# EFFECTS OF CADMIUM ON THE HEPATIC MICROSOMAL DRUG METABOLIZING SYSTEM

*A Thesis submitted for the degree of*

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*by*

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CONTENTS

CHAPTER ONE: (Contd)

## DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and to the best of any knowledge contains no material previously published by any other person, except where due reference is made in the text.

The experimental work described herein was carried out from 1974 to 1977, during which time the author was a recipient of a Commonwealth Postgraduate Research Award.

Some of the results in this thesis have been presented to the following meetings.

10th Annual Meeting of the Australasian Society of Clinical and Experimental Pharmacologists, November 24-26, 1976, Flinders Medical Centre, Adelaide, South Australia.

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## ABSTRACT OF THESIS

The thesis is concerned with the acute interaction of a single injected dose of cadmium (5 mg cadmium acetate/Kg i.p.) with the rat hepatic microsomal drug metabolizing system.

Cadmium caused a decrease in the *in vitro* metabolism of both type I and type II substrates and this was associated with a triphasic change in the cytochrome P-450 content. The biphasic decline phase lasted for approximately 48 hours and was accompanied by a similar biphasic decline in cytochrome $b_5$ and total haem. Recovery of microsomal drug oxidase activity and cytochrome P-450 content began after 2 days, but was still incomplete up to 12 days. Hepatic microsomal glucose-6-phosphatase activity fell from the 48th hour onwards, indicating a possible change in microsomal membrane integrity. However, differences in the time-course of these phenomena suggested that non-specific changes in membrane function did not contribute greatly to the inhibition of cytochrome P-450 dependent metabolic activity. Furthermore, the affects of decreased food intake and the accumulated levels of cadmium and zinc in the microsomes, were ruled out as major factors in the response. It was concluded therefore, that cadmium mediates its inhibition of the drug metabolizing system after acute administration by a specific effect on cytochrome P-450.

By comparison, the chronic, oral ingestion of cadmium did not result in any alterations to the drug metabolizing system of the rat even though a similar amount of cadmium accumulated in the liver.

This led to an investigation of the factors regulating the biosynthesis and catabolism of haem during the first 48 hours and to the possible role played by metallothionein in reversing these inhibitory effects.

It was concluded that metallothionein was not responsible for the termination of the acute inhibitory effects of cadmium at 48 hours, as the cadmium-thionein content was maximal at a time, (12 hours), when microsomal metabolic activity was still in the decline phase. Further, it was shown that while the cadmium-thionein content did not change from 12 hours to 12 days after cadmium, the synthesis of thionein continued and this bound zinc.

The activities of δ-aminolaevulinic acid synthetase, the rate limiting enzyme in haem biosynthesis, and the dehydratase were not affected during the first 48 hours after cadmium administration, whereas microsomal haem oxygenase activity was markedly stimulated. The stimulus which initiated the induction of haem oxygenase was not identified conclusively. However, cumulative additions of cadmium to microsomes *in vitro*, resulted in the quantitative conversion of cytochrome P-450 to cytochrome P-420 and it has been speculated that the denaturation of cytochrome P-450 may be such an initiating factor.

In conclusion, the increase in the rate of the catabolism of microsomal haem is possibly the primary mechanism by which both cytochome P-450 and $b_5$ are lost from the microsomes during the first 48 hours after cadmium. The fall in drug metabolic activity results from the loss of the cytochromes, in particular, cytochrome P-450.

# ABBREVIATIONS

| | |
|---|---|
| CO | carbon monoxide |
| DNA | deoxyribonucleic acid |
| EDTA | ethylenediaminetetraacetic acid |
| gm | gram weight |
| xg | times gravity; relative centrifugal force |
| i.p. | intraperitoneal |
| Kg | kilogram weight |
| mg or mgm | milligram weight |
| mls | millilitres |
| mM | millimolar |
| mRNA | messenger ribonucleic acid |
| μ | micro ..... ($1 \times 10^{-6}$) |
| MW | molecular weight |
| (n) | number of observations |
| nmoles | nanomoles ($1 \times 10^{-9}$M) |
| nm | nanometres |
| NADH | nicotinamide adenine dinucleotide (reduced form) |
| NADPH | nicotinamide adenine dinucleotide phosphate (reduced form) |
| O.D. | optical density |
| S.C. | subcutaneous |
| S.E.M. | standard error of mean |
| % (W/V) TCA | mgms trichloroacetic acid/100 mls solutions |
| V/V | volume per volume |

# GENERAL INTRODUCTION

## 1. Cadmium: history, chemistry, occurrence



Cadmium was discovered in zinc carbonate by Stromeyer in 1817. Cadmium is a bluish-white metal with an atomic number of 48 and an atomic weight of 112.41. Zinc, cadmium and mercury, commonly known as the zinc family, constitute Group 2b of the periodic table.

