RAPID QUANTITATIVE EVALUATION OF WATER QUALITY USING A MODIFIED BIOLOGICAL TEST

LH Damelin • JJ Alexander

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RAPID QUANTITATIVE EVALUATION OF WATER QUALITY USING A MODIFIED BIOLOGICAL TEST

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by

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EXECUTIVE SUMMARY

1. INTRODUCTION

There is an international movement towards the development of cheap and reliable biological toxicity assays particularly with regard to water quality assessment. Those currently in use involve the acute biological responses in fish or aquatic invertebrates, with death of the organism/s within 96 hours a measurement of acute toxicity. However, these tests, in the presence of sublethal levels of toxicants are unreliable and chronic long-term effects on populations exposed to sublethal concentrations are as important as acute toxicity.

Many tests have been proposed, and in some cases are commercially available (at great expense) which rely on various biochemical or physiological bioassays.

1.1 OBJECTIVES

1. The research was undertaken in order to develop a reliable, rapid biological assay which would enable the measurement of both acute and chronic toxicity.

2. Small volumes of water samples should be used so that duplicate or triplicate assays would be feasible.

3. Laboratory studies should be undertaken to assess the biochemical/molecular events which occur and identify the sites of action within the cells.

4. The research would primarily focus on heavy metal toxicity (with particular attention to biomarkers of chronic toxicity).

5. The effects of chelation of various heavy metals would be assessed to determine the increase or decrease in sensitivity on the test system.

6. Generalised mathematical models of biological stress would be derived.

2. In vitro **Bioassay**

A tissue culture system, using a well characterized mammalian cell line, McCoy 5A cells, was used. Cells were seeded into 96 well microtitre plates and grown in defined tissue culture medium. Stock cell cultures were maintained in tissue culture flasks and trypsinised when needed for dispersion, at fixed concentrations, into the microtitre plates.

Tetrazolium salts (white to yellow water soluble compounds) when reduced at neutral pH produce insoluble, highly coloured formazan granules. Reduction occurs due to the biological action of dehydrogenases within living cells, thus the degree of reduction is proportional to the viability and metabolic activity of energy pathways within living cells. Formazan granules can be solubilized in organic solvents and concentrated in light oils.

The highly coloured "bead" which develops can then be measured spectrophotometrically at the correct wavelength. A microtitre plate reader was used to determine absorption values.

2.1 Experimental procedure

Trypsinized McCoy cells, seeded into microtitre plates, were exposed to varying concentrations of heavy metal salts, with or without chelating agents, and incubated for periods of up to 24 hours. The tetrazolium salt (3-(4,5 dimethylthiazol-2yl)-2,5 diphenyl tetrazolium bromide (MTT) was added to the wells for 4 hours, then the formazin precipitates were solubilized and concentrated and absorbance measured at 570nm. All necessary controls were included. Electron microscopy was carried out on normal cells and cells exposed to low levels of mercury and mercury plus a ligand to identify any morphological changes which may occur.

2.2 Results and Discussion

The cytotoxicity of all metals tested had similarities in that one or more peaks of activity occurred at low concentrations of metals, frequently followed by a plateau and then a steep decline in activity at acute toxic concentrations (with the exception of Ferric chloride, which is poorly taken up by living cells). The peaks of activity were referred to as hormesis (see 3 below).

The ligand 2-mercaptoethanol in combination with mercuric chloride resulted in a 2-log

fold increase in cytotoxicity, and a less dramatic, but significant, increase in cytotoxicity of cadmium chloride.

Electron microscopy of cells exposed to low levels of mercuric chloride in the presence and absence of 2-mercaptoethanol showed that mercury alone caused a 36% reduction in cell size, margination of nuclear chromatin and had the generalised appearance of apoptotis (programmed cells death) which is a genetically controlled event. Mercury, in combination with the ligand, however, showed cells having all the hallmarks of necrosis which is a genetically uncontrolled event resulting from catastrophic injury.

The peak activity noted for each metal occurred at defined concentrations. Thus time versus concentration studies were done for each metal to investigate whether increased activity was a specific response affected by concentration in a time dependent manner. A plot of concentration versus time at which maximum activity occurred gave an exponential curve. Mathematical derivation of the curves derived for each metal tested gave rise to values which are specific for each metal group or metal/ligand complex, e.g. similarities for zinc, cadmium and mercuric chloride which belong to the same periodic group; a value for mercury/2-mercaptoethanol indicating increased toxicity, and values for ferric chloride indicating low uptake and low toxicity.

3. HORMESIS

The phenomenon of hormesis was described in the 19th Century. It is an increase in biological activity in the presence of trace amounts of toxins or inhibitors or as a response

to low levels of ionizing radiation.

As indicated above, this phenomenon was observed at low levels of toxic metals; that is, there was an increase in formazan reduction in cells exposed to "chronic" levels of metals. Hormesis was more recently noted by Roux *et al* (1993) in daphnids exposed to low levels of copper and cadmium - an increase in activity followed by a significant decrease in survival after prolonged exposure. We therefore explored the possibility that the "hormesis" we observed could provide biochemical evidence that might serve as an early and reliable indication of a stress response after exposure to low or chronic levels of toxic metals.

3.1 Heat shock proteins

All biological systems studied in the presence of stress (heat, cold, irradiation) produce increased levels of a range of stress indicators known as heat shock proteins. Prominent amongst this family is the heat shock protein 70 (Hsp 70).

3.2 Experimental procedure

Proteins and cells exposed to hormetic and higher levels of toxicants were extracted and run on polyacrylamide gels, the proteins transferred to nitrocellulose membranes (Western blotting) and reacted with mouse anti Hsp 70 monoclonal antibodies. Hsp 70 bands were visualized using enhanced chemiluminesence, and band intensity was quantified using laser densitometry.

Normal (unexposed) cells and a subline of cells rendered resistant to cadmium, were included. Since metallothionien and superoxide dismutase are also believed to protect against the cytotoxic effects of metals, assays for these proteins were included.

3.3 Results and Discussion

Pronounced protein bands were identified as the 70KDa heat-shock proteins Hsp 70 when cells were exposed to hormetic, plateau and lethal concentrations 50% (LC_{50}). Up to 10-fold concentrations were measured compared with the levels measured in untreated controls.

Metallothionein concentrations, exposed to hormetic levels of cadmium chloride, rose to double the concentrations measured in untreated controls. In time/activity assays both Hsp 70 and metallothionein reached maximum production at 16 hours post exposure.

Cadmium resistant cells, on the other hand had a constitutively high level of heat shock protein production over the range of cadmium concentrations, with a dramatic drop in production only at very high levels ($\geq$ 10μg/ml) of cadmium.

In contrast superoxide dismutase increased significantly but slightly at plateau levels of toxicant in normal cells; in cadmium resistant cells dismutase activity only increased significantly at LC_{50} and higher concentrations of cadmium.

In general, it was clear that the onset of hormesis coincided with elevated stress protein levels (particularly heat shock protein 70 and metallothionein) and that this activity was a specific response to a build-up of concentration of metal within the cell over time.

4. CONCLUSION

This study has shown that a rapid colourimetric assay can be used to quantify the degree of cytotoxicity for cells exposed to heavy metals. When metals plus ligands/chelators were used, the assay demonstrated that changes in cytotoxicity were due to the changes in metals speciation.

The sensitivity of the assay was such that, at sub-inhibitory metal concentrations, increased or hormetic activity occurred. Time/activity studies showed that hormesis was dependent on toxin concentration in an exponential level and required a critical intracellular concentration to initiate hormesis, and that this followed first order kinetics, suggesting that hormesis was a specific cellular response. This was confirmed by studies which correlated the onset of hormesis to the production of threshold levels of heat shock protein 70 and metallothionein in normal cells, and high constitutive production of these stress proteins in mutant cells rendered resistant to cadmium.

Hormesis is possibly a form of energy compensation initiated by cells exposed to extreme environmental conditions which require production of high levels of stress proteins over a short time period. That is, steady-state energy levels may be temporarily disturbed and

a burst of energy production (hormesis) serves to restore energy homeostasis to the cell.

5. FUTURE RESEARCH

5.1 This rapid, inexpensive test which requires only small volumes of water samples, could be extended to an examination of environmental waters.

5.2 Any hormetic increase in formazan production in such samples (which has been shown to correlate with increased stress protein production) would possibly indicate the presence of a toxicant at sub-toxic levels.

5.3 This test, too, will indicate an acute toxic level of biologically harmful heavy metals.

5.4 This test should be extended to examine the effects of other toxins, besides heavy metals, on the phenomenon of hormesis.

5.5 This test may serve as a generalised assay for the detection of a wide range of known toxins and also for the presence of an increasing number of xenobiotics.

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CHAPTER 1

INTRODUCTION

1.1 METAL TOXICITY

The utilization of metals such as silver prior to 2000 B.C. and the consequent exposure of smelters to toxic by products, such as lead and arsenic, possibly make metals the oldest toxins known, a belief further reinforced by descriptions of illnesses associated with metal exposure such as abdominal colic in metal extractors described by Hippocrates as early as 370 B.C. (Goyer, 1989).

Most metals which effect multiple organ systems target biochemical processes, enzymes or specific sites within the membranes and organelles of cells (Goldwater and Clarkson, 1975; Goyer, 1989). The toxic effect of a metal often involves an interaction between free metal ions and the toxicologic target and examples of such interactions include the inhibition of succinate dehydrogenase by arsenic, the inhibition of the electron transport chain by hexavalent chromium compounds as well as the non-specific binding of mercury

to the sulfhydryl groups of enzymes (Fassett, 1975; Smith, 1975 and Falchuk *et al.*, 1977).

The potential toxicity of any chemical agent is related to the concentration of the chemical to which an organism is exposed together with the quantity absorbed and the duration of exposure (Rand and Petrocelli, 1985). Therefore the dose, or quantity of metal within organs or cells manifesting a toxicologic effect, is a multidimensional concept and is a function of both time and concentration (Pfitzer, 1975; Goyer, 1989). At the cellular level, toxicity is also related to metal availability so that chemical form and ligand binding become critical factors (Jonnalagadda and Prasada Rao, 1993). Alkyl compounds, for example, such as methyl mercury, pass readily across biological membranes due to lipid solubility (Falchuk *et al.*, 1977). These compounds are also excreted from cells at slower rates relative to inorganic salts. Therefore, organometallic toxicity can be expected to differ from that of inorganic compounds (Goldwater and Clarkson, 1975).

Chelation, the formation of a metal ion complex in which the metal ion is associated with a charged or uncharged electron donor referred to as a ligand, is another important factor affecting the availability of metals (Albert, 1965; Dickerson *et al.*, 1984). Chelating agents enhance the uptake or excretion of a wide range of metals and are also able to alter the reduction potential of ions

with variable valencies. This, as well as electronic redistribution and geometrical changes upon chelation, result in a change in the reactivity of the metal ion and may lead to changes in metal toxicity (Albert, 1965; Shriver *et al.*, 1990).

1.2 TOXICITY BIOASSAYS

As the byproducts of industrial processes and mining activity, metals enter aquatic and other ecosystems via atmospheric fallout, rivers, and dumping. Heavy metals in particular present serious problems for aquatic environments with mercury, lead and cadmium generally considered to be the most hazardous to both humans and aquatic organisms while copper, zinc, silver and chromium also pose significant dangers (Forbes and Forbes, 1994).

For this reason, a need exsists for the development of rapid reproducible and economical bioassays that are able to quantify the toxic effects of metals and serve as biological indicators of effluent and receiving-water quality, particularly for the establishment of quality guidelines for aquatic environments (Roux *et al.*, 1993).

Most rapid bioassays currently in use have been designed to detect acute

responses in fish or invertebrates, where acute is defined as a condition involving a stimulus severe enough to rapidly induce a biological response (American Institute of Biological Sciences 1978). In aquatic toxicity tests, a response observed within 96 hours is typically considered an acute test with death being the most common effect observed (Forbes and Forbes, 1994). However, although acute lethality tests using Lethal Concentration 50% (LC_{50}) or similar measures as end points have formed a major part of the assessment of ecological risks for potential pollutants, and have been acclaimed as the most useful of all available toxicity tests in the past (Cairns *et al.*, 1978), the survival and growth of aquatic organisms exposed to levels of toxicants far lower than those which induce acute responses, over longer time intervals, have been clearly shown to be adversly effected by these sublethal toxicant concentrations (Roux *et al.*, 1993). It has therefore become widely accepted that the sublethal or chronic effects of pollutants are as important to the continued success of a population as have the lethal effects (Kinne, 1980).

Thus the recognition that death is a crude end point coupled with the poor correlation between acute and chronic effects (Sloof *et al.*, 1986; Forbes and Forbes, 1994), has led to the development of biochemical and physiological bioassays based on more sensitive indicators of damage, known as biomarkers (Peakall, 1992). Examples of such indicators include the measurement of lysosomal stability and mixed-function oxygenase activity particularly for the

detection of organic toxicants (Moore, 1980; Stegeman, 1980), as well as adenylate energy charge which reflects the metabolic potential available to an organism (Ivanovici, 1980). Examples of physiological indicators include the measurement of respiration or ion regulation (Hutcheson *et al.*, 1985; Forbes and Depledge, 1992), as well as the measurement of physiological energy balance to assess the degree of stress to which an organism is exposed (Koehn and Bayne, 1989).

Although all these assays have found use in research, a number of disadvantages which include low reproducibility, a need for expensive and complex equipment, specificity of response to a species or phyletic group, variation between species, laborious and time consuming methodology and very often the inability of a biomarker to provide more then a general indication of stress has meant that few have been employed in regulatory decision making which requires that assays be simple to perform and readily amenable to standardization (Forbes and Forbes, 1994).

These factors, as well as a trend to minimize the use of laboratory animals when quantitative information regarding dose and corresponding concentration of metals in cells and tissue is required, has led to the development and use of *in vitro* alternatives (Goldberg *et al.*, 1993).

1.3 *IN VITRO* TOXICITY TESTS

In vitro toxicity assays based on tissue culture model systems have proceeded rapidly over the past decade and are used to quantify the degree of cytotoxicity or cell proliferation in the presence of known or unknown toxins (Gerlier and Thomasset, 1986). These tests consist of a fixed quantity of mammalian cells which are exposed to varying concentrations of toxins for a fixed period of time.

The measurment of surviving cells is then achieved by several methods such as dye inclusion or exclusion, the degree of Cr^{51} release and the extent of incorporation of radioactive nucleotides (Carmichael *et al.*, 1987). Methods utilizing radioactivity can be partially automated, but are expensive, time consuming and hazardous. Thus, an alternative more efficient system based on the ability of dehydrogenases within viable cells to transform colourless tetrazolium salts to coloured insoluble formazan precipitates has found wide application (Möllering *et al.*, 1974).

Tetrazolium salts are 2,3,5-aromatic-substituted derivatives of 1,2,3,4-tetrazole. They can exsist in both mono or ditetrazolium forms and are generally white to yellow water soluble compounds. Irreversible reduction of

the tetrazole ring is achieved at neutral pH by the addition of hydrogen from NAD(P)H yielding deeply coloured sparingly water-soluble formazans. In practice however, NAD(P)H can only directly reduce most tetrazolium salts to a very small extent and therefore auxiliary reactions involving dehydrogenases at specific locations within the cell are required (Möllering *et al.*, 1974; Berridge *et al.*, 1996).

Formazan precipitates can be solubilized in non-polar solvents and concentrations determined spectrophotometrically while the use of multiwell scanning spectrophotometers (ELISA readers) for cells seeded in multi-well tissue culture plates, allows for the processing of a large number of samples (Mosmann, 1983).

Although tetrazolium-based colorimetric assays have been used extensively in cytotoxicity studies, having proved effective in the quantification of the degree of cytotoxicity of a number of toxicants and chemotherapeutic agents for various cell lines, (Scuderio *et al.*, 1988), little is known of the kinetics of these cytotoxic effects, or the effects of metal speciation on the sensitivity of these assays.

In addition, typical cytotoxicity curves of cellular activity versus toxin concentration often exhibit regions of high activity, with respect to control

cells, at toxin concentrations significantly lower than those which are inhibitory (Whitcutt, personal communication).

Similar observations were noted by Schulz as early as 1888 when yeast exposed to a variety of toxic agents at low concentrations exhibited increased levels of respiration (Schulz, 1888). These findings ultimately led to the establishment of the Arndt-Schulz law which stated that substances capable of inhibiting biological processes at sublethal concentrations could be expected to stimulate them at lower levels. In 1982 Stebbing referred to the stimulation of growth in organisms by low levels of inhibitors as hormesis. He suggested that this phenomenon could be the result of over-corrections by cellular control mechanisms to low levels of inhibition resulting in higher than normal growth or activity (Stebbing, 1982).

More recent toxicity studies, however, have shown that daphnids exposed to low, specific concentrations of copper and cadmium initially exhibited increased activity whereafter a significant decrease in the survival rate of test organisms was found to occur with longer exposure to these stimulatory levels of metal (Roux *et al.*, 1993). This raised the question as to whether hormesis was a specific cellular response to low but harmful levels of toxic agents and could therefore have potential use as a rapid biomarker or indicator of toxicant concentrations capable of inducing chronic toxicity.

It has therefore been the purpose of this study to investigate the cytotoxicity of heavy metals alone and in combination with chelating agents using a rapid standardized tetrazolium-based assay as well as to investigate the sites of action of metal ions in cells and the effect of chelating agents in the transport of metal ions to these sites.

It has also been the purpose of this study to investigate hormetic activity at a molecular level in an attempt to determine the mechanisms responsible for this phenomenon as well as to determine its potential use as a stress indicator in cells exposed to sublethal but damaging toxin concentrations.

1.4 AIMS

(a) To investigate the cytotoxicity and sites of action of heavy metals alone and in combination with chelating agents using a rapid standardized MTT colorimetric assay and electron microscopy.

(b) To investigate the phenomenon of hormesis in an attempt to determine its potential as an indicator of stress in cells exposed to sublethal toxin concentrations.

CHAPTER 2

AN INVESTIGATION OF THE CYTOTOXICITY AND SITES OF ACTION OF HEAVY METALS USING A RAPID MTT ASSAY AND ELECTRON MICROSCOPY

2.1 INTRODUCTION

In 1983 Mosmann described a colorimetric assay based on the tetrazolium salt 3-(4,5 dimethylthiazol-2yl)-2,5 diphenyl tetrazolium bromide (MTT) which allowed for the rapid measurement of cell proliferation and cytotoxicity (Mosmann, 1983; Gerlier and Thomasset, 1986). Although this colorimetric method had several advantages over radioactivity assays, the often incomplete solubilization of MTT formazan by isopropanol resulted in a reduction in sensitivity of the assay and it was subsequently modified by the replacement of isopropanol with other solvents like mineral oil, dimethyl sulphoxide (DMSO) or dimethyl formamide (Carmichael *et al.*, 1987).

This however required the removal of culture media from wells prior to solvent addition and attempts to use tetrazolium salts that yield water-soluble formazan

products, such as XTT or WST-1 required the addition of unstable electron coupling agents such as phenazine methosulphate while unlike MTT, which was found to be dependent on the rate of glycolytic NADH production, the reduction of these salts was shown to occur extracellularly and required dehydrogenase-independent tetrazolium reactions involving superoxide ions, which was not necessarly indicative of metabolic activity (Scudiero *et al.*, 1988; Berridge *et al.*, 1996).

Recently, a modified Mosmann assay was developed which circumvented these problems by the inclusion of a novel solubilization step that simultaneously solubilized and concentrated the resultant MTT formazan within a non-polar solvent/detergent mixture that could be added directly to the wells without prior media removal (Alexander *et al.*, unpublished results). This resulted in a significant increase in both the sensitivity and reproducibility of the assay as well as allowing for the measurement of cellular activity in the presence of toxicants at trace levels (Fig. 2.1).

However, as changes in the sensitivity of the assay were found to occur when heavy metals in combination with trace quantities of ligands or chelating agents were investigated, it was important to determine whether such changes were due to the effects of metal speciation on cytotoxicity, or due to the interference of MTT reduction by metal/ligand complexes.

Therefore in order to investigate the effects of chelating agents on the cytotoxicity of metals as well as to obtain a better understanding of the kinetics of cellular activity at low metal concentrations, the cytotoxicity of several metal chlorides was investigated alone and in combination with low

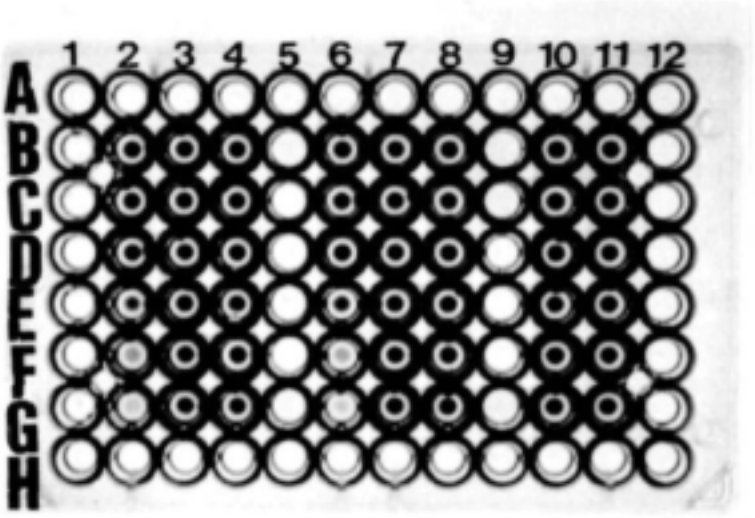

Fig. 2.1 **A modified Mosmann assay for cupric chloride.**

The addition of a non-polar solvent/detergent mixture has solubilized and concentrated the resultant MTT formazan, yielding intensely coloured beads within each test well.

