INVERTEBRATE STRESS RESPONSES AS MOLECULAR BIOMARKERS IN ECOTOXICOLOGY

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Thesis presented to the University of Nottingham for the degree of Doctor of Philosophy; September, 1994.

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Acknowledgements

I was supported by the University of Dicle (Diyarbakir) in Turkey throughout this PhD study.

I deeply acknowledge my indebtedness to Dr. David de Pomerai for his outstanding contribution to this study; for his supervision and encouragement, as well as for his understanding and patience.

I am also grateful to Jenny A. Duce for her technical assistance and friendship which provided relief in the final year, and to Mohammed Mutwakil, not only for similar support but also for his cheerful company.

I would also like to thank Drs. David Holdich, Jim Reader and David Bell for their advice and guidance within the project, and their research students Richard, Paula, Claire, Yee, Nick, Neil, Paul and Dawn for always being ready to help. Especially, I thank Richard for suggesting the use of ECL system, and letting me use many other chemicals. I would like to extend my gratitude to Drs. P.Tighe and D.Pritchard of this department for providing anti-β-galactosidase, and Professor Susan Lindquist of the University of Chicago for the provision of anti-HSP70.

Thanks also go to Mr Brian Case and Mr Roy Searcey for their patience in producing the photographs, Ms Maureen Greensmith for help with ordering facilities. I also acknowledge the help from CELE staff during the language training. Finally, I would like to thank my friends Neytullah, Beatriz, Paul and Eddie for making life in Nottingham more enjoyable.

Summary

All organisms studied so far respond to heat shock by inducing the synthesis of a number of proteins called heat shock proteins (HSPs). This universal response can also be induced by a variety of stressors, including heavy metal ions and organic and organo-metallic compounds. As a result, the stress response has recently attracted the attention of ecotoxicologists for use in environmental biomonitoring. In the present study, we have investigated the stress responses of two different organisms; namely the free-living soil nematode *Caenorhabditis elegans* (both wild-type and transgenic strains) and the freshwater crustacean *Asellus aquaticus*. We have also explored the possible use of these model systems in environmental biomonitoring, using different techniques which include metabolic labelling with subsequent one-dimensional electrophoresis and autoradiography, and one- or two-dimensional Western blotting using antibodies specific to stress protein 70.

The study with *A.aquaticus* shows that this organism exhibits a classical stress response. The exposure of asellids to heat shock-treatment (26°C); 13°C above the standard maintenance temperature or to sublethal concentrations of metal ions (Cd^{++} and Cu^{++}) resulted in the the induction of at least 5 putative HSPs which belong to several major HSP families (HSP100, HSP90 and possibly HSP60). An increase in the synthesis of smaller sizes of polypeptides (25-35 kD) should be also noted. Moreover, the time-course of heat versus heavy metal stress-responses in this organism suggests that the pattern of stress-protein synthesis changes considerably with increasing exposure time; notably the response to heat is more transient than that to heavy metals. However, HSP70 does not appear to be the major stress protein induced in this organism. The presence of low molecular weight (LMW) proteins which react with anti-HSP70 antibodies, and the apparent deficiency of classical 70 kD stress proteins in

A.aquaticus, both suggest that HSP70s in this organism are for some reason prone to degradation.

In the nematode *C.elegans*, shifting the culture temperature from 20°C to 34°C induces the synthesis of a set of HSPs corresponding to the HSP90, HSP70 and small HSP families. There are at least nine members of the *hsp70* multigene family in *C.elegans*; some members are expressed constitutively, while others are stress inducible. We have studied the effects of heat and heavy metal (cadmium) stress on the expression patterns of the HSP70 protein family in the nematodes by one- and two-dimensional Western blotting using a monoclonal anti-HSP70 antibody that recognises a conserved epitope shared by most HSP70 family members. Constitutive *C.elegans* HSP70s (expressed at 20°C) are almost undetectable on one-dimensional immunoblots, but chemiluminescent probing of two-dimensional blots reveals a complex pattern of several HSP70 spots. Mild heat shock at 31°C induces a doublet HSP70 band on one-dimensional blots, of which the heavier component (75 kD) is more prominent than the lighter (73 kD). On two-dimensional blots, this pattern is shown to be more complex, with a prominent 75 kD spot newly induced and several other spots intensified. Severe heat shock at 34°C strongly induces both 75 and 73 kD bands on one dimensional blots; two dimensional analysis reveals a series of novel and/or elevated 73 and 75 kD spots. Treatment with cadmium (16 ppm) at 31°C gives a different pattern of spots as compared with 31°C alone; several spots show enhanced while some are newly expressed, and not all of these are present at 34°C. These results indicate that related members of the HSP70 protein family in *C.elegans* are independently regulated in response to different forms of stress. The possible significance of these findings is discussed in relation to the possible use of stress responses as environmental biomonitors.

We have also utilised a stress-inducible *C. elegans* strain (CB4027) for monitoring environmental contamination. This transgenic strain carries integrated copies of the *Drosophila hsp70* promoter fused to an *E. coli lacZ* reporter gene. When exposed to heat shock or to several environmentally relevant stressors, the transgenic strain expresses the reporter product, β-galactosidase, which can easily be quantified or localised *in situ* in stained worms or on Western blots (apparently enzymatically active as a 170 kD form). We have exposed transgenic worms to a variety of toxicants at an elevated temperature (32°C) just below that required for heat shock (34°C), in order to obtain optimal transgene induction. Exposure of nematodes to several heavy metals (e.g. Cd^{++}, Hg^{++}, Zn^{++}, Sn^{++}, Mn^{++} and Ag^{+}), organometallic toxicants (tributyltin) or organic pollutants (lindane) induces β-galactosidase expression in a dose-dependent manner. Cadmium is found to be by far the strongest inducer of transgene activity amongst the agents tested, although tributyltin is an effective inducer at ppb levels. The effects of mixtures of divalent metal ions (Cd^{++}/Ca^{++}, Cd^{++}/Zn^{++} and Cd^{++}/Hg^{++}) on β-galactosidase expression have been also investigated. All three divalent ions tested in combination with cadmium significantly inhibit cadmium-induced transgene activity in comparison to cadmium alone. In the case of Cd^{++}/Ca^{++} mixtures, a marked inhibition of Cd^{++} accumulation by worm tissues has also been demonstrated, directly related to the Ca^{++} concentration. These effects may represent competition for metal-ion uptake through calcium channels. Our results show that this transgenic system works well within strictly defined assay conditions, and can detect clear responses over a 7h exposure period to environmentally relevant toxicants at sublethal concentrations well below the 24 or 48h LC50 values. However, there is a need for careful characterisation and containment of any transgenic organism if it is to be used as environmental monitoring tool.

Abreviations

AAS - atomic absorption spectrophotometry
ATP - adenosine triphosphate
BiP - immunoglobulin heavy-chain binding protein
BSA - bovine serum albumin
DNA - deoxyribonucleic acid
DTT - dithiothreitol
ECL - enhanced chemiluminescence
EDTA - diaminoethanetetra-acetic acid (disodium salt)
ER - endoplasmic reticulum
GRP - glucose-regulated protein
HI - heat-inducible
HS - heat shock
HSC - heat-shock cognate
HSE - heat-shock element
HSF - heat-shock factor
HSP - heat-shock protein
HRP - horseradish peroxidase
IEF - isoelectric focussing
IgG - immunoglobulin G
kD - kilodalton
LC - lethal concentration
LMW - low molecular weight
MT - metallothionein
NHI - non-heat-inducible
ONPG - o-nitrophenyl-β-D-galactopyranoside
PAGE - polyacrylamide gel electrophoresis
ppb - parts per billion
ppm - parts per million
PBS-T - phosphate-buffered-saline plus Tween 20
RNA - ribonucleic acid
SDS - sodium dodecyl sulphate
SP - stress protein
TEMED - NNN'N'-tetramethylethylene diamine
TBS-T - Tris-buffered-saline plus Tween 20
TBT - tributyltin
Tris - tris (hydroxymethyl) aminomethane

CHAPTER 1. INTRODUCTION

1.1 POLLUTION

Public and governmental concern about environmental pollution have recently increased because it is affecting human life as well as wildlife habitats and nature generally. Pollution is mainly caused by man; anything introduced into environment by his activities can be considered as pollution. A list of pollutants found in aquatic ecosystems is given in Table 1.1. The toxic effect of a pollutant on a target organism depends on the concentration of that toxicant and the exposure time, and this effect may be either acute or chronic. Acute effects occur rapidly, are clearly defined, often fatal and rarely reversible, whereas chronic effects develop after long exposure to low doses and may ultimately cause death. Sublethal doses often affect an organism's physiological and behavioral processes, and thus reduce overall fitness.

The effects of many pollutants on aquatic organisms have been extensively studied (see review of literature in Sheedy *et al*,1991). The most dangerous toxic materials have been listed by the environmental protection agencies (EPAs; e.g. the U.S. EPA), and their uses have been restricted by environmental acts. Major types of toxicants are listed on the basis of properties such as toxicity, persistence and potential for accumulation. Two particular classes of toxicant, namely organic compounds and heavy metals, include many listed materials which are dangerous on all these grounds and therefore have attracted the attention of ecotoxicologists.

Table 1.1 Categories of pollutants found in aquatic ecosystems*

Pollutants
Alkalis and acids (main sources; sulphur dioxide and the oxides of nitrogen from burning fossil fuels)
Detergents
Domestic sewage and farm manures
Gases (e.g. chlorine, ammonia)
Heat (electricity generating power stations)
Metals (e.g. cadmium, mercury, zinc, lead)
Nutrients, often in the form of agricultural fertilisers (phosphates, nitrates)
Oil (discharges from industry or refinery effluent)
Organic toxic wastes (phenols, formaldehydes)
Pathogens (bacteria, virus)
Pesticides (DDT, organo-tin compounds, organo-phosphorus compounds, fungicides etc.)
Radionuclides

* Based on Mason (1991)

1.1.1 Organic compounds

This group of toxicants originates from a wide variety of industrial, agricultural and some domestic sources; they are often accumulated within living tissues and some are found to be extremely toxic, causing carcinogenic, teratogenic and mutagenic effects. The detoxification of organic toxicants mainly occur through the liver detoxification enzyme

systems, whose activity and levels can be used as indicator of organic exposure (Adams *et al*, 1990). The most dangerous compounds comprise a heterogenous group of organic chemicals; many pesticides (see Murphy, 1986 for the toxic effects of pesticides) such as organophosphate (malathion) or organochlorine (DTT, lindane) insecticides, chlorophenoxy herbicides (tetrachloro-dibenzodioxin), aromatic (pentachlorophenol) or organo-mercuric (methylmercury) fungicides, petroleum hydrocarbons, and other organometallic compounds such as tributyltin (an active component of some antifouling paints) and tetraethyllead which is a very toxic petroleum additive (leaded petrol).

1.1.2 Heavy metals

Heavy metals are continually released into the aquatic environment from natural sources such as volcanic activity or weathering of rocks. However, industrial processes and some agricultural uses have greatly increased the mobilization of many metals. Because this group of pollutants has been the main subject of the present study, in this section we will concentrate on the major effects of heavy metal ions at cellular levels in some detail.

Heavy metals include both essential elements (Mn^{++}, Zn^{++}, Cu^{++} etc.) and metals with no known biological function such as Cd^{++}, Hg^{++}, Ag^{+} and Sn^{++}, some of these (Cd^{++} and Hg^{++}) are among the most toxic and important aquatic pollutants. Metal ions are usually present in tissues as divalent cations, which may be free or complexed to different classes of biological ligands. In aquatic systems, however, they are present in different forms, resulting from the equilibrium between heavy metal ions and inorganic and organic complexes (Viarengo, 1989). The speciation of heavy metals is of particular importance

because the degree of bioavailability and toxicity of these ions may depend on their chemical forms in the environment (Jonnalogadda and Rao, 1993). Heavy metal toxicity is mediated through the activity of the free metal cations in waters (Sanders *et al*, 1983; Viarengo, 1989; Wlostowski, 1992). Furthermore, some inorganic heavy metals (e.g. mercury) can be converted by microorganisms into highly toxic organometallic compounds (methyl mercury), which are more readily taken up by tissues (Jonnalogadda, 1993). Tributyltin and tetraethyllead are also highly toxic organometallics.

The mechanisms by which heavy metal ions penetrate into cells are not well known, but there is increasing evidence that metal ions enter cells through either voltage- sensitive calcium channels or by a sulfhydryl-sensitive route which involves receptor-mediated channels (Zarooigan *et al*, 1993). In addition, heavy metal uptake is thought to be the result of passive diffusion or a process of facilitated passive diffusion (Roesijadi *et al*, 1993). I will discuss metal uptake mechanisms (mainly for cadmium) in more detail in section 6.1.1.

1.1.3 Molecular mechanisms of heavy metal cytotoxicity

Although heavy metal ions affect a number of physiological processes in aquatic organisms (Viarengo, 1989), little is known regarding their fate or their effects at cellular level. It is known that heavy metals can be bound to sulfhydryl, hydroxyl, carboxyl, and amino residues of proteins, peptides and amino acids (Viarengo, 1989). However, heavy metal ions tend to form more stable complexes only with sulfhydryl residues of amino acids and polypeptides (Viarengo and Nicotera, 1991; Viarengo and Nott, 1993). Heavy

metals entering cells may therefore react with the structural and enzymatic components of membranes and organelles, or with soluble enzymes, due to the high affinity of metals for -SH groups.

The cell membranes are the first target for heavy metal effects, altering their structure and functions. One of the most important effects of metal cations (e.g. Cd^{++} and Hg^{++}) concerns the inhibition of the activity of the membrane transport enzymes such as Ca^{++},Mg^{++}-ATPases in mussels (Pivovarova *et al*, 1992; Viarengo *et al*, 1993) and Na^{+},K^{+}-ATPases (Viarengo and Nicotera, 1991). The effects of heavy metal ions on the activity of many other enzyme systems have been described, e.g. in fish (phoshatases and aminotransferases; Gill *et al*, 1990; Mayer-Gostan and Lemaire, 1991); in different yeast strains (superoxide dismutase and catalase activities; Romandini *et al*, 1992), in Pekin ducks (renal enzymes; Prasada-Rao *et al*, 1993), as well as in bacteria (β-galactosidase and dehydrogenases; Mazidji *et al*, 1992; Katayama-Hirayama, 1986).

Organelles such as mitochondria and the endoplasmic reticulum (ER) in cells are also targets for metal toxicity. Cu^{++}, but not Cd^{++}, was found to alter mitochondrial functions in mussels, either by disrupting the membranes of organelles or by altering the activity of mitochondrial enzymes (Viarengo, 1989). Similar metal effects also occur in the ER. It is well known that metal ions can inhibit enzyme systems operating in calcium homeostasis; these include plasma membrane Ca^{++}-transporting systems (ATPases), Ca^{++}, Mg^{++}-ATPases involved in Ca^{++} sequestration in the ER, and of the mitochondrial Na^{+}/Ca^{++} exchanger (Viarengo, 1989; Viarengo and Nicotera, 1991; Viarengo *et al*, 1993). A separate issue concerns the interactions between calcium and heavy metal ions in terms

of uptake, which will be discussed in Chapter 6. Moreover, heavy metals may also affect other important cellular functions, such as lysosomal activity, protein synthesis and nuclear metabolism (Viarengo and Nott, 1993).

1.2 MONITORING ENVIRONMENTAL POLLUTION

Effective environmental management requires knowledge of the fate and effects of pollutants in natural systems. Many studies have documented the effects of environmental pollutants on various aquatic organisms and the assessment of water quality by chemical sampling, or using certain aquatic organisms as bioindicators (Mason, 1991). Although pollutant levels can be measured by standard methods or by installing a computurised analyser at polluted sites, monitoring in this way will not provide information on new or unsuspected toxicants. Biological monitoring seems to be more advantageous in this respect.

There are many varieties of biological monitoring techniques, only some of which will be discussed here (see also section 3.1.1 which will deal in more detail with the application of these techniques in aquatic toxicity testing). For instance, changes in biological community structure or in certain members of a community may provide useful indicators of pollution, since not all organisms are equally sensitive to certain types of pollutants. This approach has been developed with nearly all forms of wildlife from bacteria to mammals, with macroinvertebrates being the most commonly used organisms in aquatic pollution monitoring indices (Maciorowski and Clarke, 1980; Chapman *et al*, 1982; Jeffries and Mills, 1990). The bioaccumulation of certain toxicants (e.g. heavy

metals, organometallics and organics) by internal organs such as vertebrate kidneys and livers, or by the whole organism in the case of mussels can also provide useful data on the type and nature of toxicants (Glaven *et al*, 1991; Gill *et al*, 1992; Fent and Hunn, 1993). Other ways of monitoring pollution involve the uses of ecological, morphological or physiological processes, e.g. the effects of temperature and water quality on the organism's growth (Holdich and Tolba, 1981), scope-for-growth measurements (oxygen consumption rate, food uptake etc.; Sanders *et al*, 1991), or measures of acute lethality (LC50, LD50 values; Martin and Holdich, 1986; Williams and Dusenbery, 1988,1990; Migliore and Giudici, 1990; Handy, 1994).

Recently, a number of biochemical and molecular assays have been developed, which have some practical advantages over those discribed above in environmental biomonitoring: They can provide an early warning of biological effects on organisms which could adversely affect the whole population, growth and reproduction; they are also less species specific and are often applicable to different phyletic groups as compared to other biological endpoints; they could allow monitoring of potential biological impact *in situ* using a wider range of native or transplanted organisms (Anderson, 1989; Sanders, 1990; Jenkins and Sanders, 1992). These assays generally involve monitoring changes in enzyme activity, or the effects on enzyme biosynthesis following exposure to environmental toxicants (Gill *et al*, 1990; Dutton *et al*, 1988;1990; Bitton and Koopman, 1992). More recently, molecular analysis of cellular stress responses has attracted a number of toxicologists to evaluate the use of this system as an indicator of environmental pollution. All organisms studied so far have been found to respond to a wide range of environmental stressors, physical, chemical and biological,

by synthesising a set of proteins called stress proteins. Stress proteins can be classified into two major groups; the first includes "classic" stress proteins whose synthesis is dramatically increased by heat and other stressors, the second group comprises those induced by a specific set of environmental contaminants, called stressor-specific stress proteins. Due to the particular interest of the present study, these two groups of stress proteins will be discussed in . greater detail in the following sections.

1.2.1 Heat-inducible stress proteins as environmental biomonitors

It is now well established that in most if not all organisms, a rise in ambient temperature results in the rapid induction of synthesis of a small set of proteins, called Heat Shock Proteins (HSPs). There are four major HSP families, frequently referred to as HSP90, HSP70, HSP60, and the low molecular weight HSPs (16-24 kD). The members of each protein group are closely related, have similar biochemical and immunological characteristics (Sanders, 1993). Further, it is well documented that synthesis of these proteins is also induced in response to a variety of other environmental stressors, including heavy metal ions, xenobiotics, oxidative stress, anoxia and teratogens (see both Nover, 1991a and Sanders, 1993 for review, also section 1.4). All inducers of Heat-Inducible (HI) stress proteins are united in their ability either to damage cellular proteins directly or to cause cells to produce abnormal proteins biosynthetically (Hightower, 1993). The classification and functions of HI-stress proteins will be discussed at length in section 1.3, and will not be repeated here. However, it is of particular importance to evaluate their potential as environmental monitoring tools. Because the HI-stress proteins (i) are components of cellular response which can protect against environmental stress,

(ii) are induced by a wide variety of environmental stressors, and (iii) are highly conserved through evolution in all organisms from bacteria to man, it follows that these proteins should have high potential as biomonitors of environmental contamination.

Many studies have reported the induction of HI-stress proteins by a large number of environmental contaminants as well as by heat shock. In particular, two of these proteins (namely HSP60 and HSP70) have been found to be highly inducible by a wide range of environmentally relevant toxicants (see e.g. Cochrane *et al*, 1991; Sanders *et al*, 1991; Kohler *et al*, 1992; Bradley, 1993; Dyer *et al*, 1993). The techniques most frequently used for examining stress protein induction are either (i) metabolic labelling (using radioisotopes, ^{35}S, ^{14}C or ^{3}H amino acids) with subsequent one/two-dimensional electrophoresis and autoradiography, or (ii) immunoblotting using stress-protein-specific antibodies which often cross-react with the corresponding proteins from very distantly related organisms. Quantification of accumulated stress proteins using the immunoblotting technique seems to be more reliable indicator of environmental contamination. Several anti-stress protein antibodies, especially anti-HSP70s, are now commercially available.

A number of studies using these and other molecular techniques have explored the possibility of using HI-stress proteins in biomonitoring assays (Sanders, 1990; McLennan and Miller, 1990; Odberg-Ferragut *et al*, 1991; Veldhuizen-Tsoerkan, 1991; Jenkins and Sanders, 1992; Dyer *et al*, 1993; Hightower, 1993; Sanders, 1993). The use of aquatic organisms (e.g. crustaceans, molluscs, fish) in such biomonitoring assays has been the main focus for many ecotoxicologists, and will therefore be dealt with in section 3.1.1. Several workers have agreed that ideal candidate stress proteins should meet the

following criteria : (1) their synthesis should be induced by a wide variety of stressors: (2) elevations in stress proteins should occur in organisms exposed to contaminants for long durations in their environment (persistent response rather than transient): (3) the relationship between stress protein accumulation, contaminant exposure, and organismal stress should be linked in a predictable fashion (Jenkins and Sanders, 1992; Sanders, 1993).

1.2.2 Non-heat inducible (or stressor-specific) stress proteins as biomonitors

This group of proteins shares in common the characteristic that their synthesis is induced by specific environmental contaminants, and is not substantially increased by heat. In fact, non-heat inducible (NHI) stress proteins are known to participate in specific biochemical pathways involved in the metabolism of chemicals, metabolites, or harmful by-products that are the result of a particular toxicant rather than being part of the cell's protective system in response to general cellular damage, as is the case for HI-stress proteins. Moreover, this group does not comprise a homologous group of proteins, and most are not functionally or structurally related to HI-stress proteins. Although there may be a number of proteins falling into this group, we will concentrate on those stressor-specific stress proteins which have been well characterised in many organisms, such as metallothioneins, the cytochrome P450 monooxygenase system and heme oxygenase.

(a) Metallothioneins

Metallothioneins (MTs) are a class of metal- and hormone-inducible proteins with several characteristics: they are soluble, heat stable, low molecular weight (6-7 kD; 61 aminoacids), and have a high content of cysteine (20-30%) and virtual absence of the aromatic aminoacids, histidine and methionine (Viarengo and Nott, 1993). These proteins show a high heavy-metal binding capacity due to the sulfhydryl residues of cysteine forming stable thiolate complexes with metal ions (Viarengo, 1989). MTs appear to be part of the cellular compartmentalization/ sequestration system which normally regulates the uptake, cellular metabolism and tissue distribution of essential metals such as Zn and Cu. It also interacts with non-essential "pollutant" metals such as Cd, Hg, Ag etc., and plays a role in the detoxification of excess heavy metals which penetrate into cells. It is thought that heavy metals such as Hg, Cd or Cu with a high affinity for -SH residues, may displace Zn from the physiological pool of metallothioneins, and therefore the excess of heavy metal cations, including Zn released from metallothionein, induces the synthesis of MTs. These processes chelate the heavy metal ions thus reducing their cytotoxic effects (Viarengo and Nott, 1993). In plants and many fungi, it is not metallothioneins which are induced, but rather specialised glutathione derivatives termed phytochelatins with extremely high metal binding affinity (Nover, 1991a).

Metallothioneins were first described in mammalian cells in 1957, and subsequently identified in most living organisms (Kagi and Schaffer, 1988). These proteins were initially detected in marine invertebrates (Viarengo, 1989; Viarengo and Nott, 1993), but have been further characterised in many other aquatic organisms including fish (e.g.

Hogstrand and Haux, 1991), or amphibians (Woodall and Maclean, 1992), molluscs (Veldhuizen-Tsoerkan *et al*, 1990) and crustaceans (Sanders *et al*, 1983). Both laboratory and field studies have shown an increased accumulation of metallothionein following exposure to environmentally relevant concentrations of metal ions (Hogstrand and Haux, 1991; Jenkins and Sanders, 1992; Woodall and Maclean, 1992). It is well established that MTs are a significant factor in the accumulation of pollutant metals in parenchymatous tissues (liver, kidney, pancreas and intestines) of most organisms (Kagi and Schaffer, 1988; Viarengo, 1989). Several methods such as HPLC, anion exchange chromotography and immunoassays have been developed to quantify MT concentrations in different tissues (Viarengo, 1989). In addition, analysis of the protein's bound metal content (using atomic absorption spectroscopy) may provide further information regarding the metals responsible for induction of MT synthesis. Such studies clearly indicate that the levels of these proteins in aquatic organisms may provide a useful monitoring tool for heavy metal contamination.

A few studies have already begun to evaluate the potential use of MTs in environmental monitoring (Viarengo, 1989; Sanders, 1990; Hogstrand and Haux, 1991; Jenkins and Sanders, 1992). However, all these studies point to the need for more data concerning the biological functions of these proteins. Moreover, the effects of other factors such as variations of environmental parameters (temperature, oxygen availability, salinity), sex, physiological status etc. on the tissue concentration of MT should be investigated in more detail, before utilising MT levels as a specific stress index.

(b) Cytochrome P450

The cytochrome P450s are a family of enzymes that modify the structure of a number of organic molecules, including pollutant chemicals (e.g. organometallics, pesticides, polyaromatic and chlorinated hydrocarbons), drugs, and many chemical carcinogens. It is known that the rates of synthesis and intracellular concentration of some types of P450 are increased in proportion to an organism's exposure to these compounds (Adams *et al*, 1990; Welch, 1990; Jenkins and Sanders, 1992). P450 levels in aquatic organisms may also serve as a sensitive indicator of some organic pollutants.

(c) Haeme Oxygenase

This protein was initially known as a 32 kD stress protein that was inducible by metals, sodium arsenite, and thiol reactive agents (Welch, 1990); recently, it was identified as haeme oxygenase, an enzyme which is essential for heme catabolism. Particularly, Cd and other metals such as Cu, Zn, Pb, Sn, sodium arsenite, and gold have been found to be effective inducers of this enzyme (Sanders, 1990; Jenkins and Sanders, 1992; Mitani *et al*, 1993). Recently, tributyltin, and the porphyrin complexes of Co, Zn and Sn were found to induce haeme oxygenase specifically (Zhang and Liu, 1992 and Mitani *et al*, 1993, respectively). However, it should be noted that these proteins are also heat-inducible in some organisms (e.g. rat), but not in others (e.g. human hepatoma cells and human diploid fibroblasts) (Nover, 1991a; Zhang and Liu, 1992; Mitani *et al*, 1993).

(d) Other stressor specific non-heat inducible stress proteins

These proteins include various other enzyme; some (e.g. glutathione transferase, sulfotransferase) assist in the detoxification of hydrophobic foreign compounds by linking them to endogenous molecules (glutathione and sulphate, respectively) that facilitate their excretion from cells; others are antioxidant enzymes such as superoxide dismutase, catalase, peroxidase and glutathione reductase. All of these proteins are induced by various contaminants or free radicals (Cartana *et al*, 1992; Jenkins and Sanders, 1992; Romandini *et al*, 1992). Since these enzyme sytems play important roles in contaminant metabolism and detoxification, and since their synthesis is increased by specific types of contaminants, they should also have good potential as environmental biomonitors.

1.2.3 Other molecular parameters as bioindicators of pollutant exposure

DNA damage has been proposed as a useful parameter for assessing the genotoxic properties of environmental contaminants. Many of these contaminants are chemical carcinogens and mutagens with the capacity to cause various types of DNA damage. The DNA damage by genotoxic chemicals may occur either by forming irreversible DNA adducts (e.g. polycyclic aromatic hydrocarbons), or by causing strand breaks in DNA (e.g. benzo[a]pyrene) (Adams *et al*, 1990; Shugart, 1990; Jenkins and Sanders, 1992).

Indicators of carbohydrate-protein metabolism and parameters of lipid metabolism have both been evaluated recently as indicators of contaminant exposure, since various contaminants may have direct or indirect effects in causing physiological dysfunction (Adams *et al*, 1990). Metal toxicity generally results from non-specific binding of metals

to biologically active molecules and thus resulting modification of their function. More recently, Demuynck and Dhainaut-Courtois (1994) have demonstrated that haemoglobin and myohaemerythrin are major metal-binding proteins in the polychaete worm *Nereis diversicolor*. Exposure to metals displaces iron from these preteins, which results in an increase in soluble iron concentrations that could well serve as an indicator of metal exposure. Bradley *et al* (1994) have recently tested the effects of a cationic polymer (Kymene; polyamide epichlorhydrin) and resin acids on both inducible and repressible proteins for qualitative and quantitative diagnoses of components in a complex mixture.

1.3 HEAT SHOCK RESPONSE

The discovery by Rittossa (1962) that specific puffs in the polytene chromosomes of *Drosophila* are induced by a brief heat shock was the first indication of a general cellular response to stress. There was little significant advance for a decade until Tissieres *et al* (1974) found that heat shock in *Drosophila* salivary glands induced the synthesis of a small number of polypeptides and repressed the synthesis of most proteins characteristic of normal development; they also related Ritossa's puffs to the heat-induced proteins. Scientists working on other organisms subsequently discovered that heat and many other types of stressors could induce the synthesis of similar proteins in cultured avian cells, yeast and *Tetrahymena* (Lindquist, 1986). Since then, similar responses have been reported in a wide range of organisms, including prokaryotes, eukaryotic microorganisms, plants and and animals (see review by Schlesinger *et al*, 1982; Lindquist, 1986; Lindquist and Craig, 1988; Nover, 1991a; Vierling, 1991; Sanders, 1993). The so-called heat shock response now constitutes a very active area of research in molecular biology. The heat

shock proteins (HSPs) are the most highly conserved class of proteins characterized so far (Boorstein *et al*, 1994). Although these proteins are induced by heat, most of them in fact are expressed in all cells under normal conditions, and are essential components in a number of diverse biological processes. The most prominent HSPs are generally classified into four major HSP families of 90, 70, 60 and 16-24 kD, and these are frequently referred to as HSP90, HSP70, HSP60, and low molecular weight (LMW) HSPs, respectively (Lindquist and Craig, 1988; Nover and Scharf, 1991).

1.3.1 Heat shock proteins in diverse organisms

(a) Heat shock proteins of prokaryotes

HS response has been investigated in a number of bacteria including two enteric bacteria *Escherichia coli* and *Salmonella typhimurium*, the cyanobacterium *Synechococcus* sp. and the sporulating *Bacillus subtillis*, *Mycobacterium tuberculosis* etc. (Morimoto *et al*, 1990; Nover and Scharf, 1991; Young and Garbe, 1991). Among these, the *Escherichia coli* HS response is the best characterized, and this led to the identification of a set of bacterial HSPs and the elucidation of their cellular functions (Lindquist, 1986; Hightower, 1991; Gamer *et al*, 1992; Georgopoulos and Welch, 1993).

Seventeen HSPs have been identified in *E.coli*; the well known ones include DnaK, DnaJ, GroEL, GroES, HtpG, GrpE and Lon, Clp and two RNA polymerase sigma subunits (σ^{70} and σ^{32}) (Lindquist and Craig, 1988; Nover and Scharf, 1991; Georgopoulos and Welch, 1993). Sequential increases in first σ^{32} and then σ^{70} synthesis, as well as the accumulation

of the Lon and DnaK proteins, are essential events of the control network of HSP synthesis in *E.coli* (Nover and Scharf, 1991). In most cases, cooperation between different HSPs is necessary. Genetic evidence indicates key regulatory functions for the HSPs DnaK, DnaJ and GrpE at the levels of synthesis, activity and degradation of σ^{32} (Gamer *et al*, 1992). The mechanisms by which three chaperonin proteins (DnaK, DnaJ and GrpE) regulate the activity and stability of σ^{32} involve concerted DnaK ATPase activity (Lindquist and Craig, 1988; Cyr *et al*, 1994).

DnaK (HSP70), GroEL (HSP62) and HtpG (HSP90) are structurally related to eukaryotic HSPs; i.e. DnaK is 50% identical to eucaryotic HSP70 in aminoacid sequence. GrpE is a 24 kD HSP, while the ATP-dependent Lon protease is a heat inducible protein of 94 kD that is involved in proteolysis of abnormal proteins accumulated during stress (Lindquist and Craig, 1988; Parsell and Lindquist, 1993). Immunological techniques used to identify the GroEL, DnaK protein, and the Lon protease showed that these proteins are also the major HS-induced proteins in other bacteria such as *Salmonella typhimurium* (HSPs 69, 56), *Bacillus subtilis* (HSPs 94, 76, and 66), and GroEL in *Coxiella burnetii* and *Mycobacterium tuberculosis* (Young *et al*, 1990; Nover and Scharf, 1991).

(b) HSPs in eukaryotic microorganisms and yeast

This group includes protozoa (*Plasmodium falciparum* and *Tetrahymena pyriformis*), fungi (*Claviceps purpurea* and *Aspergillus nidulans*) and yeasts (*Trichosporon pullulans* and *Saccharomyces cerevisae*) (Lindquist and Craig, 1988; Julseth and Inniss, 1990; Young *et al*, 1990; Nover and Scharf, 1991; Odumeru *et al*, 1992; Sharma, 1992; Weitzel

and Li, 1993). In all cases, prominent HSPs are found in the 70-to-90 kD region, whereas the LMW HSPs are highly variable. Both HSP90 and HSP70 of the parasitic protozoan *P.falciparum* are well characterized as immunogenic antigens (Sharma, 1992). The yeast HS response, however, is the most extensively studied in this group; the HSP pattern in yeast includes two HSP90s, a complex group of at least nine HSP70-type proteins, one HSP60 and a single small HSP of 26 kD (Lindquist and Craig, 1988; Weitzel and Li, 1993). The multiplicity of yeast HSP70s will be further discussed in Chapter 4.

(c) HSPs of plants

HS responses have been described in a wide range of plant species, including the alga *Chlamydomonas*, the dicotyledons *Arapidopsis*, *Lycopersicon* and *Pisum sativum*, and the monocotyledon *Zea mays*(Nover and Scharf, 1991; Vierling, 1991). All plant species tested produce HSPs in response to elevated temperatures. Besides the large HSPs with molecular weights of 70 and 80 kD plus a short-lived HSP90, the plant HSP pattern appears to be dominated by a highly complex group of small HSPs. Most of the small HSPs belong to the HSP20 family which forms cytoplasmic multimeric complexes. Some of these small HSPs, with molecular weights ranging from 21 to 24 kD, are localised in chloroplasts. Moreover, plants contain two types of the chaperonin HSP60 in chloroplasts and mitochondria, respectively, a DnaK-like HSP70 in mitochondria, and at least 3 different HSC70s in chloroplasts. The crossreactivity of HSP70s of higher plants is greater with anti-DnaK antibodies than with those raised against animal HSP70s. DNA sequence analysis of plant HSP90s shows 70% homology to HSP90s from other eukaryotes.

(d) HSPs of animals

The HS response has been studied very extensively in animals (see e.g. Schlesinger *et al*, 1982; Atkinson and Walden, 1985; Lindquist, 1986; Lindquist and Craig, 1988; Morimoto *et al*, 1990; Nover and Scharf, 1991; Sanders, 1993). We will briefly concentrate on animal model sytems, and on the main differences between these and other eukaryotes as well as prokaryotes with respect to their HSPs. The nematode *Caenorhabditis elegans*, the sea mussel *Mytilus edulis*, *Drosophila*, *Xenopus laevis*, chicken cells and human cells all provide well characterized examples of induced HSP synthesis in animals. *C.elegans* HS response will be dealt with in chapter 4, while the stress response in aquatic organisms including *Mytilus edulis* will be discussed in Chapter 3.

In *Drosophila*, the largest HSP is an 83 kD protein which is expressed under both control and heat shock conditions (Graziosi *et al*, 1980; Nover and Scharf, 1991). *Drosophila* HSP70 is found to be a member of a multigene family which includes the constitutively expressed HSCs 70 and 72, whose synthesis is reduced during heat shock treatment, as well as the heat-inducible HSPs 70 and 68. There are also at least 4 small HSPs with molecular weights of 22, 23, 26 and 27 kD (Pelham, 1985; Lindquist and Craig, 1988)

The pattern of HSP synthesis in vertebrate cells is similar to that in invertebrate cells; two constitutively expressed HSP90s, which are homologous to the *Drosophila* HSP83, are enhanced by heat shock. There are also two main types of protein in the 70 kD

region; one is slightly larger (72-73 kD), and is expressed constitutively but with increased synthesis during heat shock; the other is newly induced by heat and comprises one or two HSPs of 70 and/or 71 kD. Each of these HSP70 species comprises multiple isoelectric forms. The multiplicity of HSP70s in some eukaryotic species will be further discussed in chapter 4. The LMW HSPs of 25 and 27 kD in mammals and of 24 kD in chicken cells are encoded by single genes, but multiple forms occur due to postranslational phosphorylation. Because of their low methionine content, it is not possible to detect the small HSPs of vertebrates when using methionine for metabolic labelling. Other major vertebrate HSPs include the minor nucleolar HSP110 and two glucose-regulated proteins GRPs 95 and 78. The latter two are structurally related to HSPs 90 and 70, respectively, while HSPs 100 and 110 do not appear to have counterparts in *Drosophila* and *C.elegans* (Snutch and Baillie, 1983; Atkinson and Walden, 1985; Lindquist and Craig, 1988; Welch, 1990; Nover and Scharf, 1991).

1.3.2 Classification of HSPs

The analysis of HS responses in a wide range of organisms, as briefly discussed above, has shown that the HSP pattern is rather complex; it appears that HSPs are encoded by small multigene families which combine various members with divergent regulatory behaviour. One group of HSPs was found to be constitutively expressed under normal conditions, and these are often referred as to heat shock cognates (HSCs); another group of proteins, the glucose-regulated proteins (GRPs), were found to be homologous to the HSPs, but are regulated by glucose rather than by heat. Given the rapidly increasing data-set from animal and other systems, it becomes necessary to classify the HSPs and related

proteins into distinct families characterized by sequence homology, functional relatedness or intracellular localization. We will concentrate only on the four major HSP families of 90, 70, 60, and 16-24 kD which are the most prominent HSP families in *Drosophila*, mammals and most other model systems studied.

(i) The HSP90 family

This family includes abundant cytoplasmic proteins which are synthesised at increased rates under HS conditions. Examples of this protein family are the HSP90s of human, mouse, chicken, yeast, and the *Drosophila* HSP83, as well as the plant HSP80. The corresponding protein in *E.coli* is known as HtpG. Other members of the HSP90 family are the vertebrate GRP94, and a constitutively expressed surface glycoprotein of the protozoan parasite *Trypanosoma cruzi* (Lindquist, 1986; Lindquist and Craig, 1988; Morimoto *et al*, 1990; Nover and Scharf, 1991; Parsell and Lindquist, 1993).

The *Drosophila* HSP83 appears to be encoded by only one gene, while the haploid genome of yeast has two nearly identical genes of this type; one, HSC83, is constitutively expressed at a high level and moderately heat-inducible, the other, HSP83, is also constitutively expressed at a lower level and is more strongly heat-inducible. In both *Drosphila* and yeast, the members of this protein family are developmentally regulated. In vertebrate cells further diversification of the genes in this family has occured. Although at least one member of the HSP90 family, namely GRP94, is localised in the endoplasmic reticulum (ER)/golgi system, most members are abundant cytoplasmic proteins which are not significantly redistributed during the stress period (Berbers *et al*,

1988; Perdew and Whitelaw, 1991). It should be also noted that HSP90s are phosphoproteins, whereas GRP94 is classified as a glycoprotein.

Under normal conditions, HSP90 family members have been found to modulate many cellular activities by binding to target proteins, forming an inactive or unassembled complex. These target proteins include enzymes (e.g. kinases), and components of the cytoskeleton. HSP90s also forms stable complexes with several members of the nuclear steroid-hormone-receptor family, including the glucocorticoid, ostrogen and progesterone receptors (Perdew and Whitelaw, 1991). More recently, HSP90s have been found to function as chaperones without forming complexes with unfolded proteins (Parsell and Lindquist, 1993; Sanders, 1993).

(ii) The HSP70 family

This protein family is the largest of all HSP families, and has been the most extensively studied. At least 21 proteins belonging to this multigene family have been characterized (Sanders, 1993). The multiplicity of HSP70 family members in *Arabidopsis*, yeast, *Drosophila* and *C.elegans* will be discussed in Chapter 4. The HSP70 family includes heat shock-inducible (HI) and constitutive members, as well as a group of glucose-regulated GRP78 proteins known from yeast (originally called KAR2) and vertebrate cells. Distinct HSP70 family members are found in the ER, mitochondria and chloroplasts, while others are found in the nucleus and cytoplasm; some members concentrate in the cytoplasm, whereas others shuttle between the nucleolus, nucleus and cytosol. Furthermore, multicellular organisms contain several tissue-specific versions,

such as the testis-specific HSP70s (Velazquez and Lindquist, 1984; Lindquist and Craig, 1988; Morimoto *et al*, 1990; Nover and Scharf, 1991; Martin *et al*, 1993; Parsell and Lindquist, 1993).

DnaK is a well characterized HSP70 protein in *E.coli*. Additionally, two related proteins have been recently identified; one is HSC66, sharing 40% amino acid identity with DnaK, and the other is MreB which shares only 27% identity (Parsell and Lindquist, 1993). DNA sequence analysis of the HSP70 gene family has shown that bacterial DnaK proteins are markedly similar to the yeast mitochondrial SSC1 protein. Interestingly, similar DnaK-like proteins were found in mitochondria of parasitic protozoa, the nematode *C.elegans* and human cells, as well as in the chloroplasts and mitochondria of plants (Nover and Scharf, 1991). Cross hybridization and immunological techniques, as well as sequence analaysis of HSP70 genes and proteins, all evidence a remarkable degree of conservation among all members of the HSP70 family across many diverse phyla. All HSP70 proteins studied so far contain two main domains; the N-terminal domain is the most conserved and includes a high affinity ATP-binding site, whereas the C-terminal domain is less conserved and is responsible for binding substrate polypeptides (Parsell and Lindquist, 1993; Sanders, 1993).

The glucose-regulated protein GRP78 is usually classified as a member of the HSP70 family, due to its structural similarity to HSP70s. In particular, the N-terminal domain of GRP78 corresponds to the ATP-binding domain common to HSP70 proteins. GRP78s are abundant and constitutively expressed proteins, whose synthesis is moderately stimulated by mild heat shock. However, under conditions of glucose depriviation their

synthesis is greatly enhanced, and they are therefore called GRPs. A large list of GRP78 inducers has been documented, including agents interfering with glucose utilization (e.g. insulin, glucosamine, 2-deoxyglucose, and long term anoxia) or causing Ca^{++}-depriviation (e.g. calcium ionophore A23187, EGTA, and low pH), but some of them are also typical inducers of HSPs (heavy metal ions such as Cd^{++} and Zn^{++}, amino acid analogs, and heat) (Lindquist and Craig, 1988; Morimoto *et al*, 1990; Nover, 1991b). GRP78s of vertebrates were found to be associated with aberrant proteins in the ER. It is now known that they function to stabilise polypeptide subunits on their way to maturation and assembly into multimeric protein complexes, as in case of immunoglobulin IgG heavy and light chains (Welch, 1991). It should be noted that this protein was previously described as BiP, because of this latter function (Lindquist and Craig, 1988; Flyn *et al*, 1991).

(iii) The HSP60 family

The HSP60 family is a relatively new class of mitochondrial HSPs, compared to the other three classes. Members of this protein family are found in the cytosol of *E.coli* (where they are known as GroEL) and related bacteria, and also in the mitochondria and chloroplasts of eukaryotes (Lindquist and Craig, 1988; Cheng *et al*, 1989; Vierling, 1991; Welch, 1991; Parsell and Lindquist, 1993).

As a molecular chaperone, HSP60 (or chaperonin-60) binds to target proteins to facilitate protein folding and assembly. However, unlike HSP70 which binds to target proteins as a monomer, the chaperonin-60 forms an oligomeric complex comprising seven 60 kD subunits (Welch, 1991; Georgopoulos and Welch, 1993). As in case of HSP70s, HSP60s

have also an ATPase activity that increases with temperature, and the binding of ATP induces a major conformational change in the structure of the oligomer (Nover and Scharf, 1991; Parsell and Lindquist, 1993). The chaperonin functions of HSP60 are strongly dependent upon another heat inducible protein, HSP10 (known as GroES in *E.coli*, or chaperonin-10 in eucaryotic mitochondria and chloroplasts) which also forms a homo-oligomeric complex (Georgopoulos and Welch, 1993). More recently, two novel members of the chaperonin-60 family have been discovered; one is a heat-inducible protein called TF55 found in the thermophilic archaebacterium *Sulfolobus shibatae*, while the other is a non-heat inducible, eukaryotic cytoplasmic protein called TCP1, which is involved in mitotic spindle formation (Sanders, 1993).

(iv) The LMW HSP family

Unlike the three large HSP families described above, the multiplicity of LMW HSPs is much more variable between different organisms. A compiled list of LMW HSPs from a wide range of organisms showed that this protein family is more species-specific and less highly conserved than the other major HSP families. The LMW HSPs are found in prokaryotes and in eukaryotic cytosol, as well as being abundant in plants (found mainly in endomembrane systems and chloroplasts) (Nover and Scharf, 1991). It is also interesting to note that all members of this protein family examined to date are found to be related to α-crystallin proteins of the vertebrate eye, and share the ability to form multimeric higher order structures (Hockertz *et al*, 1991; Horwitz, 1992; Parsell and Lindquist, 1993).

(v) The ubiquitin family

Ubiquitin is a highly conserved LMW HSP comprising only 76 amino acid residues (7 kD). It is synthesised as a polyprotein referred to polyubiquitin, consisting of tandem repeats of the ubiquitin unit. In most organisms studied so far, small multigene families code for both the constitutively expressed and inducible ubiquitin proteins. Under heat shock conditions, enhanced synthesis of this protein provides an increased capacity for the turnover of severely damaged proteins along nonlysosomal pathways, and complements lysosomal degradation and stabilization activities of chaperonin-60 and HSP70 (Lindquist and Craig, 1988; Schlesinger, 1990; Vierling, 1991; Jentsch, 1992; Jungmann *et al*, 1993; Sanders, 1993). Interestingly, although this protein is characterized as heat inducible in many eukaryotes, polyubiquitin genes in *C.elegans* are not induced by heat shock (Nover and Scharf, 1991).

1.3.3 Conservation of HSPs

In previous sections, we have discussed the major HSP families and showed that these protein families are found in an extraordinarily diverse array of organisms. The high degree of conservation among members of most major HSP families is well established. Evidence for the conservation of HSP family members is generally derived from sequence comparisons and immunological data.

Amino acid sequence analysis of HSP90 proteins from diverse organisms has revealed that even the most distantly related eukaryotes have 50% amino acid identity and all have

greater than 40% identity with the *E.coli* homologue HtpG. In all eukaryotes, a region of extremely high negative-charge density is located at the same relative position in the HSP90 chain, although *E.coli* HtpG is missing this segment. However, all HSP90s, including the *E.coli* protein, contain another smaller conserved region towards the C-terminal. In fact, the C-terminal regions of these proteins are generally the most diverse, but the last four amino acids are the same in all eukaryotes. Moreover, HSP90s from a variety of sources have been shown to bind ATP, although they possessed very different levels of ATPase activity in different organisms (Lindquist and Craig, 1988; Parsell and Lindquist, 1993).

The HSP70 family, however, is the most highly conserved protein family studied so far; a monoclonal antibody to the *Drosophila* HSP70 cross-reacts with constitutive and/or HS-induced members of the HSP70 family in sea urchin, nematode, chicken, human and plant cells. Moreover, anti-DnaK antibodies can detect the mitochondrial HSP70 in human, HSP78 in pea chloroplasts, and DnaK-like proteins in algal chloroplasts and mitochondria (Nover and Scharf, 1991). In the C-terminal portion of eukaryotic HSP70s, a highly hydrophobic but poorly conserved sequence contains upto 50% glycine residues. This sequence ends with a more conserved oligopeptide of 9 aminoacids (markedly similar to the conserved C-terminal sequence of the HSP90 family). Interestingly, these residues have also been found in ER-localised HSP70s, where they appear to be partially responsible for retention of the protein within the ER lumen (Nover and Scharf, 1991; Gething and Sambrook, 1992; Gupta and Golding, 1993; Boorstein *et al*, 1994). Studies have revealed further biochemical similarities between related HSP70 proteins from a single organism, as well as among HSP70s isolated from diverse organisms; for example,

all HSP70s and related proteins bind ATP with high affinity (Lindquist and Craig, 1988). It appears that all of the HSP70s studied to date fold into two domains. The N-terminal domain is the most highly conserved and contains a high affinity ATP-binding site, whereas the C-terminal domain is less conserved and is responsible for binding substrate proteins and polypeptides. This organisation suggests that all HSP70s share a common mechanism for utilising energy from ATP while recognising a wide variety of substrates (Welch, 1991; Gething and Sambrook, 1992; Parsell and Lindquist, 1993).

More recently, Boorstein *et al* (1994) have analysed the nucleotide sequence of 36 HSP70 family members from 25 prokaryotic and eukaryotic species, and have found that proteins from the most distantly related species share at least 45% identity. Moreover, each group of evolutionarily similar proteins shares a common intracellular localisation and thus is presumed to be comprised of functional homologues. These protein groups include the HSP70s of cytoplasm, ER, mitochondria and chloroplasts. The authors further demonstrate that eukaryotic HSP70s, localised in the cytoplasm and ER, share approximately 50% identity with HSP70s both from prokaryotes and from eukaryotic organelles of endosymbiotic prokaryotic origin (i.e. mitochondria and chloroplasts). Members of individual eukaryotic subgroups were found to be more similar to each other, particularly those in the cytoplasm which share at least 71% identity (see also Nover and Scharf, 1991).

Bacterial GroEL and HSP60s in the mitochondria and chloroplasts of eukaryotes all share approximately 60% amino↔acid identity. Most also share a common oligomeric structure comprising two seven-membered rings, while the mitochondrial HSP60 forms a single

seven-membered ring. Like the HSP90 and HSP70 families, HSP60 also has ATPase activity (Welch, 1991; Parsell and Lindquist, 1993).

Conservation among small HSPs has also been reported; although the LMW HSPs are divergent in sequence, they are conserved in their structural properties, and characteristically form highly polymeric structures (Lindquist and Craig, 1988). The amino acid sequences of 23 small HSPs from diverse organisms including yeast, nematode, human and plants were compared, showing that this protein family is the least conserved among the major HSP families; they can be recognised by limited amino acid identity (15-20%), and a conserved hydrophobic sequence element near the C-terminal. Interestingly, the vertebrate α-crystallins contain a structurally related domain (Hockertz *et al*, 1991; Nover and Scharf, 1991; Parsell and Lindquist, 1993).

1.4 OTHER INDUCERS OF HEAT SHOCK (STRESS) RESPONSE

Following the discovery of heat shock-induced changes of gene activity by Ritossa, and the finding of HS-induced protein synthesis by Tissieres and co-workers, a bewildering multiplicity of chemical stressors has been found to induce similar effects, initially with *Drosophila* salivary glands, but soon also with vertebrate, yeast, plant, bacterial and other systems. Therefore, Hightower and White in the early 1980's suggested that HSPs be renamed cellular-stress proteins(SPs) to acknowledge the fact that a wide variety of stressors induce them (Hightower, 1993). The heat shock response is now regarded as one example of a number of inducible cellular response systems which involve the synthesis of new proteins that protect against environmental stresses. One such related response

involves the induction of GRPs (see section 1.3.2 for further description of this system). Both HSPs and GRPs are subsets of related stress proteins. There are also other types of SP which are not heat-inducible, such as metallothioneins, heme oxygenase and cytochrome P450. The use of both heat-inducible and non-heat inducible stress proteins in environmental biomonitoring has been already discussed in section 1.2.1. This section will focus only on the inducers of heat-inducible stress proteins which are listed in Table 1.2. It should be noted that this list of HSP inducers is based on those mainly compiled and discussed by Nover (1991a) and Sanders (1993; this latter study mainly focusses on environmentally relevant contaminants acting as inducers in aquatic organisms).

Table 1.2 Inducers of heat-inducible stress proteins

Oxidising agents and drugs affecting respiration and energy metabolism;
Anoxia and recovery
Antimycin A
Arsenate (10mM)
Arsenite (10-100 μM)
2,4-Dinitrophenol (0.1-1 mM)
H_2O_2 (0.05-1mM)
Hydroxylamine (10 mM)
Anion transporters;
4-Acetamido 4'-isothiocyanostilbene 2,2'-disulfonate (100 μM)
Niflumic acid (100 μM)
Flufenamic acid (100 μM)
K^+-ionophores;
Dinactin (0.1-1 μM)
Valinomycin (10 μM)

Transition series metals;

Cd^{2+} (10-100 μM)
Cu^{2+} (0.1-1 mM)
Hg^{2+} (10 μM)
Zn^{2+} (0.1 mM)

Chelating drugs;

8-Hydroxyquinoline
o-Phenanthroline
Salicylate (10 mM)

Sulphydryl reagents;

n-Ethylmaleimide (50 μM) (Cajone and Crescente, 1992)
Diamide (0.3 mM)
Iodoacetamide (10 μM)

Amino acid analogs;

Azetidine-2-carboxylic acid (5 mM)
Canavanine (0.1-1mM)
p-Fluorophenylalanine (0.2 mM)
β-Hydroxyleucine
o-Methylthreonone (3 mg/ml)
Histidinol

Inhibitors of gene expression;

Chloromphenicol (1 mM)
Gentianamycin (2 mg/ml)
Paromomycin (1 mg/ml)
Tetracycline
Cycloheximide (10 ng/ml)

Drugs affecting membrane structure;

Ethanol (2-6%)
propanol
Butanol
octanol
Lidocaine
Digitonin, Saponins, Triton-x-100, Nonidet P-40
SDS (Adamowicz *et al*, 1991; Bradley, 1993)

Steroid hormones;

Ecdysterone (1 μM)
Hydrocortisone
Methyltestosterone (10 μM)

Infection with DNA viruses;

λ-phage
Adenovirus
Herpes simplex virus
SV 40

Teratogens, carcinogens, mutagens;

Sodium arsenite (50 μM) (Honda *et al*, 1992)
Coumarin (0.1-1 mM)
Diphenylhydantoin (0.1-1 mM)
Tolbutamide (0.1-1 mM)
Diethylnitrosamine
2-Acetylaminofluorene
Methylmethane sulfonate (200 μg/ml)

Organometallics;

Tributyltin (Zhang and Liu, 1992; Steinert and Pickwell, 1993)

Organics and pesticides;

Organophosphate (Diazinon)
Organochlorine (Lindane)
Paraquat (Stringham and Candido, 1994)
Thiram
Carbamate molluscicide
Benzene
1-Chloro-2,4-dinitrobenzene
2,4-Dichloroaniline
Hexachlorobenzene
Pentachlorophenol
Trichloroethylene

Other inducers;

Abnormal proteins (Ananthan *et al*, 1986)
Ether
Hemin (erythroid cells)
High pH (pH 8.7)
Cationic polyamide epichlorhydrin, resin acids (Bradley *et al*, 1994)
Low pH (pH 5.5) (Khandjian, 1990)
Calcitonin
Prostaglandins (Santoro *et al*, 1990)
Vitamin B-6
UV irradiation
Cold shock
Nicotine

Note: Data in this table have been selected from Nover, 1991a and Sanders, 1993, unless otherwise stated. The concentration range of the chemical stressors given may represent the dose response in various systems, and for some, it may be much higher than would be present in the environment.

For most of the listed chemical stressors, specific characteristics such as the rapidity of the response and the level of induction appear to be stressor specific and probably represent consequences of the mechanism of toxicity for each contaminant. It is well known that regulation of the synthesis of heat shock proteins is dependent on the presence of damaged or denatured proteins in cells. The induction of stress-inducible HSPs is mediated by the activation and binding of the heat shock factor (HSF) to arrays of heat shock elements (HSEs) in the regulatory promoter region of the HSP gene. It is therefore suggested that the induction of HSPs by all these chemical stressors requires both the activation of HSF and the presence of the HSE. This has been demonstrated by a number of studies; for example, deletion of HSEs from HSP gene constructs eliminates their inducibility by stress, and different types of chemicals can increase the binding of the HSF to HSEs (Nover, 1991a; Hightower, 1993; Sanders, 1993; see also sections 1.6.2 and 1.6.3 for further details). In the following sections, the heat shock response will

often be referred to as the cellular stress response, and the heat-inducible stress proteins as stress proteins; both terms are to be preferred because of their wider applicability. Induction of the cellular stress response by chemical stressors is usually slower than induction by heat shock, which may reflect the fact that damage to proteins by heat shock occurs more rapidly than damage by the chemical stressors. The reason for this is because the effects of chemicals are dependent on their biological availability, uptake, and mechanisms of toxicity. The ability of chemicals to induce a stress response has been found to differ considerably between species, and this has been attributed to functional differences in physiology. Furthermore, the dose response range for stress protein (SP) induction depend in part on the ability of the test species to exclude, sequester, or detoxify chemical toxicants, as well as on tissue specificity, developmental stage, and reproductive state (Sanders, 1993).

1.5 FUNCTIONS OF HEAT SHOCK (STRESS) PROTEINS

Heat shock or stress proteins (SPs) have been implicated in several important cellular processes. SP levels have been manipulated by generating deficiency mutants or trangenics that are overexpress particular SP genes. The biochemical characterisation of SP responses in normal, mutant and transgenic lines under both normal and stressful conditions has led to an enormous volume of literature concerning their cellular functions. This section will briefly discuss the well characterised functions of the major SP families.

1.5.1 The chaperonin functions of stress proteins in protein folding, assembly and translocation

Protein folding *in vitro* has been demonstrated to be a spontaneous process dictated primarily by the linear sequence of amino acids present within polypeptides; a denatured enzyme can refold into its native conformation in the absence of other proteins, but spontaneous refolding is frequently inefficient in comparison to that observed *in vivo*. Recent studies have revealed that appropriate folding requires a class of proteins referred as molecular chaperones. A number of chaperones have been identified, and most were found to be heat heat shock or stress proteins (Welch, 1991; Gething and Sambrook, 1992). This class of proteins comprises both constitutively expressed and stress-induced members of all major (H)SP families. The constitutively synthesised chaperone proteins, often termed heat shock cognates (HSCs), play an important role in regulating protein homoestasis in cells under normal physiological conditions, and they seem to serve many functions that stem from their ability to recognise and modulate the state of folding of polypeptides. The chaperone proteins have been found to (i) modulate protein folding/unfolding, (ii) facilitate assembly/dissasembly of oligomeric structures, and (iii) direct translocation of polypeptides into appropriate cellular compartments. Under stressful conditions, however, the levels of molecular chaperones greatly increase to take on new but related roles (Parsell and Lindquist, 1993; Sanders, 1993). We will limit the discussion to the chaperonin functions of SPs 90, 70 and 60 which all have distinct cellular functions under both normal and stressful conditions.

(i) SP70 chaperones

SP70 chaperones are well known to bind to small peptides, to nascent chains on polysomes, to proteins that have been targeted to the wrong compartements, to certain protein subunits that are expressed in the absence of their partners, and to certain oligomeric proteins that are in the process of assembly or disassembly. When binding to these substrates, SP70s participate in a number of protein folding/unfolding, assembly/disassembly and translocation processes. The role of SP70s in such processes is mediated by their binding to hydrophobic surfaces of proteins, preventing them from folding incorrectly and stabilising target proteins in a fully or partially unfolded state. The hydrolysis of SP70-bound ATP was shown to promote the release of the substrate allowing it to continue folding, transport, or assembly (Schlesinger, 1986; Sadis and Hightower, 1992; Parsell and Lindquist, 1993). SP70 members implicated in these processes include:- in *E.coli*, DnaK; in yeast, the cytosolic proteins SSA1 and SSA2, the ER protein KAR2, and the mitochondrial protein SSC1; in mammalian cells, the cytosolic proteins HSP72 and HSC73, the ER protein GRP78 (also known as BiP), and GRP75 which is present in mitochondria or chloroplasts (Lindquist and Craig, 1988; Vierling, 1991; Welch, 1991; Gething and Sambrook, 1992; Georgopoulos and Welch, 1993).

SP70 chaperones under heat shock (stressful) conditions. Both constitutively expressed HSC70s and heat-induced HSP 70s migrate from the cytoplasm to the nucleus where they associate with polypeptides that form insoluble complex at the increased temperatures (Velazquez and Lindquist, 1984; Miller, 1989; Bensaude *et al*, 1990; Kabakov and Gabai, 1993). Subsequently, these proteins also migrate to the nucleolus to associate with partially assembled pre-ribosomes. It should be noted that the functional distinction between

HSP70 and HSC70 is unclear (Welch, 1990; Vierling, 1991; Gething and Sambrook, 1992).

SP70s under normal conditions. Some cytosolic forms of SP70s interact with the growing polypeptide chains emerging from ribosomes (Miller, 1989; Nelson *et al*, 1992), preventing premature aggregation, whilst others are needed for the actual transport of polypeptides to their corresponding compartments; compartment-specific SP70s bind to these transferred unfolded polypeptides as they enter their destination compartment (Welch, 1991). The constitutively expressed HSC73 may bind to nascent secretory precursors (e.g. immunoglobulins) before they fold, maintaining them in a translocation competent state before membrane penetration into organelles (Pfanner, 1990). For example, the GRP78 or BiP is abundantly present in ER and its synthesis can be further induced by the accumulation of secretory precursors such as immunoglobulins and glycoproteins in ER (Hightower, 1991). It is suggested that BiP plays a role in folding and assembly of newly synthesised proteins in ER and translocation of these proteins across ER (Welch, 1990; Nandan *et al*, 1990; Flynn *et al*, 1991; Gething and Sambrook, 1992).

Similarly, cytosolic HSC70s transfer unfolded mitochondrial proteins (encoded by nuclear genes) to mitochondrial HSP70s which probably pass at least some of these polypeptides onto HSP60 (chaperonin 60)/ chaperonin 10 for folding or assembly into oligomeric structures (Parsell and Lindquist, 1993). Chaperoning of newly synthesised proteins in eukaryotic cells, and of damaged proteins in stressed cells by SP70 and chaperonin 60 is illustrated in Figure 1.1.