There is a close resemblance between the chemistry and occurrence of cadmium and zinc, in that they form similar compounds and are more reactive than mercury. Cadmium is found in nature as an impurity in the ores of zinc, lead, and copper and today, cadmium is obtained as a by-product of the zinc industry. In the biological sphere however, the similarity does not exist; zinc is an "essential" element for some mammalian metalloenzymes while cadmium and mercury are toxic.

## 2. Cadmium and Man

The envionment in which Man lives is the most common source for his contact with cadmium. The industrial use of cadmium and as a carry-over contaminant of zinc both serve as a source for this pollutant. Environmental levels have been reported and have been summarized by Friberg et al (1974). They show that these vary from year to year, country to country and from place to place but generally, the more industrialized centres have the greatest amount of polluting cadmium in their surrounding environs.

Investigations into the toxicity of cadmium in relation to Man have been either epidemiological surveys of people exposed to cadmium or short and long term exposure to cadmium in experimental animals.

Acute effects have been investigated mainly by injecting cadmium. Such studies have indicated damage to the testes (Parizek 1956, 1957, 1960, Gunn, et al 1961, 1963), liver (Andreuzzi and Odescalchi 1958) and kidney (Favino and Nazari 1967 and Foster and Cameron 1963). Long term effects include renal tubular dysfunction as evidenced by proteinuria, glucosuria and aminoaciduria. These studies are cited to show that cadmium is a toxic substance. The various biological actions of cadmium have been more extensively reviewed by Friberg et al (1964) and Singhal et al (1976).

The literature dealing with effects of cadmium on the drug metabolizing system is not as extensive as for other systems (e.g. renal and cardiovascular), but warrants further investigation since cadmium accumulates not only in the kidney but also in the livers of exposed animals and humans.

## 3. The Drug Metabolizing System

Only a simplified summary of the components and functions of the microsomal drug metabolic enzymes will be presented at this time. A more detailed discussion of the interaction of cadmium with this system will follow later in this thesis. The drug metabolizing system has been referred to as the microsomal mixed function oxidase system (MMFO), the cytochrome-P-450 dependent or microsomal biotransformation system, or simply the microsomal enzyme system.

When a cell is ruptured during tissue homogenization, vesicular structures form from the broken endoplasmic reticulum, trapping within them many enzymes, including whole enzyme-mediated biotransformation pathways. The structures are called microsomes, and the

fraction which sediments at 105,000 x g after differential centrifugation, the microsomal fraction.

The key component of the drug metabolizing system is a membrane bound haemoprotein called cytochrome P-450. The association of this protein with membrane phospholipids is apparently required for full enzymatic activity and when this association is disrupted, cytochrome P-450 is converted to an inactive, often solubilized form, cytochrome P-420. Cytochrome P-450 has been found in a variety of plants, insects and mammalian tissues (Wichramasinghe 1975), but the greatest activity is found in the liver. Substrates include lipophilic drugs, xenobiotics such as environmental pollutants, as well as endogenous lipophilic compounds such as bile acids, fatty acids and steriod hormones. It follows therefore, that if the system's activity is altered by an exogenous substance, then its ability to perform its normal functions may be affected with possible adverse consequences to the organism.

The system is coupled to an electron transfer chain and has an absolute requirement for NADPH and molecular oxygen. Cytochrome P-450 is the terminal oxygenase; the preceding steps of the system being primarily responsible for delivering 2 electrons to the cytochrome.

Cytochrome P-450 can participate in electron transfer by means of a reversible valency change of the iron atom in the haem moiety. Under physiological conditions, cytochrome P-450 alternates between the reduced and oxidized state. When in the reduced state, carbon monoxide can bind at the position of the 6th ligand and forms a complex whose difference spectrum has a peak at 450nm; this phenomenon gives the cytochrome its name and enables its quantitative measurement.

When in the oxidized state, low spin-cytochrome P-450 can bind substrates for biotransformation. A relatively small percentage of cytochrome P-450 in microsomes is available for drug binding (Ullrich 1969), indicating that not all the cytochrome P-450 present is in the low-spin oxidized form. After substrate binding, a high spin-ferric-cytochrome P-450-substrate complex is formed. This is followed by the addition of the first electron, originating from NADPH and transferred by NADPH-cytochrome P-450-reductase, forming ferrous-cytochrome P-450-substrate complex. Molecular oxygen can bind to this complex followed by the second electron, which is required to activate the bound oxygen into a highly reactive form. Though still controversial, the experimental evidence suggests that the second electron may originate from NADH which may be transported to the ferrous cytochrome P-450-substrate-oxygen complex via cytochrome $b_5$. (Estabrook et al 1971, Mannering et al 1974).

