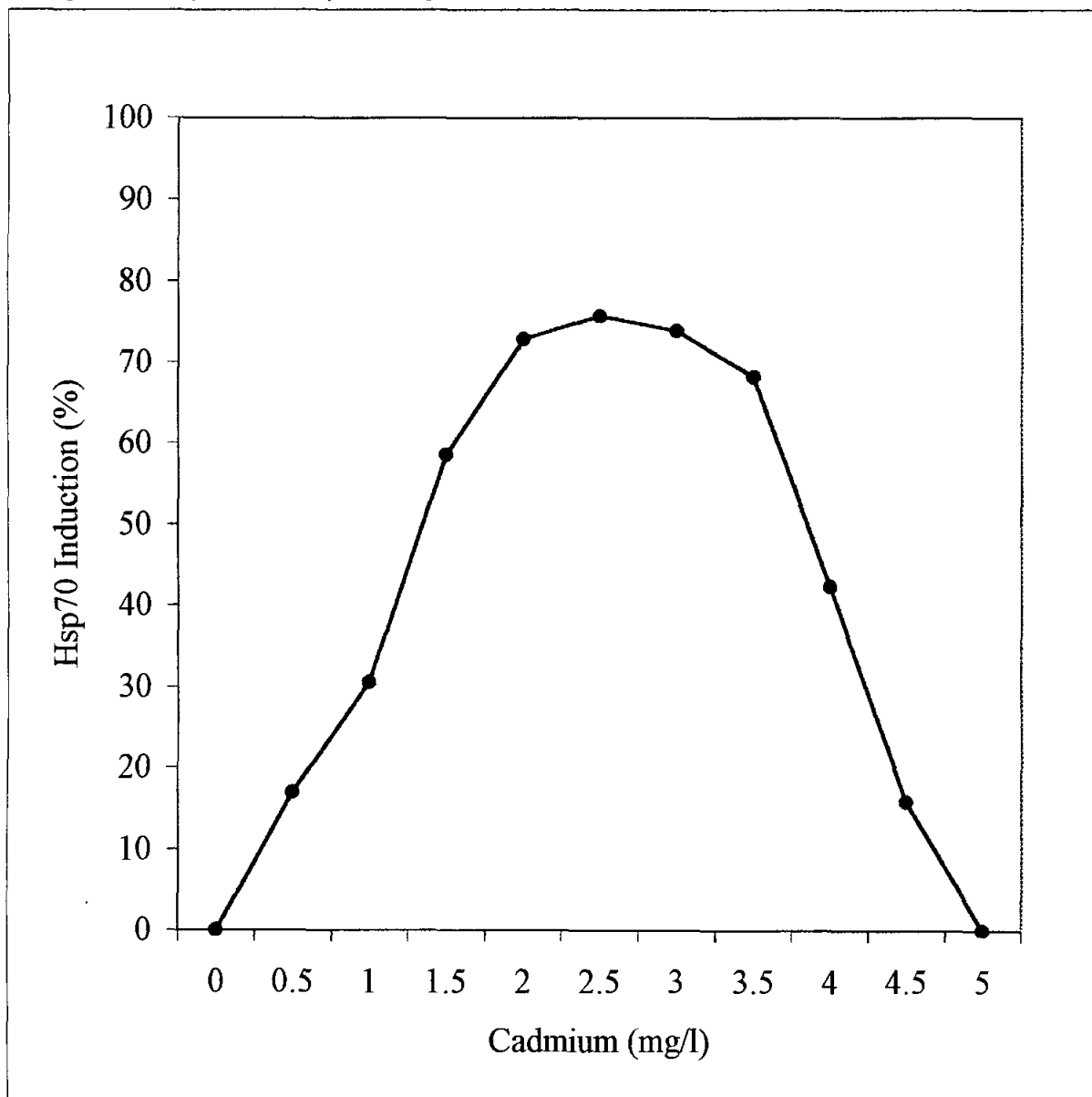
96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100µl per well of copper amended medium (0-50mg/l Cu) for 24 hours at 37°C. Copper concentrations never exceeded 10% of the media volume. Cell lysates were bound to 96 well ELISA plates. Cell lysates were probed with a mouse anti-hsp70 monoclonal antibody (clone C92F3A-5) and hsp70 detected by an alkaline phosphatase secondary antibody conjugate. Hsp70 induction (●) was expressed as a percentage of the untreated control level. The heat shock response curve shows a dose dependent increase in hsp70 induction. Hsp70 induction showed a similar response as found with 1-D electrophoresis. Measurement of hsp70 induction showed a greater response than found with measuring cell mortality using the neutral red assay.

3.8 Immunocytochemistry:

For the L929 cell line using cadmium a similar dose dependent increase in hsp70 induction as determined using Clone C29F3A-5 monoclonal antibody was observed as for both the electrophoresis and ELISA assays. At concentrations above the LC₅₀ there was a rapid decrease in hsp70 accumulation (Figure 45). At the no effect level and lowest observed effective concentration considerable hsp70 induction was observed. In control cells under normal conditions hsp70 was localised predominantly within the cytoplasm with low levels found in the nucleus (Figure 46). With increasing toxin concentration hsp70 was observed to translocate to the nucleus. At intermediate toxin concentrations a heterogeneous heat shock response was observed with some cells displaying a much stronger heat shock response than others (Figure 47). At concentrations above the LC₅₀ where few cells were alive a very strong heat shock response was observed with all cells inducing hsp70 throughout the cell (Figure 48).

Figure 45:

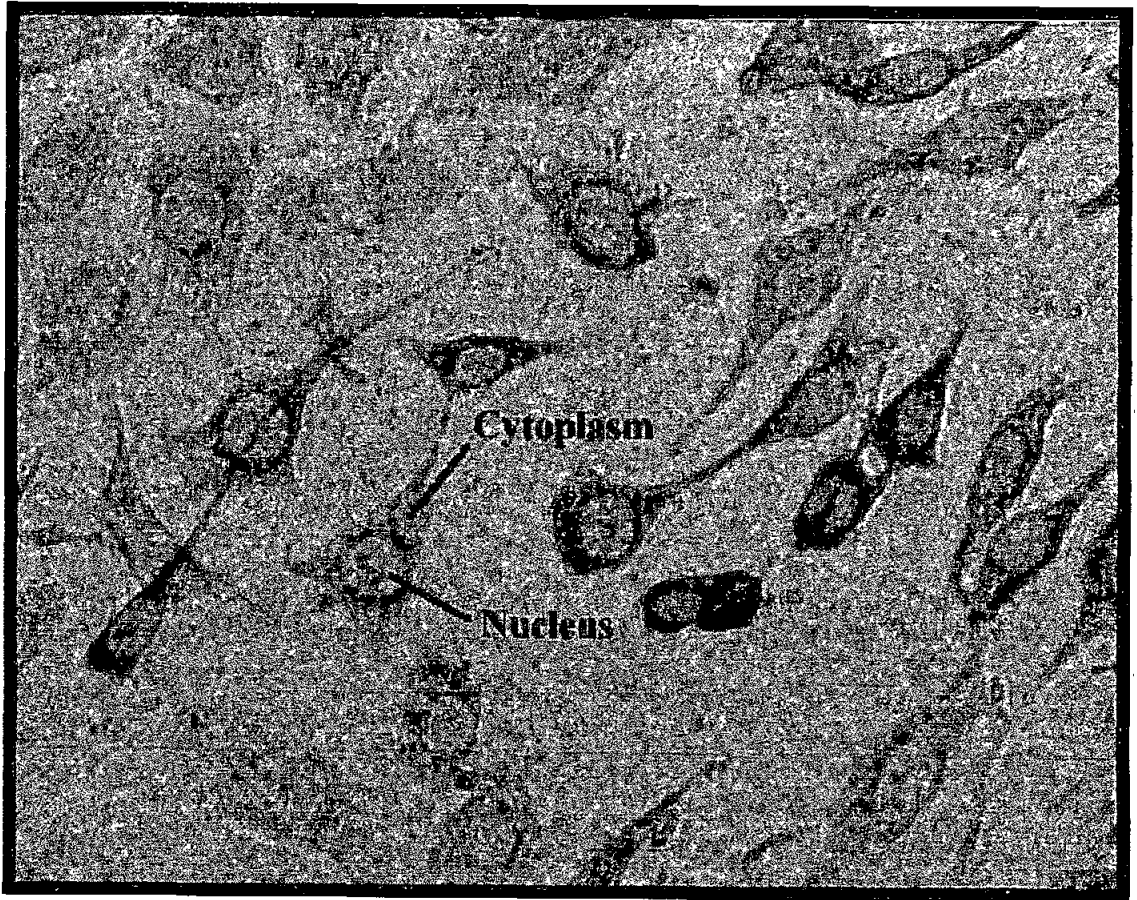
Heat shock response curve for the mouse connective tissue cell line (L929) to cadmium using immunocytochemistry technique.



Heat shock response curve for the mouse connective tissue cell line (L929) to cadmium using immunocytochemistry technique. 24 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C . The attached cells were incubated with 1000 μl s per well of cadmium amended medium (0-5mg/l Cd) for 24 hours at 37°C . Cadmium concentrations never exceeded 10% of the media volume. Cells were fixed to the plate surface using an ethanol acetone solution. Cells were probed with a mouse anti-hsp70 monoclonal antibody (clone C92F3A-5) and hsp70 detected by an alkaline phosphatase secondary antibody conjugate. Hsp70 induction (●) was expressed as a percentage of the untreated control level. The Heat shock response curve shows a dose dependent increase in hsp70 induction up to the LC_{50} value. Hsp70 induction begins to decrease above the LC_{50} value due to increased cell mortality. Below the LC_{50} value hsp70 induction increased in a similar fashion as with 1-D electrophoresis and ELISA.

Figure 46:

Heat shock protein 70 distribution in the mouse connective tissue cell line (L929).

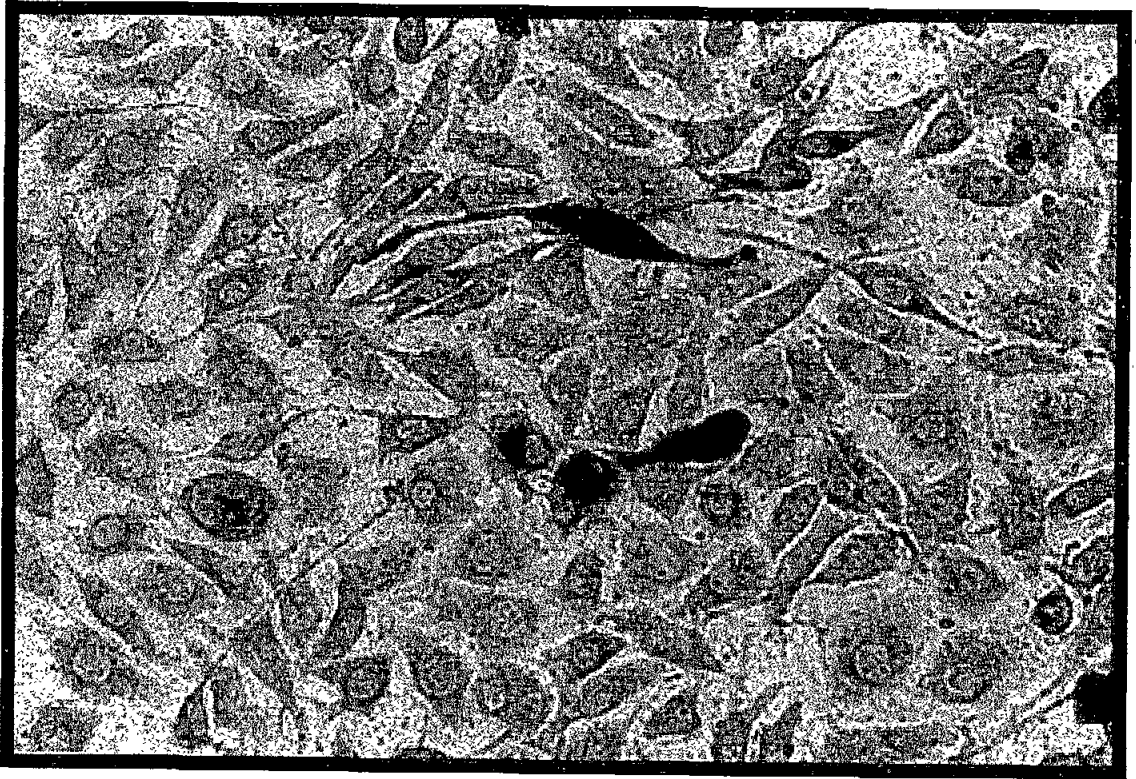


Legend:

Heat shock protein 70 distribution in the mouse connective tissue cell line (L929) as determined by immunocytochemistry. 24 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 1000µl per well of cadmium amended media (0-5mg/l Cd) for 24 hours at 37°C. Cadmium concentrations never exceeded 10% of the media volume. Cells were fixed with an ethanol acetone solution. Cells were probed with a mouse anti-hsp70 monoclonal antibody (clone C29F3A-5) and hsp70 detected by an alkaline phosphatase secondary antibody conjugate. Hsp70 was found predominantly in the cytoplasm of control cells (darker stain). Low levels of hsp70 were found in the nucleus of the control cells (lighter stain).