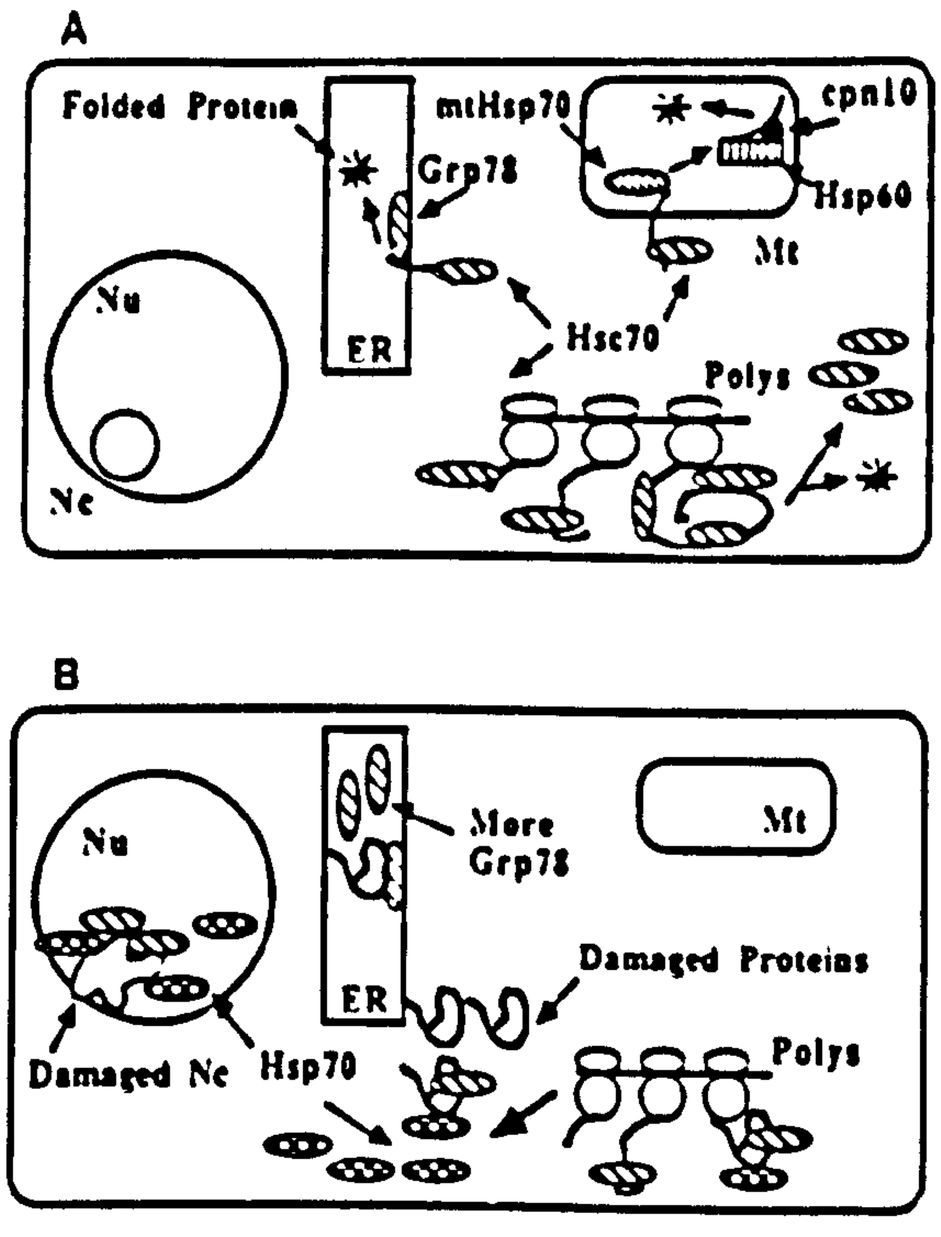


Figure 1.1 Cellular location and the proposed chaperoning functions of HSPs

(A) Chaperoning of newly synthesised proteins in normal eukaryotic cells.

(B) Chaperoning of damaged proteins in stressed cells. ER, endoplasmic reticulum; Mt, mitocondrion; Nc, nocleolus; Nu, nucleus; Polys, polysomes (from Hightower, 1991).

(ii) Chaperonin 60 (SP60/GroEL)

Members of chaperonin 60 family have been found in many prokaryotes (GroEL) and in eukaryotic organelles such as mitochondria and chloroplasts, as well as recently identified cytoplasmic counterparts (Cheng *et al*, 1989; Gatenby *et al*, 1990; Vierling,

Unlike SP70 chaperones, which bind to target proteins as a monomer, chaperonin 60 forms an olgomeric complex. It is widely believed that polypeptides bind to chaperonin in a conformation different from that of SP70s (Gething and Sambrook, 1992). Under normal conditions, this oligomeric complex binds to incomletely folded proteins and direct the folding of proteins to the correct conformation in a specific ATP-dependent fashion, as well as preventing aggregation of nascent proteins until they are competent for oligomer assembly.

SP60s under stressful conditions. The synthesis of chaperonin 60s also increases during stress, and these protein s also take on an additional role in protein repair and refolding. Increased levels of SP60s can also protect cells against protein denaturation and aggregation. However, unlike SP70s, they cannot break up existing aggregates (Sanders, 1993).

(iii) SP90 chaperones

The functions of SP90 chaperones, by contrast, appear to be more specific and diverse, as compared to the other two major classes of chaperone above.

SP90s under normal conditions. This class of proteins modulates many cellular activities by binding to target proteins, forming an inactive or unassembled complex. These target proteins include many protein kinases (such as casein kinase II and the haeme-controled eIF2α kinase), several nuclear hormone receptors (e.g. those for glucocorticoids, ostrogen and progesterone), and components of the cytoskeleton (actin and tubulin) (Schlesinger, 1986; Rose *et al*, 1987; Lindquist and Craig, 1988; Hightower, 1991; Sanders, 1993). For instance, constitutively expressed SP90s bind to transmembrane kinases after synthesis,

forming inactive complexes, until they reach their appropriate destination, after which they are phosphorylated and their kinase domains activated (Lindquist and Craig, 1988). This protein group also functions to inhibit the activation and translocation of steroid receptors into the nucleus in the absence of ligand. Binding of steroid hormone promotes dissociation of SP90 from the complex and results in the transformation of the receptor into an active transcription factor. The binding and release of target proteins by SP90 chaperonins are not well understood, although their weak ATP-binding properties have been repeatedly demonstrated. Little is known about the chaperonin functions of GRP94 which is present abundantly in ER. Like GRP78, it is also induced by accumulation of unfolded proteins in the ER, suggesting that it may function with BiP to assist the assembly of nascent polypeptides (Gething and Sambrook, 1992; Georgopoulos and Welch, 1993; Parsell and Lindquist, 1993). Although SP90 members have been found in association with other SPs, suggesting some role in a "superchaperone" assembly (see also next section), their relevance to stress-related phenomena is not well understood.

One of the most recent developments in the analysis of small (LMW) SPs is that they too display elements of chaperone function *in vitro*. Unlike other SPs, the formation of stable complexes between small SPs and protein substrates has not been reported, and their chaperonin activities also seem to be independent of ATP. Vertebrate lens α-crystallin is related in structure and sequence to the small SPs, and has recently been shown to function as a molecular chaperone (Horwitz, 1992).

(iv) Cooperation between chaperone protein families

In most cases, SP10 (bacterial GroES) and ATP are required for further folding of the substrate protein and release from chaperonin 60 (or GroEL; Parsell and Lindquist, 1993). It has recently been shown that in *E.coli*, the genes for GroEL and GroES are located in the same operon; both proteins together form an oligomeric complex (a chaperone machine) which is involved in the oligomerisation of other proteins (Georgopoulos and Welch, 1993). As previously mentioned, cooperation between SP70 and chaperonin 60 is thought to occur during polypeptide import and subsequent protein folding and oligomersation in mitochondria *in vivo* (Pfanner, 1990). These interactions between SP70s and DnaJ-like proteins are also known to take place *in vitro*. Therefore, it is suggested that DnaJ, SP70 (DnaK) and chaperonin 60 (GroEL) work together to provide a pathway for protein folding (Gething and Sambrook, 1992; Georgopoulos and Welch, 1993; Parsell and Lindquist, 1993). In *E.coli*, it has been further shown that DnaJ and GrpE serve as regulatory factors to modulate the chaperone activity of DnaK (Gamer *et al*, 1992; Cyr *et al*, 1994). Interestingly, recent evidence demonstrates that associations between SPs 90, 70 and 60 may exist during the assembly of the progesterone hormone receptor complex. Indeed, these three proteins may together constitute a functional "super-chaperone machine" (Perdew and Whitelaw, 1991; Parsell and Lindquist, 1993).

1.5.2 The function of stress proteins in degradation of damaged proteins

We have already discussed how the major SPs function as molecular chaperones to form complexes with nascent polypeptides under normal conditions, or with proteins that become misfolded or unfolded during stress, hence rescuing these proteins from

aggregation, irreversible damage and degradation. But not all damaged proteins can be chaperoned and rescued in this way, and it is well established that stressed cells can activate several components of a proteolytic degradation system to help cope with the stress. This enzymatic pathway exists in all prokaryotic and eukaryotic organisms. The degradation of damaged proteins in these organisms is rapid, and helps to (1) reduce the possibility of deleterious interactions between these polypeptides and functional proteins, (2) prevent the formation of insoluble protein aggregates, and (3) release the aminoacids and peptides contained in nonfunctional polypeptides for synthesis of new proteins (Schlesinger, 1990; Kabakov and Gabai, 1993; Parsell and Lindquist, 1993).

(a) Turnover of aberrant proteins in prokaryotes

In *E.coli*, the cytosolic degradation of abnormal proteins is accomplished by two proteases, namely Lon (94 kD stress protein) and Clp proteases (Lindquist and Craig, 1988). Both of these enzymes employ the energy of ATP hydrolysis in protein degradation. As discussed in section 1.3.1, Lon protease-like proteins are found in many other prokaryotes, and represent a novel class of serine proteases. Lon plays two important roles; one is a "specific" role in catalysing the turnover of some key regulatory proteins, the other is a "general" role in promoting the degradation of abnormal proteins. Besides Lon, other SPs such as DnaK, DnaJ, GrpE and GroEL/ES are shown to be important for the ability of *E.coli* to degrade abnormal proteins. It has been suggested that these SPs promote intracellular proteolysis in two different ways, both of which employ their ability to function as chaperones; they either interact directly with protease complexes where substrates are maintained in a protease-sensitive conformation, or else

they function separately from the proteases by preventing the aggregation of unfolded proteins, so increasing the concentration of susceptible substrates, and thereby indirectly promoting proteolysis.

Experiments show that the degradation of 50% of abnormal proteins is accomplished by Lon protease, and 15% by Clp proteases. Clp protease is a 750 kD hetero-oligomer composed of two unrelated subunits which belong to distinct, highly conserved protein families containing both prokaryotic and eukaryotic members. The Clp family is also referred to as SP100 family. SP104 in *S.cerevisiae* is a well known member of this protein family (Parsell and Lindquist, 1993).

(b) Turnover of abnormal proteins in eukaryotes

Many proteins degraded in the eukaryotic cytosol are proteolysed by the ubiquitin-dependent system. The presence of ubiquitin seems to be restricted to eukaryotes as no related protein family has been found in bacteria (Jentsch, 1992). A complex set of enzymes degrades not only abnormal proteins, but also a number of important short-lived regulatory proteins. Increased levels of ubiquitin during stress reflect an increased demand for removal of damaged proteins. The role of ubiquitin itself in proteolysis is to serve as a tag marking out substrates to be degraded (Schlesinger, 1986; 1990; Miller, 1989; Vierling, 1991; Jentsch, 1992). As shown in Figure 1.2, the ubiquitin-dependent degradation pathway involves several steps. First, ubiquitin is activated by attachment to E1 enzyme (ubiquitin activating enzyme) in an ATP-dependent reaction. Second, activated ubiquitin is then transferred to E2 proteins (ubiquitin conjugating enzymes).

Third, the E2 enzymes catalyse the transfer of ubiquitin to a lysine residue on the protein to be degraded. For some substrates, the action of an additional enzyme, E3 (ubiquitin protein ligase) is required for substrate recognition. Following the first attachment of a single ubiquitin molecule to the substrate, ubiquitin itself is prone to further ubiquitinations (multiubiquitination). Finally, the damaged protein is targeted for degradation by a multi-subunit ATP-dependent protease (26S protease) or proteasome (Hershko, 1988; Lindquist and Craig, 1988; Schlesinger, 1990; Jentsch, 1992; Jungmann *et al*, 1993; Parsell and Lindquist, 1993).

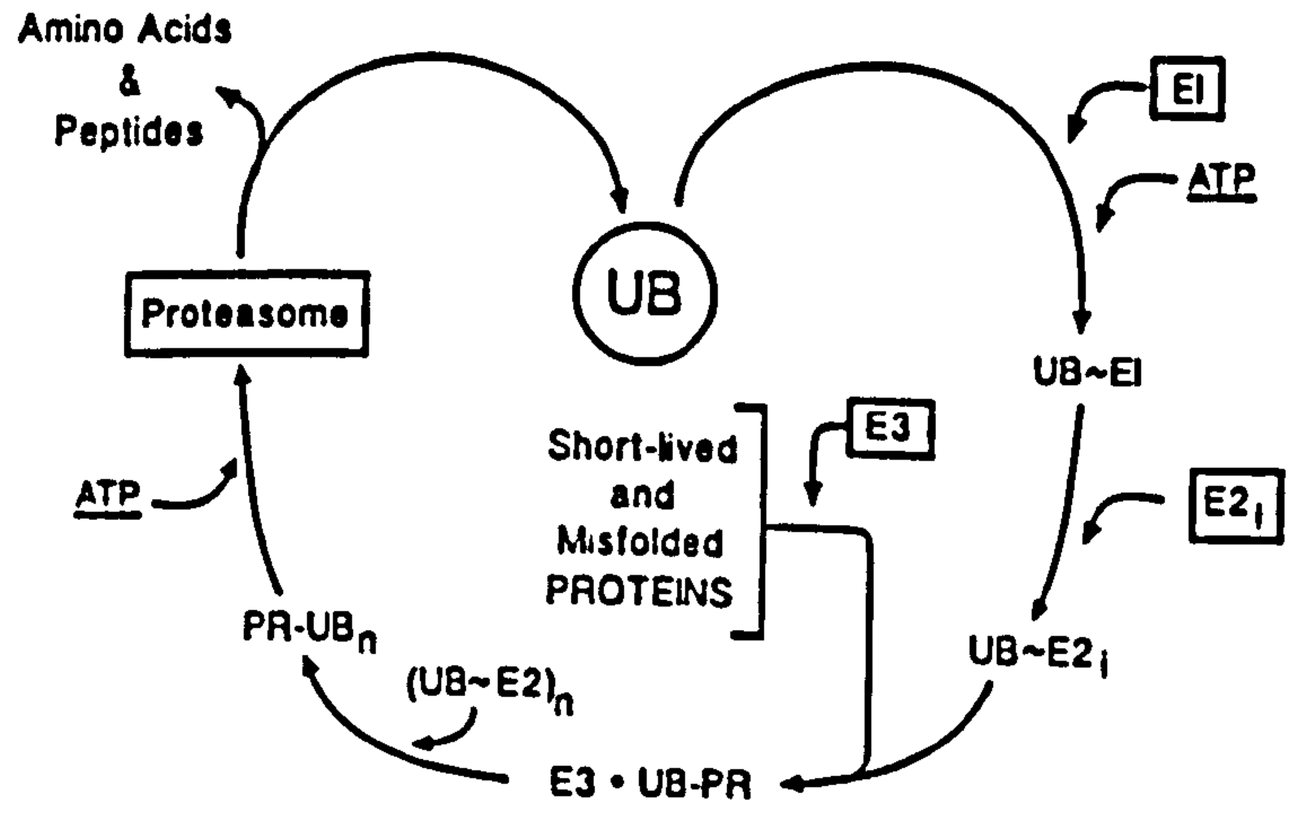


Figure 1.2 Enzymatic pathway for ubiquitin-dependent proteolytic degradation in eukaryotes. E1, ubiquitin activating enzyme; E2i, ubiquitin conjugating enzymes; E3, ubiquitin protein ligase; Ub-PR, mono-ubiqutinated protein; PR-UBn refers to polyubiqutinated protein (modified from Schlesinger, 1990).

Although polyubiquitin genes are characterized as heat inducible in many eukaryotes, these genes in *C.elegans* are not induced by heat shock (Nover and Scharf, 1991).

Interestingly, the yeast ubiquitin conjugating enzyme (UBC7) is induced by cadmium, but again not by heat shock (Jentsch, 1992). Moreover, the levels of ubiquitin conjugating enzymes have been shown to increase in chicken embryo fibroblasts exposed to heat, organic solvents, and oxidants (Schlesinger, 1990). Jungmann *et al* (1993) has recently shown that the ubiquitin-dependent proteolysis pathway in yeast is activated in response to cadmium, and mutants deficient in the conjugating enzyme, UBC7 or 26S protease (also known as proteasome) are hypersensitive to cadmium, suggesting that cadmium resistance is mediated in part by degradation of abnormal proteins. E3 enzyme targets certain proteins for ubiquitin-dependent degradation. It is also believed that eukaryotic SPs with chaperonin functions might serve in an E3-like capacity, and both compete to bind to unfolded proteins. It is interesting to note that mammalian 26S protease shares weak homology with an *E.coli* Clp protein member in the ATP-binding region (Schlesinger, 1990; Parsell and Lindquist, 1993).

Ubiquitin-dependent protein degradation appears to function in the stress response in two ways: first, some of the key reactions in this pathway are catalysed by heat-inducible proteins; second, the ubiquitin system appears to be responsible for much of the turnover of eukaryotic stress-damaged polypeptides (Parsell and Lindquist, 1993). Ubiquitin is known to be involved in the nonlysosomal degradation of severely damaged intracellular proteins through a well defined pathway. However, a large fraction of proteins are degraded in a non-selective manner in lysosomes (Jentsch, 1992; Sanders, 1993). It is speculated that ubiquitin conjugation may also participate in lysosomal functions (Jentsch, 1992). A similar partial role has also been suggested for a constitutive member of the SP70 family, which apparently binds to long-lived proteins containing a specific

sequence, and thus targets them to lysosomes during starvation. Although proteolysis in ER is not well understood, damaged proteins are rapidly proteolysed in this compartment, and BiP may be involved in this process, as BiP is shown to increase in response to heat shock and to the presence of unfolded proteins in ER (Parsell and Lindquist, 1993). It should be also noted that SP70 itself has been reported to have protease activity (Mitchel *et al*, 1985).

1.5.3 Stress protein functions in stress resistance and thermotolerance

Acquired thermotolerance is a phenomenon whereby a mild conditioning temperature confers tolerance to subsequent higher temperatures that would otherwise be lethal. Thermotolerance has been studied extensively, and has been demonstrated to exist in many organisms, varying from bacteria to mammals (for review see Lindquist, 1986; Lindquist and Craig, 1988; Black and Subjeck, 1990; Nover, 1991b; Weber, 1992; Kampinga, 1993; Parsell and Lindquist, 1993). It is still not known whether this heat resistance is due to some kind of protective mechanism leading to less initial damage or whether it is due to better repair (in terms of recovery) of heat-induced damage. However, it is well established that the induction of SPs and the development of thermotolerance are correlated in a wide variety of systems, and virtually every major class of SPs has now been implicated in acquired thermotolerance (Julseth and Inniss, 1990; Nover, 1991b; Odumeru *et al*, 1992; Sanchez *et al*, 1992; Kampinga, 1993; Parsell and Lindquist, 1993). In eukaryotic cells, a large number of stressors that induce SPs, including ethanol, cadmium, arsenite and hydrogen peroxide have also been shown to induce thermotolerance (Plesset *et al*, 1982; Li, 1983; Mizzen and Welch, 1988; Nover,

1991b; Sanchez *et al*, 1992); however, heat pretreatment does not usually confer resistance to chemical stressors (though there have been few reported exceptions; Nover, 1991b).

In most organisms, SP70s are among the most prominent proteins induced by heat. It is now clear that the heat-inducible forms of SP70 play a central role in stress tolerance, by promoting growth at high temperatures and/or protecting organisms from killing at extreme temperatures. In *E.coli* and yeast, the heat-inducible SP70s are primarily responsible for helping cells grow at moderately high temperatures, rather than for helping them survive at extreme temperatures (Parsell and Lindquist, 1993). In *Drosophila*, however, SP70s appear to be the major proteins involved in tolerance to extreme temperatures (Hightower, 1991). The role of heat-inducible SP70s in vertebrate cells has also been studied, and several lines of evidence support the role of SP70s in thermotolerance:- e.g.

(1) in several different cell types exposed to a wide variety of tolerance-inducing treatments, there is a closer correlation between induction of thermotolerance and the accumulation of SP70s than for the accumulation of any other SPs;

(2) hyperthermic cells obtained through long temperature cycles are found to overexpress SP70;

(3) microinjection of anti-SP70 or of SP70 mRNA produces opposite effects on thermotolerance, in that the former decreases while the latter increases cell survival during heat shock;

(4) transfection of rat cells and monkey cells with constitutively expressed human SP70 genes dramatically increases thermotolerance (Li, 1983; Mizzen and Welch, 1988;

Nover, 1991b; Li *et al*, 1992; Kampinga, 1993; Parsell and Lindquist, 1993).

While much is known about the general biochemical activities of SP70 and its importance in protecting organisms at high temperatures, little is yet known about the mechanisms whereby these proteins lead to thermotolerance. However, it is believed that the chaperonin function of SP 70 may well be important in thermotolerance, by protecting proteins and protein complexes from denaturation and aggregation during stress, as well as directing severely damaged proteins to lysosomes for breakdown. Under stressful conditions, SP70s mainly localise in the nuclei and nucleoli where they associate with sensitive structures such as pre-ribosomes, ribosomes and spliceosomes to protect RNA processing and protein synthesis, which in turn may allow organisms to recover from environmentally induced damage (Black and Subjeck, 1990; Welch, 1990). It is now clear that not only SP70s play a role in stress tolerance, but that other classes of SPs also play important roles. Because of the role of stress proteins in regulating protein homeostasis at the cellular level and their role in acquired tolerance at the organismal level, SPs seem to play an important ecological role in the physiological adaptation of organisms to their environment (Sanders, 1988; Sanders, 1993).

One of the most interesting aspects of SPs function in thermotolerance is that different organisms appear to employ different SPs in response to similar types of stress to acquire such tolerance. For example, SP70 is very important for thermotolerance in mammals and *Drosophila*, whereas it plays a minor role in yeast and *E.coli*. Similarly, members of the SP100 family are very important for surviving extreme stress in yeast and *E.coli*, and perhaps in mammals too, but seem quite unimportant in *Drosophila* which does not even

synthesise this protein in response to heat shock (Nover, 1991b; Sanchez *et al*, 1992; Parsell and Lindquist, 1993; Weitzel and Li, 1993). A temperature-sensitive species of *Hydra* does not induce the synthesis of stress proteins at elevated temperatures, whereas a closely related species that can induce the synthesis of SP60 (chaperonin) but not SP70 displays thermotolerance (Bosch *et al*, 1988). Moreover, the synthesis of small SPs promotes thermotolerance in *Drosophila* and in mammalian cells, but appears to have little or no effect in yeast (Hightower, 1991; Parsell and Lindquist, 1993; Sanders, 1993). It is thought that SPs may play specialised roles in different cell types in protecting large structural complexes from disruption by stress; alternatively, this diversity of SPs employed in stress tolerance may reflect the different sensitivities of different targets to damage in different organisms (Parsell and Lindquist, 1993). Besides all this, many other factors may play a role in stress tolerance; for example, the accumulation of trehalose in yeast, of glycerol in vertebrates, and of prolin in plants in all cases closely parallels the acquisition of a stress-tolerant state (Nover, 1991b; Parsell and Lindquist, 1993). In yeast, it has been further shown that SP70 negatively modulates heat-shock-induced accumulation of trehalose to a greater extent than SP90, and promotes recovery from heat stress (Hottiger *et al*, 1992; Cheng *et al*, 1993).

1.5.4 Stress proteins and the immune response

The ubiquitous distribution of SPs and their high sequence conservation make them excellent immune targets. The possible role of SPs in physiological and pathological activities related to immunity has been implicated in many recent studies (for review see Kaufmann, 1990; 1992; Young *et al*, 1990; Aquino and Brosnan, 1992). These include:

antibody assembly and antigen processing; host-cell protection against "immune stress"; participation in tumor surveillance and rejection; relation to T-cell recognition; dominant antigenicity for the immune response against microbial pathogens; and a possible role in autoimmunity.

As previously discussed, a member of SP70 family, BiP, binds to newly synthesised unfolded heavy chains immonoglobulins prior to folding and linkage with light chains, and it is also believed that BiP may contribute to immunoglobulin assemblies. Moreover, due to their involvement in unfolding, translocation and degration of proteins, SPs may play an important role in the processing of foreign antigens (Kaufmann, 1990; 1992). Unlike the antigen-specific activity of T-cells, large lymphocytes (macrophages) are well known to express non-specific killer activity, producing toxic molecules such as reactive oxygen metabolites in order to kill infectous microorganisms. These macrophages have been shown to produce increased levels of SPs to protect themselves from their own "killer" mechanisms (Kaufmann, 1992). Increased levels of SPs, particularly SP90 and 70 family members, in virus-transformed and chemically-induced tumor cells have been observed in several studies, and evidence suggests the involvement of stress proteins in the surveillance of tumor cells by recognising and subsequently rejecting such transformed cells (Kaufmann, 1992).

It is now well established that SPs are major antigens in many pathogens including bacteria, protozoa and nematodes. The immune response to many infectious diseases is shown to be dominated by antibodies against members of different stress protein families (Young *et al*, 1990; Nover, 1991c; Young and Garbe, 1991; Sharma, 1992). GroEL-like

stress proteins (65 kD) have been identifed as the most common antigen of bacterial infections, especially for *Mycobacteria* (Young *et al*, 1990; Young and Garbe, 1991), whereas antibodies against SP70 have been found to predominate in host responses to protozoa (e.g. *Plasmodium falciparum*; Sharma, 1992) and in nematodes (Nover, 1991c). However, an interesting aspect of stress protein involvement in immune system is that they may have a role in autoimmunity. Since highly conserved stress proteins (e.g. human SP65 cognate shares 65% sequence homology with SP65 of *M.tuberculosis*) are targets for immune recognition, the immune system must guard against mounting a response that could be directed to homologous self-proteins. This control is thought to be at the level of T-cell recognition. It should be noted that antibody responses to self stress proteins is an observed phenomenon in patients suffering from several autoimmune diseases (Young *et al*, 1990; Kaufmann, 1992).

1.5.5 Other reported functions of SPs

Although SPs are clearly important in all of the cellular functions described before, they may also participate in a number of other biological processes. *E.coli* SP70, DnaK is well known to facilitate bacteriophage λ DNA replication; the initiation of λ DNA requires first the assembly of various proteins at the origin of replication, and subsequently the disassembly of this complex. SPs are thought to play similar roles in both prokaryotic and eukaryotic DNA replication events (Lindquist and Craig, 1988; Georgopoulos and Welch, 1993). Another important function of SPs, in particular SP90, is that involved in the structure and function of the cytoskeleton. A recently identified eukaryotic chaperonin (TCP-1) has also been shown to facilitate the the synthesis of tubulin and actin, as well

as a role in mitotic spindle formation (Sanders, 1993). SPs are likely to play important roles in physiological processes which involve the rapid breakdown and reorganisation of tissues such as larval settling, molting, and breakdown of symbiosis. It is also thought that they may be involved in triggering developmental changes in response to changes in environmental conditions. The stress-response of the green alga *Volvox* is a good example, where the induction of a stress protein has been shown to trigger a change from asexual to sexual reproduction (Nover, 1991c; Sanders, 1993). It is interesting to note that in *C.elegans* dauer larvae, the levels of SP70 and polyubiquitin mRNA rise sharply within 75 minutes of recovery from the dauer stage, whereas SP90 mRNA levels decline sharply during this recovery time (Dalley and Golomb, 1992). The expression of SPs in many eukaryotes is known to be regulated during development (Vierling, 1991), and the role of stress proteins in stress-induced teratogenesis and other developmental effects has been also reported (see review by Nover, 1991c). Furthermore, the uncoating ATPase is a member of SP70 family, whose function in bovine brain is to release clathrin triskelions from coated vesicles in an ATP-dependent manner (Ungewickell, 1985; Chappell *et al*, 1986; Lindquist and Craig, 1988). Recent evidence suggests that chaperone proteins, especially SP70 and SP90, not only function in folding or assembly of newly synthesised proteins, but may also modulate the activation or deactivation of mature polypeptides by phosphorylation and dephosphorylation respectively (Georgopoulos and Welch, 1993). In fact, as discussed in section 1.5.1, SP90 is well known to bind to target protein kinases and to activate them by phosphorylation as active kinases.

1.6 GENETIC REGULATION OF STRESS PROTEIN EXPRESSION

1.6.1 Stress signals

Control of stress protein expression induced by extracellular physical (e.g. heat) or chemical stressors is usually mediated by a number of signal systems. These include receptor proteins, proteins involved in signal transformation which eventually lead to the generation of "second messengers", and regulatory proteins that trigger the response. Increasing recent evidence suggests that the accumulation of abnormal proteins in stressed cells is an essential element of the signal system, and triggers the activation of heat-inducible genes.

(a) Abnormal proteins as a stress signal

There have been a number of findings which support the central role of abnormal proteins serving as a stress signal. As discussed before, the synthesis of heat inducible proteins is activated by heat or a variety of other metabolic stresses (e.g. amino acid analogs, heavy metals, arsenite), most if not all of which are united by their ability to cause damage to intracellular proteins. This damage has been referred to as "proteotoxicity" (Hightower, 1991). Ananthan and co-workers (1986) have shown that injection of abnormal proteins into *Xenopus* oocytes activates transcription from a *Drosophila* or human *hsp70* promoter, and further demonstrated that a common regulatory mechanism exists for the activation of *hsp70* expression by heat shock and by abnormal proteins. Recently, Kabakov and Gabai (1993) have further demonstrated that

heat and other stresses (e.g. oxidative stress, ATP depletion) cause a rapid aggregation of cell proteins, suggesting that stress-induced protein aggregation may be one of the key control points in HSP expression. Moreover, the addition of deuterium oxide or 1M glycerol (both of which are known to stablize proteins) inhibits activation of the *hsp90* and *hsp70* genes in chicken embryos at the heat shock temperature (Nover, 1991a). More interestingly, a yeast mutant defective in the genes for two heat-shock-inducible ubiquitin-conjugating enzymes has been shown to express a constitutive, high level of HSP synthesis due to the inefficient degradation of short-lived and abnormal proteins (Nover, 1991a).

(b) Search for other signal systems

The complex pattern of cellular reprograming during the heat shock response implicates other signal systems in addition to that involved in *hsp* gene activation (i.e. the activation of HSF, which will be discussed in section 1.6.2). An excellent review of the network of signal systems involved has been written by Nover (1991a), which will be briefly discussed here. Upon heat stress, a large number of metabolic systems are known to be affected. One of these is mitochondrial respiration; because many chemical stressors capable of inducing heat shock puffs in *Drosophila* are respiratory inhibitors, it was thought that mitochondria might be primary targets of stress attack generating a heat shock signal. However, several studies have revealed that this system is not involved in signal transformation. The breakdown of cytoskeletal structures may be involved indirectly in the induction of heat shock genes, by forming cytoplasmic protein aggregates (Welch, 1990; Nover, 1991a; Kabakov and Gabai, 1993). The role of

intracellular Ca^{+2} levels has been also evaluated in this respect. Although there is no direct evidence for the involvement of Ca^{+2} levels in heat shock phenomena (Welch, 1990), it is well known that HSP90 and HSP70 bind to calmodulin and control the interaction of this protein with cytoskeletal proteins in a Ca^{+2}-dependent manner (Nover, 1991a). Moreover, Gaestel *et al* (1992) have recently shown that the small heat shock protein (HSP25) in mouse is dephosphorylated by a calcium/calmodulin-dependent protein phosphatase. Other systems implicated in signal transformation systems include phosphoinositide metabolism, polyamine levels, cyclic AMP and diadenosine tetraphosphate (see Nover, 1991a for further details). Another system that must be considered is a rapid cytoplasmic accumulation of trehalose in yeast following heat stress. Cheng *et al* (1993) have examined the effects of such accumulation on heat shock induction, and suggested that the levels of HSP70s, but not HSP90, may be an important factor in controlling both HSP and trehalose synthesis in the yeast heat shock response.

(c) A role for HSP70 in the stress signal system

As discussed in section 1.5.1, the SP70 family is among the most prominent and well characterised molecular chaperones, and includes both stress-inducible and constitutively expressed (cognate) protein members. Under normal conditions, HSC70 transiently binds to nascent polypeptide chains, facilitates translocation of certain proteins across organelle membranes, and helps in protein folding and assembly. In stressed cells, however, increased levels of denatured proteins caused by physical or chemical stressors stimulate the induction of new HSP70 synthesis to cope with the imposed stress. According to a popular hypothesis, a decrease in the level of free HSP70 in stressed cells may lead to

the activation of heat shock transcription factor (HSF), which then binds to the regulatory DNA regions of heat-inducible genes (*hsp* promoters) to stimulate their transcription (Sorger, 1991; Cheng *et al*, 1993; Kabakov and Gabai, 1993). It has been further suggested that HSC70 in normal cells is reversibly associated with HSF, but upon stress the sequestration of HSP70 with denatured proteins releases HSF, which in turn allows the transcription of heat inducible genes (Hightower, 1991; Georgopoulos and Welch, 1993). Recently, Gamer *et al* (1992) has reported the associations of the DnaK (SP70), DnaJ and GrpE proteins with σ^{32} (corresponding to bacterial HSF) *in vivo*, supporting the sequestration model above. They also speculate that, in *E.coli*, the cellular thermometer which receives intracellular stress signals is not primarily DnaK, but rather a coordinate activity of DnaJ and DnaK. Furthermore, functional *in vitro* and *in vivo* interactions between mammalian HSF and cytosolic HSP70 have been recently demonstrated (Abravaya *et al*, 1992; Georgopoulos and Welch, 1993; Hatayama *et al*, 1993).

1.6.2 Activation of heat shock factor (HSF)

The gene encoding HSF was first isolated from *S.cerevisiae* (Sorger and Pelham, 1987), and subsequently from many other prokaryotic and eukaryotic species (Sorger, 1991). Electrophoretic analyses of HSF from *S.cerevisiae*, *Drosophila*, and human cells clearly showed that the HSFs have molecular sizes of 150, 110, and 83 kD, respectively (Wu *et al*, 1990; Zimarino *et al*, 1990). It is now known that upon heat shock a preexisting pool of inactive HSF is converted into an active form capable of efficiently stimulating the transcription of heat shock genes (Pelham, 1985; Nover, 1991a; Sorger, 1991; Westwood *et al*, 1991; Westwood and Wu, 1993). This posttranslational activation of HSF is not

only induced by heat shock, but also by a wide variety of reagents that favor the dissociation and denaturation of protein complexes (Sorger, 1991), including heavy metals and organometallics (Zhang and Liu, 1992; Hatayama *et al*, 1993; Mitani *et al*, 1993). *In vitro* activation of HSF can be obtained by elevated temperatures, calcium ions, low pH, urea, non-ionic detergents, 4-hydroxynonenal (a highly cytotoxic product of lipid peroxidation), and interaction with antibodies (Zimarino *et al*, 1990; Cajone and Crescente, 1992). Many if not all of these agents have been shown to induce conformational changes in HSF, suggesting that HSF itself is the sensory machinery which recognizes various forms of stress. Kimura *et al* (1991) have demonstrated that the inactive and the active forms of human HSF can be separated on the basis of different sedimentation coefficients estimated by sucrose density gradient centrifugation, indicating that the activation of HSF correlates with an alteration in its molecular form upon stress shock.

It is now established that the transcription of heat-inducible genes is mediated through the binding of activated HSF to a conserved DNA sequence known as the heat shock element (HSE), found upstream of heat-inducible genes. The HSE is the site of interaction with HSF, and thereby controls the transcriptional activation of heat-inducible genes (Bienz, 1985; Wu *et al*, 1990; Sorger, 1991; Lis and Wu, 1993; see also the next section for further details). Despite the highly conserved sequences of both heat-inducible proteins (HSPs; see section 1.3.3 above) and HSEs in eukaryotes, the protein sequences of HSFs are strikingly divergent, except for the DNA-binding domain and adjacent hydrophobic residues, or leucine-zipper motifs (Westwood *et al*, 1991). It is also interesting to note that the temperature required for HSP induction varies greatly among

species. This difference is largely determined by the species-specific stress transformation chains, including the activation of HSF. For example, using a *Drosophila* HSE-containing promoter fusion, reporter gene induction has been achieved at 25°C in sea urchin eggs, at 34°C in *C.elegans*, at 40°C in tobacco cells and at 42-43°C in mammalian cells (Fire, 1986; Nover, 1991a). These results also provide evidence for the conservation of at least the DNA-binding domain of HSF.

The regulation of HSF activity is believed to occur at two separate levels, DNA binding and transcriptional competence. Interestingly, this regulation appears to differ between higher and lower eukaryotes. As mentioned before, in unstressed cells the HSF protein is present in an inactive form that possesses high affinity binding to DNA following a stress stimulus. This binding, in *Drosophila*, is accomplished by a stress-induced oligomerisation (Westwood *et al*, 1991). However, the binding of HSFs to DNA in the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* seems to be constitutive and independent of any stress stimulus, bypassing the first level of regulation (Sorger, 1991). The second stage of HSF activation common to both prokaryotes and eukaryotes involves a stress-induced phosphorylation of the factor, which thus becomes competent for transcriptional activity (Wu *et al*, 1990; Lis and Wu, 1993; Westwood and Wu, 1993). The high-affinity binding of HSF to HSE target sequences has been shown to be dependent on multimerization, although the quaternary structure of active HSF is not completely known. However, Westwood and Wu (1993) have recently provided the evidence that the inactivated form of HSF in eukaryotes (except yeast) acquires high-affinity DNA-binding activity upon heat shock through a conformational change associated with a monomer-to-trimer transition. Trimer and multimer (hexamer) active forms have been also obtained

with *S.cerevisiae* HSF (Hightower, 1991). Like the DNA-binding domain, the trimerization domain (consisting of hydrophobic residues) is both conserved and located in the N-terminal region of HSF (Lis and Wu, 1993). It should also be noted that inactive HSF in *Drosophila* is localised in the nuclei of cultured cells and on polytene chromatin, and that it becomes redistributed to heat-shock puff sites as well as to developmental loci during heat stress, suggesting a role for HSF as a repressor of normal gene activity during stress (Westwood *et al*, 1991).

Recent experiments further indicate that HSF in the yeasts *S.cerevisiae* and *K.lactis* is encoded by a single-copy gene, whereas two or three related HSF genes have been found in higher eukaryotes (Hightower, 1991; Lis and Wu, 1993). The latter may reflect functional diversification, and in fact, increasing evidence strongly suggests that this might be the case. For example, the study by Mitani *et al* (1993) have suggested that HSF from human hepatoma cells comprises two different transcription factors, a haem-responsive transcription factor activated by porphyrin complexes of $CoCl_2$ which is only responsible for induction of haem oxygenase, and a metal ($SnCl_2$)-responsive transcription factor which activates the transcription of both haem oxygenase and *hsp70* genes. It has also been shown that tributyltin increased the abundance of heme oxygenase and *hsp70* mRNAs , but heat shock did not have any effect on the levels of the former (Zhang and Liu, 1992). Moreover, human HSF1 mediates the response to heat shock, while HSF2 is activated by haemin during erythroid differentiation (Lis and Wu, 1993).

1.6.3 HSF binding to HSEs, and transactivation of heat-inducible genes

Heat-inducible genes possess a promoter region comprising multiple copies of short and highly conserved DNA sequences called heat shock elements (HSEs). The first functional analysis of these regulatory regions was reported for the *Drosophila hsp70* gene by examining the expression of a series of deletion mutations. Sequence comparison of the *hsp70* and other *Drosophila* heat shock genes further showed the existence of the TATA box at -30 and a consensus 14 bp sequence CnnGAAnnTTCnnG (n refers to any nucleotide) 20 to 30 nucleotides upstream of the TATA box (Bienz, 1985; Pelham, 1985, 1987; Morimoto *et al*, 1990a). Similar regulatory regions have been found upstream of a variety of heat-inducible genes in organisms as diverse as yeast and human (Amin *et al*, 1988; Lis *et al*, 1990). Close inspection of these sequences reveals a simple repeating sequence; periodically arranged GAA segments, repeated at 2-nucleotide intervals and in alternating orientations, are key feature of heat shock regulatory elements (Nover, 1991d). Analysis of these sequences by deletion mutations clearly showed that a functional heat-shock regulatory element, at least in the case of the *hsp70* promoter, includes a minimum of three GAA segments arranged as contiguous inverted repeats (nGAAnnTTCnnGAAn or nTTCnnGAAnnTTCn; Amin *et al*, 1988; Nover, 1991d; Lis and Wu, 1993). However, in many natural heat-shock regulatory elements, substitutions have occurred in some of the GAA/TTC blocks (e.g. replaced by GAG/CTC; Amin *et al*, 1988), but they are still recognisible and functional. The strong conservation of this regulatory sequence explains why the *Drosophila hsp70* promoter is able to function in many eukaryotic species including sea urchins, *C.elegans* (note that reporter expression in the CB4027 transgenic strain used in this study is regulated by a *Drosophila hsp70* promoter; see section 5.1.2),

amphibians and mammals (Pelham, 1987). An excellent review of homologous and heterologous expression systems based on the use of fusion constructs that contain heat shock promoter regions linked to a reporter gene (usually the *E.coli lacZ* gene) has been written by Nover (1991d).

It should also be noted that maximum heat shock inducibility of many genes in different kinds of organisms depends on multiple HSEs frequently scattered over several hundred base pairs in the promoter region (for review see Pelham, 1987; Amin *et al*, 1988; Nover, 1991d). For example, the cascades of HSEs extend up to -350 bp for the *Drosophila hsp26* promoter and to -2100 bp for the *hsp27* promoter. It has been shown that in optimum combinations, HSE arrays can act as transcriptional enhancers; they act irrespective of their direction of insertion or distance from the transcription start (Nover, 1991d). Interestingly, the analysis of heat-induced expression of *Xenopus hsp70* gene constructs demonstrated that the wild-type gene containing three overlapping HSE dimers .NG (nGAAnnTTCn) and two CAT (or CCAAT) boxes in front of the TATA box is highly expressed in HeLa cells. Removal of one HSE dimer has no effect, irrespective of whether it is distal or proximal to the TATA box. Moreover, 215 bp of foreign DNA can be inserted between the HSE-containing region and the TATA box without influencing heat inducibility, unless the TATA-proximal helper element (CAT-box 1) is also removed. Remarkably, two overlapping HSE dimers in combination (provided they are moved closer to the TATA box) function perfectly without CAT-box 1. Therefore, it becomes clear that the enhancer-like activity of HSE combinations need to be complemented by TATA-proximal helper elements, either a CAT box or a HSE monomer itself (Pelham, 1987; Nover, 1991d). Similar results were obtained by transforming HeLa

cells with a construct containing a β-globin gene and *Drosophila hsp70* promoter/enhancer element. This suggests that the presence of heat shock elements alone does not necessarily indicate heat inducibility, but there is rather a need for additional promoter elements, such as the TATA-box and TATA-proximal helper elements, and also for their proper interaction. Over and above the position and probable role of HSEs in heat-inducibility, the heat shock promoters are extremely complex, including several other consensus elements with transcriptional functions unrelated to heat-shock (Nover, 1991d).

The simplest explanation for the long-range effects discussed above is that HSF interacts simultaneously with its distant binding sites and with proteins bound near the TATA box, and thus takes an appropriate position to direct RNA polymerase II to the transcriptional start site. The role of the TATA-proximal CAAT boxes would thus be to hold enhancer-bound HSFs close to the TATA box (Pelham, 1987; Morimoto *et al*, 1990a). The binding of HSFs to these structurally distinct sites as well as to HSE arrays with larger numbers of 5 bp segments (nGAAn) is not resolved yet. However, it is believed that the answer may lie in the oligomeric nature of the heat shock factor (see also below). Although heat-shock factors from yeast and higher eukaryotes have been shown to form trimers upon heat-shock activation, it is not known whether HSF exists *in vivo* primarily as a trimeric, hexameric, or possibly even larger complex (Sorger, 1991; Westwood and Wu, 1993). Several studies have indicated that each subunit of a HSF multimer may bind to a single nGGAn unit, thus a trimer would bind to HSEs composed of at least three such 5 bp units. A three-stranded α-helical coiled-coil structure has been proposed for the formation of interface between HSF monomers. This structure would allow the flexibility to HSF monomers to act on differentially oriented nGAAn units, forming large complexes (e.g.

hexamers) which are certainly important for the activation of heat inducible genes *in vivo*. The model for the interaction of the coiled-coil HSF trimers with different arrays of 5 bp units is shown in Figure 1.3 (Lis *et al*, 1990; Sorger, 1991; Lis and Wu, 1993).

It is well known that in uninduced *Drosophila* cells, heat shock promoters are poised for a rapid change in transcription by assembly in an accessible chromatin structure that is depleted of histones and hypersensitive to nuclease digestion. Important roles are played at least two transcription factors, namely the GAGA factor bound to GA/CT repeats in many *Drosophila* genes, and the TATA-binding protein TBP (which binds to available TATA boxes), as well as a paused RNA polymerase II molecule which appears to be stalled close to transcription start site (Lis and Wu, 1993). Upon heat shock, the major change observed is the rapid targeting of HSF to the HSEs, and the accesible chromatin structure may be important for this rapid binding of HSF. Unfortunately, the precise mechanism by which binding of HSF trimers leads to the stimulation of transcription is not yet known. However, it is thought that HSF acts directly or indirectly to accelerate the rate of escape of the paused RNA polymerase (see Figure 1.4; Lis *et al*, 1990; Sorger, 1991; Lis and Wu, 1993).

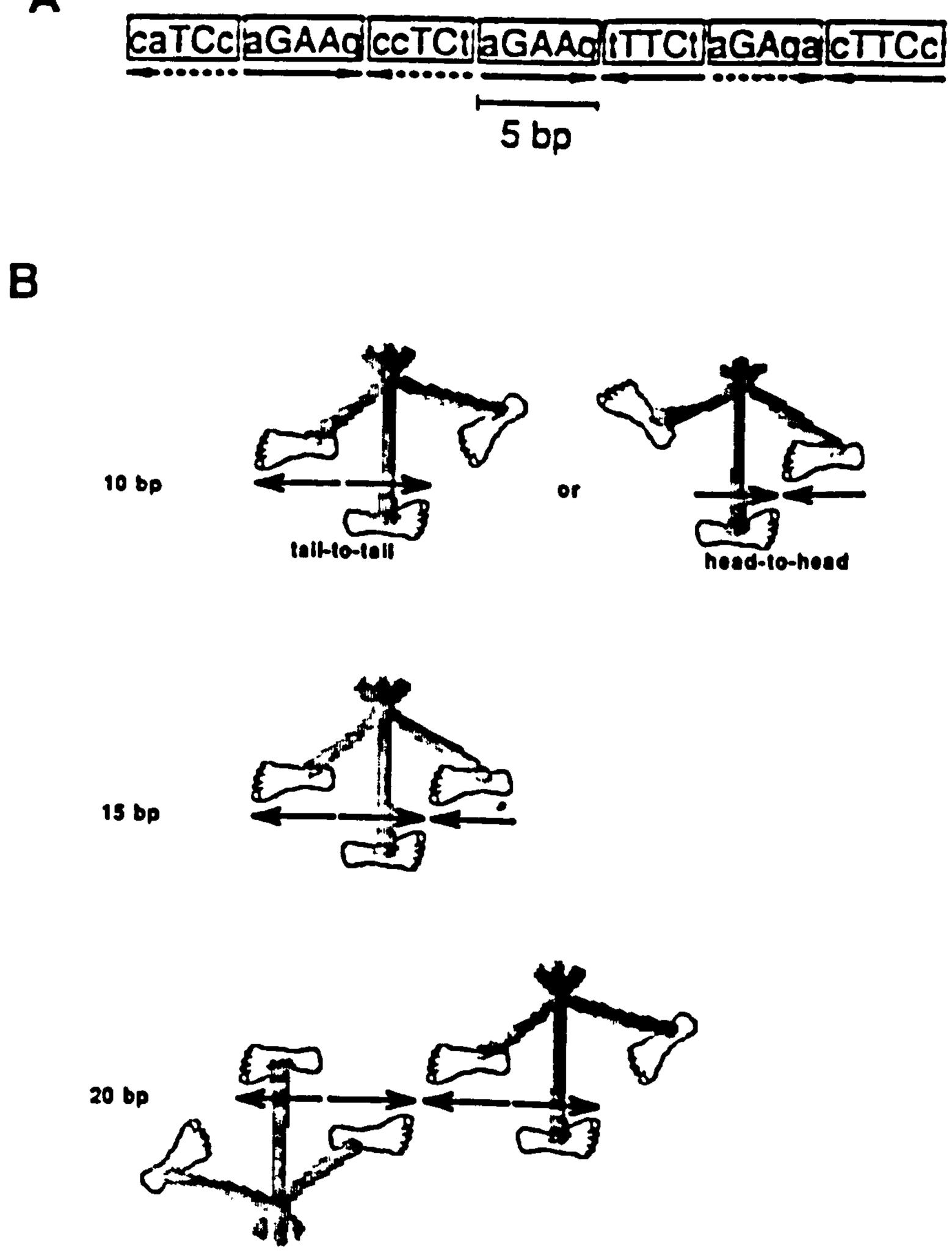


Figure 1.3 (A) Heat shock elements are arrays of 5 bp units. The HSE sequence shown is from the *Drosophila hsp83* promoter. Arrows indicate the orientation of the units, and the dashed lines denote imperfect matches to the consensus the nGAAn sequence (From Sorger, 1991). (B) Model for the interaction of HSF trimers with different arrays of 5 bp units. The foot represents the DNA-binding domain of HSF, and the legs join the subunits of the trimer (With permission from Morimoto *et al*, 1990).

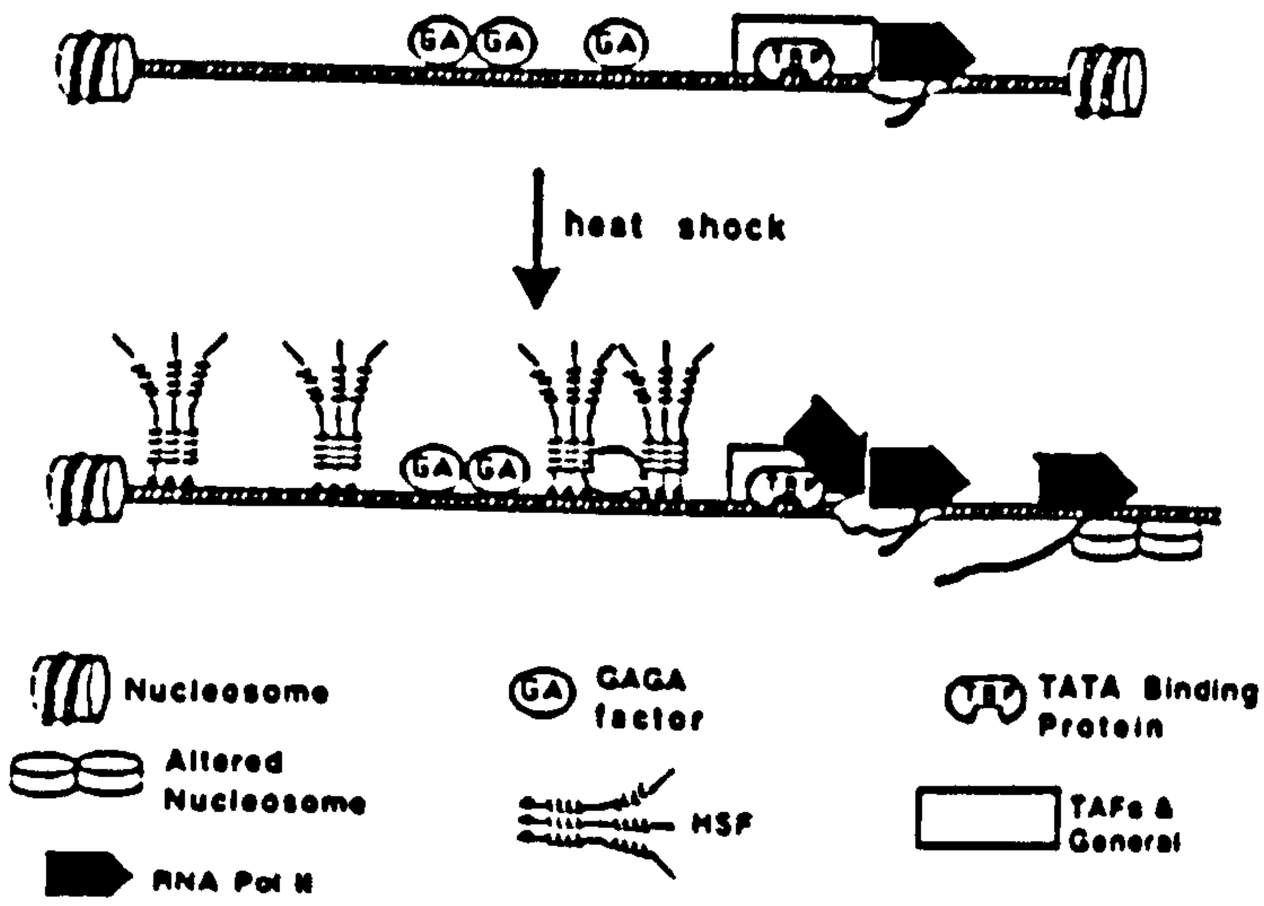


Figure 1.4 The *in vivo* architecture of the *hsp70* promoter before and after heat shock. The uninduced heat shock promoter possesses a paused RNA polymerase II, a TATA binding protein, and an upstream factor(s) that binds to the GAGA sequence elements. TBP-associated factors (TAFs) and at least some of the general transcription factors are also presumed to be present at normal conditions. Upon heat shock, HSF binds to HSE (From Lis and Wu, 1993).

In the present study, we have examined the stress responses of the crustacean *Asellus aquaticus* and the nematode *Caenorhabditis elegans* (both wild type and transgenic strains) to a wide range of toxicants, and have explored the possible use of these organisms in biomonitoring. The biology of the crustacean *A.aquaticus* and its use in biomonitoring will be dealt with in section 3.1.1 (see also figure 1.5). The biology of the nematode *C.elegans* and its use in toxicity testing will be discussed separately in sections 4.1.1 and 5.1.1, respectively (see also figure 1.6 for the life cycle of this organism).

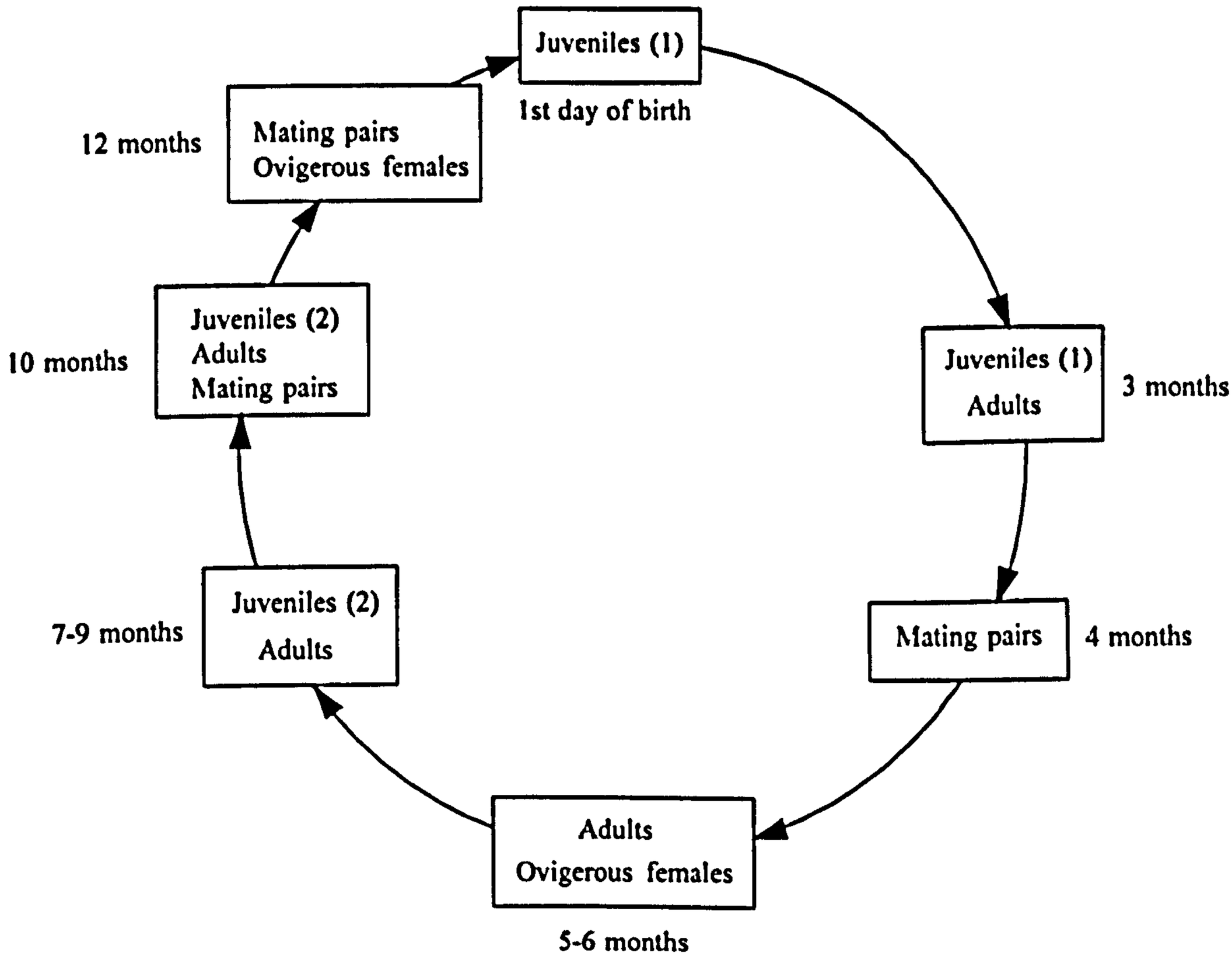


Figure 1.5 Life history of *Asellus aquaticus* (from the first day of birth) under laboratory conditions at 13°C over a 12 month period (modified from Hijji, 1986).

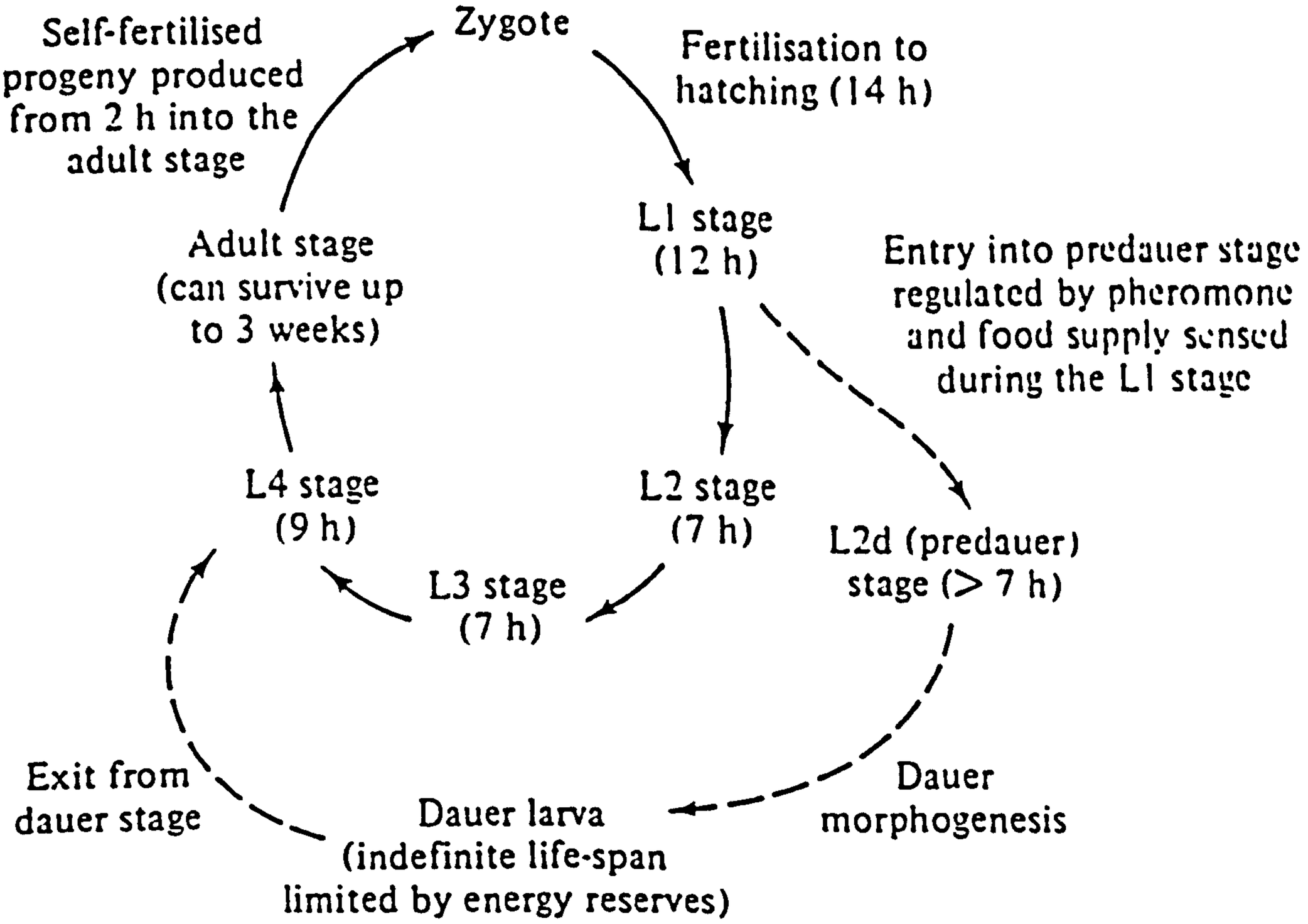


Figure 1.6 Life cycle of *Caenorhabditis elegans* (From de Pomerai, 1990).

Times refer to growth at 25°C. ——→ normal life cycle: - - - - - - - →, dauer pathway (facultative diapause)

CHAPTER 2. MATERIALS AND METHODS

2.0 MATERIALS

(i) Nematode strains

The wild type (N2) and transgenic (CB4027 and CB4028) *C. elegans* strains were obtained from Drs. J.Hodgkin and A.Chisholm (MRC Cambridge), as was the *lac*-operon deleted strain of *E. coli* (P90C) used as a food source.

(ii) Culture media disposables

Petri dishes --- Sterilin

Multiple well plates --- Corning

1-10 ml sterile pippettes --- Appleton Woods

50 ml sterile flasks --- Nunclon

10 ml centrifuge tubes --- Sarstedt

50 ml centrifuge tubes --- Falcon

(iii) All solutions were made up in ultrapure water with >17 MQ

(iv) Heavy metal ions and other toxicants

All metal ions were from BDH-AnalaR, except the ones listed below:

Mercuric chloride, Chromium chloride and Silver nitrate --- Sigma

(v) Other chemicals and reagents

All chemicals and reagents were from Sigma Ltd. and all solvents were obtained from BDH-AnalaR, except for those listed below:

Acetone, Sodium carbonate and Potassium tartarate --- Fisons

Amonium persulphate --- BDH-AnalaR

Agarose LE --- Seakem

Yeast extract --- Oxoid

Bactotryptone --- Difco

Scintillation fluid,Emulsfier --- Safe-Packard

Ampilfy --- Amersham

X-ray films and X-ray film developer --- Kodak Ltd.

Fixative --- Ilford Photographic

(vi) Electrophoresis and Western blotting materials

The enzyme β-galactosidase, prestained protein markers, ampholines and all antibodies were purchased from Sigma Ltd., except rat anti-HSP70 which was obtained from Affinity BioReagents, and rabbit anti-β-galactosidase which was a gift from Dr Tighe of this department.

The PVDF transfer membrane was from DuPont-PolyScreen, and Biotrace nitrocellulose membranes from Gelman Sciences. Both radioactive[^{35}S]-methionine (37) and enhanced

chemiluminescence (ECL) kits were from Amersham Ltd.

2.1 MAINTENANCE OF *CAENORHABDITIS ELEGANS*

Agar media (solid) and S-medium (liquid) were mainly used to culture the nematodes.

2.1.1 Agar media

Both wild-type and transgenic strains were cultured on NGM agar medium (appendix 1) in 9 cm sterile Petri dishes at 15-20°C, feeding on *Escherichia coli* (strain P90C) and handled as described by Brenner (1974). The worms were subsequently used after 5 days of culture when they were predominantly adults. Leaving the worms longer than 2-3 weeks results in dauer larvae formation because of an increase in the number of worms and starvation (Cassada and Russell, 1975) . The worms in dauer larvae stage were maintained without feeding for several months after which they were transferred to new agar plates with bacteria; under these conditions, dauer larvae recover forming L4 larvae and fertile adults.