(Left to right) - Columns 2, 3, 4 and 6, 7, 8 contain doubling dilutions of cupric chloride from 50μg/ml (G2 and G6) to $3.5x10^{-3}\mu$g/ml (B4 and B8). Columns 10 and 11 are untreated controls.

levels of chelating agents/ligands using the above mentioned rapid MTT-based colorimetric assay at fixed and varying time intervals, while the sites of action of mercury and the effect of low levels of the ligand, 2-mercaptoethanol on the transport of this metal to those sites was investigated using transmission electron microscopy.

2.2 MATERIALS AND METHODS

2.2.1 Cell Lines and Culture

McCoy 5A mouse cells obtained from Highveld Biological Association were maintained in mimimum essential medium (MEM) (Highveld Biological) supplemented with glutamine, Earle's salts, 5% fetal calf serum and 100μg/ml penicillin, 100μg/ml streptomycin solution (Highveld Biological) at 37°C. Cell cultures were split once a week in a 1:6 ratio using a 0.2% v/v trypsin solution in phosphate buffered saline (PBS) combined with a 0.1% w/v ethylene diamine tetraacetic acid (EDTA) solution in PBS, in a 1:1 ratio to detach cells from culture flasks.

2.2.2 *Colorimetric MTT Assay*

McCoy 5A cells trypsinized as above, were seeded into 96 well tissue culture plates (Nunclon) at 8000 cells per well in 100μl MEM supplemented as above. Plates were incubated for 18 hours at 37°C and 5% CO_2 in air. Heavy metal salt solutions to be tested were two fold serially diluted in a master plate with MEM and 100μl of each dilution was then further reduced to 50% of its original concentration when transferred to a corresponding well of the tissue culture plate. The cells were then incubated as before for a further 24 hours. Following incubation, 10μl of a 5mg/ml solution of 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) (Sigma) in 0.01M PBS was added to each well and the incubation continued for an additional 4 hours. Cells were then solubilized by the addition of 10μl 3N HCL and 10μl of 10% NP-40 solution (Unilab) to each well. Following this the formazan precipitate was solubilized by the addition of 25μl of a 2:1 paraffin oil : carbon tetrachloride solution containing 0.1% v/v NP-40. Plates were stored at 4°C for 18 hours and read on a Biorad ELISA plate reader using a 570nm filter. The absorbance results were plotted using non treated control samples to determine the 100% activity level.

2.2.3 Mercury Assay

A 40μg/ml solution of mercuric chloride in PBS was serially diluted to a final dilution $6*10^{-4}$ μg/ml and each dilution utilized in an MTT assay as described

in 2.2.2. In addition an identical test was carried out but with 10μl of a $8*10^{-7}$ M 2-mercaptoethanol solution in PBS, added to each well. Both tests were carried out in triplicate.

2.2.4 Copper Assay

A 100μg/ml solution of cupric chloride in PBS was serially diluted to a final dilution of $7*10^{-4}$ μg/ml and each dilution utilized in an MTT assay as described in 2.1.2. In addition, an identical test was carried out but with 10μl of an $8*10^{-7}$ M solution of ethylene diamine added to each well. Tests were performed in triplicate.

2.2.5 Zinc and Cadmium Assays

100μg/ml Solutions of cadmium and zinc chloride were diluted to a final volume of $7*10^{-4}$ μg/ml and then each dilution utilized in MTT assays alone and in combination with 2-mercaptoethanol as described for mercury.

2.2.6 Ferric Assay

Ferric chloride was utilized in an MTT assay as described for copper however

ethylene diamine was substituted by EDTA.

2.2.7 Time/Activity Assays

The relationship between the onset of hormetic activity and metal concentration was determined by MTT assays described in 2.2.2 using cupric, mercuric or cadmium chloride at concentrations of 0.4, 0.8, 1.6 and 3.2μg/ml. Plates were incubated for 12 hours after which 10μl of a 5mg/ml solution of MTT in PBS was added to succesive wells at 72 minute intervals over a further 12 hour period. Solubilization of cells in succesive wells was carried out 2 hours after each MTT addition.

2.2.8 Electron Microscopy

McCoy 5A cells were incubated as before in 25cm^2 flasks for 3 days until 50-60% confluency was obtained. Cells were then given fresh medium spiked with 1.25μg/ml mercuric chloride or 0.625μg/ml mercuric chloride in combination with $8*10^{-7}$M 2-mercaptoethanol while additional flasks given medium free of toxicant served as controls. All flasks were incubated at 37°C for 24 hours. Cells were prepared for transmission electron microscopy by a modification of the method described by Hayat (1972). Cells were prefixed with 4% gluteraldehyde in 0.01M PBS (pH 7.2) on ice for 1 hour and removed from the

surfaces of flasks with a cell scraper. The resulting suspensions were pelleted with a bench top centrifuge at 1200rpm for 2 minutes and pellets were washed 5 times with ice cold PBS. Cells were postfixed with 1% osmium tetroxide solution in PBS for 1 hour and washed a further 5 times with ice cold PBS. Pellets were dehydrated in a graded series of ethanol and propylene oxide solutions and then embedded in araldite epon resin. Blocks were allowed to harden at 60°C for 18 hours and were sectioned on a microtome with a glass knife. Sections were double stained with 0.5% w/v uranyl acetate and lead citrate solutions and then examined with a model JEM-100S transmission electron microscope at an operation voltage of 80KV.

2.3 RESULTS AND DISCUSSION

2.3.1 Cytotoxicity Assays for Metals Alone and in Combination with Chelating Agents

The cytotoxicity curves of absorbance versus the log of metal concentration (μg/ml) for ferric, cupric, cadmium, zinc and mercuric chloride alone and in combination with chelating agents are presented in figures 2.2-2.6 respectively.

The cytotoxicity curves for all metals tested exhibited similar characteristics,

and consisted of one or more peaks with activities at least 10-15% greater than that exhibited by non-treated control cells. These peaks were present within an approximate metal concentration range of 0.002 to 0.2μg/ml and were generally followed by plateaux or troughs with activities at or below that of the control. The succesion of peaks and troughs was followed by a steady decline in activity with increasing metal concentration for all metals with the exception of ferric chloride which exhibited low toxicity at concentrations as high as 100μg/ml indicating poor cellular uptake of ferric ions (Fig 2.2).

This is in aggreement with the findings that iron in its ferrous state is required for cellular uptake via transferrin (Goyer, 1989). It is therefore possible that ferrous ions formed during the assay from the reduction of ferric ions by media constituents may have been sufficient to stimulate cellular activity but was not present at a high enough concentration to elicit significant toxicity via the formation of reactive oxygen intermediates (Fehér *et al.*, 1987; Cairo *et al.*, 1996). The stimulation of cells by ferrous ions is further supported by the cytotoxicity curve for ferric chloride in the presence of EDTA (Fig 2.2) which exhibited no significant activity over the entire concentration range. As EDTA forms an extremely stable chelate with ferric ions ($K_{my} = 1.3*10^{25}$) (West *et al.*, 1992), the reduction of ferric ions in the presence of this chelating agent would have been significantly inhibited resulting in little or no ferrous ion formation (Albert, 1965), and thus the absence of increased cellular activity.

The presence of trace amounts of the ligands ethylene diamine with cupric chloride and 2-mercaptoethanol with mercuric, cadmium and zinc chloride was found to either have a significant or minimal effect on the cytotoxicity of these metals.

Ethylene diamine induced a region of constitutive activity over a cupric chloride concentration range of 0.001 to 0.7μg/ml (approximately 8% greater than non-treated control cells) as well as a significant reduction in cytotoxicity at higher copper concentrations when compared to the cytotoxicity curve for cupric chloride alone (Fig 2.3). As copper toxicity has been attributed to the oxidation of the sulphhydryl groups of proteins by the metal in its divalent state as well as to the production of reactive oxygen intermediates (Steinebach and Wolterbeek, 1994), the presence of ethylene diamine most likely prevented these deleterious effects by the formation of stable complexes with cupric ions which have significantly lower reduction potentials than the ion itself (Albert, 1965), thus greatly reducing its oxidising ability as well as making less ions available for the catalysis of superoxide formation.

2-Mercaptoethanol exserted little effect on the cytotoxicity of both cadmium and zinc chloride as the curves for each respective metal with trace quantities of the ligand appeared almost identical to those of the metals alone (Fig 2.4 and

2.5), with the only notable difference being the presence of a third peak or a slightly extended but irregular plateau just prior to the steady decline in activity at high cadmium concentrations, suggesting a slight decrease in toxicity of this metal chloride in the presence of 2-mercaptoethanol.

The effect of the ligand on mercuric chloride however resulted in a dramatic increase in cytotoxicity which was almost 2-log fold greater than that observed for mercuric chloride alone. Since the affinities of zinc, cadmium and mercury for thiol groups follow the order Zn< Cd< Hg (Hunziker and Kägi, 1985), it seems likely that zinc would have had a weak association with the thiol group of 2-mercaptoethanol which may have been further compromised by the acidity of the media due to the often high build up of CO_2 during incubation periods, resulting in competition of metal ions with protons for available thiol groups. The slight decrease in toxicity of cadmium suggests that a greater affinity of the ion for sulphhydryl groups compared to zinc resulted in some ligand/metal complex formation which in turn may have inhibited cellular uptake to a degree.

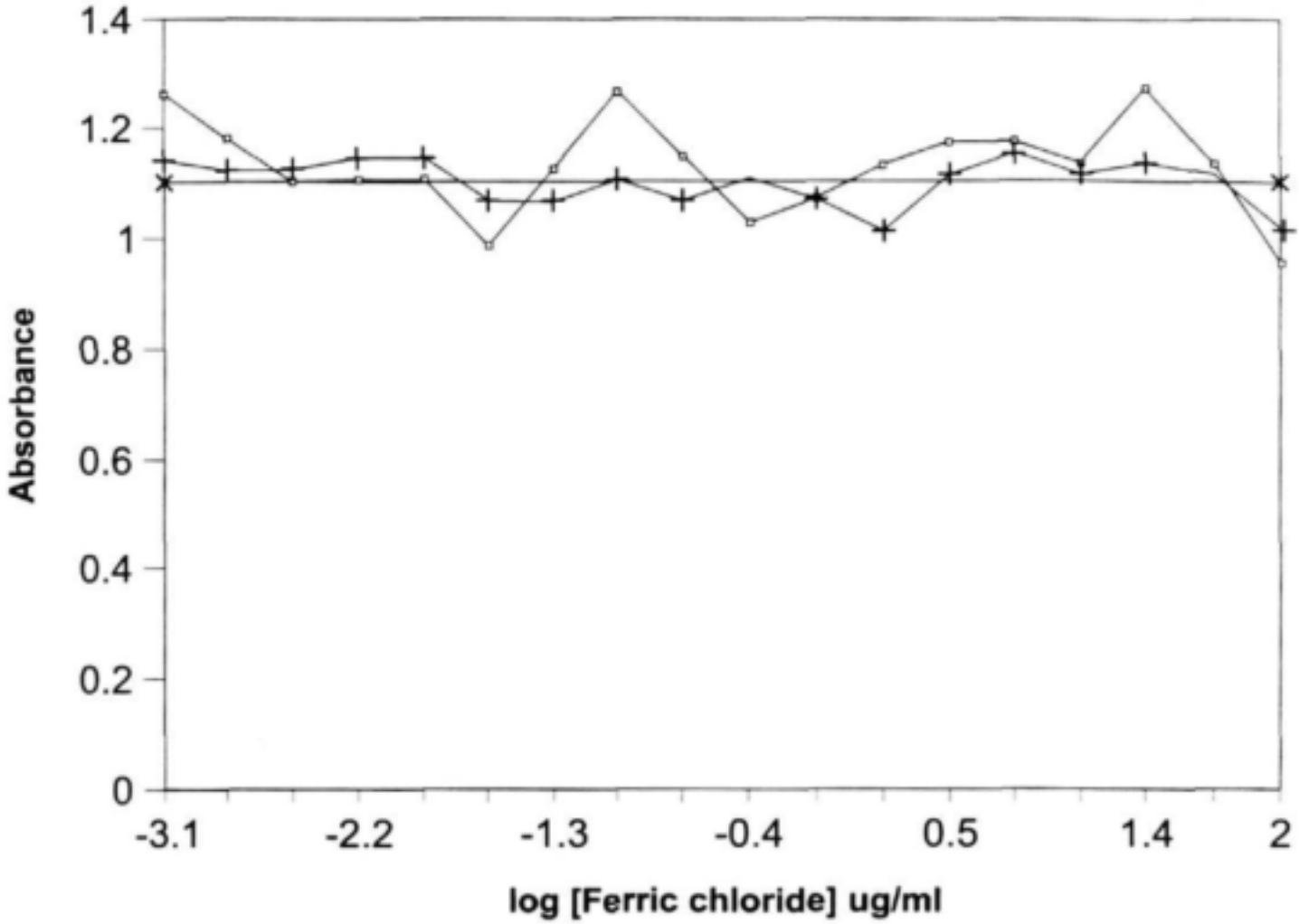

Fig. 2.2 **Cytotoxicity curve for ferric chloride alone and in combination with EDTA.**

(□) Ferric chloride, (+) Ferric chloride and EDTA ($8x10^{-7}$M).

(×) 100% control activity.

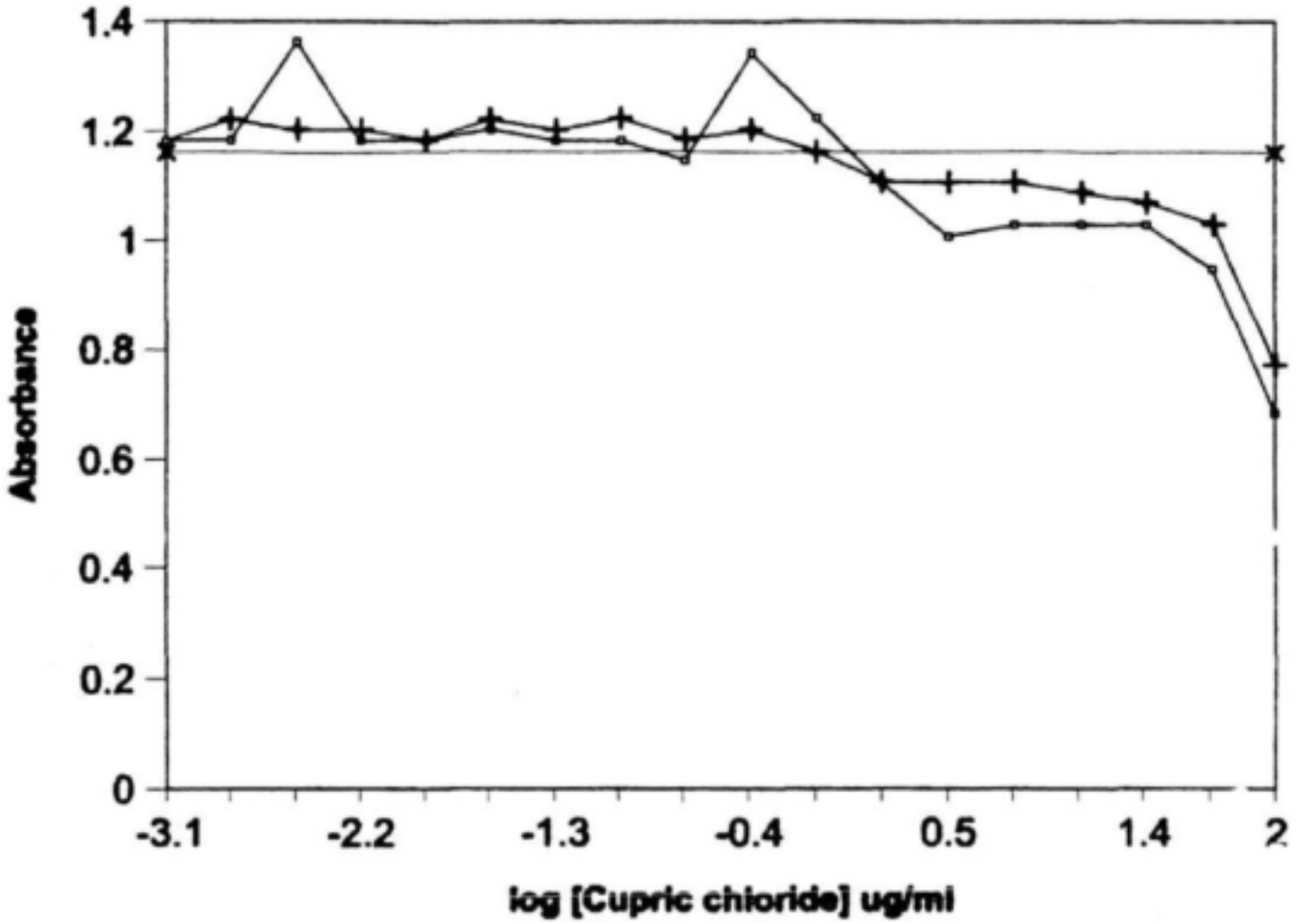

Fig. 2.3 Cytotoxicity curve cupric chloride alone and in combination with ethylene diamine

(□) cupric chloride, (+) cupric chloride and ethylene diamine ($8x10^{-7}$M). (×) 100% control acitivy.

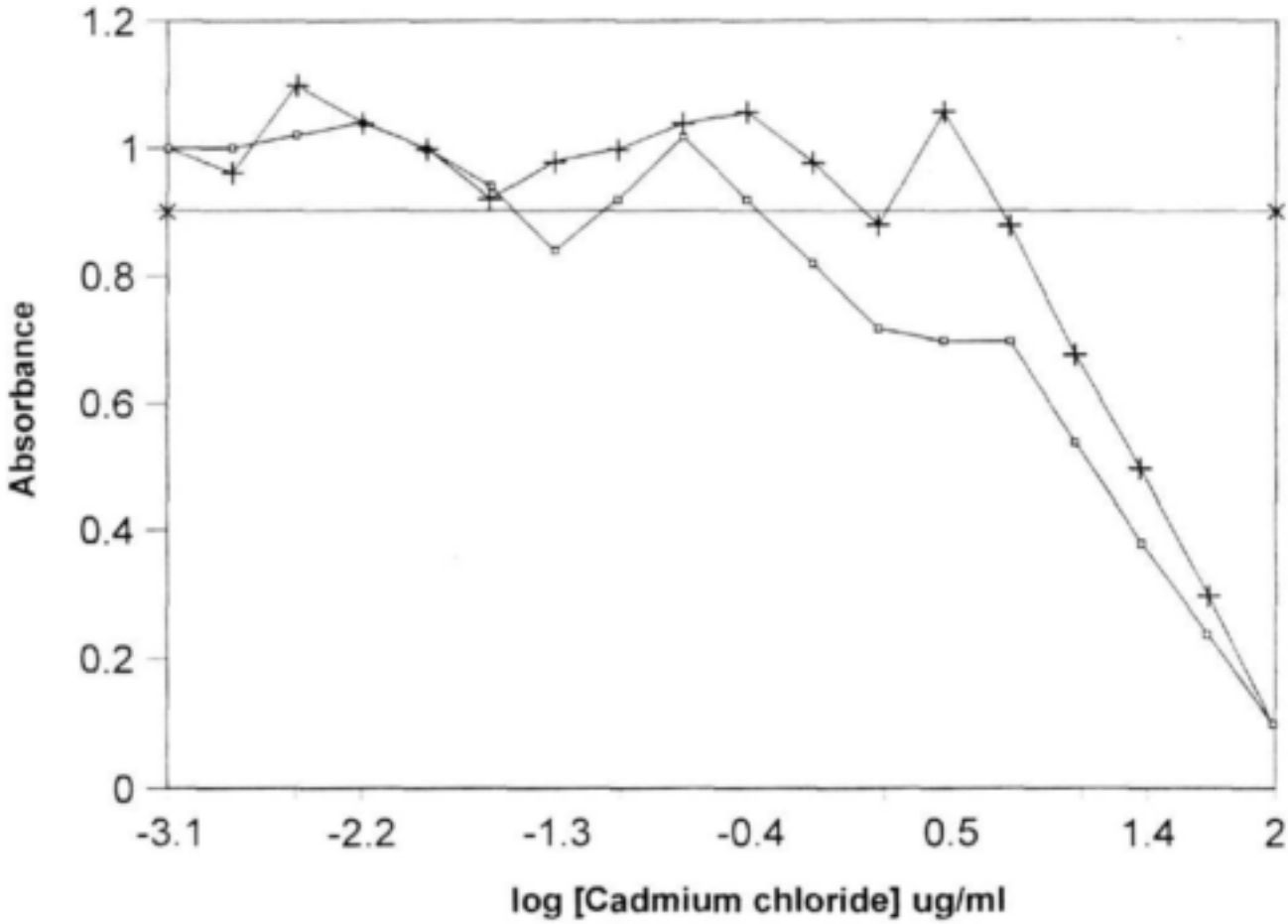

Fig. 2.4 **Cytotoxicity curve for cadmium chloride alone and in combination with 2-mercaptoethanol.**

(□) cadmium chloride, (+) cadmium chloride and 2-mercaptoethanol ($8x10^{-7}$), (×) 100% control activity.