Figure 47:

Heat shock protein 70 distribution in the mouse connective tissue cell line (L929) exposed to cadmium.

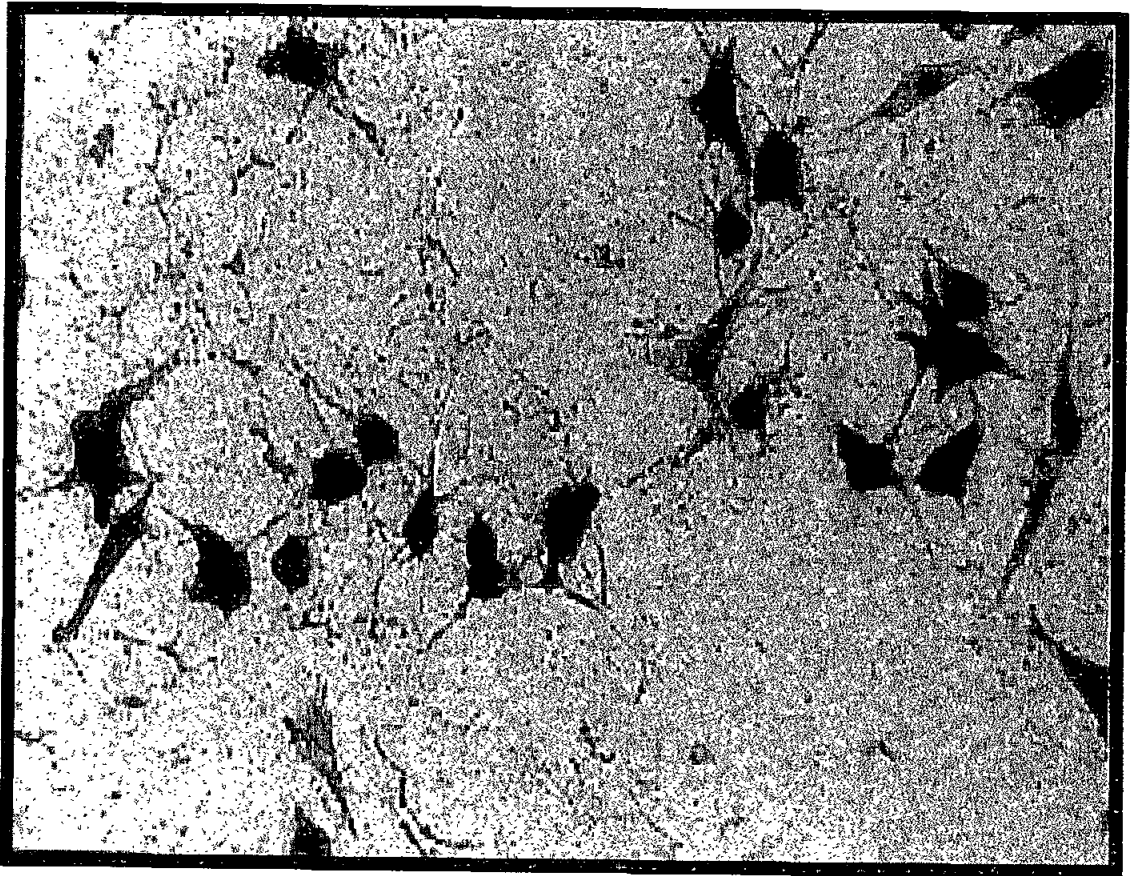


Legend:

Heat shock protein 70 distribution in the mouse connective tissue cell line (L929) as determined by immunocytochemistry. 24 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 1000 μ l per well of cadmium amended media (0-5mg/l Cd) for 24 hours at 37°C. Cadmium concentrations never exceeded 10% of the media volume. Cells were fixed with an ethanol acetone solution. Cells were probed with a mouse anti-hsp70 monoclonal antibody (clone C29F3A-5) and hsp70 detected by an alkaline phosphatase secondary antibody conjugate. Hsp70 was found to translocate from the cytoplasm to the nucleus with increasing concentrations of cadmium. At intermediate cadmium concentrations a heterogeneous stress response was observed with some cells displaying a much stronger induction of hsp70 than others (darker stained cells).

Figure 48:

Heat shock protein 70 distribution in the mouse connective tissue cell line (L929) exposed to lethal concentrations of cadmium.



Legend:

Heat shock protein 70 distribution in the mouse connective tissue cell line (L929) as determined by immunocytochemistry. 24 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C . The attached cells were incubated with $1000\mu\text{l}$ per well of cadmium amended media ($0\text{-}5\text{mg/l}$ Cd) for 24 hours at 37°C . Cadmium concentrations never exceeded 10% of the media volume. Cells were fixed with an ethanol acetone solution. Cells were probed with a mouse anti-hsp70 monoclonal antibody (clone C29F3A-5) and hsp70 detected by an alkaline phosphatase secondary antibody conjugate. Hsp70 was found to translocate from the cytoplasm to the nucleus with increasing concentrations of cadmium. At concentration above the LC_{50} the cells that were still alive possessed a very strong stress response with Hsp70 induction found throughout the cell structure (dark stain).

3.9 2-Isobutyl piperidine:

3.9.1 Toxicity Data:

Toxicity data was established for the mouse connective tissue cell line (L929). Dose response curves were constructed using cell mortality versus toxin concentration. From these dose response curves the traditional measures of toxicity the median lethal concentration value and the no effect level were estimated. For the L929 cell line the dose response curve for 2-Isobutyl piperidine showed a typical sigmoidal shape with toxicity increasing in a dose dependent fashion (Figure 49). The median lethal concentration (LC_{50}) as well as the lethal concentrations at 20% (LC_{20}) and 80% (LC_{80}) cell mortality are presented in Table 23. The no effect level of 2-Isobutyl piperidine to the L929 cell line was estimated to be 0.25%.

Table 23. Lethal concentration values of 2-Isobutyl piperidine to the L929 cell line.

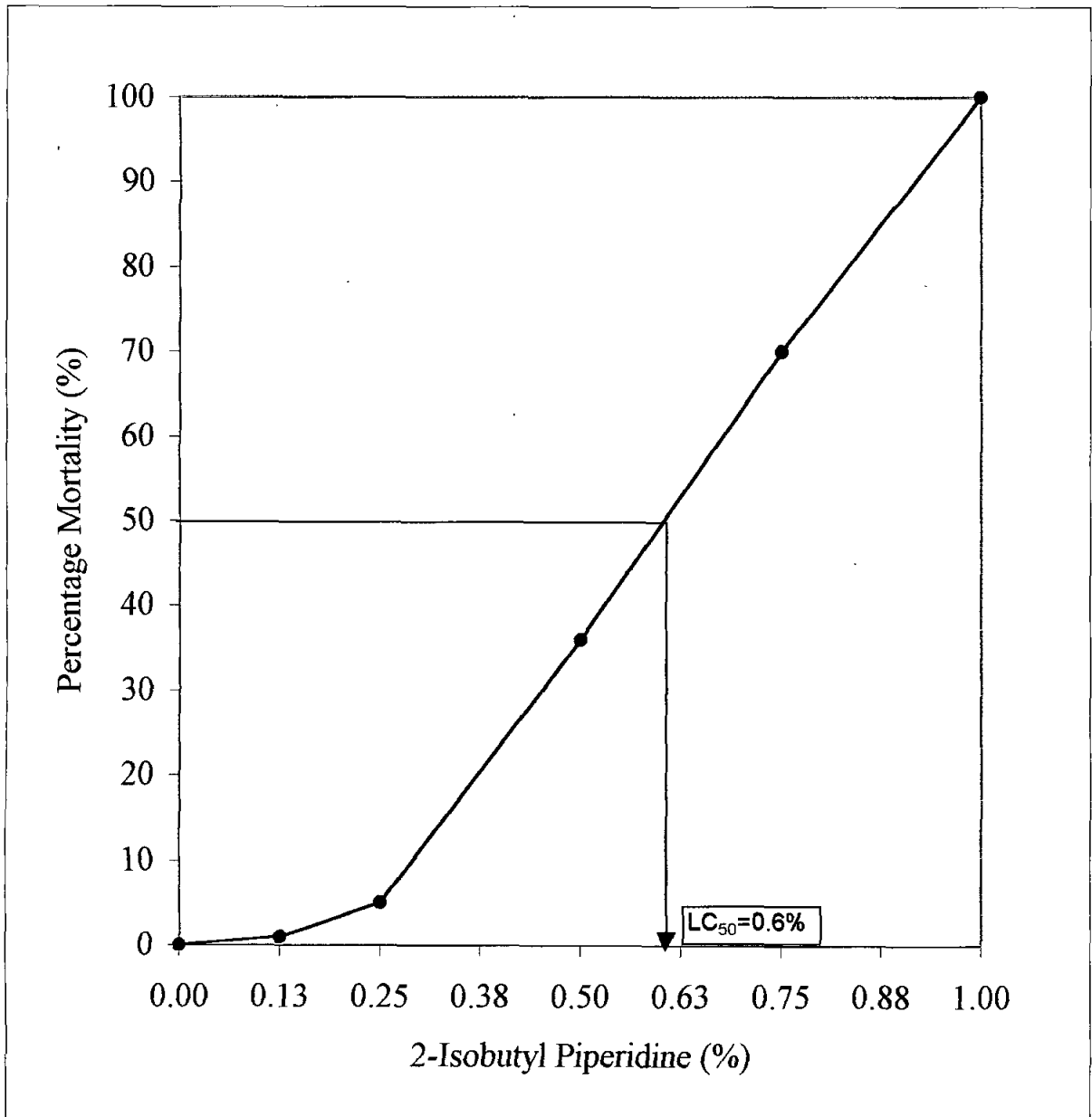
Toxin	LC_{20}	LC_{50}	LC_{80}	Slope
2-Isobutyl piperidine	0.37 %	0.6 %	0.84 %	0.0078

3.9.2 Heat shock response data:

Under identical test conditions, as the neutral red assay, cells were exposed to 2-Isobutyl piperidine at concentrations ranging from sub-lethal to lethal. Using this concentration range allowed the total profile of heat shock protein induction to be examined on a single gel.