2.1.2 S-Medium

The worms grown on 5-10 agar petri dishes were washed into M-9 buffer (appendix 2), pelleted and then transferred to 1 litre of S-Medium (as described by Sulston and Brenner, 1974) with a significant amount of bacteria (50 ml slurry) present.

S-Medium:

S buffer; 0.1 M NaCl, 0.05 M Potassium phosphate (pH 6.0) and 1ml of a saturated ethanolic solution of cholesterol (5 mg/ml) was added to 1 L total volume.

After autoclaving, the sterile solutions of the following were added in order;

S buffer	1 litre
1 M Potassium citrate pH 6.0	10 ml
1 M $CaCl_2$	3 ml
1 M $MgSO_4$	3 ml
Trace Metal Solutions	10 ml

(Trace Metal Sol. 2.5 mM $FeSO_4$, 1 mM $MnCl_2$, 1 mM $ZnSO_4$ and 0.1 mM $CuSO_4$)

The aerated worm cultures were incubated in a constant temperature incubator at 20 °C. After 5 days of growth, mg quantity of worms were allowed to settle at 4 °C for 10 minutes. This temperature slows down worm movement and helps to pellet them easily. They were then sieved through a 50 µm mesh to separate the sensitive adult worms from the less sensitive larval stages.

2.1.3 Freezing the worms indefinetely

The wild type and transgenic strains were washed into 5 ml of M9 buffer, mixed with equal volume of freezing solution (appendix 3), and stored at -70°C in 0.5 ml freezing vials. When needed, the worms were thawed and regrown on NGM agar plates.Only early

larval stages could survive freezing and thawing.

2.2 GROWING *ESCHERICHIA COLI* CULTURES

Both wild type (OP50) and *lac*-operon-deleted (P90C) strains were grown in 50 ml sterilized Luria-Bertani (LB) medium overnight at 37°C. Under sterile conditions, the bacteria were spread on agar plates and allowed to grow overnight at 37°C.

LB Medium :

1% (w/v) Bactotryptone, 0.5% (w/v) Yeast extract, 0.08 M NaCl, 0.002 M NaOH (pH 7.0).

2.3 COLLECTION AND MAINTENANCE OF *ASELLUS AQUATICUS*

A. aquaticus and other crustacean species used in this study were collected from either Attenborough Nature Reserve and Nottingham canal ? which are thought to be relatively unpolluted, or else from a polluted site at Water Orton on the River Tame. They were brought to the laboratory, placed in aerated perspex tanks containing fresh water from their original habitat and acclimated at 13°C for at least 1 week before the test exposure.

2.4 EXPOSURE OF *C.ELEGANS* TO HEAT AND TO TOXICANTS

The worms were exposed to stress conditions either in K-Medium (as described by Williams and Dusenbury,1990; appendix 4) or in agar medium on which the nematodes

reside in a thin layer of K-Medium. Mainly, the experimental design was as described by Williams and Dusenbury (1990), except that in this study mixed populations of the worms were used instead of young adults derived from dauer larvae preparations.

During the assay, the worms grown on agar plates were washed into K-Medium, centrifuged at 3000 xg for 2 minutes and then diluted 10-20x with new K-Medium. This leaves only a limited amount of bacteria in the worm suspension as a food source during exposure; clearly, starvation of the worms during exposure could add to their stress, but on the other hand excessive numbers of bacteria might sequester much of the toxicant and make it less available to the worms. The worms were then placed in either 24-well tissue culture plates or petri dishes (volume varied between 1 to 10 ml). 50-500 x concentrated solutions of metal ions or other reagents were added to the required final concentrations. The plates or dishes (including controls with no additions) were finally transferred to LEEC-constant incubators, depending on the nature of exposure, for 7 hrs. A temperature of 31-32°C proved to be the upper limit at which most transgenic control worms remained unstained following histochemical staining for β-galactosidase. Additionally, transgenic worms were heat-shocked at 34°C as described by Fire (1986).

12 inorganic heavy metal ions were tested at the following concentrations (in ppm):-

Metal ions		ppm
Cadmium($CdCl_2$)	;	0.05-24
Zinc($Zn\ SO_4.7H_2O$)	;	0.05-24
Mercury($HgCl_2$)	;	0.1-24
Copper($CuSO_4.5H_2O$)	;	0.5-10
Manganese($MnCl_2.4H_2O$)	;	1-50
Lead [$Pb(NO_3)_2$]	;	0.1-8
Silver($AgNO_3$)	;	2-100
Tin($SnCl_2.2H_2O$)	;	2-50
Strontium($SrCl_2.6H_2O$)	;	2-50
Chromium($CrCl_3.6H_2O$)	;	0.1-50
Chromium($K_2Cr_2O_7$)	;	0.1-50
Cobalt($CoCl_2.6H_2O$)	;	5-50

Concentrations chosen are mainly in the range of the LC 50 values for wild type *C. elegans* over 1-4 days of exposure to these heavy metal ions (Williams and Dusenbury, 1990). All concentrations were calculated on the basis of mg/ml of the metallic ion.

For the organic toxicants, initial range finding experiments were performed using order-of-magnitude differences in concentrations. In cases where ethanol was used to solubilise the toxicant [lindane or tributyltin chloride (TBT)], the final solvent concentrations did not exceed 0.1% (v/v), and controls included ethanol alone at the same concentration.

The concentrations tested were 0.005-1.000 ppm for TBT and 0.005-2.000 ppm for lindane. The concentrations of TBT were again calculated as mg.L (ppm) of inorganic tin, whereas for lindane, ppm values were based on the total molecular weight.

The nematodes were exposed to certain heavy metal ions in the presence of Ca^{++} or Ca-related agents such as calcium ionophore (A23187), calcium uptake inhibitors (verapamil, nifidipine or Lanthanum). All these agents except Lanthanum were also dissolved in ethanol. The required concentrations of each agent were added to the final concentrations.

2.5 EXPOSURE OF *A. AQUATICUS* TO HEAT AND TO TOXICANTS

The asellids were heat shocked at 26°C for between 2-20 hrs, whereas controls were incubated at 14°C. Time course experiments were also carried out for the asellids exposed to each heavy metal concentration at 14°C for 2-20 hrs. The heavy metal concentrations used were as listed;

Metal ions	ppm
Cadmium	; 0.0018-2.000
Copper	; 0.0025-2.000
Mercury	; 0.2
Zinc	; 20
TBT	; 0.03
TPL	; 0.03

The asellids were placed in 1-50 ml volume of filtered water from their source site,

aerated well and the heavy metals were then added to the desired concentrations. Inorganic heavy metal concentrations used were close to 96 h LC50 values for *A. aquaticus* (Martin and Holdich,1986; Migliore and Giudici,1990). For the organometallic compounds, the concetrations chosen were in the range of the organic exposure for *C. elegans* (see section 2.4). All concentrations were calculated on the basis of mg/L of the metallic ion.

2.6 DETERMINATION OF METAL CONTENTS OF *C. ELEGANS* TISSUE SAMPLES AND SUPERNATANTS

After exposure, the worms were washed 4-5x with fresh K-Medium (appendix 4) and twice with ultrapure water. 20-100 mg (wet weight) of CB4027 worms were pelleted at 4,000 xg for 5 minutes in prewashed metal-free plastic tubes. The tubes were reweighed both before and after drying the pellet at 50°C for 48 hrs to determine wet and dry weights. On average, the latter represents 12% of the former. Dried pellets were then digested with 1 ml of concentrated Nitric acid (HNO_3, BDH Analar Grade) at 60 °C overnight or until completely dissolved. The digested tissue samples were diluted to 4 ml final volume with ultrapure water. These samples were analysed for Cd^{++} content by Atomic Absorption Spectrophotometry (AAS) using a Pye-unicam SP9 instrument. Sn^{++} determinations were carried out in the Department of Animal and Plant Sciences, Sheffield University, using AAS with a graphite furnace.

For supernatant metal determinations, the worms, bacteria and debris were removed by centrifugation after exposure and 10 ml supernatant was mixed with HNO_3 (25% (v/v)

final). Then, the heavy metal contents were determined by AAS, using standards of the same metallic salt dissolved in K-Medium.

2.7 HISTOCHEMICAL DETECTION OF β-GALACTOSIDASE IN *C. ELEGANS*

The histochemical detection of β-galactosidase was performed by using two different staining methods, one as described by Fire (1986), the other using X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Small aliquots of worms washed with M9 buffer (appendix 2) were transferred onto slides coated with 0.1% BSA (Sulston and Hodgkin, 1988), and diluted with 100 µl of dd water. They were then dehydrated under a laminar airflow hood for approximately 1 hr, fixed and finally stained using either method.

Following fixation with two drops of acetone, the staining with Fire's method was performed by rehydrating the worms with 100 µl of staining solution. The staining solution was prepared as follows: 0.2 M Sodium phosphate pH 7.5 1 mM Dithiothreitol (DTT) was saturated at 65°C with 6-bromo-2-naphthyl-D-galactopyranoside, and SDS was added to a final concentration of 0.004%. After 1 hr in the staining solution, 20-50 µl of a fresh 0.125% solution of Fast blue B diazonium salt was added. The substrate is cleaved by β-galactosidase to yield a local insoluble precipitate of 6-bromo-2-naphthol, which reacts with the diazonium salt to form a blue dye.

In the other staining technique performed using X-gal as substrate, the worms were fixed with 1% glutaraldehyde for 5-10 minutes at room temperature, washed 3x with M9 buffer (appendix 2) to remove the glutaraldehyde. Then, X-gal reaction mixture (appendix 5)

was added and incubated at 37°C for 1 hr to overnight. In this case, the hydrolysis of X-gal and subsequent dimerization of photochrome results in deposition of blue pigments.

The stained worms were mounted in 60% (v/v) glycerol, covered with cover slips and then sealed off with nail polish for permanent records. They were finally photographed under direct light using a Leitz inverted phase microscope. Film used was from Kodak Ltd.

2.8 ASSESSMENT PROTOCOL FOR SCORING THE STAINED WORMS

A simple point system has been adopted to compare the degree of staining between different runs as well as different conditions, as described in more detail in Chapter 2 Sections 2 (iv) and 3.3. This system gives a consistent gap between positive and negative controls (e.g. 16 ppm Cd^{++} + 32°C and 32°C alone, respectively).

2.9 EXTRACTION OF *C. ELEGANS* SOLUBLE PROTEINS AND β-GALACTOSIDASE ASSAYS

After exposure, worms were centrifuged at 4,000 xg for 2 minutes, washed with 30 vols M9 buffer (appendix 2) at least twice to remove excess bacteria and heavy metal ions. A small aliquot of worms was removed for staining as previously described in section 2.7, then 200 µl of M9 buffer (appendix 2) containing 1 mM DTT and 0.1% (w/v) SDS or (v/v) Triton X-100 was added to the remaining worms in each Eppendorff tube. The worms were homogenised thoroughly with a tight-fitting pestle, and centrifuged at 12,000

xg for 3 minutes to remove the debris.

40-60 µl of each sample was subsequently used for each enzyme determination. Duplicate β-galactosidase activities were determined spectrophotometrically at 410 nm (using a LKB UV spectrophotometer) after 15 hrs at 30 °C by measuring o-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolysis as described by Fire (1986). Duplicate 20 µl aliquots of each sample were also used for determining soluble protein concentrations using the method of Lowry *et al* (1951). A purified preparation of *E. coli* β-galactosidase (Sigma) was used to provide a standard curve for each experiment. Standard curves were produced for each set of assays using duplicate standards, containing 0, 0.0005, 0.001, 0.002, 0.004, 0.006, 0.008, 0.01, 0.03 and 0.05 units/ml of β-galactosidase. One unit is defined as the amount of enzyme required to hydrolyse 1 nmol of ONPG/min under the assay conditions used (Fire, 1986).

2.10 EXTRACTION OF *A. AQUATICUS* SOLUBLE PROTEINS

After test exposure, the asellids were removed from the culture, anesthetised with chloroform and washed with filtered water (from their habitat) or a large amount of extraction buffer (appendix 6). They were then either frozen at -70°C or transferred to a homogenizer. 150 µl of the extraction buffer was added to the homogenizer in which each animal was homogenized throughly, and the homogenate was transferred to a clean Eppendorff tube, centrifuged in a MSE microcentrifuge at 12,000 xg for 2-5 minutes. 20 µl aliquots of the supernatant were used for determination of total soluble protein concentration, by the method of Lowry *et al* (1951) and also 5 µl for determination of

radioactive incorporation by precipitation with trichloroacetic acid (TCA; see Section 2.13 below). Samples were stored at -20°C.

2.11 DETERMINATION OF PROTEIN CONCENTRATION

Soluble protein concentrations for both *C. elegans* and *A. aquaticus* were determined according to the method of Lowry *et al* (1951) with bovine serum albumin (BSA) as standard. 20 µl samples of supernatants were used for each determination. In each set of assays, duplicate standards containing 0, 10, 20, 30, 40, 50, 70, 100 and 200 µg of BSA were used to produce a standard curve.

1 ml of Lowry mixture (appendix 7) was added to each sample and left for 20 minutes at room temperature. 25 µl of Folin and Ciocalteau reagent diluted 1:1 with distilled water was added and held for a further 20 minutes. The O.D. of sample relative to the blank was then determined at 595 nm using a LKB UV spectrophotometer. The protein concentration of each sample was worked out on the standard curve.

2.12 ACETONE PRECIPITATION OF PROTEINS

Asellid proteins were precipitated by acetone as described by Dunn (1992).

1 volume of ice-cooled sample and 4 volumes acetone at -20°C were mixed well and transferred to -70°C overnight. After precipitation was complete, the mixture was centrifuged at 11,000 xg for 5 minutes at 4°C. Following this, the supernatant was

removed and the pellet was left to dry on ice for about 45 minutes, after which it was mixed well with the desired volume of IEF sample buffer (appendix 8). The precipitated sample was finally applied to isoelectric focusing gels (see Section 2.18.1).

2.13 RADIOACTIVE LABELLING OF THE ANIMALS AND DETERMINATION OF (^{35}S) METHIONINE INCORPORATION

Both *A. aquaticus* and *C. elegans* were labelled with 3.0 MBq/ml ^{35}S-methionine (Amersham). After incubation (control or toxicant treatments), the animals were removed, washed 3 times and homogenized throughly as in Sections 2.9 and 2.10.

Following centrifugation, 5 µl of each supernatant containing radioactively labelled proteins and 1 ml of 2% Casein hydrolysate / 10% TCA were mixed together in an Eppendorff tube, left on ice for 1 h and then filtered under suction onto glass fibre filters (Whatman GF/C). Precipitated proteins on the filter were washed with 2x20 ml of 6% TCA / 4% Sodium pyrophosphate, then briefly with distilled water and finally ethanol. The filters were pinned out on marked pyrofoam boxes to dry, then transferred to 5 ml of scintillation fluid (Emulsifier-Safe, Packard) and finally counted using a Packard Liquid Scintillation analyzer. These counts enabled similar amounts of radioactivity, varying from $2x10^4$ - $1x10^6$ cpm, to be loaded onto each lane for subsequent gel electrophoresis.

2.14 PROTEIN DOT BLOTS

Appropriate amounts of protein sample were spotted onto nitrocellulose membranes and allowed to dry in room temperature. They were then either blocked with a blocking solution for immunoreaction (see Section 2.17 or 2.19.2), or stained for enzyme activity (see section 2.7).

2.15 ANALYSIS OF PROTEINS BY ONE-DIMENSIONAL GEL ELECTROPHORESIS

2.15.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Laemmli (1970) using an LKB-Pharmacia mini gel apparatus, and a Biorad 200/2.0 power supply.

7-12.5% acrylamide gel solutions (appendix 9) were prepared for the resolving gels. Polymerization was catalysed by adding freshly prepared Ammonium persulphate solution (approx 0.1% w/v) and NNN'N'-tetramethyl ethylenediamide (TEMED). During the polymerization, the gels were overlaid with Propan-2-ol which excludes oxygen and produces a smooth interface. After polymerization, propan-2-ol was removed by aspiration, and the stacking gel solution (appendix 10) was poured over the top. A comb was inserted gently to form a number of wells and the stacking gel was allowed to polymerize for about 1 hr. After removing the comb, the wells were washed briefly with d.water and the gels were assembled onto the gel apparatus. Finally, the tanks were filled

with electrophoresis buffer (appendix 11).

Samples applied to each well contained between 100 and 200 µg of total protein for Coomassie blue staining, or $2x10^4$ - $1x10^6$ cpm of ^{35}S methionine-label for the autoradiography in a volume of 10-50 µl. Each sample was mixed with 10 or 20 µl of SDS-PAGE sample buffer (appendix 12) and placed in water at 100°C for 1-5 minutes to ensure complete dissociation of non covalently bound subunits. 5 µl of [^{35}S]-methionine labelled chick lens protein solution* was used to provide molecular weight markers for autoradiographs (principally, the major δ-crystallin band at 50 kD and α-crystallin band at 20 kD). Additionally, 7 µl of Sigma prestained protein markers were run in most cases; these markers had approximate molecular weights of 116, 84, 64, 48, 37 and 30 kD. The samples and the protein markers mixed with sample buffer were then loaded into the wells and electrophoresed at 120 V for 3 h. (prepared from day-old chick lenses incubated for 24 h in medium 199 containing 1 $mCi.ml^{-1}$ of [^{35}S]-methionine; sample prepared by D de Pomerai)

2.16 LOCALIZATION OF PROTEIN BANDS IN SDS-POLYACRYLAMIDE GELS

2.16.1 Detection of β-galactosidase activity in the gels

For staining enzyme activity *in situ*, polyacrylamide gels were prepared as described above except that some variations were introduced to the SDS-PAGE sample buffer (appendix 12); sample buffer contained only 0.1% SDS, and 1 mM DTT instead of 3%

β-mercaptoethanol. In addition to these changes, the samples were not boiled before loading. A detailed procedure for localization of enzyme activity in the gels are described in Dottin *et al* (1987).

After electrophoresis, the gels were removed from the electrophoresis tank and placed in 0.2 M Sodium phosphate buffer (pH 7.5) for 10 minutes. They were then transferred to 50 ml staining solution (Section 2.7) for 45 mins, before adding a 0.125 % solution (w/v) of Fast blue B diazonium salt to visualise the bands. The stained gels were photographed immediately as the proteins in gels diffuse with time. Permanent and better results were obtained following transfer onto a nitrocellulose support matrix by Western blotting ,and staining the blots with the same procedure as described above.

2.16.2 Coomassie staining

Gels containing unlabelled proteins were fixed and stained by soaking 3 hrs or overnight in 0.01% (w/v) Coomassie Blue R250 in 10% (v/v) acetic acid and 45% (v/v) ethanol. Gels were destained by diffusion in several changes of destain solvent (the same mixture without Coomassie Blue R250) for about 1 hr with constant agitation. The gels were then placed in gel wash solution (7% acetic acid) overnight. Finally, the destained gels with stained protein bands were photographed for permanent records.

2.16.3 Autoradiography

The gels were stained and destained as described above and then treated with Ampilfy

(Amersham) for 30 mins. They were dried down onto Whatman 1M paper at 80°C using a Biorad gel drier. The dried gels were subsequently exposed to X-ray films (Kodak-X-OMAT) and stored at -80°C for upto 8 weeks. The autoradiographs were processed using D92 Kodak developer for 2-3 mins and Ilford Photographic Fixative.

Additionally, two computerised image analysers (one from Oxford Positron Systems and the other a Biorad phosphor image analyser) were used to compare the results with conventional autoradiographs. Histograms of the radioactively labelled proteins on each individual lane were displayed, printed and analysed for comparison.

2.17 LOCALISATION OF PROTEIN BANDS ON TRANSFER MEMBRANES BY IMMUNOBLOTTING

After SDS-PAGE, the proteins in polyacrylamide gels were transferred to either nitrocellulose (Gelman Sciences-BioTrace) or PVDF (DuPont-PolyScreen) transfer membranes and probed immunologically according to the method of Towbin *et al* (1979).

Prior to the transfer, gels and membranes were assembled in electroblot pads as follows: Membranes were cut to fit the gels and Whatman 3MM filter papers were cut slightly larger than the size of the membranes. Both gels and membranes were soaked in electroblot buffer (appendix 13) for 10 mins, whereas pads and filter papers were wetted with the buffer just before the assembly. In case of PVDF membranes which are extremely hydrophobic, they were prewetted in 100% ethanol for at least 1 minute,

followed by one rinse in distilled water for 2-3 mins and finally equilibrated in the electroblot buffer for 10 mins. After incubation, the gels and membranes were sandwiched between two pieces of Whatman paper (the membrane was always placed onto the anode-facing side of the gel) and positioned vertically between the electrodes of the transfer unit (LKB 2051 mini gel multiblot), which was filled with the electroblot buffer (appendix 13).

Protein transfer was achieved using a current of 150 mA for 4 hrs, supplied by a Biorad 200/2.0 power supply. The blots were blocked overnight in 250 ml Tris Buffered Saline (TBS; see appendix 14) containing 0.05% (v/v) Tween-20 and 5% (w/v) dried milk powder (or casein) at 4 °C, to saturate all remaining protein-binding sites on the membranes. After blocking, the blots were washed 4 x with TBS/0.05% Tween-20 (TBS-T), and probed with a primary antibody, which was either a monoclonal rat anti-HSP70 or mouse anti-HSP70, or a polyclonal rabbit anti-β-galactosidase in TBS-T containing 5% blocking solution for 3 hrs at room temperature. After 4 washes with TBS-T, the blots were incubated with either peroxidase-linked antibodies or alkaline phosphatase-coupled antibody, as secondary antibodies. A simple diagram summarising the procedures for one dimensional immunoblotting has been drawn to explain variations that resulted from using different primary and secondary antibodies (see Fig. 2.1).

(i) Peroxidase-linked antibodies

The peroxidase-linked antibodies used in the study were an anti-rat IgG (1:500) against rat anti-HSP70 (1:1000) and anti-rabbit IgG (0.1%) to rabbit anti-β-galactosidase

(1:1000), diluted in TBS-T (or PBS-T used in variation 2 below) / 5% blocking solution. Following 2 hrs treatment with the secondary peroxidase-linked antibody at room temperature, the membranes were washed 3 x with TBS-T or PPS-T and finally treated with different detection systems as described below and illustrated in Figure 2.1.

Variation 1. The membranes were directly stained for peroxidase, using the following mixture : 10 ml of freshly prepared solution of 4-chloro-1-naphthol in methanol (3 mg/ml) and 50 ml TBS. The washed membranes (protein side up) were placed in this mix and then 0.1 ml of 6% Hydrogen peroxide (H_2O_2) was added to start the reaction. The blots were gently swirled until a brown staining became apparent, localising the target or related proteins clearly without too much background. The reaction was stopped by transferring the membranes to 1% acetic acid solution. This procedure was used to probe *C. elegans* endogenous HSP70s (with rat anti-HSP70) and transgene β-galactosidase (with rabbit anti-β-galactosidase).

Variation 2. The PVDF membranes (used in this system instead of nitrocellulose) were treated with BLAST amplification system (purchased from DuPont) before immunohistochemical staining for peroxidase as described above. The rat anti-HSP70 was used here to probe asellid HSP70s. Following incubation in the HRP-linked secondary (anti-rat), the BLAST system was applied to the membranes following the manufacturer's instructions. This system is designed to amplify the signal generated by horseradish peroxidase (HRP) on western blots. The blotting amplification system utilises both biotinyl-tyramide and Streptavidin-HRP enzyme. The former reacts with immobilized HRP antibody on the

Figure 2.1 The diagram showing one-dimensional immunobloting procedures that were carried out using different primary and secondary antibodies

Fig. 2.1

Electrophoresis

Blotting
(PVDF/nitrocellulose membs)

Blocking (5% sol.)

wash 4x (TBS-T/PBS-T)

Incubation in
primary antibody

monoclonal rat
anti-HSP70

wash 4x

peroxidase linked-
secondary antibody

wash 4x

(variat.1)
Direct staining
for peroxidase

(variat.2)
Blast amplification
system

wash 4x

Direct staining
for peroxidase

monoclonal mouse
anti-HSP70

wash 4x

alkaline phosphatase
secondary ant.

wash 4x

(variat.3)
Direct staining
for phosphatase

polyclonal rabbit
anti-β-galactosidase

wash 4x

peroxidase linked-
secondary antibody

wash 4x

(variat.1)
Direct staining
for peroxidase

membrane and the subsequent addition of the streptavidin-HRP causes the binding of an additional enzyme to the whole complex. This principally results in some signal amplification.

(ii) Alkaline phosphatase-coupled antibody

It can be seen from the diagram (see Figure 2.1) that in addition to rat anti-HSP70, a mouse monoclonal anti-HSP70 (1:600) was also used to probe *C. elegans* HSP70 proteins. In this case, an anti-mouse alkaline phosphatase-coupled antibody (1:250 diluted in PBS-T (appendix 15) containing 5% BSA) was employed against the mouse anti HSP70, after which the same extensive washing was repeated for the blots.

Variation 3. The following procedure was performed for histochemical staining of alkaline phosphatase-coupled antibody as described in (Sambrook *et al*, 1989): For the staining, following solutions were prepared first;

NBT (Nitroblue tetrazolium):

0.5 gr NBT dissolved in 10 ml 70% dimethylformamide.

BCIP (5-Bromo-4-chloro-3-indolyl phosphate):

0.5 gr of BCIP disodium salt was dissolved in 10 ml of 100% dimethylformamide.

Alkaline phosphatase buffer:

100 mM NaCl

5mM $MgCl_2$

100 mM Tris-Cl (pH 9.5)

Then, the blots were stained as follows;

(1) 66 µl of NBT stock was mixed well with 10 ml of alkaline phosphate buffer, and then 33 µl of BCIP stock was added to this solution.

(2) The washed nitrocellulose membrane was immediately transferred to the above mixture (0.5 ml of substrate mixture per square centimeter of the membrane), and incubated at room temperature with gentle agitation.

The substrates BCIP and NBT are converted *in situ* into a dense blue compound by immunolocalized alkaline phosphatase. When the immunolocalized bands develop to the desired intensity, which normally takes 20 mins, the blots were transferred into a plastic box containing 0.002% EDTA (pH 8.0) in PBS (appendix 15) + 0.1% Triton X-100. The stained membranes were finally photographed to provide a permanent record of the experiment.

2.18 ANALYSIS OF PROTEINS BY TWO-DIMENSIONAL GEL ELECTROPHORESIS

This technique was performed using the method devised by O'Farrell (1975). The technique utilises isoelectric focusing gels to separate proteins in the first dimension on the basis of their isoelecric points, and SDS-PAGE slab gels in the second dimension on the basis of molecular mass of the proteins. A practical guide for the methods was mainly followed as in Rickwood *et al* (1990).

2.18.1 First-dimensional separation by isoelectric focussing

The proteins were separated first on the basis of their isoelectric points, using a LKB 2050-600 tube gel kit on which up to six capillary tubes could be employed for each run. The tubes were cleaned by boiling in 0.1 M HCl for 30 mins, followed by two rinses in double distilled water and one rinse in ethanol and then left to air dry. Before inserting the tubes into tube gel adaptor, the bottom of each tube was wrapped tightly with two layers of parafilm.

(i) Preparation of samples

The homogenized samples were prepared for both animals as described in Section 2.9 and 2.10, except that non-ionic detergent Triton-X-100 was employed to solublise proteins instead of SDS which can lead to the formation of artefactual bands in the first dimension, or to the loss of proteins from the tube gels (Rickwood *et al*, 1990).

In the experiments with *A.aquaticus*, known protease inhibitors such as Phenylmethylsulfonyl fluoride (PMSF; 1 mM), Benzamidine (1 mM) and EDTA (0.2 mM) were added to the extraction buffer (appendix 6) before homogenization.

(ii) Preparation of the first dimensional gels

IEF gel solutions were prepared using the method of O'Farrell (1975) and the procedure followed was as described in Rickwood *et al* (1990).

The gel mixture solution (3-4% acrylamide-bisacrylamide rod gels containing 2% ampholines (Sigma) comprised of 1.6% pH range 5 to 7 and 0.4% pH range 3 to 10.5; appendix 16) was transferred to each tube using a 1 ml syringe with a plastic tubing attached to the needle. The tubes were filled with approximately 200 µl of the gel solution to within 10-15 mm of the top of the tube (which were 75 mm long and had inner diameter of 1.5 mm). The tubes that contained air bubbles were discarded. The remaining ones were overlaid with 10 µl of Ultrapure water and allowed to polymerize for 1 h, after which the water was removed by aspiration and overlaid with 15 µl of IEF sample buffer (appendix 8) for a further hour. While the gels were polymerizing, the following electrolyte solutions were prepared ;

Anolyte : 10 mM H_3PO_4

Catholyte: 20 mM NaOH

Lower reservoir (anode chamber) was filled with H_3PO_4 solution and the rod gels (without parafilm seals) were lowered into it, avoiding possible air bubble trapping at the bottom of the tubes.

(iii) The sample loading and electrofocusing of the gels

After removing the sample buffer by aspiration, the estimated volume of each sample (prepared as described above) was applied to each gel; usually 25-150 µg of proteins in 5-25 µl of samples depending on the detection method used. For example, 25 µg of *C. elegans* samples in 5 µl volume was sufficent for detection of the HSP70s which were

separated by two dimensional SDS-PAGE and immunolocalized using the ECL system. The samples were then overlaid with 10 µl of Overlayer buffer (appendix 17). Following this, the gels were covered carefully with fresh 20 mM NaOH to the top and then the upper reservoir was filled with the latter solution to cover the gel tubes.

The apparatus was finally connected to the power supply (Pharmacia 3000/150) and the proteins were electrophoresed at the run conditions listed below;

Voltage applied	Time (hr)
100	1
200	2
300	2
400	8
600	1.5 (this helps to sharpen the focused protein bands)

Total run time was 5000 (V.h) approx.

At the end of the run, the tube gels were removed from the adaptor and they were either placed on ice ready to run for the second dimension, or stored at -20°C.

(iv) Coomassie staining of IEF gels

Before proceeding to the second dimensional SDS-PAGE, one or two of the IEF gels were Coomassie-stained to localize the electrofocused bands. The IEF gels were removed from thier glass tubes by gentle water pressure, placed in a petri dish containing Ultrapure water

for 30 seconds and then transferred to 12.5% (w/v) TCA for 30 mins. Following this, they were placed in coomassie stain for 1/2 hr, destain for 1 hr and finally subjected to gel wash as described in Section 2.16.2.

2.18.2 Second-dimensional separation by SDS polyacrylamide gel electrophoresis

Gradient separating gels were employed for the second dimensional SDS-PAGE, because higher resolution is usually obtained with these gels.

(i) Preparation of the second dimensional gels

10-16% gradient separating gels were prepared using a simple gradient gel maker. All gels were in a multi-casting unit using the same solution to produce identical gels.

Light (appendix 18) and dense (appendix 19) solutions were poured into two different chambers (the dense solution to the reservoir and the light solution to the mixing chamber) and then mixed by adjusting the tap between two chambers so as to ensure production of linear gradient gels. The gels were overlaid with propan-2-ol. After polymerization, the propan-2-ol was removed and the surface of the gels were washed with Ultrapure water and finally drained well. 6-8 % stacking gel solution (appendix 10) was poured onto each slab upto within 5 mm of the top, overlaid with propan-2-ol again and left to polymerize. A comb was inserted into one of the slab gels in the the casting unit to perform one dimensional separation of proteins by SDS-PAGE for comparison. After polymerization, the gels were covered with 3 ml 0.1% SDS in Tris-HCl (pH 6.8),

wrapped in Saran wrap and stored at 4 °C to be used the next day.

(ii) Equilibration of IEF gels for the second dimension

Prior to the equilibration of the IEF gels, the sealing gel solution was prepared to attach the rod gels to the second dimensional gel. The sealing gel solution was prepared as follows; 0.15% agarose was melted in 0.125 M Tris-HCl (pH 6.8) and allowed to cool down to 45-55°C before adding a tenth volume of 20% SDS (appendix 20).

The IEF gels left on ice were removed from their glass tubes by pushing them out with gentle water pressure (using 50 ml syringe attached to the gel tubes with silicone rubber tubing). The rod gels were extruded into petri dishes containing dd water for 20 seconds to redissolve any precipitated urea. The distilled water was removed and each gel was covered with 15 ml of equilibration buffer as below.

Equilibration buffer:

2.5% SDS

2 mM DTT

0.125 mM Tris-HCl(pH 6.8)

10% Glycerol

0.04% Bromophenol blue

They were incubated in the buffer solution for 45 mins at room temperature with gentle

shaking. At the mean time, the estimated volume of samples and protein markers were mixed with 20 µl of the equilibration buffer and left for the same period of time to provide identical conditions for one- and two-dimensional SDS-PAGE slab gels.

(iii) Loading the equilibrated IEF gels onto the second dimensional gels

At the end of equilibration time, the buffer was removed and the rod gels were tipped onto a piece of parafilm (Nesco film) and then straightened out with a clean spatula. Through holding the parafilm the gels were rolled into the gap above the second dimensional gels. In case of difficulty, the rod gels were gently pushed into the gap by using a spatula, leaving approximately 1 mm space between two gels.

The gradient slab gels with IEF gels were mounted on the electrophoresis tank, and the molten agarose sealing solution, as previously described, was poured into the space between two gels using a 5 ml syringe with a wide-bore needle (19 gauge). Only enough of the agarose gel was injected to fill the space. When air bubbles were introduced, they were removed immediately by a syringe needle.

While the sealing gels were polymerizing, which normally takes about 5 minutes, the samples and protein markers left in 20 µl equilibration buffer were applied to gradient sample wells for one dimensional protein analysis as explained above.

The electrophoresis tanks were filled with electrophoresis buffer (appendix 11) and the gels were finally run at a constant current of 15 mA for each slab gel for 3 hrs. When

the running was complete, the gels were processed for analysis of proteins either by silver staining or by Enhanced Chemiluminescence (E.C.L.) system.

2.19 LOCALIZATION OF PROTEINS ON TWO-DIMENSIONAL GRADIENT GELS

The proteins separated by isoelectric focusing in the first dimension and by SDS-PAGE in the second dimension as previously described were localized using two detection systems, namely silver staining and enhanced chemiluminescence.

2.19.1 Silver staining

It is well known that the silver staining procedure is upto 100 times more sensitive than Coomassie blue R-250. This method was applied to the second dimensional gradient gels to determine whether the proteins were properly focused. For these purposes, two different silver stain kits were purchased ; one was from Sigma, and the other from Bio Rad. In both cases, the proteins were fixed in the gels before staining. They were then subjected to the staining procedures following the manifacturer's instructions for each kit.

2.19.2 Immunoblotting

Proteins were transferred from second dimensional polyacrylamide gels to nitrocellulose membranes and probed immunologically by using the method described by Towbin *et al* (1979). They were then subjected to (ECL) detection system.

The assembly of polyacrylamide gels and membranes were performed as previously described in Section 2.17. The electroblot buffer used contained the same components as in Appendix 13, but with half of the Glycine and Tris-Cl concentrations.

The transfer was achieved using a current of 250 mA at 150 V for 2-3 hrs supplied by a Bio-Rad 200/2.0 power supply. When transfer was complete, the blots were placed in a plastic box containing 7% BSA blocking solution in TBS (appendix 14) at 4°C overnight to saturate all remaining protein-binding sites on the nitrocellulose membranes. After blocking, the blots were washed at least four times for 5 minutes with TBS/0.05% Tween-20, and incubated in rat anti-HSP70 primary antibody (1:1000 diluted in TBS-T plus 5% BSA) for 5 hrs at 4 °C after which the blots were extensively washed with TBS-T 5x5' to remove excess and non-specifically-bound antibody. The membranes were then transferred to anti-rat IgG (peroxidase linked-secondary antibody diluted 1:500 in TBS-T/5% BSA). Then, the same extensive washing was repeated to prepare the blots for the final ECL detection system.

This detection method is a light emitting non-radioactive method used for the detection of immoblized specific antigens, conjugated directly or indirectly with horseradish peroxidase-linked antibodies. Chemiluminescence occurs when the energy from a chemical reaction is emitted in the form of light. Two ECL detection products (Du Pont or Amersham) were used for these purposes, and in both cases the same procedure was followed. All steps were carried out in a dark room as follows;

The ECL regent was prepared by mixing equal volumes of detection reagent 1 and

detection reagent 2 immidiately before use (at least 0.250 ml per square centimeter of the membrane). The TBS-T-washed blots were drained from the excess buffer by holding them vertically and touching them against tissue paper. The blots were then placed in the ECL mix and incubated with gentle shaking for 1 minute at room temperature. After incubation, the nitrocellulose membranes were drained off the excess mix solution and wrapped in Saran wrap. When air pockets were introduced, they were gently smoothed out with tissue paper. Following this, the blots with protein side up were immediately placed in the film cassettes. From here on, the lights were switched off, using only red safelights, and the films were carefully placed on top of the membranes. The films were exposed for 2 to 10 minutes and developed immediately. On the basis of their appearance, the other exposure were varied from 3-30 mins. The exposed films were developed in the developer solution for 2-3 mins till the bands develop to the desired intensity, placed in the fixer solution for 1-2 mins and finally rinsed in tap water for at least 2 mins.

2.20 APPENDIX

(1) NGM agar

NaCl	3 g
agar	17 g
peptone	2.5 g
choleserol (5 mg/ml in ethanol)	1 ml
MQ water	975 ml

Autoclaved and cooled down to 50-60°C, and then using sterile technique, the following was added by mixing well after each addition.

$CaCl_2$ 1M	1 ml
$MgSO_4$ 1M	1 ml
potassium phosphate 1 M pH 60	25 ml

(2) M9 Buffer

22 mM	KH_2PO_4
42 mM	Na_2HPO_4
85 mM	NaCl
1 mM	$MgSO_4$
MQ water	1 litre

(3) Freezing solution

NaCl	5.85 g
KH_2PO_4	6.8 g
glycerol	300 g
1 M NaOH	5.6 ml
MQ water	1 litre

Autoclaved, and then using sterile technique, the following was added

0.1 M $MgSO_4$	3 ml

(4) K-Medium

53 mM	NaCl
32 mM	KCl
1 litre	MQ water

(5) X-gal reaction mixture (60 ml)

10 mM KCl	
0.1 M sodium phosphate pH 7.5	50.04 ml
1 M $MgCl_2$	60 µl
50 mM $K_4[Fe(CN)_6]$	3,6 ml
50 mM $K_3[Fe(CN)_6]$	3,6 ml
10% (v/v) Triton X-100	600 µl
2% (w/v) x-gal in dimethylformamide	1.5 ml

(6) Extraction buffer (100 ml)

20x TBS [2.2 M NaCl, 200 mM Tris-HCl pH 7.5]	5 ml
10% (w/v) SDS	2 ml
100 mM DTT (or 0.5 ml 2-mercaptoethanol)	2 ml
Distilled water	91 ml

(7) Lowry mixture (100 ml)

Lowry solution [2% Na^2CO^3, 0.4% NaOH]	98 ml
2% potassium tartrate	1 ml
1% copper sulfate	1 ml

(8) IEF Sample buffer

9.5 M urea

2 mM DTT

2% Nonidet P-40 (NP40)

1.6% ampholines pH 5-7

0.4% ampholines pH 3.5-10

(9) SDS-Polyacrylamide resolving gel solution

0.375 M Tris-HCl pH 8.8

7-12.5% (w/v) acrylamide-methylene-bis acrylamide (30:0.8)

0.1% (w/v) SDS

0.03% (w/v) $(NH_4)_2\ S_2O_8$

0.1% (v/v) NNN'N'-Tetramethylethylene-diamide

(10) SDS-Polyacrylamide stacking gel solution

0.125 M Tris-HCl pH 6.8

6-8% (w/v) acrylamide-methylene-bis acrylamide (30:0.8)

0.1% (w/v) SDS

0.06% (w/v) $(NH_4)_2\ S_2O_8$

0.1% (w/v) NNN'N'- tetramethylethylenediamide

(11) SDS-PAGE Electrophoresis buffer

0.025 M Tris-HCl

0.2 M glycine

0.1% SDS

(12) SDS-PAGE Sample buffer

0.08 M Tris-Cl pH 6.8

1.66% (w/v) SDS

3% (v/v) 2-mercaptoethanol

0.3% (w/v) bromophenol blue

(13) Electroblot buffer

25 mM Tris base

185 mM glycine

20% (v/v) methanol

(14) Tris-buffered saline (TBS)

10 mM Tris-HCl pH 7.5

110 mM NaCl

(15) Phosphate-buffered saline, 10x (PBS 10x)

15 mM $NaH_2PO_4.2H_2O$

81 mM Na_2HPO_4

1.45 M NaCl

pH of 1xPBS:7.3-7.5

(16) IEF Gel mixture solution

9 M urea

3-4% (w/v) acrylamide-methylene-bis acrylamide (30:0.8)

2% (v/v) Nonidet P-40

1.6% ampholines pH 5-7

0.4% ampholines pH 3.5-10

0.05% (v/v) NNN'N'-tetramethylethylenediamide

0.01% (w/v) $(NH_4)_2S_2O_8$

(17) IEF Overlayer buffer

9 M urea

0.8% ampholines pH 5-7

0.2% ampholines pH 3.5-10

1.5 mM DTT

(18) Gradient SDS-PAGE light solution

0.375 M Tris-HCl pH 8.8

10% (w/v) acrylamide-methylene-bis acrylamide (30:0.8)

0.1% (w/v) SDS

0.035% (w/v) $(NH_4)_2S_2O_8$

0.03% (v/v) NNN'N'-Tetramethylethylenediamide

(19) Gradient SDS-PAGE dense solution

0.375 M Tris-HCl pH 8.8

16% (w/v) acrylamide-methylene-bis acrylamide (30:0.8)

0.1% (w/v) SDS

15% glycerol

0.035% (w/v) $(NH_4)_2S_2O_8$

0.03% (w/v) NNN'N'-Tetramethylethylenediamide

(20) Sealing gel solution

0.125 M Tris-HCl pH 6.8

0.15% (w/v) agarose

2% (w/v) SDS

CHAPTER 3. STRESS RESPONSES OF THE FRESHWATER CRUSTACEAN *ASELLUS AQUATICUS* TO HEAT SHOCK AND TO SUBLETHAL CONCENTRATIONS OF HEAVY METALS

3.1 INTRODUCTION

3.1.1 THE BIOLOGY OF *ASELLUS AQUATICUS* AND ITS USE IN BIOMONITORING

Asellus aquaticus is an aquatic isopod crustacean; it is found in large numbers and widely dispersed throughout Europe, because this species is relatively tolerant to many kinds of pollution, especially thermal and organic pollution. For this reason, *A.aquaticus* is a useful indicator organism that can be used effectively to measure water quality. Males and females of this organism have a life cycle of 18 months; there are five marsupial stages (denoted as A-E), free-living juveniles and adults, but no larval stages in this species. Upon egg fertilisation, the eggs are deposited into the marsupium beneath a female. Each egg is enclosed by an outer membrane until the embryo becomes a free-living juvenile which is released from the marsupium, mostly in July (Holdich and Tolba, 1981).

Temperature has been found to affect the developmental rate and survival of fertilised eggs outside the marsupium of ovigerous females (Holdich and Tolba, 1981). *A.aquaticus* is exposed not only to thermal pollution, but also to environmental

contamination of various types in fresh water. Although there are numerous accounts of increases in the population densities of this animal with increases in organic pollution (Aston and Milner, 1980; Mason, 1991), *A.aquaticus* is extremely sensitive to heavy metals such as Cd^{++}, Cu^{++} and Zn^{++} (Martin and Holdich, 1986; Migliore and Giudici, 1990). It has also been established that *A.aquaticus* has a high potency to accumulate Cd, especially from dietary sources (van Hattum *et al*, 1989). More interestingly, Green *et al* (1988) have found that increased temperatures decrease the toxicity of phenol (measured by three response criteria; immobilisation, paralysis and death) at relatively high concentrations in asellids. Since *A.aquaticus* is (i) subjected to both thermal and heavy-metal stresses in fresh water, (ii) is widely distributed and (iii) plays a key role in food webs, it should be an ideal organism for use in biomonitoring.

3.1.2 THE USE OF AQUATIC ORGANISMS IN TOXICITY TESTING

Aquatic organisims have been widely used to assess environmental pollution because of their ecological and economic importance and their morphological, physiological and ecological diversity in aquatic habitats. The use of these organisms in toxicity tests is also essential in the protection of aquatic ecosystems. Many studies have been carried out on the effects of environmental pollutants on various aquatic organisms, and on biological assessment of water quality using certain indicator organisms (see Sheedy *et al*,1991 for extensive review). Most toxicity tests have been concerned with measures of acute lethality; the results are expressed as a concentration or dose of toxicant at which a specified percentage (e.g. LC50, LD70) of the test organisms are killed over a standard period of time (e.g. 24,48,72 or 96 hr) (Mason,1991; Martin and Holdich,1986; Williams

and Dusenbery,1988,1990; Green *et al.*,1988; Migliore and Giudici,1990; Handy,1994).

Others have been concerned with ecological, morphological or physiological processes, e.g. the effects of temperature and water quality on the development and survival of tested organisms (Holdich and Tolba,1981), scope-for-growth measurements to assess the whole organism's physiological condition (Sanders *et al*,1991), or toxicant accumulation assays. Thus, the uptake and accumulation of known pollutants by internal organs such as vertebrate kidneys, or by the whole organism in the case of mussels, can provide useful data on the type and nature of toxicants (Bryan,1976; van Hattum *et al*, 1989; Glaven *et al.*,1991; Hogstrand and Haux,1991; Veldhuizen-Tsoerkan *et al.*,1991; Gill *et al.*,1992; Fent and Hunn,1993; Handy,1994).

The accumulation and detoxification of several metal toxicants (such as cadmium) involves binding to and induction of metalothionein proteins. Therefore, these proteins and their levels in aquatic organisms have attracted the attention of many ecotoxicologists for use as biomarkers of environmental contamination (Sanders *et al*,1983; Glaven *et al.*,1991; Hogstrand and Haux,1991; Viarengo and Nott,1993; Handy,1994; and also see section 1.2.2). Recently, attention has turned to biochemical and molecular analysis of this and other responses in order to investigate the effects of sublethal concentrations of known pollutants on aquatic organisms. Because enzymes are key catalysts of metabolic reactions in cells, their use has been explored as a basis for toxicity testing. These assays involve monitoring changes in enzyme activity following exposure to environmental toxicants (Gill *et al.*,1990; Bitton and Koopman,1992; Mazidji *et al.*,1992; Viarengo *et al*,1993). Futhermore, the effects of chemical contaminants on

the biosynthesis of enzymes have been of particular interest (Hirayama,1986; Dutton *et al.*,1988,1990; Gill *et al*,1990; Bitton and Koopman, 1992).

More recently, molecular analysis of cellular stress responses has attracted a number of toxicologists to evaluate the use of this system as an indicator of environmental pollution. Since this system has been the focus of the work described here, it will be discussed in detail in the following sections.

3.1.3 STRESS RESPONSE INDUCTION IN AQUATIC ORGANISMS

The cellular stress response protects organisms from damage caused by a wide variety of environmental stressors, including heat shock, heavy metal ions, oxidative stress, ethanol or aminoacid analogs (for review see Nover,1991a; Sanders,1993; and also see section 1.4). Organisms can respond to environmental insults by synthesising different groups of proteins. One such group are the metal-induced proteins known as metalothioneins. The metalothioneins are cysteine-rich proteins of low molecular weight, which are involved in the binding and detoxification of metal ions (see section 1.2.2). The other major group of induced proteins are the stress proteins (or heat shock proteins). Only this latter group will be the subject of this section. A related response involves the induction of glucose-regulated proteins (grp's) in response to a partially overlapping range of stress conditions (Nover, 1991b; see also section 1.3.2).

Stress proteins are known to be highly conserved and have been found in all organisms studied to date, ranging from bacteria to man. These proteins and their role in repair of

and protection from environmentally induced damage, have been discussed in chapter 1. Using the stress response as an environmental monitoring tool has provided an important focus in recent years, particularly in aquatic organisms (McLennan and Miller,1990; Hightower,1993; Sanders,1993).

The stress response has been examined in aquatic protozoa, echinoderms, crustaceans, molluscs and fish (see Sanders,1993 for recent review). Studies of the stress response in aquatic organisms have also shown a high degree of conservation among the stress proteins induced. Moreover, all major classes of stress proteins are stress-inducible in most aquatic species. SP70 proteins of protozoa, crustaceans, molluscs, echinoderms and fish crossreact with antibodies raised against SP70s from *Drosophila* and humans (Sanders,1993). Fish have been extensively studied using both whole animals and cell cultures (Nover and Scharf,1991; Dyer *et al.*,1993; Sanders,1993). Exposure of fish to elevated temperatures or to other stressful conditions results in expression of the major stress protein families; SP90, SP70, chaperonin-60 and low molecular weight proteins. Especially, first two have been found to be highly conserved, and crossreact with antibodies against SP90 and SP70 from other species.

A few studies on molluscan stress responses have been also reported (Veldhuizen-Tsoerkan *et al.*,1990, 1991; Nover and Scharf,1991; Sanders, 1988, 1993; Sanders *et al*, 1994). The induction of stress proteins by several stressors with particular environmental relevance has been reported in the sea mussel *Mytilus edulis*. Veldhuizen-Tsoerkan *et al.*(1990), using metabolic labelling techniques, have demonstrated that exposure of sea mussels to cadmium followed by heat shock treatment resulted in a strong inhibition of

the radioactive ^{35}S methionine incorporation, but differentially enhanced the expression of the stress proteins. They have further shown that the exposure to cadmium resulted in a dose-dependent inhibition of radioactive aminoacid incorporation (both ^{35}S methionine and ^{35}S cysteine) into gill soluble proteins. Veldhuizen-Tsoerkan *et al.* (1991) have also examined the effect of heat shock on stress protein synthesis in unpolluted sea mussels following transplantation to contaminated sites for a long period of time, demonstrating that field exposure to contaminated sites alters the response of mussels to other kinds of stress such as elevated temperature and anoxia. Moreover, exposing sea mussels to a range of copper concentrations results in accumulation of stress protein-60. The quantification of this protein showed a significant correlation between SP60 accumulation and Cu concentrations in the sea water (Sanders *et al*,1991). Sanders *et al* (1994) have also examined the expression and accumulation of two major stress proteins of 70 and 60 kD in the gill and mantle of blue mussels, *Mytilus edulis*, exposed to a range of Cu concentrations for 7 days, and have shown that tissue-specific differences in accumulation of stress proteins occur in this organism. Steinert and Pickwell (1993) have recently investigated the stress protein induction by TBT ingested by mussels using both metabolic labelling and Western blotting methods. They have found a linear increase in 70kD protein concentrations following exposure to a range of TBT concentrations.

Exposure of the rotifer *Brachionus plicatilis* to sublethal doses of $CuSO_4$ resulted in maximum SP58 induction (4-5 fold increase in SP58 levels over controls) at a dose that is approximately 5 % of the LC50 for that compound. A similar response was seen with TBT at 20-30 µg/L. However, rotifers' response was not observed following exposure to

$HgCl_2$, $AlCl_3$, $ZnCl_2$ or SDS (Cochrane *et al.*,1991).

There is even less published work available on the stress response in crustaceans. One of the first such studies (Miller and McLennan, 1988) involved a comparison of thermotolerance and heat shock responses in the stress-resistant embryonic cysts and nauplius larvae of the brine shrimp *Artemia*, indicating that this crustacean at least exhibits a classical stress response and possesses some major classes of stress proteins, such as SP90 and SP70. Hakimzadeh and Bradley (1990) examined variation in the heat shock response among the copepod *Eurytemora affinis* (Poppe) individuals raised at different temperatures. They also investigated differences in stress protein induction in males and females of this species. The pattern of stress protein induction in *Erytemora affinis* was found to be extremely variable between copepods raised at different temperatures and then exposed to heat shock. This observation was in agreement with their early findings demonstrating significant differences in tolerance to high temperatures in copepods raised at different temperatures. In the same study, they have also looked at the effects of TBT on the stress response induction in the copepod *Eurytemora affinis* (Poppe) and found that the pattern of protein induction by TBT was found to be different from that following heat shock, but was similar to the pattern following parasite infection (Hakimzadeh and Bradley,1990). More recently, the stress response induction in the planktonic crustacean *Daphnia magna* has been evaluated for use as a biomarker of environmentally induced stress (Bradley,1993). Bradley (1993) has also presented evidence that stress proteins of 90kD and 70kD are synthesised in greater quantities in response to increasing concentrations of both AgCl and SDS. The effects of sublethal concentrations of two pesticides (lindane and malathion) on stress protein induction in

D.magna have been also examined using the methods of metabolic labelling, immunoblotting and both one and two dimensional gel electrophoresis (Bond and Bradley, 1993).

The techniques most frequently used for examining the stress responses are either metabolic labelling (using radioisotopes, ^{35}S, ^{14}C or ^{3}H) with subsequent electrophoresis and autoradiography or immunoblotting using stress protein-specific antibodies. Quantification of stress proteins using the latter technique seems to be the more reliable indicator of environmental contamination. Because of the highly conserved nature of SP70s, antibodies against these proteins often cross-react with the corresponding proteins from very distantly related organisms (Kurtz *et al*, 1986). A number of anti-SP70 antibodies have been developed and some are commercially available (e.g. rat monoclonal anti-SP70 from Affinity BioReagents). In the present study, we have investigated the heat shock responses in some of freshwater crustaceans such as *Asellus aquaticus, Crangonyx* sp., and *Gammarus* sp., exposed to heat and sublethal concentrations of heavy metals. Only one of them, namely *A.aquaticus* showed a clear cut stress response during heat or heavy metal stress. Our study clearly indicates that this organism also exhibits a classical stress protein induction. Exposure of asellids to heat shock and heavy metals (cadmium and copper) stress induces major stress protein families of SP100, SP90 and possibly SP60. However, SP70 is not the major stress protein induced in this organism. Moreover, the time course of heat/heavy metal stress response reveals that the pattern of stress protein synthesis changes considerably with increasing time of incubation (see section 3.1 and 3.2).

3.2 MATERIALS AND METHODS

(i) The animals used in the radioactive labelling experiments described in section 1 and 2 were from a clean site (Nottingham Canal), whereas in the rest of experiments, asellids from both polluted and unpolluted sites were used to compare their stress responses. The collection and maintanence of *A.aquaticus* and other crustacean species were as described in Chapter 2, Section 3.

(ii) The asellids were labeled with 3.0 MBq/ml ^{35}S-methionine (Amersham). The experimental design and conditions were as detailed in Chapter 2, Section 5.

(iii) The asellids were homogenized in a homogenizer of 1 ml volume using the extraction buffer described in Appendix 6 (see Chapter 2, Section 10). However, for two dimensional sample preparation SDS was replaced with non-ionic detergent Triton X-100, and β-mercaptoethanol with DTT before homogenization. Known protease inhibitors such as PMSF, Benzamide and EDTA were also added to the extraction buffer in some experiments (see also Chapter 2, Section 18).

(iv) Protein separation by SDS-PAGE; asellid proteins were separated by one-and two-dimensional SDS-PAGE, using the techniques developed by Laemmli (1970) and O'Farrell(1975), respectively. One dimensional separation of proteins were performed on 12% acrylamide gels, whereas 10-16% gradient separating gels were employed to separate proteins in the second dimension of two-dimensional SDS-PAGE (isoelectric focusing was used to separate proteins in the first dimension). Both techniques are

performed as described in Chapter 2, Sections 15 and 18.

A computerised image analyser from Oxford Positron Systems was used to compare radioactive band patterns with conventional autoradiographs. The histograms of radioactively labelled proteins on each individual lane are displayed for comparison.

(v) The proteins were transferred onto membranes using the method devised by Towbin et al. (1979) (See Chapter 2, Section 17 for a brief description of the technique). The Blast Amplification System (purchased from DuPont) was applied to one-dimensional Western blots, whereas the ECL detection system was used on two-dimensional Western blots (as described in Sections 2:17 and 2:19, respectively).

3.3 RESULTS

3.3.1 INDUCTION OF *A.AQUATICUS* HEAT SHOCK PROTEIN SYNTHESIS BY THERMAL STRESS

Figure 3.1 shows the autoradiogram and Autograph histograms of newly synthesised proteins in control (14°C) and heat-shocked (26°C) asellids. Exposure of the asellids to heat shock induces the synthesis of at least five putative (H)SPs of approximate molecular weights, 110, 96, 89, 60 and 52 kD respectively (determined by reference to radioactively labelled chick lens standards on the same gel).

Figure 3.1 also shows time course comparisons of (H)SP synthesis with increasing time of incubation at 26°C alongside controls at 14°C. *Asellus* responded to heat shock even after a 2 hr incubation (lanes 2 and 3); however, a clear heat-shock response was seen at 4 hr (lanes 4 and 5). Some HS proteins were still being synthesised after 20 hr incubation at that temperature (lanes 7-12). It is interesting to note that a protein band of apparent molecular weight 110 kD is induced at 2 hr, but its synthesis declines to basal levels from 4 hr up to 20 hr incubation. On the other hand, a band corresponding to the 60 kD stress protein family is newly induced after 4 hr incubation, and is present up to 20 hr of incubation. A weak stress response is also detectable in 20 hr controls [lane 13, see also lane 6 (4 hr control) for comparison]. Moreover, it can clearly be seen that there are individual variations in the pattern of protein synthesis in the asellids exposed to the same conditions. The histograms of radioactively labelled proteins on each individual lane are also displayed in the figure for comparison with the autoradiograms.

Figure 3.1 Time-course comparison of HSP synthesis with increasing time of incubation at 26°C.

Figure 3.1 (A)

Autoradiography of HSP synthesis ;

Lane 1 - Standard chick lens proteins (SD).

Lanes 2 and 3 - 2 hrs heat shock at 26°C.

Lanes 4 and 5 - 4 hrs heat shock at 26°C.

Lane 6 - 4 hrs control at 14°C (C-4h).

Lanes 7 and 8 - 8 hrs heat shock at 26°C.

Lanes 9 and 10 - 12 hrs heat shock at 26°C.

Lanes 11 and 12 - 20 hrs heat shock at 26°C.

Lane 13 - 20 hrs control at 14°C (C-20h).

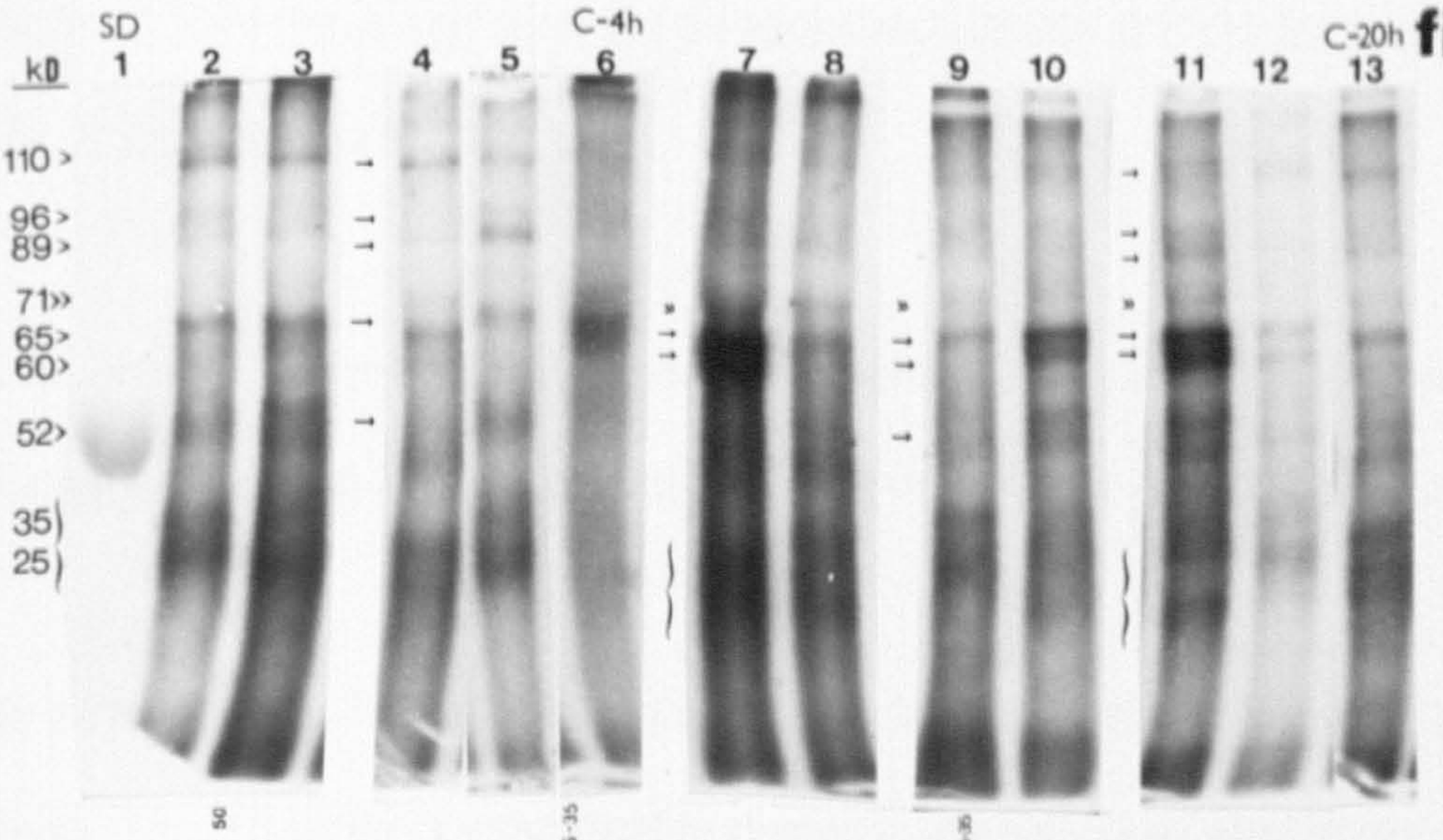
All samples were run in duplicate on identical 12% acrylamide gels.