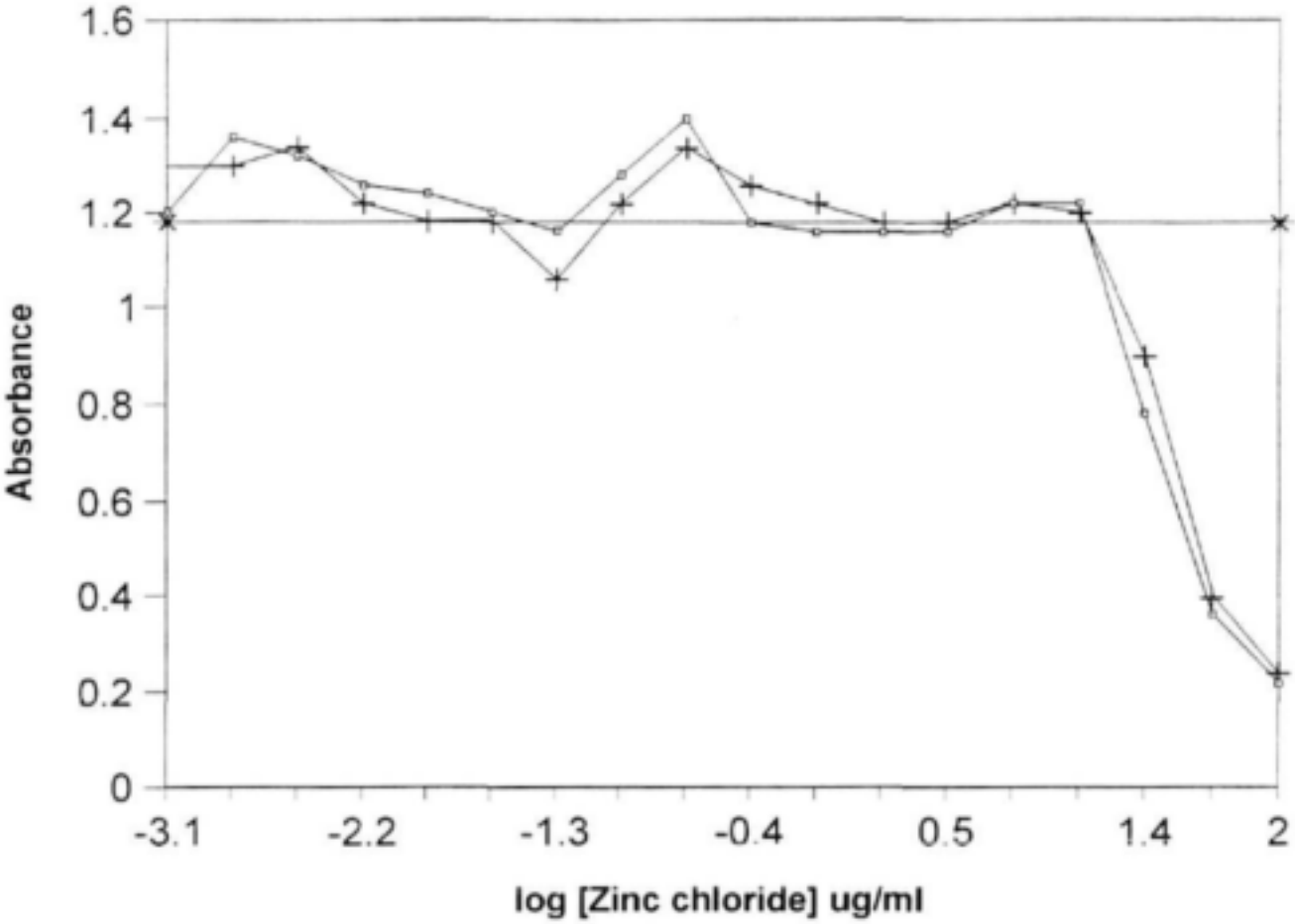

Fig. 2.5 **Cytotoxicity curve for zinc chloride alone and in combination with 2-mercaptoethanol.**

(□) zinc chloride, (+) zinc chloride and 2-mercaptoethanol ($8x10^{-7}$M), (×) 100% control activity.

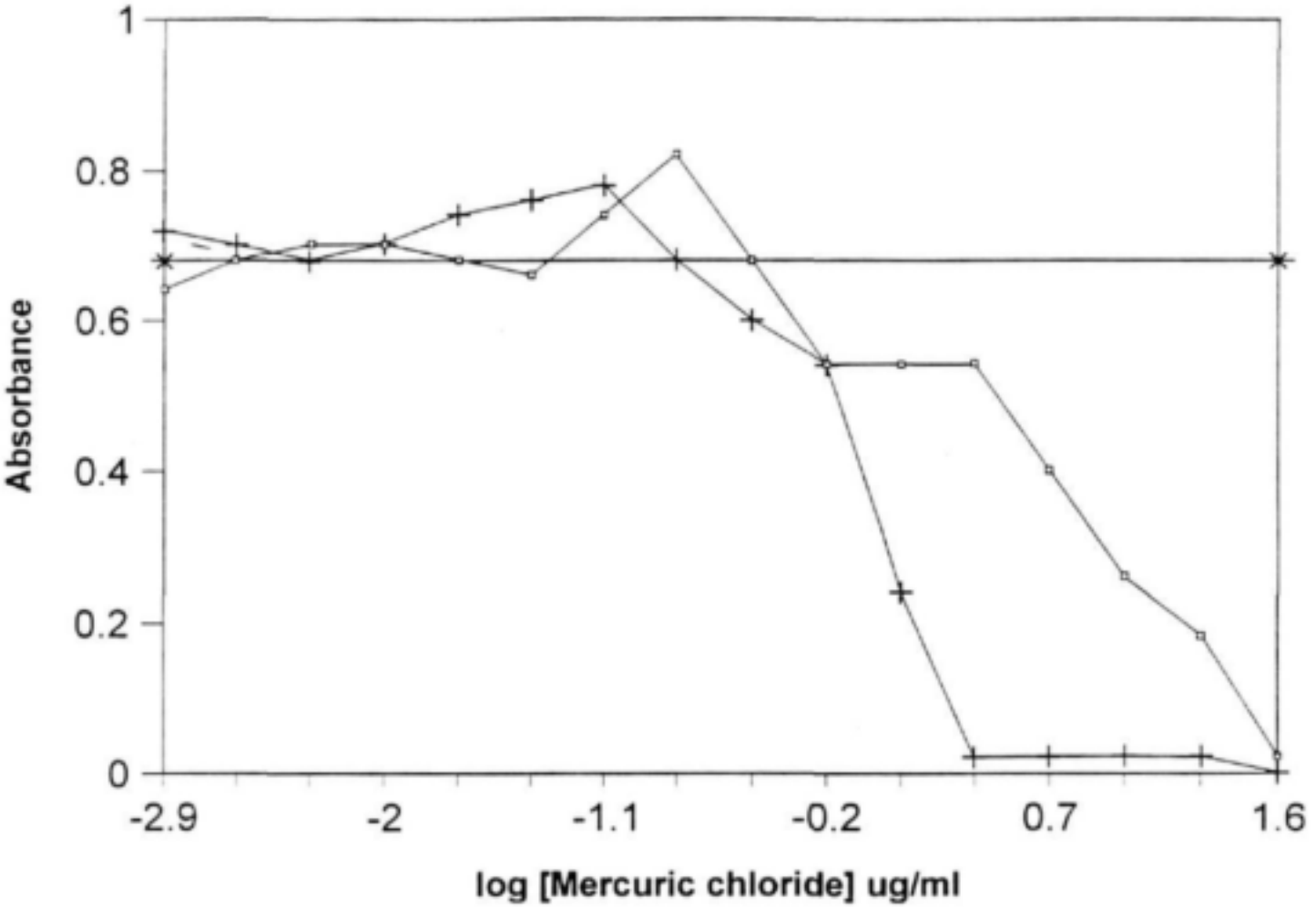

Fig. 2.6 **Cytotoxicity curve for mercuric chloride alone and in combination with 2-mercaptoethanol.**

(□) mercuric chloride,(+) mercuric chloride and 2-mercaptoethanol ($8x10^{-7}$M), (×) 100% control activity.

The fact that mercuric toxicity increased so significantly in the presence of 2-mercaptoethanol hovever, is not only indicative of the much higher affinity of the ion for thiol groups in comparison to zinc and cadmium but may have also been due to the tendency of the ion to form highly stable covalent bonds with soft electron donors, (Pauling, 1970; Shriver *et al.*, 1991) resulting in mercaptide complexes, which like organomercuric compounds, were polar but lipid soluble and were thus able to readily diffuse across cellular membranes, resulting in an increased toxicity at lower metal concentrations.

2.3.2 An Investigation of the Sites of action of Mercuric Chloride Alone and in the Presence of 2-Mercaptoethanol by Electron Microscopy

In order to determine whether the apparent increase of mercuric chloride toxicity in the presence of 2-mercaptoethanol exhibited by the MTT assay was due to greater uptake of the compound via passive diffusion of mercuric/ligand complexes across cellular membranes, or due to the inhibition of mechanisms involved in MTT reduction, electron microscopy was used to examine cells exposed to mercuric chloride alone and in combination with 2-mercaptoethanol.

Micrographs of non-treated control cells and those exposed to mercuric chloride

at a concentration of 1.25μg/ml as well as 0.625μg/ml in combination with 2-mercaptoethanol are presented in figures 2.7-2.9 respectively.

Cells exposed to mercuric chloride alone or in combination with 2-mercaptoethanol exhibited a number of distinct morphological changes when compared to control cells (Fig 2.7).

Cells exposed to mercury alone exhibited a reduction in size of the cytoplasm and nucleus by approximately 36% (Fig. 2.8), while the chromatin of these cells were often condensed and or marginalized and more electron dense than that of the control. In addition cells exhibited an invagination of the nuclear membrane as well cytoplasmic membrane blebbing while the cytoplasms of these cells although intact contained dark grey electron dense bodies not present in the control micrographs.

Cells exposed to mercuric chloride and 2-mercaptoethanol exhibited even more dramatic morphological changes when compared to control cells (Fig. 2.9). The chromatin of these cells was extremely electron dense and clumped in an irregular fashion while focal disruption of the nuclear membrane and disintegration of the cytoplasmic membrane was also evident. Organelles while still integral were markedly swollen while inclusion bodies were also found in the nuclei of some cells.

As electron microscopy was performed on cells exposed to concentrations of mercuric chloride and mercuric chloride/2-mercaptoethanol which corresponded to regions on the cytotoxicity curves for each respective assay at which the onset of decreased activity was evident (Fig. 2.6), cytotoxicity was expected. However cell and nuclear shrinkage, membrane blebbing as well as chromatin condensation and margination apparent in cells exposed to mercuric chloride alone was not just indicative of non-specific cytotoxic effects, but has been clearly shown to be associated with apoptosis, the deliberate and genetically controlled form of cell death, which can be activated by environmental stresses that induce cellular and/or DNA damage (Kerr *et al.*, 1994; Grimm *et al.*, 1996; Verheij *et al.*, 1996). As mercury has been found to associate efficiently with both proteins and chromosomal DNA (Falchuk *et al.*, 1977), it is likely that such associations resulted in DNA structural damage as well the inactivation of essential cellular proteins thus triggering apoptosis in cells exposed to these concentrations of toxicant.

Interestingly, the irregular clumping of chromatin as well as the focal disruption and disintegration of membranes and swelling of organelles in cells exposed to mercuric chloride and 2-mercaptoethanol have been shown to be the morphological changes associated with another distinct form of cell death: necrosis, a genetically uncontrolled outcome resulting from catastrophic injury to cells (Kerr *et al.*, 1994).

This finding strongly suggests that the presence of 2-mercaptoethanol did indeed fascilitate greater uptake of mercuric ions, possibly by the passive diffusion of lipid soluble mercaptide complexes as an increase in toxicant uptake in this manner would have very likely resulted in a dramatic increase in toxicity at lower concentrations of metal to the extent that even cellular

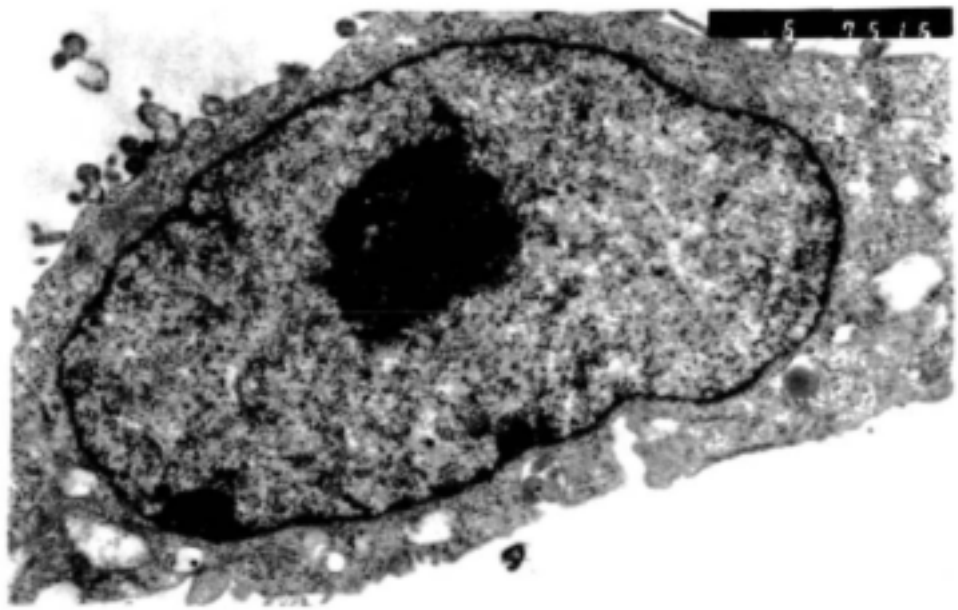

Fig. 2.7 Electron micrograph of an untreated control cell (6000x magnification).

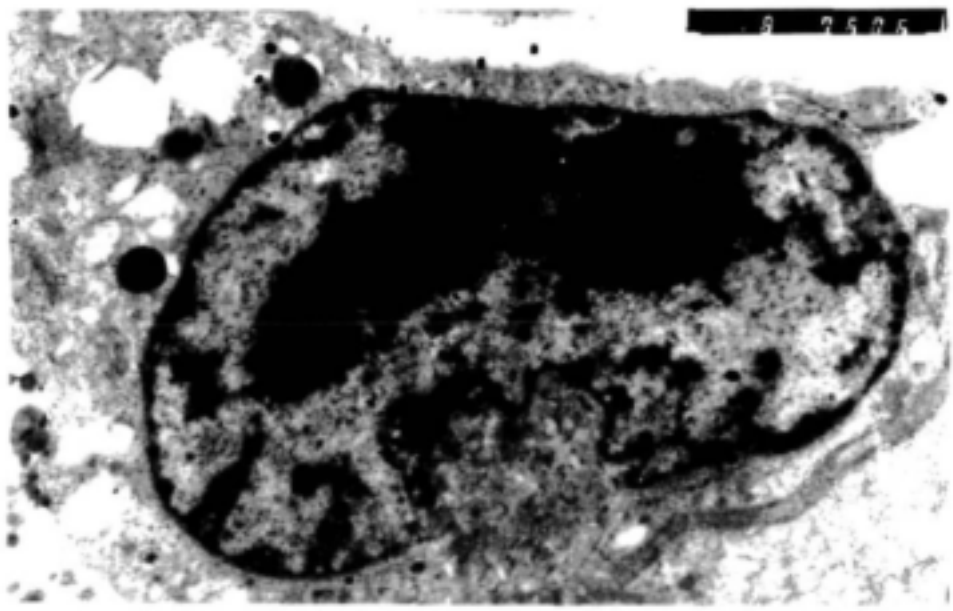

Fig. 2.8 Electron micrograph of a cell exposed to 1.25μg/ml mercuric chloride for 24 hours (8000x magnification).

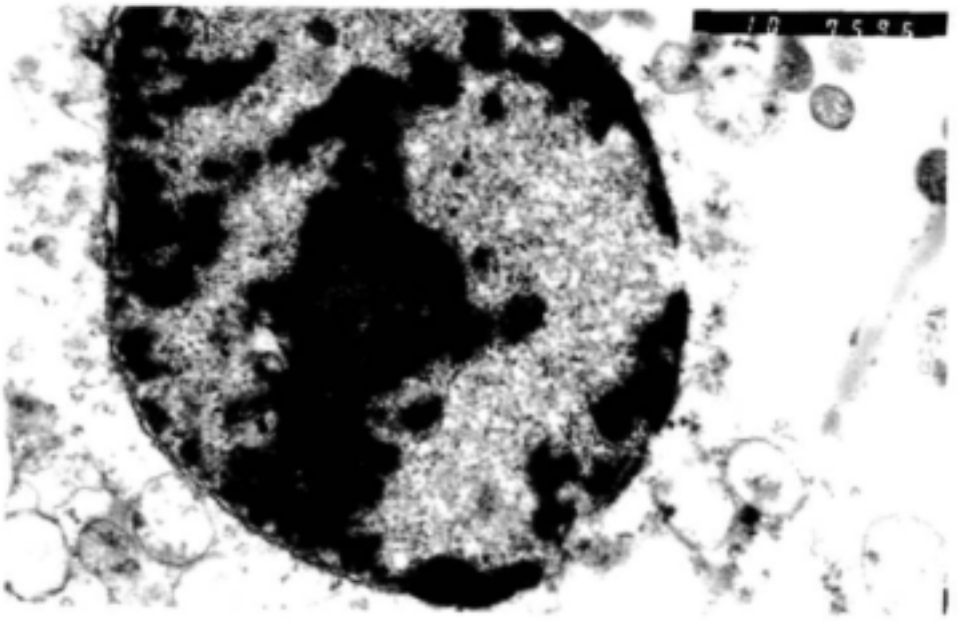

Fig. 2.9 Electron micrograph of a cell exposed to 0.62μg/ml mercuric chloride in combination with $8x10^{-7}$M 2-mercaptoethanol for 24 hours. (10000x magnification)

components involved in programmed cell death would be rapidly and irreparbly damaged. This is further reinforced by the increased electron density of the chromatin in comparison to control cells and those exposed to mercuric chloride alone, indicating considerably higher intracellular levels of the metal associated with the DNA of cells exposed to mercuric chloride and 2-mercaptoetanol.

2.3.3 An Investigation of the Dependence of Increased Cellular Activity on Toxicant Concentration and Exposure Time

Increased or 'hormetic' activity observed at low concentrations of toxicant in Figs. 2.2-2.6 has, as discussed previously, (Chapter 1) been described as a phenomenon caused by the non-specific inhibition of metabolic pathways by low levels of toxicants, resulting in over corrections by cellular control mechanisms and therefore higher than normal cellular activity (Stebbing, 1982). However, cells exposed to a broad concentration range of toxicant would then, according to this hypothesis exhibit increased activity at and beyond a specific threshhold concentration until the onset of toxicity. Yet, as evident from the cytotoxicity curves for all metal salts tested, a succession of peaks and plateax or troughs were present prior to the onset of toxicity, with peaks occuring at specific concentrations, suggesting that this increased activity was a specific response, possibly affected by concentration in a time dependent manner.

Time/activity studies strongly agreed with this hypothesis as cells exposed to 0.4μg/ml cupric chloride exhibited peak hormetic activity at 23.8 hours however by increasing metal concentration to 0.8μg/ml this peak activity regressed to 22.6 hours. At 1.6μg/ml hormetic activity regressed further to 21.4 hours while at 3.2μg/ml cupric chloride, hormetic activity occured at 20.2 hours and then peaked again at 23.8 hours clearly indicating the dependence of hormetic onset time on concentration (Fig. 2.10).

A plot of concentration versus the time at which maximum activity was observed yielded an exponential curve (Fig. 2.11), which indicated that for a narrow concentration range, metal concentration (C) and the onset time of maximum activity (t_{max}) were exponentially related such that:

$$t_{max} := (-\ln C/C_0)/k \qquad (1)$$

Where k is a rate constant and C_0 is a theoretically large concentration at which the onset of hormetic activity would occur instantaneously ($t=0$). This suggested that a critical concentration of toxicant was required within cells to elicit increased activity and that the time taken to reach this critical concentration was dependent on the concentration of toxicant to which cells where exposed, in a first order manner (Tuey, 1980).

In addition, as more than one activity peak was found for most of the metals tested, and these were often separated by a broad concentration range, (cadmium chloride induced peaks at 0.003 and 0.2μg/ml) it was further suggested from these findings that such critical concentrations could be reached more than once over 24 hours exposure period, thus resulting in multiple peak activities increasing in frequency with increasing concentration of toxicant.

The form of the curves in Fig. 2.10 also suggested that at fixed metal concentrations, cellular activity tended to a maximum in a sigmoidal fashion with curves increasing in steepness as metal concentration increased. This suggested that activity peaks could be described by the sigmoidal equation:

$$A(t): = hA_{max}/(1+e^{a-bt}) \qquad (2)$$

where activity A tends over time t to a theoretical maximum A_{max} and where the steepness or order of the curve is described by b, while a is a constant, dependant on control or 100% cellular activity. As it was not a certainty that the change in activity with time remained sigmoidal close to A_{max}, the constant h was included such that activity could then be said to tend over time to hA_{max} were h is believed to tend to 1.

In order to determine whether the change in activity with time could be

described in this manner, equation (2) was rearranged to yield the straight line equation:

$$\ln(hA_{max}/A - 1) = -bt + a \qquad (3)$$

and $\ln(hA_{max}/A - 1)$ was plotted against time values for each time activity curve in figure 2.10 using time intervals in which curves displayed sigmoidal characteristics. An average A_{max} value of 1.16 was used while h was assumed to be 1.