Figure 49:

Dose response curve for the mouse connective tissue cell line (L929) to 2-Isobutyl piperidine.



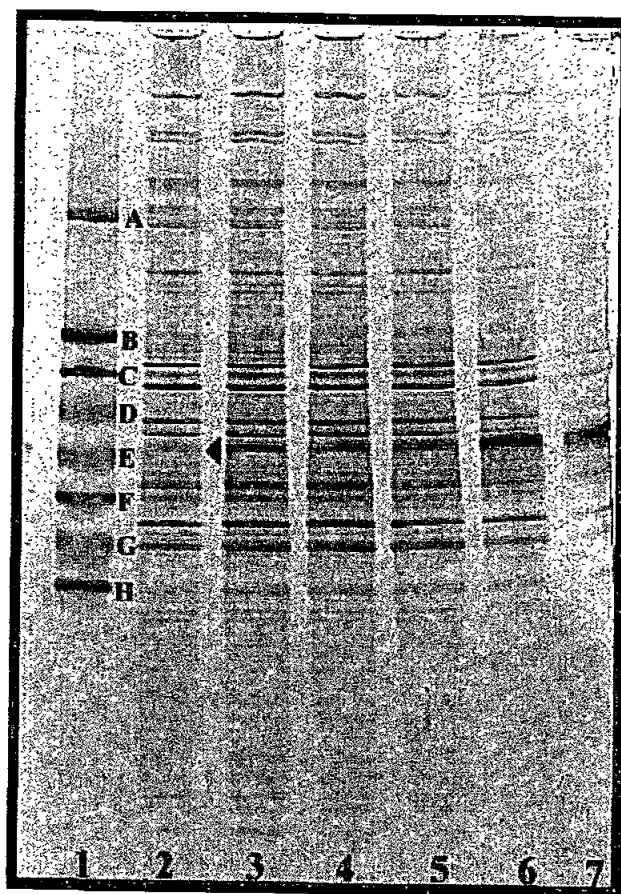
Dose response curve for the mouse connective tissue cell line (L929) to 2-Isobutyl piperidine. 96 well plates were seeded with 4×10^4 cell's per ml for 24 hours at 37°C. The attached cells were incubated with 100µls per well of 2-Isobutyl piperidine ammended medium (0-1% 2-Isobutyl piperidine dissolved 1:4 in 1% acetone) for 24 hours at 37°C. 2-Isobutyl piperidine concentrations never exceeded 10% of the media volume. A solution of 1% acetone was used as a solvent control. Cell mortality (●) was estimated by the neutral red assay. Neutral red optical density readings were expressed as a percentage of the untreated control. The dose response curve for 2-Isobutyl piperidine shows a typical sigmoidal shape with toxicity increasing in a dose dependent fashion. The median lethal concentration was estimated by regression of the linear portion of the curve to be 0.6%. The no effect level was estimated to be 0.25%.

Hsp70 levels increased in a dose dependent fashion, above the LC₅₀ this increase began to plateau. Even with a reduced rate of induction hsp70 becomes the dominant protein above the LC₅₀ concentration. For the L929 cell line at the LC₅₀ value hsp70 represented 16.5% of the total cellular protein content for 2-Isobutyl piperidine. Repression of normal protein synthesis was also observed. Normal protein synthesis was repressed in a dose dependent manner, below the LC₅₀ repression was less severe becoming more pronounced with increasing toxin concentration.

To allow comparison between the neutral red assay and heat shock protein assay the LC₅₀ was used as a gauge to assess sensitivity. Heat shock protein induction at the LC₅₀ was calculated by regression of the linear portion of the heat shock response curve. For the L929 cell line the heat shock response curve for 2-Isobutyl piperidine showed a dose dependent increase in hsp70 induction (Figures 50 & 51). Hsp70 induction for 2-Isobutyl piperidine at the LC₅₀ was estimated to be 56%. This represents an 11% greater response than found with measuring cell mortality using the neutral red assay. The L929 cell line showed a 40% increase in hsp70 induction at the no effect level. The heat shock response assay identified hsp70 induction at concentrations that were nearly 2½ times lower than the median lethal concentration. Hsp70 induction at the lowest observed effective concentration (0.125% 2-Isobutyl piperidine) was estimated to be 35%, identifying hsp70 induction at concentrations that were 2 times lower than the no effect level. Comparing the toxicity data established using the neutral red assay and the heat shock response assay shows that the heat shock response assay is more sensitive (Figure 52).

Figure 50:

One-dimensional SDS-PAGE separation of mouse connective tissue cell line (L929) exposed to increasing concentrations of 2-Isobutyl piperidine.

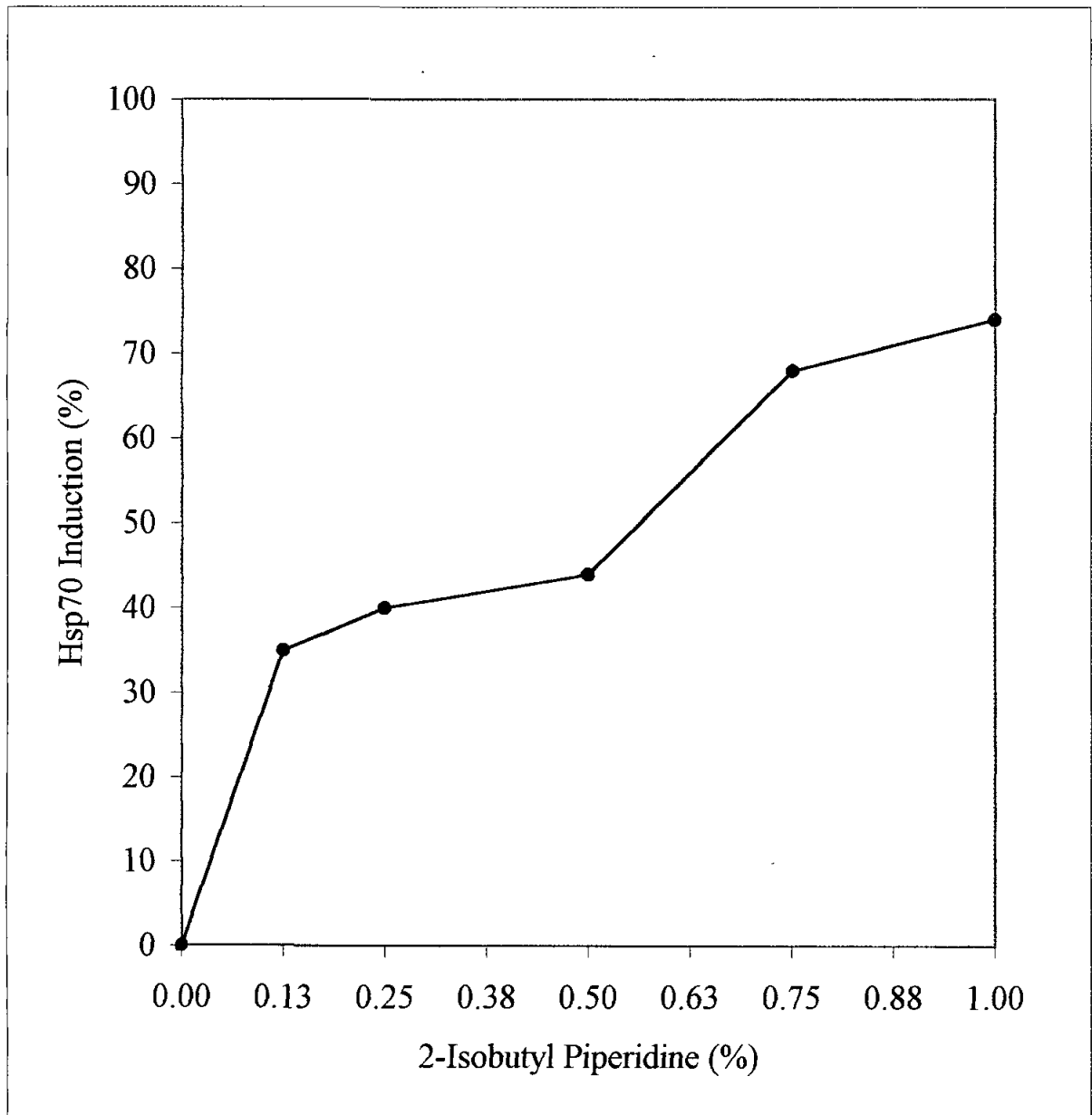


Legend:

One-dimensional SDS-PAGE separation of the mouse connective tissue cell line (L929) exposed to increasing concentrations of 2-Isobutyl piperidine. 96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100µl per well of 2-Isobutyl piperidine amended medium (0-1% 2-Isobutyl piperidine dissolved in 1% acetone) for 24 hours at 37°C. 2-Isobutyl piperidine concentrations never exceeded 10% of the media volume. A solution of 1% acetone was used as a solvent control. Cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Lane 2 represents the separation of L929 cells exposed to unamended media. Lanes 3-7 represent separations of L929 cells exposed to increasing concentrations of 2-Isobutyl piperidine (0.125, 0.25, 0.5, 0.75 & 1.0% 2-Isobutyl piperidine). Lane 1 represents separation of molecular weight markers (A=200 kDa, B=116 kDa, C=97 kDa, D=84 kDa, E=66 kDa, F=55 kDa, G=45 kDa & H=36 kDa). Arrowhead (◄) indicates the relative position of hsp70. Hsp70 induction showed a dose dependent increase. Repression of normal protein synthesis occurred with increasing 2-Isobutyl piperidine concentration.

Figure 51:

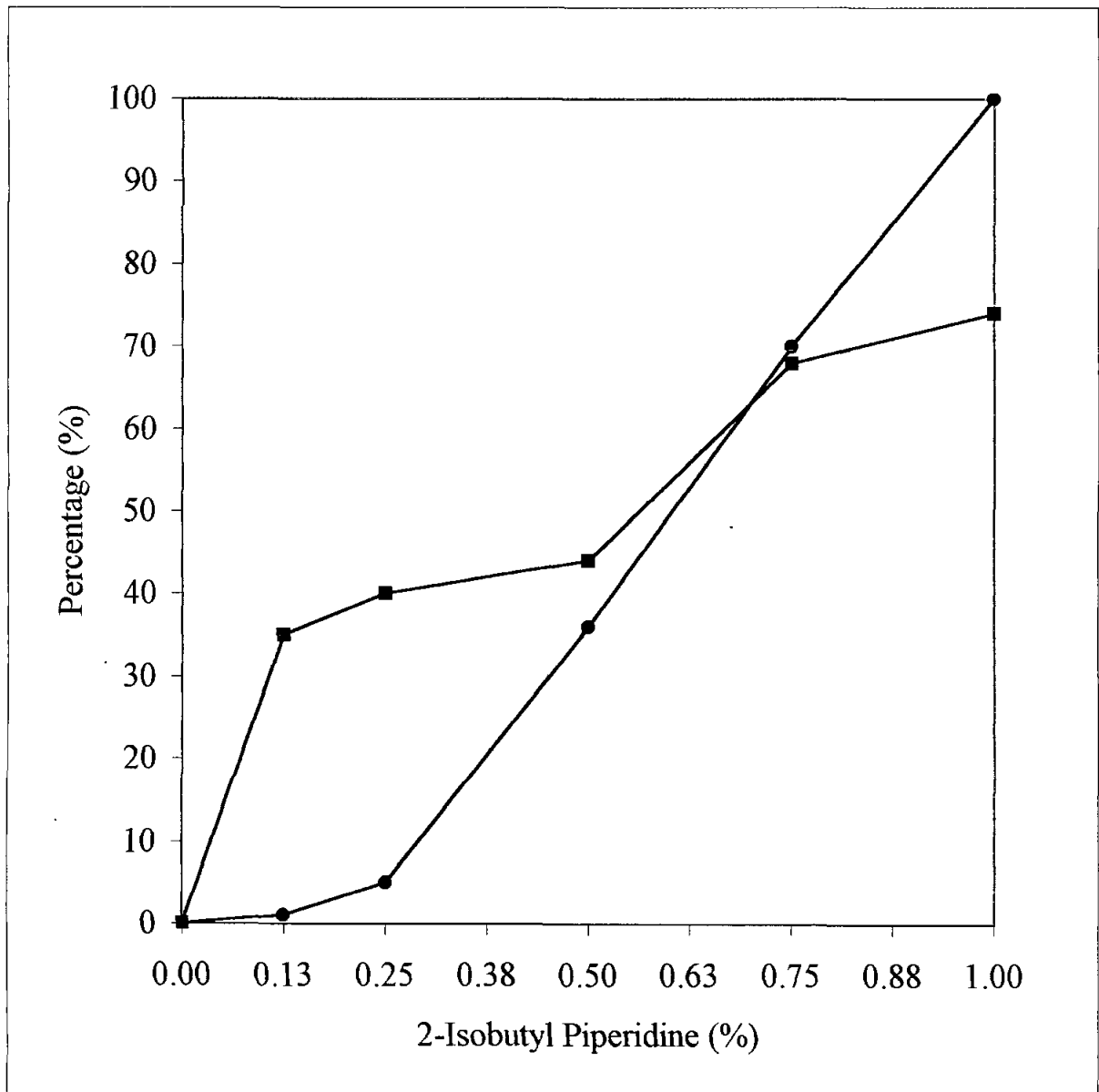
Heat shock response curve for the mouse connective tissue cell line (L929) to 2-Isobutyl piperidine.