Figure 3.1 (B)

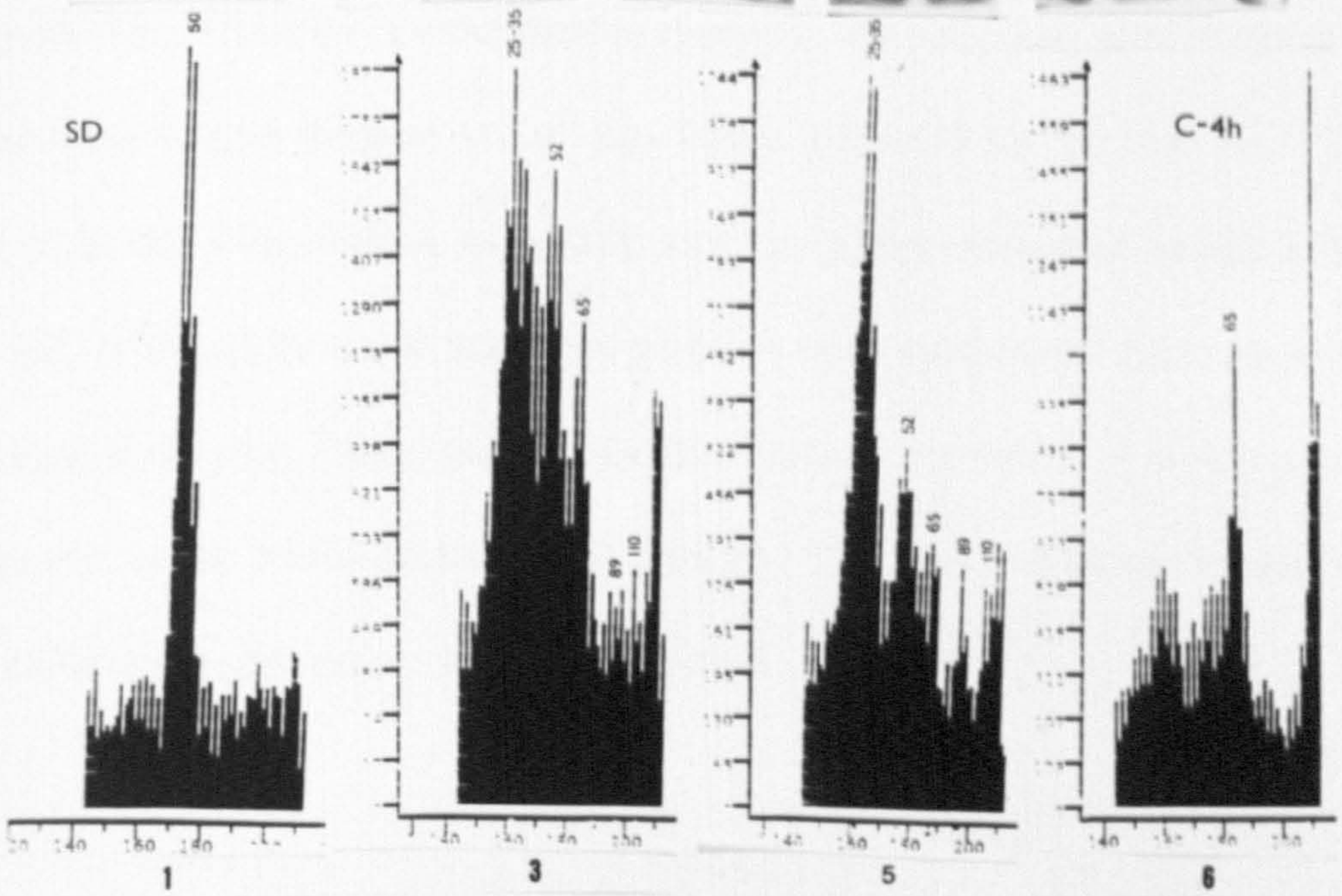
Histograms of radioactively labelled proteins on each individual lane shown in the autoradiograms. Only one lane is displayed for each sample.

fig.3.1

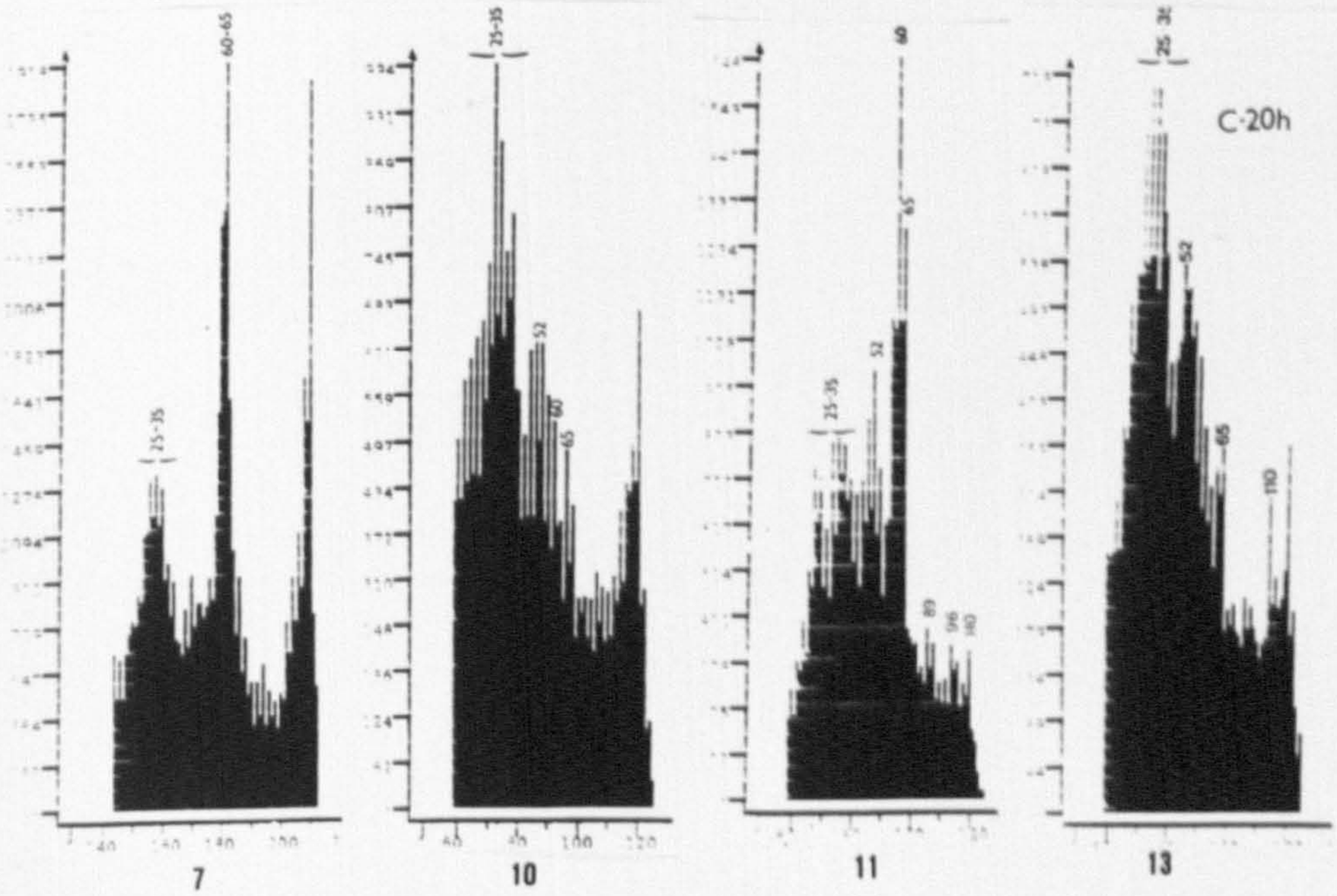
A



B



LANES:



LANES:

3.3.2 INDUCTION OF *A.AQUATICUS* STRESS PROTEINS BY HEAVY METAL IONS, CADMIUM AND COOPER

As can be seen in figures 3.2 and 3.3, *Asellus aquaticus* responds to heavy metal stress by inducing a similar set of stress proteins of 110, 96, 89, and probably 60 and 52 kD.

As with thermal stress, the asellids begin to respond to cadmium and copper after 2 hr of exposure time. However, a considerable change in the pattern of protein synthesis was observed from 8 hrs in the case of Cu^{++} (see figure 3.2) or 12 hrs the case of Cd^{++} (figure 3.3) up to 20 hrs of incubation, especially with the higher molecular weight SPs of 110 and 96 kD. It should be noted that these proteins were synthesised more abundantly in the Cu-treated samples. Unlike the heat shocked animals, induction of 60 kD protein was not clear-cut in the heavy-metal treated animals. The stress response is also variable among individuals exposed to heavy metal stress.

Figure 3.2 Time course study of SP synthesis in *A.aquaticus* exposed to the heavy metal ion Cu^{++} (0.5 ppm) at 14°C.

Figure 3.2 (A)

Autoradiograms of SP synthesis induced by copper stress;

Lanes 1 and 2 - 2 hrs incubation.

Lanes 3 and 4 - 4 hrs incubation.

Lane 5 - 4 hrs control at 14°C (C-4h).

Lanes 6 and 7 - 8 hrs incubation.

Lanes 8 and 9 - 12 hrs incubation.

Lanes 10 and 11 - 20 hrs incubation.

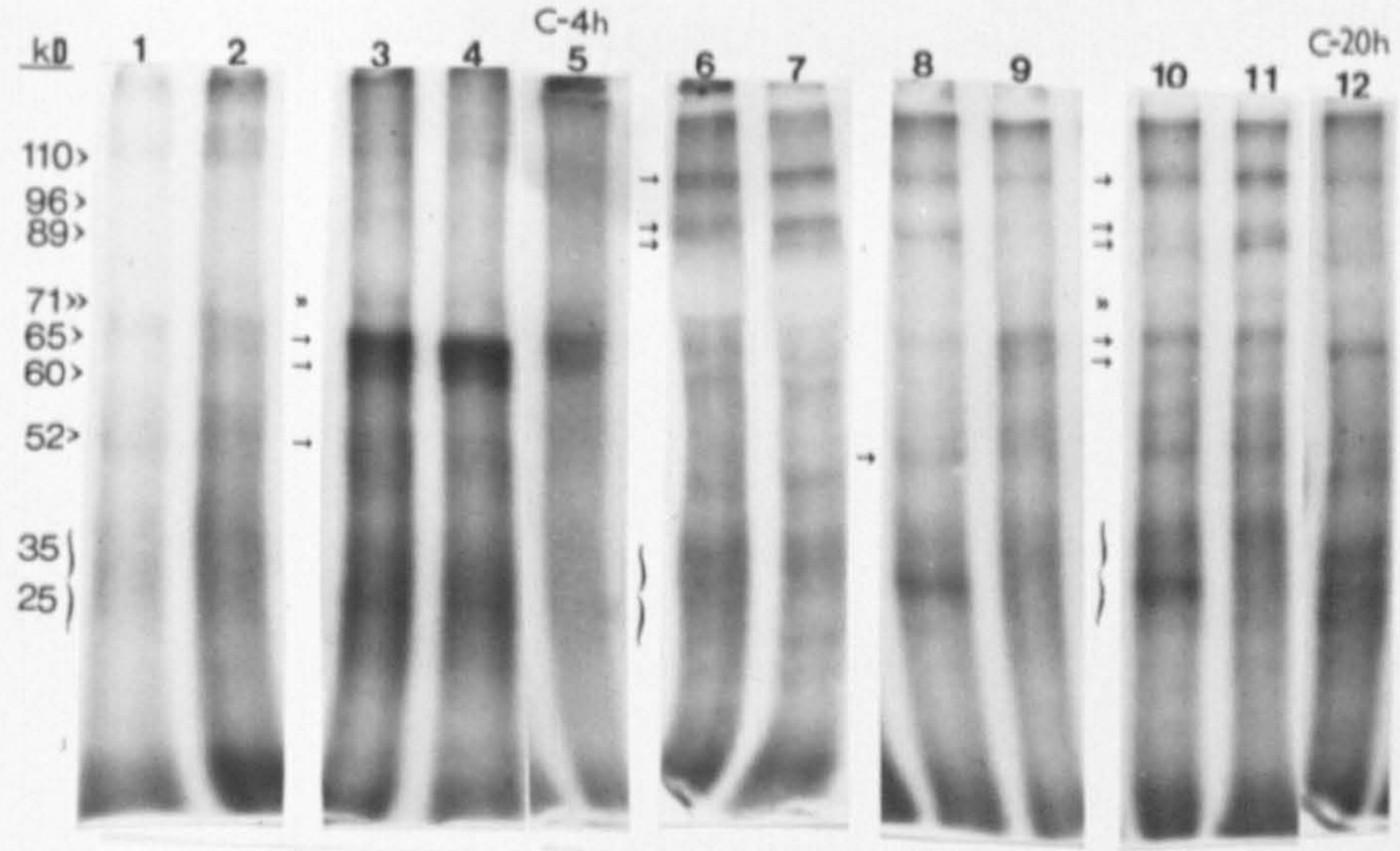
Lane 12 - 20 hrs control (C-20h).

Figure 3.2 (B)

Histograms of the labelled proteins on each individual lane are also displayed for comparison.

fig.3.2

A


kD
1
2
C-4h
3
4
5
6
7
8
9
C-20h
10
11
12
110
96
89
71
65
60
52
35
25

B

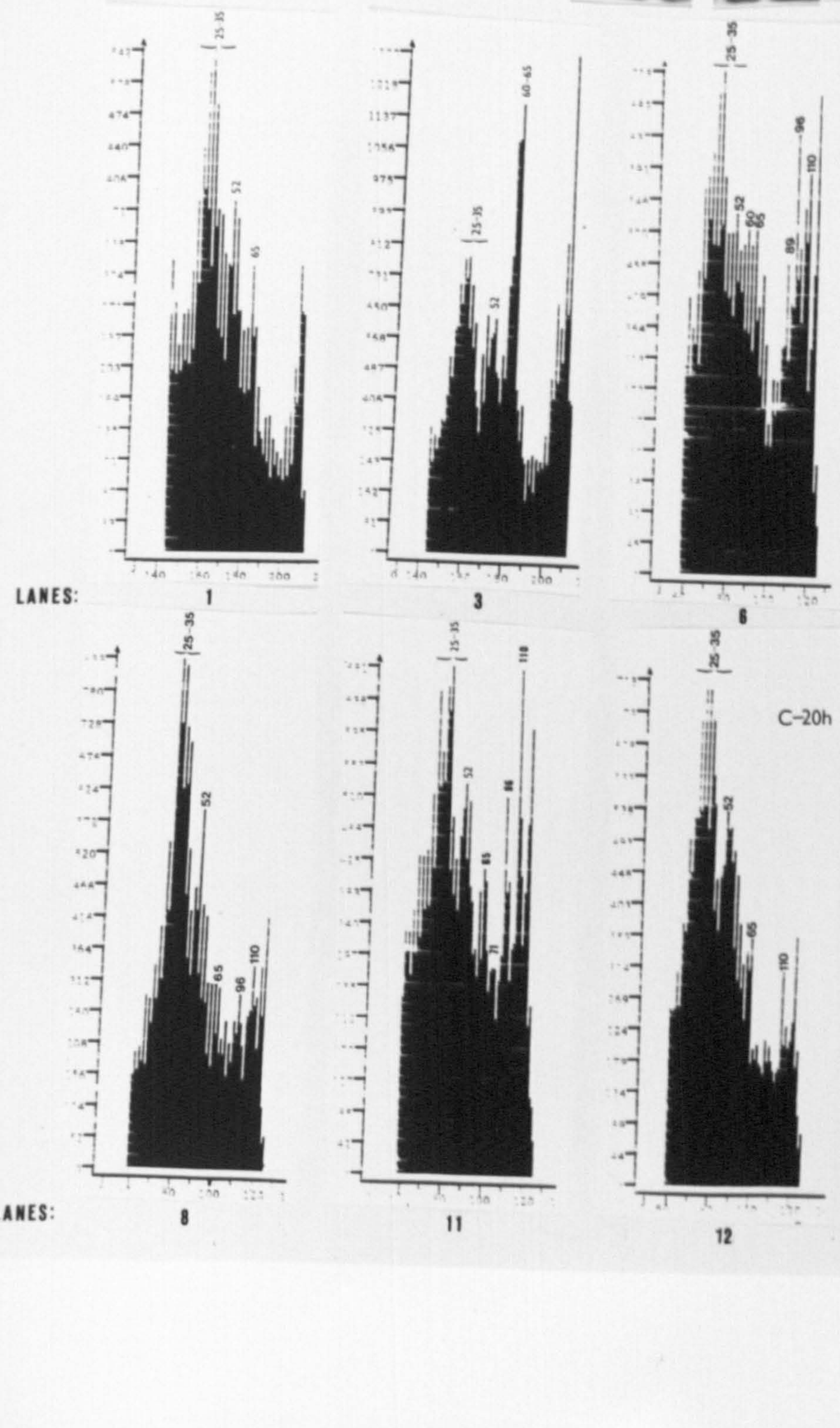

LANES:
1
3
6
8
11
12
C–20h
25-35
52
65
60-65
96
110
89
71
80

Figure 3.3 Time-course comparison of SP synthesis in *A.aquaticus* in response to the heavy metal ion Cd^{++} (0.35 ppm) at 14°C.

Figure 3.3 (A) Autoradiography of cadmium stress protein induction;

Lanes 1 and 2 - 2 hrs incubation.

Lanes 3 and 4 - 4 hrs incubation.

Lane 5 - 4 hrs control at 14°C (C-4h).

Lanes 6 and 7 - 8 hrs incubation.

Lanes 8 and 9 - 12 hrs incubation.

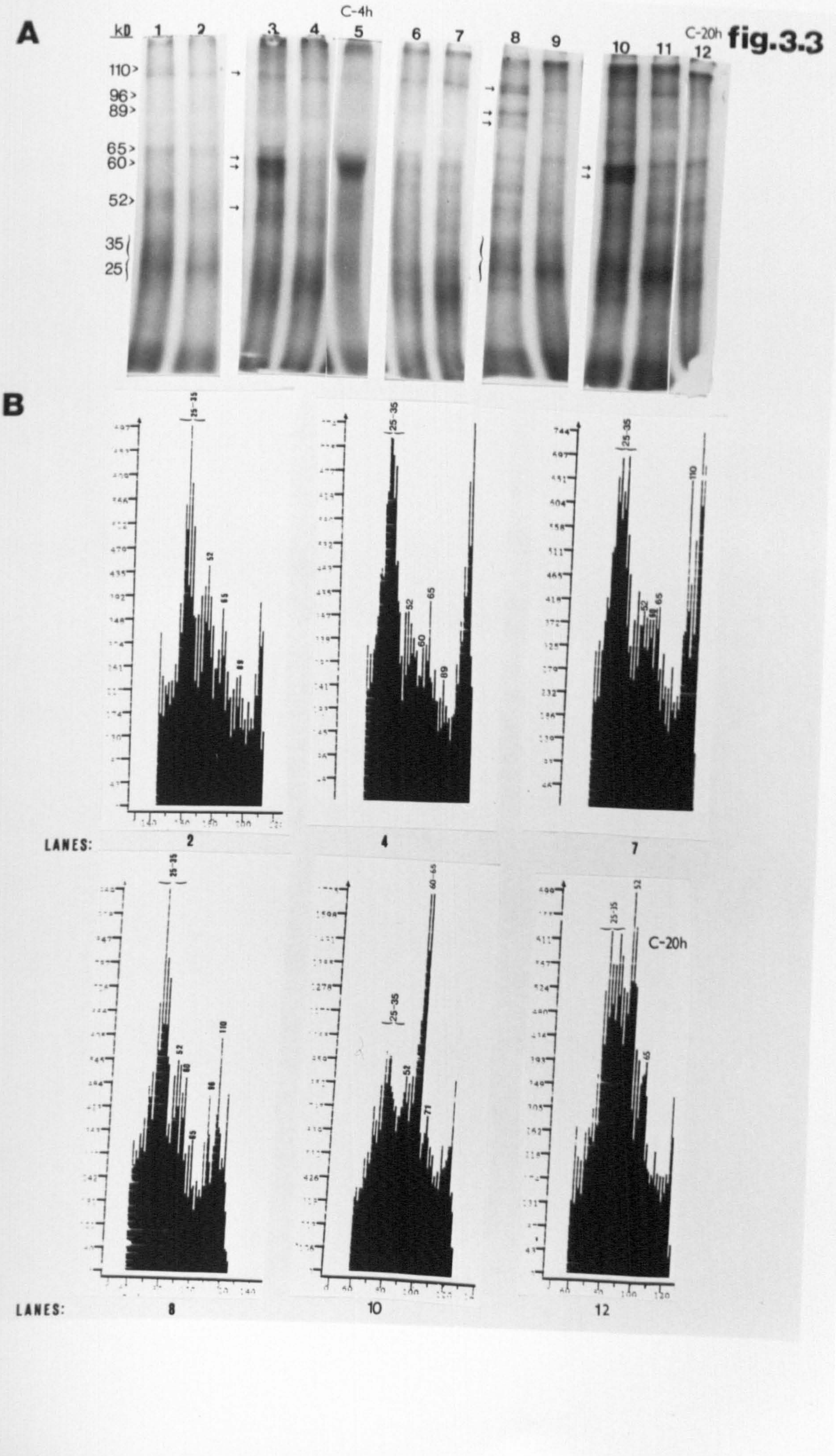
Lanes 10 and 11 - 20 hrs incubation.

Lanes 12 - 20 hrs control (C-20h).

The samples are run as duplicates on identical 12% gels.

Figure 3.3 (B) Histograms of radioactively labelled proteins on individual lanes are displayed. Each histogram displayed represents the same lane number shown on the autoradiograms.

fig.3.3


A
kD
1
2
3
4
C-4h
5
6
7
8
9
10
11
12
C-20h
110
96
89
65
60
52
35
25
B
25-35
52
65
60
89
110
71
C-20h
LANES:
2
4
7
8
10
12

3.3.3 DIFFERENTIAL RADIOACTIVE ^{35}S-METHIONINE INCORPORATION INTO *A.AQUATICUS* PROTEINS AS INDICATORS OF ENVIRONMENTAL STRESS

Initial experiments with *in vivo* labelling of asellids have shown that there were considerable differences in the radioactive incorporation into soluble proteins of animals exposed to various stressful conditions. From this point of view, we have used asellids to investigate differential radioactive methionine incorporation into *Asellus aquaticus* proteins as a possible indicator of environmental contamination. Both polluted and unpolluted site asellids were exposed to heat shock and to heavy metal ions such as Cd, Cu, Zn, Hg, as well as the organometallic agents tributyltin (TBT) and tetraphenyl lead (TPL) in the presence of radioactive ^{35}S-methionine.

Figure 3.4 shows that heat shock exposure resulted in a significant increase in radioactive incorporation into the soluble proteins of animals from both sites (more significant in clean site asellids), whereas most of the heavy metals tested clearly inhibited the radioactive incorporation compared to controls and heat shock treated animals. Organometallic compounds had much less effect, but were used at very much lower concentrations.

Furthermore, the radioactive incorporation in polluted site animals was relatively higher than in unpolluted animals, especially for control and organometal treatments. However, inorganic metal ions inhibited radioactive incorporation into soluble proteins to approximately the same extent in animals from both sites. Overall, methionine incorporation into soluble proteins is significantly reduced by all heavy metal ions tested

(Cd, Cu, Zn, Hg), but not by organometallic compounds at relatively toxic concentrations.

It should be noted that in the present experiments the asellids were heat-shocked at 26 °C for 5 hr alongside 5 hr-controls, whereas 10 hr incubation was chosen for the metal exposures as our previous *in vivo* labelling experiments have shown that more clear-cut responses are obtained over the longer period of time.

Figure 3.4 Differential ^{35}S-methionine incorporation into *A.aquaticus* soluble proteins.

1 - 5 hrs control at 14°C (C-5h).

2 - 5 hrs heat shock at 26°C.

3 - 10 hrs control (C-10h).

4 - 0.1 ppm Hg^{++}.

5 - 1 ppm Cd^{++}.

6 - 1 ppm Cu^{++}.

7 - 20 ppm Zn^{++}.

8 - 0.03 ppm Tributyl tin.

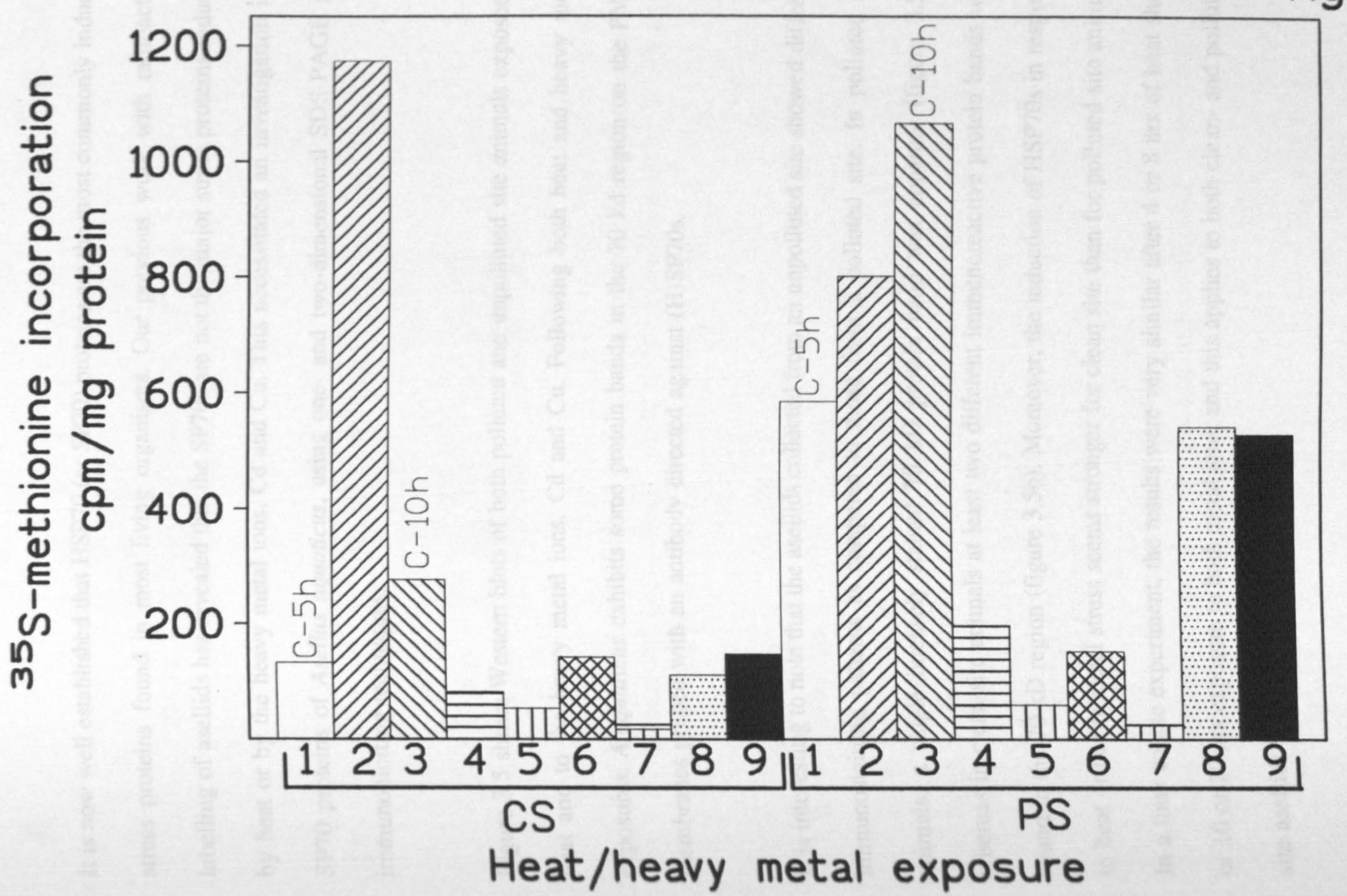
9 - 0.03 ppm Tetraphenyl lead.

The asellids were exposed to all heavy metal ions for 10 hrs of incubation as described in chapter 2.5. Each data point is the mean from two determinations.

CS : Clean site asellids.

PS : Polluted site asellids.

fig.3.4


^{35}S-methionine incorporation
cpm/mg protein
1200
1000
800
600
400
200
C-5h
C-10h
C-5h
C-10h
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
CS
PS
Heat/heavy metal exposure

3.3.4 HSP70 PROTEINS OF *A.AQUATICUS*: PROTEINS PRONE TO PROTEASE BREAKDOWN?

It is now well established that HSP70 (or SP70) proteins are the most commonly induced stress proteins found in most living organisms. Our previous work with radioactive labelling of asellids has revealed that the SP70s are not the major stress proteins induced by heat or by the heavy metal ions, Cd and Cu. This necessitated an investigation into SP70 proteins of *Asellus aquaticus*, using one- and two-dimensional SDS-PAGE and immunoblotting techniques.

Figure 3.5 shows Western blots of both polluted and unpolluted site animals exposed to heat and to the heavy metal ions, Cd and Cu. Following both heat and heavy metal exposures, *A.aquaticus* exhibits some protein bands in the 70 kd region on the PVDF membranes probed with an antibody directed against (H)SP70s.

It is interesting to note that the asellids collected from an unpolluted site showed different immunostaining patterns as compared to those from a polluted site. In polluted site animals, a single intensely stained 70 kD protein band was apparent (figure 3.5a), whereas in clean-site animals at least two different immunoreactive protein bands were found in the 70 kD region (figure 3.5b). Moreover, the induction of HSP70s in response to heat or heavy metal stress seems stronger for clean site than for polluted site animals. In a time course experiment, the results were very similar after 4 or 8 hrs of heat shock or 10 or 20 hrs exposure to both metal ions, and this applies to both clean- and polluted-site asellids (see figure 3.5a,b).

fig. 3.5

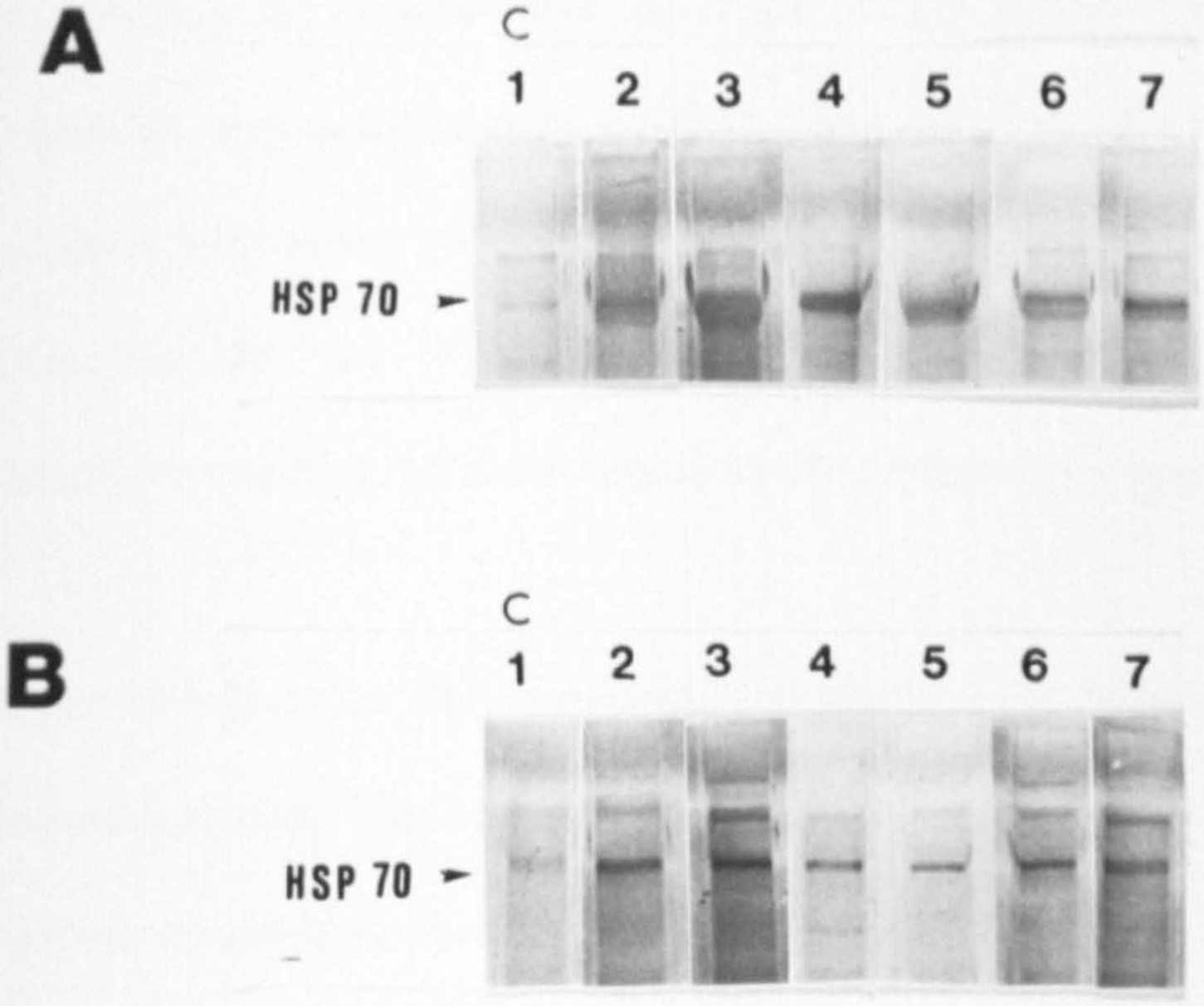


Figure 3.5 Western blots of both polluted and unpolluted site asellids exposed to heat and heavy metal stress, then probed with anti-HSP70s and subjected to the Blast Amplification System.

A : Unpolluted site.
B : Polluted site.

Lane 1 - control (C)
Lane 2 - 4 hrs HS
Lane 3 - 8 hrs HS
Lane 4 - 10 hrs Cd^{++} (1 ppm).
Lane 5 - 20 hrs Cd^{++} (1 ppm).
Lane 6 - 10 hrs Cu^{++} (1 ppm).
Lane 7 - 20 hrs Cu^{++} (1 ppm).

The estimated volumes of samples (100 μg total protein for each sample) were run on 12% acrylamide gels and immunoblotted as described in chapter 2.15 and 2.16, respectively.

Although faint bands corresponding to the SP70 proteins of *A.aquaticus* were picked up by using the sensitive Blast Amplification System, we have experienced great difficulties in detecting the signals from the target 70 kD protein family in this organism. No detectable response was seen in blots subjected to the normal histochemical detection methods with either peroxidase- or alkaline phosphatase-linked secondary antibodies. Therefore, the use of other sensitive detection systems such as ECL (enhanced chemiluminescence). has been necessary for further investigation.

Figure 3.6a,b and c show one- and two-dimensional Western blots subjected to ECL detection system. The one dimensional immunoblots of the control and stress-induced samples demonstrate that the monoclonal antibody directed against a conserved region of SP70 proteins crossreacted with low molecular weight polypeptides, which appear to be of 20-40 kD, but no reaction was observed in the 70 kD region. Two-dimensional blots also clearly show several immunoreactive spots in the low molecular weight region, indicating that these small polypeptides may in fact be products of *in vitro* degradation of HSP70 proteins.

In the following experiments, known protease inhibitors such as PMSF, benzamide and EDTA in combination have been employed to halt the possible degradation by proteases. Using such inhibitors did not appear to halt protein degradation; however, with the clean site asellids, a weak band was observed in the region of 70 kD (especially in heatshocked samples), in addition to those of low molecular weight region (see figure 3.6d).

Figure 3.6 The demonstration of *A.aquaticus* HSP70 degradation.

Figure 3.6 (a) and (b) Two dimensional immunoblots of the clean site control and heat shock samples, respectively; (c) and (d) One dimensional immunoblots of both clean site and polluted site asellids exposed to heat and heavy metal stress. Protease inhibitors (PMSF,benzamide and EDTA) were added to the samples just before homogenization only in part (d) in comparison to others. 75 µg of total protein was loaded onto each lane on 10-16% gradient polyacrylamide gels. The methods were carried out as detailed in chapter 2.18 and 2.19.

LMWs : Low molecular weight proteins.

CS : Clean site asellids

PS : Polluted site asellids

S - Sigma standard protein markers

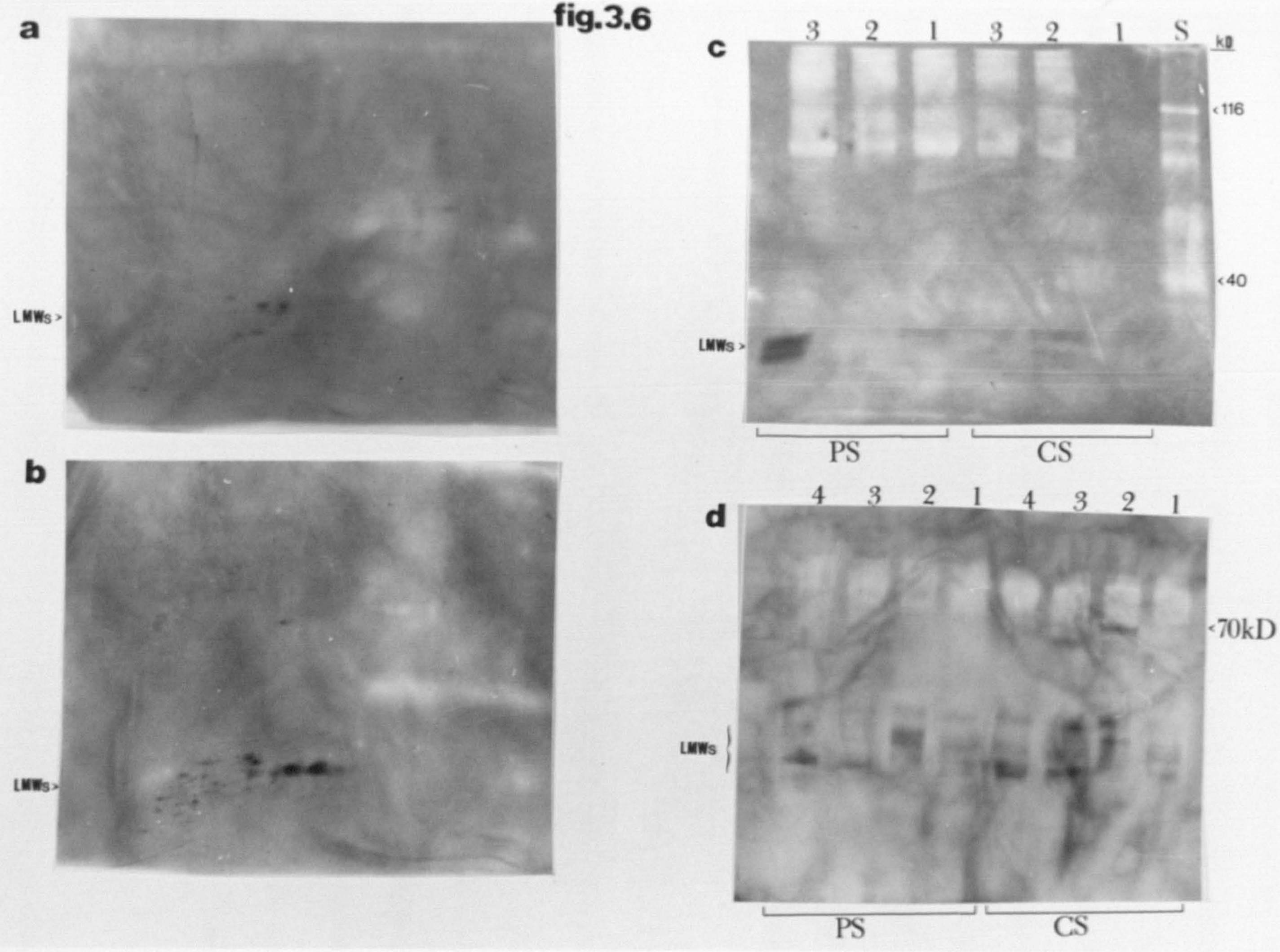
Lane 1 - control at 14°C.

Lane 2 - 5 hrs heat shock at 26°C.

Lane 3 - 10 hrs 1 ppm Cd^{++} at 14°C.

Lane 4 - 10 hrs 1 ppm Cu^{++} at 14°C.

fig.3.6


a
LMWs >
b
LMWs >
c
3 2 1 3 2 1 S
kD
< 116
< 40
LMWs >
PS
CS
d
4 3 2 1 4 3 2 1
< 70kD
LMWs
PS
CS

3.4 DISCUSSION

In the present study, the stress response of the crustacean *Asellus aquaticus* to heat and to heavy metal ions such as Cd^{++} and Cu^{++} at sublethal levels has been investigated, as well as the possible use of this system as indicator of environmental contamination. This organism responded to heat shock-treatment (26°C) by synthesising at least 5 putative HSPs which are either not detectable or are manufactured at low levels in controls at 14°C. The heat shock proteins induced were of approximate molecular weights 110, 96, 89, 60 and 52 kD respectively (section 3.1; figure 3.1). An increase in the synthesis of smaller sizes of polypeptides (25-35 kD) should also be noted. However, these proteins of low molecular weights are likely to be the degradation product of HSP70 proteins as is shown in section 3.3.4 (figure 3.6).

Exposure of the asellids to sublethal concentrations of Cd^{++} or Cu^{++} resulted in the induction of a set of proteins of similar sizes to those HSPs induced by heat (figures; 3.2 and 3.3). Although there were differences in the pattern of stress protein synthesis shown in the autoradiograms, the Autograph analysis of stress responses showed that these proteins were clearly induced by both Cd^{++} and Cu^{++}. A protein band of apparent molecular weight 65 kD is also present under all conditions (including controls); this protein is constitutively synthesised, but appears to be enhanced in response to some stressful conditions.

The time-course experiments with the asellids for 2, 4, 8, 12 and 20 hrs revealed that the pattern of stress protein synthesis changes dramatically with increasing time of incubation

at 26°C (figure 3.1). Similar results were obtained by Nickells *et al.* (1989), indicating that there are some effects of different temperatures and lengths of heat shock on the pattern and intensity of HSP synthesis in *Xenopus laevis* embryos. *Asellus aquaticus* showed some response to heat shock after about 2 hrs, but a clearer heat shock response is apparent after 4 hrs. A protein band at 110 kD is induced following heat shock treatment after 2 hrs, but its synthesis declines to control levels after 4 hrs of incubation, while a protein of 60 kD molecular weight is newly induced and abundant present from 4 to 20 hrs. The nature of this transient response is not clear.

Hakimzadeh and Bradley (1990) monitored such transient responses in the copepod *Eurytemora affinis*, indicating that with a continuation of heat-shock conditions some HSPs gradually disappear and normal protein synthesis is restored. They have further shown that after 24 hrs of heat shock, the proteins of higher molecular weights (109 and 98 kD) were no longer present, while the smaller HSPs were still being synthesised. Interestingly, a 60 kD protein in *Hydra attenuata* was found to be the major HSP induced in response to high temperatures. This protein was shown to play an important role in thermotolerance in this species. However, several other *Hydra* species were unable to synthesise detectable levels of this 60 kD protein, and therefore, were unable to acquire thermotolerance (Bosch *et al*,1988). Our findings are generally in line with these results, suggesting that such responses may be a pre-requisite of acclimation; e.g. as a consequence of regulation among the stress proteins themselves to counteract the effects of longer exposures to stressful conditions.

Like thermal stress, the response to heavy metal ions (Cd^{++} and Cu^{++}) is detectable after

2 hrs of incubation, but a striking difference was observed in the pattern of protein synthesis from 8 hrs onwards for Cu^{++} (figure 3.2) or from 12 hrs in the case of Cd^{++} (figure 3.3), especially in the proteins with higher molecular weights. In particular, the induction of these proteins is much clearer in Cu^{++}-treated animals, as can be seen in figure 3.2. The putative SPs with molecular weights of 110 and 96 kD are abundantly present from 8 hrs up to 20 hrs, suggesting that the response to copper is both continuous and dynamic (a phenomenon also observed under severe heat shock conditions; Lindquist, 1986). Unlike thermal stress, the induction of a 60 kD SP by heavy metal stress in *A.aquaticus* is not clear-cut.

However, the cadmium response is less clear cut in the asellids, although some bands corresponding to the SPs are detectable. In fact, such heavy metal responses are widely believed to be transient. For example, Heikkila *et al* (1982) found that the SP levels in fish declined towards control levels after prolonged exposure to Cd^{++} ions. Ideally, the test organisms should be subjected to various heavy metal concentrations over longer periods of time in order to determine time limits for these responses. Additionally, clear and rapid induction of SP's is needed if the *A.aquaticus* stress response is to be useful as an indicator of pollution. In order to establish a clear response to heavy metal stress, it may be advantageous to combine moderate increases in the ambient temperatures (e.g. 20°C) with low concentrations of metal ions, since seasonal temperature fluctuations in aquatic ecosystems are also possible (Huey and Bennett, 1990) . Veldhuizen-Tsoerkan *et al* (1990) pointed out that exposure to Cd^{++} followed by heat shock differentially enhanced the expression of SPs in the sea mussel *Mytilus edulis*. The same authors (Veldhuizen-Tsoerkan *et al,* 1991) also demonstrated that heat shock alters stress protein

induction significantly in unpolluted sea mussels following transplantation to contaminated sites for a long period of time. Li (1983) further showed that, sodium arsenite or ethanol treatment could confer resistance to subsequent heat shock in chinese hamster fibroblast cells, leading to greater cell survival as well as enhanced SP synthesis. As can be seen in figures 3.1, 3.2 and 3.3, although *A.aquaticus* clearly responds to environmental stress, the pattern of SP synthesis is somewhat variable among individuals exposed to the same conditions over the same exposure time. These variations may result from the different sex, age, nutritional state, or metabolic rate of each individual. Several studies have demonstrated that there are differences in stress response among individuals because of the influence of the factors mentioned above (Lindquist,1980; Nickells *et al*,1989; Hakimzadeh and Bradley,1990; Sanders,1993). In the present study, the use of small eppendorf tubes, each containing two individuals in 1 ml of radioactive medium, might have caused additional stress for the animals. The apparent induction of some (H)SP bands in controls after 20 hrs of incubation could suggest stress caused by the small medium volumes and/or lack of oxygen.

It is now well known that the ubiquitous SP-70 family is the most highly conserved and largest of all the stress protein families found in all most living organisms, ranging from bacteria to human. It is also known that SP70s have important biochemical functions such as protein folding, transport and repair. On the other hand, a few exceptions have been reported in the literature to the effect that this protein family is not inducible in some organisms. For example, Bosch *et al* (1988) examined eight different *Hydra* species and found that the synthesis of the major stress protein, HSP70 was absent in those species examined. More recently, Gellner *et al* (1992) have showed that the inability of some

species of *Hydra* to synthesise HSP70 in response to stress was in part due to reduced stability of HSP70 mRNAs during heat shock. The absence of a 70 kD stress protein was also observed in the rotifer *Brachionus plicatilis* (Cochrane *et al*,1991).

The radioactive labelling and subsequent electrophoresis of *A.aquaticus* proteins has revealed that SP70 proteins were also not the major class of stress proteins induced in this organism. Using several immunoblotting techniques, the SP70 family in the asellids from both polluted and unpolluted sites has been investigated in order to determine whether this protein family is inducible. With normal immunoblotting detection systems, we have been unsuccessful in detecting the presence of SPs of this molecular weight. However, using more sensitive detection systems such as the Blast amplification system or ECL, we were able to detect immunoreactive band(s) in the region of 70 kD, as well as bands in the lower molecular weight range.

As can be seen from figure 3.5, following heat or heavy metal exposure, the asellids collected from both polluted and unpolluted sites exhibit some protein bands at 70 kD on blots treated with the Blast amplification system. Moreover, the asellids from an unpolluted site had different immunoreactive patterns as compared to those from a polluted site. Time course experiments have shown that there were no great differences in protein pattern in response to different stressful conditions over a period of time, and this was true for animals from both sites. The levels of immunodetectable SP70 proteins induced by heat as well as by heavy metal ions are relatively low. However, levels of accumulated protein will vary much more slowly than the rates of synthesis measured by metabolic labelling. The asellid pattern is thus somewhat similar to those reported in a

rotifer and in some species of *Hydra* (Cochrane *et al*,1991; Bosch *et al*,1988). Two explanations can be offered here regarding the apparent lack of SP70 proteins: (i) few if any 70 kD SPs are inducible by stress in these animals (though they are present as cognate proteins), and/or (ii) these proteins are degraded somehow *in vivo* or *in vitro*.

Further investigation of SP70s, using both one- and two-dimensional SDS-PAGE followed by immunoblotting, has clearly shown that these proteins are indeed prone to degradation (figure 3.6a-d). The anti-HSP70 monoclonal antibody, which reacts with denatured 70 kD proteins in variety of different species, crossreacts with proteins in a low molecular weight range (20-40 kD) on the one and two dimensional blots, but shows no detectable reaction in the 70 kD range at all. Lindquist (1986) and Miller and McLennan (1988) also observed proteolytic degradation products of similar molecular weight. Moreover, Cochrane *et al* (1991) have recently observed weak reaction between a 40 kD protein induced by heat and a monoclonal antibody directed against a conserved region of SP70, indicating that this protein might be a product of *in vitro* degradation. In fact, both instability and degradation of the 70 kD stress proteins have been reported before. For example, Mitchell *et al* (1985) presented evidence that the 70 kD HSP of *Drosophila* decays *in vivo* at a much faster rate than other abundantly labelled proteins. They have also found that SP70 degradation occurs *in vitro*, even during electrophoresis; this is not mediated by a general protease, but rather the SP70 protein has a slow proteolytic action upon itself.

We have used several known protease inhibitors in combination in order to examine the nature of the apparent SP70 degradation in *A.aquaticus*. As can be seen in figure 3.6d,

a weak band was observed at 70 kD only in clean site asellids exposed to heat or Cd^{++} ions, even when the inhibitors were added to the extraction buffer just before homogenization (CS;lanes 2 and 3). Immunoreactive proteins in the smaller molecular weight range were present under all stressful conditions as well as in controls , and this applies to animals from both sites. This provides suggestive evidence that the degradation of SP70s in asellids may not be due to general protease action alone, but rather to a combination of factors including self-degradation or even the electrophoresis process itself. For whatever reason, these particular proteins appear to be rather unstable, and a great deal of work still needs to be done before considering the use of *A.aquaticus* (H)SP70s as an indicator of environmental pollution.

However, it may be more advantageous to evaluate other stress proteins of this organism as environmental biomarkers. For example, we have shown that the stress-inducible proteins of 110, 89 or even 60 kD are clearly present following exposure to stressful conditions. In particular, the last two groups of proteins are immunologically well-characterized and a range of monoclonal or polyclonal antibodies directed against them are commercially available. The differential ^{35}S-methionine incorporation into *A.aquaticus* soluble proteins in response to heat shock and heavy metal ions was also shown to be an important stress indication of heavy metal (figure 3.4) that could be used alongside other parameters.

CHAPTER 4. THE EFFECTS OF HEAT AND HEAVY METAL STRESS ON THE INDUCTION OF HSP70s IN THE NEMATODE *C. ELEGANS*

4.1 INTRODUCTION

4.1.1 THE BIOLOGY OF *CAENORHABDITIS ELEGANS*

Caenorhabditis elegans is a small, free-living soil nematode, which can be found commonly in many parts of the world. In the mid-1960's, Sydney Brenner chose this organism as a promising model organism for biological studies. Since then, a great deal of research has been done on this species. *C.elegans* offers many advantages as an experimental organism (Brenner,1974; Sulston and Brenner,1974; Byerly *et al.*,1976; Wood, 1988; de Pomerai, 1990; Hope, 1994), including:-

(i)- simple culture methodology at 15-25°C on agar media or in liquid cultures, feeding on *Escherichia coli* bacteria;

(ii)- rapid growth and small size (adults c. 1 mm long; many thousands per culture plate);

(iii)- a short generation time of about 3 days with 250-350 progeny on average per hermaphrodite adult under normal conditions;

(iv)- reproduction by either self-fertilisation (within XX hermaphrodites) or cross-fertilisation (between rare XO males and hermaphrodites);

(v)- deep-frozen stocks can be maintained indefinitely without any genetic drift;

(vi)- relative transparency, allowing nuclei to be visualised by Nomarski optics.

These features have attracted a large number of workers to work with this species. *C. elegans* is now probably the most completely characterised metazoan in terms of its

anatomy, genetics and development. Additionally, its genome is also likely to be the first to be mapped and fully sequenced (Coulson *et al.*,1986; Lewin, 1990; Sulston *et al.*,1992; Wilson *et al*, 1994).

Both hermaphrodites and males are each about 1 mm in length, but they are unlike each other in appearance. Most natural populations are predominantly composed of hermaphrodites. Adult males have 1031 somatic cells, whereas adult hermaphrodites have only 959. Moreover, *C.elegans* has a small haploid genome size, only 20 times that of *E.coli* and about half that of *Drosophila* (Nicholas, 1984). This organism has the typical nematode body plan, with an outer tube comprising cuticle, hypodermis, neurons, and muscles surrounding a pseudocoelomic space that contains the gonads and the inner tube of intestine. The cuticle of *C.elegans* is an extracellular structure that covers the outermost surfaces of all hypodermal cells and also lines the pharynx and rectum. The organism feeds through the pharynx, pumping food into the intestine; food then passes through the intestine and out from the anus (Barnes, 1980; Wood, 1988).

In spite of its anatomical simplicity, *C.elegans* has a wide range of specialised cell types carrying out diverse functions during the development of the organism. The life cycle of *C. elegans* involves development from the egg, through several larval stages to the adult, taking about 3.5-days under ideal conditions at 20°C. Fertilisation of oocytes occurs internally in an adult hermaphrodite, and eggs are held in uteros for approximately 3 hrs, and then released to the outside through the vulva to undergo various stages of embryogenesis. The first stage larva (L1) hatches from the egg 14 hrs after fertilisation, and then can feed and grow independently. The L1 develops to an adult through three

further larval stages, referred to L2, L3 and L4. Under unfavourable conditions (e.g. crowding and/or lack of food) sensed during L1, larvae can proceed via a modified L2 stage (called L2d) to enter a facultative diapause called the dauer larva, which is resistant to many environmental stresses and can survive for prolonged periods with minimal metabolism (Cassada and Russel, 1975; Wharton, 1986; Wood, 1988). If food becomes available or crowding decreases, dauer larvae are triggered to exit from this diapause state and to reenter the normal developmental cycle at L4; adult survival times are apparently unaffected by the length of time spent as a dauer larva. The advantages of using *C.elegans* in toxicity testing will be discussed in section 5.1.1.

4.1.2 HEAT SHOCK RESPONSE IN *C. ELEGANS*

When the culture temperature is shifted from 20°C to temperatures above 29°C, the nematode stops growing, does not reproduce and dies slowly. Exposure of *C. elegans* to elevated temperatures of 31-35°C induces the synthesis of at least eight polypeptides of 81, 70, 41, 38, 29, 19, 18 and 16 kDs (Snutch and Baillie, 1983; Heschl and Baillie,1990a). The synthesis of a wide range of normal proteins is also repressed upon heat shock. Interestingly, hsp29, hsp19 and hsp16 first become apparent at 29°C (only the synthesis of hsp16 gradually decreases as the severity of the stress increases), while the synthesis of HSPs, 81, 70, 41 and 38 is enhanced during heat stress at higher temperatures. Moreover, HSP induction in *C. elegans* is not dependent on developmental stage, i.e. this organism does not exhibit stage-specific differences in HSP expression. Dauer larvae also display a heat shock response and synthesise a similar set of HSPs to that inducible during normal development. In addition, however, heat-shocked dauer larvae show at least one other inducible

polypeptide of molecular weight 50 kD, which is not detectable in growing worms.

Thus *C. elegans* possesses a classic heat shock response, inducing the synthesis of stress proteins corresponding to the HSP90, HSP70 and small HSP families characteristic of other species. Most of the genes encoding these last two protein groups in the nematode have been cloned and characterised (Russnak and Candido,1985; Jones *et al.*,1986; Heschl and Baillie,1989;1990a;1990b). The major low molecular weight HSPs in *C. elegans* are HSPs16 and 18. Both classes of HSPs consist of multiple isoforms (e.g. the 16 kD HSPs of this organism are encoded by four highly similar genes), and are highly homologous to the small HSPs of *Drosophila* and the α-crystallins of the vertebrate eye lens (Russnak *et al.*,1983; Hockertz *et al.*,1991; Dixon *et al.*,1990).

There are, however, at least nine members of the HSP70 multigene family in *C. elegans*. Most of these genes have been characterised and assigned to three different subfamilies; namely HSP-1, HSP-3 and HSP-6 (Snutch *et al.*,1988; Heschl and Baillie, 1989; 1990a; 1990b). The HSP-1 subfamily consists of hsp-1 and hsp-2ps genes; the former encodes a heat inducible *hsc70* (possible product [pp] HSP70A), whereas the latter is found to be closely related to the former, but no trancripts of this gene have been detected (appears to be a pseudogene). The HSP-3 subfamily comprises the hsp-3 and hsp-4 genes; the hsp-3 gene (*grp78*-like gene; encoding pp HSP70C) is constitutively expressed and not heat inducible, whereas the hsp-4 gene product (HSP70D) is not detectable under normal conditions, but its synthesis is enhanced upon heat shock by as much as 50-fold above control levels. The HSP-6 subfamily comprises the hsp-6 gene (a mitochondrial *DnaK*-

like gene; pp HSP70F) which is constitutively expressed and also heat inducible (Heschl and Baillie,1989; 1990a;1990b). Although most members of the HSP70 multigene family have been characterised in the nematode, many questions still remain unanswered concerning the regulation and roles of HSP70s during development or under stressful conditions. In this context, we have investigated the differential expression of HSP70s in *C. elegans* strains exposed to heat shock and to heavy metal stress at different temperatures, using one- and two-dimensional SDS-PAGE, radioactive labelling and Western blotting techniques.

4.1.3 THE MULTIPLICITY OF HSP70 FAMILY

The HSP70 (or SP70) family is the most highly conserved and largest of the stress protein families. Although *hsp70* genes were first characterised in *Drosophila melanogaster*, homologous genes have been found in both prokaryotic and eukaryotic species studied so far (Nover and Scharf, 1991; Boorstein *et al*, 1994). The multiplicity and regulation of the *hsp70* gene family in various organisms is not yet fully analysed. A large number of proteins encoded by this multigene family have been characterised. Members of this protein family include heat shock (stress)-induced and constitutive members as well as glucose regulated 78 kD proteins (GRP78s); the genes encoding these proteins were all found to have a high degree nucleotide-sequence similarity in organisms ranging from mammals, insects and nematodes to bacteria (Nover and Scharf, 1991; see also section 1.3.3). More recently, Boorstein *et al* (1994) have analysed the nucleotide sequences of 30 *hsp70* genes from 30 eukaryotic and procaryotic species, and have demonstrated a high degree of conservation of the HSP70 protein sequence from even

the most distantly related species (at least 45% identity). Moreover, organisms from such diverse phyla as vertebratess and molluscs share similar antigenic and ATP-binding domains (Gething and Sambrook, 1992; Sanders, 1993). A rat monoclonal antibody (as used in this study) against a conserved epitope of *Drosophila* HSP70 cross-reacts with many inducible and constitutive HSP70 protein-family members from plant, fungal and animal sources (Kurtz *et al*, 1986). The Intracellular localisation and function of HSP70-type proteins have been extensively studied and a great deal is known concerning the biochemical functions of HSP70 family members in protein folding, degradation, repair and translocation (Nover and Scharf, 1991; Gething and Sambrook, 1992; Kampinga, 1993; Parsell and Lindquist, 1993; and also see section 1.5). However, there is much less information concerning the differential regulation of these closely-related *hsp70* genes under normal conditions or in response to different stressors.

The most extensively studied *hsp70* and related genes are those from *Drosophila*, *Saccharomyces cerevisiae* and *C. elegans*. In *Drosophila*, 6 different HSP70 family members have been identified; these comprise two HS-induced members (HSPs70 and 68) plus two related constitutive proteins (HSCs70 and 72), and two minor constitutively expressed genes. Protein products from the latter two have not been identified as yet, but the remaining four are all detected by a monoclonal antibody generated by Velazquez *et al* (Nover and Scharf, 1991). The yeast HSP70 family, however, is the best studied of all HSP70 families identified so far. Eight members of this protein family have been identified (Boorstein *et al*, 1994) and their properties have been defined by studying strains that contain mutations in particular *hsp70* gene family members (Werner-Washburne *et al*, 1987), or that contain *hsp* promoter-lacZ fusion constructs (Elwood and

Craig, 1984; Kirk and Piper, 1991). Five to eight members of the HSP70 protein family from *Arabdopsis* were detected by two-dimensional gel analyses, and there also appears to be one or more *hsp70* pseudogenes (see review in Nover and Scharf, 1991).

4.2 MATERIALS AND METHODS

(i) Maintenance and stress exposure of *C. elegans*

The wild type (N_2) and transgenic (CB4027) nematode strains, as well as the *lac*-operon deleted strain of *E. coli* (P90C) were obtained from Drs J.Hodgkin and A.Chisholm (MRC Cambridge). Worms were grown on NGM agar plates at 20°C for 4-5 days and then washed into K-medium where they were exposed to heavy metal ions at 20 or 31°C, or heat shocked at 34°C, as described in chapter 2 sections 1 and 4. The chosen Cd^{++} and other heavy metal concentrations were in the range of the LC50 values for wild-type *C. elegans* over a 24 to 96 h period (Williams and Dusenbery, 1990) [Chapter 5 describes the effects of these and other stressor conditions on transgene induction in the transgenic (CB4027) nematode strain].

(ii) Extraction of *C. elegans* soluble proteins

Following exposure, worms were centrifuged, washed 2-3x with M9 buffer (appendix 2) and left in the buffer for 45 minutes after which all remaining bacteria in the nematode's gut are likely to be digested. A detailed method is described in section 2.9. Worms were homogenised in 150 µl of M9 buffer containing 1 mM DTT + 0.1 % (w/v) SDS for one dimensional separations, or 1mM DTT + 0.1 % (v/v) Triton-X-100 for two-dimensional separations, and then centrifuged to remove the debris. The determination soluble protein concentrations was carried out using the method of Lowry *et al* (1951) as described in section 2.11.

(iii) Radioactive labelling of worms and determination of (^{35}S)-methionine

The nematodes were labelled with 3.0 MBq/ml ^{35}S-methionine (Amersham) and homogenized as described in section 2.9. Following centrifugation, radioactively labelled proteins in 5 µl of each supernatant were precipitated on to glass fibre filters (Whatman GF/C), washed extensively and counted using a Packard Liquid Scintillation counter as described in section 2.13. $1x10^6$ cpm of total labelled protein was loaded onto each lane of an SDS polyacrylamide gel subsequent electrophoresis.

(iv) Analysis of proteins by one- and two-dimensional SDS-PAGE

One dimensional SDS-PAGE was performed according to the method of Laemmli (1970) mainly using an LKB-Pharmacia mini gel apparatus. 10 or 12.5% acrylamide gels were employed for separation of proteins by one dimensional SDS-PAGE as described in section 2.15.1. Samples applied to each well contained 200 µg of total protein for Coomassie blue staining and for one dimensional western blots, or $1x10^6$ cpm of ^{35}S-methionine for autoradiography, in a volume of 10-50 µl. Localisation of protein bands on one dimensional gels and blots is described in sections 2.16 and 2.17, respectively. Both rat anti-HSP70 (1:1000) and mouse anti-HSP70 (1:600) were used to probe one dimensional blots.

Two dimensional separation of proteins was carried out using the method devised by O'Farrell (1975), which utilises isoelectric focussing gels (4% acrylamide gels containing 1.6% pH 5-7 and 0.4% pH 3-10.5 ampholines) to separate proteins in the first dimension,

and SDS-PAGE slab gels (10-16% gradient gels) in the second dimension, as described in section 2.18. Immunoblotting of two dimensional gels was as described in section 2.19.2. Rat anti-HSP70 primary antibody (Affinity BioReagents) at a dilution of 1:1000 in TBS-T plus 5% BSA was used as a probe for the target HSP70 proteins. Antibody binding to target protein spots was detected using ECL detection reagents and ECL Hyperfilm (Amersham) according to the manufacturer's instructions.

4.3 RESULTS

4.3.1 RADIOACTIVE LABELLING OF *C. ELEGANS* PROTEINS DURING EXPOSURE TO CADMIUM AND HEAT SHOCK

As described in section 4.1.1, a classic heat-shock response involving at least eight polypeptides is induced in *C. elegans* in response to elevated temperatures of 31-35°C (Snutch and Baillie, 1983). Our initial studies with the transgenic nematode strain (CB4027) has suggested that exposure to cadmium ions at 20°C does not induce a detectable transgene response; an accompanying temperature of 31-32°C is required for maximal induction of β-galactosidase activity by cadmium during a 7 h exposure, (see section 5.3). These transgene responses should be compared with those of the endogenous *hsp* genes, whose induction by heat and Cd^{++} ions has been examined in the radioactive labelling study described here.