Plotting the data in this manner yielded curves analogous to hill plots, (Palmer, 1995) where graphs consisted of linear central portions of slope b with deviations from linearity at each end (Fig. 2.12) clearly indicating that the change in activity with time was indeed sigmoidal within the chosen time intervals.

Since it was also apparent from activity/time plots that curve steepness increased with increasing metal concentration (Fig. 2.10). The manner in which b was related to concentration was investigated. A plot of the log of cupric chloride concentrations used in figure 2.10 versus the b values obtained from each corresponding curve exhibited a linear relationship (Fig. 2.13) and thus could be described by:

$$\log C_i = mb + \log C_{b_0} \qquad (4)$$

where m is the slope of equation 4 and C_{b_0} is a theoretically small concentration at which b is zero. Thus it was now possible to substitute

equation (4) into (2) yielding:

$$A(t) = hA_{max}/(1 + e^{s-[t\log(C-C_{b_0})]m}) \qquad (5)$$

which described the change in activity with time, where steepness of the slope b is expressed in terms of toxicant concentration.

The above findings culminating in equation (5) were significant as from these results it was possible to describe the increase in activity evident in Figs. 2.2-2.6 as composite curves where each log concentration value could be seen to represent a sigmoidal curve of specific order b, tending to a maximum activity which would be reached at a specific t_{max} value, itself dependent in an exponential manner on toxicant concentration (eqn 1).

Thus at 24 hours a specific concentration of toxicant C_a would correspond to maximum activity on a sigmoidal curve with a specific order b_a. At a lower

concentration C_b the resultant activity would then correspond to another sigmoidal curve of order b_b that would only reach maximum activity at 24 + x hours (where $x > 0$). Therefore this activity value would not only correspond to another curve but would also represent an activity value somewhere below maximum activity as at 24 hours this curve would have only partially reached hA_{max}.

It could therefore be concluded from these findings that this increased activity could be described as a succession of increasing log concentration values within a narrow concentration range corresponding to an activity curve where each increasing activity is derived from a sigmoidal curve with a specific and increasing order and where peak activity corresponds to the activity value on the curve which, for that specific concentration, reaches maximum activity at 24 hours.

In addition as (m), the change in log concentration with b is constant for all sigmoidal curves within the composite curve, it should also therefore be possible to calculate m from any activity value and corresponding log concentration within this region, and such m values should be specific for the toxicant used as rearranging equation 4 yields:

$$b = \log(C/C_0)/m \qquad (6)$$

which indicates that the sigmoidal curves of different toxicants which exhibit different steepnesses within the same concentration range would have distict m values, dependent on the intrinsic qualities of the the toxicant or toxicant/ligand complex which determine its ability to enter the cell and interact with toxicological targets. These m values could thus have potential use as indicators of specific toxicants or toxicant combinations in unknown samples which exhibit hormetic peaks.

b_2 Values (analogous to the half lifes ($t_{1/2}$) of isotopes), which using equation 6, can be calculated from:

$$b_2 = (\log 2)/m \qquad (7)$$

would therefore also be unique for specific toxicants and could further prove usefull in determining the effects of metal speciation on cellular uptake as an increase or decrease in metal uptake in the presence of low levels of ligands or chelating agents over the same concentration range would alter b_2 in a proportional manner.

In order to determine whether this mathematical description of hormesis agreed with the changes in cytotoxicity observed for the metals or metal/ligand

combinations tested, equations (5) and (7) were used to derive m and b_2 values respectively, from the hormetic peaks of all metals assays within a concentration range of 0.03 to 3μg/ml (Fig. 2.2-2.6).

The m and b_2 values for mercuric chloride alone and in combination with 2-mercaptoethanol as well as for cupric, cadmium, zinc and ferric chloride, shown in Table 2.1, were indeed found to be specific for each metal or metal/ligand complex and also exhibited similarities for zinc, cadmium and mercuric chloride which belong to the same periodic group and were expected to exhibit similar modes of toxicity.

In addition the b_2 value for mercuric chloride in combination with 2-mercaptoethanol was almost double that of mercuric chloride alone and this agreed with the increased toxicity observed for the metal salt in combination with this ligand and further substantiated the hypothesis that 2-mercaptoethanol facilitated in the greater uptake of mercuric chloride by the cell.

Finally, the hypothesis that low ferric chloride toxicity, even at high concentrations, was due to poor uptake of the toxicant agreed with the b_2 value for ferric chloride which was the lowest of all toxicants tested.

Thus it was clear from these findings that the mathematical description of hormesis did indeed correlate with the changes in cytotoxicity observed for the

metals and metal-ligand combinations tested and that this model had potential use as an indicator of specific toxicants which induce hormesis as well as a method for the investigation the effects of metal speciation on cytotoxicity.

It is important to specify however that the above mentioned kinetics can only be seen to apply to regions of hormetic peaks which increase with increasing log concentration values for although it may be very likely that the steady decrease in activity evident after peak activity may also be described as a composite curve, it can not be assumed that this curve tends to control activity in a symmetrically sigmoidal manner.

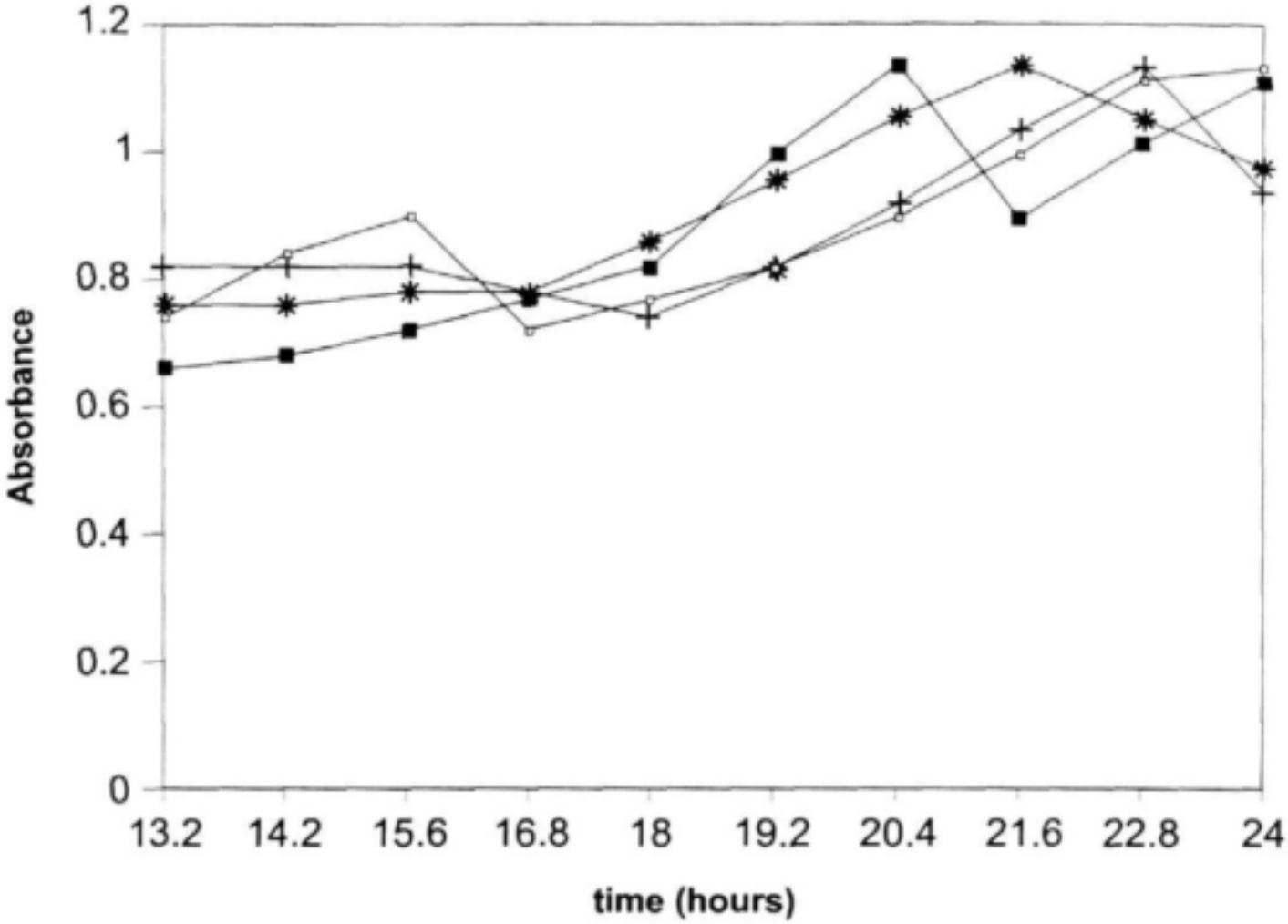

Fig. 2.10 **Time/activity assay for cells exposed to cupric chloride.**

(□) 0.4μg/ml, (+) 0,8μg/ml, (✳) 1.6μg/ml, (■) 3.2μg/ml $CuCl_2$.

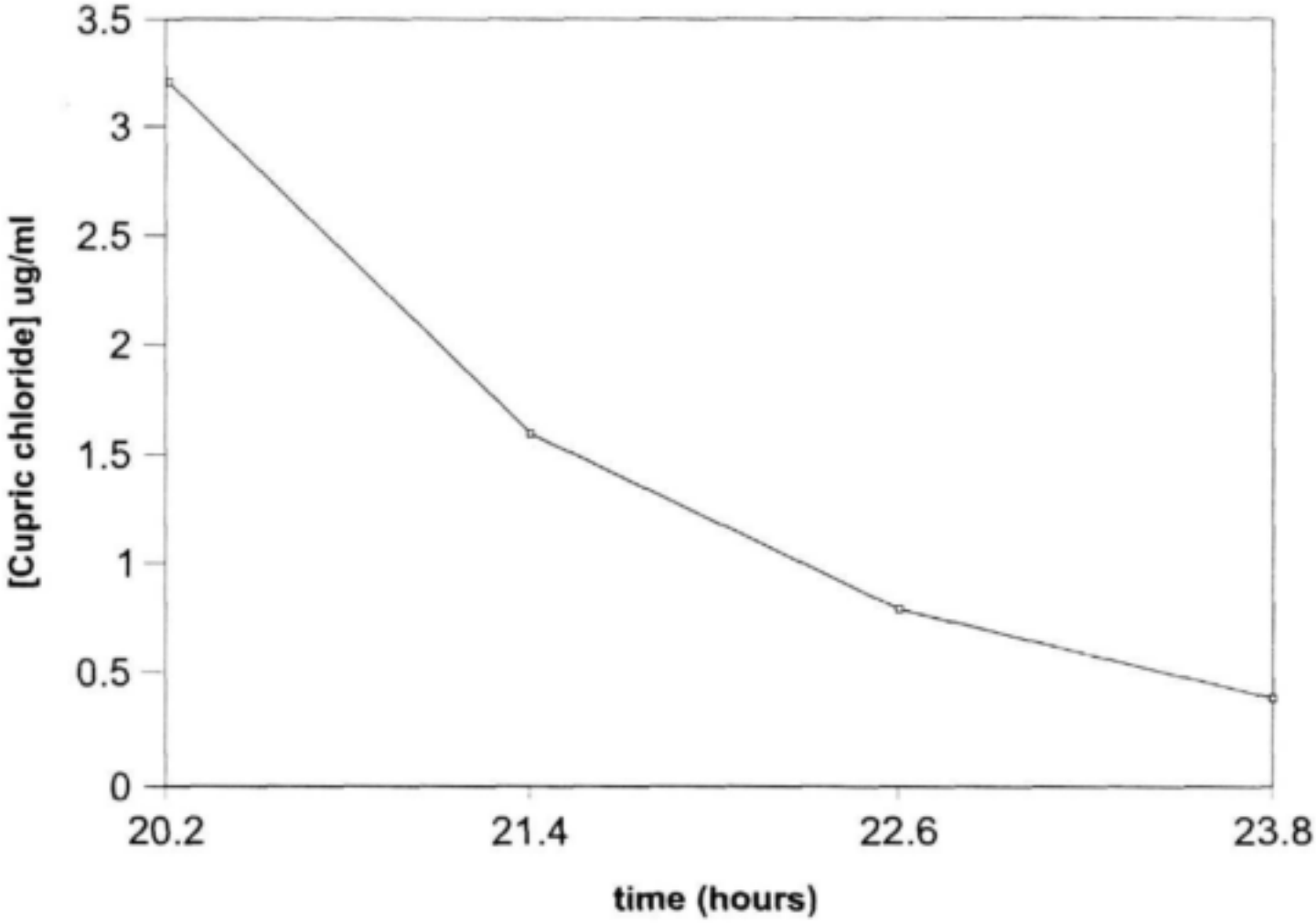

Fig. 2.11 Concentration versus hormetic onset time (t_{max}) for cells exposed to cupric chloride.

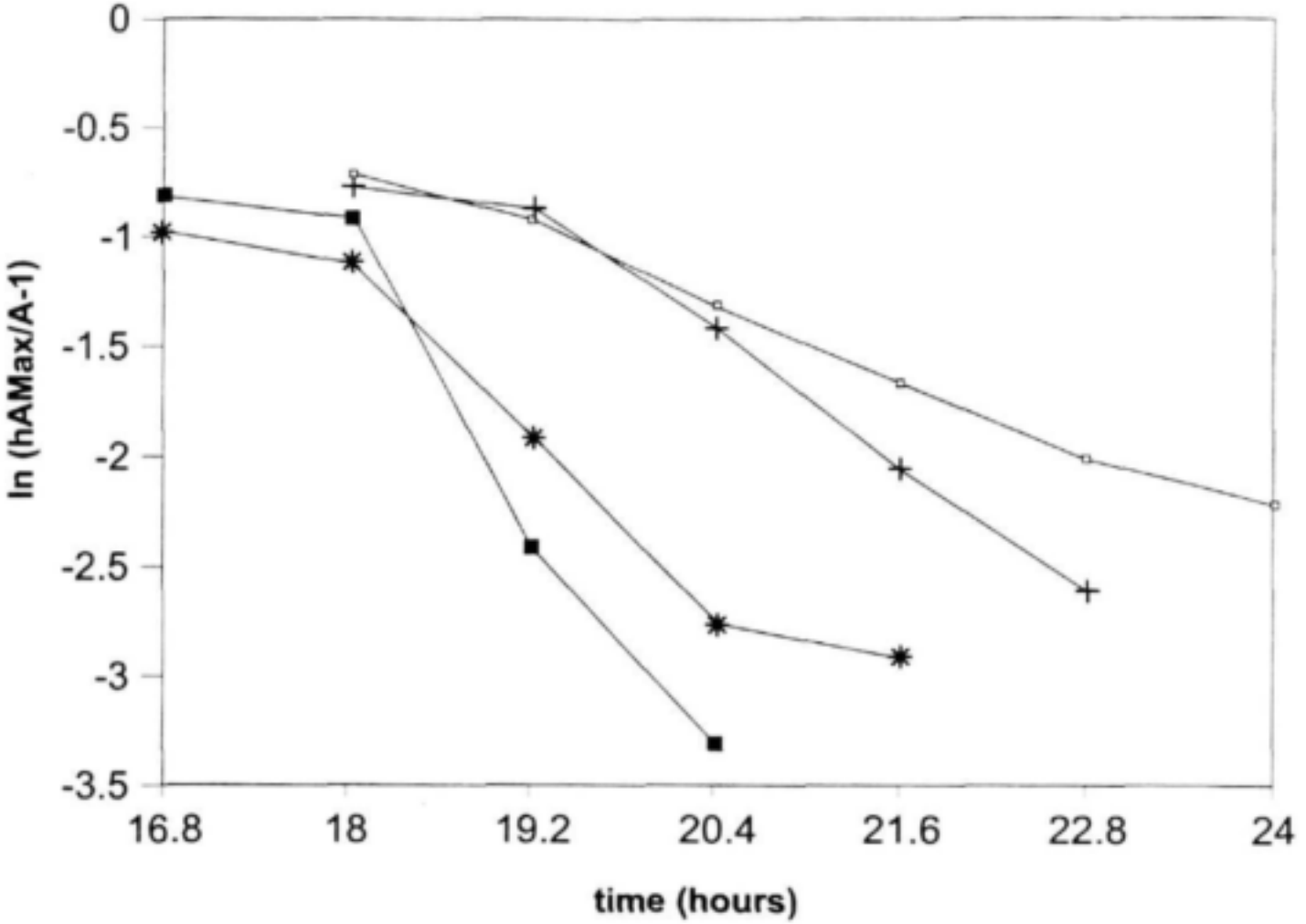

Fig. 2.12 **$\ln(h\Lambda_{max}/\Lambda - 1)$ versus time for cells exposed to cupric chloride.**

(□) 0.4μg/ml, (+) 0.8μg/ml, (✳) 1.6μg/ml, (■) 3.2μg/ml cupric chloride.

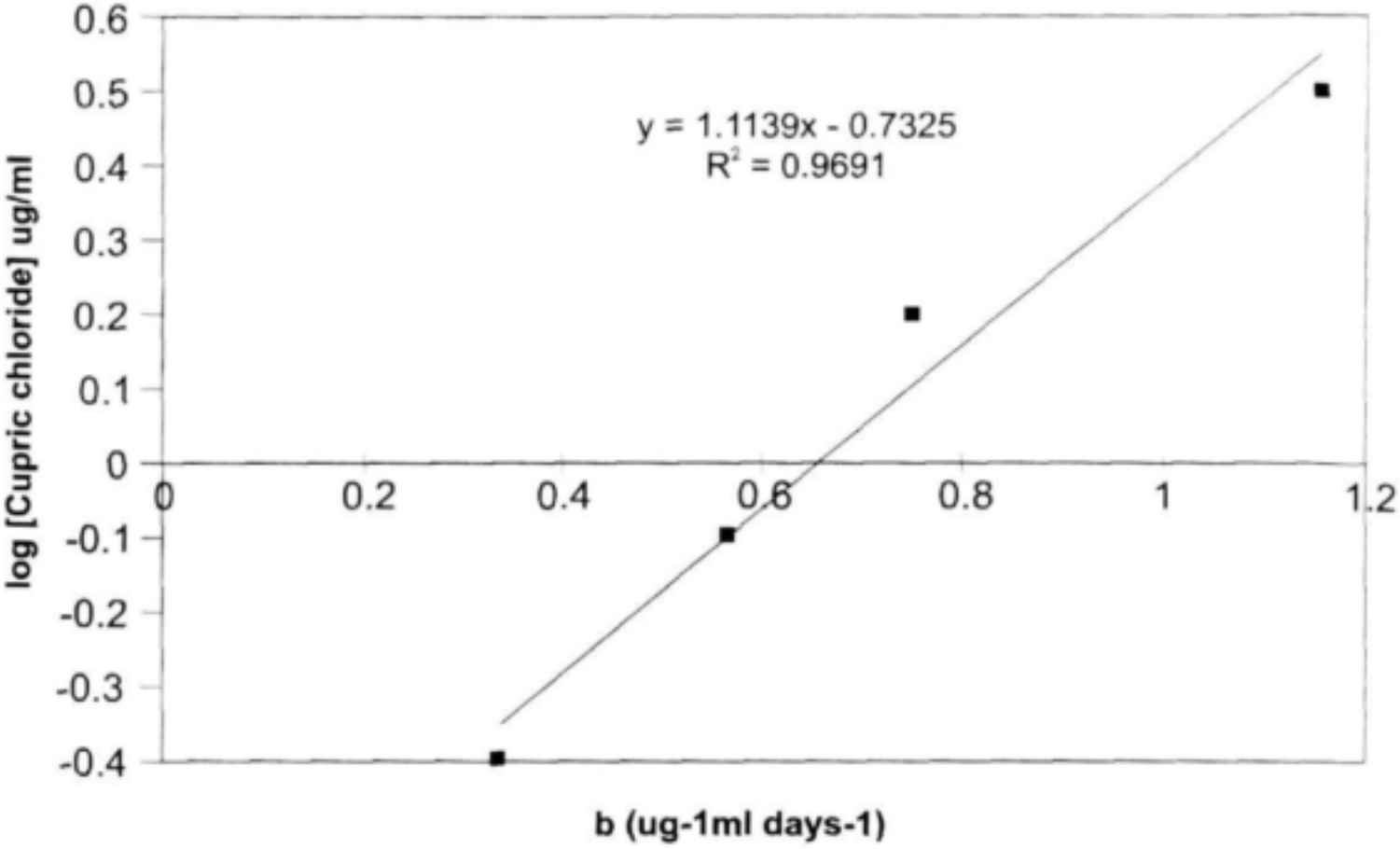

Fig. 2.13 log concentration versus b values for cells exposed to cupric chloride.

Table 2.1: m and b_2 Values for Metals and Metal/Ligand Complexes

Metal/Metal-ligand	m* (μgml^{-1}days)	b_2 (μg^{-1}mldays^{-1})
$HgCl_2$	2.55	0.12
$HgCl_2$ + 2-ME**	1.72	0.2
$CuCl_2$	1.83	0.16
$CdCl_2$	2.15	0.14
$ZnCl_2$	2.38	0.12
$FeCl_3$	3.33	0.09

* m Values were derived from equation 5 using h= 1, C_{to} = 1* 10^{-5} μg/ml and t= 1 day while all curves were standardized using a common control activity (A_c) of 0.8. The constant a, was estimated by $a=\ln(hA_{max}/A_c - 1)$, where a maximum A_{max} value of 1.16 was used and activity was assumed to be A_c at t=0.