Heat shock response curve for the mouse connective tissue cell line (L929) to 2-Isobutyl piperidine. 96 well plates were seeded with 4×10^4 cells per ml for 24 hours at 37°C. The attached cells were incubated with 100µls per well of 2-Isobutyl piperidine amended medium (0-1% 2-Isobutyl piperidine dissolved 1:4 in 1% acetone) for 24 hours at 37°C. 2-Isobutyl piperidine concentrations never exceeded 10% of the media volume. A solution of 1% acetone was used as a solvent control. Cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp70 induction (●) was expressed as a percentage of the untreated control level. The heat shock response curve shows a dose dependent increase in hsp70 induction. Measurement of hsp70 induction at the median lethal concentration showed a 11% greater response than found with measuring cell mortality using the neutral red assay. Hsp70 induction was observed at the no effect level.

Figure 52:

Comparison of cell mortality and hsp70 induction for the mouse connective tissue cell line (L929) to 2-Isobutyl piperidine.



Comparison of cell mortality and hsp70 induction for the mouse connective tissue cell line (L929) to 2-Isobutyl piperidine. Cell mortality (●) was estimated by the neutral red assay. The median lethal concentration was estimated by regression of the linear portion of the curve.


For the heat shock response assay cell lysates were separated by 1-D SDS-PAGE and detected by silver staining. Hsp70 induction (■) was expressed as a percentage of the untreated control level. Measurement of hsp70 induction at the median lethal concentration, the no effect level and at the lowest concentration tested showed a 11%, 40% and 35% greater response than found with measuring cell mortality using the neutral red assay.

4 Discussion.

The purpose of this study was to assess the potential of heat shock protein 70 as an *in vitro* measure of toxicity. The demand on animal toxicity testing has led to alternative *in vitro* assays being developed. Although several *in vitro* toxicity assays involving cell culture have been developed, they typically rely on measuring cell death or the characteristics leading to cell death, an all or nothing process, and thus are incapable of identifying sub-lethal toxicity. The heat shock response was discovered by Ritossa (1962), it is a conserved cellular defence mechanism found across a broad range of taxonomic classes. Induction of heat shock proteins occurs as a direct consequence of cellular stress and allows changes in gene expression to be assessed. It has been suggested that the heat shock response has potential as a measure of toxicity and several applications have already been developed that use electrophoresis, Western blotting and transgenic organisms. For the heat shock response to find application in routine toxicity testing, it must be shown to be more sensitive than existing assays, have biological relevance and be applicable to a wide range of organisms. In addition, an easy to use, rapid, economical, sensitive and reproducible assay is desirable.

4.1 Toxicity Data.

In this study, toxicity data was established for two cell lines, the mouse connective tissue cell line (L929) and the normal rat kidney cell line (NRK). Initially a broad range of toxin concentrations was screened followed by a narrower range. This ensured dose response curves were constructed using concentrations that ranged from non-lethal to lethal, thus encompassing the total toxicity profile of the compound. Toxicity data for



median lethal and no effect concentrations as defined by Trevan (1927) and Rand and Petrocelli (1985) were established using the neutral red assay. Previous comparative studies have shown good correlation between different *in vitro* cytotoxicity assays (Shopsis and Eng, 1985; Borenfreund and Puerner, 1985; Riddell *et al*, 1986; Babich and Borenfreund, 1987; Mazziotti *et al*, 1990) suggesting that the choice of assay pertains more to personal preference and ease of use than absolute sensitivity. The neutral red assay has been applied extensively to various aspects of toxicity testing (Borenfreund and Puerner, 1985; Riddell *et al*, 1986; Stark *et al*, 1986; Hunt *et al*, 1987; Benford and Good, 1987; Babich and Borenfreund, 1987) and shows excellent correlation with several whole animal toxicity tests such as the *in vivo* Draize test for the ranking of surfactants (Borenfreund and Puerner, 1985; Shopsis and Eng, 1985), rat oral LD₅₀ values ($r=0.77$) (Benford and Good, 1987), *in vitro* and *in vivo* data for the Bluegill sunfish ($r=0.988$) and fathead minnow ($r=0.835$) (Babich and Borenfreund, 1987) and corneal opacity scores ($r=0.84$) (Dierickx and Gordon, 1990). In this study, the neutral red assay was used to establish toxicity data for three heavy metals. This toxicity data was used as the benchmark against which the performance of the heat shock response was gauged.

The median lethal concentration value (LC₅₀) is defined as the concentration of chemical that results in the death of 50% of the population within a defined period (Trevan, 1927). The use of mortality is nowadays considered a crude measure of toxicity as it provides little information on the chronic and sub-lethal effects of the chemical. The median lethal concentration is a misunderstood measure and frequently misapplied. It is not a biological constant but unique for individual experiments and

therefore its use in legislation and chemical classification is questionable. Factors that affect the median lethal concentration value in animal tests include, species, age, weight, sex, genetic strain, health, diet, mode of administration and temperature (Zbinden and Flury-Roversi, 1981). Using a cell detachment assay Garza-Ocanas *et al*, (1990) estimated the LC₅₀ of mercury chloride to be 1.3mg/l for NRK-52E cells whilst Mazziotti *et al*, (1990) using Hep-2 cells and a total protein assay estimated the LC₅₀ of mercury chloride to be 55mg/l. This represents a 40 fold difference in LC₅₀ values. A 380 fold difference in LC₅₀ values for cadmium chloride was found between the findings of Clothier *et al*, (1988) and Brandao *et al*, (1992). A kenacid blue assay with 3T3-L1 cells gave an LC₅₀ value of 0.2mg/l (Clothier *et al*, 1988), whilst Brandao *et al*, (1992) estimated the LC₅₀ of cadmium chloride using a neutral red assay to be 76mg/l for FHM cells. Using the kenacid blue assay Clothier *et al*, (1988) estimated the LC₅₀ of copper sulphate to be 5mg/l for 3T3-L1 cells whilst Brandao *et al*, (1992) using FHM cells and a neutral red assay estimated the LC₅₀ of copper sulphate to be 800mg/l. This represents a 160 fold difference between LC₅₀ values. With such high variation the use of the median lethal concentration as a measure of toxicity except as a constant within experiments must be questioned. Reporting absolute median lethal concentration values are of little use when comparing the toxicity of different compounds, as comparative studies inevitably use varying cell lines, media, incubation temperatures, serum concentrations and assays. Therefore, to allow for comparison it is necessary to rank the toxins relatively in increasing order of toxicity.

In this study, using the median lethal concentration, the three metals were ranked cadmium being the most toxic, mercury been slightly less toxic and copper the least

toxic. Other studies using cell cultures concur with these rankings (Clothier *et al*, 1988; Scarino *et al*, 1988; Mazziotti *et al*, 1990; Ryan and Hightower, 1994; Schmalz *et al*, 1997) (Table 24). In some studies, mercury has been found to be more toxic than cadmium (Shopsis and Eng, 1985; Brandao *et al*, 1992). No explanation for this difference has been offered. Whole animal toxicity data reported for rat and mouse intra peritoneal rankings (Shopsis and Eng, 1985; Clothier *et al*, 1988) and for fish and invertebrate rankings (Verma *et al*, 1981; Brandao *et al*, 1992) agree with results in this study. One problem associated with simply ranking chemicals is that the difference in toxicity between two chemicals may be assumed equivalent. Thus, in this study, as well as ranking the metals their relative toxicity is reported. For the two cell lines, cadmium was found to be one fold more toxic than mercury. Cadmium was found to be 8 fold more toxic than copper for the NRK cell line and 17 fold for the L929 cell line. For each metal it is the cation that elicits the toxic effect (Hulme *et al*, 1989). When the LC₅₀ values for the three metals were compared between the two cell lines, it was shown that both the L929 and NRK cell lines have similar sensitivity to cadmium and mercury. However, the toxicity of copper to each cell line differs greatly. The LC₅₀ of copper for the L929 cell line was estimated at 39.95mg/l whereas the LC₅₀ for the NRK cell line was estimated at 18.48mg/l. The NRK cell line was found to be over twice as sensitive to copper than the L929 cell line. The coefficients of variation (%CV) found between replicate tests would indicate experimental error was not at fault and that the different sensitivity to copper was due to the cell line. The pathway through which copper elicits its toxicity may differ between the two cell lines. Kfir and Prozesky (1981) found a similar response for HeLa cells when assaying the toxicity of cadmium, mercury,

Table 24. Median lethal concentrations (LC₅₀) for cadmium, mercury and copper.

Method	Cells	Cadmium	Mercury	Copper	Reference
72hr Kenacid Blue	3t3-L1	0.2 mg/l	2 mg/l	5 mg/l	Clothier <i>et al</i> , 1988
24 hr Total protein	IEC-17	0.25mg/l	n/a	60mg/l	Scarino <i>et al</i> , 1988
24hr Total protein	Hep-2	18.1mg/l	55mg/l	n/a	Mazziotti <i>et al</i> , 1990
66hr Neutral red	PLHC-1	10.8mg/l	n/a	32.4mg/l	Ryan and Hightower, 1994
96hr Neutral red	Flounder kidney	9.1mg/l	n/a	28.8mg/l	Ryan and Hightower, 1994
24hr MTT test	HaK	1.5mg/l	4mg/l	6mg/l	Schmalz <i>et al</i> , 1997

n/a (Metal was not measured).

copper and lead. Examining three different cell lines, buffalo green monkey, mouse lymphoma and HeLa cells they observed HeLa cells were least sensitive to cadmium, mercury and copper but the most sensitive to lead. They suggested the lower sensitivity of HeLa cells than that of the other two cell lines may be due to the long history of cultivation *in vitro* coupled with the high growth rate and higher resistance of tumour cells. They did not suggest any reasons for HeLa cells higher sensitivity to lead.