The change in the spectrum (absolute and difference) of cytochrome P-450 as well as the spin state of the haem iron are thought to result from an alteration in the nature of the 6th ligard about the haem iron. Drugs which bind have been broadly divided into two classes depending on the type of difference spectra they produce. They are called type I or type II substrates and produce a type I or II spectra respectively. A type I spectrum has a $\lambda$ max. in the range of 385 - 390 nm and $\lambda$ min. 418-427 nm, while type II is an approximate mirror image in that its $\lambda$ max. extends from 425 - 435 nm and the $\lambda$ min. 390 - 405 nm. The nature of the binding sites differ also. Type I substrates are thought to bind to the apoprotein or an area associated with membrane phospholipids. (Chaplin and Mannering 1969, 1970). Type II substrates are thought to react directly by ligating

with the haem-iron at the position of the 6th ligand, creating a specific ferrihaemochrome complex. (Schenkman et al 1967). Therefore, a change in the ligand state occurs either by direct intervention or mediated by a conformational change in the apoprotein after substrate binding.

The formation of a cytochrome P-450-substrate complex is a requisite step for the initiation of metabolism (Ullrich 1977) but the production of a binding spectrum does not guarantee metabolism, nor does the absence of a binding spectrum preclude metabolism.

Some compounds designated as type II often possess some affinity for the type I binding site and have been termed "modified type II" compounds (Schenkman et al 1967). This leads to the suggestion that compounds tend to bind to both sites but generally bind preferentially to one of the sites and from this they receive their designation as being type I or II. Further, it follows that compounds which bind equally to both sites, cancel out the peak and trough effect and produce no nett binding spectra. Similarly, cytochrome P-450 species exist which may have one binding site defective or altered. For example, polycyclic hydrocarbons induce the synthesis of a modified cytochrome P-450, termed cytochrome $P_1$-450 or P-448 which is thought to have a defective type I binding site. (Mannering 1971, Remmer 1972).

It has been deduced that the substrates to be metabolized displace the 6th ligand of iron and that oxygen binds at the position of the 5th ligand. (Chevion et al 1977). It becomes conjecture as to how the oxygen can get into a sufficiently close

position to react with the substrate, in view of the fact that the 5th and 6th ligands project in opposite directions about the haem iron. Conformational changes in the cytochrome molecule, in response to substrate and NADH binding, have been proposed and perhaps this brings the two reactants together. However, the exact mechanism of action is still the subject of investigation and will not be elaborated upon in this thesis.

## 4. Acute Effects of Cadmium on the Drug Metabolizing System

Wagstaff (1973) examined the effects of cadmium given in the diet (100 - 5,000 ppm) for 15 days in female rats and reported a dose - dependent decrease in hexobarbital sleeping time and stimulation in the oxidative cleavge of O-ethyl-p-nitrophenylphenylphosphonothioate and O-demethylase activity. These data suggest that enzyme induction has occured. In contrast, although in another species, Unger and Clausen (1973) found that on the 4th day after a single intraperitoneal injection of cadmium nitrate (3 mg/Kg) in male mice, O-demethylase activity was inhibited. It was suggested that inhibition could have resulted from the formation of a cadmium-chelate with the thiol groups (cysteine) present in haemoproteins. Alternatively, cadmium could have bound to glutathione and inhibited epoxide-glutathione transferase.

Further studies supported the view that acute administration of cadmium led to enzyme inhibition, rather than induction. Schnell et al (1974) reported that a single injection of 2mg cadmium/Kg i.p. in male rats resulted in the potentiation of hexobarbital sleeping time which began on day 1, while peak activity extended from day 2 to 5.

Johnston et al (1974) and Hadley et al (1974) both reported that cadmium treatment did not alter the dose of intravenously infused hexobarbital necessary to suppress EEG activity and it was suggested that impairment of drug disposition rather than an alteration in the sensitivity of the CNS was a major factor in the prolongation of sleeping time. Hadley et al (1974) also reported that cadmium inhibited the *in vitro* metabolism of aminopyrine, hexobarbital, p-nitroanisole and zoxazolamine. Johnston et al (1975) reported that the pharmacological response to hexobarbital after cadmium treatment was related to tissue levels of the drug and that cadmium-induced changes could only be observed in rats with intact livers.

From these papers, the following can be concluded. Firstly, acute cadmium administration inhibits the drug metabolizing system of the liver, resulting in the potentiation of the pharmacological effect of substrates dependent on this system for elimination. Secondly, the degree of potentiation of hexobarbital sleeping time is a time-dependent phenomenon and will differ at various times after cadmium treatment.

These reports have also raised a number of further issues. Hadley et al (1974) suggested that a decrease in food consumption might explain some changes in microsomal activity, although this was not a significant factor in their experiments. On the other hand, Wagstaff (1973) also reported that the consumption of cadmium-supplemented feed was less than in the controls. Since Marselos and Laitinen (1975) found that rats starved for a period of 72 hours had a greater p-nitroanisole demethylase activity than control fed animals, it is possible that decreased food consumption may have been a contributing factor to the enzyme stimulation reported by Wagstaff (1973).