** 2-ME = 2-mercaptoethanol

CHAPTER 3

PROTEIN PRODUCTION IN CELLS EXPOSED TO HORMETIC, PLATEAU AND LETHAL CONCENTRATIONS 50% (LC_{50}) LEVELS OF CADMIUM AND MERCURIC CHLORIDE

3.1 INTRODUCTION

The findings of Chapter 2, clearly showed that the onset of hormesis was dependent on concentration in a first order manner. This suggested that increased activity was a specific response to a build up of critical levels of heavy metals within cells and was further reinforced by the sigmoidal manner in which activity tended to a maximum over time, as sigmoidal kinetics are often indicative of the amplified responses observed for cascade systems like kinase pathways, which trigger specific responses in cells exposed to a host of environmental stimuli and stress inducers (Kyriakis *et al.*, 1994; Palmer, 1995).

Thus in light of these findings, it was necessary to further investigate hormesis

at the molecular level in an attempt to determine whether this increased activity could be correlated to changes in protein synthesis or to the elevated production of specific proteins like metallothioneins and superoxide dismutase, which have been found to protect cells against the toxic effects of heavy metals (Hatayama *et al.*, 1991; Steinebach and Wolterbeek, 1994).

In addition, if hormesis was a specific response to critical levels of heavy metals in cells, it would also be important to investigate the effect of metal resistance on this response as well as to compare protein synthesis in normal and resistant cells exposed to heavy metals.

Therefore the production of metallothionein and superoxide dismutase as well as the protein profiles of McCoy 5A and McCoy 5A cadmium resistant mutant cells (CRM) were investigated after exposure for various time intervals to mercuric chloride and/or cadmium chloride at concentrations corresponding to hormetic, plateau, and lethal concentration 50% (LC_{50}) regions of the cytotoxicity curves for each metal chloride.

3.2 MATERIALS AND METHODS

3.2.1 Establishment of McCoy 5A Cadmium-Resistant Mutant Cells

McCoy 5A mouse cells were grown in MEM supplemented as described in (2.2.1) until 80% confluency was obtained. Cells were then given fresh medium with 1.5μg/ml phenazine methosulphate (Sigma) and incubated at 37°C for 18h. Surviving cells where given fresh medium with 0.8μg/ml cadmium chloride and incubated for 2 weeks at 37°C. Cadmium resistant mutant cells (CRM) were then maintained and cultured as in (2.2.1) but with MEM additionally supplemented with 1.3μg/ml cadmium chloride.

3.2.2 MTT Colorimetric Assays for CRM Mutant Cells

Cadmium chloride assays using CRM mutant cells and normal cells were performed in the manner described in (2.2.2) and (2.2.5), while time activity assays for both normal and CRM cells exposed to hormetic levels of cadmium chloride (0.2μg/ml) were performed in the manner described in (2.2.7).

3.2.3 Protein Profiles

McCoy 5A cells and CRM mutant cells were grown in 25cm^2 flasks for 2-3 days until 60% confluency was obtained. Normal cells were then given 10ml fresh medium spiked with mercuric chloride at 0.2, 1.3 and 6.2μg/ml while both normal and CRM cells were given 10ml MEM with cadmium chloride at 0.2, 1.6 and 6.2μg/ml respectively corresponding to hormetic, plateau and LC_{50} concentrations for each metal. Additional flasks of normal cells were exposed to 0.003, 0.012 and 0.02μg/ml cadmium chloride to determine protein production at low toxicant concentrations while flasks given medium free of toxicant, served as controls.

Cells were then incubated for 24 hours at 37°C or for 0, 8, 16 and 24 hours at hormetic concentrations (0.2μg/ml), when protein production over 24 hours was investigated. Following this, cells were washed twice with 2ml ice cold PBS and lysed by the addition of 1ml triple detergent lysis buffer, containing 150mM sodium chloride, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate and 0.02% sodium azide in 50mM Tris.Cl solution (pH 8.0). After incubation on ice for 20 minutes, cell lysates were transferred to Eppendorf tubes and centrifuged at 12000g for 3 minutes at 4°C in a microfuge. Supernatants were then stored at -20°C until further use. Protein concentrations were determined using the Bicinchoninic acic (BCA) method as described by Smith *et al.*, (1985).

Protein profiles for cells exposed to cadmium and mercuric chloride were obtained by SDS-polyacrylamide gel electrophoresis modified for the detection of Hsp 70 and metallothionein by Hatayama *et al.*, (1991), and protein bands were visualized with silver staining using a quick silver staining kit (Amersham).

3.2.3 Quantification of Heat Shock Protein 70 (Hsp 70)

Hsp 70 was identified and quantified by western blotting and enhanced chemiluminesence (ECL). Proteins separated on 12% (w/v) polyacryamide gels as described by Hatayama *et al.*, (1991), were transferred electrophoretically to ECL nitrocellulose membranes (Amersham) using a Hoefer western blotting apparatus, at a constant voltage of 45V for 16 hours at 4°C. After protein transfer, filters were dried on blotting paper at room temperature for 30 minutes, rehydrated in deionized water and stained in a 0.2% (w/v) solution of ponceau S (Merck) containing 3% (w/v) trichloroacetic and sulphosalicylic acids (Merck), in order to visualize transferred proteins. Filters were then washed in 4 changes of deionized water and incubated for 1.5 hours at room temperature in 15ml blocking solution containing 5% (w/v) nonfat dried milk, 0.01% (v/v) Antifoam A (Sigma) and 0.02% sodium azide in PBS.

After blocking, filters were transferred to fresh blocking solution containing a

dilution anti-bovine Hsp 70 monoclonal antibody (Sigma) at a dilution of 1:3000 and incubated for 2 hours at room temperature. Filters were then washed 3 times for 10 minutes in 250ml PBS and once for 10 minutes in 200ml Tris-saline (150mM NaCl, 50mM Tris.Cl pH 7.5), then transferred to 15ml blocking solution containing 5% nonfat dried milk and 0.01% (v/v) Antifoam A in Tris-saline with horseradish peroxidase-coupled goat anti-mouse polyclonal antibody (Binding Site, UK) at a dilution of 1:500 and incubated at room temperature for a further hour.

Filters were then washed 4 times for 10 minutes in 200ml Tris-saline, incubated for 1 minute in an equal volume of ECL detection reagents (Amersham) and exposed to Curix X-ray film (Agfa) for 1 to 5 minutes. Band intensity was quantified using laser densitometry and expressed as area under curve (mm^2).

3.2.4 Quantification of Metallothionein

Metallothionein levels in both normal and CRM cells exposed to 0.2, 1.6, 6.2μg/ml cadmium chloride for 24h and 0.2μg/ml cadmium chloride for 0, 8, 16 and 24 hours as described in (3.2.3) were quantified and compared to levels in untreated controls by a modification of the assay described by Bartsch *et al.*, (1990).

After incubation, cells in flasks were washed twice with 5ml ice cold PBS, harvested with a cell scraper, resuspended in 1ml 10mM Tris.Cl, 85mM NaCl (pH 7.4) and transferred to eppendorf tubes. Cells were then lysed by rapid freezing and thawing (5 times) in liquid nitrogen and the resultant lysate was centrifuged at 100000*g* for 1hour at 4°C in a Beckman TL 100.2 ultra-centrifuge. The BCA method was used to quantify the protein concentrations of the supernatants and these were stored until further use at -70°C.

For the assay, appropriate volumes of supernatant containing 36μg of protein were made up to a final volume of 200μl with 10mM Tris.Cl, 85mM NaCl (pH 7.4). Following this 11μl of a 20μCi/ml solution of $^{109}CdCl_2$ (1000μCi/μgCd) (Amersham) was added to each 200μl sample. After 10 minutes incubation at room temperature, 100μl of a 66% w/v solution of chelex 100 (Biorad) in 10mM Tris.Cl, 85mM NaCl (pH 7.4) was added to each sample and these were incubated for a further 30 minutes with shaking every 10 minutes.

Samples were then spun briefly at 12000*g* and 180μl volumes were transferred to eppendorf tubes containing 20μl of a 10% w/v sucrose solution. These 200μl aliquots were then chromatographed on a Sephadex G-50 column (1.0*20cm) equilibrated with 10mM Tris.Cl, 85mM NaCl (pH 7.4) at a flow rate of 1.5ml/min. The eluate was collected in 20 1ml fractions and these were analysed for ^{109}Cd with an Autogamma 5000 counter (Packard).

Metallothionein-containing fractions were taken as those which accurately corresponded to the peak activity fractions of a 10μg sample of purified metallothionein from rabbit liver (Sigma), prepared and chromatographed in an identical manner.

3.2.5 Superoxide Dismutase Activity

Superoxide dismutase activity was quantified in normal and CRM cells by a modification of the method described by Ewing and Janero (1995). McCoy 5A cells seeded into flat bottom 96 well tissue culture plates at 20000 cells per well in 100μl MEM were incubated for 18 hours at 37°C and 5% CO_2 in air. Cells were then exposed to mercuric and cadmium in the same way as was described for the rapid MTT colorimetric assays carried out for each metal (Chapter 2; Sections 2.2.2, 2.2.3 and 2.2.5).

After 24 hours of incubation MEM in the wells was removed and the cells solubilized by the addition of 25μl 1% (v/v) NP-40 in 50mM phosphate buffer (pH 7.4), followed by the addition of 200μl 50mM phosphate buffer containing 0.1mM EDTA, 62μM nitroblue-tetrazolium chloride (NBT) and 98μM NADH. A further 25μl of 50mM phosphate buffer containing 0.1mM EDTA and 33μM phenazine methosulphate was added to each well and the plates incubated for 5 minutes.

The reaction was stopped by the addition of 25μl 3N HCl to the wells followed by the further addition of 25μl isopropanol to solubilize the NBT formazan. Plates were read on a Biorad ELISA plate reader using a 570nm filter, where the level of inhibition of superoxide mediated NBT reduction was used as an indicator of increased superoxide dismutase activity when compared to non-treated control cells.

3.3 RESULTS AND DISCUSSION

3.3.1 Protein Profiles of Cells Exposed to Hormetic, Plateau and LC_{50} Levels of Mercuric and Cadmium Chloride

The protein profiles of cells exposed to hormetic levels of cadmium chloride (0.2μg/ml) for 8, 16, and 24 hours (Fig. 3.1A) and hormetic, plateau and LC_{50} levels of mercuric chloride (figure 3.1B) and cadmium chloride (Fig. 3.1C) for 24 hours, exhibited a significant increase in protein synthesis when compared to the profiles of control cells, with pronounced protein bands clearly visible at and above the 66kDa molecular weight marker. Western blotting with ECL detection identified this 70kDa protein as heat shock protein 70 (Hsp 70), the highly conserved molecular chaperone and stress protein which has been shown to be an essential component of the rapid stress response exhibited by cells

exposed to adverse environmental conditions and toxic agents such as elevated temperatures, heavy metals, ionizing radiation and ionophores (Lindquist, 1986; Welch, 1992; Mager and De Kruijff, 1995).

Quantification of the ECL bands by laser densitometry showed that levels of Hsp 70 were 10 fold and 2 fold higher in cells exposed to 0.2μg/ml mercuric chloride and cadmium chloride respectively, as compared to non-treated controls, (Figs. 3.2 and 3.3), while cells exposed to hormetic levels of cadmium chloride for 8, 16 and 24 hours showed that this increase in Hsp 70 synthesis was hyperbolic over the 24 hour exposure period, with maximum protein production reached after 16 hours of exposure and maintained at those levels up until 24 hours (Fig. 3.4).

Cells exposed to low concentrations of cadmium chloride which induced increased activity after 24 hours of exposure (0.003μg/ml) exhibited a 20% increase in Hsp 70 levels when compared to non-treated controls, and interestingly, these levels increased by a further 34%, 43% and 50% in cells exposed to 0.012μg/ml, 0.02μg/ml and 0.2μg/ml respectively (Fig. 3.5).

At plateau concentrations of mercuric chloride and cadmium chloride Hsp 70 levels were extremely high with protein for cadmium chloride at 1.6μg/ml found to be more than triple that of the control, these levels then dropped

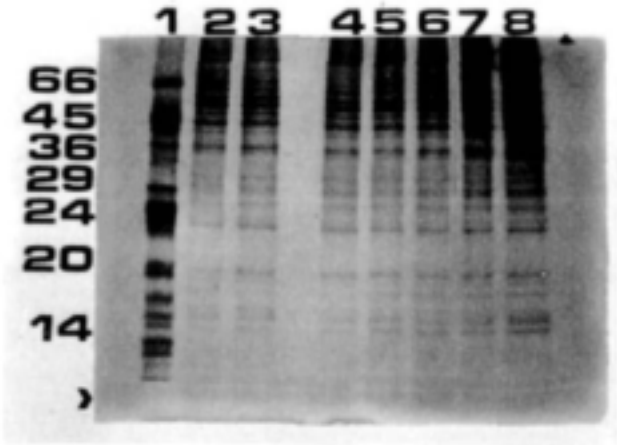

Fig. 3.1A **Silver stained SDS-PAGE gel exhibiting the protein profiles of cells exposed to 0.2μg/ml cadmium chloride for 8, 16 and 24 hours.**

Lane 1: molecular weight markers (kDa); Lanes 2, 3: 24 hours; Lanes 4, 5: 16 hours; Lane 6: 8 hours; Lanes 7, 8: Control; Arrow indicates metallothionien.

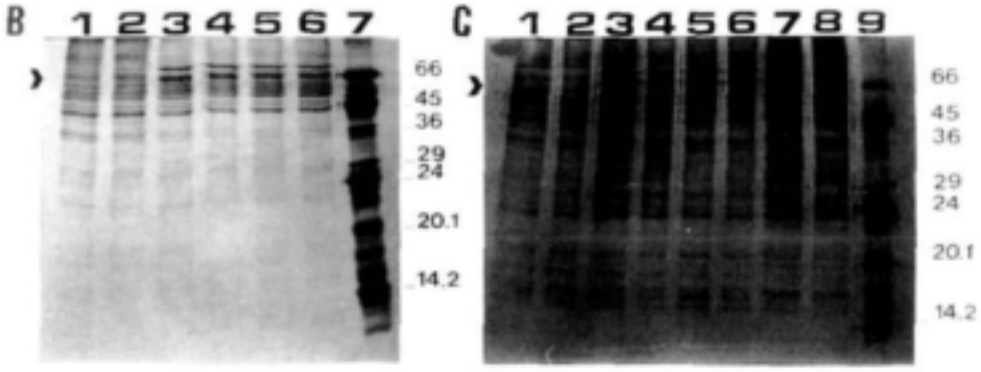

Fig. 3.1B **Silver stained SDS-PAGE gel exhibiting the protein profiles of cells exposed mercuric chloride for 24 hours at concentrations corresponding to hormetic (0.2μg/ml) and plateau (1.3μg/ml) levels of activity.**

Lanes 1, 2: Control; Lanes 3, 4: Hormetic activity (0.2μg/ml); Lanes 5, 6: Plateau activity (1.3μg/ml); Lane 7: Molecular weight markers (kDa).

Fig. 3.1C **Silver stained SDS-PAGE gel exhibiting the protein profiles of cells exposed to cadmium chloride for 24 hours at concentrations corresponding to hormetic (0.2μg/ml), plateau (1.6μg/ml) and LC_{50} levels of activity.**

Lanes 1, 2: Control; Lanes 3, 4: Hormetic activity (0.2μg/ml); Lanes 5, 6: Plateau activity (1.6μg/ml); Lanes 7, 8: LC_{50} activity (6.2μg/ml); Lane 9: Molecular weight marker (kDa).

Arrows indicate Hsp 70.

somewhat at LC_{50} concentrations but were still more than double that of control cells (Fig. 3.2 and 3.3).

3.3.2 Metallothionein Production in Cells Exposed to Hormetic, Plateau and LC_{50} Levels of Cadmium Chloride

Metallothioneins (MT) are ubiquitous, cysteine rich, low molecular weight proteins (6kDa) that have high affinities for heavy metals and are involved in the regulation of copper and zinc homeostasis (Hamer, 1986). As they have been found to be strongly induced in cells exposed to heavy metals, genotoxic agents and UV radiation, MT is believed to play an important role in the protection of cells against the toxic effects of metals and reactive oxygen species (Carter *et al.*, 1984; Whitacre, 1996).

Metallothionein production in cells exposed to hormetic levels of cadmium chloride (0.2μg/ml) for 24 hours rose to more than double that of the untreated controls but unlike Hsp 70 began to decline at 1.6μg/ml (plateau) and then dropped to below control levels at 6.2μg/ml (LC_{50}) (fig 3.6). This is in agreement with previous findings which showed that (MT) production in hepatocytes exposed to cupric chloride began to decline at high copper concentrations (Steinebach and Wolterbeek, 1994).

Over 8, 16 and 24 hours however, cells exposed to 0.2μg/ml cadmium chloride exhibited a sigmoidal increase in the protein, which like Hsp 70 reached a maximum at 16 hours and plateaued at 24 hours (Fig. 3.7).

3.3.3 Time/Activity Assay for Cells exposed to 0.2μg/ml Cadmium Chloride

As both maximum Hsp 70 and metallothionein production in cells exposed to hormetic levels of cadmium chloride (0.2μg/ml) were reached at 16 hours and maintained up until 24 hours where protein production was then found to level off, it was necessary to determine the manner in which the change in activity with time corresponded to this protein production.

Time/activity assays for cells exposed to 0.2μg/ml cadmium chloride showed that the onset of increased activity occured after 20 hours and reached a maximum at 24 hours (Fig. 3.8). Thus hormesis did not only correspond to significantly increased levels of stress protein but more specifically, the onset of peak activity corresponded to maximum Hsp 70 and MT production.

3.3.4 A Comparison of the Cytotoxicity Assays and Stress Protein Production in Normal and Cadmium Resistant Mutant Cells

Since hormesis appeared to be a specific response induced at a time when the ability of the cell to deal with increasing toxicant concentration had reached a threshhold level. This raised the question as to how metal resistance would affect these activity peaks. Therefore cadmium resistant mutant cells (CRM) were selected and used in identical cytotoxicity and protein studies as those carried out for normal cells.

The MTT assay performed with CRM cells exhibited a dramatically different cytotoxicity curve in comparison to normal cells (Fig. 3.9) and displayed a constitutively increased and oscillating level of activity over the entire concentration range of cadmium chloride, with a dramatic decline in activity only apparent at very high metal concentrations (> 10μg/ml).

CRM cells exposed to MEM with no cadmium chloride exhibited high constitutive levels of both Hsp 70 and metallothionein when compared to the untreated controls of normal cells. Hsp 70 dropped slightly but remained significantly high after exposure to hormetic (0.2μg/ml) and plateau (1.6μg/ml) concentrations of cadmium chloride for 24 hours and then rose to high levels again at the LC_{50} concentration (6.25μg/ml) (Fig. 3.10), while metallothionein

in CRM cells remained high and almost constant for all three concentrations (3.11).

In addition both Hsp70 and metallothionein in CRM cells exposed to 0.2μg/ml of cadmium chloride for 8, 16 and 24 hours exhibited plateaued protein levels, which corresponded to the high constitutive activity of these cells over the same time period (Fig. 3.12). Thus clearly showing that stress protein production in CRM cells was both high and constitutive and corresponded to the constitutive hormetic activity displayed by these cells.

Therefore from the above findings it was clear that the onset of hormesis not only coincided with elevated stress protein production but more specifically with discrete stress protein levels, indicating that this increased activity was indeed a specific response coinciding with a threshhold level of stress protein synthesis in response to a build up of a critical concentration of metal within the cell over time.

In addition, the fact that Hsp 70 levels in cells, which exhibited hormetic activity at 0.003μg/ml, was approximately 20% lower than that observed for cells which exhibited hormesis at 0.2μg/ml, coupled with the finding that a steady increase in Hsp 70 levels was apparent in cells exposed to 0.012 and 0.02μg/ml cadmium chloride, suggested that the concentration of toxicant to

which the cells were exposed not only affected the time at which hormetic activity was initiated (which occured prior to 24 hours for 0.012 and 0.02μg/ml) but also influenced the magnitude of stress protein production over that time period.

Thus an increase in metal concentration resulted in an increased level of stress protein production over time and this agreed with the findings of chapter 2 where the steepness or order of the hormetic response (b) was also found to be proportional to log toxicant concentration over the same time period (2.2.3, equation 4), therefore further showing that hormesis was a clear indicator of the stress response induced in cells when exposed to sublethal toxicant concentrations.