Fig.4.1A demonstrates the inducibility by both heat and Cd^{++} stress of polypeptides whose apparent sizes correspond to HSP85, HSP78, HSP72 and HSP45. The induction of HSP85 appears to be gradually enhanced as the severity of stress increases. It is essentially undetectable in 20 or 31°C controls; some induction is seen with 2 ppm Cd^{++} at 31°C, but higher levels of HSP85 are detectable with 16 ppm Cd^{++} at 31°C or at the heat shock temperature of 34°C. In contrast, HSP78 is detectable in all samples , including controls. HSP72 is also detectable in controls, but is significantly enhanced under all stressful conditions. The apparent 45 kD protein is absent in controls (20°C), but is clearly synthesized following exposure to stress. We have also used a Biorad

Figure 4.1 **Stress protein induction by cadmium and heat in *C. elegans***

Radioactive labelling of wild-type N2 worms was as described in section 2.13. Radioactively labelled proteins ($1x10^6$ cpm) in appropriate volume of each sample were mixed with sample buffer (appendix 12) containing 0.3% (w/v) SDS and 1 mM DTT. After boiling for 5 minutes in water, the samples as well as non-radioactive Sigma pre-stained protein markers were run on 12.5% SDS-PAGE gels as described in chapter 2.15.

Part A shows an autoradiogram of stress protein induction by cadmium and heat.

Lane 1 - 20°C control.

Lane 2 - 31°C control.

Lane 3 - 2 ppm Cd^{++} at 31°C.

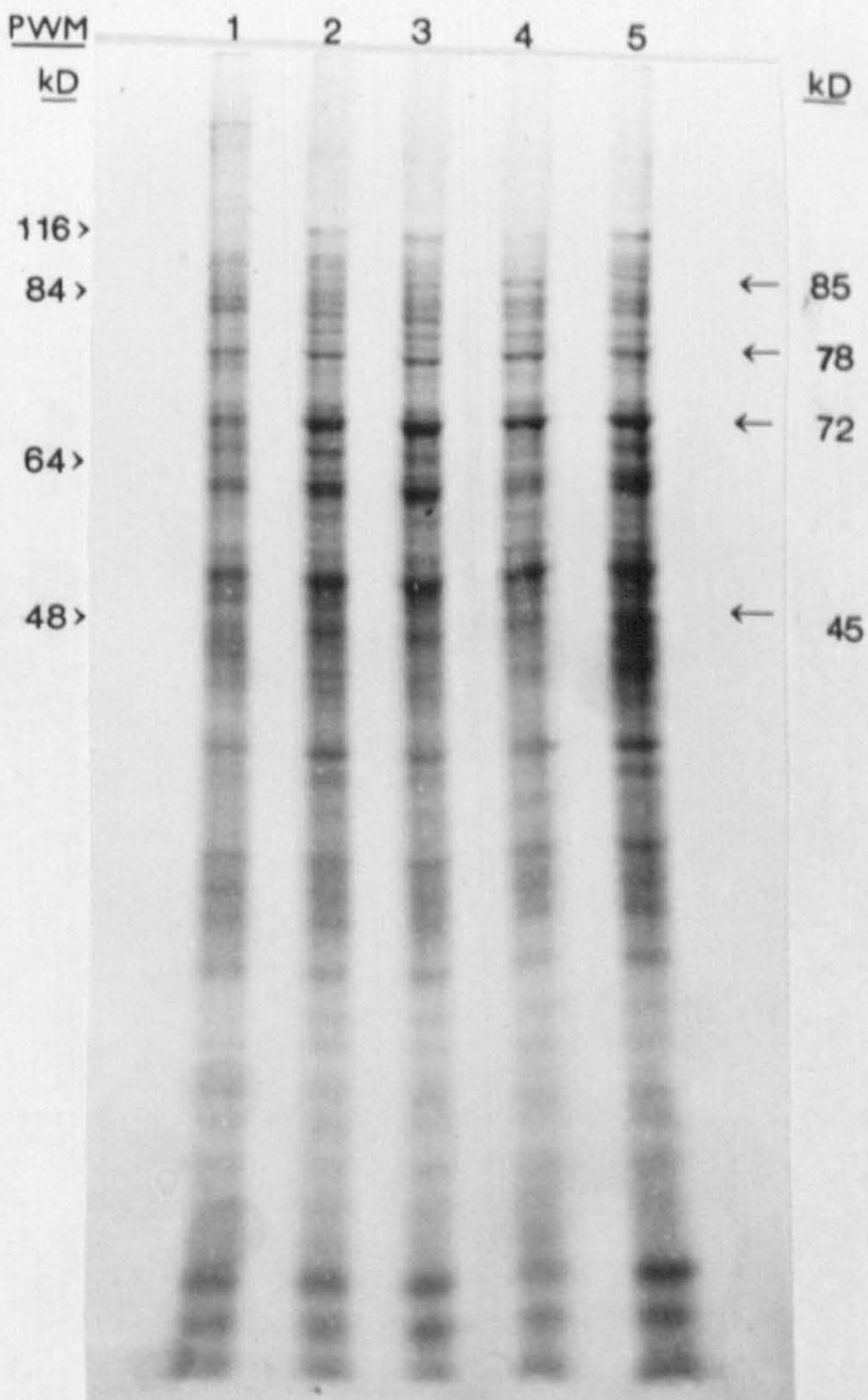
Lane 4 - 16 ppm Cd^{++} at 31°C.

Lane 5 - 34°C heat shock.

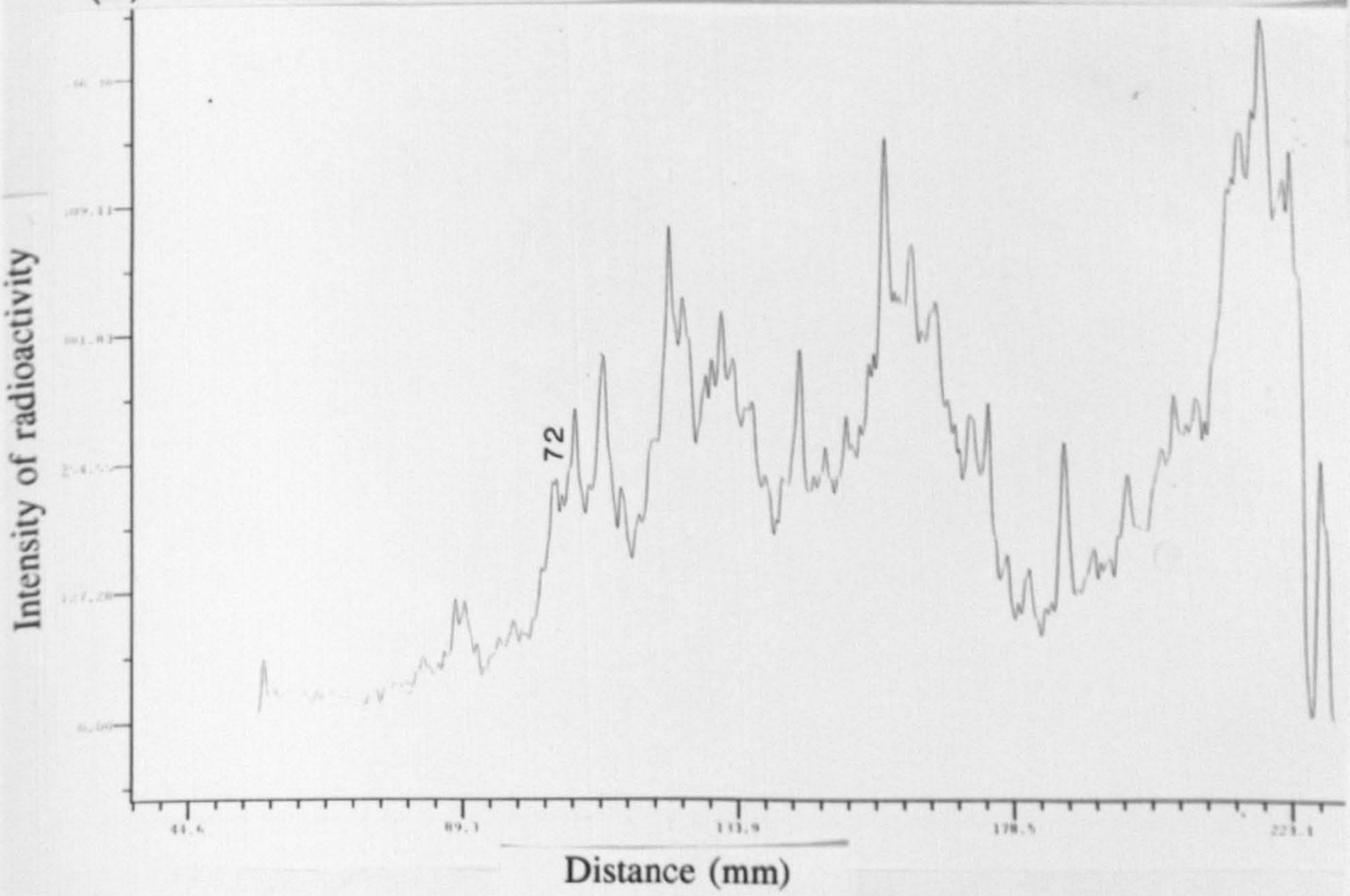
Part B shows the histograms of radioactively labelled proteins on each individual lane (the numbers correspond to the lane numbers shown in the autoradiogram). A Biorad phosphor image analyser was used for comparison. Identified peaks (85, 78 and 72 kD) clearly differed between lanes when transparencies of the gel traces (part B) were superimposed.

Fig. 4-1

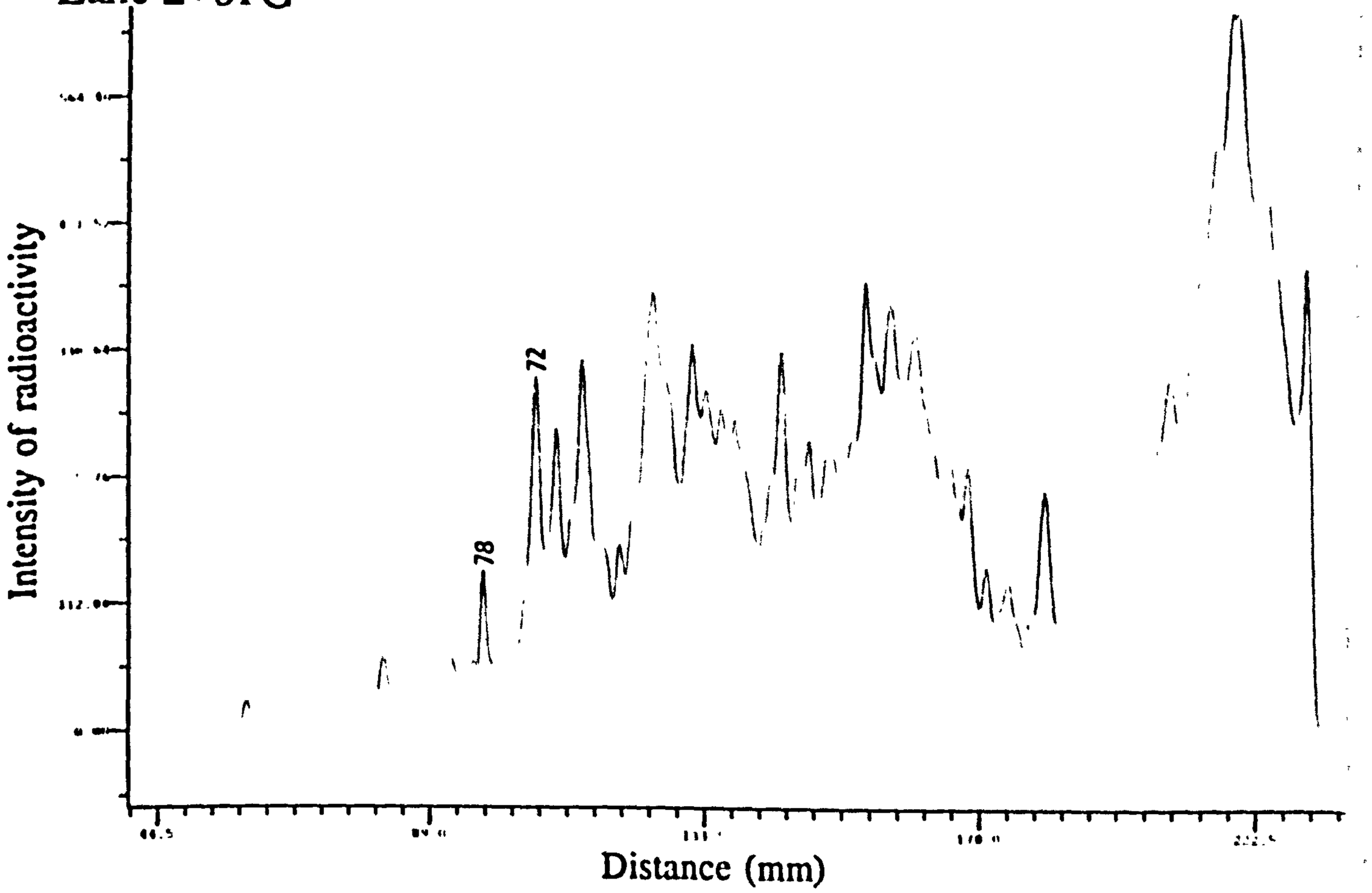
(A)



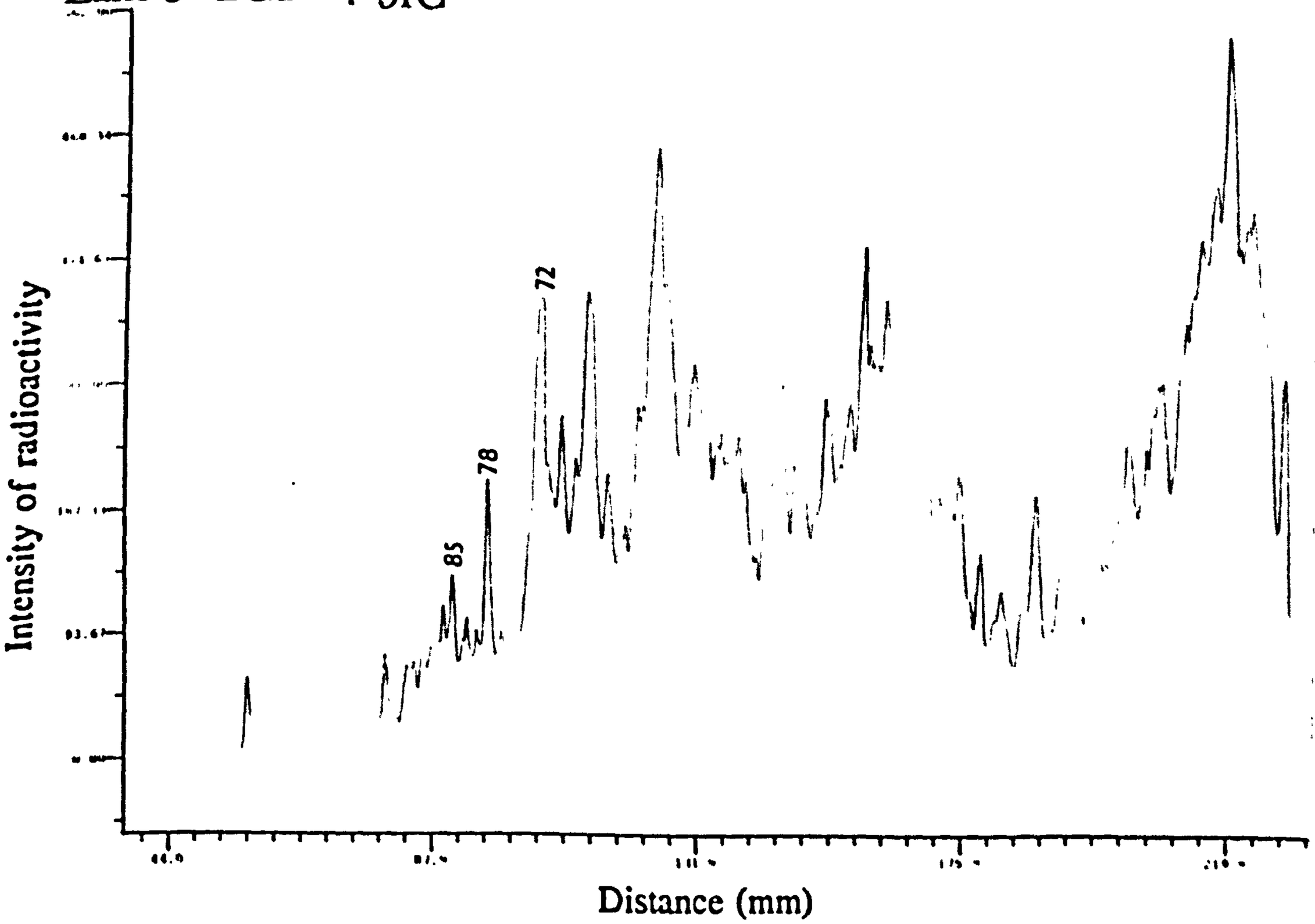
(B) Lane 1: 20℃



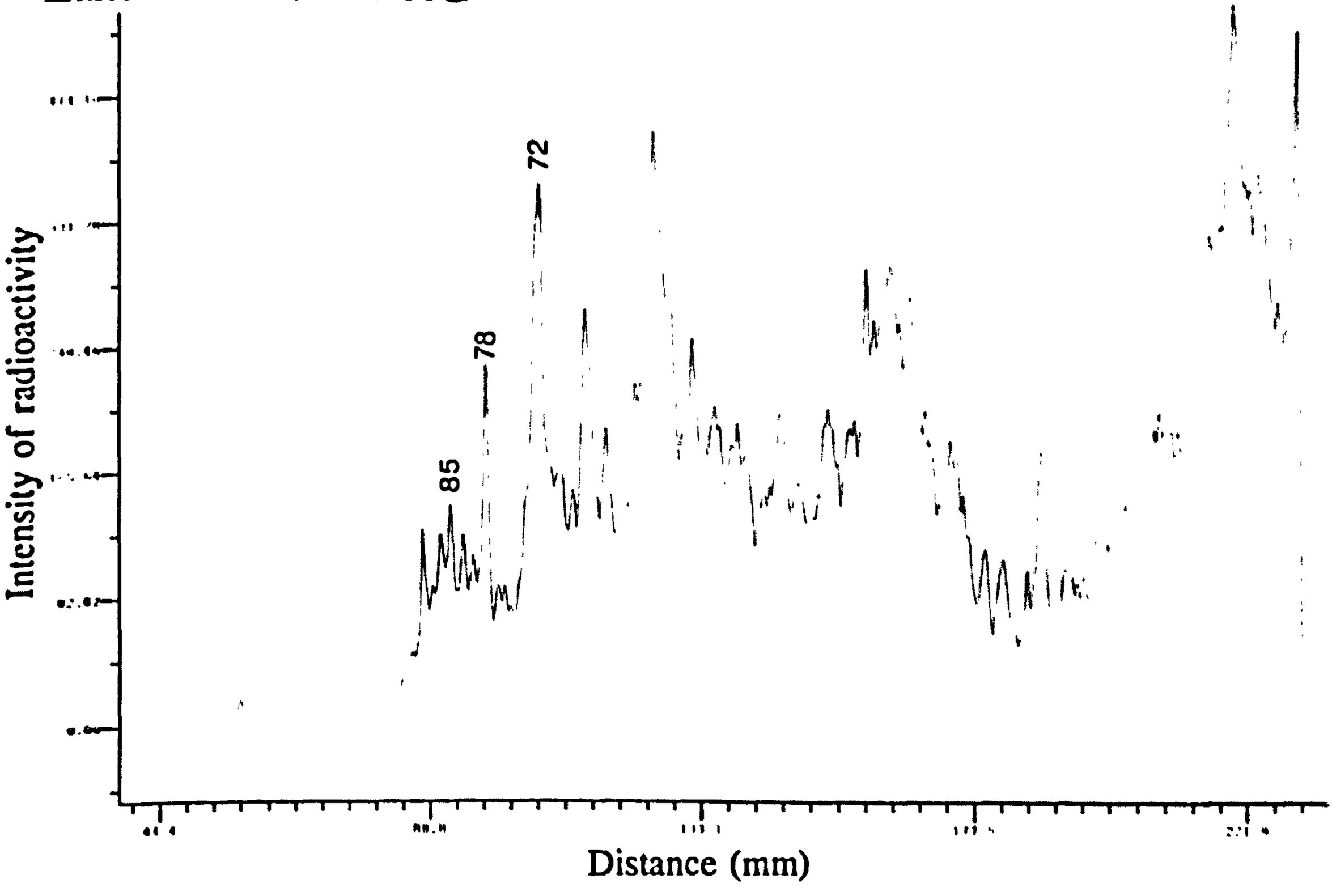
Lane 2 : 31°C



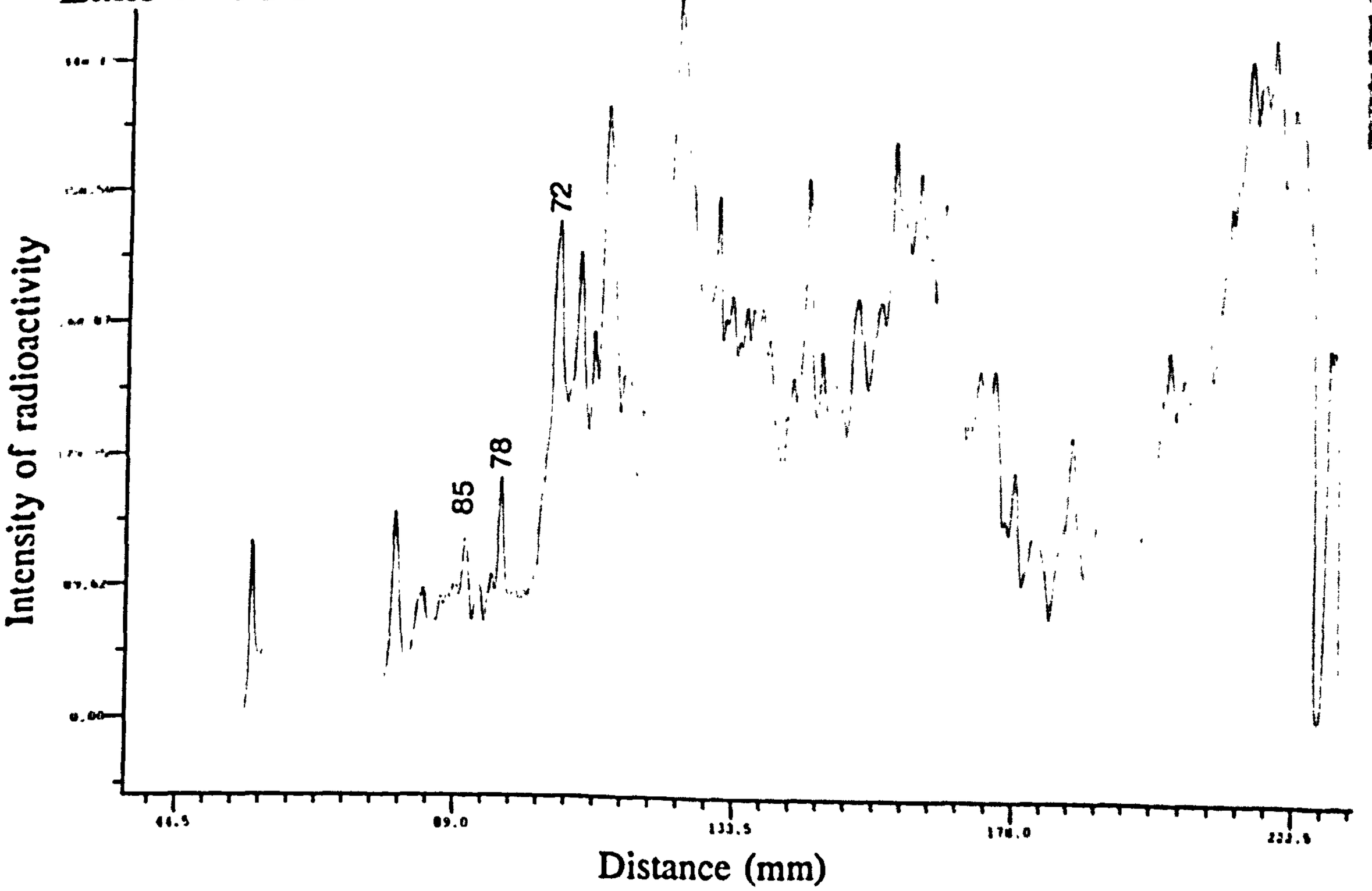
Lane 3 : $2Cd^{++}$ + 31°C



Lane 4 : 16 Cd^{++} + 31℃



Lane 5 : 34℃



phosphor image analyser to compare the results with conventional autoradiograms, as shown in Fig.4.1B.

4.3.2 70 KD STRESS PROTEINS OF THE NEMATODE *C.ELEGANS* EXPOSED TO HEAT SHOCK AND TO HEAVY METAL IONS

We have analysed the nematode HSP70s following separation on one dimensional SDS-PAGE and Western blotting with two different monoclonal anti-HSP70 antibodies that cross-react with most HSP70 family members. Fig.4.2B shows the crossreactivity of 70 kD nematode stress proteins with a rat monoclonal anti-HSP70; constitutive or induced members of this protein family are practically undetectable at 20°C (note that constitutively expressed HSP70s are detectable using this antibody with more sensitive ECL detection system; Fig.4.3a and f), mild heat shock at 31°C induces a doublet HSP70 band of which the larger 75 kD component is more prominent than the smaller 73 kD species. Cd^{++} ions (16 ppm) at 31°C clearly enhance the induction of the larger component, while both species are greatly intensified by heat shock at 34°C.

However, we have obtained somewhat different band patterns when using a mouse clonal anti-HSP70 followed by treatment with alkaline phosphatase-linked secondary antibody (Fig.4.2C). Because this detection system is more sensitive than the one used above (immunohistochemical staining for peroxidase), constitutive members of this protein family are detectable at 20°C (although the staining developed more slowly in the control as compared to stressed lanes). It is interesting to note that an additional protein with an apparent molecular weight of 80-85 kD is present following all treatments (including the

Figure 4.2 One dimensional SDS-PAGE and immunoblotting analysis of 70 kD stress proteins in *C. elegans* following exposure to heat and cadmium

Extraction of proteins from nematodes was as described in section 2.9, and 200 µg of total protein was loaded onto each lane for SDS-PAGE (with 12% acrylamide gels) and immunoblotting, as described in sections 2.15 and 2.17, respectively. 5 µl/lane of prestained molecular weight markers (Sigma) were also run in parallel; the apparent molecular sizes of these proteins are 116 (β-galactosidase), 84, 64, 48, 37 and 30 kD.

All parts were run with identical protein samples.

Part A shows total *C. elegans* proteins stained with Coomassie blue (staining was carried out as described in 2.16).

Part B shows blot probed with monoclonal rat anti-HSP70 and peroxidase-linked anti-rat IgG.

Part C shows blot probed with monoclonal mouse anti-HSP70 and alkaline phosphatase-linked anti-mouse IgG.

Lane 1 - prestained MW markers.

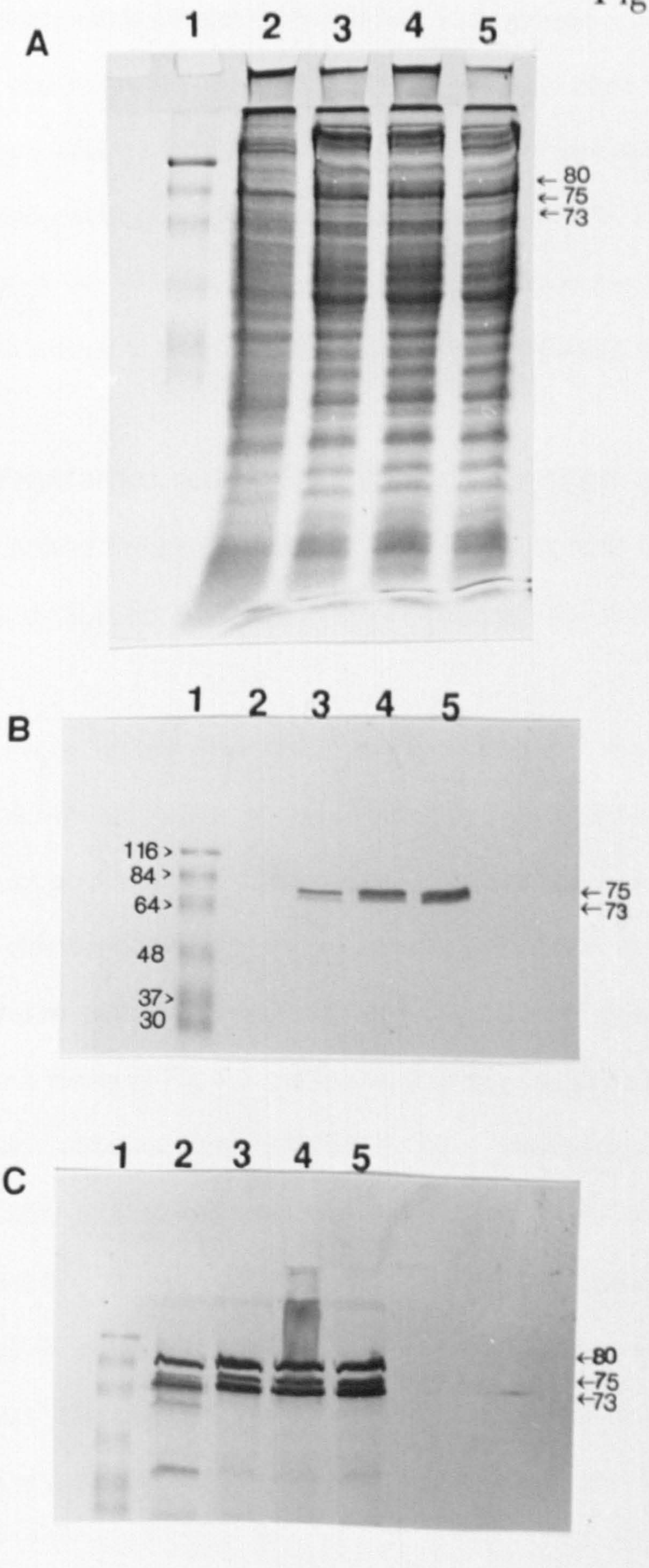
Lane 2 - 20°C control (7 h incubation).

Lane 3 - 31°C control (7 h incubation).

Lane 4 - 31°C + 16 ppm Cd^{++} for 7 h.

Lane 5 - 34°C heat shock for 7 h.

Fig. 4.2



20 and 31°C controls); this is not recognised by the previous rat anti-HSP70. The identity of this larger protein remains unclear. It may well represent a divergent HSP70 family member similiar to the mammalian GRP78 which is larger than HSP70/72. It is interesting to note that Heschl and Baillie (1990a;1990b) have identified a gene encoding GRP78 like protein (possible product is designated as HSP70C) which is constitutively expressed in the nematode. Fig.4.2A shows the Coomassie stained gel on which equivalent amount of samples were run, and the corresponding bands are indicated.

4.3.3 DIFFERENTIAL EXPRESSION OF HSP70 PROTEINS IN *C.ELEGANS* FOLLOWING EXPOSURE TO HEAT AND CADMIUM, AS REVEALED BY USING TWO-DIMENSIONAL SDS-PAGE

The resolution of one-dimensional electrophoresis and blotting is insufficient to distinguish between proteins of similar size belonging to the same family. Using two dimensional gel electrophoresis followed by immunoblotting and detection with a highly sensitive chemiluminescent system, we have resolved each of the doublet bands (73 and 75 kD) shown on the one dimensional blots (Fig.4.2B) into several spots differing in pI value. As is shown in Fig. 4.3, there are at least 12 identified spots, some of which are constitutive and some stress inducible. Arbitrary numbers have been assigned to the major HSP70 spots detected, starting from the first anodal spot of the 75 kD band (designated 1) to the most cathodal spot (designated 8), whereas spots in 73 kD range are designated as spots 9, 10, 11 and 12. This number closely corresponds to the known number of *C. elegans* HSP genes (Heschl and Baillie, 1990a). It should be noted that the identities of spots assigned are based on distances from spot 1, thus the identification

Figure 4.3 Two dimensional blotting analysis of stress-induced HSP70 proteins

30 µg protein samples from wild-type N2 worms were subjected to 2D electrophoresis (IEF followed by SDS-PAGE using 10-16% gradient acrylamide gels; section 2.18), blotting and immunoprobing (with the rat monoclonal anti-HSP70 antibody) and ECL chemiluminescent detection. The last three procedures were carried out as described in section 2.19. Chemiluminescence was detected on ECL Hyperfilm (Amersham) with exposures varying from 2 to 4 minutes.

Part a. worms incubated at 20°C (controls) for 7 h, 4 min exposure.

Part b. worms exposed 7 h at 31°C (sub heat shock), 2 min exposure.

Part c. worms exposed 7 h to 16 ppm Cd^{++} at 31°C, 2 min exposure.

Part d. worms exposed 7 h at 34°C (severe heat shock), 2 min exposure.

Part e. worms exposed 7 h to 16 ppm Cd^{++} at 20°C, 2 min exposure.

Part f shows one dimensional blotting analysis of the same samples. The last two blots (parts e and f) were run on different occasions as compared to the others; 2 min exposure.

Lane 1 - Sigma prestained protein markers with molecular sizes of 190, 125, 88, 68, 56, 38 and 33.5 kD.

Lane 2 - 20°C controls.

Lane 3 - 20°C + 16 ppm Cd^{++}.

Lane 4 - 31°C sub heat shock.

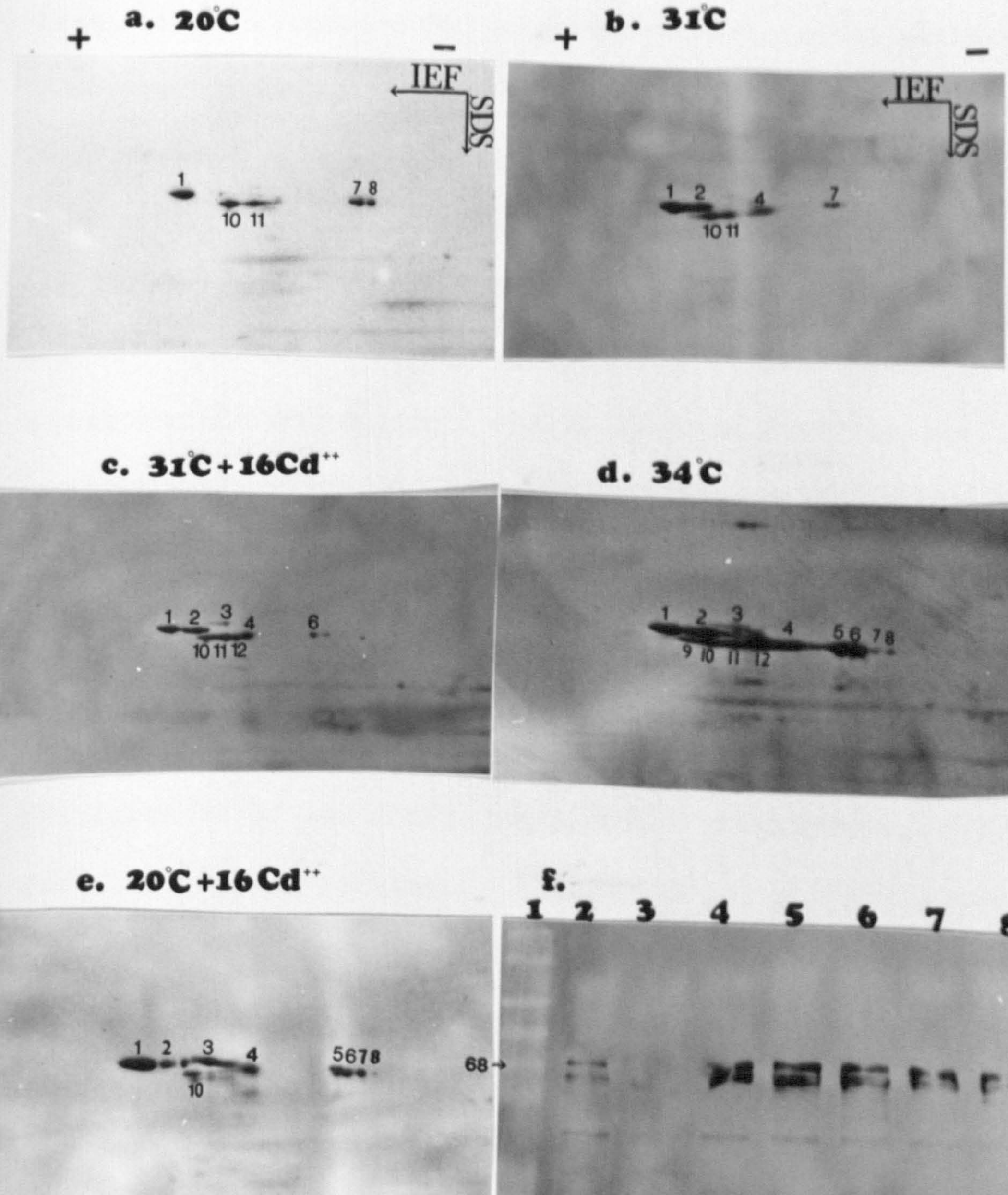
Lane 5 - 31°C + 16 ppm Cd^{++}.

Lane 6 - 34°C heat shock.

Lane 7 - 31°C + 16 ppm Zn^{++}.

Lane 8 - 31°C + 16 ppm Hg^{++}.

Fig. 4.3


a. 20°C
+
−
IEF
SDS
1
10 11
7 8
b. 31°C
+
−
IEF
SDS
1 2
4
7
10 11
c. 31°C + 16Cd++
1 2 3 4
6
10 11 12
d. 34°C
1 2 3 4 5 6 7 8
9 10 11 12
e. 20°C + 16Cd++
1 2 3 4
10
5678
68
f.
1 2 3 4 5 6 7 8

of the most cathodal spots (5-8) remains provisional. It can be seen from Fig.4.3 at least 5 identified HSP70 isoforms (spots 1, 7, 8, 10 and 11) are expressed under control conditions at 20°C (Fig.4.3a), and several others are newly induced following heat and cadmium exposure. Only two newly induced spots (2 and 4; Fig.4.3b) are detectable at the mild heat shock temperature (31°) (as compared with 20°C controls), whereas spot 8 has apparently disappeared. Cd^{++} (16 ppm) at 31°C seems to induce two more additional spots (3 and 6; Fig.4.3c; note that the former spot is slightly larger with molecular size of approx. 78 kD), and spot 12 is also relatively intensified. The pattern of HSP70 spots significantly changes at the severe temperature of 34°C, with the number of strongly induced spots reaching about 6 (spots 2, 3, 4, 5, 6, and 9; Fig.4.3d). Spot 4 is greatly intensified under all stressful conditions, and appears to merge with spot 12 in most cases (contrast Fig.4.3a with Fig.4.3b,c and d). Spots corresponding to the positions of 7 and 8 are present but relatively faint. Furthermore, Fig. 4.3e shows the protein pattern following treatment of worms with 16 ppm Cd^{++} at 20°C (which was run on different occasion in parallel with one dimensional blots, shown in Fig. 4.3e and f); here the spot corresponding to 2 is extremely weak, while spots 3 and 4 are clearly detectable. This suggests that Cd^{++} ions affect the pattern of HSP70 protein spots even at 20°C, although the effect is more pronounced at 31°C (Fig.4.2B).

The one-dimensional immunoblots shown in Fig.4.3f confirm that Cd^{++} ions induce HSP70 expression strongly at 31°C (though not at 20°C), where the bands appear even fainter than in 20°C controls; compare lanes 5 and 2 with 3). The same HSP70 doublet is observed in Zn^{++}- or Hg^{++}-treated worms, although the blot shown does not provide evidence of clear HSP70 induction by these ions (lanes 7 and 8) over that observed in 31°C controls (lane 4).

4.4 DISCUSSION

A comparison between the one- and two-dimensional blots shown in Figs. 4.2 and 4.3 demonstrates that the HSP70 responses to different forms of stress are quite complex and cannot be analysed simply by one-dimensional separations. Many features are revealed only following two-dimensional analysis. Although at least 5 members of the *hsp70* gene family have been cloned and analysed in *C. elegans* (Heschl and Baillie, 1990a;1990b), it remains to be seen whether other genes remain to be discovered. We here demonstrate that increasing levels of stress (34°C as opposed to 31°C) induce a more complex array of HSP70s, while 16 ppm Cd^{++} at 31°C induces several spots, some of which are weak or not present in the 31°C control. Our work further shows that the inducibility of the doublet band (clearly seen on one-dimensional blots in Fig.4.2; see also Fig. 4.3f for comparison) differs in response to different stressors. Thus different stressors and/or degrees of stress may differentially induce particular subsets of HSP70 proteins, and at least part of this diversity probably reflects the known divergences between members of the *C. elegans hsp70* gene family (both in terms of their sequences and in their patterns of constitutive versus stress-inducible expression; Heschl and Baillie, 1990a;1990b). In fact, the differential regulation of *hsp70* gene family has also been observed in *S. cerevisiae* under heat shock conditions, and this was found to be the result of the differential transcription from their *hsp70* promoters (Elwood and Craig, 1984). The authors further indicate that the three yeast *hsp70* genes compared share extremely high homology in their coding sequences, but promoter sequences upstream of these structural genes are not similarly conserved. More recently, Young and Craig (1993) have specifically studied one of these yeast *hsp70* genes (designated as SSA1), which is

expressed at moderate levels under basal conditions and shows a rapid increase in expression following heat shock; this study shows that the differential interaction of HSF with particular members of the multiple HSEs present in the promoter region of the SSA1 gene largely control the differential expression of this gene under both basal and heat-shock conditions. It is, therfore, possible that similar regulatory mechanisms might also be responsible for the differential induction of HSP70 family members in *C. elegans* in response to different stressors.

Many workers have advocated the use of stress-based assays as biomonitors of environmental pollution, and this has been greatly facilitated by the availibility of class-specific anti-HSP antibodies, in particular anti-HSP70s (Sanders, 1993). We here show that significant details of the HSP70 induction pattern can be revealed only by two-dimensional blotting analysis; these details are obscured or lost when analysing by the simpler technique of one dimensional blotting (e.g. Kohler *et al*, 1992; Steinert and Pickwell, 1993; Bradley, 1993). Moreover, two-dimensional blotting has several significant advantages over metabolic labelling, which reveals a complex array of total newly synthesised proteins (e.g. see Adamowicz *et al*, 1991). However, it remains to be seen whether the complex patterns of both constitutive and stress-inducible *hsp70* gene products and the significant technical demands required in two dimensional blotting analysis (shown in this study) will ever rival the simple transgenic approach (described in the next chapter) as a pollution monitoring tool. We therefore suggest that future studies should focus more closely on those *hsp70* gene-family members whose expression is induced by a broad range of toxicant stresses. Reporter gene constructs utilising the promoters of such genes have the potential to generate transgenic strains

which are significantly more sensitive to induction by pollutants than currently available strains (e.g. CB4027, see chapter 5; or the *hsp16*-promoter/*lacZ* strains used by Stringham and Candido, 1994).

CHAPTER 5. EVALUATION OF STRESS-INDUCIBLE TRANSGENIC NEMATODE STRAINS AS BIOLOGICAL INDICATORS OF ENVIRONMENTAL STRESS

5.1 INTRODUCTION

5.1.1 ADVANTAGES OF USING THE NEMATODE *CAENORHABDITIS ELEGANS* IN TOXICITY TESTS

Because of the wealth of descriptive information now available (see section 4.1.1 for a brief description of this organism) and the simplicity of maintaining animal stocks in the laboratory, the free-living nematode *C.elegans* could be an ideal organism for use as a bioindicator of environmental pollution.

There have been a few toxicological studies pointing out the usefulness of *C. elegans* in toxicity testing. For example, Williams and Dusenbery (1988,1990) determined LC50 values for a range of metal ions over 1-4 days of exposure, and have suggested that the nematode could be used to predict mammalian acute lethality to these metals at less than 10% of the cost for comparable tests on vertebrates. Donkin and Dusenbery (1993) have recently developed a new method for recovering nematodes effectively from soils. They have also shown that 24h LC50s for copper ions in soil were close to the corresponding LC50 for a 2-week earthworm soil toxicity test. Thus a 24-hr nematode toxicity test may be comparable to 2-week earthworm test in terms of sensitivity. Van Kessel *et al.* (1989) studied the effects of cadmium chloride on growth and reproduction in *C. elegans*, and

developed a simple short-term bioassay to investigate the toxicity of environmental pollutants on soil organisms.

Some other assays have also been developed to screen for mutagens or carcinogens based on mutant reversion to wild-type size (Lew *et al.*,1983) or motility (mentioned by Mlot,1991); such methods are clearly applicable to environmental toxicants with known genotoxic effects, but would be inappropriate for other classes of toxicant. The striking effects of certain neurotoxins on motility have suggested the possible use of *C. elegans* as a preliminary screen for such agents (Williams and Dusenbery,1987). We ourselves have observed increased motility of *C. elegans* treated with sub-nanomolar concentrations of dioxin (Guven and de Pomerai, data not shown). In the present work, by taking the advantage of this well defined organism, we have utilised a stress-inducible nematode strain for a rapid aquatic toxicity testing.

5.1.2 TRANSGENIC ORGANISMS AS TOXICOLOGICAL INDICATORS

In recent years, genetic engineering has generated a wide range of transgenic animals, mostly using recombinant DNA techniques to construct gene fusions in these organisms. In this type of work, the regulatory region of interest is fused to a second, easily assayable reporter gene. *lacZ* fusion constructs are extremely popular, because both quantitative and *in situ* localisation studies of the reporter product are simple to carry out in a wide range of organisms, from yeast to mammalian cells (Kirk and Piper,1991; Fire,1986;1992; Hall *et al.*,1983; Fire *et al.*,1990; Draber *et al.*,1992). Transgenic organisms generated in this way are used mainly for molecular studies of gene expression

and regulation (Hope, 1994). Recently, the utility of such transgenic organisms as toxicological indicators has attracted some attention (see e.g. Anderson,1989). We have used established transgenic strains (CB4027 and CB4028; Fire,1986) of *C. elegans*, carrying integrated copies of a stress-inducible reporter gene, as indicators of environmental contamination. This construct comprises a *Drosophila hsp70* promoter fused to an *E. coli lacZ* structural gene encoding β-galactosidase (Fig. 5.1).

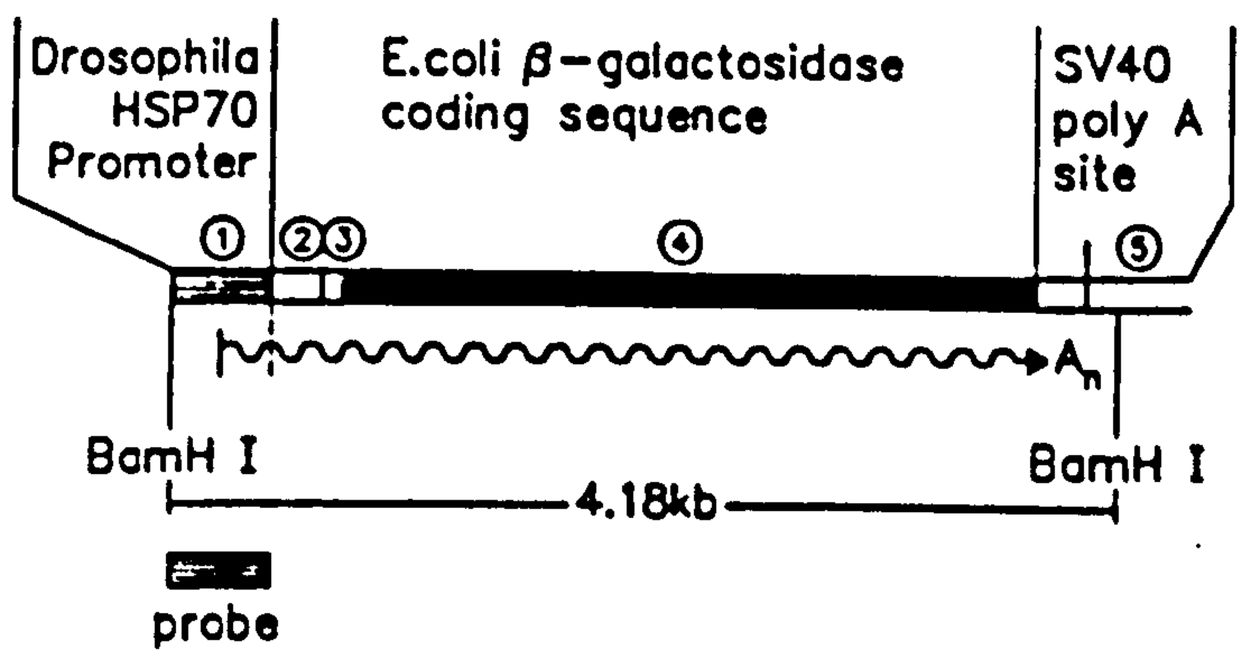


Fig. 5.1 Map of plasmid pShZ1 showing fragment used as a probe of the sequences derived from the *Drosophila* HSP70 promoter element. This fusion construct carries a *Drosophila* HSP70 promoter (designated as 1) and an *E. coli lacZ* reporter gene (designated as 4), as well as the SV40 polyadenylation regions (5) (from Fire, 1986).

Generally, clustered heat-shock elements (HSEs) present in *hsp* promoter sequences direct transcriptional activation of the associated hsp genes (Pelham, 1987; Lis and Wu, 1993). This occurs following heat shock or during exposure to certain other kinds of environmental stress (reviewed by Bienz,1985; Sorger,1991; Lis and Wu, 1993; see also sections 1.6). Under such conditions, the HSEs are bound by an activated (oligomeric) form of the heat shock transcription factor (HSF; Zimarino and Wu,1987; Zimarino *et*

al.,1990; Westwood and Wu, 1993; see section 1.6), which can then switch on *hsp* gene transcription. HSF protein is present at all times in non-stressed metazoan cells, but rapidly oligomerises, translocates into the nucleus and binds to DNA following its post-translational activation by stress (Westwood *et al.*,1991). The HSE consensus sequences and their spacing are well conserved among the heat-inducible *hsp* genes analysed to date, but this is less true of the HSF protein sequence. Nevertheless, many HSFs can efficiently activate heterologous *hsp* promoters, implying conservation of at least the DNA-binding domain. As a case in point, the *Drosophila hsp70* promoter used in constructing the transgenic *C. elegans* strains used in this study is clearly recognised by the nematode's endogenous HSF, so transactivating *lacZ* gene expression (Fire,1986); this happens under the same conditions that switch on the nematode's own heat-inducible *hsp70* genes (Snutch and Baillie,1983; Heschl and Baillie,1990a; see also chapter 4).

There have been several other reports on the construction of fusions between the HSE and *lacZ* genes. For example, *HSE-lacZ* constructs in yeast have been used to characterise *HSE-lacZ* activity in *S. cerevisae* cells of different physiological states and differing genetic backgrounds (Kirk and Piper,1991), and to determine differential regulation of the three *hsp70* gene and related genes in *S. cerevisae* following heat shock treatment (Ellwood and Craig, 1984). Stringham *et al.* (1992) have examined the expression of the *hsp16* gene family in *C. elegans* by introducing *hsp16-lacZ* fusions in to this organism; in this study, the nematode's own *hsp16* promoter is fused to the reporter gene *lacZ*. Furthermore, the effect of abnormal proteins on the stress response was directly studied by monitoring the expression of an *hsp70-lacZ* fusion in *Xenopus* oocytes following injection with native or denatured proteins (Ananthan *et al.*,1986) Organisms carrying

such stress-inducible gene constructs could potentially provide an early warning of environmental stress (Anderson,1989; Miller,1989), but clearly both the system's sensitivity and the range of effective inducers must first be carefully evaluated. In the present study, we show that several environmentally relevant heavy metal ions and other toxicants, as well as heat shock, induce β-galactosidase expression in the transgenic *C.elegans* strain, CB4027. Our results suggest: (i) that care is required to optimise the assay conditions for maximal sensitivity; (ii) that cadmium is a far more effective inducer than any other toxicant tested; and (iii) that some toxic agents induce detectable stress responses only across a certain range of concentrations, with negative results from above as well as below this range. These findings underline the need for careful characterisation of any transgenic organism if it is to be used for environmental monitoring, and suggest that any such use should be as an adjunct to existing methods.

5.2 MATERIALS AND METHODS

(i) Growing *C. elegans* cultures

Growing and maintaining the nematode cultures were as described in section 4.2. Worms were routinely used after 4 or 5 days, by which time responsive adults tend to predominate over less-responsive larvae. Because up to 20% of the total worm mass in our mixed cultures comprised larvae expressing no detectable transgene activity under most conditions tested, the induction values are somewhat lower than those expected from adults-only cultures. The procedures for preparing worms for the toxicant exposure in K-medium are described in section 2.4.

(ii) Exposure of nematodes to heat and toxicants

Exposure of worms grown at 15 or 20°C to metal ions and other toxicants was carried out as described in section 2.4. The chosen heavy metal concentrations ($mg.L^{-1}$ for the metallic ion, i.e. ppm) were in the range of the LC50 values for wild-type *C. elegans* over a 24 to 96 h period (Williams and Dusenbery, 1990). However, for organic pollutans, initial range finding experiments were performed using order-of-magnitude differences in concentration; in cases where ethanol was used to solubilise the toxicant, the final solvent concentration did not exceed 0.1% (v/v), and controls included ethanol alone at the same concentration.

(iii) Determination of metal contents of tissue samples and supernatants, and β-

galactosidase assays were carried out as described in sections 2.6 and 2.9, respectively.

(iv) Histochemical detection of β-galactosidase and the assessment protocol for scoring stained worms

Histochemical detection of the reporter gene product was performed mainly using the method described by Fire (1986). Stained worms mounted in 60% (v/v) glycerol and photographed under direct light on a Leitz inverted phase microscope. To compare the degree of staining between different runs, we have adopted a simple point system which gives a fairly consistent gap between positive and negative controls (16 ppm Cd^{++} and 32°C alone, respectively). We assign arbitrary intensity values of 0 for unstained worms, 2 for pink/red staining and 4 for intense purple/blue staining. Since limited embryo staining within worms can occur even in 32°C controls, this is assigned as a location value of 1, as compared to values of 2 for staining in the phraynx (which is never seen in 32°C controls), and of 3 for staining in both embryos and pharynx or in the entire worm. We assess all adult worms present (generally 30-50) in each of several microscope fields examined; each worm is assigned a value for staining intensity (SI) and for staining location (SL). For each of the staining classes we derive a score by multiplying its frequency x SL x SI. These scores are then added together and divided by the total number of worms assessed. Because it is possible that some worms might be killed by the toxicant before transgene expression becomes detectable, we divide the final staining score by the proportion of worms left alive at the end of the assay. For most conditions, less than 10% of the worms were dead after the incubation time; if high mortality (> 20%) was observed, the experiment was always repeated using lower concentrations of

toxicant.

(v) SDS-PAGE and Western blotting

Protein samples were run on 0.1% SDS-PAGE gels containing 7% acrylamide as described by Laemmli (1970) and blotted onto Biotrace (Gelman) membranes according to the method of Towbin *et al* (1979). Both methods are described in sections 2.15 and 2.17, respectively. Blots were either stained histochemically as in section 2.16.1 or else probed for 3 h at room temperature with a rabbit polyclonal antibody (a gift from Dr. P.Tighe of this department) against β-galactosidase (1:1000 in TBS-T) followed by extensive washing and then treated with peroxidase-linked anti-rabbit IgG (0.1% in TBS-T) as described in section 2.17.

5.3 RESULTS

5.3.1 CHARACTERIZATION OF A STRESS-INDUCIBLE TRANSGENIC NEMATODE STRAIN (CB4027) EXPOSED TO ELEVATED TEMPERATURES AND TO VARIOUS CONCENTRATIONS OF HEAVY METAL IONS

We have initially started to study two transgenic *hsp70-lacZ* lines (CB4027 and CB4028) of the nematode *C. elegans*. The CB4027 line was found to respond to the stressors more effectively, as monitored by β-galactosidase assays or *in situ* staining. Therefore this transgenic strain was used throughout the study described here.

(a) The effects of temperature on *HSE-lacZ* reporter transgene

The heat shock response of wild type *C. elegans* has been studied, and the number of HSPs induced by heat shock is well defined (Snutch and Baillie,1983; Heschl and Baillie,1990a; Hockertz *et al.*,1991). *C. elegans* is unusual in that a large rise in ambient temperature is required to induce a full heat shock response; for worms grown at 20°C, optimal induction of hsp70 occurs only at 34°C (Snutch and Baillie,1983). The transgenic *hsp-lacZ* strain was grown at 15°C, but transgene expression shows a similar heat-induction profile to that of the endogenous *hsp70* gene (see Fig. 4.2). Fig. 5.2 shows the effects of temperature on β-galactosidase activity in transgenic worms exposed in two different media. Transgene expression is minimal up to 32°C (see also Fig. 5.5a), with significant induction at 33°C rising to about 10-fold above controls at 34°C.

Heat induction of transgene activity is slightly more effective for worms exposed on agar

Fig.5.2

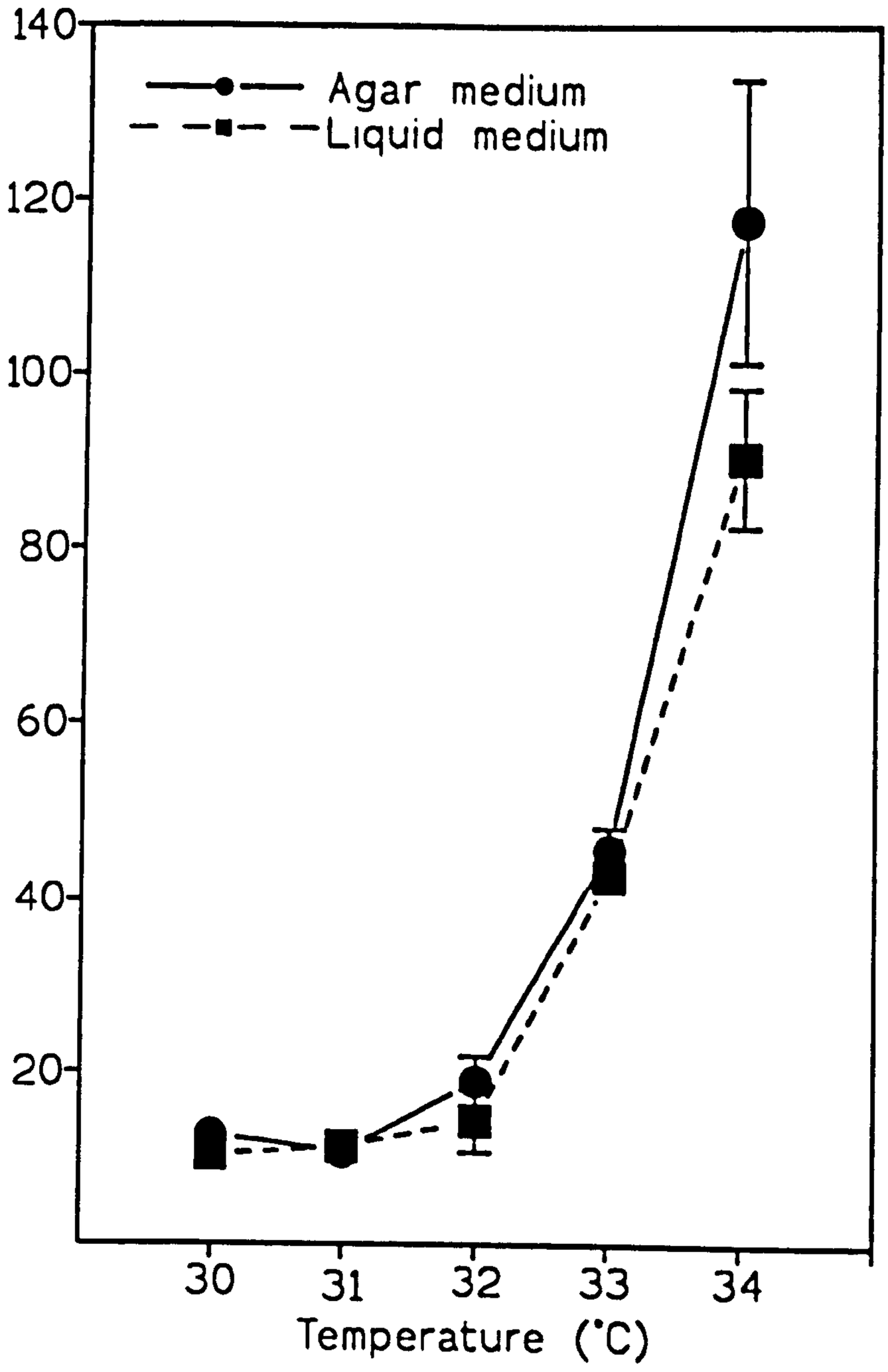


Figure 5.2 Heat induction of β-galactosidase activity.

Worms were grown at 15°C and then exposed to temperatures of 30-34°C for 7 h in liquid medium or on agar plates. β-galactosidase assays and determination of protein concentrations were carried out as described in chapter 2 sections 9 and 11, respectively. The mean and standard deviation (bar) from at least 4 determinations are shown.

plates as compared to those in liquid medium. The growth temperature of 15°C was chosen for two main reasons; first, we found unexpectedly that the level of β-galactosidase expression at 32°C is actually lower for nematodes maintained at 15°C as opposed to 20°C (see control values in Fig. 5.6); second, growth at 15°C seems to give a much tighter stress induction curve , as compared to worms grown at 20°C (see Fig. 5.6).

(b) Reporter transgene expression in the nematode *C. elegans* following exposure to heavy metal ions at elevated temperatures

The nematodes were initially exposed to several heavy metal ions (e.g. Cd, Zn and Cu) across a range of concentrations at 20°C. We found that these heavy metals alone at 15°C or 20°C do not detectably induce β-galactosidase, but if an accompanying temperature of 29-32°C is used during heavy metal exposure over a 7 h period, then dose-dependent transgene expression is observed. The maximum exposure temperature at which most control worms remained unstained following histochemical staining for the reporter gene product, β-galactosidase is 32°C (Fig. 5.5a). As is also shown in section 4.3 (Fig.4.2), Cd^{++} induction of endogenous HSP70 is also significantly enhanced at a sub-heat shock temperature of 32°C. Cd^{++} was found to be the strongest inducer of transgene activity among ten or more heavy metals tested, and therefore *C. elegans* was mainly exposed to this particular metal ion in the following experiments.

(c) The effects of two different media on cadmium-induced transgene expression

Aquatic and agar media were compared as regards to β-galactosidase induction in the worms exposed to the heavy metal ion, Cd^{++} (Fig.5.3). Unlike heat shock treatment, the transgene induction caused by cadmium ions was much higher in liquid K-medium as opposed to agar cultures (e.g. at 16 ppm Cd^{++}, β-galactosidase activity in aquatic medium was almost 3-fold higher than in agar plates). In the case of agar cultures, the bacterial lawn was on the surface and the worms resided in a thin film of K-medium; it follows that metal ions will be absorbed into the agar and taken up by bacterial cells. For this reason, all subsequent toxicant treatments were carried out in liquid K-medium, with only a small proportion of bacteria present (see section 2.4).

(d) Investigation of the time course of cadmium induction

The transgenic worms grown at 15°C were exposed to cadmium ions at 32°C over 4, 7 and 12 h. Fig. 5.4 shows that β-galactosidase induction by cadmium is highest over a 7 h period as compared to either 4 or 12 h exposures. It should be noted that a large number of worms (mainly adults) are found dead following 12 h exposures, and this is the most likely reason for the fall in transgene product activity between 7 and 12 h. Therefore, our standard assay conditions for this study involved toxicant treatment for 7 h at 32°C.