Median lethal concentrations have not only been reported but also lethal concentrations for 20% and 80% cell mortality. Using the 20, 50 and 80% lethal concentration values the increase in toxicity with increasing toxin exposure can be assessed (Riddell *et al*, 1986; Clothier *et al*, 1988). In this study copper was found to be the least toxic of the three metals. However, examining the linear slope of the 20, 50 and 80% lethal concentrations indicated the toxicity of copper increased more rapidly with increasing exposure than either mercury or cadmium. The no effect level represents the lowest detection limit of the assay and was estimated using the Students T-test as the concentration of toxin that resulted in no cell mortality as assessed by the neutral red assay. The neutral red assay measures cell mortality and thus is incapable of measuring sub-lethal effects that occur below the no effect level.

4.2 Electrophoresis:

The use of 4-22.5% gradient SDS-PAGE in this study was designed to maximise the resolution of proteins in the 50-100kDa range. The extent of the linear relationship between log molecular weight and relative mobility is limited for a homogeneous gel concentration, with gradient gels the relationship is much wider ranging from 10^4 - 10^6

Daltons (Lambin, 1978). In addition, the use of SDS gives a uniform charge surface density and provides an overall conformation close to random coil structure allowing more accurate estimation of molecular weight (Lambin, 1978). Using SDS-PAGE gradient gels, coefficients of variation as low as 2.9-4.2% for molecular weight estimates have been found (Poduslo, 1981). The reproducibility of SDS-PAGE after 12 different separations was estimated to range from 5-10%, with new batches of acrylamide sometimes observed to alter the relative mobility of proteins (Weber and Osborn, 1969). In this study, the coefficient of variation (%CV) for control samples separated on replicate gels was estimated to be 5%. The variation observed in this study may be attributed to the pouring of individual gradient gels and variability of the silver stain technique. Using SDS-PAGE gradient gels the separation of the cells proteins on a single gel was optimised allowing their molecular weights to be estimated.

Silver stain proved to be a highly sensitive detection system detecting between 25-30 protein bands in the normal protein pattern of the two cell lines. Marshall (1984b) using this stain on two-dimensional electrophoretic gels detected over 200 polypeptide spots in unconcentrated human urine, 180 spots in human serum and 150 spots in a single human fingerprint. In this study, the silver stain was modified by altering the concentration of citric acid in the developer. The original concentration of citric acid was 0.005% however increasing the concentration to 0.1% reduced the rate of staining and destaining allowing for more control over the process. The use of this silver stain with its higher sensitivity (100 times) over traditional protein stains such as coomassie brilliant blue allowed the total protein profile of the cells to be easily detected.

Due to the toxicity and pungent odour of mercaptoethanol, the effectiveness of the alternative reducing agent dithiothreitol was assessed. In control samples no difference could be ascertained between the two reducing agents. However, where cells had been exposed to the metals the ability of dithiothreitol to reduce the proteins was inhibited. This inhibition resulted in the expression of an apparent 60kDa protein with no 70kDa protein. One of the major heat shock proteins has a molecular weight of 60kDa, therefore, it was initially thought that the cell line had altered its heat shock response. Running samples with the two reducing agents on a single gel, it was observed that for mercaptoethanol a 70kDa protein was resolved and for dithiothreitol a 60kDa protein was resolved. An interfacial zone indicating that they were related connected the two proteins. Allore and Barber (1984) investigating intramolecular disulphide bonding produced similar results. Running samples in reduced and non-reduced forms on a single gel resulted in the same interfacial zone that allowed the disulphide bonding of proteins to be tracked. When proteins are reduced, the internal disulphide bonded domain unfolds resulting in an expanded random coil structure of longer effective chain length and lower electrophoretic mobility than that of the structurally more compact non-reduced form of the protein (Allore and Barber, 1984; Marshall, 1984b). Upon reduction the 60kDa protein gave an apparent molecular weight of 70kDa, i.e. hsp70. This differential response to full and intermediate reduction could be used as a characteristic response in the identification of these proteins as suggested by Marshall (1984b).

One difficulty encountered with the heat shock response assay was the use of the electrophoresis sample buffer which was found to inhibit the Lowry protein assay. The

sample buffer is not only an essential component of the electrophoresis system but also lyses the cells. Sodium hydroxide was used as an alternative to lyse the cells. Prior to electrophoresis the appropriate volume of lysate was mixed with double strength sample buffer ensuring normal electrophoretic conditions. The low concentration of NaOH used did not interfere in the assay.

4.3 Repression of normal proteins:

One of the advantages of the heat shock response assay is the induction of heat shock proteins coupled with the corresponding repression of normal protein synthesis. That is, as the heat shock protein levels increase, normal proteins that could potentially interfere with the assay are repressed allowing for the easier assessment of the heat shock proteins. In this study, normal protein synthesis was repressed with increasing toxin concentration in an apparent dose dependent manner (Figures 15, 17, 19, 21, 23 & 25) this finding concurs with the findings of Ritossa (1962), Tissieres *et al*, (1974) and Grosvik and Goksyor (1996). The repression of normal protein synthesis was first observed in *D. busickii* after 30 min incubation at 30°C (Ritossa, 1962). This repression has been attributed to the break down of pre-existing polysomes (McKenzie *et al*, 1975) and the subsequent selective transcription of heat shock protein mRNA, demonstrating transcriptional and translational control (Mirault *et al*, 1978). This repression is so pronounced that at the median lethal concentration (LC₅₀) hsp70 represents between 4-19% of the total cell protein content. Tissieres *et al*, (1974) showed hsp70 accumulated to 15-19% of the total protein content. Below the median lethal concentration repression of normal protein synthesis is less pronounced as both normal and heat shock mRNA are transcribed.

4.4 Heat shock response:

The study of the heat shock response originated when Ritossa (1962) observed alterations in the puffing pattern of *D. busckii*. Subsequently Tissieres *et al*, (1974) showed the induced puffs corresponded to the induction of a set of polypeptides and the repression of others. Since its discovery, the heat shock response has been demonstrated to occur in organisms across a broad range of taxonomic classes (Table 25). To assess if the cell lines used in this study exhibited a heat shock response, a classic heat shock experiment was conducted (Ritossa, 1962). With increasing temperature, the two cell lines L929 and NRK showed increasing levels of hsp70 coupled with the repression of normal protein synthesis. Our study concurs with the findings of Tissieres *et al*, (1974) who observed hsp70 induction with heat shock, accompanied by a reduction in other protein bands. The two cell lines were very sensitive demonstrating hsp70 induction with only a 3°C increase in temperature, the NRK cell line was, however, slightly more sensitive demonstrating a greater response. The induction of heat shock protein 70 with only a 3°C increase, a temperature typical of fever, has also been demonstrated in brain, heart, kidney and in the tissues of foetuses of thermo-regulating animals (Brown *et al*, 1982). Therefore, it could be concluded that the heat shock response is a relevant natural cellular response to physiological stress in the intact animal. The potential clinical importance of monitoring heat shock protein induction is obvious in cases of fever and chemotherapy allowing acceptable levels to be ascertained and optimum treatment times to be assessed (Brown *et al*, 1982; Low-Friedrich *et al*, 1991). The induction of heat shock proteins with increasing temperature appears to be a cellular defence mechanism (Key *et al*, 1982; Loomis and Wheeler, 1982) as the response is dependent on attaining a critical temperature not merely an increase of 5-6°C (Ritossa, 1962) and this critical

Table 25: Heat shock proteins induced in a variety of organisms by heat shock

Species	Molecular Weights of Proteins induced (kDa)	Reference
Crustaceans		
<i>Mytilus edulis</i>	72,70,60,58	Sanders, 1990
	120,45,25,20	Brown <i>et al</i> , 1995
Cell cultures		
RTG-2	87,70,62,42,32,30	Kothary <i>et al</i> , 1984
Mouse liver	87,70,68	Zatloukal <i>et al</i> , 1988
Mouse cardiac myocytes	70	Low-Friedrich <i>et al</i> , 1991
HeLa	70	Steels <i>et al</i> , 1992
Opossum kidney	73,72,70	Nissim <i>et al</i> , 1993
Human fibroblasts	70	Luce <i>et al</i> , 1993
3t3-L1	72	Marshall and Kind, 1994
Mm96e	70	" " " "
mm253c1	70	" " " "
HT29	110,90,70,68	Delmas <i>et al</i> , 1995
Astroglial	70, 30	Opanashuk and Finhelstein 1995
Fish		
<i>Salmo gairdneri</i>	87,70,42,32,30	Kothary <i>et al</i> , 1984
Channel catfish	70,68	Abukhalaf <i>et al</i> , 1994
Bacteria		
<i>Saccharomyces cerevisiae</i>	104,70	Vogel <i>et al</i> , 1995
Plants		
Zea may seedlings	96,84,82,74,72,18	Bonham-Smith <i>et al</i> , 1988
Pondweed	102,95,93,90,83,78	Siesko <i>et al</i> , 1997
Invertebrates		
<i>Daphnia magna</i>	70	Bond and Bradley, 1995
<i>Chironomus tentans</i>	72	Karouna-Renier and Zehr, 1999
Mammals		
Mice	70	Zatloukal <i>et al</i> , 1988
Bovine	70	Gutierrez and Guerriero, 1991
Chicken	23,70,90	Miller and Qureshi, 1992

temperature coincides with protein denaturation (Hightower, 1988). As a potential measure of toxicity, the heat shock proteins are unique: appropriate damage causes a dramatic increase in their synthesis. Induction results from specific mRNA synthesis and not merely from the effect elevated temperature has on translation or subsequent processing (Tissieres *et al*, 1974). Thus, there is an opportunity to measure a responsive aspect of gene expression itself (Anderson *et al*, 1982). The protective nature of the heat shock response was observed by Bonham-Smith *et al*, (1988) through the induction of heat shock proteins with a gradual increase in temperature resulting in less repression of normal protein synthesis. In this study, the treatment temperature was achieved rapidly by immersing the cells in a water bath at the appropriate temperatures. Substantial repression of normal cell proteins was observed at the highest temperatures. Anderson *et al*, (1982) suggested that the rapid attainment of the treatment temperature results in greater repression of normal proteins due to the time required by the cell to synthesise the protective heat shock proteins. Several investigators have observed the protective nature of the heat shock response through the induction of heat shock proteins that confer protection to cells exposed to a subsequent more aggressive treatment and have termed this effect thermotolerance (Li *et al*, 1982; Subject *et al*, 1982; Steels *et al*, 1992). Tissieres *et al*, (1974) observed that temperatures that induce heat shock proteins are within those known to produce phenocopies in *Drosophila* and are possible in nature. Thus, it may be that temperature variations have a profound effect on development and selection due to differential modulation of gene activities (Tissieres *et al*, 1974). Several investigators have suggested that heat shock proteins protect against such developmental abnormalities (Peterson and Mitchell 1982; Buzin and Bournias-Vardiabasis 1982). The rapid induction of heat shock proteins as thought in response to

an emergency, the temperature required for induction which mimics that found in the environment and their role in thermotolerance all point to the heat shock response as a protective mechanism in cells (Lindquist and Craig, 1988). As a potential measure of toxicity, the heat shock response represents as ideal candidate, occurring in response to stimuli not as a consequence.

4.5 Heat shock protein 70 induction versus toxicity data:

The heat shock response has been applied to various aspects of toxicology such as the adaptation of organisms to various types of environment (Brown *et al*, 1995) and the possibility of differentiating between natural and anthropogenic stressors (Gonzalez and Bradley, 1994). Numerous studies have compared the heat shock response with existing measures of toxicity and the three metals used in this study have been frequently assessed. Grosvik and Goksoyr (1996) investigated the heat shock response to cadmium as a biomarker of toxicity. Vedel and Depledge (1995) examined the heat shock response as a biomarker for copper pollution in the gills of shore crabs. Delmas *et al*, (1995) investigated the heat shock response suggesting it would provide a sensitive, rapid and universal measure of environmental aggression. Sanders (1990) found the heat shock response to be a more sensitive measure than scope for growth in *Mytilus edulis* exposed to copper. The heat shock response has been used to assess metal toxicity in a number of distinct environments. Terrestrial pollutants and environmental conditions that affect plant growth and yields have been investigated (Kelley and Freeling, 1982; Bonham-Smith *et al*, 1988; Wollgiehn and Neumann, 1995). Werner and Nagel (1997) investigated the potential of the heat shock response to measure the toxicity of cadmium pollution in sediments. The heat shock response as a biomarker of

aquatic pollution has also been investigated (Sanders, 1993; Siesko *et al*, 1997). Muller *et al*, (1995) examined the toxicity of river water using the heat shock response. To expand and develop on the research already carried out, various techniques including electrophoresis, Western blotting, ELISA and immunocytochemistry were used to assess the potential of heat shock protein 70 to measure *in vitro* toxicity.