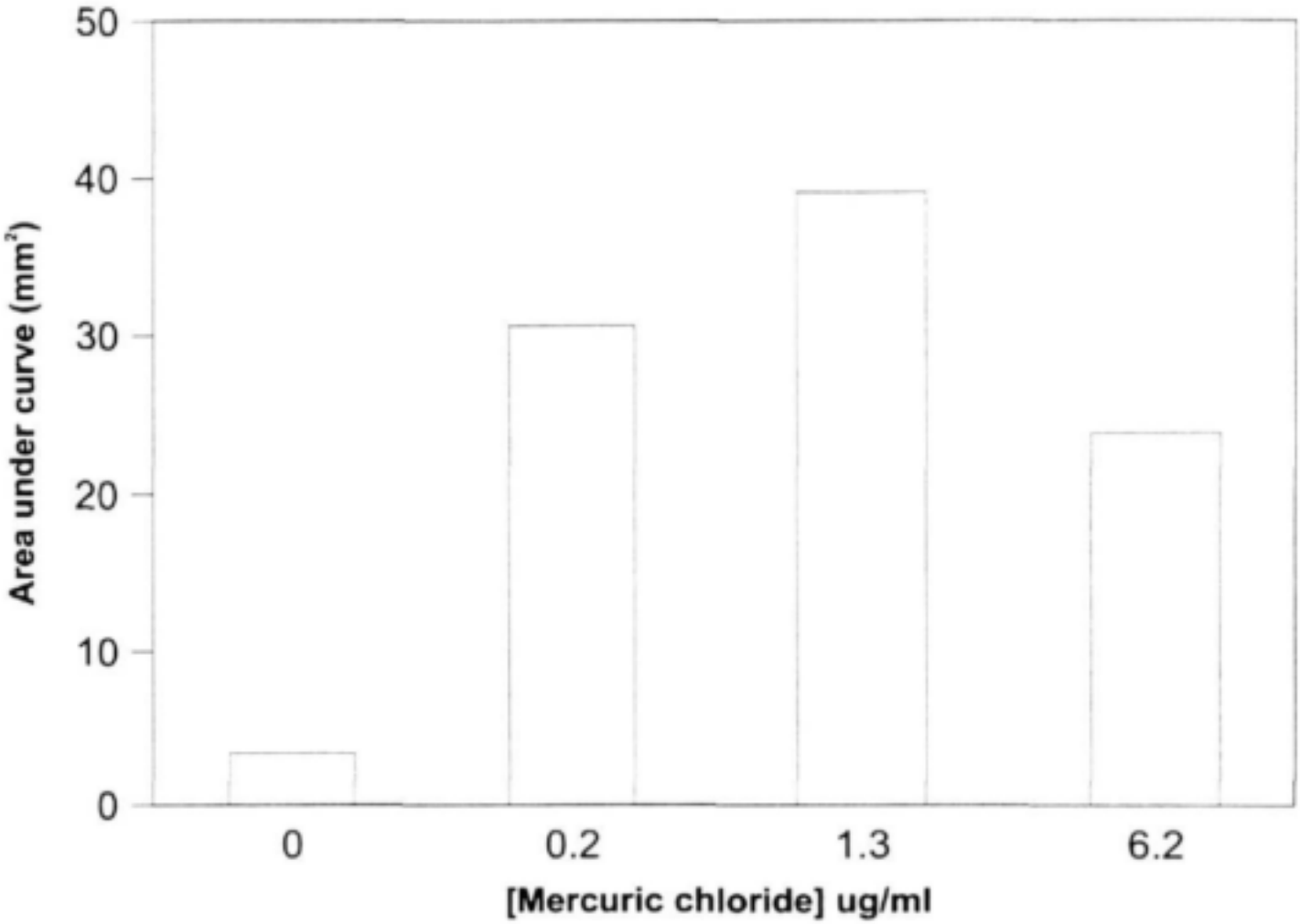

Fig. 3.2 Hsp70 levels in cells exposed to hormetic (0.2μg/ml), plateau (1.3μg/ml) and LC_{50} (6.2μg/ml) concentrations of mercuric chloride for 24 hours.

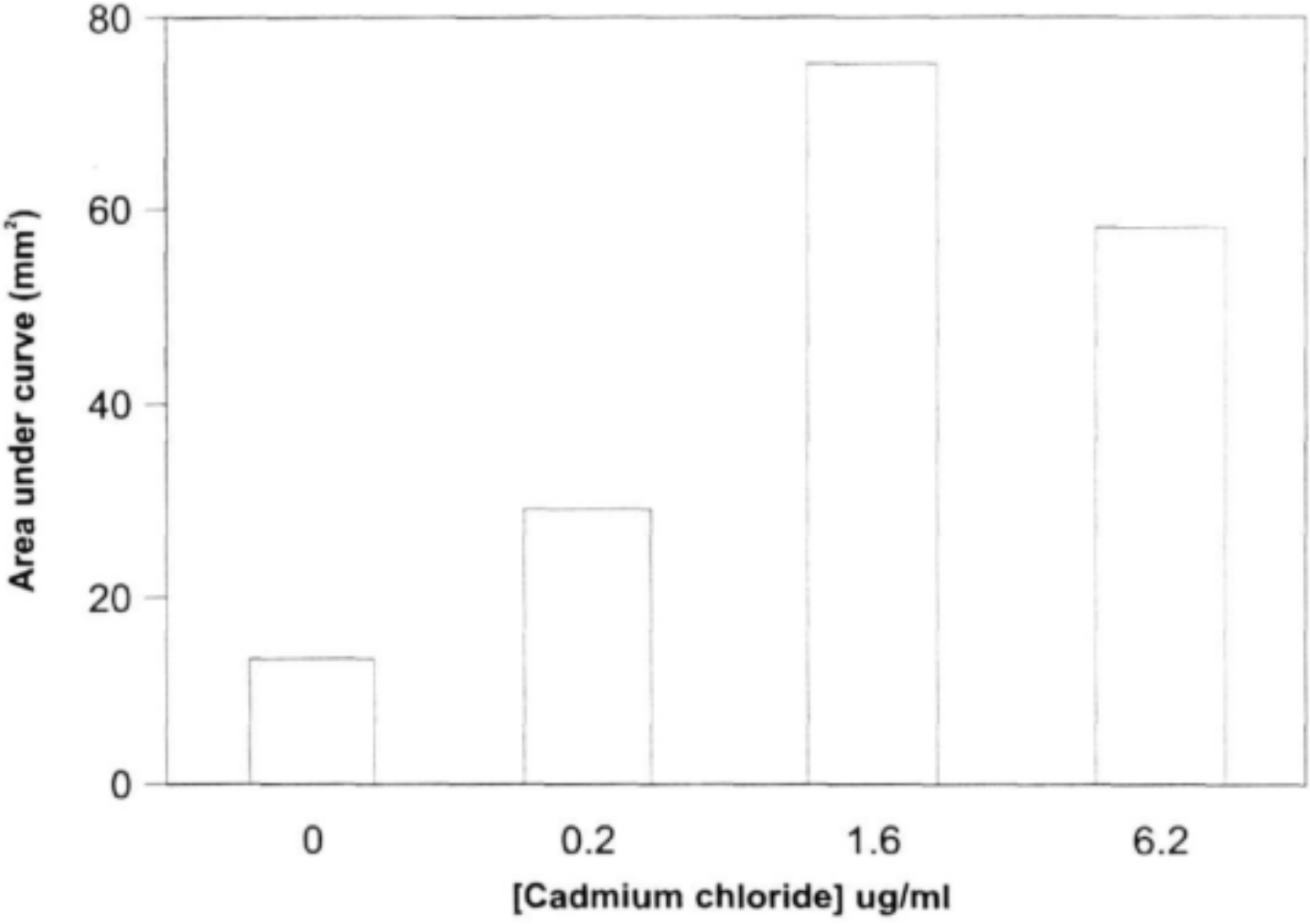

Fig. 3.3 Hsp70 levels in cells exposed to hormetic (0.2μg/ml), plateau (1.6μg/ml) and LC_{50} (6.2μg/ml) concentrations of cadmium chloride for 24 hours.

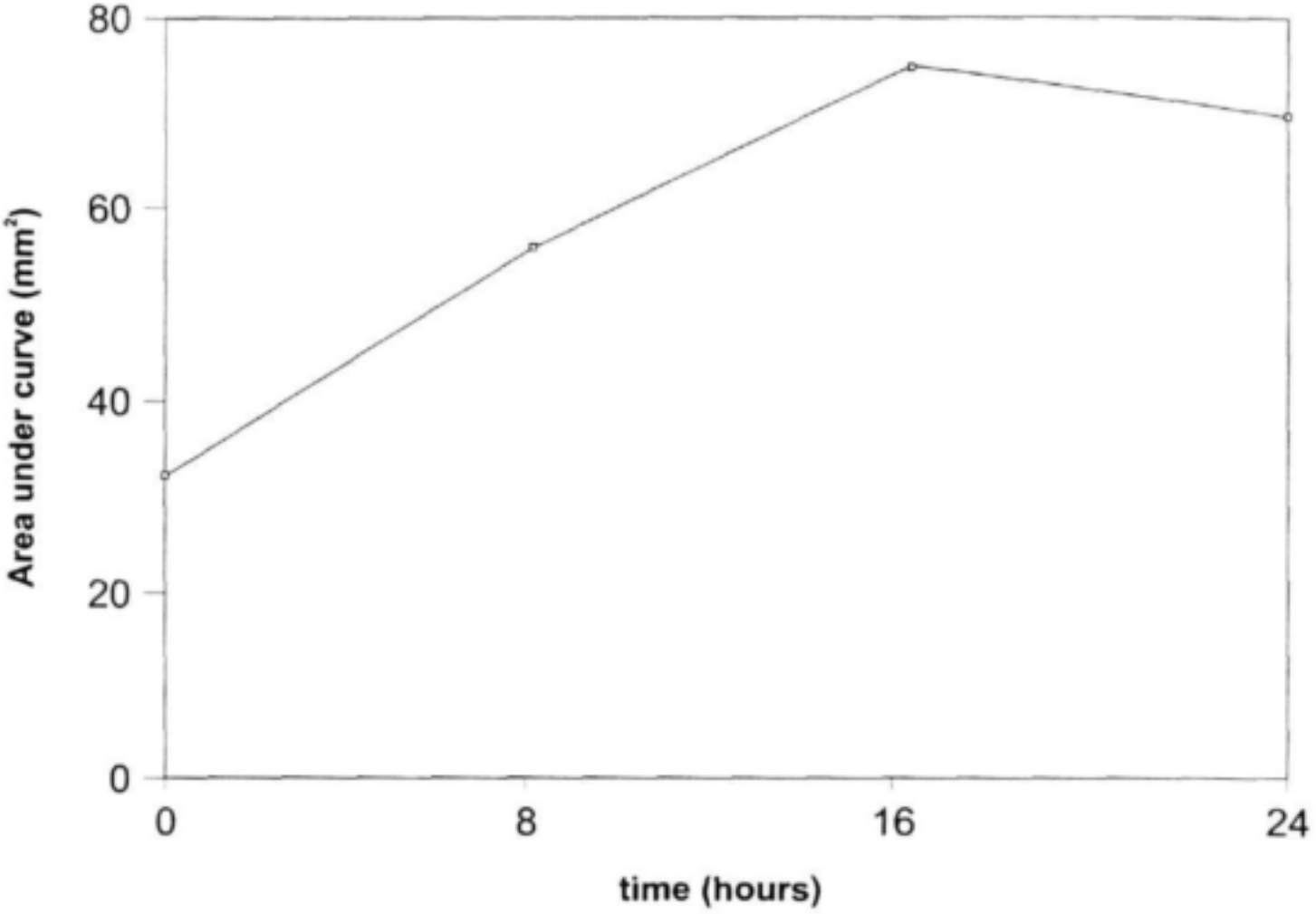

Fig. 3.4 Hsp70 levels in cells exposed to 0.2μg/ml cadmium chloride over 8, 16, 24 hours.

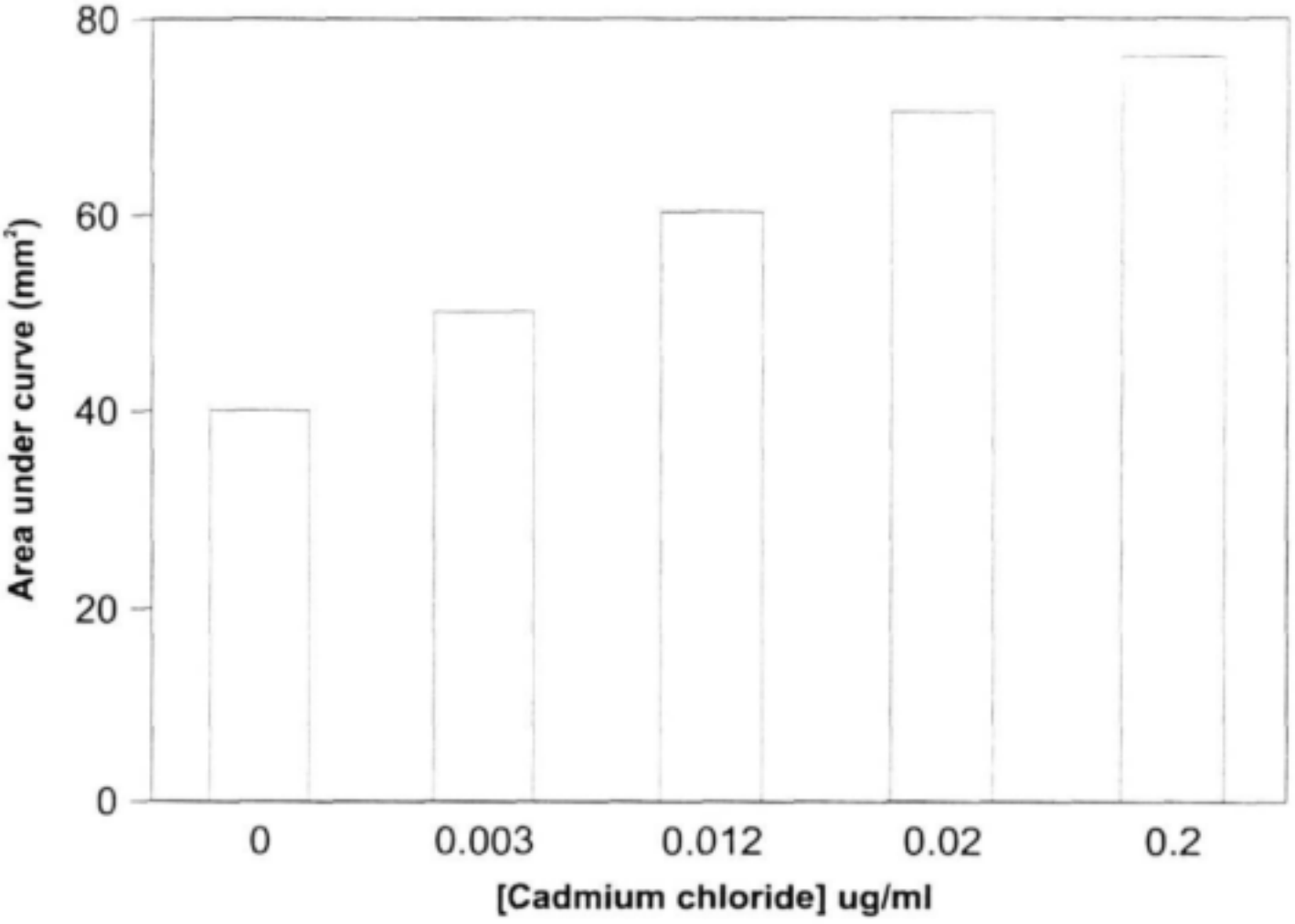

Fig. 3.5 Hsp70 levels in cells exposed to 0.003, 0.012, 0.02 and 0.2μg/ml cadmium chloride for 24 hours.

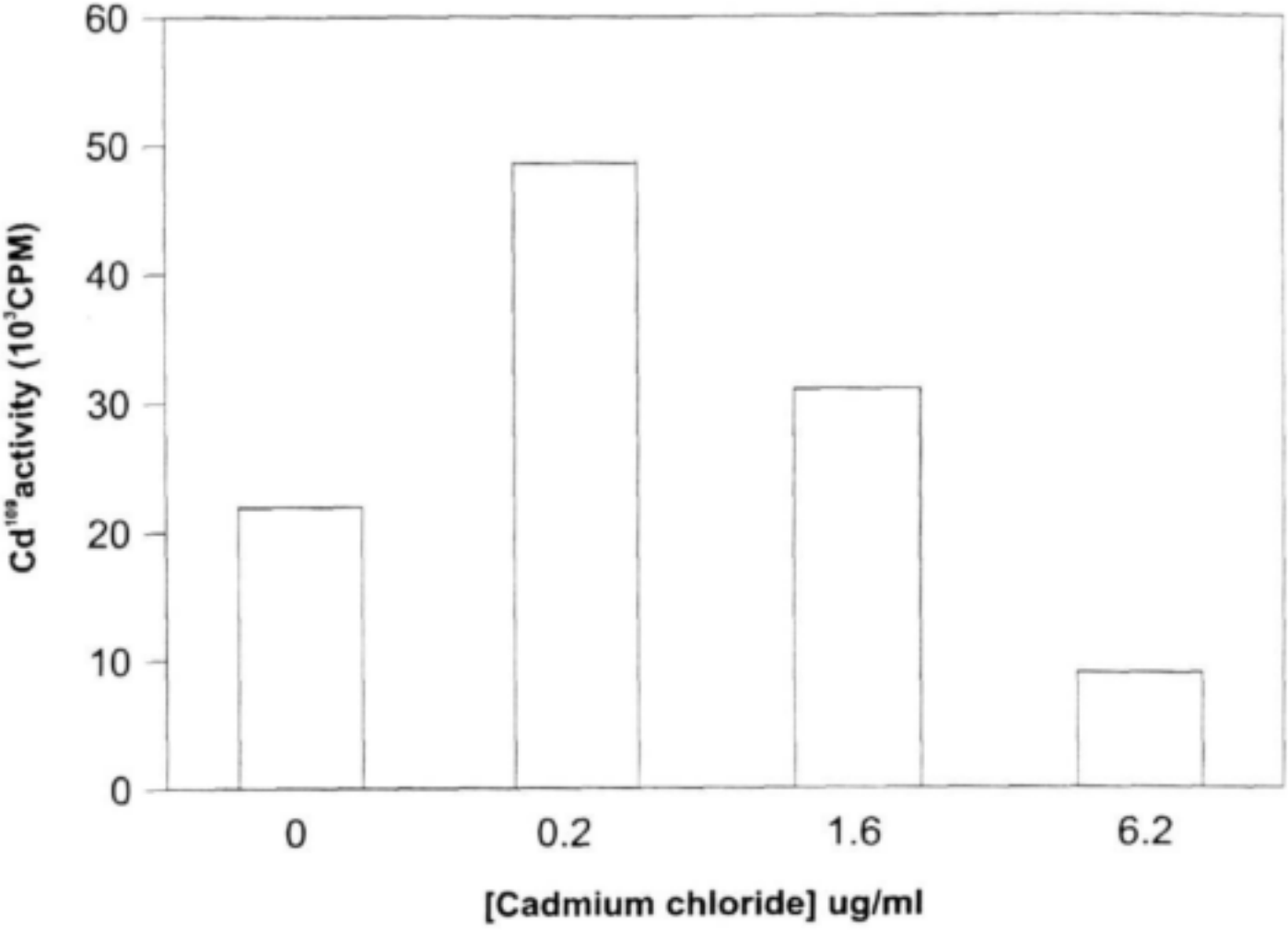

Fig. 3.6 Metallothionein levels in cells exposed to hormetic (0.2μg/ml), plateau (1.6μg/ml) and LC_{50} (6.2μg/ml) concentrations of cadmium chloride for 24 hours.

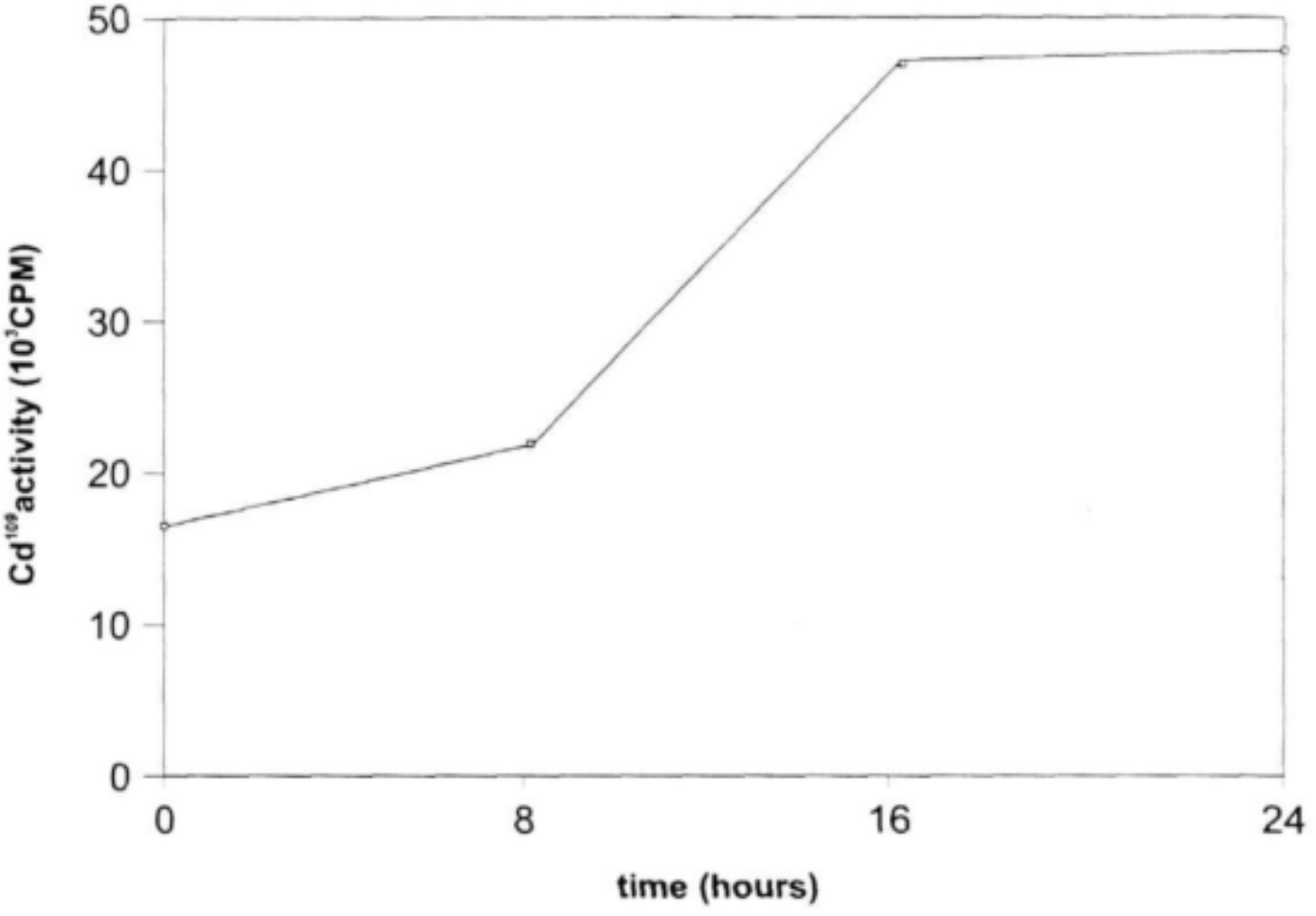

Fig. 3.7 Metallothionein levels in cells exposed to 0.2μg/ml cadmium chloride over 8, 16 and 24 hours.