Figure 5.3 **The effects of aquatic and agar media on cadmium-induced β-galactosidase activity**

The determination of both β-galactosidase activity and protein content was as the same as for Fig. 5.2. Each data point shown in the Figure represents the mean of two replicates. In this experiment, the worms were grown at 20°C and then exposed to cadmium at 32°C.

Figure 5.4 **Time course of cadmium-induced transgene expression**

The worms grown at 15°C were exposed to Cd^{++} concentrations (0-16 ppm) over 4, 7 and 12 h at 32°C. The mean value for each data point is derived from three determinations. A large number (≥ 40%) of worms were dead after 12 h of exposure.

Fig.5.3

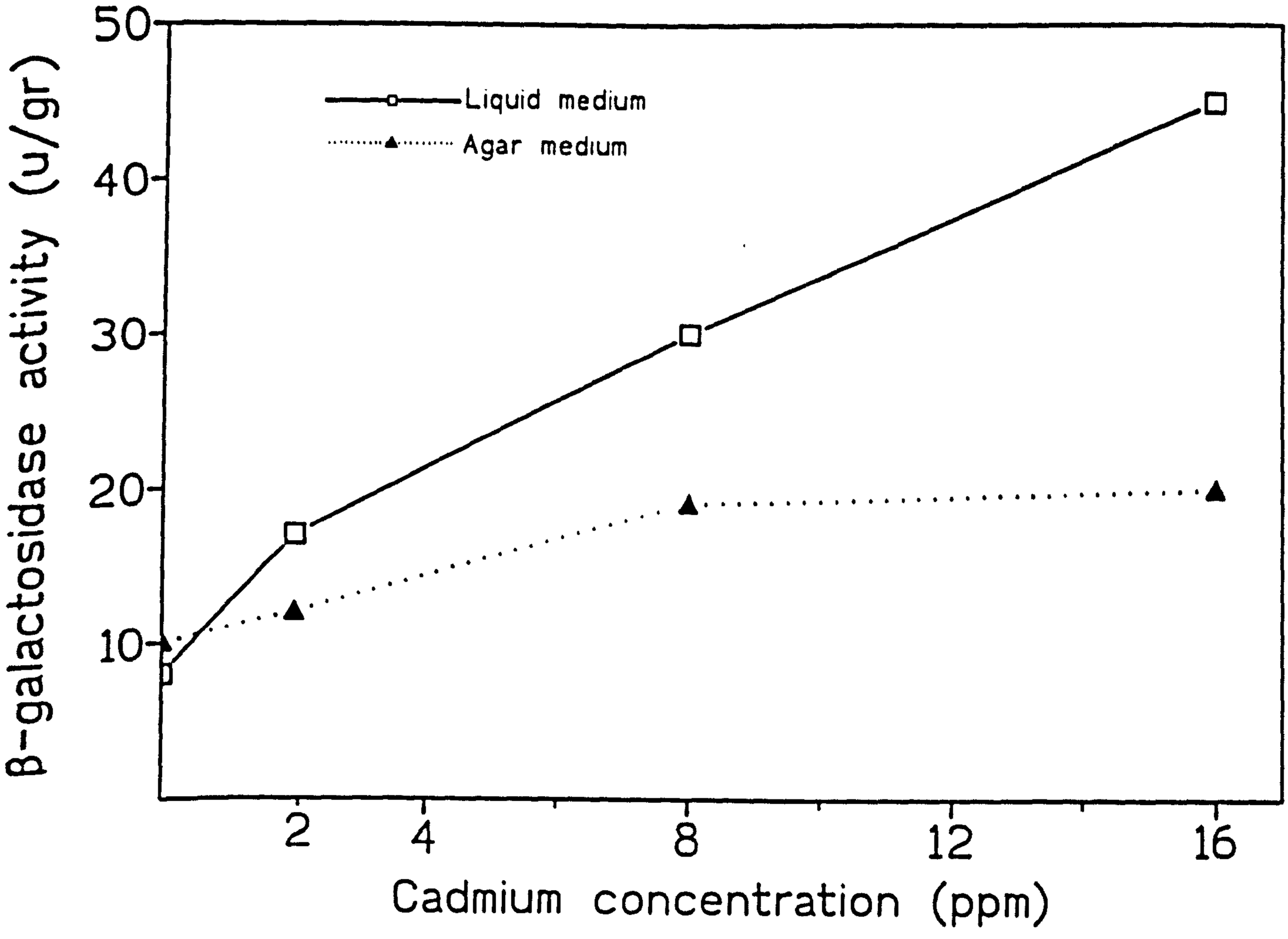
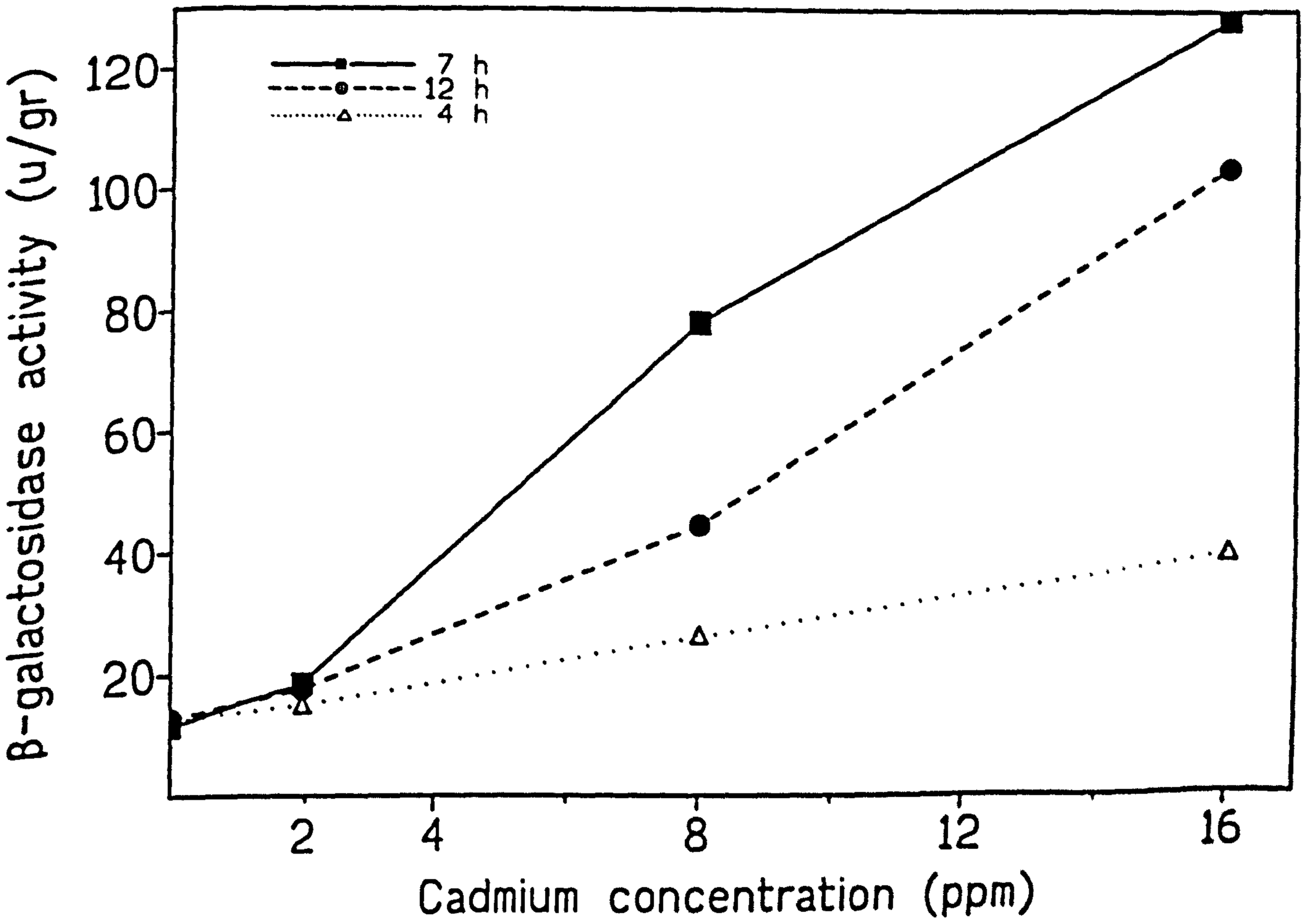

Liquid medium
Agar medium
β-galactosidase activity (u/gr)
50
40
30
20
10
2
4
8
12
16
Cadmium concentration (ppm)

Fig.5.4


7 h
12 h
4 h
β-galactosidase activity (u/gr)
120
100
80
60
40
20
2
4
8
12
16
Cadmium concentration (ppm)

(e) Developmental stage-specific stress response in the nematodes

Heavy metal ions such as Cd^{++} apparently act by aggravating the stress already caused by the incubation temperature of 32°C. This can be clearly seen in Fig.5.5; whereas control worms are unstained at 32°C (Fig.5.5a), they are strongly stained throughout at 34°C (Fig.5.5i). At 33°C, adults show moderate staining but larvae remain unstained (Fig.5.5g). However, exposure of worms to 2 ppm Cd^{++} at 33°C results in strong staining of larvae as well as adults (Fig.5.5h; compare with 2 ppm Cd^{++} at 32°C in Fig.5.5c), also note that the smaller larvae remain unstained even in 16 ppm Cd^{++} at 32°C (Fig.5.5f).

We suggest that both the heat (32 or 33°C) and heavy metal stress co-operatively activate the endogenous HSF to switch on both *hsp* genes and the *lacZ* transgene, by binding to HSEs. Snutch and Baillie (1983) have previously showed that HSP induction by heat shock was not stage-dependent, since all developmental stages showed similar protein patterns following electrophoretic analaysis of samples. However, the results discussed above indicate the induction of developmental stage-specific stress responses by cadmium in *C. elegans*. Possible reasons for this difference are discussed later.

Figure 5.5 ***In situ*** **histochemical staining of transgenic worms**

Histochemical staining of acetone-fixed worms was performed as described in section 2.7. The stained worms were photographed using an inverted Leitz photomicroscope. Heat and toxicant treatment was for 7 h in all cases.

(a) 32°C control; no staining (red embryos occur rarely).

(b) 32°C + 0.5 ppm Cd^{++}; some pharyngeal staining (solid arrow).

(c) 32°C + 2 ppm Cd^{++}; stronger phryngeal staining in many adults.

(d) 32°C + 8 ppm Cd^{++}; strong staining in and beyond pharynx.

(e) 32°C + 16 ppm Cd^{++}; strong staining throughout in most adults.

(f) 32°C + 16 ppm Cd^{++}; weaker staining in large (L4) larvae, but none in smaller (L1/L2) larvae (open arrows; contrast with part h).

(g) 33°C heat; staining in adults but not larvae (open arrow).

(h) 33°C + 2 ppm Cd^{++}; small (L1/2) larvae as well as adults are are strongly stained throughout (solid arrow); compare with parts c and g.

(i) 34°C heat shock; strong staining throughout most worms.

(j) 32°C + 2 ppm Zn^{++}; slight phryngeal staining.

(k) 32°C + 16 ppm Zn^{++}; strong pharyngeal staining in most adults.

(l) 32°C + 16 ppm Hg^{++}; strong pharyngeal staining in most adults.

(m) 32°C + 50 ppm Sn^{++}; some staining in pharynx and embryos.

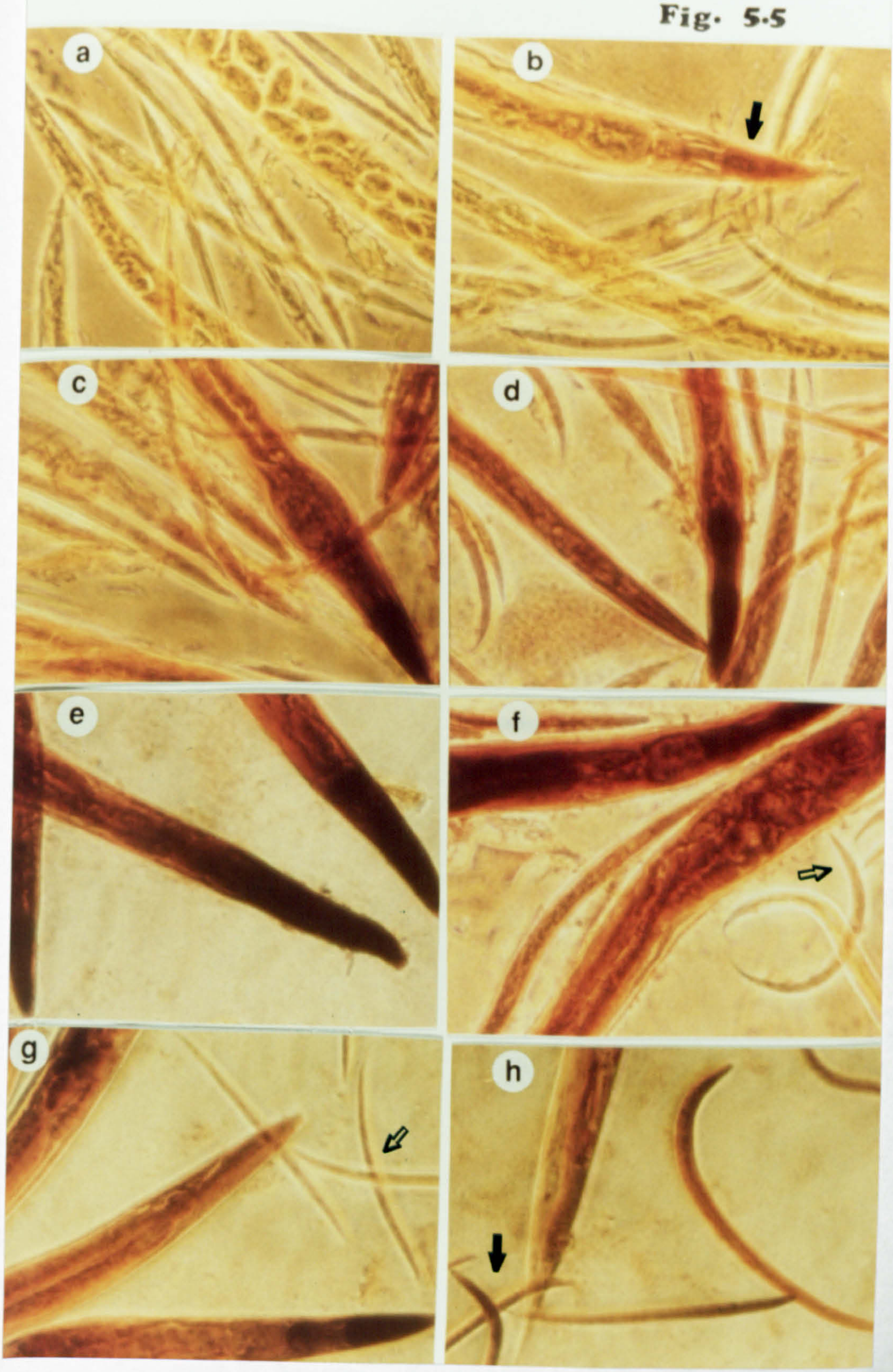
(n) 32°C + 10 ppm Mn^{++}; moderate staining of embryos in adults.

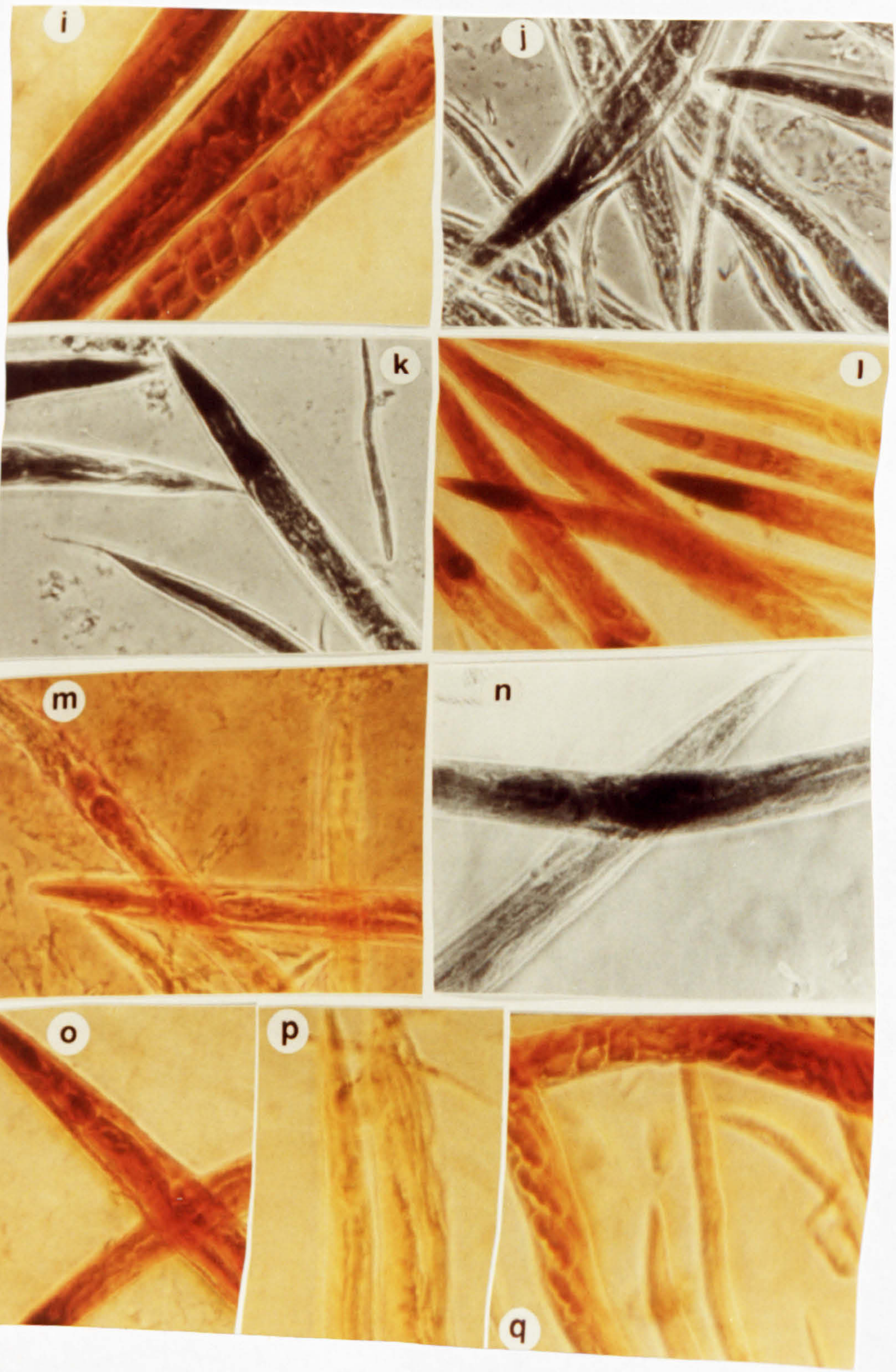
(o) 32°C + 50 ppm Ag^{+}; pharyngeal staining in some adults.

(p) 32°C + 100 ppm Ag^{+}; no staining at all.

(q) 32°C + 50 ppb TBT; red staining in most worms.

Fig. 5.5




i
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5.3.2 THE EFFECTS OF LOWER GROWTH TEMPERATURES ON THE HEAVY METAL-INDUCED β-GALACTOSIDASE ACTIVITY IN TRANSGENIC NEMATODES

The normal growth temperature for wild type worms is 15-25°C. As previously mentioned, cadmium alone at 15 or 20°C does not detectably induce β-galactosidase, but if the temperature is raised to 32°C during the heavy metal exposure over 7 h period, then concentration dependent transgene expression is observed (Fig. 5.6; Fig. 5.5a-e; Table 5.2A). As shown in Fig. 5.6, background expression due to heat (32°C) alone is significantly lower for worms grown at 15°C, in comparison to those grown at 20°C ($p < 0.005$). It further shows that the effect of increased cadmium concentrations on β-galactosidase expression is significantly higher for worms grown at the lower temperature. Apart from the crossover point at 2 ppm Cd^{++}, where β-galactosidase activities are virtually identical, the two curves shown in Fig. 5.6 differ significantly ($p < 0.005$) throughout. The transgene induction by 16 ppm Cd^{++} is as much as 14-fold over controls for worms grown at 15°C, as compared to only 3-fold induction for those grown at 20°C (see the insert histogram in Fig. 5.6, where the data are normalised to the respective 32°C control values as 100%).

We have also investigated the effect of growth temperature on cadmium accumulation by the worms. As is shown in Table 5.1A, the results are quite interesting; worms grown at 20°C accumulate similar levels of Cd^{++} whether exposure takes place at 15, 20 or 32°C. However, worms grown at 15°C accumulate less Cd^{++} when exposed at 15 or 20°C, but Cd^{++} accumulation is increased > 2-fold during exposure at 32°C. Probably,

this is the reason why Cd^{++} induction of transgene activity is higher for worms grown at 15°C rather than 20°C. The controls included in Table 5.1A show little (< 2-fold) accumulation of Cd^{++} by dead worms, and suggest that much of the Cd^{++} (> 3/4 of total) within live worms remains in the soluble fraction after homogenisation.

As a further check, we have shown that following standard exposures of worms to heavy metals at 32°C for 7 h, the concentration of metal ion in the medium decreases by 12.97 ± 3.01% over the assay period, irrespective of the metal ion or its initial concentration. This figure represents metal accumulation within worms, as well as adsorption or non-specific binding to worm cuticles, remaining bacterial cells and plastic dishes (see Table 5.1B). Thus it is clear that the worms are exposed to more than 80% of the original test concentration of metal ion for the duration of the assay period.

Fig. 5.6

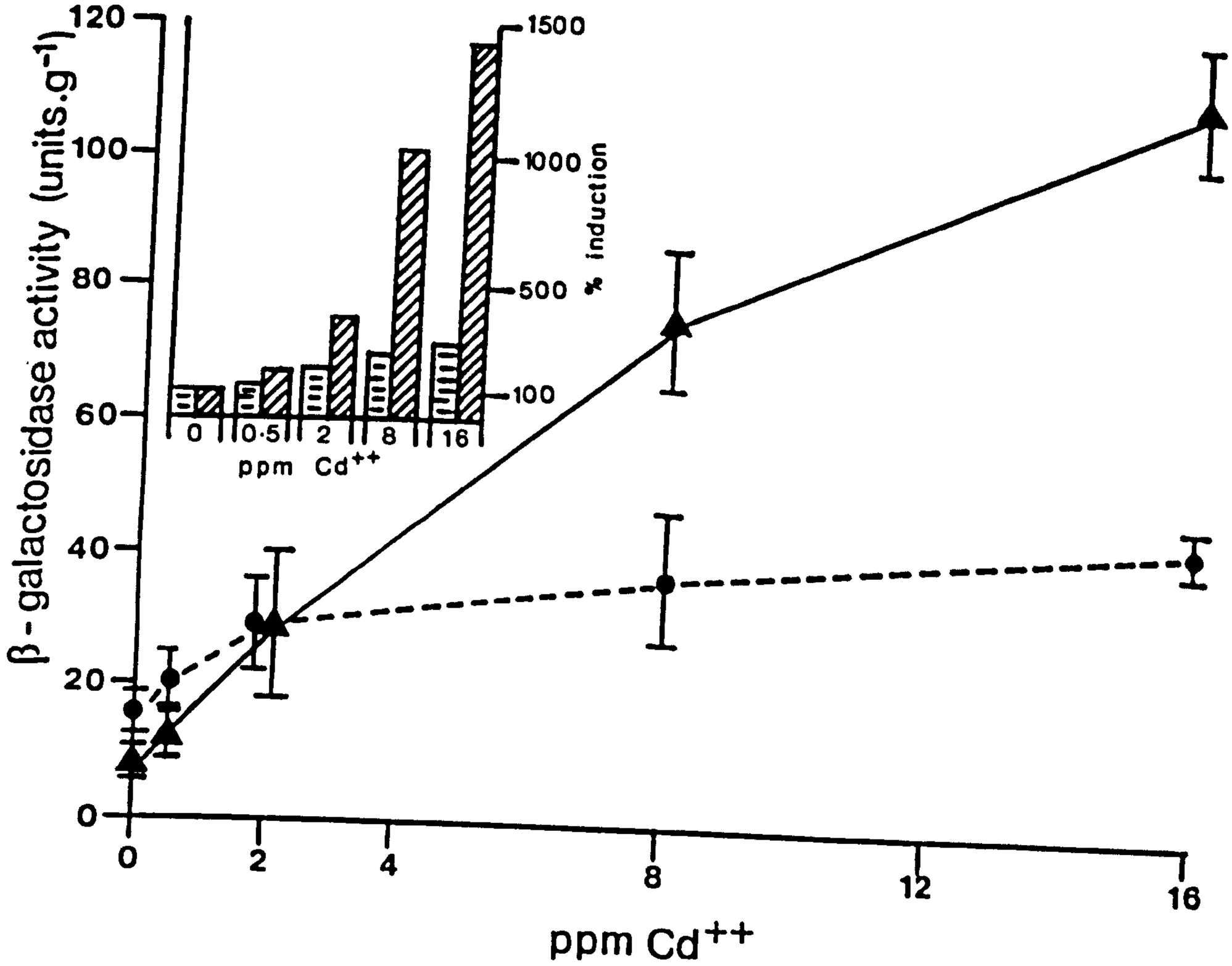


Figure 5.6 Cadmium induction of β-galactosidase activity in transgenic worms grown at different temperatures

The main Figure shows the mean β-galactosidase activity (± standard error) determined for worms exposed to Cd^{++} concentrations, ranging from 0 to 16 ppm, for 7 h at at 32°C. β-galactosidase activity and protein content were determined as described in sections 2.9 and 2.11, so as to calculate the specific enzyme activity in units g^{-1}. The mean and standard error (bar) for each data point were determined from at least 6 replicates. Results are compared between worms grown at 20°C (solid circles, dashed line) and those grown at 15°C (solid triangles, unbroken line). The insert Figure shows the same data normalised to the respective control values (32°C, in the absence of Cd^{++}) as 100%. Hatched bars represents worms grown at 15°C, while dashed bars are for worms grown at 20°C.

Table 5.1 Worms were grown at 15°C (except 20°C in top line of Table 5.1A), and exposed to the indicated metals for 7 h under standard conditions at 32°C unless otherwise indicated (Table 5.1A). All metal concentrations were determined by AAS, using a graphite furnace in the case of tin, as described in section 2.6. Each entry in Table 5.1B represents the mean of 3 determinations on assay supernatants relative to the initial test solutions; an overall mean ± SEM for relevant data on percentage losses is given as the final entry. After exposure, worms were pelleted, washed and prepared for AAS as desribed in section 2.9. In a series of control determinations, we found that the dry mass of worms represents on average 12.05% of the wet weight of pelleted worms after removal of all visible supernatant. Therefore, apparent concentrations of metal per unit dry weight have been divided by 8.3 to obtain concentrations per unit wet weight, which can be more readily compared to the initial concentrations in the external medium (Table 5.A and C). Apparent accumulation by the worm pellet is roughly estimated by dividing the concentration in wet worms by the appropriate external concentration. Since it is possible that metal ions might be bound nonspecifically by the nematode cuticle or bacterial cells as well as being taken up into worm tissues, we have included the controls shown in the last 2 lines of Table 5.1A. Both controls indicate that such sources of error do not affect our results significantly. For Table 1 parts A and C, each entry gives the mean + SEM values from 4-8 determinations. The Sn determinations in Table 1C were carried out by Mr A. Fairburn of the analysis unit in the department of Animal and Plant Sciences, Sheffield University.

Table 5.1 Metal contents of worms and supernatants.

5.1A Effect of growth temperature on cadmium accumulation by worms.

Worms grown at:	Exposed 7h at:	ppm Cd^{++} in test medium	ppm Cd^{++} in wet worms	Mean accumulation
20°C	15 / 20 / 32°C	16	408 ± 52	25.5-fold
15°C	15 / 20°C	16	280 ± 18	17.5-fold
15°C	32°C	16	614 ± 9	38.4-fold
Dead worms	32°C	16	29 ± 6	1.83-fold
Homogenised worm pellet	32°C	16	179 ± 25	11.2-fold

5.1B Concentrations of metal ions in medium before and after standard assays.

	ppm metal before assay	ppm metal after assay	Percentage loss of metal ion
Mercury (Hg^{++})	1.0	0.938	9.1%
Mercury (Hg^{++})	5.0	4.27	16.3%
Zinc (Zn^{++})	1.0	0.882	11.8%
Zinc (Zn^{++})	5.0	4.17	16.5%
Cadmium (Cd^{++})	1.0	0.875	12.5%
Cadmium (Cd^{++})	5.0	4.52	9.7%
Cadmium (Cd^{++})	16.0	13.62	14.9%
Mean for all metals and concentrations	-	-	12.97 ± 3.01%

5.1C Accumulation by worms of inorganic tin versus tributyltin chloride.

Test Toxicant	ppm of metal in test medium	ppm of metal in wet worm pellet	Mean accumulation
Sn^{++}	5.0	44.8 ± 10.66	8.95-fold
Sn^{++}	0.25	2.13 ± 0.92	8.51-fold
Sn^{++}	0.05	0.49 ± 0.14	9.89-fold
Tributyltin-Cl	0.25	32.6 ± 1.15	130.4-fold
Tributyltin-Cl	0.05	6.13 ± 0.144	122.6-fold
Tributyltin-Cl	0.005	1.09 ± 0.036	217.1-fold

5.3.3 THE HSE-*LACZ* TRANSGENE IS INDUCED BY A VARIETY OF OTHER TOXICANTS INCLUDING HEAVY METALS, ORGANOMETALLIC AND ORGANIC COMPOUNDS, AS WELL AS A PESTICIDE

As can be seen from Table 5.2 parts A and B, two criteria are used for assessing transgene induction under a wide range of experimental conditions. One is based on quantitating the activity of the reporter β-galactosidase gene, and the other is a scoring protocol for *in situ* histochemical staining which gives a fairly consistent gap between positive and negative controls (16 ppm Cd^{++} and 32°C alone, respectively). A detailed description of this scoring protocol is given in section 2 (iv). This simple points system has been adopted for two reasons ; firstly, it has the advantage of requiring less time and fewer worms, and secondly, this protocol is applicable to several toxicants whose effects on transgene activity are not clearly detectable by β-galactosidase assays. Using this system, the mean score for controls over a series of at least 10 runs was 0.65 ± 0.17 (SEM), whereas for 16 ppm Cd^{++}, the corresponding score was 7.98 ± 0.44. This suggests approximately 12-fold induction by 16 ppm Cd^{++} over controls, which is close to the previous 14-fold induction of transgene enzyme activity (see Fig. 5.6 insert). We subtract the control score from the corresponding 16 ppm Cd^{++} score to obtain a consistent gap score of 7.34 ± 0.37 (Table 5.2A). The use of gap scores overcomes the need to quote separate control values for each run. We then used the same approach to obtain a mean gap score of 2.72 ± 0.25 for 2 ppm Cd^{++}, which represents a 4-fold induction over controls; the corresponding value for β-galactosidase induction is 3.8-fold (Fig. 5.6 insert). Since the staining patterns given by 2 ppm and 16 ppm are quite different (Fig. 5.5c versus e and f), our scoring system seems broadly to reflect genuine β-galactosidase

activity. As is shown in Table 5.2A and B, this is confirmed by the general agreement between gap scores and β-galactosidase activities for several other toxicants. It was found that mean gap scores from independent observers assessing the same set of stained worms differed by around 10%. Typical gap scores for a variety of toxicants are assessed, and tabulated in Table 5.2 parts A and B, together with the corresponding mean β-galactosidase activities where available.

(a) The effects of Group 2B metal ions on transgene induction

Using the data obtained from both β-galactosidase assays and the scoring protocol, we have compared the effects of Group 2B metal ions (Cd^{++}, Zn^{++} and Hg^{++}) as inducers of the transgene activity (Table 5.2A; and also see Fig.5.5e, k and l). We find that Zn^{++} is the least effective of the three, while Hg^{++} is comparable in effectiveness with Cd^{++} at 0.5 ppm, about 50% as effective at 2 ppm, and only 14% as effective at 16 ppm. We note that Cd^{++} is toxic to *C. elegans* over prolonged periods, with a high 24 h LC50 value of 904 ppm decreasing to 22 ppm after 48 h and to 1.5 ppm after 72 h, whereas the relevant 24 and 48 h LC50 values are 202 and 2 ppm for Zn^{++}, 10 and 3 ppm for Hg^{++} (Williams and Dusenbery, 1990).

(b) The effects of other metal ions and organic and organo-metallic compounds

Table 5.2B shows the effects of other heavy metal ions and organic and organometallic compounds as inducers of transgene expression. Ag^{+} seems to be one of the better heavy

Table 5.2 Worms were exposed for 7 h to the indicated concentrations of each test toxicant under standard conditions at 32°C. Staining gap scores are given for all entries; where available, corresponding β-galactosidase activities (net units per gram above 32°C controls) are quoted within square brackets. Histochemical staining was performed as described in section 2.7 and stain-scores (relative to parallel controls without toxicant) were assessed as described in sections 5.2(iv) and 5.3.3. Each entry gives the mean of 4 to 8 such gap scores assessed on 2 or more occasions. Larger batches of toxicant-treated worms were processed for β-galactosidase assays as described in section 2.9; each value quoted in Table 2A and within square brackets in Table 2B represents the mean net activity (i.e. greater than parallel 32°C controls) in units per gram derived from 4-10 such assays. The SEM values are shown for the cadmium data in Fig.5.6. Generally speaking, there is good agreement between the staining gap scores and β-galactosidase activities recorded following different toxicant treatments. Entries marked Tox showed substantial (>20%) mortality among treated worms, which may reduce the staining gap scores.

Table 5.2 Gap scores and ß-galactosidase activities induced by various toxicants.

2A. Comparison of the zinc, cadmium and mercury induction of the transgene.

Test Toxicant	Gap score 0.15 ppm	ß-gal (u/g) 0.15 ppm	Gap score 0.5 ppm	ß-gal (u/g) 0.5 ppm	Gap score 2.0 ppm	ß-gal (u/g) 2.0 ppm	Gap score 16 ppm	ß-gal (u/g) 16 ppm
Zinc	0	0	0.24	0	1.54	4.8	2.64	7.93
Cadmium	0.23	0	0.86	2.25	2.07 ±.22	11.04	7.34 ±.37	92.8
Mercury	0.20	0	0.94	3.20	2.21	6.05	2.96	12.8

2B. Other toxicants as inducers of transgene expression.

Concentration of test toxicant in ppm or ppb.

Test Toxicant	100 ppm	50 ppm	20 ppm	10 ppm	2 ppm	500 ppb	150 ppb	50 ppb	15 ppb	5 ppb
Ag^{+}	-0.5 Tox?	3.5 [32]	2.7	2.4 [14]	1.0 [4.3]	0.8	0.5	0.1	ND	ND
Sn^{++}	ND	2.2 [7.4]	1.7	1.2	0.7 [1.7]	ND	0.3	0	ND	ND
Mn^{++}	ND	2.4 [9.6]	2.0	1.6 [5.4]	1.0 [4.7]	ND	0.4	0	ND	ND
Lindane	ND	ND	ND	ND	0.9 Tox	ND	1.7 [11]	ND	1.35	ND
Tributyl-tin-Cl	ND	ND	ND	ND	ND	ND	1.3 Tox	3.2 [28]	2.0 [14]	1.8 [11]

metal inducers amongst those studied, giving clear concentration dependent transgene induction up to 50 ppm. The 24 h LC50 value for Ag^{+} is only 5 ppm, decreasing to 2 ppm after 48 h (Williams and Dusenbery, 1990). Dose response experiments over a wide range of Ag^{+} concentrations have shown that some response to this metal ion can be detected even at 0.15 ppm in our assay sytem. However, we find that Ag^{+} at very high concentrations (e.g 100 ppm) gives a negative gap score, even though most worms appear to survive for 7 h under these highly toxic conditions. The reason for this anomaly is possibly due to metal binding to the induced transgene product and consequently inhibiting its enzyme activity (see Mazidji *et al*, 1992), or heavy metal inhibition of the transcription/translation apparatus resulting in lower transgene expression. Mazidji *et al.* (1992) have studied the effects of heavy metal ions on the β-galactosidase, finding that Cd^{++}, Hg^{++}, Zn^{++}, Cu^{++} and Ni^{++} inhibit β-galactosidase activity, whereas Pb^{++} and As (as arsenate) have no effect or even stimulate the enzyme. Although we wash our worms routinely before staining, this will remove only metal ions in the external medium, and is unlikely to effect those already internalised (see Table 5.1 parts A and C for metal accumulation by worms). Whatever the underlying reason, the sharp decrease in transgene expression from high at 50 ppm Ag^{+} to zero at 100 ppm (Table 5.2B; Fig. 5.5o and p) suggests caution when using this transgenic system at high toxicant concentrations. We have also observed substantially lower transgene expression with several other toxicants at high concentrations which killed off a significant proportion (>20%) of the worms during the exposure period (see 2 ppm entry for lindane and 150 ppb for TBT in Table 5.2B).

Table 5.2B also shows that both organic (lindane) and organo-metallic (tributyltin

chloride (TBT)] pollutants induce transgene expression at considerably lower concentrations than those required for comparable induction by inorganic metal ions. For example, inorganic Sn^{++} is a relatively weak inducer (50 ppm giving a gap score of 2.2, and an enzyme activity of 7.4 units.g^{-1}; Fig.5.5m and Table 5.2B), whereas the organometallic toxicant tributyltin (TBT) is effective at 5 to 50 ppb (β-galactosidase activities of 11 and 28 u.g^{-1}, respectively, Table 5.2B; see also Fig.5.5q for 50 ppb TBT-induced staining). One possible explanation for the different effects of inorganic and organic forms of tin on transgene induction may be related to poor cellular uptake of inorganic Sn^{++} as compared to ready penetration of membranes by TBT; alternatively, the organometallic form may cause much greater damage to cellular proteins than the inorganic ion, thus inducing a greater stress response. Interestingly, we find that the net accumulation of tin (concentration in wet worms relative to the external medium) over 7 h at 32°C is more than an order of magnitude greater for TBT than for inorganic Sn^{++} (see Table 5.1C). There are other reports that document the induction of heat shock (stress) proteins by TBT in the mussel *Mytilus edulis* (Steinert and Pickwell, 1993), the rotifer *Brachionus plicatilis* (Cochrane *et al.*,1991) and in human diploid fibroblasts (Zhang and Liu, 1992). We note that TBT induces medium-intensity staining throughout the whole worm, affecting most worms at all stages of development, rather than the more intense but localised anterior or phryngeal staining seen mainly in young adult worms following heavy metal treatments (Fig. 5.5q). Lindane induces the nematode transgene at 15 to 150 ppb (gap scores of 1.35 and 1.7), though higher concentrations cause marked toxicity and a decrease in the gap score. For several toxicants we note that there is relatively little change in the gap score recorded across a wide range of concentrations. In case of Mn^{++}, a 25-fold increase in Mn^{++} concentration barely doubles both the gap score and the mean

β-galactosidase activity (Table 5.2B; Fig.5.5n). However, such induction is still clearly greater than in 32°C controls, and so provides information relevant to the purposes of this assay. Other toxicants which have been shown in our laboratory to induce transgene expression in CB4027 worms at 32°C include the metal ions Pb^{++}, Ni^{++}, Co^{++}, Cu^{++}, V^{3+}, V^{4+} and Al^{3+} (but not Ca^{++}, Mg^{++}, Fe^{++} or Fe^{3+}), the anthelminthic ivermectin (at concentrations down to 10^{-11} M), several commercial fungicides (e.g. prochloraz), tetraphenyl-lead, phenol and DDT (data not shown).

5.3.4 WESTERN BLOTTING OF STRESS-INDUCED β-GALACTOSIDASE

Fig. 5.7 shows that the induced β-galactosidase activity can be localised to one or two protein bands on Western blots following *in situ* histochemical staining or probing with a polyclonal anti-β-galactosidase antibody. We note that both commercial *E. coli* β-galactosidase and the induced transgene product are enzymatically active as a 170 kD form, whereas the 116 kD β-galactosidase monomer band included among the prestained molecular weight (MW) markers is immunodetectable (Fig. 5.7B), but has no detectable enzyme activity at all (Fig.5.7A). This difference is likely to be due to the high concentrations of denaturant present in the Sigma prestained MW marker preparation (e.g. 4 M urea, 2% SDS and 10 mM DTT); β-galactosidase is normally active as a tetramer (Wallenfels and Weil, 1970), but the 170 kD form produced under mild denaturing conditions (only 0.1% SDS in our extraction buffer and SDS-PAGE system) seems to retain significant activity (Muga *et al*, 1993; Fig. 5.7A). In fact, this active form of the reporter gene product appear to have a slightly higher molecular size than the corresponding form of the purified *E. coli* β-galactosidase, as shown more clearly in Fig.

5.7B (gel distortion obscures this difference in Fig. 5.7A). This size difference presumably results from the inclusion of some additional sequences at the N-terminal of the *E. coli* β-galactosidase coding region in the *lacZ* fusion construct used by Fire (1986) (see also Hope, 1991). The yield of the transgene product induced in response to heat or cadmium is roughly estimated as around 0.01 % of *C. elegans* total soluble protein.

Figure 5.7 **Western blotting analysis of induced β-galactosidase.**

Worm homogenates were prepared (as described in section 2.9) and analysed by SDS-PAGE and Western blotting as described in sections 2.15 and 2.17, respectively. The two blots shown in Parts A and B were both run with identical protein samples to demonstrate transgene induction following stress exposure. Part A shows the blot subjected to *in situ* histochemical staining, as described in section 2.16. Part B shows the blot immunoprobed with polyclonal anti-β-galactosidase (as detailed in section 2.17).

In Parts A and B, the lanes were loaded as follows:-

Lane 1, Sigma prestained SDS-PAGE MW markers; only that part of the blot containing the β-galactosidase monomer (116 kD) is shown.

Lane 2, 0.1 units of commercial (Sigma) purified β-galactosidase.

Lane 3, 0.2 units of commercial (Sigma) purified β-galactosidase.

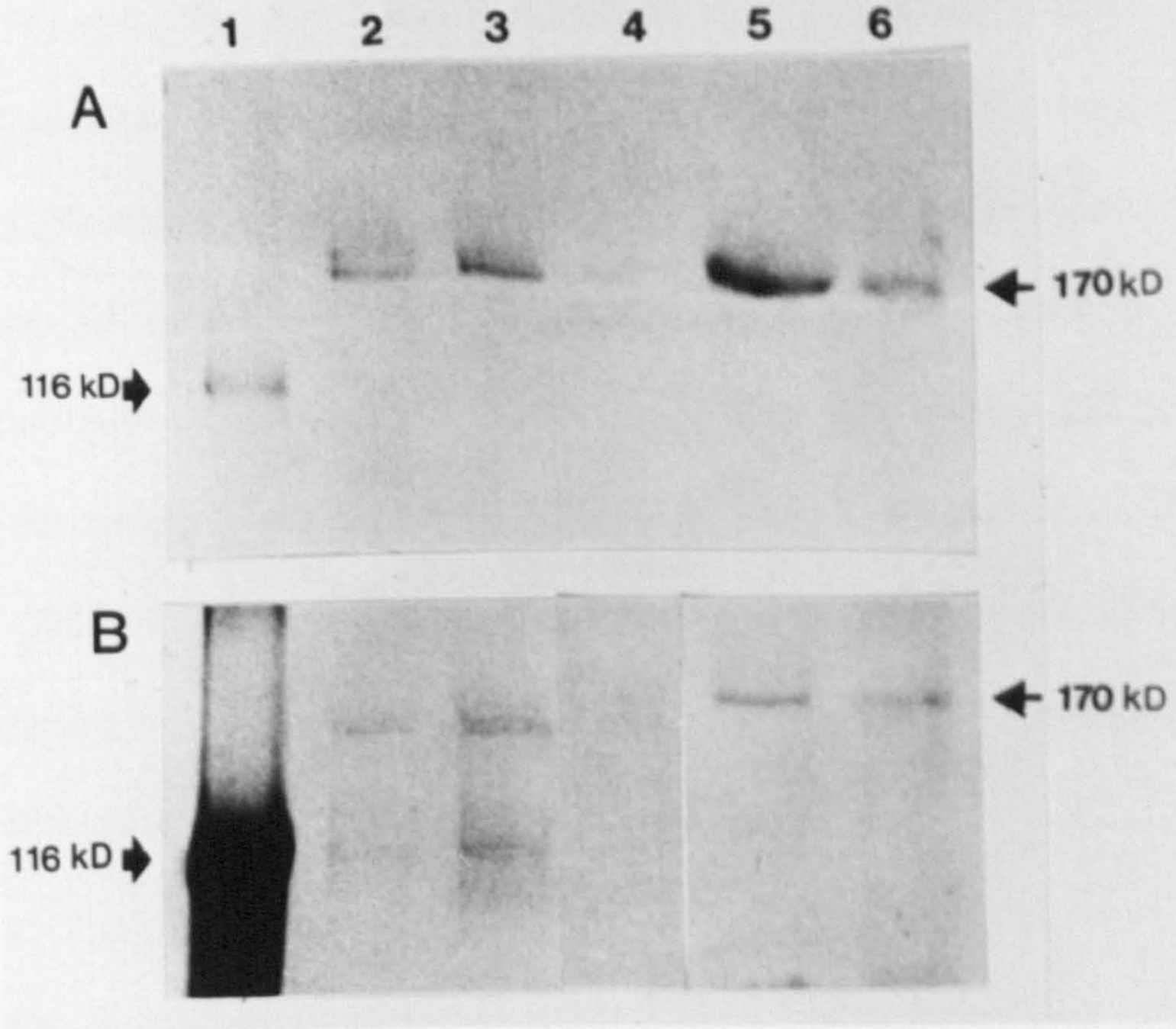
Lane 4, control (7 h at 32°C).

Lane 5, 16 ppm Cd^{++} (7 h at 32°C).

Lane 6, Heat shock (7 h at 34°C).

Lanes 4-6 were each loaded with approximately 200 μg total protein. Arrows point to the β-galactosidase monomer (116 kD) and active form (170 kD); both are detected as immunoreactive (Part B), but only the latter is enzymically active (Part A). In Part B, the transgene product (lanes 5 and 6) appears slightly larger than the purified enzyme (lanes 2 and 3), though this difference is obscured by band distortion in Part A. In the purified enzyme lanes (2 and 3), both monomer and active-form bands react faintly with the antibody (shown in Part B), but only the latter is enzymically active (Part A).

Fig. 5.7



5.4 DISCUSSION

Our results clearly show that the exposure of transgenic nematodes to a variety of toxicants can induce transgene expression, and therefore this system can provide a quick (about 8 h) and simple assessment of aquatic toxicity caused by these agents. However, careful control of the assay conditions is necessary for obtaining reproducible data; for example, incubators with temperature control accurate to within ± 0.1°C are essential. Although our staining and assessment procudures can provide a quick guide to the effects of various toxicants (Fig. 5.5, Table 5.2A and B), full β-galactosidase assays are needed for proper quantitative comparisons (Fig. 5.6 and Table 5.2A and B), which takes about 30 h in total. As far as practical applications are concerned, these transgenic worms must not be released to the environment, so they can not be exposed to potentially contaminated water on site; instead, water samples must be brought back to the laboratory for testing. We note that these transgenic strains are classed as relatively non-hazardous (ACGM class 1), so their use and maintainance does not in itself impose high costs on a testing laboratory; nonetheless, some restrictions and care are appropriate when handling such animals.

In this study, the transgenic nematode strain (CB4027) was extensively tested in order to obtain optimal sensitivity in the transgene response to the potent pollutants documented here. For instance, an accompanying sub-heat shock temperature (29-32°C) is required in order to detect significant transgene induction by the toxicants tested. We also found that transgene induction by Cd^{++} is 3-fold higher in aquatic medium as compared to agar medium, whereas the reverse is true for induction by heat shock temperatures (Figs. 5.3

and 5.2, respectively). One possible explanation is that the aquatic environment provides greater effective exposure to the heavy metal ions, whereas adsorption in to agar medium and uptake by excess bacteria are both possible on agar plates (Williams and Dusenbery, 1988). Stage-specific stress responses, observed mainly in adult but not larval stages of *C. elegans* following heavy metal exposure (Fig.5.5), imply that there might be other cellular defense mechanisms acting during the larval stages to enable these larvae to cope with the toxic effects of heavy metal ions. Metallothioneins, which are low-MW metal-binding proteins, are induced in many animals by toxic levels of cadmium, copper or zinc. It is also known that they are involved in the detoxification of heavy metals (Kagi and Schaffer, 1988; Hogstrand and Haux, 1991; see also section 1.2.2). Indeed, recent findings by Freedman *et al* (1993) strongly suggest the involvement of such proteins in the stage-specific stress responses described above for *C. elegans*. They have investigated the inducibility of metallothionein gene expression in transgenic *C. elegans* strains that carry a *lacZ* reporter gene fused to one of two metallothionein gene promoters. One of these genes (CeMT2) is abundantly expressed in the intestinal cells of larvae and adult nematodes following heat or cadmium stress but is not detectable in controls, whereas the other (CeMT1) is constitutively expressed in the larval pharynx and is also induced by cadmium and heat shock in the intestinal cells of *C. elegans* larvae. These results suggest that CeMT2 provides a first line of defence against toxic metals in all developmental stages including the adults, whereas the stage-specific expression of CeMT1 seems to augment this function in the nematode larvae. Thus this may well be the possible explanation for our finding of larvae being less susceptible to the toxic effects of metal ions, since constitutive CeMT1 expression in the pharynx during larval stages may confer resistance to heavy metal stress, whereas this is no longer true in

adults.

Many of the effects we have documented here are paralleled in other systems. Thus lower growth temperatures prior to heat shock exposure improve the induction response of a transgenic yeast strain carrying a heat-inducible *lacZ* construct (Kirk and Piper, 1991), although basal activities were constant in the yeast study. This could be explained in terms of a greater temperature rise (17 as opposed to 12°C) from growth to assay conditions causing more extensive oligomerisation of the endogenous HSF, which in turn could enhance transgene induction. Apart from this possible effect, there is an apparent increase (2-fold) in Cd^{++} accumulation when worms grown at 15°C (but not 20°C) are then transferred to 32°C (Table 5.1A); this observation as yet remains unexplained. Garty *et al* (1986) have also observed that the rate of cadmium uptake into rat red blood cells at 25°C was one-fourth of that at 37°C. They have further shown that some metabolic inhibitors did not reduce cadmium uptake into red blood cells, suggesting the possibility that cadmium uptake by rat red cells occurs by passive transport. In fact, it is widely believed that heavy metal uptake in most animals is the result of passive transport (Bryan, 1976; Roesijadi & Unger,1993), either by voltage-sensitive (Hinkle *et al*,1987) or receptor-mediated channels (Garty *et al*,1986; Roesijadi & Unger,1993). However, different metal accumulation levels in *C. elegans* grown at the two different temperatures cannot simply be attributed to temperature-dependent increases in passive transport, since no such changes are observed with worms grown at 20°C and then exposed to metal ions at 32°C. Our data on combined temperature and heavy metal effects (Fig. 5.5a-i) strongly suggest that both factors work together additively or synergistically to activate transgene expression. The subheat-shock temperature of 32°C therefore sensitises the worms to any

additional stress imposed by test toxicants.

We have also compared the effects of several heavy metal ions and two organo-metallic compounds on transgene expression, as well as the accumulation of these agents by worms. These studies show that organo-metallic toxicants are effective at ppb levels, whereas all metal ions studied are only effective at ppm levels (Table 5.2B). Furthermore, organometallic compounds are accumulated in greater amounts by worms than are the corresponding inorganic metal ions (Table 5.1C; similar indications also for tetraphenyl-lead versus Pb^{++}, data not shown). Metal-ion inhibition of β-galactosidase (Mazidji *et al*, 1992) and/or generalised metabolic toxicity may explain why high concentrations of certain toxicants cause lower apparent induction (Table 5.2B). The former might be overcome by using immunoassays to quantitate β-galactosidase protein levels, but in the latter case, lower rates of gene expression would also affect the amounts of enzyme produced.

Several other groups have advocated measuring stress-protein induction or accumulation as a biomarker of environmental contamination by various pollutants (Anderson, 1989; Miller, 1989; Hakimzadeh and Bradley, 1990; Sanders, 1990; Sanders *et al*, 1991; Kohler *et al*, 1992; Hightower, 1993; Bradley, 1993; Steinert and Pickwell, 1993). The validity of this approach remains open to question, since only a single molecular response is being investigated. Validation of such systems (including our transgenic system described here) will require clear-cut responses to a wide range of toxicants at concentrations comparable to or much lower than their LC50 values. In this study, we show that metal ions induce the reporter gene at concentrations comparable to their 24-96 h LC50 values,

providing a more rapid indication of metal toxicity (Table 5.2 parts A and B) than current LC50 assays using *C. elegans* (Williams and Dusenbury, 1990). Even so, it remains to be seen whether assays based on the heat-shock sytem will respond to toxicants with a sensitivity comparable to that shown by complex physiological processes such as respiration, growth or motility. Most assays for evaluating stress proteins as biomarkers have been based on quantifying stress-protein accumulation by using specific antibodies. HSP60 and 70, in particular, have been the focus of several studies because they are highly inducible by a wide variety of toxicants, and are highly conserved in most organisms (Sanders, 1990; Cochrane *et al*, 1991; Kohler *et al*, 1992; Bradley, 1993; Steinert and Pickwell, 1993; Sanders, 1993). The procedures involved in metabolic labelling, electrophoresis and subsequent autoradiography are also frequently used to examine the entire translational profile in response to stressful conditions, which can be useful for identifying new inducible proteins (e.g. Hakimzadeh and Bradley, 1990; Odberg-Ferragut *et al*, 1991; Veldhuizen-Tsoerkan *et al*, 1990 and 1991; Kohler *et al*, 1992). However, the use of two-dimensional gel electrophoresis becomes essential for separating stress inducible proteins from heat-shock cognates (HSCs) which are expressed constitutively in non-stressed cells. Even if the heat-shock-based assays discussed above become accepted for particular environmental monitoring purposes, it should be borne in mind that these assays make significant technical demands which may limit their usefulness, and are rather complex as compared to the transgenic system described here (see chapter 3 and 4). Thus this transgenic approach offers the advantages of technical simplicity and the ease of processing multiple parallel samples for staining or enzyme assays which can be conducted by individuals who have no particular expertise in *C. elegans* development or anatomy.

However, there are several disadvantages of this transgenic system that should also be considered. Firstly, the transgenic organisms cannot be exposed to contaminated water on site, but must be contained within the laboratory, thus imposing some additional costs and restricting field usefulness. Secondly, although *C. elegans* is an ideal organism for monitoring both water and soil pollution (Williams and Dusenbery, 1990; Donkin and Dusenbery; 1993), the transgenic strain seems relatively unresponsive to low levels of many toxicants. Indeed, this may be the reason why a large accompanying temperature rise is needed to maximise sensitivity to all toxicants tested. Sub-heat-shock temperatures should ideally be avoided in order to eliminate possible interactions between heat and toxicant effects. Therefore, strictly toxicant-inducible transgenic strains are needed in order to accurately interpret the test response as positive or negative. The transgenic *C. elegans* strains carrying the *hsp16-lacZ* transgene seems to be a better system in this respect (Stringham *et al*, 1992). Because the HSP16s are induced at a lower temperature (29°C) than the HSP70s (34°C; Snutch and Baillie, 1983), this transgene is inducible by many toxicants at the normal growth temperature of 20°C, but on the negative side, only some of the tested animals expressed this transgene in response to metal inducers (Stringham and Candido, 1994). Thirdly, the nematode *C. elegans* itself appears relatively insensitive to many heavy metal ions (Williams and Dusenbery, 1990), as compared to LC50 values for e.g. *Daphnia magna*. It is therefore unlikely that any transgenic nematode strain can overcome this insensitivity, although the greater sensitivity shown to organic and organo-metallic toxicants in both systems is perhaps more hopeful. Among all those toxicants tested, only Cd^{++} gives a strong dose response relationship (Fig. 5.6; Table 5.2A). Arsenite and cadmium are classical chemical inducers of the heat shock or stress response in most metazoans, and cadmium is known to activate the ubiquitin-

dependent proteolysis pathway in yeast, implying that Cd^{++} toxicity is mediated through cadmium-induced formation of abnormal proteins (Jungmann *et al*, 1993). It may be that the other toxicants tested simply do not damage cellular proteins to the same extent as Cd^{++}, perhaps because their primary toxicity in *C. elegans* is directed elsewhere. It is therefore necessary to select for transgenic strains which respond to a wide range of toxicants acting by different mechanisms. One possible approach would utilise constructs carrying the *hsp* promoters from toxicant-inducible genes fused to a reporter gene (*lacZ*, or *lux*AB coding for bacterial luciferase; for the latter, see Boylan *et al*, 1989), as discussed in the previous chapter. In conclusion, more research is required before the usefulness of transgenic animals as toxicological indicators can be accurately evaluated.

CHAPTER 6. THE EFFECTS OF CALCIUM AND CALCIUM-CHANNEL RELATED AGENTS ON CADMIUM ACCUMULATION AND ON CADMIUM-INDUCED STRESS IN THE TRANSGENIC NEMATODE STRAIN

6.1 INTRODUCTION

6.1.1 CADMIUM ACCUMULATION AND ITS INHIBITION BY CALCIUM

Cadmium is known to be a highly toxic metal ion, which strongly reactswith thiol groups of proteins (Pivovarova *et al*,1992) and can substitute for other metal ions such as zinc in certain enzymes. Cadmium is found to be accumulated by internal organs such as vertebrate kidneys and livers or by the whole body in case of aquatic organisms. The internal disposition or accumulation of cadmium often involves the induction of novel cysteine rich-proteins called metallothioneins which bind to metal ions with high affinity and protect cells from cadmium toxicity (Woodall and Maclean,1992; Wlostowski,1992). Granules in membrane-bound vesicles are also known to retain a large fraction of absorbed cadmium (Viarengo,1989; Roesijadi and Unger,1993). Although general models have been proposed for metal flux accross cellular membranes of aquatic organisms (Roesijadi and Unger,1993), little is known about the mechanisms by which cells take up specific metal ions (see review of the metal uptake mechanisms in marine invertebrates; Viarengo, 1989).

Interactions between calcium and cadmium uptake have been reported for several aquatic

organisms; it is now well known that the toxicity of cadmium greatly varies with water hardness (Gill and Epple,1992), and that heavy metals can alter the physiological status of the cells by affecting the mechanisms involved in calcium homeostasis (Viarengo and Nicotera, 1991). Verbost *et al* (1989) demonstrated that cadmium inhibits the erythrocyte Ca^{++}-pump competitively, and hence disturbs intracellular Ca^{++} homeostasis. Recent findings further indicate that cadmium uptake into cells occurs through either voltage-sensitive calcium channels (Hinkle *et al*,1987) or by a sulfhydryl-sensitive route which involves receptor-mediated channels (Garty *et al*,1986; Zaroogian *et al*,1993). Moreover, experiments with several species have shown that cadmium uptake did not require metabolic energy, and was unaffected by temperature or O_2 (Garty *et al*,1986; Zaroogian *et al*,1993). Thus cadmium uptake was thought to be the result of passive diffusion, or a process of facilitated passive diffusion (ATP-dependent) (Roesijadi and Unger,1993; Zaroogian *et al*,1993).

There have been several reports demonstrating the involvement of calcium channels in cadmium uptake, and inhibition of the uptake by calcium channel blockers. Verapamil, nifedipine and diltiazem are the best known antagonists of voltage-sensitive and receptor-operated calcium channels (Hosey and Lazdunski,1988). Each of these blockers binds to a different site in the channels (Roesijadi and Unger,1993). Hinkle *et al* (1987) have demonstrated that secretory cells were highly protected against cadmium toxicity in the presence of organic calcium channel blockers such as verapamil or nifidipine. More recently, it has been shown that such calcium channel blockers only partially inhibit both calcium (c. 20%) and cadmium uptake (c. 55%) in the oyster gill, indicating that other major pathways for calcium entry might exist in this system (Roesijadi and Unger, 1993).

The present study provides evidence for the existence of calcium channels also in the nematode *Caenorhabditis elegans* and suggests their involvement in cadmium uptake. We have used the transgenic system, which has been extensively characterised in the previous chapter, to demonstrate dose-dependent inhibition by Ca^{++} ions of Cd^{++}-induced transgene activity. This is directly related to the levels of Cd^{++} accumulated in the worms, which is also inhibited by Ca^{++} ions. Moreover, Cd^{++}-induced transgene activity is also partially inhibited by calcium channel blockers in our system, suggesting that Cd^{++} levels in cells largely determine the extent of transgene activation, and that much of the Cd^{++} enters via calcium channels. Calcium ionophore (A23187) does not greatly increase transgene induction by 16 ppm Cd^{++} (nor in controls), but strongly stimulates transgene induction by 10 ppm Mn^{++}; notably this ionophore has a very high affinity for Mn^{++}. We further show that similar interactions may also occur between cadmium and other heavy metal ions such as zinc and mercury.

6.2 MATERIALS AND METHODS

(i) Maintenance of *C. elegans* cultures

Growth and maintenance of worm cultures were as described in sections 4.2 and 5.2.

(ii) Worm exposure to heat and toxicants and determination of metal contents

The nematodes were exposed to heat and toxicants as described in sections 2.4 and 5.2. Ethanol was used to solubilise the calcium channel-related reagents (e.g. nifedipine or calcium ionophore). The final solvent concentrations did not exceed 0.1% (v/v), and controls included ethanol alone at the same concentration. Determination of metal contents of tissue samples was as detailed in section 2.6. The Cd^{++} content was determined using atomic absorption spectrophotometry (Pye-Unicam SP9 instrument). Concentrations of Cd^{++} per unit wet weight of worms were divided by the external Cd^{++} concentration (routinely 16 ppm) to provide a rough estimate of the net accumulation of Cd^{++} by worms.

(iii) Histochemical staining and β-galactosidase assays

The procedures followed for both methods were as described in sections 2.7 and 2.9, respectively.

(iv) Statistical analysis

The significance of differences between appropriate pairs of data-sets was assessed by the Bonferroni test. Linear regression was used to fit a line to the data in Figs. 6.1a,b and 6.2d.

6.3 **RESULTS**

6.3.1 CALCIUM AS A MODULATOR OF CADMIUM INDUCTION

(a) Effect of calcium on cadmium-induced transgene activity

As shown in chapter 5, Cd^{++} ions are strong inducers of the transgene under standard assay conditions, giving dose-dependent β-galactosidase induction at ppm levels (4-fold above controls at 2 ppm; 14-fold at 16 ppm). When carrying out these experiments, we have noticed on a few occasions a much-reduced response to Cd^{++}, and in each case this has been traced to improperly deionised water containing significant concentrations of Ca^{++}. Therefore, in the present study, we have investigated the effect of Ca^{++} on Cd^{++} induction.