The toxicity data established using the neutral red assay in this study was used as the benchmark against which the performance of the heat shock response was gauged. Under identical test conditions, as those used in the neutral red assay, cells were exposed to metallic stresses at concentrations ranging from sub-lethal to lethal encompassing their total toxicity profile. Since the discovery of the heat shock response, numerous studies have examined the response upon exposure to cadmium, mercury and copper (Table 26). For both cell lines using the three metals hsp70 was observed to increase in a dose dependent fashion. This concurs with the findings of Fischbach *et al*, (1993), Stringham and Candido (1994) and Ryan and Hightower (1994). At the LC₅₀ hsp70 is the predominant protein being produced. The results of this study concur with those of Levinson *et al*, (1980). Assessing the heat shock response for metals using chicken fibroblast cells, they observed the induction of several heat shock proteins. They demonstrated that the cation was the active ion as equivalent concentrations of sodium sulphate did not induce the heat shock response. Levinson *et al*, (1980) observed that the heat shock proteins induced, the levels attained and their rate of induction varied with the metal used. Cadmium was found to be the most effective inducer on both a mass and kinetics basis. They observed that agents that induced the heat shock response had a high affinity for sulphhydryl containing molecules.

Table 26: Heat shock proteins induced by cadmium, mercury and copper.

Species	Proteins induced by cadmium (kDa)	Reference
<i>Drosophila</i>	70	Bunch <i>et al</i> , 1988
Mouse cardiac myocytes	71,68,30	Low-Freidrich <i>et al</i> , 1991
HeLa cells	70	Steels <i>et al</i> , 1992
Human fibroblasts	70	Luce <i>et al</i> , 1993
NIH/3T3 cells	70	Fischbach <i>et al</i> , 1993
HeLa cells	32	Marshall and Kind, 1994
Flounder/ PLHC-1 cells	75,70,35,32,27,25	Ryan and Hightower, 1994
Nematode	16	Stringham and Candido, 1994
Tomato	70,17	Wollgiehn and Neumann, 1995
Astroglial cells	30	Opanashuk and Finhelstein, 1995
<i>Gammurus oceanicus</i>	70	Brown <i>et al</i> , 1995
<i>Gammurus dubeni</i>	70	" " " "
<i>Mytilus edulis</i>	70	" " " "
Salmon hepatocytes	70,30-32,45-46,15	Grosvik and Goksoyr, 1996
Pondweed	162,142,122,82,61	Siesko <i>et al</i> , 1997
Amphipods	60,70	Werner and Nagel, 1997
<i>Aedes albopictus</i>	120,110,108,98,70	Braeckman <i>et al</i> , 1999
Endothelial cells	70,60,32,27	Wagner <i>et al</i> , 1999
Hepatocytes	73,72	Dilworth <i>et al</i> , 2000
HeLa cells	70	Ait-Aissa <i>et al</i> , 2000

Species	Proteins induced by Mercury (kDa)	Reference
Mouse cardiac myocytes	70,30	Low-Friedrich <i>et al</i> , 1991
NIH/3T3 cells	70	Fischbach <i>et al</i> , 1993
Nematode	16	Stringham and Candido, 1994
Tomato	70,17	Wollgiehn and Neumann, 1995
HeLa cells	70	Oshima <i>et al</i> , 1997
HeLa cells	70	Ait-Aissa <i>et al</i> , 2000

Species	Proteins induced by Copper (kDa)	Reference
<i>Drosophila</i>	70	Bunch <i>et al</i> , 1988
<i>Mytilus edulis</i>	72,70,68,60,	Sanders, 1990
NIH/3T3 cells	70	Fischbach <i>et al</i> , 1993
Nematode	16	Stringham and Candido, 1994
Flounder/ PLHC-1 cells	75,70,32,27	Ryan and Hightower, 1994
Crab	70	Vedel and Depledge, 1995
FHM-2 cells	70	Sanders <i>et al</i> , 1995
Hep G2/ <i>Xenopus laevis</i>	70	Camatini <i>et al</i> , 1999
Zebra mussels	70,60	Clayton <i>et al</i> , 2000

As so many other stimuli elicit the heat shock response and do not affect sulphhydryl containing molecules, the abnormal protein theory of Hightower (1980) is taken to be the more likely stimulus for heat shock protein induction. Ananthan *et al*, (1986) demonstrated that injecting denatured protein into a cell was sufficient to induce the heat shock response. Metal concentrations sufficient to cause protein denaturation therefore result in the induction of heat shock proteins.

In this study, a dose dependent increase in hsp70 occurred and corresponded with increasing cellular toxicity as determined using the neutral red assay, thus demonstrating biological significance. Ryan and Hightower (1994) observed similar findings using the neutral assay to measure toxicity. Fischbach *et al*, (1993) also observed that the induction of hsp70 has physiological significance. They ranked metals based on their ability to induce the hsp70 promoter gene fused to human growth hormone in 3t3 cells. They found a correlation between hsp70 induction for cadmium, mercury and copper with carcinogenesis data, mutagenesis data, unscheduled DNA synthesis and *in vitro* cell transformation studies; induction of hsp70 by mercury correlated with chromosome aberrations and sister chromatid exchange data. Stringham and Candido (1994) developed transgenic lines of nematodes that produce β -galactosidase when hsp16 is induced. A tissue specific heat shock response was observed for different metals. Mercury showed exclusive heat shock protein staining within the gut, copper in the muscle and neurons of the anterior end of the pharynx and cadmium stained heat shock proteins in muscle, nerve and epidermal cells of the pharynx and occasionally the intestine. Such tissue specificity suggests the possibility of identifying target organ toxicity using the heat shock response.

In this study, it was observed that above the LC_{50} hsp70 induction begins to plateau. Cell mortality at these concentrations is extreme and outweighs hsp70 induction. Fischbach *et al*, (1993) also observed a reduced response at higher metal concentrations with excessive metal concentrations resulting in cell lysis. Although numerous studies have used cadmium, mercury and copper to induce heat shock proteins, differing objectives and methods of data analysis prevent comparison. The majority of studies either identify the appearance of hsp's without quantifying levels of induction, or assess the lowest concentration of chemical to induce hsp's without identifying biological significance or toxicity data. In this study, comparing the toxicity data established using the neutral red assay directly with hsp70 induction allowed the sensitivity of the heat shock response to be assessed. Hsp70 induction was calculated relative to its control level allowing the magnitude of the response to be assessed. At the LC_{50} hsp70 induction was found to have a greater response than that of the neutral red assay. This concurs with the findings of Ryan and Hightower (1994) who demonstrated approximately a 14% greater heat shock response above that of the neutral red assay and of Werner and Nagel (1997) who demonstrated approximately a 37% greater response over a standard 24hr LC_{50} assay for Amphipods. The L929 cell line showed a 25% greater response for cadmium and copper and the NRK cell line showed a 25% greater response for cadmium and mercury. For mercury the L929 cell line showed a greater response of 5% and the NRK cell line showed a 16% lower response for copper. This lower response was caused by increased cell mortality or denaturation of hsp70 at the LC_{50} . The greater response shown by the induction of hsp70 above that of the neutral assay for several of the metals indicates the heat shock protein response is a more sensitive assay allowing easier assessment of toxicity.

Reporting heat shock protein 70 induction at the median lethal concentration, the sensitivities of the two cell lines to the toxins can be compared. When heat shock protein induction at the LC₅₀ values for the three metals were compared, between the two cell lines, it was shown that both the L929 and NRK cell lines had similar sensitivities to cadmium. The NRK cells were found to be more sensitive to mercury than the L929 cells inducing a greater level of hsp70 and the NRK cell line was found to be less sensitive than the L929 cells to copper inducing less hsp70. Kfir and Prozeskey (1981) observed that HeLa, BGM (buffalo green monkey) and ML (mouse lymphoma) cells had different sensitivities to chemicals. HeLa cells were the least sensitive and BGM cells the most sensitive. Other investigators have also found cell lines of varying sensitivities including PLHC-1 cells, flounder kidney cells (Ryan and Hightower, 1994), human epithelial cells, RTG-2 cells (Marion and Denizeau, 1983), Fathead minnow cells and Bluegill sunfish cells (Babich and Borenfreund, 1987). Such variations in sensitivities have been partly attributed to different incubation temperatures and serum requirements (Marion and Denizeau, 1983; Ryan and Hightower, 1994).

Several investigators have reported heat shock protein induction below a toxins median lethal concentration suggesting the potential to identify sub-lethal toxicity (Ryan and Hightower, 1994; Stringham and Candido, 1994; Werner and Nagel, 1997). Ryan and Hightower (1994) estimated the recovery threshold between lethal and sub-lethal effects for cadmium to be between 12.5mg/l and 15mg/l. Concentrations below the LC₅₀ were taken to be sub-lethal as cells could recover. Stringham and Candido (1994) using transgenic lines of nematodes that produce β -galactosidase when hsp16 is induced observed heat shock proteins were induced at concentrations below the LC₅₀ for

cadmium, mercury and copper respectively. Werner and Nagel (1997) using Western blotting observed heat shock protein induction using cadmium in three species of Amphipod occurred below the median lethal concentration (LC_{50}). However, to date no studies have demonstrated heat shock protein induction at toxin concentrations that result in no cell mortality.

The no effect level was taken as the concentration at which the neutral red assay was unable to detect any cell mortality, any measurable response by the heat shock response assay would indicate a more sensitive assay capable of identifying sub-lethal toxicity. The results of this study are more sensitive than those of Ryan and Hightower (1994), Stringham and Candido (1994) and Werner and Nagel (1997), as sub-lethal concentrations were below the no effect level. The heat shock response identified hsp70 induction below the no effect level and at substantial concentrations ranging from 28-48% above control levels. Measuring sub-lethal toxicity at concentrations as much as three times lower than the LC_{50} value demonstrates the potential of the heat shock response. The sensitivity of the heat shock response assay to identify sub-lethal toxicity was assessed by examining hsp70 induction at the lowest observed effective concentration. The high levels of induction at the lowest observed effective concentrations ranging from 11-23% above the control indicate the sensitivity of the heat shock response assay for measuring sub-lethal toxicity. The heat shock response identified sub-lethal toxicity at concentrations 5 times lower than the no effect level and as much as 8 times lower than the LC_{50} value.

Comparing the toxicity data established using the neutral red assay and the optimised heat shock response assay it is evident that the heat shock response assay is more sensitive. At the LC₅₀ values measuring hsp70 induction the heat shock response assay identified a greater response than the neutral red assay. For several of the toxins this response was 25% greater than that observed when measuring cell mortality. However, as hsp70 induction begins to plateau around the LC₅₀ hsp70's increase is less pronounced. The true potential of the heat shock response assay lies in the assessment of sub-lethal toxicity. Substantial hsp70 induction was observed at the no effect level and lowest observed effective concentration identifying sub-lethal toxicity at concentrations several times below the LC₅₀ and where the neutral red assay failed to demonstrate any cell mortality.