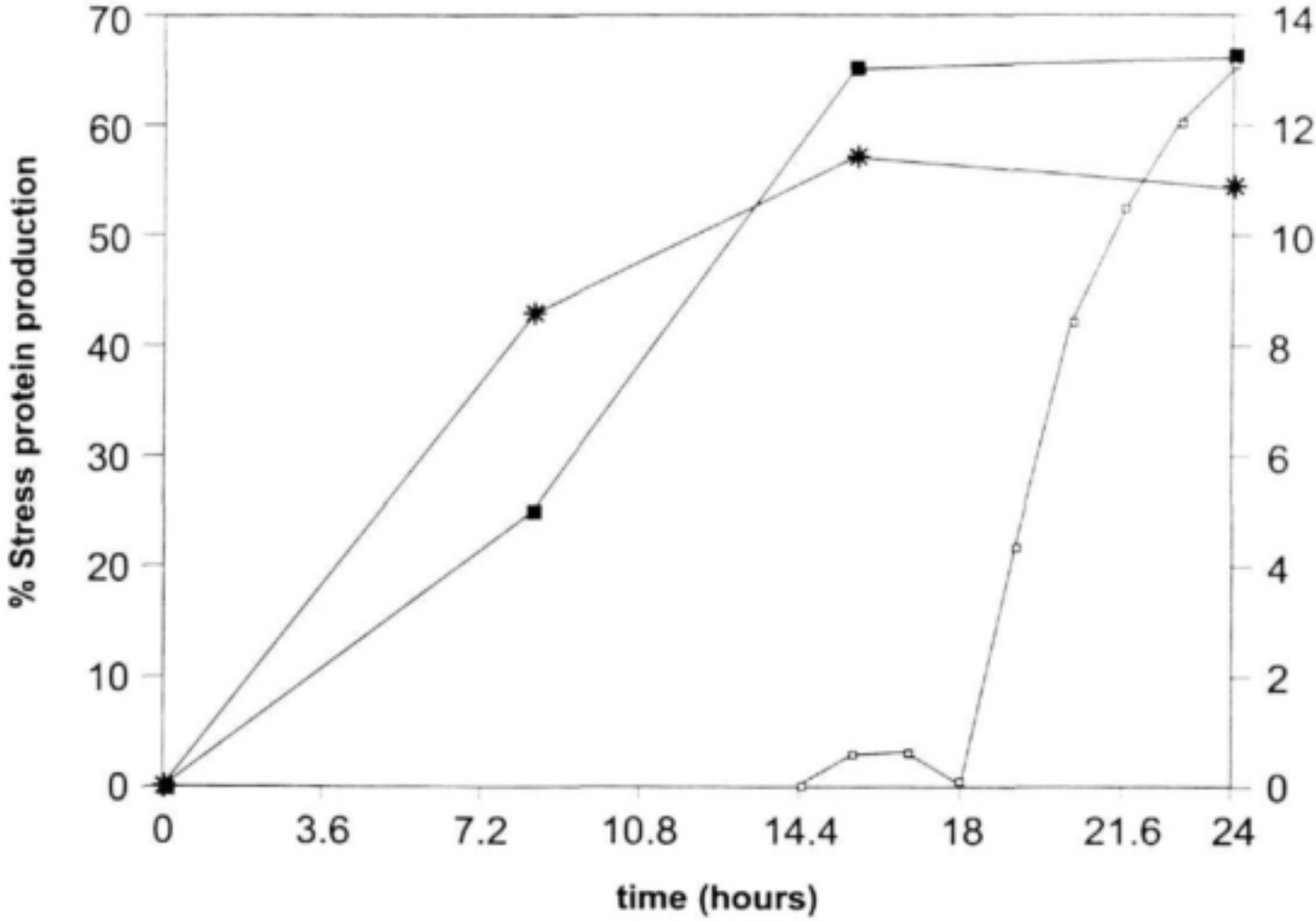

Fig. 3.8 **Time/activity assay and corresponding stress protein levels for cells exposed to 0.2μg/ml cadmium chloride over 24 hours.**

Cellular activity and protein levels are relative to that of untreated control cells. (✳) Hsp70 (area under curve mm^2), (■) metallothionein (Cd^{109} activity 10^3 CPM), (□) Absorbance (570nm).

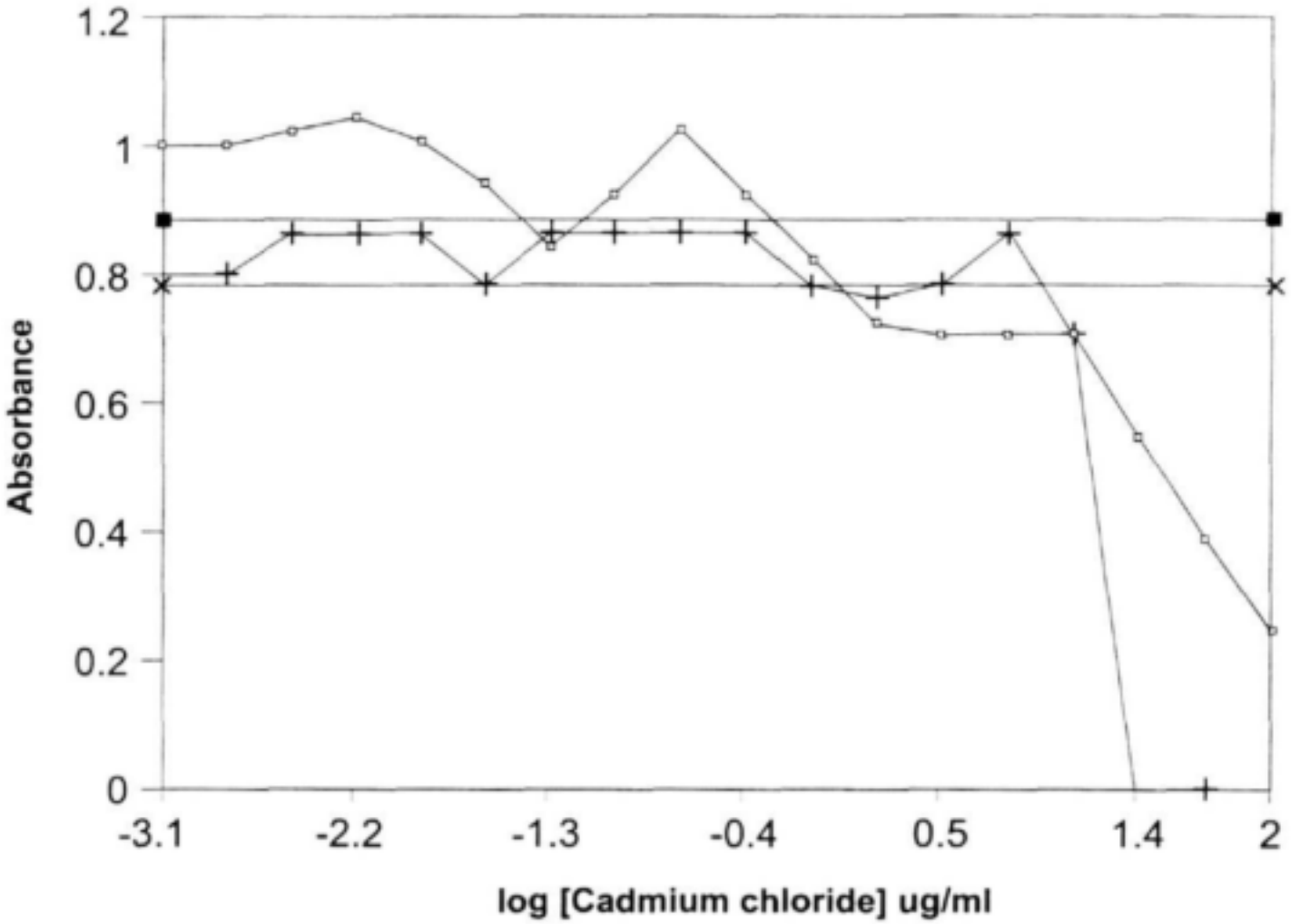

Fig. 3.9 **A comparison of the cytotoxicity curves of CRM and normal cells exposed to cadmium chloride for 24 hours.**

(□) normal cells, (■) 100% control activity for normal cells, (+) CRM cells, (×) 100% control activity for CRM cells.

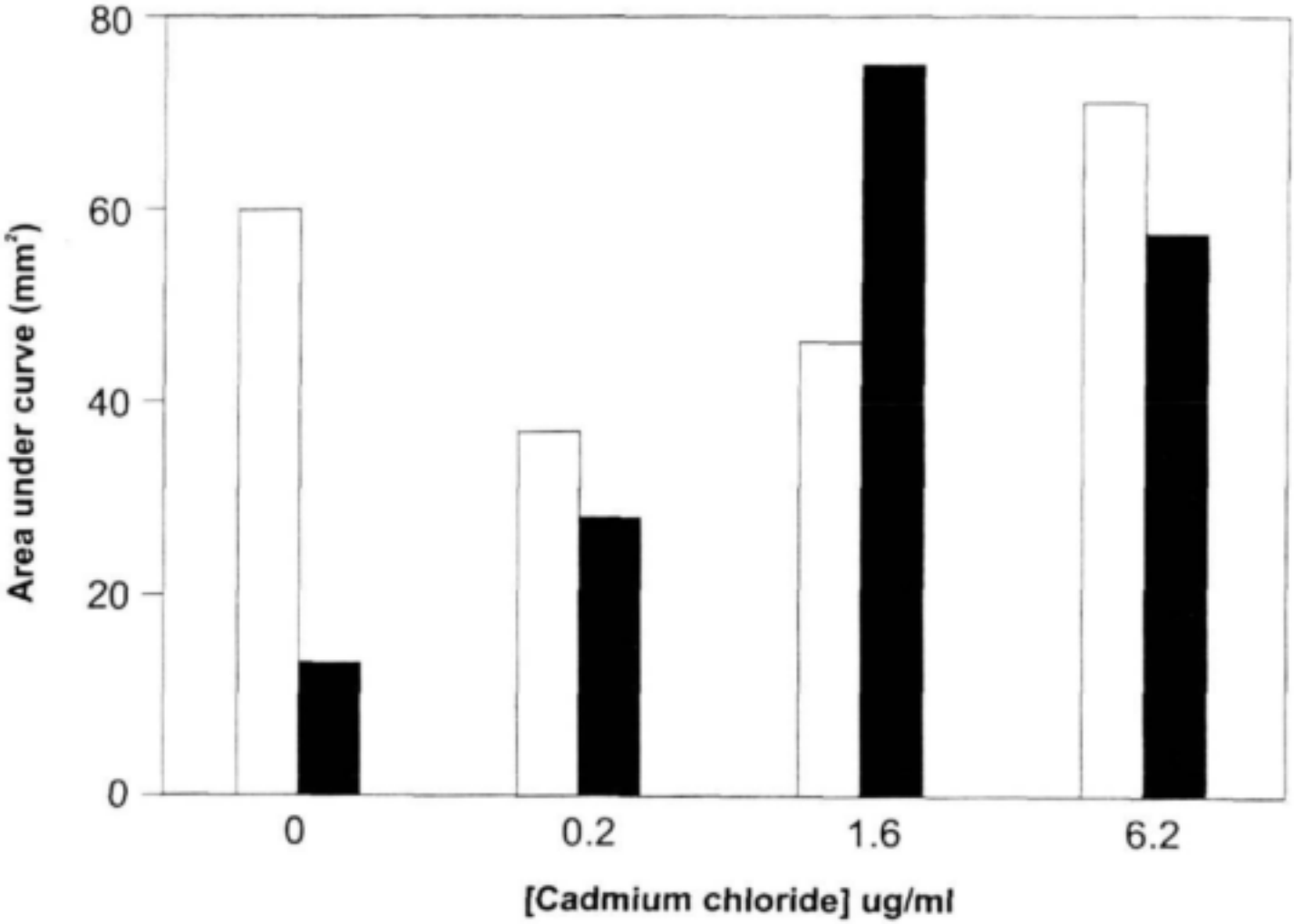

Fig. 3.10 **A comparison of Hsp70 levels in normal and CRM cells exposed to hormetic (0.2μg/ml), plateau (1.6μg/ml) and LC_{50} (6.2μg/ml) concentrations of cadmium chloride for 24 hours.**

Normal cells (black bar), CRM cells (white bar).

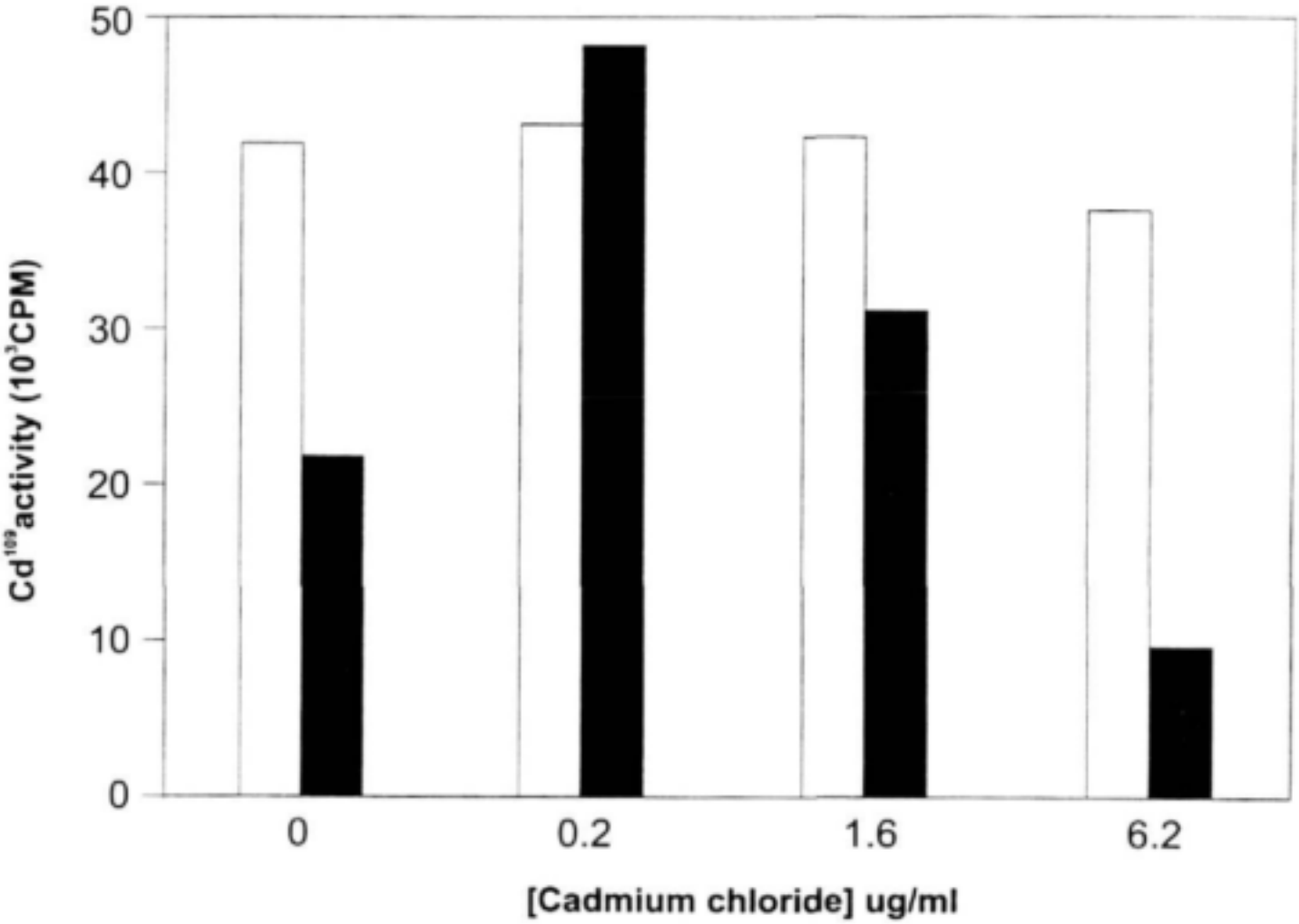

Fig. 3.11 **A comparison of metallothionein levels in normal and CRM cells exposed to hormetic (0.2μg/ml), plateau (1.6μg/ml) and LC_{50} concentrations of cadmium chloride for 24 hours.**

Normal cells (black bar), CRM cells (white bar).

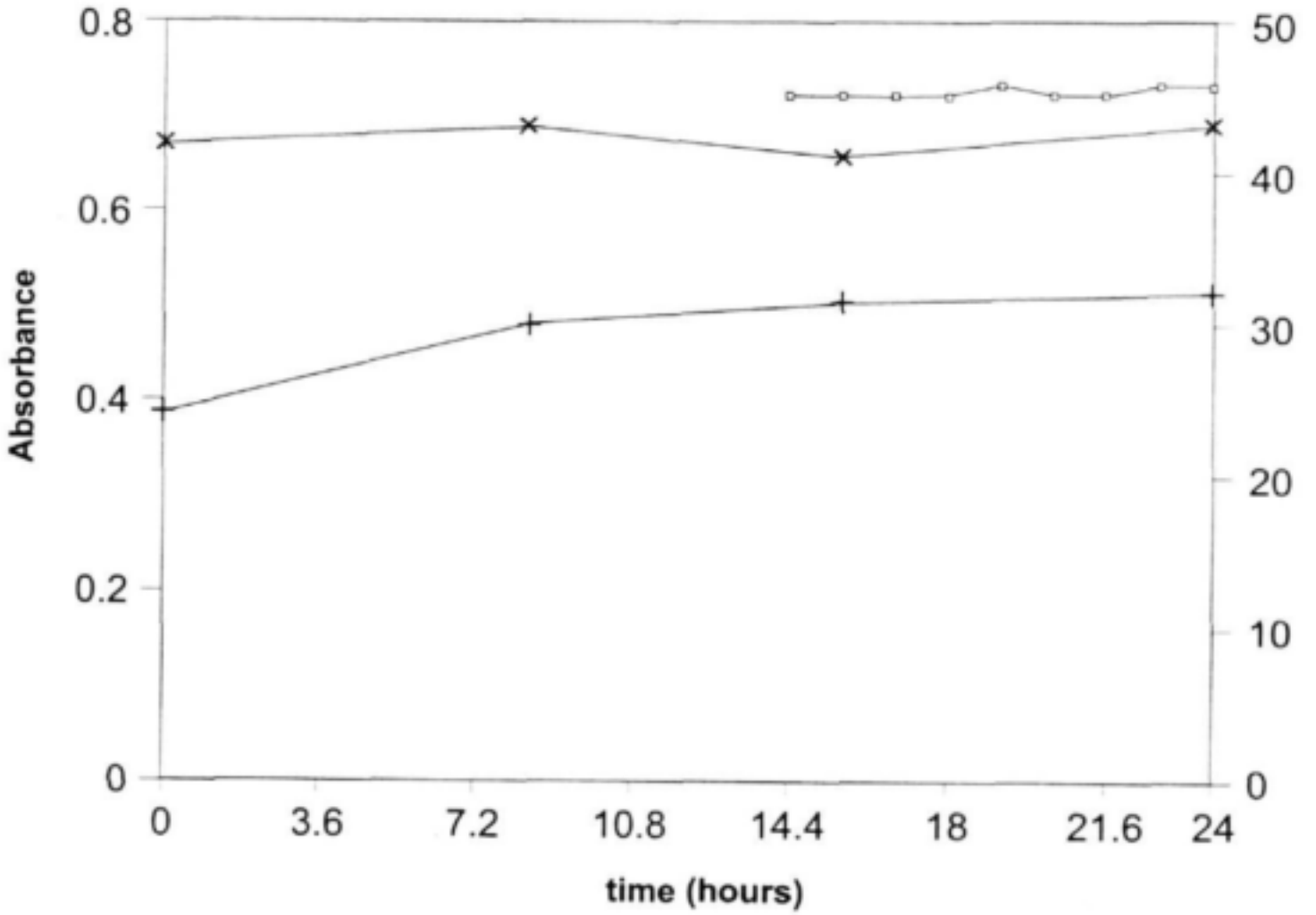

Fig. 3.12 **Time/activity assay and corresponding levels of stress proteins for CRM cells exposed to 0.2μg/ml cadmium chloride over 24 hours.**

(□) Cellular activity (absorbance 570nm), (+) Hsp70, (Area under curve $mm^2/100$), (×) metallothionein.