Fig. 6.1a shows a linear decrease in 16-ppm-Cd^{++}-induced transgene activity with logarithmically increasing concentrations of Ca^{++} ions. The Ca^{++} concentrations used were in 0.1 to 500 ppm range. Ca^{++} ions alone (even at 500 ppm) did not induce the β-galactosidase expression. The net induction of β-galactosidase activity by 16 ppm Cd^{++} is virtually eliminated by 200 or 500 ppm Ca^{++} (Fig.6.1a). For comparison, we have also included a histogram in Fig.6.1a, which shows mean β-galactosidase activities measured in the same experiment for controls (32°C alone) and for worms treated with 16 ppm Cd^{++} in the absence of Ca^{++}. We have analysed and compared these data statistically; transgene activities induced by 16 ppm Cd^{++} in the presence of 0.1 or 1 ppm Ca^{++} differ insignificantly from 16 ppm Cd^{++} alone ($p > 0.05$), whereas

Figure 6.1 **The effect of calcium on cadmium induction**

Part a : Calcium inhibition of transgene induction by 16 ppm cadmium chloride

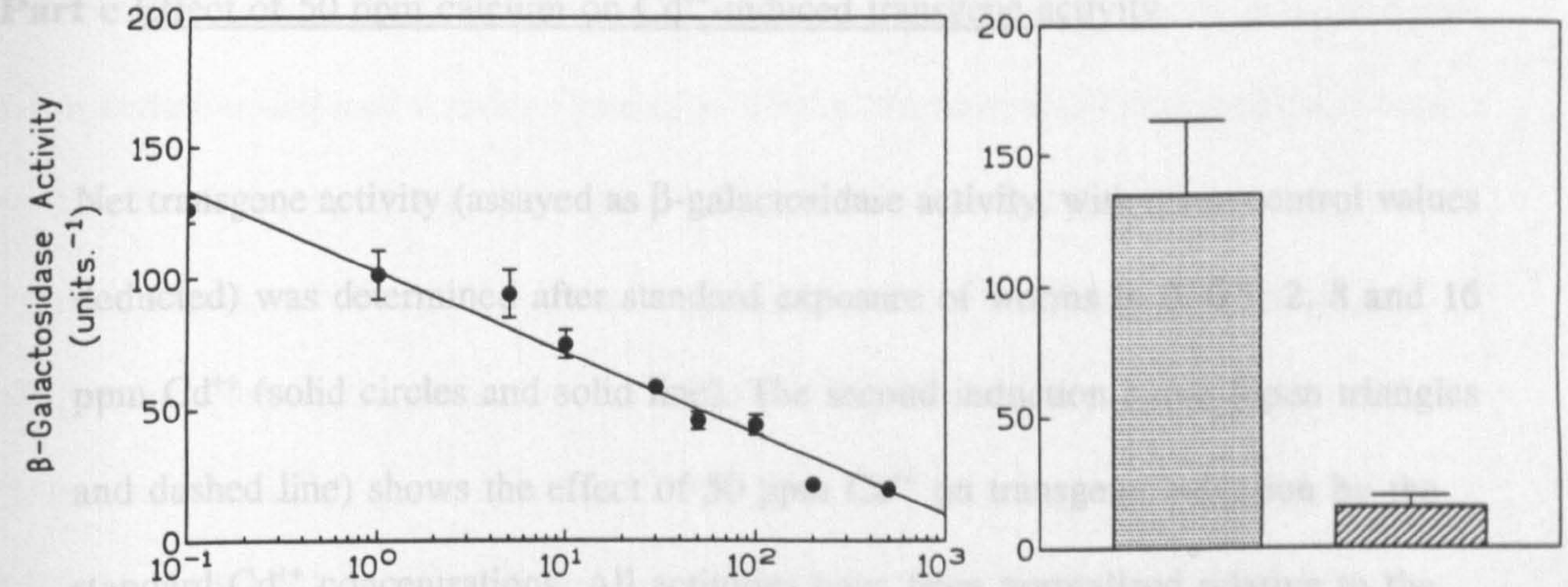
Transgenic worms (CB4027) were exposed to 16 ppm Cd^{++} ($CdCl_2$) plus various concentrations of calcium ($CaCl_2$; from 0.1 to 500 ppm) for 7 h at 32°C. then replicate β-galactosidase assays were performed as described in section 2.9. The mean and SEM, derived from 6 to 8 determinations in 3 separate experiments, is given for each point, and is plotted against the Ca^{++} concentration on a log scale; a linear regression line has been fitted to the data. The histogram shows comparable data (mean ± SEM) for worms treated with 16 ppm Cd^{++} in the absence of Ca^{++} (stippled bar), and for 32°C control worms (hatched bar).

Part b : Calcium inhibition of cadmium accumulation by worms

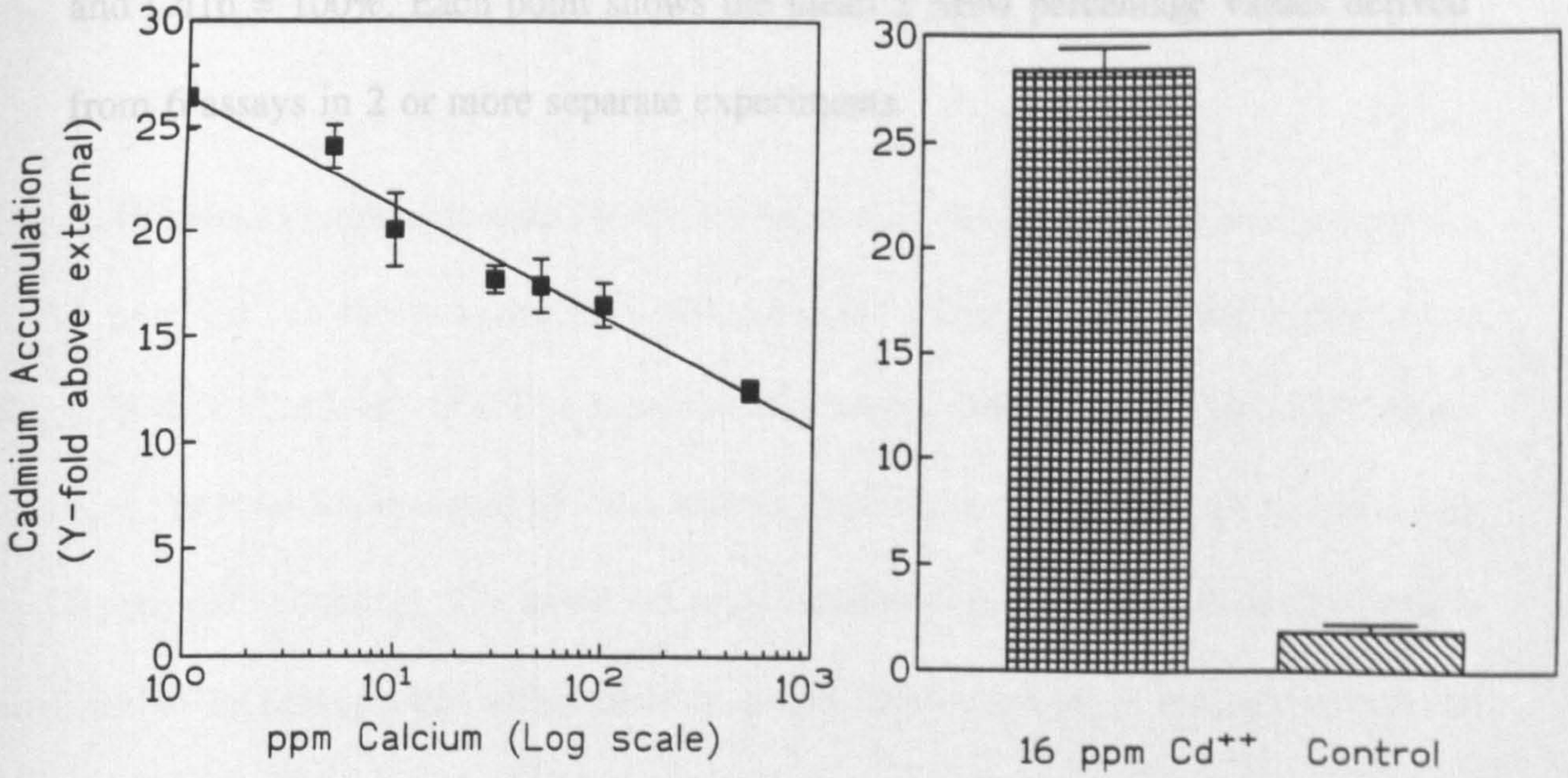
Worms were exposed to 16 ppm Cd^{++} plus various Ca^{++} concentrations (from 1 to 500 ppm) for 7 h at 32°C. Cadmium contents were determined in replicate samples by atomic absorption spectrophotometry, as described in section 2.6. The mean and SEM, derived from 4-6 separate determinations in 3 separate experiments. is given for each point, and is plotted against the Ca^{++} concentration on a log scale; again a linear regression line has been fitted to the data. The histogram shows comparable data (mean ± SEM) for worms treated with 16 ppm Cd^{++} in the absence of Ca^{++} (cross-hatched bar), and for dead worms (frozen overnight, then similiarly exposed to 16 ppm Cd^{++} for 7 h at 32°C; reverse hatched bar).

Fig.6.1

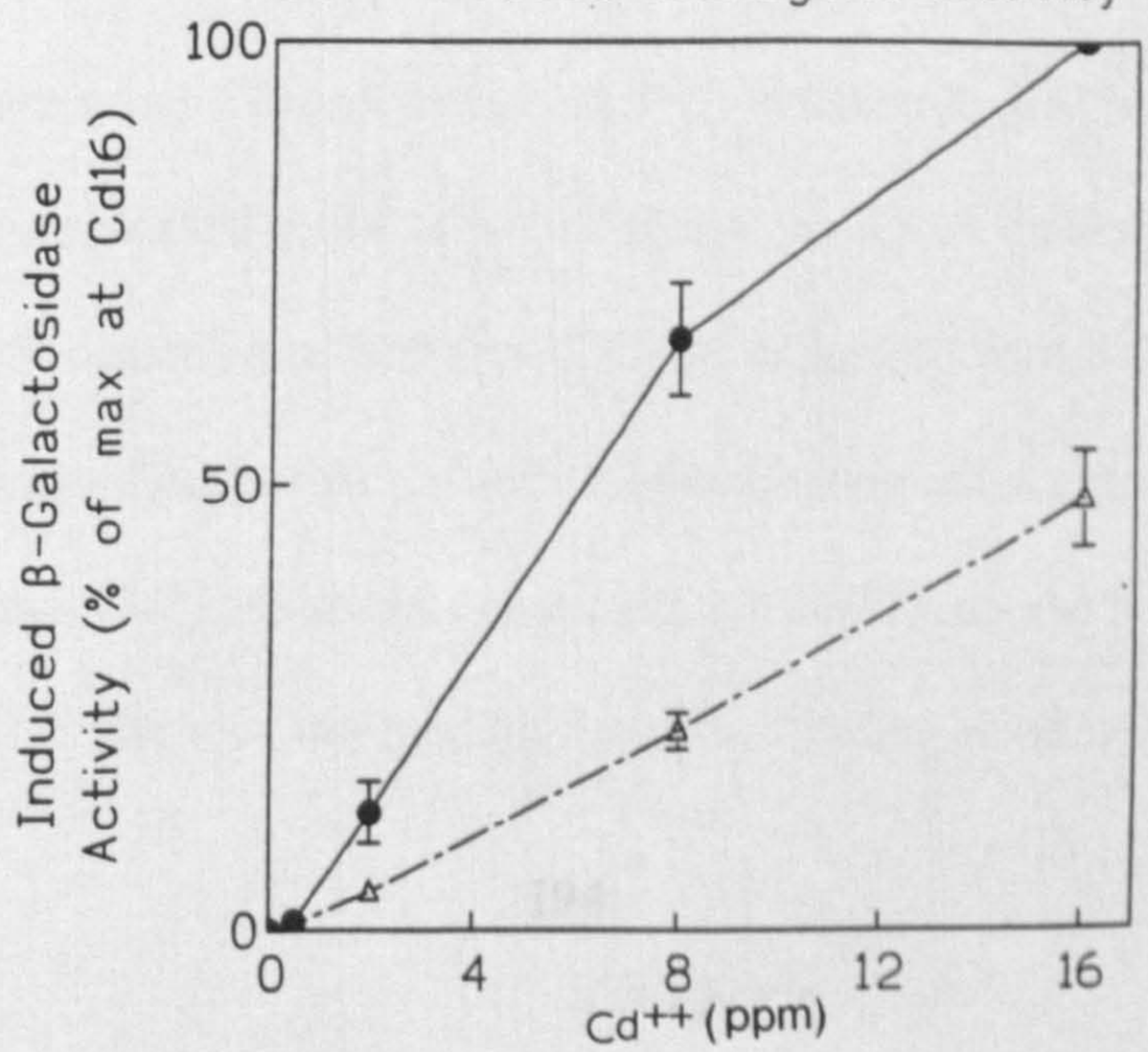
a) Calcium Inhibition of Transgene Induction by 16 ppm Cadmium Chloride.



b) Calcium Inhibition of Cadmium Accumulation by CB4027 Worms.



c) Effect of 50 ppm Calcium on Cd^{++}-induced Transgene Activity



Part c Effect of 50 ppm calcium on Cd^{++}-induced transgene activity

Net transgene activity (assayed as β-galactosidase activity, with mean control values deducted) was determined after standard exposure of worms to 0, 0.5, 2, 8 and 16 ppm Cd^{++} (solid circles and solid line). The second induction curve (open triangles and dashed line) shows the effect of 50 ppm Ca^{++} on transgene induction by the standard Cd^{++} concentrations. All activities have been normalised relative to the maximum activity induced by 16 ppm Cd^{++} alone (as 100%). Thus controls = 0% and Cd16 = 100%. Each point shows the mean ± SEM percentage values derived from 6 assays in 2 or more separate experiments.

Cd-induced activity in the presence of 10-500 ppm Ca^{++} is very significantly different from cadmium-induced activity alone ($p < 0.001$). We have also compared these values with controls; no significant differences between Cd^{++}-induced transgene activity at Ca^{++} concentrations of 100 to 500 ppm and control values ($p > 0.05$), the differences at 30 or 50 ppm are just significant above control ($p < 0.05$), but are very significantly different from controls at Ca^{++} concentrations of 0.1, 1, 5 and 10 ppm ($p < 0.001$). As expected, Cd16 alone is also statistically significant from control values ($p < 0.0001$).

(b) Effect of calcium on cadmium accumulation

Fig. 6.1b shows comparable data for the levels of Cd^{++} accumulated by worms exposed to 16 ppm Cd^{++} in the presence of 1-500 ppm Ca^{++}. The accompanying histogram in Fig.6.2b shows the levels of Cd^{++} accumulated by worms treated with 16 ppm Cd^{++} alone, and also the level accumulated by dead worms (frozen overnight) exposed to identically to 16 ppm Cd^{++} (control). The small net accumulation observed in these dead worms is attributable to non-specific metal binding to the cuticle or other biological material (bacterial cells etc) present in the worm pellet. As with Cd^{++}-induced transgene activity, we observe a linear decrease in the net accumulation of Cd^{++} by worms with logarithmically increasing concentrations of Ca^{++}. Although Cd^{++}-induced transgene activity is completely blocked at 500 ppm Ca^{++} (Fig.6.1a), approximately 12-fold net Cd^{++} accumulation is still detectable at 500 ppm Ca^{++} (as compared with 2-fold in dead worm controls), which may reflect our crude method of estimating net accumulation. However, it is also possible that Ca^{++} ions do not completely block Cd^{++} uptake (e.g. Cd^{++} ions may compete for intracellular calcium-binding proteins; Hinkle *et al*,1987), even at Ca^{++}

concentrations which totally inhibit Cd^{++}-induced transgene activity. Statistical analysis of calcium inhibition of cadmium uptake demonstrates very significant differences in cadmium accumulation by worms in the presence of 10-500 ppm Ca^{++} (note similar values for calcium inhibition of cadmium-induced transgene activity), as compared to Cd^{++}-accumulation in the absence of calcium ($p < 0.001$).

(c) The effects of 50 ppm Ca^{++} on the transgene's dose response to Cd^{++}

Fig. 6.1c shows that net induction of transgene activity is inhibited by 60-70% following exposure to the standard concentrations of Cd^{++} (0, 0.5, 2, 8 and 16 ppm) in the presence of a constant 50 ppm of Ca^{++}. This finding is consistent with the data shown in Fig.6.1a.

6.3.2 THE EFFECTS OF CALCIUM CHANNEL BLOCKERS

As shown in Fig.6.2, we have tested the effect of three known calcium channel blockers on Cd^{++}-induced transgene activity. The organic calcium channel blockers nifedipine (constant 10 μM; Fig.6.2a) and verapamil (constant 20 μM; Fig. 6.2b) both inhibit transgene activity by 20-30%. It should be noted that higher concentrations of both agents were tested, but were found to induce significant transgene activity in controls without Cd^{++}; in any case, neither of these blockers completely inhibits Cd^{++} or Ca^{++} into mollusc gill tissue (Roesijadi and Unger,1993), even at 10-fold higher concentrations. Furthermore, we have combined the inducer (Cd^{++}) with equimolar concentrations of La^{+++} as a calcium transport inhibitor (Perry and Flik, 1988); we

Figure 6.2 **Effects of calcium uptake inhibitors on Cd^{++}-induced transgene activity**

Worms were exposed to standard Cd^{++} concentrations of 0.5, 2, 8 and 16 ppm, then replicate β-galactosidase assays were performed as described in section 2.9. Net β-galactosidase activities (with mean control values deducted) have been normalised relative to the maximum activity induced by 16 ppm Cd^{++} alone in each experiment (shown as 100%). Separate Cd^{++} dose-response curves are shown for each of the sets of experiments in each panel (solid circles and solid lines throughout), and mean ± SEM percentage values are shown for 0.5, 2 and 8 ppm Cd^{++}. The second induction curve in each panel shows the effect of one inhibitor of Cd^{++}/Ca^{++} uptake when combined with the standard Cd^{++} concentrations. For all data presented, each point shows the mean ± SEM derived from 6 or more assays in 2 or more separate experiments.

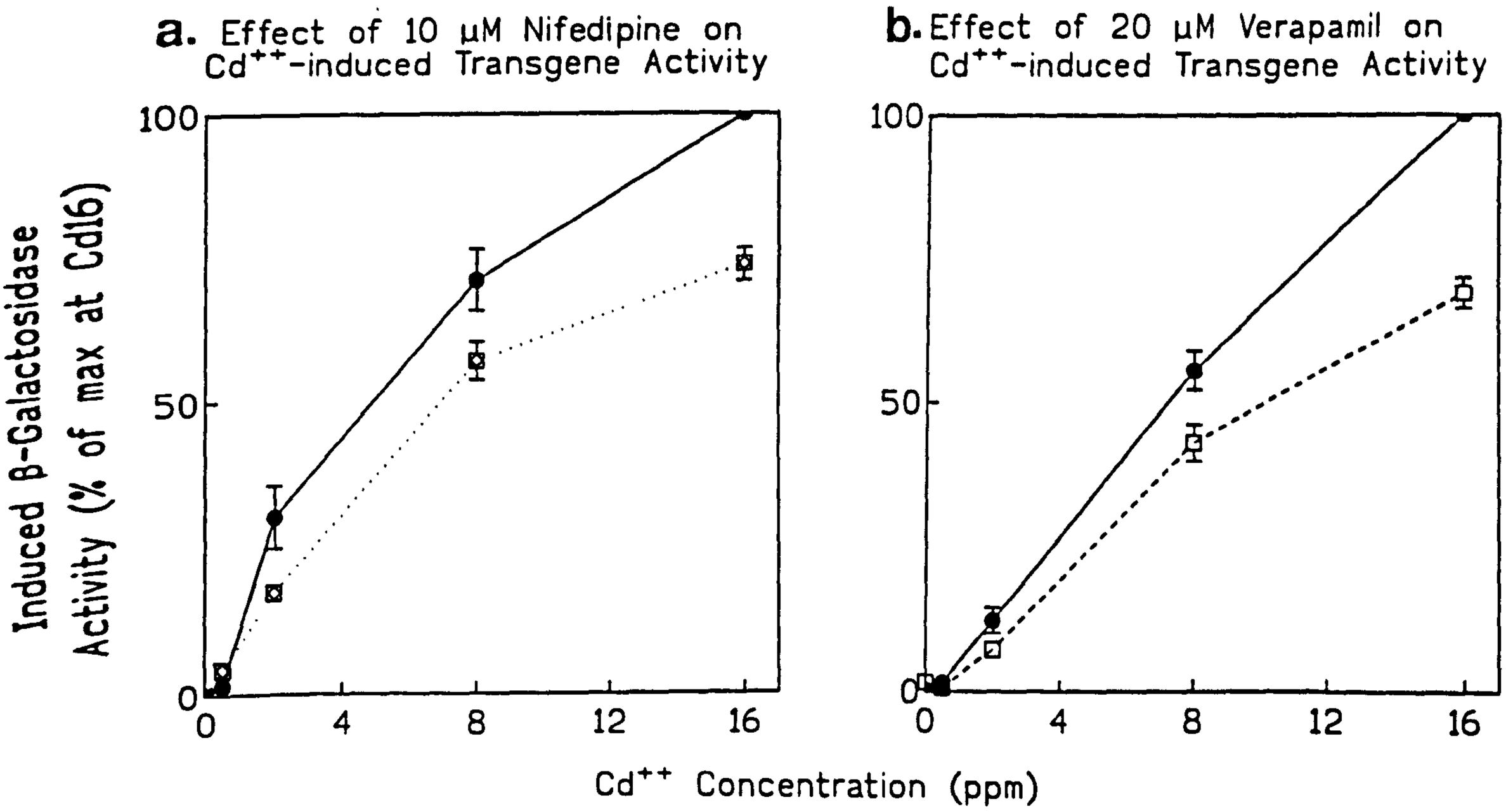
Part a : Effect of 10 μM Nifedipine (constant) on Cd^{++} induction; open symbols, dotted line.

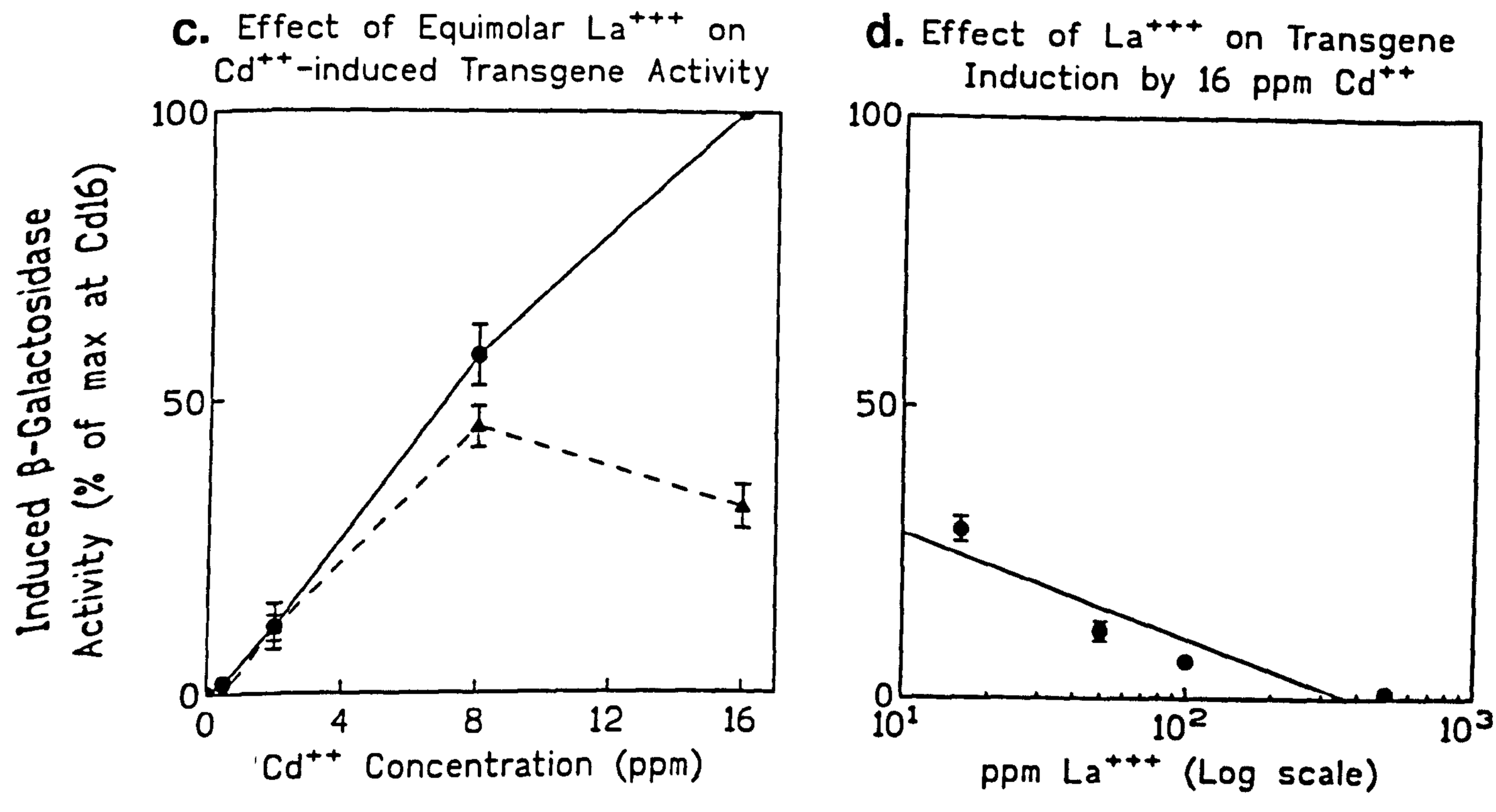
Part b : Effect of 20 μM Verapamil (constant) on Cd^{++} induction; open squares, dashed line.

Part c : Effect of equal metal-ion concentrations of La^{+++} on Cd^{++} induction; solid triangles, dashed line.

Part d : Effect of La^{+++} on transgene induction by 16 ppm Cd^{++}; here all data points have been plotted against the La^{+++} concentration on a log scale, and a linear regression line has been fitted to the data.

Fig.6.2


a. Effect of 10 µM Nifedipine on Cd++-induced Transgene Activity
b. Effect of 20 µM Verapamil on Cd++-induced Transgene Activity
Induced β-Galactosidase Activity (% of max at Cd16)
100
50
0
0
4
8
12
16
Cd++ Concentration (ppm)


c. Effect of Equimolar La+++ on Cd++-induced Transgene Activity
d. Effect of La+++ on Transgene Induction by 16 ppm Cd++
Induced β-Galactosidase Activity (% of max at Cd16)
100
50
0
0
4
8
12
16
Cd++ Concentration (ppm)
10¹
10²
10³
ppm La+++ (Log scale)

found no detectable effect at 2 ppm for each ion, about 20% inhibition at 8 ppm each, >60% inhibition at 16 ppm each (Fig. 6.2c). Higher concentrations of La^{+++} combined with 16 ppm Cd^{++} caused further inhibition (Fig. 6.2d), but also caused precipitation problems above 50 ppm La^{+++}.

6.3.3 THE EFFECTS OF CALCIUM IONOPHORE (A23187) ON TRANSGENE INDUCTION BY A RANGE OF HEAVY METAL IONS

Taken together, the evidence cited above strongly suggests that Ca^{++} and calcium channel blockers interfere with Cd^{++}-induction of transgene activity by reducing Cd^{++} accumulation within worm tissues; this implies that calcium channels exist in nematodes, and that Cd^{++} uptake occurs at least in part via calcium channels. We therefore reasoned that agents which increase calcium transport into cells, such as calcium ionophore A23187 (see e.g. Inagaki *et al*,1985; Pohl *et al*,1990), might also increase the transgene response to heavy metal ions in our system. As shown in Fig. 6.3, we have tested the effect of this ionophore on two divalent metal ions, Cd^{++} and Mn^{++}. The net effect of 20 μM A23187 (Calbiochem) on transgene induction by 16 ppm Cd^{++} is a slight (10-15%) but consistent stimulation (barely significant; $p < 0.05$). However, the same concentration of A23187 also causes a slight (though not statistically significant; $p > 0.05$) increase in transgene activity under control conditions (Fig. 6.3). Calcium ionophores are also known to induce stress responses in other systems (see Nover,1991b and Lindquist,1986). Additionally, we have tested the effects of A23187 on transgene induction by a range of other divalent metal ions, including Mn^{++}, Hg^{++} and Zn^{++}, and find consistent and significant stimulation only in the case of Mn^{++} (Fig.6.3).

Figure 6.3 **Effect of 20 μM calcium ionophore (A23187) on transgene activity**

Mean (± SEM) β-galactosidase activities were measured in 6-8 determinations under the specified conditions. In each pair of bars, the first shows the activity measured in the absence of A23187 (CaI), while the second shows the activity measured in the presence of 20 μM CaI during the exposure period. **Bars 1 and 2** (both blank), 32°C controls and controls + CaI. **Bars 3 and 4** (horizontal & vertical lines), 10 ppm Mn^{++} and 10 ppm Mn^{++} + CaI. **Bars 5 and 6** (reverse hatched & hatched), 16 ppm Hg^{++} and 16 ppm Hg^{++} + CaI. **Bars 7 and 8** (diagonal & upright cross-hatching), 16 ppm Zn^{++} and 16 ppm Zn^{++} + CaI. **Bars 9 and 10** (dotted & solid), 16 ppm Cd^{++} and 16 ppm Cd^{++} + CaI.

Effect of 20 μM Calcium Ionophore
(A23187) on Transgene Activity

Fig.6.3

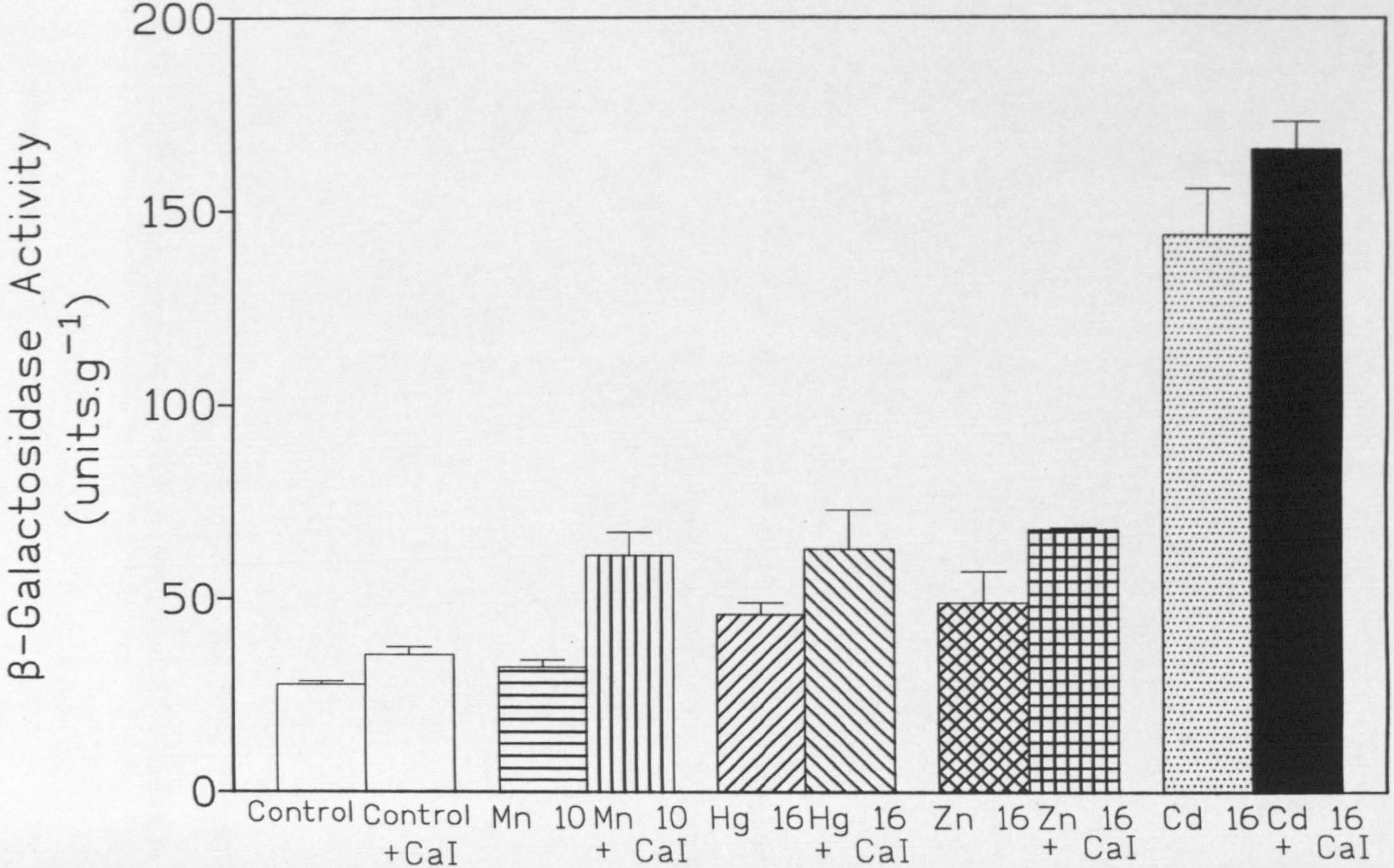


Figure 6.4 **Effect of Calcium Ionophore (A23187) on histochemical staining for transgene product**

CB4027 worms were stained *in situ* for β-galactosidase activity as described in section 2.7, then examined and photographed under direct illumination. Bar shows 0.1 mm.

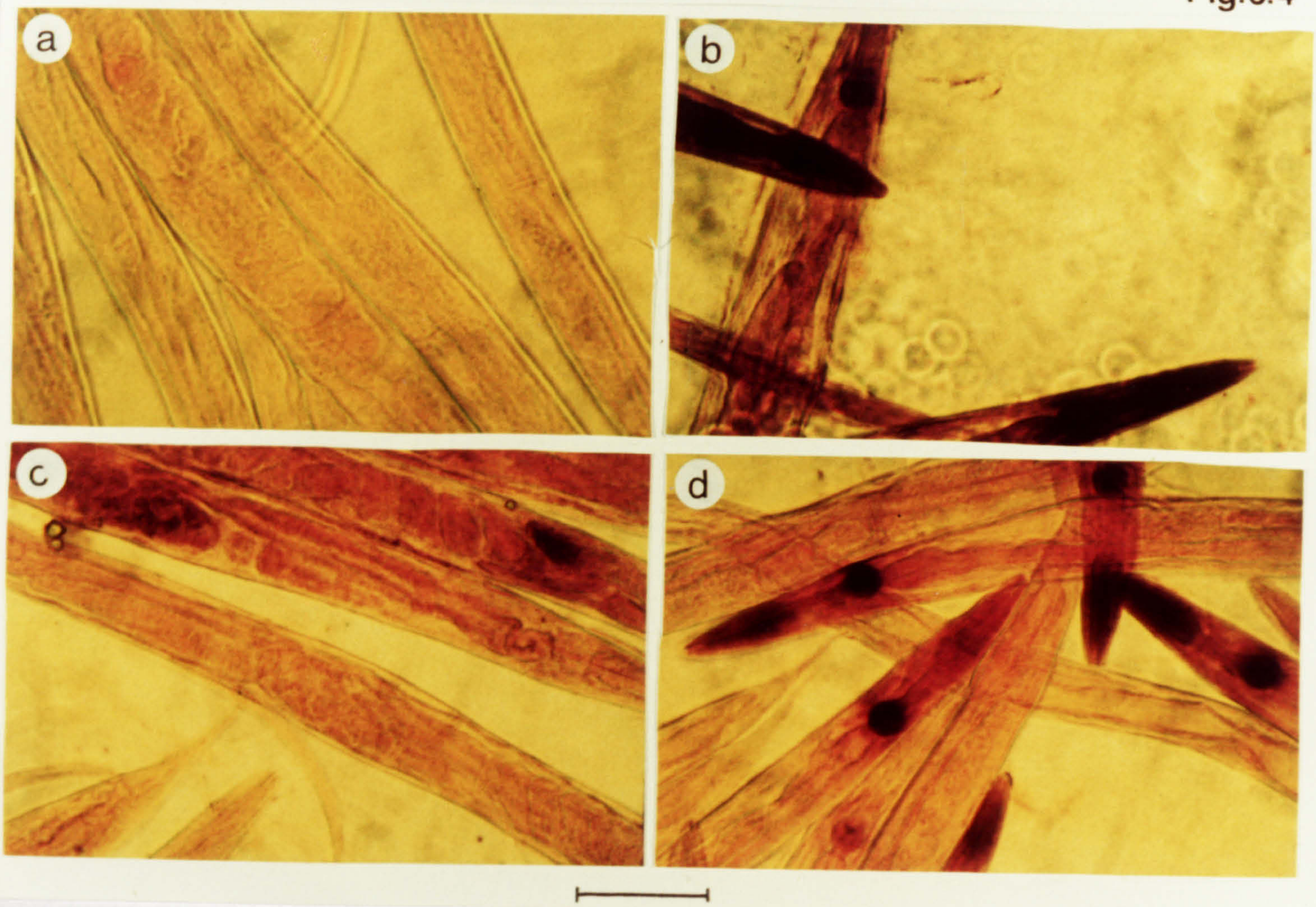
Part a : Control worms (32°C); no staining.

Part b : Worms exposed to 16 ppm Cd^{++} at 32°C; intense staining of pharynx and anterior.

Part c : Worms exposed to 10 ppm Mn^{++} at 32°C; weak staining of embryos.

Part d : Worms exposed to 10 ppm Mn^{++} plus 20 μM A23187; strong staining of the pharynx in almost all worms, demonstrating increased transgene induction.

Fig.6.4



10 ppm Mn^{++} is a poor inducer, giving a < 20% increase in β-galactosidase activity relative to controls (a difference of only 5 units g^{-1}, and not statistically significant; Fig.6.3; and also see Table 5.2 in chapter 5). Inclusion of 20 μM A23187 during exposure to 10 ppm Mn^{++} significantly ($p < 0.001$) stimulates β-galactosidase activity (up to 35 units g^{-1} above control levels; Fig.6.3). It should be noted that Mn^{++} has an affinity for A23187 which is some 80-fold greater than its affinity for Ca^{++} (Calbiochem technical information bulletin supplied with this product). It is therefore likely that Mn^{++} accumulation is stimulated by A23187 to a much greater extent than the accumulation of Cd^{++} and other metal ions tested, though this point has yet to be verified by determining the Mn content of worms in the presence or absence of A23187. This point is also confirmed by the histochemical staining panels shown in Fig. 6.4; controls are virtually unstained (panel a), whereas worms treated with 16 ppm Cd^{++} show intense staining of the pharynx and other anterior structures in the majority of worms (panel b). Worms treated with 10 ppm Mn^{++} are mostly unstained, with a minority staining positively in eggs or embryos within adults (panel c). However, strong pharyngeal staining is observed in most worms treated with the combination of 10 ppm Mn^{++} in the presence of 20 μM A23187 (panel d). The predilection for embryonic and pharyngeal staining in heat-induced CB4027 worms has been discussed in chapter 5 (see also Fire, 1986).

6.3.4 HEAVY METAL IONS OTHER THAN CALCIUM CAN ALSO REDUCE THE TRANSGENE INDUCTION BY CADMIUM

As shown in Table 6.1, metal ions such as Zn^{++} and Hg^{++} can also reduce the extent of

transgene induction by Cd^{++} ions. Combinations of Cd^{++} with equal metal-ion concentrations of Zn^{++} slightly stimulate transgene induction at 0.5 or 2 ppm each (though these differences are not statistically significant; $p > 0.05$); however, at 8 or 16 ppm each these ions show antagonistic effects, with a reduction of transgene induction (47.2% and 42.6%, respectively) as compared with Cd^{++} alone. We further show that Zn^{++} at all concentrations tested (2, 8 and 16 ppm) inhibits 16-ppm- Hg^{++}-induced transgene activity, with the greatest inhibition seen at Zn^{++} concentration of 16 ppm (68.3%; Fig.6.5). Similar effects have been observed with Cu^{++} ions in combination with Cd^{++} (data not shown). We have also tested the effect of equal concentrations of Hg^{++} ions on Cd^{++}-induced transgene activity; we interestingly find that Hg^{++} ions reduce the transgene induction at all concencentrations tested, but the reduction at 8 and 16 ppm each is to a greater extent (58.5% at 8 ppm each and 66% at 16 ppm each; Table6.1).

Table 6.1 **Effect of heavy metal ions (Cd^{++}, Zn^{++} and Hg^{++}) alone and in combination on transgene activity**

	Mean β-galactosidase activity (units.g^{-1})				
Concentration of each metal ion	0.0 ppm	0.5 ppm	2.0 ppm	8.0 ppm	16.0 ppm
Cd^{++} alone	11.45 ± 1.97	20.08 ± 3.12	26.50 ± 2.38	71.45 ± 4.75	121.3 ± 11.4
Zn^{++} alone	11.45 ± 1.97	14.95 ± 2.61	20.45 ± 1.27	24.25 ± 2.22	27.71 ± 3.07
Hg^{++} alone	11.45 ± 1.97	____	21.55 ± 2.15	26.10 ± 3.2	29.79 ± 2.98
Zn^{++} plus Cd^{++}	11.45 ± 1.97	24.33 ± 1.30	32.95 ± 0.79	37.75 ± 4.64	69.63 ± 3.74
Hg^{++} plus Cd^{++}	11.45 ± 1.97	____	21.90 ± 2.60	29.65 ± 3.53	41.20 ± 3.82

Table 6.1 Worms were exposed to the indicated concentrations of Zn^{++}, Hg^{++} and Cd^{++} alone or in combination (at equal metal-ion concentrations) for 7 h at 32°C, then β-galactosidase activities were measured in replicate samples, as described in section 2.9. Each entry gives the mean and SEM from at least 4 such assays.

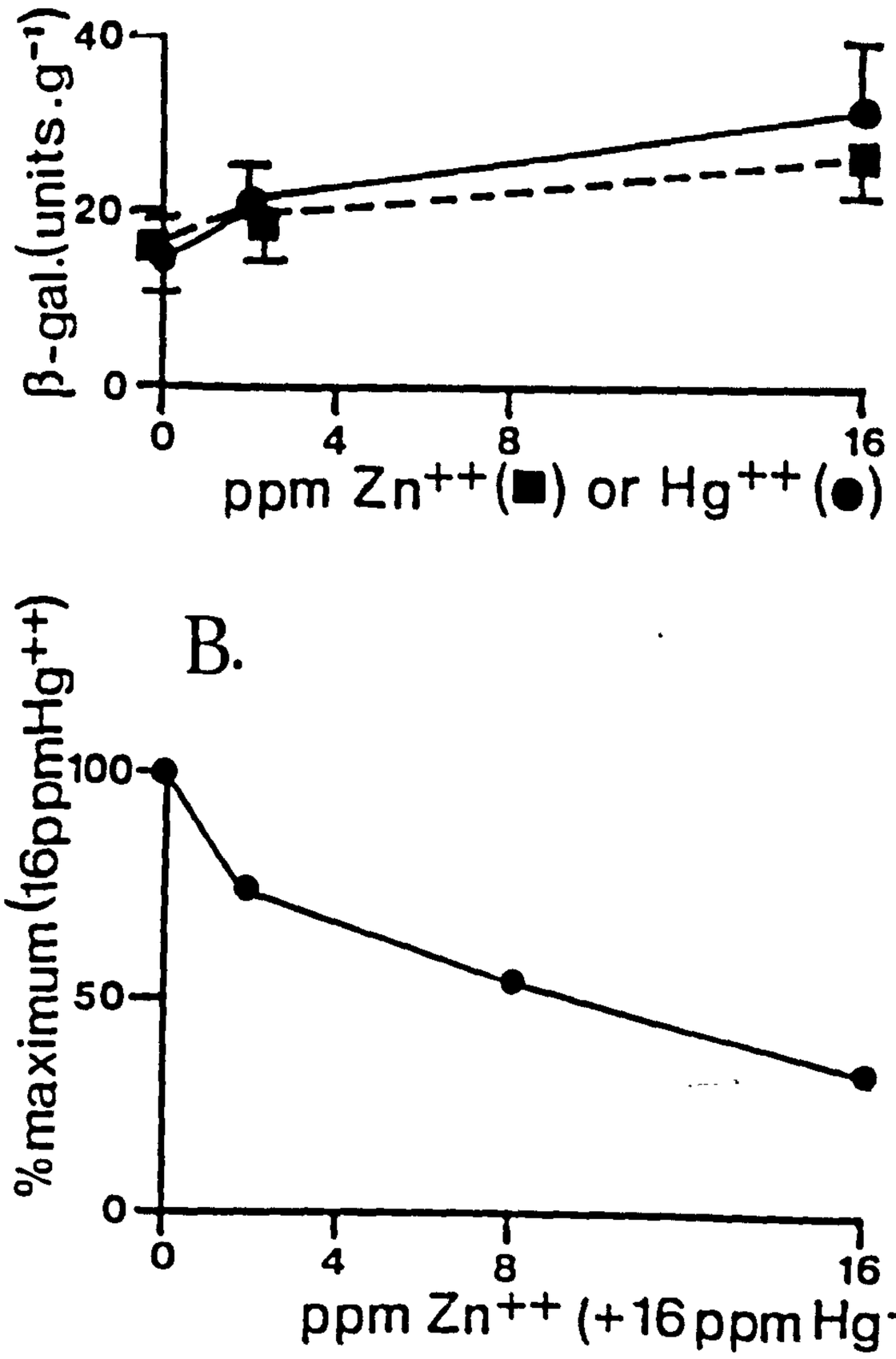


Fig.6.5 The effect of Zn^{++} on transgene induction by Hg^{++}

Part A shows the induction of β-galactosidase activity caused by increasing concentrations of Zn^{++} (solid squares, dashed line) or of Hg^{++} (solid circles, unbroken line). 16 ppm Hg^{++} alone gives approximately 2-fold induction of β-galactosidase above controls.

Part B shows the effect of combining different concentrations of Zn^{++} with a constant 16 ppm of Hg^{++}. The induction value for 16 ppm Hg^{++}, shown in part A, is normalised to 100% to show the inhibition of induction caused by adding Zn^{++}. The mean and standard error for parts A and B are derived from 4 separate determinations.

6.4 DISCUSSION

Previous results presented in chapter 5 have shown that stress-inducible transgenic nematodes can provide a rapid assessment of aquatic toxicity at toxicant concentrations that comparable to LC50 values for 1-4 days. In the present study, we have extended these findings to mixtures of metal ions rather than single toxicants. We have used Cd^{++}-induced transgene activity as the basis for our studies described here because this ion is by far the best inducer among the 12 or so metals studied to date (see chapter 5). In fact, cadmium is known to induce a heat shock response in many other systems, for reasons which have been discussed before (chapter 5; Jungmann *et al*, 1993). A number of studies have demonstrated that heavy metal toxicity is greatly reduced in hard water, which has been associated with the presence of Ca^{++} ions (see e.g. Gill and Epple, 1992). Conversely, the cytotoxicity of many heavy metals appears to be mediated via disruption of Ca^{++} homeostasis (reviewed by Viarengo, 1989; Viarengo and Nicotera, 1991; see also section 1.1.3). The evidence from several systems shows that Cd^{++} accumulation occurs via calcium channels, either by voltage-sensitive or receptor-operated calcium channels (Hinkle *et al*, 1987; 1992; Zaroogian *et al*, 1993). Our results are also in broad agreement with these previous studies, demonstrating parallel declines in 16-ppm-Cd^{++}-induced transgene activity (Fig. 6.1a and c) and in net Cd^{++} accumulation (Fig. 1b) in the presence of Ca^{++} ions.

We find that various calcium channel blockers also inhibit transgene induction by Cd^{++} (Fig. 6.2), albeit to varying extents. It is possible that the drugs verapamil and nifedipine may not work equally effectively throughout the worm, since their uptake and possible

metabolism in different worm tissues have not been studied. Published data using these organic calcium channel blockers suggest half-maximal inhibition at concentrations ranging from 4 μM (for verapamil; Hinkle *et al*, 1987) to >250 μM; moreover, Cd^{++} uptake is blocked more effectively than Ca^{++} uptake by both agents (Roesijadi and Unger, 1993). Thus the modest degree of inhibition of Cd^{++}-induced transgene activity (c. 25%) observed in Fig. 6.2a and b with 10 μM nifedipine or 20 μM verapamil, respectively, is in keeping with published findings, and is nevertheless indicative of Cd^{++} uptake via calcium channels. Although the inhibition of cadmium uptake by organic calcium channel blockers such as verapamil appears to be a noncompetitive inhibition (Blazka and Shaikh, 1991), concentrations of nifedipine greater than 10 μM, or of verapamil greater than 20 μM, do induce detectable transgene activity in controls and make it difficult to evaluate such data relative to the dose-response curves with Cd^{++} alone. In case of calcium transport inhibitor La^{+++}, we were able to use relatively high concentrations of this inorganic inhibitor, since it does not on its own induce transgene expression in controls. At 16 ppm, La^{+++} reduced the transgene activity by 16 ppm Cd^{++} to a greater extent than both nifedipine and verapamil (c. 60% versus 25%, respectively). In whole trout, La^{+++} but not nifedipine was found to inhibit Ca^{++} influx via gills (Perry and Flik, 1988).

Calcium ionophore stimulates a range of Ca^{++}-dependent processes (Mastro and Smith, 1983; Inagaki *et al*, 1985), apparently by facilitating Ca^{++} entry into cells (Pohl *et al*, 1990). This ionophore is also known to transport other divalent cations across biological membranes (Geller *et al*, 1987). Although its effects on controls and Cd^{++}-induced transgene activities, as well as on Hg^{++} and Zn^{++}, are minimal in the transgenic nematode strain, a striking stimulation of transgene activation by 10 ppm Mn^{++} was observed in the

presence of 20 μM A23187 (Figs. 6.3 and 6.4). This is consistent with the known affinities of A23187 for various divalent metal ions, which are in the order $Mn^{++} > Ca^{++} > Mg^{++} > Cd^{++}$ (Calbiochem technical bulletin for the product). The greater affinity of A23187 and its derivatives for Mn^{++} as compared to Ca^{++} is also the basis for using Mn^{++} ions to quench quin-2 fluorescence in bromo-A23187-treated cells when calibrating free cytoplasmic Ca^{++} (Deber *et al*, 1985). We here attribute the effect of A23187 on Mn^{++} induction to enhanced accumulation of this ion within ionophore-treated worms, and more research is required to test this hypothesis by using radioactive Mn^{++} and measuring worm accumulation of this ion in the presence and absence of A23187.

The effects of Zn^{++} and Hg^{++} on Cd^{++}-induced transgene activity suggest possible interference at the level of uptake into worm tissues. Notably, low concentrations (0.5 or 2 ppm) of both Cd^{++} and Zn^{++} ions combined give an additive effect (albeit insignificant statistically), whereas higher concentrations (8 or 16 ppm of each ion) show an inhibition by Zn^{++} relative to Cd^{++} alone (Table 6.1). Somewhat similar effects of Zn^{++} counteracting Cd^{++} toxicity have been documented in ectomycorrhizal fungi (Colpaert and van Assche, 1991) and in cultured osteoblasts (Iwami *et al*, 1992). All concentrations of Hg^{++} tested, however, reduce transgene induction by Cd^{++}, which suggests competition with Cd^{++}. It is interesting to note that inorganic Hg^{++} has recently been found to increase the voltage-dependent Ca^{++} current through calcium channels at nanomolar concentrations, but caused inhibition at micromolar concentrations (Rossi *et al*, 1993). Endo and Shaikh (1993) have also found that cadmium uptake by primary cultures of rat renal cortical epithelial cells was reduced by other metal ions such as Zn^{++}, Cu^{++} and Hg^{++}. We also show that Zn^{++} inhibits Hg^{++}-induced transgene activity upto the controls levels. All these findings

strongly suggest that the transport of cadmium and even mercury may involve processes designed for essential metal ions. We conclude that further research is required to test whether net Cd^{++} accumulation by worms is reduced in parallel with transgene induction by the additional presence of these metal ions.

CHAPTER 7. GENERAL DISCUSSION

Because stress proteins are part of the primary cellular protective response during environmental stress, they are induced by a wide variety of biologically harmful agents, and are highly conserved in all organisms. As a result, the stress response has recently come under intense investigation as a basis for biomonitor systems to assess environmental contamination. A number of techniques are available for examining the stress responses in various organisms; the most frequently used techniques include metabolic labelling with subsequent one or two dimensional electrophoresis, and one- or two-dimensional Western blotting using stress- protein-specific antibodies. Another promising technique involves the use of transgenic organisms as biomonitors; in this approach, the transgenic organism carries a fusion construct containing a stress-inducible gene promoter plus a foreign reporter gene, which allows the monitoring of stress protein induction. In the present study, using all these techniques, we have examined the stress responses of two different organisms; one is the free living soil nematode *Caenorhabditis elegans* (both wild-type and transgenic strains), and the other is the freshwater crustacean *Asellus aquaticus*, both of which are well known-invertebrate model systems. We have also explored the possible use of these systems in environmental biomonitoring, and the results have been discussed with relevance to different techniques applied.

In order to evaluate the potential of using stress proteins as biomarkers of environmental stress, elevated stress proteins must accumulate in organisms under realistic environmental conditions in response to a wide variety of environmental stressors. Metabolic labelling studies on *Asellus aquaticus* clearly show that a stress response is

induced at concentrations as low as 0.35 ppm for Cd^{++} and 0.5 ppm in the case of Cu^{++} ions, which are well within the ranges measured in the environment. However, the induction of stress responses in nematodes involves exposure to heavy metal ions at relatively high concentrations (e.g. 2-16 ppm for clear-cut induction in the case of Cd^{++}), although these concentrations are well below 24 h LC50 values for this species. *C.elegans* appears to be intrinsically insensitive to many metal ions, as evinced by the high 24-96 h LC50 values measured by several authors (Williams and Dusenbery, 1988, 1990; Stringham and Candido, 1994; Mutwakil and de Pomerai, unpublished observations), as compared to many aquatic invertebrates such as *Daphnia* or the sea mussel *Mytilus edulis*. In *Daphnia*, Ag^{+} concentrations as low as 0.125 ppb have been shown to induce the synthesis of characteristic stress proteins (SPs 70 and 90; Bradley, 1993). Furthermore, exposure of *M.edulis* to 3.2-100 ppb Cu^{++} over 7 days has been demonstrated to induce significant amounts of a 60 kD stress protein in *Mytilus* (Sanders *et al*, 1991). It is probable that the relative insensitivity of *C.elegans* to heavy metal ions will limit its usefulness in toxicity testing. In part, this lack of sensitivity may be due to a high threshold requirement for HSF activation or for HSF binding to HSEs in *C.elegans*, since a large increase in temperature is needed to activate the heat-shock genes (from 20°C to 29°C for *hsp16* and to 34°C for *hsp70*; Snutch and Baillie, 1983). But it is unlikely that this lack of sensitivity can be attributed entirely to the heat-shock response system itself. One additional reason may be that metal ions are absorbed primarily via the gut and probably do not penetrate the cuticle readily. The abundance of metal-binding proteins (metallothioneins) in the gut of nematodes may also help to detoxify incoming metal ions efficently (Freedman *et al*, 1993). Moreover, adult *C.elegans* appear to be more sensitive to metal stress than are the larval (L1-L4) stages,

possibly because of constitutive metallothionein gene expression in the larval pharynx which may serve to detoxify metal ions more efficiently (Freedman *et al*, 1993). To test this, the promoters of heat shock protein and metallothionein genes in this organism could be fused to two different reporter genes so as to monitor the expression of both transgenes following toxicant exposure. Ideally, responses would be compared between wild-type and strains which are null mutants for either or both of the metallothionein genes (these latter should be more sensitive to metal toxicity). In fact, transgene constructs containing metallothionein promoters fused to reporter gene could be used more effectively to screen for heavy metal contamination in toxicity tests, since metallothioneins are specifically induced by many metal ions.

As mentioned above, *C.elegans* is unusual in that a large temperature rise in ambient temperature is required to induce a full heat-shock response (Snutch and Baillie, 1983). For worms grown at 20°C, optimal induction of *hsp70* occurs at 34°C, though *hsp16* is induced at 29°C (Snutch and Baillie, 1983; Stringham *et al*, 1992). This may be the reason why we need to combine the effect of the toxicants under study with a sub-heat-shock temperature increase, in order to provide adequate sensitivity in our transgenic system. Ideally, such combinations should be avoided in order to eliminate possible interactions between heat and toxicant effects. A promising model system in this respect is the transgenic *C.elegans* construct carrying a homologous *hsp16* promoter instead of the heterologus *hsp70* promoter used in the study. Because *hsp16* genes are activated at a lower temperature than any other stress-induced genes in *C.elegans*, β-galactosidase activity is inducible by several toxicants at the normal growth temperature of 20°C (Stringham and Candido, 1994). But due to the intrinsic insensitivity of this species to

heavy metals (discussed above), even this *hsp16*-based transgenic system remains relatively insensitive to such ions. For example, Cd^{++} induction of the *hsp16/lacZ* transgene occurs at quite high (μM) concentrations, which correspond to approximately 3 ppm for minimal and 11 ppm for maximal induction. In the case of Hg^{++}, maximum transgene activity is obtained at about 4 ppm. Although these values are slightly lower than comparable figures for the transgenic strain CB4027 used here, and also avoid the need for a concomitant temperature increase, these concentrations are still environmentally unrealistic. Another negative aspect of this transgenic system is that only a fraction of the exposed nematodes responded to toxicant exposures (e.g. 27% in case of 11 ppm Cd^{++}), whereas the entire population responded to heat shock. It seems unlikely that any *C.elegans* transgenic construct can wholly overcome this intrinsic insensitivity, although the greater sensitivity shown to several organic and organo-metallic toxicants such as lindane and tributyltin (this study; Guven *et al*, 1994) is perhaps more promising. It would, however, be interesting to test whether the *hsp16/lacZ* transgenic strain could be sensitised to heavy metal toxicants by raising the exposure temperature from 15 or 20°C to 25°C, which is still in the range of normal growth temperatures for this species.

Despite the metal ion-insensitivity of *Caenorhabditis elegans*, there are many advantages of using this organism in toxicity testing. These include; (i) its small size and the ease of handling large number of nematodes in the laboratory, (ii) its suitibility for use in both soil (Donkin and Dusenbery, 1993) and aquatic toxicity testing, (iii) its genetic homogeneity populations (due to the predominance of self-fertilisation within hermaphrodites). Particularly, the latter feature of nematodes would eliminate much of

the variation between individuals which was observed very often in *Asellus aquaticus*. It is well known that stress responses to certain toxicants may vary between the two sexes (Hakimzadeh and Bradley, 1990) or between different developmental stages (Miller and McLennan, 1988). Selecting individuals of one sex and at the same developmental stage should help to reduce variability in the asellid results. The individual variations observed in asellids may also indicate that precautions should be taken when using the stress response as an indicator of environmental contamination, since there is the possibility of inducing stress responses simply by handling the organisms during collection or exposure (limited volumes of ^{35}S-methionine containing media may themselves cause stress). Dietary influences also appear to modify the stress response; differential ^{35}S-methionine incorporation into stress proteins were found in rainbow trout fed different diets (Sanders, 1993).

Much current research is focused on the use of stress responses in other organisms, which are far more sensitive to a wide variety of toxicants, as a basis for detecting environmental contamination. The model organisms selected for this purpose should meet several other criteria, such as small size, and ease of handling a large number of organisms. Both *Daphnia* and perhaps *Gammarus* are freshwater crustaceans which seem to fit these criteria. In this laboratory, both of these organisms have recently been shown by immunoblotting to induce 70 kD stress proteins at very low (ppb) metal concentrations (Child, Guven, Reader and de Pomerai, unpublished observations). Unfortunately, these organisms are not well characterised in terms of their genetics, and do not at present lend themselves to transgenic studies. Thus, stress-inducible transgene constructs will need to be studied in a range of more genetically amenable systems, in order to provide highly

sensitive bioindicators of toxicant stress. For example, transgene constructs containing a human *hsp70* promoter fused to a human growth hormone gene and transfected into cultured mouse cells, have been succesfully used for toxicity screening with a large number of metal ions (Fischbach *et al*, 1993).

Clearly, the applicability of this system to aquatic ecosystems for biomonitoring would be limited, but might be ethically less objectionable than toxicity testing on living mammals. The use of transgenic fish, either whole organisms or derived cell lines, in environmental biomonitoring has recently attracted the attention of some ecotoxicologists, but little success has been reported to date.

Another stress response-based assay is to measure stress-protein induction or accumulation as a sensitive assay for environmental contamination by various toxicants. The techniques most frequently used in such assays involve the use of metabolic labelling and stress-protein-specific antibodies. The metabolic labelling technique provides information on the entire translational profile in response to a stressor. However, under continuous exposure to moderate stress conditions, changes in translational patterns are transient and translational activity in time reverts to patterns similar to those found in controls. Such a transient response appears to occur in *A.aquaticus* following stress exposure (as discussed in chapter 3). Another variant of the stress-protein approach is to use antibodies to measure the accumulated levels of stress proteins; this quite accurately reflects the level of stress experienced by organisms exposed to moderate toxicant perturbations over long periods of time. This approach has been notably successful in the case of *Mytillus edulis*, where Cu^{++} treatment induces *hsp60* (Sanders *et al*, 1991) and tributyltin treatment induces *hsp70* (Steinert and Pickwell, 1993), and in *Daphnia magna*

where low concentrations of AgCl induce both *hsp70* and *hsp90* (Bradley, 1993). One problem that may arise here is the presence of heat-shock protein cognates (e.g. HSC70) expressed constitutively in controls, which could mask low-levels of stress-induced protein isoforms. The use of two-dimensional electrophoresis should overcome this problem, but this adds in a further complex technique and so becomes quite demanding. This approach has been applied successfully to the nematode *C.elegans* exposed to cadmium (see chapter 4). However, the problem of SP70 degradation observed in *A.aquaticus* (as discussed in chapter 3) and in other systems (Lindquist, 1986; Miller and McLennan, 1988; Cochrane *et al*, 1991) together with non specific antibody binding on Western blots both serve to limit the usefulness of this technique in environmental biomonitoring. Furthermore, it is not easy to process a large number of samples by one- or especially two-dimensional Western blots, nor to make accurate quantitative comparisons between them. In view of these limitations, the transgenic approach discussed above offers the advantages of technical simplicity and the ease of processing multiple parallel samples for staining or enzyme assays.

Increasing evidence suggests that any environmental contaminant which can damage proteins would be expected to induce a classic stress response. The extent of such stress induction by a single toxicant will be dependent on the levels of toxicant accumulation (uptake minus any efflux) and by the mechanisms of toxicity in the organism under test. Many studies have shown a dose-response relationship between toxicant concentration and the levels of accumulated stress proteins (Sanders *et al*, 1991; Hightower, 1993), or of reporter protein in transgenic systems (Guven *et al*, 1994; Stringham and Candido, 1994; discussed in chapter 5). In general, stress response-based assays, as discussed in

this study and elsewhere, seem to work well when screening responses to a single toxicant, but the situation becomes more complex when testing the mixtures of toxicants which are often found in the environment. The study with transgenic nematodes clearly shows that Ca^{++} as well as several other metal ions (such as Zn^{++} and La^{+++}) interfere with Cd^{++} accumulation, and thereby reduce the extent of transgene induction by Cd^{++} ions. In this case, the level of stress response does not reflect the levels of toxicant (i.e. metal ions) present in the external medium.

Indicators of stress from different levels of biological organisation carry inherent strengths and weaknesses, and it is important to determine how information from each level can be coordinated and most effectively used in biomonitoring programs. From this point of view, stress response-based assays could be used for preliminary screening as indicators of "general stress", since they are nonspecific in their response and react to most major classes of contaminants (see Sanders, 1990; Jenkins and Sanders, 1992). The use of stress-protein biomarkers provides little information as to the causative factor or factors involved, as few if any stressor-specific features have been identified to date (but see chapter 4 on possible stressor-specific features of the 2D pattern of HSP70 proteins induced by heat and Cd^{++}. For this purpose, other molecular parameters such as stressor-specific responses could be used to determine the causal factors responsible for the positive results of the general stress response. Some of these stressor-specific indicators are the metallothioneins induced by heavy metal ions, and the cytochrome P450s, which are synthesised in response to a number of polyaromatic and chlorinated hydrocarbons. Future studies will no doubt identify other stressor-specific stress proteins which also play roles in protecting or repairing cellular sites of damage caused by specific stressors, and

this may open up new avenues of research for biomonitoring purposes.