4.6 Western blotting:

Specific antibodies using Western blotting were used to confirm that the 70kDa protein identified in this study was heat shock protein 70. The ATP binding characteristics of heat shock proteins allow for their purification by ATP-agarose column chromatography permitting the production of specific antibodies (Margulis *et al*, 1991). Levinson *et al*, (1980) found proteins similar but not identical to those of chick embryo fibroblasts (Kelley and Schlesinger, 1978) and to those of *Drosophila* cells (Tissieres *et al*, 1974) suggesting commonality between induction of hsp's in species as diverse as flies, birds and humans. Schlesinger *et al*, (1982) appear to be one of the first research groups to develop antibodies to heat shock proteins and use them in Western blotting studies. They found a polyclonal chicken anti-hsp70 antibody reacted with species as diverse as slime moulds, rodents and humans. Abukhalaf *et al*, (1994), using a

polyclonal anti-hsp70 antibody raised in catfish, found cross-reactivity with liver, muscle and gill tissues of fathead minnows, red shiners, black bass and bluegill sunfish. Because of the highly conserved nature, it has been suggested that heat shock proteins might be broadly cross-reactive in species from diverse phyla, a characteristic that would enhance their usefulness in toxicity testing.

The initial antibody used in the study was clone BRM-22 monoclonal anti-hsp70. This antibody identifies both the constitutive and inducible forms of hsp70. Although the inducible form of hsp70 is not constitutively found in most cells low levels were observed in the control samples for the L929 and NRK cell lines. It is not known whether its presence was due to adverse conditions in culturing or sampling or if the inducible form of hsp70 is found normally in these two cell lines. Kothary *et al*, (1984) using Western blotting also observed inducible hsp70 in control samples. In stressed cells the inducible form was observed to have a much greater induction than the constitutive form. No cross reactivity was observed with other cell proteins, molecular weight markers were included as negative controls to ensure non-specific binding did not occur. The second type of antibody used for Western blotting was clone C92F3A-5 monoclonal anti-hsp70. This antibody identifies only the inducible form of hsp70 and was chosen to allow ELISA and immunocytochemistry techniques to be explored also. As with clone BRM-22 the inducible form of the antibody was identified in control samples at low levels but was observed to increase with metal concentration. Again no cross reactivity was observed with other proteins. Sanders *et al*, (1994) observed that C92 cross-reacted with mammalian hsp70 but not with algae, invertebrates or fish. The BRM-22 antibody reacted with a 70kDa protein in all fish tested and in three species of crustacean, in one of the fish it also bound to a 78kDa protein and in several

invertebrates it only bound to a 78kDa protein. Thus, heat shock proteins appear to be less conserved in fish and invertebrates than mammals. Levinson *et al*, (1980) observed different proteins were induced in chicken fibroblasts and human fibroblasts indicating species specificity. Lewis *et al*, (1975) found hsp molecular weights varied between species. Thus, it would appear that before using antibodies across broad taxonomic classes careful characterisation of the antibody is essential. Polyclonal antibodies may be more suitable to routine investigations having the advantage of being more cross-reactive and cheaper to produce than monoclonal antibodies. Although quantitative increases in hsp70 were observed, Western blot results were used only as a qualitative tool to confirm hsp70 induction.

4.7 Toxin interaction:

Several combinations of the metals were examined to assess the potential of the heat shock response to measure hsp70 induction in complex mixtures as found in the environment. Sprague (1970) proposed that a simple approach to classifying the interaction of two toxicants was to use half the concentration of toxicant required to achieve a set response. If the two toxicants just cause the response their interaction has an additive effect, a higher response means that their interaction has a more than additive effect. If the response is the same as would be achieved with one toxin there is no interaction and if the response is lower than that achieved by one toxin then there is an antagonistic effect. Antagonism between cadmium and nickel and cadmium and zinc has been observed. Nickel has been shown to reduce the toxicity of cadmium (Babich and Borenfreund, 1990) and cadmiums toxicity is also reduced by zinc (Christian *et al*, 1973). To assess the toxicity effect of the mixtures on cell mortality the neutral red

assay was used. Cell mortality estimates for the mixtures were compared to those for the individual metals and an additive effect was observed. All mixtures produced a higher cell mortality than that achieved by any individual metal. For the heat shock response to have application in environmental monitoring it would also have to identify the increased toxicity elicited by the metal mixtures. For the lower concentrations of metal mixtures the heat shock response also identified the additive effect. Here as was found for the individual metals the response was greater than that found when measuring cell mortality. This indicates not only the ability of the heat shock response to identify interaction between toxins in a mixture but also the higher sensitivity of the heat shock response. Very few studies have been conducted to examine the effects of toxin interaction. Verripoulos *et al*, (1987) showed a synergistic effect for cadmium and copper and for copper and zinc. Kohler and Eckwert (1997) observed that cadmium and lead as well as cadmium and zinc act as synergists and cause super-additive effects on the heat shock response in woodlice (*Oniscus asellus*).

4.8 ELISA:

Enzyme linked immunosorbent assays (ELISA) were first described in the early 1970's. These assays have often replaced more conventional systems not only because they offer greater sensitivity and specificity but also because they are simple to perform and readily automated. In order for the heat shock response to optimise its potential in toxicology, it is essential that the assay is easy to use, cheap, rapid, sensitive and in as far as possible automated. Silver stained SDS-PAGE produces a wealth of information with regard to the total protein profile, however it is time consuming, technically demanding and labour intensive. For routine application, induction of a single heat

shock protein may provide sufficient information avoiding the necessity to analyse the total heat shock protein profile. This study agrees with the findings of Sanders (1993) who suggested hsp70 as a suitable candidate as a biomarker, as it is highly conserved and typically the most abundant heat shock protein induced. Initially, the heat shock response and ELISA appeared incompatible as the sample buffer used for the electrophoresis prevented the cell lysates binding to the ELISA plate and also inhibited the enzyme substrate colorimetric endpoint. To overcome this, sample buffer was excluded from the assay without any adverse effects with regard to antibody antigen recognition.

The single band isolated by the C92F3A-5 anti-hsp70 monoclonal antibody using Western blotting indicates that the results generated, using the ELISA technique, are for the inducible form of hsp70. Margulis *et al*, (1991) were one of the first researchers to use ELISA in the heat shock response. Using competitive and sandwich ELISA they characterised several forms of hsp70. Gutierrez and Guerriero (1991) developed a competitive ELISA to measure the total pool of hsp70 present in different bovine tissues. In this authors knowledge this study represents the first use of ELISA in conjunction with the heat shock response to assess toxicity. The results of the ELISA assay were found to be very similar to those of the SDS-PAGE separations indicating both methods were suitable for identifying toxicity. The slightly higher induction of hsp70 for several of the toxins below the LC₅₀ value may indicate that the ELISA assay is more sensitive at these concentrations avoiding the potential interference from other proteins and the background associated with SDS-PAGE separations. The highly conserved nature of the heat shock response throughout evolution benefits this assay

allowing single antibodies to be used across taxonomic classes thus reducing the expense. The potential for automation of the ELISA technique, using multi-well plate washers, reagent dispensers and plate reading spectrophotometers makes the ELISA heat shock protein assay very attractive and viable for routine application. SDS-PAGE should be considered as a front line assay used initially to characterise the heat shock response when a new organism or compound is assessed. In this study it was shown that at very toxic concentrations there is a decreased heat shock response thus, the possibly arises of false negative results occurring. That is, where very toxic compounds inhibit hsp70 induction resulting in the perception that the compound was non-toxic. Using SDS-PAGE, such false negatives can be identified as not only will hsp70 induction decrease but there will also be a corresponding and dramatic decrease in normal protein synthesis. With the ELISA technique serial dilution of the compound is necessary to avoid this prozone effect.

4.9 Immunocytochemistry:

Identifying the location of heat shock proteins within the cell under normal and stressed conditions is important as it may provide an indication to their function. To this authors knowledge this study represents the first use of immunocytochemistry in conjunction with the heat shock response to assess toxicity. Two approaches were adopted in order to determine the potential of immunocytochemistry technique. Using an insoluble coloured endpoint that precipitates at the location of hsp70, qualitative data regarding the location and translocation of hsp70 could be ascertained. The second approach used a soluble coloured endpoint that allowed the level of hsp70 to be quantitatively determined. The single band isolated by the C92 anti-hsp70 monoclonal antibody using

Western blotting supports the suggestion that the results generated using the immunocytochemistry technique are for the inducible form of hsp70. The advantage of using immunocytochemistry is that no transfer of cells out of the 96 well culture plates is necessary, reducing the variability of the assay. Immunocytochemistry has been previously used to identify heat shock protein localisation and translocation within cell cultures. Schlesinger *et al*, (1982) was one of the first teams to develop antibodies to heat shock proteins and apply them to immunocytochemistry techniques. Using monolayers of chicken embryo fibroblastic cells, they found no antigens present on the cell surface. However, cells fixed with methanol, to allow penetration, showed widespread intense staining. Hsp70 was distributed in both nuclear and cytoplasmic compartments with hsp90 been strictly cytoplasmic. Welch *et al*, (1982) also observed cytoplasmic and nuclear staining in several cell lines using a polyclonal anti-hsp70 antibody. Hsp100 has been observed to associate with the golgi apparatus and may be involved in its catabolic and metabolic functions (Lin *et al*, 1982; Welch *et al*, 1982). In this study, the inducible isoform of hsp70 was observed to be located predominantly within the cytoplasm and to a lesser degree within the nucleus in control samples. Upon metal exposure hsp70 was observed to increase dramatically within the nucleus suggesting membrane translocation as reported by other investigators (Welch *et al*, 1982; Lin *et al*, 1982). Marshall and Kind (1994) observed staining was minimal under normal conditions but upon stress hsp70 was observed to translocate from the cytoplasm to the nucleus and return to the cytoplasm during recovery.

In this study using intermediate metal concentrations the reaction was heterogeneous with only a portion of the cells showing hsp70. Similar observations have been made with regard to the heterogeneous nature of the heat shock response and have suggested a

dependency on cell specific factors in hsp induction (Luce *et al*, 1993; Marshall and Kind, 1994). The heterogeneous response identified using immunocytochemistry may account for some of the variability observed when using SDS-PAGE to demonstrate hsp70 induction. Vedel and Depledge (1995) found a high degree of variability in hsp70 induction of *M. edulis* with coefficients of variation (%CV) ranging from 48-66%. They proposed that this variation is masked in other studies that pool organisms/samples. This study supports their proposal. An increase hsp70 induction was observed in a dose dependent manner using pooled cells. Stringham and Candido (1994) demonstrated the tissue specific nature of the heat shock response. They observed mercury showed exclusive staining within the gut, however only 43% of animals showed the response, copper induced staining in the muscle and neurons of the anterior end of the pharynx with only 38% of animals showing the response and cadmium stained muscle, nerve and epidermal cells of the pharynx and occasionally the intestine with only 27% of animals showing the response. Using a non-toxic dye they eliminated the possibility that the animals were not feeding due to the presence of the metals and concluded that the lack of response was due to the heterogeneous nature of the heat shock response.

At concentrations above the LC₅₀ value hsp70 induction occurred throughout the cell. Marshall and Kind (1994) also observed this ubiquitous response and suggested that the intracellular distribution of hsp70 depended on the applied stimulus and recovery time. In this study, it was seen that the few cells alive at these lethal concentrations possessed a very strong heat shock response. Using the soluble coloured endpoint, hsp70 inductions similar to those found using SDS-PAGE and ELISA were observed. However, above the LC₅₀ there was a rapid decline in hsp70 due to the increased rate of

cell mortality. The true potential of the heat shock response is at sub-lethal concentrations.