3.3.5 Superoxide Dismutase Activity in Cells Exposed to Mercuric and Cadmium Chloride

Superoxide dismutases (SOD) are metalloenzymes present in both the cytosol and mitochondria. They play an important role in the removal of superoxide anions (O_2^-) by catalysing the reaction:

$$2O_2^- + 2H \rightarrow H_2O_2 + O_2$$

and are therefore believed to protect cells against some of the cytotoxic effects caused by extracellular metal exposure (Fehér *et al.*, 1987; Steinebach and Wolterbeek, 1994).

The superoxide dismutase activity of cell extracts exposed to mercuric and cadmium chloride for 24 hours was determined by the degree of inhibition of O_2^- mediated nitro-blue tetrazolium reduction. Normal cells exposed to mercuric and cadmium chloride exhibited significant SOD activity which was found to occur slightly down stream from hormetic concentrations for both metals (Fig. 3.13). SOD activity then peaked at plateau levels of toxicant but decreased somewhat with increasing metal concentration although still remaining higher in comparison to untreated control cells.

Superoxide dismutase activity in CRM cells exposed to cadmium chloride for 24 hours however, only increased significantly at LC_{50} and higher concentrations of the toxicant (Fig. 3.13).

The high SOD activity in cells exposed to plateau levels of metal is in agreement with previous research which found that moderate to high levels of copper also elicited a significant increase of superoxide dismutase activity in rat hepatocytes (Wolterbeek and Steinebach, 1994).

However, if high SOD activity was soley in response to increased levels of reactive oxygen species, it would have been expected that SOD activity in CRM cells be constitutive as they were maintained at plateau levels of cadmium chloride (1.3μg/ml). Yet SOD activity only became significantly high in CRM cells, at metal concentrations which were toxic to them, suggesting that superoxide dismutases could have other functions in cells exposed to toxic levels of stress inducers.

One possible role for these enzymes could be the production of hydrogen peroxide (H_2O_2) the reactive oxygen intermediate which is believed to act as a second messenger in signal tranduction pathways and has also been shown to activate the nuclear factor kappa B (NF-kB) which has been found to be potently induced in SOD-overexpressing cell lines (Schulze-Osthoff *et al.*,

1995; Sundaresan *et al.*, 1995).

As it has recently been shown that NF-kB participated in the induction of apoptosis in cell lines deprived of serum (Grimm *et al.*, 1996), it may also be possible that at toxic metal concentrations, high levels of SOD activity could produce sufficient levels of hydrogen peroxide to activate NF-kB which in turn would fully or partially participate in events leading to apoptosis.

This hypothesis lends support from the electron microscopy studies discussed in Chapter 2 which showed that at 1.25μg/ml mercuric chloride, cells exhibited morphological features typical of apoptosis (Fig. 2.8). While from the superoxide dismutase assay carried out for mercuric chloride, this concentration clearly corresponded to a region of maximum SOD activity (Fig. 3.13).

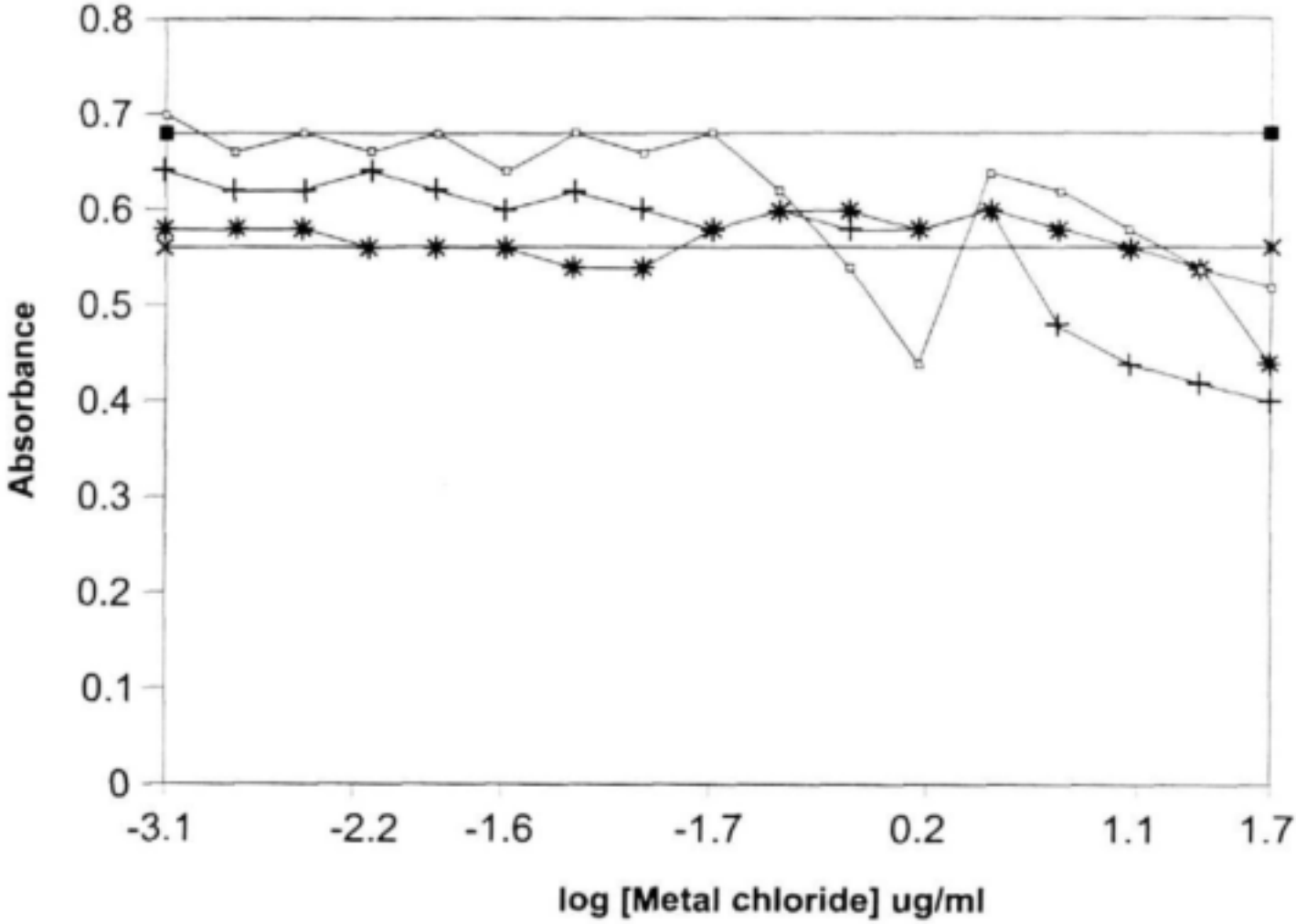

Fig. 3.13 **Superoxide Dismutase activity for cells exposed to mercuric chloride and cadmium chloride for 24 hours.**

(□) Normal cells exposed to mercuric chloride, (■) control activity for mercuric chloride SOD assay, (+) normal cells exposed to cadmium chloride, (✳) CRM cells exposed to cadmium chloride, (×) CRM control activity.

CHAPTER 4

4.1 CONCLUSION

In this study it was shown that a rapid MTT-based colorimetric assay could be used to quantify the degree of cytotoxicity for cells exposed to heavy metals and that the changes in sensitivity exhibited by this assay, when metals were used in combination with trace quantities of ligands or chelating agents, were the result of changes in toxicity due to metal speciation rather than the inhibition of MTT reduction (Chapter 2, 2.3.1-2.3.2).

Furthermore the high sensitivity of the assay allowed for an investigation of the effects of low levels of toxicants on cellular activity and in particular, the increased or hormetic activity exhibited by cells exposed to sub-inhibitory toxicant concentrations.

Time/activity studies revealed that the onset of hormesis was dependent on toxicant concentration in an exponential manner. This indicated that a critical intracellular concentration of toxicant was required to initiate hormesis and that the time taken to reach this concentration was dependent on the toxicant

concentration, to which the cells were exposed, in an first order manner (Chapter 2, 2.3.3).

In addition, activity was found to tend to a maximum in a sigmoidal fashion, where the steepness or order of this sigmoidal response was proportional to log concentration values. Thus, it was possible to describe a hormetic peak as a composite curve which for a narrow concentration range was composed of a series of sigmoidal curves of increasing steepness, each tending to a maximum activity at a specific time (Chapter 2, 2.3.3).

The dependence of hormesis on specific toxicant concentrations and the manner in which activity tended to a maximum over time strongly suggested that this phenomenon was a specific cellular response to a build up of critical levels of metal within cells.

This was confirmed by protein studies which correlated the onset of hormetic activity to the production of threshhold levels of metallothionein and Hsp 70 in normal cells and showed that high constitutive expression of these stress proteins in cadmium resistant mutant cells corresponded to constitutive hormetic activity (Chapter 3, 3.3.1-3.3.4).

In addition, it was shown that the level of stress protein production over time

was proportional to toxicant concentration and this correlated with the increase in the steepness or order of hormesis observed for increasing log toxicant concentrations (Chapter 3, 3.3.4).

These findings therefore clearly showed that hormesis was indeed a specific indicator of toxicant concentrations capable of inducing a stress response in cells and that the order of this hormetic activity was proportional to the magnitude of the induced stress response. Furthermore, the uniform kinetics of hormetic activity allowed for a mathematical description of this increased activity making it potentially useful for the identification of unknown toxicants at concentrations which induce a stress response in cells, as well for the investigation of the effects of metal speciation on cytotoxicity (Chapter 2, Table 2.1).

Although the above findings clearly show that hormesis is an indicator of stress, the precise mechanisms responsible for its initiation and regulation still remain unclear.

Yet, as the magnitude of the induced stress response was shown to be proportional to the order of the hormetic slope and inversly proportional to the onset of hormesis, with increasing toxicant concentrations, it is possible that this phenomenon is a form of energy compensation initiated by cells exposed to extreme environmental conditions which require the production of high levels

of stress proteins over a short period of time and where the degree of stress protein production is proportional to the severity of the stress to which the cells are exposed.

Under these conditions steady-state energy levels may be temporarily disturbed thus triggering a rapid burst of energy production (hormesis) which serves to restore energy homeostasis in the cell.

However, the sigmoidal kinetics of hormetic activity, which is often indicative of the amplified responses observed for cascade systems and the effects of allosteric modifiers on biochemical pathways (Palmer, 1995), further suggests that this increased activity may be coupled to or regulated by specific stress-induced pathways such as the stress-activated protein kinase (SAPK)/c-Jun amino-terminal kinase (JNK) signaling pathway which has been found to be potently induced by protein synthesis inhibitors, tumor necrosis factor α (TNFα), heat shock and UV radiation (Coso *et al.*, 1995), and is also believed to coordinate apoptosis in cells exposed to severe environmental stress (Verheij *et al.*, 1996).

Therefore an investigation of the relationship between hormesis and stress-induced signaling pathways and its possible relationship with apoptosis (shown in cells exposed to moderate levels of mercuric chloride, Chapter 2, 2.3.2), as

well as the effects of metal resistance on these relationships would be some of the aims of future research on this phenomenon.

REFERENCES

Albert A (1965). *Selective Toxicity*. Methuen and Company Limited, London.

American Institute of Biological Sciences (1978). Criteria and rationale for decision making in aquatic hazard evaluation (third draft). In: *Estimating the Hazard of Chemical Substances to Aquatic Life, ASTM Special Technical Publication 657, Aquatic Hazards of Pesticides Task Group of the American Institute of Biological Sciences.* (Eds. Cairns J Jr., Dickson K L and Maki A W). pp 241-274. American Society for Testing and Materials, Philadelphia, USA.

Bartsch R, Klein D and Summer K H (1990). The Cd-Chelex assay: a new sensitive method to determine metallothionein containing zinc and cadmium. *Archives of Toxicology* 64: 177-180.

Berridge M V, Tan A S, McCoy K D and Wang R (1996). The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts. *Biochemica* 4: 15-20.

Cairns J Jr., Dickson K L and Maki A W (Eds.) (1978). *Estimating the Hazard of Chemical Substances to Aquatic Life*. American Society of Testing and Materials, Philadelphia, USA.

Cairo G, Castrusini E, Minotti G and Bernelli-Zazzera A (1996). Superoxide and hydrogen peroxide-dependent inhibition of iron regulatory protein activity: a protective strategem against oxidative injury. *The FASEB Journal* 10: 1326-1335.

Carmichael J, De Graff W G, Gazdar A F, Minna J D and Mitchell J B (1987). Evaluation of a tetrazolium-based colorimetric assay: assessment of chemosensitivity testing. *Cancer Research* 47: 936-942.

Carter A D, Felber B K, Walling M, Jubier M, Schmidt G J and Hamer D H (1984). Duplicated heavy metal control sequences of the mouse metallothionein-I gene. *Proceedings of the National Academy of Sciences USA* 81: 7392-7396.

Coso O A, Chiariello M, Yu J, Teramoto H, Crespo P, Xu N, Miki T and Gutkind J S (1995). The small GTP-binding protein Rac 1 and Cdc 42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* 81: 1137-1146.

Dickerson R E, Gray H B, Darensbourg M Y and Darensbourg D J (1984). *Chemical Principles*. Benjamin Cummings, London.

Ewing J F and Janero D R (1995). Microplate superoxide dismutase assay employing a nonenzymatic superoxide generator. *Analytical Biochemistry* 232: 243-248.

Falchuk K H, Goldwater L J and Vallee B L (1977). The biochemistry and toxicology of mercury. In: *The Chemistry of Mercury* (Ed. McArliff C A). pp. 261-264. Macmillan, Canada.

Fasset D W (1975). Cadmium. In: *Metallic Contaminants and Human Health* (Ed. Lee D H K). pp. 98-117. Academic Press, London.

Fehér J, Csomos G and Vereckei A (1987). *Free Radical Reactions in Medicine*. Springer-Verlag, Berlin.

Forbes V E and Depledge M H (1992). Cadmium effects on the carbon and energy balance of mud snails. *Marine Biology* 113: 263-269.

Forbes V E and Forbes T L (1994). *Ecotoxicology in Theory and Practice*. Chapman and Hall, London.

Gerlier D and Thomasset N (1986). Use of MTT colorimetric assay to measure cell activation. *Journal of Immunological Methods* 94: 57-63.

Goldberg A M, Frazier J M, Brusick D, Dickens M S, Flint O, Gettings S D, Hill R N, Lipnick R L, Renskers K J, Bradlaw J A, Scala R A, Veronesi B, Green S, Wilcox N L and Curren R D (1993). Framework for validation and implementation of *in vitro* toxicity tests. *In vitro Cell Developmental Biology* 29A: 688-692.

Goldwater L J and Clarkson T W (1975). Mercury. In: *Metallic Contaminants and Human Health*. (Ed. Lee D H K). pp. 17-24. Academic Press, London.

Goyer R A (1991). Toxic effects of metals. In: *Casarett and Doul's Toxicology, the Basic Science of Poisons, 4th ed.* (Eds. Amdur M O, Dow J and Klassen C D). pp. 623-673. Pergamon Press, New York.

Grimm S., Bauer M K A, Baeuerle P A and Schulze-Osthoff K (1996). Bcl-2 Down-regulates the activity of transcriptional factor NF-kB induced upon apoptosis. *The Journal of Cell Biology* 134: 13-23.

Hamer D H (1986). Metallothionein. *Annual Reviews in Biochemistry* 55: 913-951.

Hatayama T, Tsukimi Y, Wakatsuki T, Kitamura T and Imahara H (1991). Different induction of 70,000-Da heat shock protein and metallothionein in HeLa cells by copper. *Journal of Biochemistry* 110: 726-731.

Hayat M A (1972). *Basic Electron Microscopy Techniques*. van Nostrand Reinhold, New York.

Hunziker P E and Kägi J H R (1985). Metallothionein. In: *Metalloproteins*. (Ed. Harrison P M). pp. 149-181. Macmillan Press, London.

Hutcheson M, Miller D C and White A Q (1985). Respiratory and behavioral responses of the grass shrimp *Palaemonetes pugio* to cadmium and reduced disolved oxygen. *Marine Biology* 88: 59-66.

Ivanovici A M (1980). Application of adenylate energy charge to problems of environmental impact assessment in aquatic organisms. *Helgoländer Meeresunters* 33: 556-565.

Jonnalagadda S B and Prasada Rao P V V (1993). Toxicity, bioavalability and metal speciation. *Comparative Biochemistry and Physiology* 3: 585-595.

Kerr J F R, Winterford C M and Harmon B V (1994). Apoptosis, its significance in cancer and cancer therapy. *Cancer* 73: 2013-2026.

Kinne O (1980). 14th European marine biology symposium "Protection of life in the sea": summary of symposium papers and conclusions. *Helgoländer Meeresunters* 33: 732-761.

Koehn R K and Bayne B L (1989). Towards a physiological and genetic understanding of the energetics of the stress response. *Biological Journal of the Linnean Society* 37: 157-171.

Kyriakis J M, Banerjee P, Nikolakaki E, Dal T, Ruble E A, Ahmad M F, Avruch J and Woodgett J R (1994). The stress-activated protein kinase subfamily of C-Jun kinases. *Nature* 369: 156-160.

Lindquist S (1986). The heat shock response. *Annual Reviews in Biochemistry* 55: 1151-1191.

Mager W H and De Kruyff A J J (1995). Stress-induced transcriptional activation. *Microbiological Reviews* 59: 506-531.

Möllering H, Wahlefeld A W and Michal G (1974). Visualization of NAD(P)-dependent reactions. In: *Methods of Enzymatic analysis, Vol. 1.* (Eds. Bergmeyer H V and Gawehn K). pp. 136-143. Verlag Chemie Weinheim, Academic Press, London.

Moore M N (1980). Cytochemical determination of cellular responses to environmental stressors in marine organisms. *Rapp.P.-Y. Réun. Cons. Int. Explor. Mer.* 179: 7-15.

Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* 65: 55-64.

Palmer T (1995). *Understanding Enzymes*. Prentice Hall/Ellis Horwood, London.

Pauling L (1970). *General Chemistry*. Dover Publications, New York.

Peakall D (1992). *Animal Biomarkers as Pollution Indicators*. Chapman and Hall, London.

Pfitzer A (1975). Concepts of environmental toxicology. In: *Metallic Contaminants and Human Health*. (Eds. Lee D H K). pp.... 1-12. Academic Press, London.

Rand G M and Petrocelli S R (Eds.) (1985). *Fundamental Aquatic Toxicology*. Hemisphere, New York.

Roux D J, Kempster P L, Truter E and van der Merwe L (1993). Effect of cadmium and copper on survival and reproduction of *Daphnia pulex*. *Water S A* 19: 268-280.

Schulz H (1888). *Pflügers Arch. Ges. Physiol.* 42: 517.

Schulze-Osthoff K, Los M and Baeuerle P A (1995). Redox signalling by transcription factors NF-kB and AP-1 in lymphocytes. *Biochemical Pharmacology* 50: 735-741.

Scudiero D A, Shoemaker R H, Paull K D, Monks A, Tierney S, Nofziger T H, Currens M J, Seniff D and Boyd M R (1988). Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Research* 48: 4827-4833.

Shriver D F, Atkins P W and Langford C H (1991). *Inorganic Chemistry*. Oxford University Press, Oxford.

Sloof W, van Oers J A M and De Zwart D (1986). Margins of uncertainty in ecotoxicological hazard assessment. *Environmental Toxicology and Chemistry* 5: 841-852.

Smith G R (1975). Five of potential significance. In: *Metallic Contaminants and Human Health*. (Ed. Lee D H K). pp. 17-24.

Smith P K, Krohn R I, Hermanson G T, Mallia A K, Gartner F H, Provenzano M D, Fujimoto E K, Goeke N M, Olson B J and Klenk D C (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* 150: 76-85.

Stebbing A R D (1982). Hormesis - the stimulation of growth by low levels of inhibitors. *The Science of the Total Environment* 22: 213-234.

Stegeman J J (1980). Mixed function oxygenase studies in monitoring for effects of organic pollution. *Rapp. P. -V Réun. Cons. Int. Explor. Metr.* 179: 33-38.

Steinbach O M and Wolterbeek H Th (1994). Effects of copper on rat hepatoma HTC cells and primary cultured rat hepatocytes. *Journal of Inorganic Biochemistry* 53: 27-48.

Sundaresan M, Yu Z, Ferrans V J, Irani K and Finkel T (1995). Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. *Science* 270: 296-299.

Tuey D B (1980). Toxicokinetics. In: *Introduction to Biochemical Toxicology*. (Eds. Hodgson E and Guthrie F E). pp. 40-65. Blackwell Scientific Publications, Oxford.

Verheij M, Bose R, Lin X H, Yao B, Jarvis W D, Grant S, Birrer M J, Szabo E, Zon L I, Kyriakis J M, Haimovitz-Friedman A, Fuks Z and Kolesnick R N (1996). Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. *Nature* 380: 75-79.

Welch W J (1992). Mammalian stress response: cell physiology, structure/function of stress proteins and implications for medicine and disease. *Physiological Reviews* 72: 1063-1081.

West D M, Skoog D A and Holler F J (1992). *Fundamentals of Analytical Chemistry*. 6th ed. Saunders College Publishing, London.

Whitacre C M (1996). Application of Western blotting to the identification of metallothionein binding proteins. *Analytical Biochemistry* 234: 99-102.

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