In conclusion, stress responses have the potential to make great contributions in the area of ecotoxicology. More research is required on the stress responses of many other model organisms, which respond to a wide range of toxicants at environmentally realistic concentrations. However, it remains an open question as to whether the stress response-based indicators are more sensitive to toxicant perturbation than are complex processes such as respiration, growth or motility, and could ever rival the established whole-organism approaches to environmental monitoring.

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Publications

1- Guven, K., Duce, J.A., de Pomerai, D.I. (1994).

Evaluation of a stress-inducible transgenic nematode strain for rapid aquatic toxicity testing. **Aquatic Toxicology** 29, 119-37.

2- Guven, K., Duce, J.A., de Pomerai, D.I.

Calcium moderation of cadmium stress explored using a stress-inducible transgenic strain of *Caenorhabditis elegans*. **Comp. Biochem. Physiol. Part C** (in press).

3- Guven, K. & de Pomerai, D.I.

Differential induction of HSP70 proteins by heat and cadmium in *Caenorhabditis elegans*. **J. Therm. Biol.** (submitted)

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Aquatic Toxicology 29 (1994) 119–137

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(Received 1 April 1993; revised received 15 November 1993; accepted 25 February 1994)

Abstract

A transgenic strain of the nematode *Caenorhabditis elegans* which carries a stress-inducible *lacZ* reporter gene has been evaluated in terms of its response to several environmental toxicants. Optimal sensitivity is obtained by exposing these worms to toxicants at a temperature just below that required for heat induction of the transgene. Under these circumstances, several heavy metals (Cd^{2+}, Zn^{2+}, Hg^{2+}, Mn^{2+}, Sn^{2+}, Ag^{+}) cause dose-dependent transgene expression, which can be monitored as β-galactosidase enzyme activity or by in situ histochemical staining. A simple assessment procedure has been developed so that staining patterns can be compared between runs. The induced enzyme activity is localised in a single band (of apparent size 170 kD) on Western blots, as shown both by histochemical staining and immunoprobing. Endogenous heat-shock proteins (*hsp70*) are optimally induced under the same assay conditions, but modest induction is also apparent under control conditions (sub-heat-shock temperatures alone). Our system requires relatively high concentrations (ppm) of metallic ions for clear-cut induction, but is apparently more sensitive to certain organic and organo-metallic compounds (lindane and tributyltin are effective at ppb levels). This system works well within strictly defined assay conditions, but some toxicants are more effective inducers than others (e.g. Cd^{2+} versus Mn^{2+}), while some give paradoxical dose–response curves possibly due to enzyme poisoning at high toxicant concentrations (e.g. Ag^{+}). However, similar problems are likely to be encountered with any transgenic assay system based on the heat-shock response when used to monitor environmental pollution.

Key words: *Caenorhabditis elegans*; Transgenic; Toxicity testing; Heavy metals

1. Introduction

The free-living soil nematode, *Caenorhabditis elegans*, offers many advantages as an experimental organism, including its detailed genetics and complete cell lineage, an

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SSDI 0166-445X(94)00018-L

unusual mode of reproduction by either self- or cross-fertilisation, and a rapid generation time of only 3 days at 20°C when grown on agar plates or in liquid culture using *Escherichia coli* as food source (Brenner, 1974). Indeed, *C. elegans* is probably the most completely characterised metazoan in terms of its anatomy, genetics and development (see Wood, 1988); its genome is also likely to be the first to be fully sequenced (initial findings in Sulston et al., 1992).

However, few toxicological studies using *C. elegans* have been reported. Williams and Dusenbery (1988, 1990) determined LC50 values for a range of metal ions over 1–4 days of exposure, and have suggested that *C. elegans* could be used to predict mammalian acute lethality to these metals at less than 10% of the cost for comparable tests on vertebrates. Assays have also been developed to screen for mutagens or carcinogens, based on mutant reversion to wild-type size (Lew et al., 1983) or motility (mentioned by Mlot, 1991); such methods are clearly applicable to environmental pollutants with known genotoxic effects, but would be inappropriate for other classes of toxicant. The striking effects of certain neurotoxins on motility have suggested the possible use of *C. elegans* as a preliminary screen for such agents (Williams and Dusenbery, 1987). We ourselves have noted increased motility of *C. elegans* treated with sub-nanomolar concentrations of dioxin (Guven and de Pomerai, unpublished observations).

In recent years, genetic engineering has generated a wide range of transgenic animals, used mainly for molecular studies of gene expression and regulation. The utility of such transgenic organisms as toxicological indicators has attracted some attention (see e.g. Anderson, 1989). We have used an established transgenic strain (CB 4027; Fire, 1986) of *C. elegans*, carrying integrated copies of a stress-inducible reporter gene. This construct comprises a *Drosophila hsp70* promoter fused to an *E. coli lacZ* structural gene encoding β-galactosidase. Generally, clustered heat-shock elements (HSEs) present in *hsp* promoter sequences direct transcriptional activation of the associated *hsp* genes; this occurs following heat shock or during exposure to certain other kinds of environmental stress (reviewed by Bienz, 1985; Sorger, 1991). Under such conditions, the HSEs are bound by an activated (oligomeric) form of the heat-shock transcription factor (HSF; Zimarino and Wu, 1987; Zimarino et al., 1990), which can then switch on *hsp* gene transcription. HSF protein is present at all times in non-stressed metazoan cells, but rapidly oligomerises, translocates into the nucleus and binds to DNA following its post-translational activation by stress (Westwood et al., 1991). The HSE consensus sequences and their spacing are well conserved among the heat-inducible *hsp* genes analysed to date, but this is less true of the HSF protein sequence. Nevertheless, many HSFs can efficiently activate heterologous *hsp* promoters, implying conservation of at least the DNA-binding domain. As a case in point, the *Drosophila hsp70* promoter used in constructing the transgenic *C. elegans* strain used here is clearly recognised by the latter's endogenous HSF, so *trans*-activating *lacZ* gene expression (Fire, 1986); this happens under the same conditions that switch on the nematode's own heat-inducible *hsp70* genes (Snutch and Baillie, 1983; Heschl and Baillie, 1990; see also Fig. 3C below).

Organisms carrying such stress-inducible gene constructs could potentially provide an early warning of environmental stress (Anderson, 1989; Miller, 1989), but clearly

both the system's sensitivity and the range of effective inducers must first be evaluated carefully. We here report on the effects of several heavy metals and other toxicants as inducers of β-galactosidase expression in the transgenic *C. elegans* strain CB 4027. Our results suggest: (a) that care is required to optimise the assay conditions for maximal sensitivity; (b) that Cd^{2+} is a far more effective inducer than any other toxicant tested; and (c) that some toxic agents induce detectable stress responses only across a certain range of concentrations, with negative results from above as well as below this range. These findings underline the need for careful characterisation of any transgenic organism before it can be used for environmental monitoring, and suggest that any such use should be as an adjunct to existing methods.

2. Materials and Methods

The transgenic *C. elegans* strain CB 4027 (=332-2 of Fire, 1986) and the *lac*-operon deleted *E. coli* strain P90C were generous gifts from Dr J. Hodgkin and Dr A. Chisholm of the MRC Molecular Biology Laboratory, Cambridge, UK. All chemicals were from Sigma-Aldrich Ltd, Poole, UK, unless otherwise stated. The anti-β-galactosidase antibody (polyclonal, raised in rabbits) was a generous gift from Dr P. Tighe of this department, while the anti-*hsp70* family antibody (rat monoclonal MA3-001) was purchased from Affinity Bioreagents (Neshanic Station, New Jersey, USA). The metallic salts used in this study (all ACS grade) were $CdCl_2 \cdot 2½H_2O$, $MnCl_2 \cdot 4H_2O$, $AgNO_3$, $SnCl_2 \cdot 2H_2O$, $ZnSO_4 \cdot 7H_2O$, and $HgCl_2$.

Worm culture

CB 4027 worms were cultured at 15°C or 20°C on NGM Agar plates (Sulston and Hodgkin, 1988), using *E. coli* strain P90C as a food source. Worms were routinely used after 3 or 4 days, by which time responsive adults tend to predominate over less-responsive larvae. For the purposes of our study, synchronous cultures of young adults derived from dauer larvae (as used by Williams and Dusenbery, 1990) offered no significant advantages, although we estimate that up to 20% of the total worm mass in our mixed cultures comprised larvae expressing no detectable β-galactosidase under most conditions tested. Thus the induction values given in Fig. 2 are somewhat lower than those which might have been expected from adults-only cultures. For assay purposes, worms were washed off the plates with K medium (53 mM NaCl, 32 mM KCl), centrifuged briefly (4000 *g*, 2 min) and resuspended in an appropriate volume of fresh K medium in Petri dishes or multiwell plates (Corning Cell-Wells). This procedure leaves a few bacteria in the worm pellet, providing a food source during subsequent exposure, but this bacterial contamination is insignificant compared to the mass of worms present during the assay. Since P90C bacteria have their own *lacZ* gene deleted, all detectable β-galactosidase activity must derive from the worms.

Exposure of worms to heat and toxicants

Exposure of worms (grown normally at 15°C) to metal ions and other toxicants was carried out by adding appropriate volumes of 50× to 500× concentrated solutions

to each well or dish containing the worms suspended in 1 or 10 ml of K medium. Exposure was routinely continued for 7 h at 32°C (see Results section). The chosen heavy metal concentrations (mg L^{-1} for the metallic ion, i.e. ppm) were in the range of the LC50 values for wild-type *C. elegans* over a 24–96 h period (see Williams and Dusenbery, 1990). For organic pollutants, initial range-finding experiments were performed using order-of-magnitude differences in concentration; in cases where ethanol was used to solubilise the toxicant (lindane and tributyltin chloride), the final solvent concentration did not exceed 0.1% (v/v), and controls included ethanol alone at the same concentration. The concentration of tributyltin chloride (TBT) was again calculated as mg L^{-1} (ppm) of tin. For lindane, ppm values were based on the total molecular weight.

Determination of metal contents of tissue samples and supernatants

Between 20 and 100 mg wet weight of CB 4027 worms were exposed to toxicants as above, then the worms were removed by centrifugation, washed 5× with fresh K medium and briefly with ultrapure water. The worms were spun down in pre-weighed metal-free plastic tubes and air dried at 50°C for 48 h. The tubes were reweighed to measure the dry weight of worms present (control determinations showed that the dry mass of worms represents 12% of the wet weight after pelleting at 12 000 *g* and carefully removing all supernatant fluid). The dried pellets were each digested with 1 ml of concentrated HNO_3 (B.D.H. Analar grade) at 60°C until completely dissolved (usually overnight), and then diluted to 4 ml total volume with ultrapure water. These samples were analysed for heavy metal content by atomic absorption spectrometry (with graphite furnace for Sn determinations, carried out in the Department of Animal and Plant Sciences, Sheffield University). After centrifugation to remove worms, bacteria and debris, 10 ml assay supernatants were mixed with HNO_3 (25% v/v final) and heavy metal determinations were performed by atomic absorption spectrometry, using standards of the same metallic salt dissolved in K medium containing 25% (v/v) of concentrated HNO_3.

β-*Galactosidase assays*

After exposure, worms were centrifuged (4000 *g* for 2 min) and washed with 20 volumes of M9 buffer (85 mM NaCl, 42 mM Na_2HPO_4, 22 mM KH_2PO_4, 1 mM $MgSO_4 \cdot 7\ H_20$) at least 3 times to remove bacteria and toxicant. A small aliquot of worms was removed for staining as below, then 200 μl of M9 buffer containing 1 mM dithiothreitol (DTT) and 0.1% (w/v) sodium dodecyl sulphate (SDS) was added to the remainder. The worms were homogenised thoroughly with a tight-fitting pestle, and the homogenate centrifuged (12 000 *g* for 3 min) to remove debris. β-Galactosidase activity was determined spectrophotometrically at 410 nm after 15 h at 30°C by measuring ONPG (*o*-nitrophenyl β-D-galactopyranoside) hydrolysis, as described by Fire (1986). A purified preparation of *E. coli* β-galactosidase (Sigma) was used to provide a standard curve for each experiment; one unit is defined as the amount of enzyme required to hydrolyse 1 nmol of ONPG/min under the assay conditions used (Fire, 1986). Protein concentrations were determined according to the method of Lowry et al. (1951).

Histochemical detection of β-galactosidase

This was also carried out as described by Fire (1986); aliquots of worms were acetone-fixed onto slides and then stained using 6-bromo-2-naphthyl-D-galactopyranoside and Fast Blue B diazonium salt. Stained worms were mounted in 60% (v/v) glycerol and photographed under direct light on a Leitz inverted phase microscope. Our assessment protocol for comparing the degree of staining between different runs is described in the Results section.

SDS-Polyacrylamide gel electrophoresis and Western Blotting

Protein samples were run on 0.1% SDS polyacrylamide gels containing 7% or 10% acrylamide as described by Laemmli et al. (1970), using an LKB-Pharmacia Minigel system. Prestained molecular weight markers (Sigma) were also run on each gel; notably, the largest of these is β-galactosidase (116 kD). Blotting onto Biotrace (Gelman) membranes was carried out using an LKB Miniblot system at 4°C for 4 h. The membrane was blocked overnight in 10 mM Tris-HCl pH 7.5/154 mM NaCl/0.02% Tween-20 (TBS-T) containing 5% dried milk powder at 4°C. Blots were either stained histochemically as above (Fire, 1986; Fig. 3A) or else probed for 3 h at room temperature with a rabbit polyclonal antibody against β-galactosidase (0.1% in TBS-T; see Fig 3B). After three washes with TBS-T, the blot was treated for 3 h with peroxidase-linked anti-rabbit IgG (0.1% in TBS-T), then washed 3× with TBS-T and finally stained using 4-chloro-1-naphthol and H_2O_2. The same blotting and immunoprobing

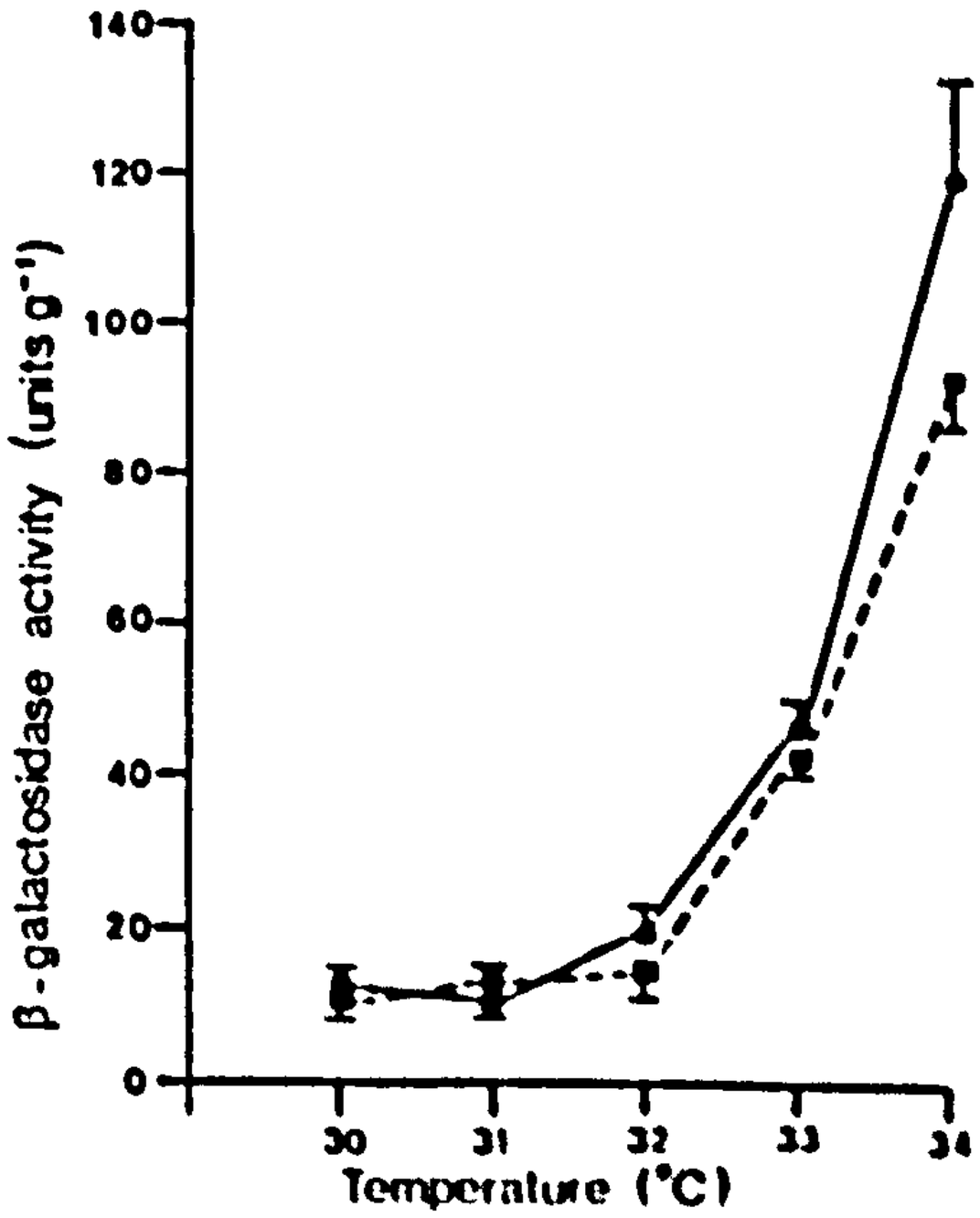

Fig. 1. Heat induction of β-galactosidase activity. Worms were exposed for 7 h to temperatures of 29–34°C in liquid medium (solid squares, dashed line) or on agar plates (solid circles, unbroken line). β-Galactosidase activity and protein content were determined as described in Methods for all samples, so as to calculate the specific enzyme activity in units g^{-1}. The mean and standard error (bar) from at least six determinations are shown.

protocol was employed to detect endogenous *hsp70* proteins, using a 1:500 dilution of a commercial anti-*hsp70* family antibody (rat monoclonal MA3-001 from Affinity Bioreagents), which reacts with an epitope of denatured *hsp70*'s from a wide range of animal, plant and fungal species (Fig. 3C); in this case the secondary antibody was 0.1% (v/v) peroxidase-linked anti-rat IgG.

3. Results

C. elegans is unusual in that a large rise in ambient temperature is required to induce a full heat-shock response (Snutch and Baillie, 1983). For worms grown at 20°C, optimal induction of *hsp70* occurs at 34°C, though *hsp16* is induced at 29°C (Snutch and Baillie, 1983; Stringham et al., 1992). The transgenic *hsp70-lacZ* strain used in this study was grown at 15°C, but gives a similar heat induction profile to that of the endogenous *hsp70* (see also Fig. 3C). As shown in Fig. 1, transgene expression (assayed as β-galactosidase activity) is minimal up to 32°C (see also Fig. 4a, with significant induction at 33°C rising to about 10-fold above controls at 34°C. Heat induction of transgene activity is slightly more effective for worms grown on agar

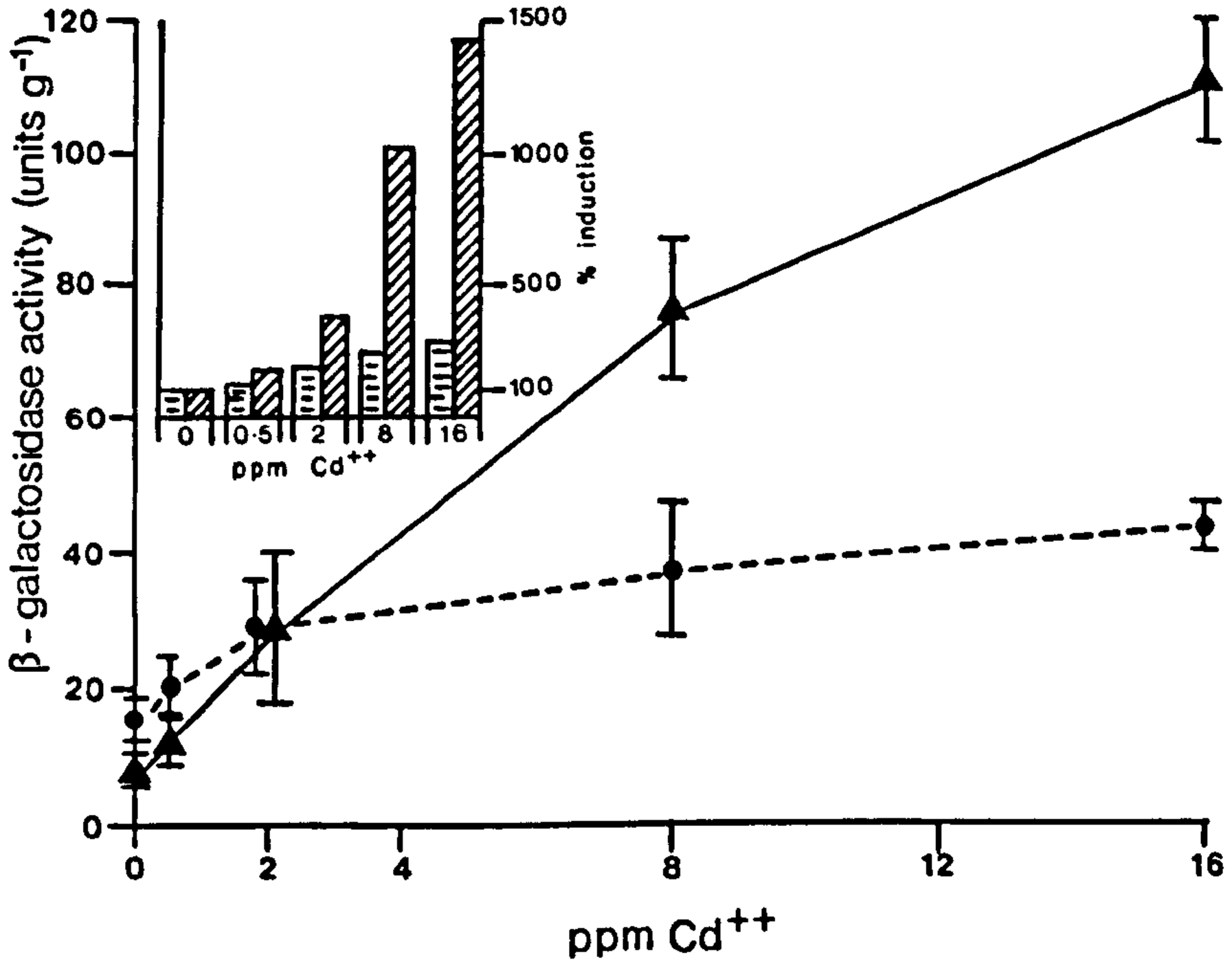

Fig. 2. Cadmium induction of β-galactosidase activity in transgenic worms grown at different temperatures. The main figure shows the mean β-galactosidase activity (± standard error) determined for worms exposed to Cd^{2+} for 7 h at 32°C, across the range 0–16 ppm Cd^{2+}. Determination of β-galactosidase activity and numbers of replicates were the same as for Fig. 1. Results are compared between worms grown at 20°C (solid circles, dashed line) and those grown at 15°C (solid triangles, unbroken line). The insert figure shows the same data normalised to the respective control values (32°C, no Cd^{2+}) as 100%. Hatched bars, worms grown at 15°C. Dashed bars, worms grown at 20°C.

Table 1
Staining gap scores and β-galactosidase activities induced by various toxicants

(A) *Comparison of zinc, cadmium and mercury as inducers of transgene expression*

Test toxicant	Gap score 0.15 ppm	β-gal (μ/g) 0.15 ppm	Gap score 0.5 ppm	β-gal (μ/g) 0.5 ppm	Gap score 2.0 ppm	β-gal (μ/g) 2.0 ppm	Gap score 16 ppm	β-gal (μ/g) 16 ppm
Zinc	0	0	0.24	0	1.54	4.8	2.64	7.93
Cadmium	0.23	0	0.86	2.25	2.07 ± .22	11.04	7.34 ± .37	92.8
Mercury	0.20	0	0.94	3.20	2.21	6.05	2.96	12.8

(B) *Other toxicants as inducers of transgene expression*

Staining gap scores are given for all entries; where available, corresponding β-galactosidase activities (net units per g above 32°C controls) are quoted within square brackets.

Test toxicant	Concentration of test toxicant in ppm or ppb: ppm					ppb				
	100	50	20	10	2	500	150	50	15	5
Ag^{+}	−0.5 Tox?	3.5 [32]	2.7	2.4 [14]	1.0 [4.3]	0.8	0.5	0.1	ND	ND
Sn^{2+}	ND	2.2 [7.4]	1.7	1.2	0.7 [1.7]	ND	0.3	0	ND	ND
Mn^{2+}	ND	2.4 [9.6]	2.0	1.6 [5.4]	1.0 [4.7]	ND	0.4	0	ND	ND
Lindane	ND	ND	ND	ND	0.9 Tox	ND	1.7 [11]	ND	1.35	ND
Tributyl-tin-Cl	ND	ND	ND	ND	ND	ND	1.3 Tox	3.2 [28]	2.0 [14]	1.8 [11]

Worms were exposed to the indicated concentrations of each test toxicant under standard conditions (7 h at 32°C). Histochemical staining was performed as described in Methods, and stain-scores (relative to parallel controls without toxicant) were assessed as described in the Results section. Gap scores were determined by subtracting the mean control score from each toxicant-treated score (see Results). Each entry gives the mean of 4 to 8 such gap scores assessed on two or more occasions. Larger batches of toxicant-treated worms were processed for β-galactosidase assays as described in Methods; each value quoted in Table 1A and within square brackets in Table 1B represents the mean net activity (i.e. greater than parallel 32°C controls) in units per g derived from 4–10 such assays. Typical SEM values are shown for the cadmium data in Fig. 2. Generally speaking, there is broad agreement between the staining gap scores and β-galactosidase activities recorded following different toxicant treatments. Entries marked Tox showed substantial (>20%) mortality among treated worms, which may reduce the staining gap scores. The unusual case of 100 ppm Ag^{+} (marked Tox? because most worms survived the treatment but showed even less staining than 32°C controls) is discussed in the Results section. ND, not determined.

plates as compared to liquid medium. We find unexpectedly that the level of β-galactosidase expression at 32°C is actually *lower* for worms grown at 15°C as opposed to 20°C (see control values in Fig. 2). Thus growth at 15°C seems to give a much tighter temperature induction curve (Fig. 1), as compared to worms grown at 20°C. A similar effect of growth temperature on heat inducibility has been reported for yeast strains carrying a heat-inducible *lacZ* transgene (Kirk and Piper, 1991).

Turning to the effects of heavy metals, cadmium is by far the strongest inducer of transgene activity among those tested. Because metal ions will be absorbed into the

Table 2
Metal contents of worms and supernatants

(A) *Concentrations of metal ions in medium before and after standard worm assays*

	ppm metal before assay	ppm metal after assay	Percentage loss of metal ion
Mercury (Hg^{2+})	1.0	0.938	9.1%
Mercury (Hg^{2+})	5.0	4.27	16.3%
Zinc (Zn^{2+})	1.0	0.882	11.8%
Zinc (Zn^{2+})	5.0	4.17	16.5%
Cadmium (Cd^{2+})	1.0	0.875	12.5%
Cadmium (Cd^{2+})	5.0	4.52	9.7%
Cadmium (Cd^{2+})	16.0	13.62	14.9%
Mean for all metals and concentrations	–	–	12.97 ± 3.01%

(B) *Effect of growth temperature on cadmium accumulation by worms*

Worms grown at	Exposed 7 h at	ppm Cd^{2+} in test medium	ppm Cd^{2+} in wet worms	Mean accumulation
20°C	15/20/32°	16	408 ± 52	25.5-fold
15°C	15/20°	16	280 ± 18	17.5-fold
15°C	32°	16	614 ± 9	38.4-fold
Dead worms	32°	16	29 ± 6	1.83-fold
Homogenised worm pellet	32°	16	179 ± 25	11.2-fold

(C) *Accumulation by worms of inorganic tin versus tributyltin chloride*

Test toxicant	ppm of metal in test medium	ppm of metal in wet worm pellet	Mean accumulation
Sn^{2+}	5.0	44.8 ± 10.66	8.95-fold
Sn^{2+}	0.25	2.13 ± 0.92	8.51-fold
Sn^{2+}	0.05	0.49 ± 0.14	9.89-fold
Tributyltin-Cl	0.25	32.6 ± 1.15	130.4-fold
Tributyltin-Cl	0.05	6.13 ± 0.144	122.6-fold
Tributyltin-Cl	0.005	1.09 ± 0.036	217.1-fold

agar and taken up by bacterial cells, the results of cadmium treatment are more clearcut in liquid as opposed to agar cultures (data not shown). For this reason, *all* toxicant treatments were carried out in liquid K medium, in the absence of agar and with only a small proportion of bacteria present (see Methods). Cadmium alone at 15°C or 20°C does not detectably induce β-galactosidase, but if the temperature is raised to 32°C during cadmium treatment over a 7 h period, then concentration-dependent transgene expression is observed (Fig. 2; Fig. 4d, e, g, h; Table 1A). Background expression due to heat (32°C) alone is significantly lower for worms grown at 15°C, as compared to those grown at 20°C ($p < 0.005$; Fig. 2). Moreover, the effect of increased cadmium concentrations on β-galactosidase expression is significantly greater for worms grown at the lower temperature. Apart from the crossover point at 2 ppm Cd^{2+} (where enzyme activities are virtually identical), the two curves shown in Fig. 2 differ significantly ($p < 0.005$) throughout. The insert histogram in Fig. 2 shows the same data normalised to the respective control values as 100% (i.e. at 32°C in the absence of Cd^{2+}). This demonstrates a 14-fold induction by 16 ppm Cd^{2+} over controls for worms grown at 15°C, as compared to only 3-fold induction for those grown at 20°C. Time-course experiments showed higher induction by Cd^{2+} over a 7 h period as compared to either 4 or 12 h exposures (data not shown). For this reason, our standard assay conditions used in this study involved toxicant treatment for 7 h at 32°C of worms grown at 15°C. Table 2A shows that under these conditions the concentration of heavy metal in the medium decreases by 12.97 ± 3.01% over the assay period, irrespective of the metal ion or its initial concentration; this represents metal accumulation within worms plus adsorption/non-specific binding to worm cuticles, remaining bacterial cells and plastic dishes. Thus the fluid pumped through the

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All metal concentrations were determined by atomic absorbtion spectrometry (AAS), using a graphite furnace in the case of tin; metal-ion standards were made up in K medium (Table 2A) or 25% concentrated nitric acid (Tables 2B and C) as appropriate. Worms were grown at 15°C unless otherwise indicated (20°C in top line of Table 2B), and exposed for 7 h to the indicated metals under standard conditions (32°C except for some data included in Table 2B). In all cases, assays were scaled up 10-fold so as to increase both the mass of worms and volume of supernatant. Each entry in Table 2A represents the mean of 3 determinations on assay supernatants relative to the initial test solutions; since the percentage losses recorded varied from 9% to 17% independent of the ion or concentration used, an overall mean ± SEM for all relevant data is given as the final entry. After exposure, worms were pelleted, washed and prepared for AAS as described in the Methods. In a series of control determinations, we found that dry mass of worms represents on average 12.05% of the wet weight (measured after pelleting at 14000 *g* and removal of all visible supernatant). Hence apparent concentrations of metal per unit dry weight have been divided by 8.3 to obtain concentrations per unit wet weight, which can be more readily compared to the initial concentrations in the external medium (Tables 2B and C). Apparent accumulation by the worm pellet was determined simply by dividing the concentration in wet worms by the appropriate external concentration. We accept that this is only a crude estimate, including metal bound non-specifically by the nematode cuticle or by bacterial cells as well as metal actually taken up into worm tissues. However, the controls included in Table 2B show that these errors do not affect our results significantly. Dead worms (frozen overnight) accumulate Cd^{2+} less 2-fold above the external medium (e.g. from adsorption to cuticle etc). Crude homogenisation of Cd^{2+}-exposed worms releases some 70% of the accumlated Cd^{2+} into the soluble fraction, only 30% being retained in the pellet. For Table 2 parts B and C, each entry gives the mean of 4–8 determinations; SEM values are given in the penultimate column.

pharynx throughout the assay period contains >80% of the original test concentration of metal. Moreover, the controls included in Table 2B show little (< 2-fold) accumulation of Cd^{2+} by dead worms, and much of the Cd^{2+} within live worms remains in the soluble fraction after homogenisation. We also note an interesting effect of growth temperature on cadmium accumulation by the worms (Table 2B). Worms grown at 20°C accumulate similar levels of Cd^{2+} whether exposure takes place at 15, 20 or 32°C. Worms grown at 15°C accumulate less Cd^{2+} when exposed at 15°C or 20°C, but Cd^{2+} accumulation is increased >2-fold during exposure at 32°C. Since an increase in non-specific metal binding seems unlikely (no such effect for worms grown at 20°C), this may help to explain why Cd^{2+} induction of transgene activity is higher for worms grown at 15°C rather than 20°C.

As shown in Fig. 3, the induced β-galactosidase activity can be localised to a single protein band on Western blots following in situ staining or probing with a polyclonal anti-β-galactosidase antibody. We note that both commercial *E. coli* β-galactosidase and the induced enzyme seem to react optimally as a 170 kD form (see Muga et al., 1993), whereas the 116 kD β-galactosidase band included among the prestained molecular weight (MW) markers is immunodetectable (Fig. 3B), but has no detectable enzyme activity (Fig. 3A). We provisionally ascribe this difference to the high concen-

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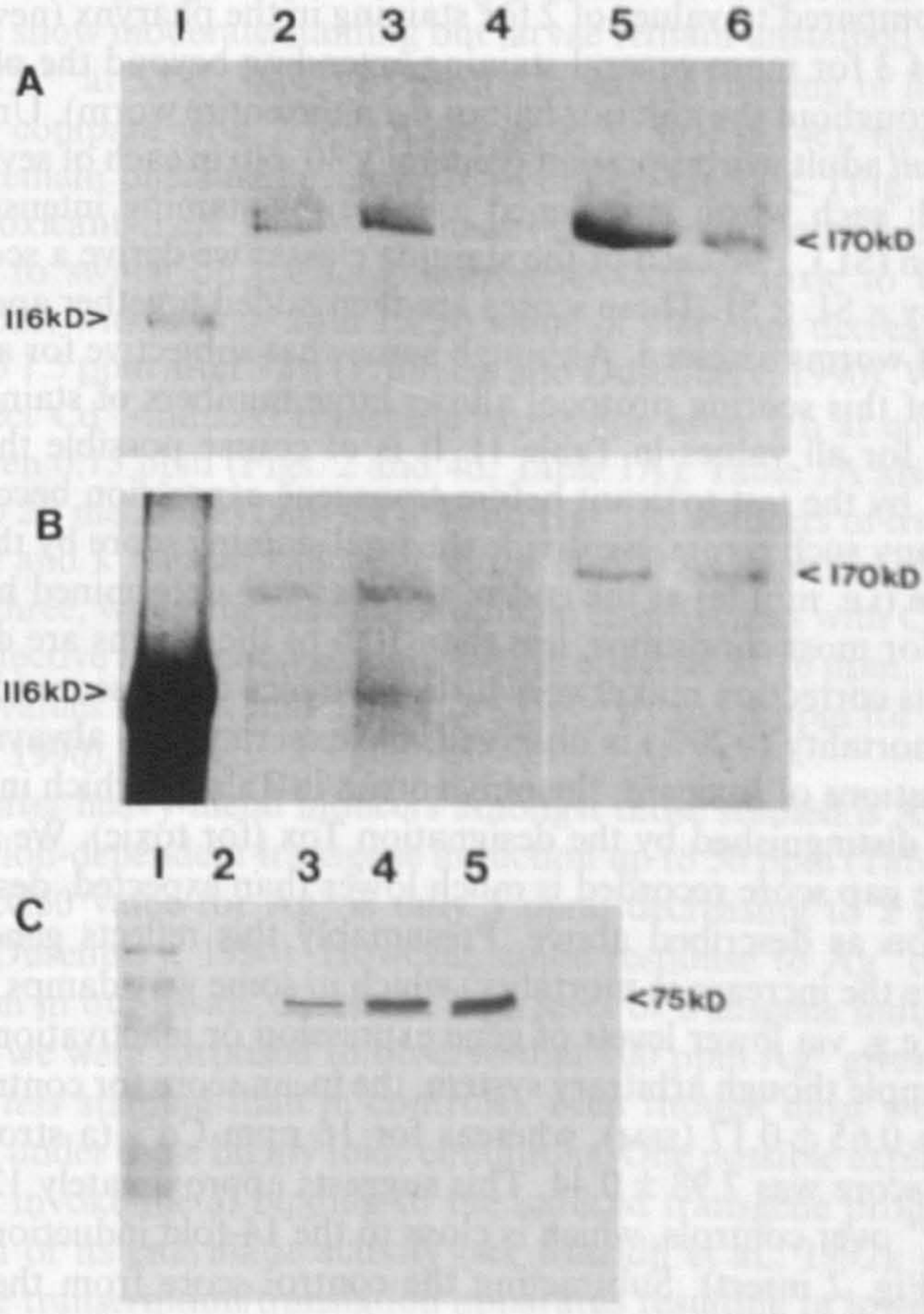
Fig. 3. Western blotting analysis of induced β-galactosidase. Worm homogenates were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting as described in Methods. The two blots shown in Parts A and B are both run with identical protein samples to demonstrate β-galactosidase induction. A third blot is shown in Part C to demonstrate induction of endogenous *hsp70* under the same conditions. **Part A:** Blot subjected to in situ histochemical staining (Methods) to detect renatured and enzymically active β-galactosidase. **Part B:**Blot immunoprobed with polyclonal anti-β-galactosidase (details in Methods). In Parts A and B, the lanes were loaded as follows: Lane 1, Sigma prestained SDS-PAGE MW markers; only that part of the blot containing the β-galactosidase monomer (116 kD) is shown. Lane 2, 0.1 unit of commercial (Sigma) purified β-galactosidase. Lane 3, 0.2 unit of commercial (Sigma) purified β-galactosidase. Lane 4, homogenate of control worms (7 h at 32°C). Lane 5, homogenate of cadmium-treated worms (7 h at 32°C+16 ppm Cd^{2+}). Lane 6, homogenate of heat-shocked worms (7 h at 34°C). Lanes 4–6 were each loaded with approximately 20 μg total protein. Arrows point to the β-galactosidase monomer (116 kD) and active form (170 kD); both are detected as immunoreactive (Part B), but only the latter is enzymically active (Part A). Note that the 116-kD monomer is immunoreactive in the MW marker lane (Part B) but is devoid of enzyme activity (Part A; the faint band seen at this position is due to the prestain). In Part B, the worm transgene product (lanes 5 and 6) appears slightly larger than the purified enzyme (lanes 2 and 3), though this difference is obscured by band distortion in Part A. In the purified enzyme lanes (2 and 3), both monomer and active-form bands react faintly with the antibody (Part B), but only the latter is enzymically active (Part A). **Part C:** In this case the blot was probed as in Part B with a commercial rat monoclonal anti-*hsp70* family antibody (Affinity Bioreagents), which was detected with a peroxidase-linked anti-rat IgG secondary antibody (Sigma); 20 μg of total protein was loaded onto lanes 2–5. Lane 1, prestained molecular weight markers (Sigma), whose approximate sizes in kD are 116 (top), 84, 64, 48, 36.4 and 29.7. Lane 2, homogenate of worms incubated for 7 h at 20°C (no detectable *hsp70*). Lane 3, homogenate of worms incubated for 7 h at 32°C (some *hsp70* induction). Lane 4, homogenate of worms incubated for 7 h at 32°C with 16 ppm Cd^{2+} (strong *hsp70* induction). Lane 5, homogenate of worms heat-shocked for 7 h at 34°C (strong *hsp70* induction). Note the presence of a doublet *hsp70* band, the larger of which is more strongly induced at 32°C than is the smaller (lane 3). These may represent the products of differentially heat-inducible *hsp70* genes (see Heschl and Baillie, 1990). We estimate the apparent molecular size of these bands to be about 75 kD (arrowhead), slightly larger than the expected 70 kD.

trations of denaturant present in the MW marker preparation; β-galactosidase is normally active as a tetramer, but the 170 kD form produced under mild denaturing conditions (0.1% SDS in our extraction buffer and SDS-PAGE system) seems to retain significant activity (Muga et al., 1993; Fig. 3A). In fact, this active form of the transgene product has a slightly higher apparent molecular size than the corresponding form of the purified *E. coli* enzyme, as shown in Fig. 3B (gel distortion obscures this difference in Fig. 3A). This presumably results from the inclusion of some additional sequences at the ends of the *E. coli* β-galactosidase coding region in the *lacZ* fusion construct used by Fire (1986). Fig. 3C shows parallel results using a commercial anti-*hsp70* monoclonal antibody (Affinity Bioreagents) to detect endogenous *hsp70*'s induced in CB 4027 worms under the same induction conditions as those used in Figs. 3A and 3B. We note that *hsp70* is essentially undetectable at 20°C, but that moderate induction can be detected at 32°C (control conditions giving barely detectable transgene expression; Figs. 3A and 3B). However, both 34°C heat-shock and 16 ppm Cd^{2+} treatment at 32°C give very much stronger induction of endogenous *hsp70* (particularly the smaller component of the approximately 75 kD immunoreactive doublet band in Fig. 3C), closely mirroring the transgene response. Thus transgene



expression may be more difficult to activate than the endogenous heat-shock response (perhaps because the nematode HSF does not recognise the heterologous *Drosophila hsp70* promoter as readily as its own *hsp* promoters), but this disadvantage is more than outweighed by the greater ease of detecting and quantifying the transgene product.

Both β-galactosidase assays and in situ histochemical staining provide simple and reliable means for assessing transgene induction under a wide range of experimental conditions, the latter with the added advantage of requiring less time and far fewer worms. However, it is difficult to quantitate staining data impartially so as to make valid comparisons between runs. We have adopted a simple points system which gives a fairly consistent gap between positive and negative controls (16 ppm Cd^{2+} and 32°C alone, respectively). Although the majority of control worms (32°C alone) remain unstained, in many runs a few embryos within adults are stained pink or red. As Fire (1986) has noted, there is a predilection for staining of embryos and/or pharyngeal tissue in adult CB 4027 worms, possibly dependent upon the site(s) of fusion-gene insertion into the *C. elegans* genome. We assign arbitrary intensity values of 0 for unstained worms, 2 for pink/red staining and 4 for intense purple/blue staining. Since limited embryo staining can occur even in 32°C controls, we assign this a location value of 1, as compared to values of 2 for staining in the pharynx (never seen in 32°C controls) and of 3 for more general staining extending beyond the pharynx and embryos (often throughout the anterior half or even the entire worm). Under each condition, we assess *all* adult worms present (generally 30–50) in each of several microscope fields examined; each worm is assigned a value for staining intensity (SI) and for staining location (SL). For each of the staining classes we derive a score by multiplying its frequency × SL × SI. These scores are then added together and divided by the total number of worms assessed. Although somewhat subjective for any given worm, the simplicity of this scoring protocol allows large numbers of stained worms to be assessed (>100 for all values in Table 1). It is of course possible that some worms might be killed by the test toxicant before transgene expression becomes detectable. To correct for any such errors, we divide the final staining score by the proportion of worms left alive (i.e. motile) at the end of the assay, as determined by microscopical examination. For most conditions, less than 10% of the worms are dead after 7 h of exposure, so this correction makes very little difference to the score (divided by 0.9 or 0.95). If high mortality (>20%) is observed, the experiment is always repeated using lower concentrations of toxicant; the only entries in Table 1 which involve high mortality rates are distinguished by the designation Tox (for toxic). We note that for all such entries the gap score recorded is much lower than expected, despite application of the correction as described above. Presumably this reflects generalised toxicity (over and above the increase in mortality) which in some way damps down the transgene response (e.g. via lower levels of gene expression or inactivation of the HSF).

Using this simple though arbitrary system, the mean score for controls over a series of 10 runs was 0.65 ± 0.17 (SEM), whereas for 16 ppm Cd^{2+} (a strong inducer) the corresponding score was 7.98 ± 0.44. This suggests approximately 12-fold induction by 16 ppm Cd^{2+} over controls, which is close to the 14-fold induction of β-galactosidase activity (Fig. 2 insert). Subtracting the control score from the corresponding

16 ppm Cd^{2+} score gives a consistent *gap score* of 7.34 ± 0.37 (Table 1A), the use of which overcomes the need to quote separate control values for each batch of worms. We repeated this exercise using 2 ppm Cd^{2+} as the positive control, and obtained a mean value of 2.72 ± 0.25 (gap score 2.07; Table 1A), representing a 4-fold induction over controls; the corresponding value for enzyme induction under identical conditions is 3.8-fold (Fig. 2 insert). Since the staining patterns given by 2 ppm and 16 ppm Cd^{2+} are quite different (Fig. 4e versus h, i), we believe that our scoring system broadly reflects genuine β-galactosidase activity. This is confirmed by the general agreement between gap scores and β-galactosidase activities for several diverse toxicants, as shown in Table 1. We find that mean gap scores from independent observers assessing the same set of stained worms differ by around 10%, though low values (<1) are generally less reliable and can only be regarded as indicative. Typical gap scores for a variety of toxicants are tabulated in Table 1, together with the corresponding mean β-galactosidase activities where available. Representative staining patterns for worms exposed to some of these toxicants are shown in Fig. 4. Metal ions such as Cd^{2+} apparently act by aggravating the stress already caused by the incubation temperature of 32°C. This is shown clearly in Fig. 4a–f; whereas control worms are unstained at 32°C (Fig. 4a), they are strongly stained throughout at 34°C (Fig. 4b). At 33°C, adults show moderate staining but larvae remain unstained (Fig. 4c). Inclusion of 2 ppm Cd^{2+} at 33°C, however, results in strong staining of larvae as well as adults (Fig. 4f; compare with 2 ppm Cd^{2+} at 32°C in Fig. 4e); note also that the smaller larvae remain unstained even in 16 ppm Cd^{2+} at 32°C (Fig. 4i). We suggest that both the toxicant (here Cd^{2+}) and heat (32°C) *co-operatively activate* the endogenous HSF to switch on the *lacZ* transgene. Cd^{2+} is toxic to *C. elegans* over prolonged periods, with a high 24 h LC50 value of 904 ppm decreasing to 22 ppm after 48 h and to 1.5 ppm after 72 h (Williams and Dusenbery, 1990). We note that our system can detect Cd^{2+}-induced transgene expression after 7 h at concentrations as low as 0.5 or even 0.15 ppm (Figs. 2 and 4d; Table 1A). Table 1A also compares the effects of Group 2B metal ions (Zn^{2+}, Cd^{2+} and Hg^{2+}) as inducers of transgene activity (see also Fig. 4j and k for Zn^{2+} and Fig. 4l for Hg^{2+}). As expected, Zn^{2+} is the least effective of the three, while Hg^{2+} is comparable in effectiveness with Cd^{2+} at 0.5 ppm, about 50% as effective at 2 ppm, and only 14% as effective at 16 ppm. The relevant 24 and 48 h LC50 values are 202 and 2 ppm for Zn^{2+}, 10 and 3 ppm for Hg^{2+} (Williams and Dusenbery, 1990).

One of the better heavy-metal inducers amongst those studied is Ag^{+}, which gives clear concentration-dependent transgene induction up to 50 ppm (Table 1B). We note that the 24 h LC50 value for Ag^{+} is only 5 ppm, decreasing to 2 ppm after 48 h (Williams and Dusenbery, 1990). However, some response to Ag^{+} can be detected even at 0.15 ppm in our assay. Given the high level of transgene induction observed at 50 ppm Ag^{+}, we were surprised to observe that 100 ppm Ag^{+} gives a *negative* gap score (i.e. with *less* staining than in controls), even though most worms appear to survive for >7 h under these highly toxic conditions. One possible explanation for this anomaly would invoke metal binding to the induced transgene product and consequent inhibition of its enzymatic activity (see Mazidji et al., 1992), or heavy-metal inhibition of the transcription/translation apparatus resulting in lower transgene ex-

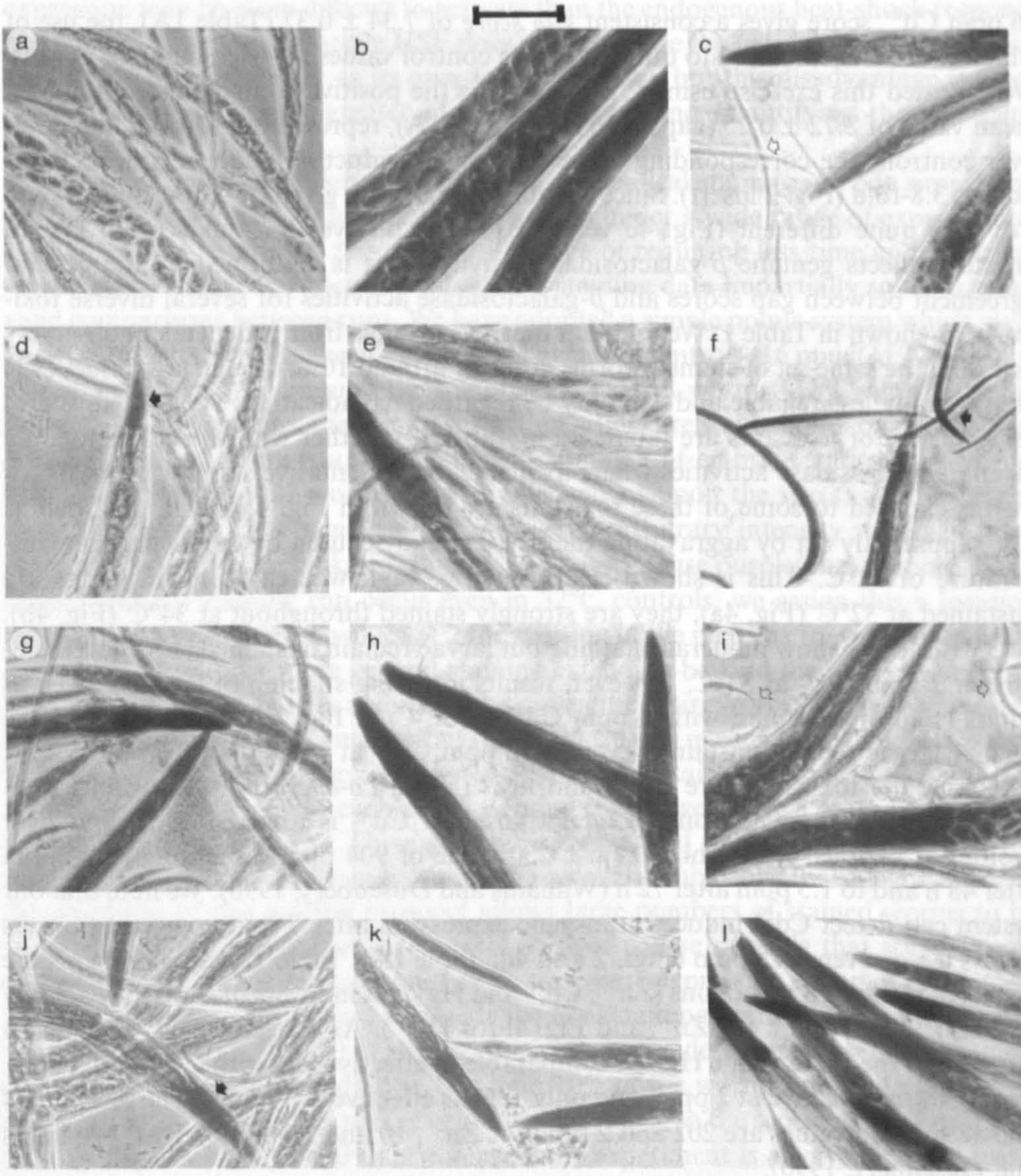


Fig. 4. Photographs of transgenic worms after in situ staining. Histochemical staining of acetone-fixed worms was performed as described in Methods. Selected fields were photographed under direct illumination on an inverted Leitz photomicroscope. Dark areas represent red to purple/blue staining. Heat and toxicant treatment was for 7 h in all cases. Bar: 100 μm. (a) 32°C control; no staining (pink/red embryos occur rarely). (b) 34°C heat shock; strong staining throughout most worms. (c) 33°C heat; staining in adults but not larvae (open arrow). (d) 32°C+0.5 ppm Cd^{2+}; some pharyngeal staining (solid arrow). (e) 32°C+2 ppm Cd^{2+}; stronger pharyngeal staining in adults. (f) 33°C+2 ppm Cd^{2+}; small (L1/2) larvae as well as adults are strongly stained (solid arrow; compare with parts c and e). (g) 32°C+8 ppm Cd^{2+}; strong staining in and beyond pharynx. (h) 32°C+16 ppm Cd^{2+}; generally strong staining in anterior half. (i) 32°C+16 ppm Cd^{2+}; strong staining in most adults, weaker staining in large (L4) larvae, but none in smaller (L1/2) larvae (open arrows; contrast with part f). (j) 32°C+2 ppm Zn^{2+}; slight pharyngeal staining (solid arrow). (k) 32°C+16 ppm Zn^{2+}; strong pharyngeal staining in many adults. (l) 32°C+16 ppm Hg^{2+}; strong pharyngeal staining in many adults.

pression. Such effects might vary from metal to metal; thus Cd^{2+}, Hg^{2+}, Zn^{2+}, Cu^{2+} and Ni^{2+} are all known to inhibit β-galactosidase activity, whereas Pb^{2+} and As (as arsenate) have no effect or even stimulate the enzyme (Mazidji et al., 1992). Although we wash our worms routinely before staining, this will only remove metal ions in the external medium, and is unlikely to affect those already internalised or adsorbed by the cuticle, since the worms remain intact until acetone fixation. In the specific case of Ag^{+}, which binds readily to many proteins, it is possible that this metal remains largely bound to proteins other than β-galactosidase during assays on 50 ppm-treated worms, but that substantial binding to the transgene product can occur when using 100 ppm-treated worms. In any case, the sharp decrease in transgene expression from high at 50 ppm Ag^{+} to zero at 100 ppm (Table 1B) suggests caution when using this system at high toxicant concentrations. With several other toxicants we have observed substantially lower-than-expected gap scores under toxic conditions which kill off a significant proportion (>20%) of the worms during the 7 h exposure period (see 2 ppm entry for lindane and 150 ppb entry for TBT in Table 1B).

Table 1B includes an organic and an organo-metallic pollutant, both of which induce transgene expression at considerably lower concentrations than those required for comparable induction by metal ions. Although ethanol is a known inducer of the heat-shock response in many animal systems, we find that concentrations between 0.1% and 1% induce staining only in a few isolated embryos, and the gap scores do not differ significantly from K-medium controls (data not shown). Whereas inorganic Sn^{2+} is a relatively weak inducer (gap score of 2.2 at 50 ppm Sn^{2+}), the potent organometallic toxicant tributyltin chloride (TBT) is effective at 5–50 ppb (gap scores of 1.8 and 3.2, respectively). This contrast may be related to poor cellular uptake of inorganic Sn^{2+} as compared to ready penetration of membranes by TBT. Consistent with this, we find that the net accumulation of tin (concentration in wet worms relative to the external medium) over 7 h at 32°C is more than an order of magnitude greater for TBT than for inorganic Sn^{2+} (see Table 2C); note, however, that no correction has been made for possible differential adsorption by the cuticle. Steinert and Pickwell (1993) have recently documented the induction of *hsp70* proteins by TBT in the mussel *Mytilus edulis*. TBT induces medium-intensity staining throughout the whole worm, affecting most worms at all stages of development, rather than the more intense but localised anterior or pharyngeal staining seen mainly in young adult worms following heavy-metal treatments (Fig. 4). Lindane is also an effective inducer of the nematode transgene at 15 to 150 ppb (gap scores of 1.35 and 1.7), though higher concentrations cause marked toxicity and a decrease in the gap score (as is the case for TBT). For several toxicants we note that there is relatively little change in the gap score recorded across a wide range of concentrations. Typically, the intensity scores do not exceed 2 and the location scores may not exceed 1, hence only low gap scores are possible even if the majority of worms are affected. This does not appear to be an artefact of our staining assessment protocol, since similarly shallow dose-response relationships are evident in the β-galactosidase activity data. Thus in the most extreme case, a 25-fold increase in Mn^{2+} concentration barely doubles both the gap score and the mean β-galactosidase activity (Table 1B). Nevertheless, such induction is clearly greater than in 32°C controls, and so provides information relevant to the

purposes of this assay. It is likely that induction of a stress-response by these agents is largely incidental to their main toxic effects.

4. Discussion

The above results demonstrate that transgenic nematodes carrying stress-inducible reporter genes can provide a quick (about 8 h total) and simple assessment of aquatic toxicity by a variety of agents. However, precise assay conditions are critical for obtaining reproducible data; for instance, incubators with temperature control accurate to within ± 0.1°C are essential. But whilst our staining and assessment procedures can provide a quick guide to the effects of various toxicants (Fig. 4, Table 1), full β-galactosidase assays are needed for proper quantitative comparisons (Figs. 1 and 2), and this requires some 30 h in total. Because these worms are transgenic and must not be released into the environment, they cannot be exposed to potentially contaminated water on site; rather, water samples must be brought back to the laboratory for testing. Because the strain used here (CB 4027) is fully characterised and carries a transgene encoding a harmless protein, it is classed as relatively non-hazardous and can be handled at containment level 1 under current UK regulations (essentially, good microbiological practice). Thus the use of transgenic test animals does not in itself impose high costs on the testing laboratory, though clearly some restrictions and care are appropriate when handling such animals.

Many of the effects documented here are paralleled in other systems. Thus lower growth temperatures improve the induction response of a transgenic yeast strain carrying a heat-inducible *lacZ* construct (Kirk and Piper, 1991), although basal activities were constant in the yeast study. This could be explained in terms of a greater temperature rise (17°C as opposed to 12°C) from growth to assay conditions causing more extensive oligomerisation of the endogenous HSF, with consequent enhancement of transgene induction. Superimposed upon this, there is also an apparent increase in Cd^{2+} accumulation when worms grown at 15°C (but not 20°C) are transferred to 32°C (Table 2B); this observation remains as yet unexplained. As mentioned above, metal-ion inhibition of β-galactosidase enzyme activity (Mazidji et al., 1992) and/or generalised metabolic toxicity may explain why high concentrations of certain toxicants cause lower apparent induction (Table 1B). The former might be overcome by using immunoassays to quantitate β-galactosidase protein levels, but in the latter case, lower rates of gene-expression would also affect the amounts of enzyme produced.

Several groups have advocated measuring stress-protein induction or accumulation as a sensitive assay for environmental contamination by various toxicants (Anderson, 1989; Miller, 1989; Hakimzadeh and Bradley, 1990; Sanders et al., 1991; Kohler et al., 1992; Hightower, 1993; Bradley, 1993; Steinert and Pickwell, 1993). The validity of this approach remains open to question, since only a single molecular response is being investigated. Validation of such systems (including that described here) will require clear-cut responses to a wide range of toxicants at concentrations comparable to or (preferably) much lower than their LC50 values. Even so, it remains

to be seen whether the heat-shock system is more sensitive to toxicant perturbation than are complex processes such as respiration, growth or motility. The relative effectiveness of different assay methods will no doubt vary according to the organism under investigation, and in this respect the heat-shock system may be sub-optimal for *C. elegans*, since a large temperature rise is required to induce a stress response. Finally, even if heat-shock-based assays become accepted for particular environmental monitoring purposes (Hakimzadeh and Bradley, 1990; Kohler et al., 1992; Bradley, 1993; Steinert and Pickwell, 1993), it should be borne in mind that these assays make significant technical demands which may limit their usefulness. Thus the procedures involved in radiolabelling test animals and subsequent autoradiography to examine the profile of newly synthesised proteins (e.g. Hakimzadeh and Bradley, 1990; Kohler et al., 1992) are considerably more complex and time-consuming than those used here. Another variant of the stress-protein approach is to use antibodies to measure the accumulated levels of heat-shock proteins in a static test species after long-term exposure to toxicants. This has been notably successful in the case of *Mytilus edulis*, where Cu^{2+} treatment induces *hsp60* (Sanders et al., 1991) and TBT treatment induces *hsp70* (Steinert and Pickwell, 1993); again, however, this approach is quite demanding technically. One problem which may arise here is the presence of heat-shock cognates (hsc's) expressed constitutively in non-stressed cells; these could mask low-level induction of genuine *hsp70* by a test toxicant. The use of two-dimensional gel electrophoresis should overcome this problem, but only at the cost of introducing additional technical demands. In view of these limitations, the transgenic approach described here offers the advantages of technical simplicity and the ease of processing multiple parallel samples for staining or enzyme assays.

However, several significant disadvantages should also be considered. Firstly, the containment problem posed by transgenic indicator organisms is not trivial, although this need not impose large additional costs (see earlier). Secondly, although *C. elegans* is a convenient organism for monitoring both soil and water pollution (Williams and Dusenbery, 1988, 1990), the transgenic strain used here seems relatively unresponsive to low levels of many toxicants, as compared with LC50 data for e.g. *Daphnia magna*. This may be an incidental effect of the large temperature rise needed to induce a full heat-shock response in *C. elegans*; in order to provide adequate sensitivity in our assay, we need to combine the toxicant under study with a sub-heat-shock temperature increase. Ideally, this should be avoided, in order to eliminate possible interactions between heat and toxicant effects. One model system showing great promise in this respect is a transgenic *C. elegans* construct carrying a homologous *hsp16* promoter linked to a *lacZ* reporter gene (Stringham et al., 1992). Because the *hsp16* genes are induced at a lower temperature (29°C) than the *hsp70* and other heat-shock genes (34°C; Snutch and Baillie, 1983), β-galactosidase activity is inducible by many toxicants at the normal growth temperature of 20°C (Stringham and Candido, 1994). Thirdly, the nematode *C. elegans* itself appears relatively insensitive to many heavy metal ions, as revealed by its 24–96 h LC50 values which almost all lie in the ppm rather than ppb range (Williams and Dusenbery, 1990). It is unlikely that any *C. elegans* transgenic construct can wholly overcome this intrinsic insensitivity, although the greater sensitivity shown to several organic and organo-metallic toxicants (only

two of which are included in this study) is perhaps more hopeful. Here, however, weak dose-reponse relationships raise an acute problem; indeed, among all the toxicants tested, only Cd^{2+} gives a strong dose-response relationship (Fig. 2). Arsenite and Cd^{2+} are the classical chemical inducers of the heat-shock (stress) response in most metazoans, and Cd^{2+} is known to activate the ubiquitin-dependent proteolysis pathway in yeast (Jungmann et al., 1993), which is in turn linked to the stress response. Thus Cd^{2+} toxicity within cells is probably mediated through Cd^{2+}-induced formation of abnormal proteins (Jungmann et al., 1993). It may be that the other toxicants tested simply do not damage cellular proteins to the same extent as Cd^{2+}, perhaps because their primary toxicity is directed elsewhere. Since there is evidently some activation of the stress response by these agents (even if only as an incidental effect), it remains possible that a more stress-sensitive transgenic construct would give clearer responses to them (see above). In order to optimise a stress-inducible transgenic system for use in toxicity testing, it will be necessary to select for transgenic strains which respond more strongly than does CB 4027 to a wide range of toxicants acting by different mechanisms. This study demonstrates that the potential of transgenic animals as toxicological indicators has yet to be fully realised, but even when the problems outlined above have been overcome, it remains an open question as to whether such systems could ever rival established whole-organism approaches to environmental monitoring.

Acknowledgements

The authors would like to thank Mr B. Case for preparing the photographs and the Government of the Republic of Turkey for a research studentship to K.G.. The University of Nottingham Research Fund and the Natural Environment Research Council (grant GR9/800) are thanked for financial support. Drs J. Reader and P. Crittenden of this department are thanked for help with atomic absorption spectrometry, as is Mr A. Fairburn of the Department of Animal and Plant Science analysis unit at the University of Sheffield.

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