4.10 2-Isobutyl piperidine:

The metals used in this study were chosen as they were known to be toxic and thus their toxicity could retrospectively be measured using the neutral red assay allowing direct comparisons to be made with the heat shock response assay. However, realistically the heat shock response assay would be used to screen chemicals of unknown toxicity. To assess the ability of the heat shock response to determine the potential toxicity of an unknown chemical a novel chemical 2-Isobutyl piperidine was used. 2-Isobutyl piperidine was synthesised and kindly provided by Professor Janus Szfrank at the University of Gdansk in Poland. It is similar in structure to a toxin found in the Colorado potato beetle and was therefore considered potentially toxic. The fact that it is insoluble in water added another variable to the test allowing the ability of the heat shock response to measure the toxicity of chemicals immiscible with water to be assessed. Acetone was used as a solvent (Werner and Nagel, 1997) at a concentration (1%) that neither caused cell mortality or heat shock protein induction. 2-Isobutyl piperidine reacted as a typical toxin with a dose dependent increase, producing a sigmoidal shaped dose response curve. Examining lethal concentrations at 20, 50 and 80% cell mortality showed a linear increase in toxicity.

The heat shock response was also typical of the metal toxins with repression of normal protein synthesis occurring in a dose dependent manner. At the LC_{50} hsp70 represented nearly 17% of the cells total protein. Above the LC_{50} hsp70 induction was reduced as

cell mortality overcame the system, however at this concentration hsp70 induction had an 11% greater response than that of cell mortality. The heat shock response assay showed the most potential in identifying sub-lethal toxicity. At the no effect level hsp70 induction was 40% above the control, this concentration was 2½ times lower than the LC₅₀. The response was very sensitive, hsp70 at the lowest observed effective concentration was 35% above the control measuring sub-lethal toxicity at concentrations 2 times lower than the no effect level. The identification of sub-lethal toxicity by the heat shock response for a novel compound would appear to confirm its potential in the field of toxicology.

4.11 Potential of the heat shock response to assess toxicity:

The repression of normal protein synthesis with increasing toxin concentration enables the easier assessment of hsp70. Cell lines were sensitive to temperature shock inducing hsp70 at physiological relevant temperatures. Induction of hsp70 with cadmium, mercury and copper compared well with other studies. Hsp70 induction by metals has been found to correlate with several studies investigating cellular abnormalities including cancer (Fischbach *et al*, 1993), demonstrating the physiological relevance of the heat shock response. Studies showing tissue specificity by heat shock proteins for metals suggest the possibility of identifying target organ toxicity (Stringham and Candido, 1994). Results generated using the neutral red assay have been shown to correlate with those of animal studies and other cell culture endpoints (Borenfreund and Puerner, 1985; Babich and Borenfreund, 1987). Here it was observed that the induction of hsp70 generated a greater response than that found using the neutral red assay. The induction of hsp70 below the no effect level demonstrated its ability to identify sub-lethal toxicity and the superior sensitivity of the heat shock response compared to that of the neutral red assay. The induction of hsp70 at metal concentrations several times lower than their no effect level demonstrated the potential of the heat shock response for measuring sub-lethal toxicity. Toxins in the true environment are found in complex mixtures with the possibility for interaction increasing the overall toxicity. Induction of hsp70 identified increasing toxicity due to interaction of the metals demonstrating its potential for environmental monitoring. The conserved nature of heat shock proteins particularly hsp70 makes Western blotting an ideal technique allowing for their identification across taxonomic classes. The use of ELISA and immunocytochemistry techniques in conjunction with the heat shock response support and expand the findings

to assess toxicity. The ELISA assay was potentially more sensitive than SDS-PAGE as interference from surrounding proteins and background staining is avoided. The possibilities for automation make this assay very attractive for routine application. The immunocytochemistry assay had the advantage of measuring qualitative or quantitative changes in hsp70 induction, although the assay was found to be suitable for sub-lethal toxicity studies only. A heterogeneous heat shock response was identified at intermediate toxin concentrations which was masked using SDS-PAGE and may account for some of the variability in those results. Finally, the heat shock response was used in an attempt to identify the toxicity of a novel compound. The compound 2-Isobutyl piperidine has a structure similar to a toxin found in the Colorado potato beetle and as such was presumed toxic. Induction of hsp70 was found to have a greater response than that using the neutral red assay. The induction of hsp70 below the no effect level demonstrated the ability to identify sub-lethal toxicity and the superior sensitivity of the heat shock response compared to that of the neutral red assay. The induction of hsp70 at concentrations several times lower than the no effect level demonstrated the potential of the heat shock response for measuring sub-lethal toxicity.

The study of the heat shock response to assess toxicity is in its infancy. The findings of this study suggest the heat shock response has considerable potential in the field of toxicology. The ELISA and immunocytochemistry techniques employed here are more sensitive than existing assays, have biological relevance and are applicable to a wide range of organisms. In addition, they are easy to use, rapid, economical, sensitive and reproducible. Using these techniques the relationship between the heat shock response and carcinogens/mutagens as suggested by Fischbach *et al*, (1993), teratogens as

suggested by Buzin and Bournias-Vardiabasis (1982) and autoimmune diseases as suggested by Watson (1990) could be further elucidated. The immunocytochemistry technique appears to have potential in the study of heat shock protein functions. Ananthan et al, (1980) demonstrated injecting denatured protein in to a cell was all that was required to induce heat shock proteins, possibly by using the immunocytochemistry technique the induction of heat shock proteins could be tracked.

Although numerous studies have examined the heat shock response with respect to medicine and toxicology, very few applications have been developed. Perhaps the development of the ELISA and immunocytochemistry techniques with their potential for automation will result in the heat shock response finding a role in routine applications.

Publications & Presentations:

The use of stress proteins as a biomarker of sub lethal toxicity: Induction of heat shock protein 70 by 2-Isobutyl piperidine and transition metals at sub lethal concentrations. E. Gibney, J. Gault & J. Williams. *Biomarkers*, In press.

Toxicity testing using production of stress proteins in cultured cells as an indicator. E. Gibney, J. Gault & J. Williams. *The Irish Scientist Year Book*, pp 72, 1997.

7th Annual Environmental Researchers Colloquium, University of Limerick, 1997.
Expression of stress proteins in cultured cells, a measure of heavy metal toxicity.
E. Gibney, J. Gault & J. Williams.

2nd Annual Science Research Colloquium, Institute of Technology, Sligo, 1997. Stress proteins as biomarkers of environmental pollution. E. Gibney, J. Gault & J. Williams.

Academy of Biomedical Sciences Annual National Conference. 1996. The potential of stress proteins to measure of environmental pollution. E. Gibney, J. Gault & J. Williams.

Appendix 1.

Table 1: Types of experiments using animals.

Experiment	Reference
Kidney Function	Tisher, 1970
Atherosclerosis	Howard et al, 1972
Aggression	Bammer and Eichelman, 1983
Acute Toxicity Test	Humane Society of the USA, 1984
Poisoning	Seale <i>et al</i> , 1984
Hypothermia/hyperthermia	Vincent <i>et al</i> , 1984
Induction of cancer	Dagle <i>et al</i> , 1984
Heart disease	Kohn and Barthold, 1984
Diseases	Dobson, 1987
Burns/fire-related injury	Wretland, 1987
Hypertension	Eichberg and Shade, 1987
Cancer	Lave <i>et al</i> , 1988
Blasts	Phillips, 1988
Hepatitis B	Ponzetto <i>et al</i> , 1988
Sensory deprivation	Mriganka <i>et al</i> , 1988
Stroke	Wiebers <i>et al</i> , 1990
AIDS	Ward <i>et al</i> , 1991
Reproduction	Steinetz <i>et al</i> , 1992
Inflammation of the eye	Wentworth <i>et al</i> , 1993
Radiation	Geraci <i>et al</i> , 1993
Smoking	Penn and Snyder, 1993
Electrical shock	Woodmansee <i>et al</i> , 1993
Gastrointestinal ulcers	Yi <i>et al</i> , 1993
Swimming in freezing water	Paré, 1994
Dissection	National Anti-Vivisection Society, 1994
Sleep deprivation	Porkka-Helskanen <i>et al</i> , 1997

Appendix 2.

Table 2. Number of animals used in the European Union in 1996.

Country	Numbers
Austria	204,825
Belgium	1,515,867
Denmark	350,226
Finland	110,659
France	2,609,322
Germany	1,509,619
Greece	19,280
Ireland	77,107
Italy	1,094,185
Luxembourg	1,003
Netherlands	652,300
Portugal	49,520
Spain	506,837
Sweden	786,012
United Kingdom	2,659,368
Total	12,146,130

Adapted from European Commission (1999).

Appendix 3.

Table 5: Applications of the heat shock response:

Application	Reference
Gene expression	Mirault <i>et al</i> , 1978
Heavy metal toxicity	Levinson <i>et al</i> , 1980
Fever	Brown <i>et al</i> , 1982
Acquired tolerance	Subjeck and Sciandra, 1982
Thermotolerance	Li <i>et al</i> , 1982
Viral activation	Yonemoto <i>et al</i> , 1982
Effects of pesticides	Werner <i>et al</i> , 1987
Alcoholism	Walsh and Crabb, 1989
Chronic liver disease	Zatloukal <i>et al</i> , 1988
Environmental monitoring	Sanders, 1990
Oxidative stress	Watson, 1990
Cardiotoxicity	Low-Friedrich <i>et al</i> , 1991
Affects of aging	Luce <i>et al</i> , 1993
Cellular metabolism	Nissim <i>et al</i> , 1993
Target organ toxicity	Stringham <i>et al</i> , 1994
Environmental pollution	Muller <i>et al</i> , 1995
Genetic defects	Vogel <i>et al</i> , 1995
Environmental Adaptation	Brown <i>et al</i> , 1995
Chemical ingestion	Witzmann <i>et al</i> , 1995

Appendix 4:

Mouse connective tissue cell line: (L929)

Mouse connective tissue cell line is fibroblastic-like (Figure 3A). An inoculum of $6-8 \times 10^5$ cells in 3ml of Dulbeccos modified Eagles media multiplies approximately 8-9 fold within 7 days at 37°C provided the medium is renewed 3 times weekly and the pH is maintained at 7.3. Subcultures are prepared by the trypsinisation method.

Clone 929 was derived in 1948 from the parental strain established in 1940 by W.R. Earle (Sanford *et al*, 1948). Strain L was one of the first strains to be established in continuous culture, and clone 929 was the first cloned strain developed. The parent L strain was derived from normal subcutaneous areolar and adipose tissue of a 100 day old, male C3H/AN mouse, and clone 929 was established from the 95th subculture generation of the parent strain. Karyology, chromosome frequency distribution 100 cells: $2n = 40$, long metacentric chromosome with secondary constriction noted in 77/100 cells. The species was confirmed as mouse by mixed agglutination and hemagglutination tests. the L929 cell line is susceptible to pseudorabies virus and vesicular stomatitis virus but not to poliovirus type 1, Coxsackie virus type B-5 or polyoma virus. No tumours were observed in non irradiated mice after intramuscular injection however 11/18 tumours were produced in x-irradiated mice. The cells are reverse transcriptase positive (Sanford *et al*, 1948).

Appendix 5:

Normal rat kidney cell line: (NRK-52E)

Normal rat kidney cell line is epithelial-like (Figure 3B) and was cloned from a mixed culture of normal rat kidney cells. Since its origin in 1978, the cell line has been cultured in 95% Dulbecco's modified Eagles medium with non essential amino acids and 5% calf serum. The NRK-52E cells are distinct from their the NRK-49F cells being much more stable in culture, the latter having a fibroblastic morphology. The cell line was validated by cytogenetics and isoenzymes. The NRK-52E were found to be reverse transcriptase negative (DeLarco and Todaro, 1978).

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