Addition of cadmium to microsomal preparations *in vitro* provides another aspect to the response. In concentrations of 5 x $10^{-7}$M to 5 x $10^{-4}$M, cadmium inhibits the metabolism of p-nitroanisole, aminopyrine and zoxazolamine. (Hadley et al 1974). This concentration range is based on the levels of cadmium (6µg/g) found in the livers of rats after injection of an inhibitory dose of cadmium. However, this concentration represents total liver concentration and the cadmium content of the microsomal fraction was not determined. Hadley et al (1974) were the first to report that the amount of cytochrome P-450 in the cadmium treated rats was less than that in controls. Therefore, a new and important factor was introduced.

Hadley et al (1974) did not propose any specific mechanism for the inhibition, but they speculated that the "transitory nature" of the phenomenon is due to the synthesis of metallothionein which leads to an intrahepatic redistribution of cadmium. Unger and Clausen (1973) also suggested that the inhibition was related to the accumulation of cadmium and that it was initiated when the capacity of metallothionein to bind cadmium was exceeded.

This was the extent of the published literature at the time when the experiments in Chapter 1 were begun. Since the completion of the experiments, Yoshida et al (1976) reported on the time-course of aminopyrine-N-demethylase activity in the 9,000 x g supernatant fraction derived from male mice injected with cadmium chloride (3mg/Kg). Maximum inhibition occurred 48 hours after injection while activity returned almost to control level by 72 hours. A similar time-course of activity may also be deduced from the combined data of Schnell et al (1974) and Hadley et al (1974). However, a

close examination of Schnell et al's (1974) data in rats indicates that hexobarbital sleeping times were still prolonged beyond the 72nd hour after injection. It is not known if this discrepancy represents a true species difference.

Yoshida et al (1976) reported that 48 hours after cadmium injection, (3 mg/Kg) aniline hydroxylase activity was also decreased in mice and together with the aminopyrine-N-demethylase data, concluded that the extent of the inhibition by cadmium was dose-dependent. Further, they proposed that inhibition was a result of a direct action of cadmium upon the drug metabolizing system. It was also suggested that inhibition could have resulted from the decrease in the cytochrome P-450 content possibly by cadmium impairing the activity of δ-aminolaevulinic acid dehydratase, though cadmium had reduced the activity of the latter enzyme by only 20% in these studies.

Recently, Teare et al (1977) found that aniline hydroxylase and nitroreductase activities were inhibited and the level of cytochrome P-450 was lowered after a single, intraperitoneal injection of 2.5 or 3.75 mg cadmium chloride/Kg in male Wistar rats. O-demethylase activity was not inhibited by either dose of cadmium when the activity was measured in 0.05M phosphate-buffered incubation system. However, the activity of this enzyme was inhibited when measured in a Tris-buffered system. It is not known what caused the "buffer effect". Unger and Clausen (1973) and Hadley et al (1974) both used Tris-buffers. Wagstaff (1973) did not mention the buffer used.

## 5a. Metallothionein

Early investigations into the biochemistry of metals led to the recognition that mammalian kidneys and livers accumulated high concentrations of cadmium which also had a long half-life (Schroeder 1956). A cadmium containing metalloprotein, discovered by Margoshes and Vallee (1957) in equine renal cortex, provides a possible explanation for this. Kagi and Vallee (1960, 1961) isolated and characterized the protein and named it metallothionein because of its metal and sulphur content. (5.9% cadmium, 2.2% zinc and 8.5% sulphur). Most of the sulphur is from cysteine residues and exists as thiol groups capable of binding cations in a tridendate complex. The metal-free protein is called thionein and contains 16.3% nitrogen and 9.3% sulphur (Kagi and Vallee 1961).

33% of the amino acids in metallothionein are cysteine, while lysine, serine and glycine each account for approximately 10-11% of the total residues. Phenylalanine, tyrosine, tryptophan and histidine are absent. A direct result of these properties is that metallothionein absorbs strongly at 250 nm due to the energy of the cadmium-sulphur bond and absorbs weakly at 280 nm due to its lack of aromatic amino acids. The *in vitro* addition of cadmium to metallothionein results in a dose-related increase in the absorbance at 250 nm. The addition of acid results in the decrease of 250 nm absorbance due to the displacement of cadmium from its binding sites by the protons. Cadmium, zinc and copper bind to metallothionein but its affinity for cadmium is by far the greatest and as such it can displace zinc and copper from their binding sites.

These then are the properties of metallothionein and some have been used by other workers in subsequent years for the identification and comparison of isolated metalloproteins.

## 5b. Nomenclature

The term metallothionein will be used to refer to the "metallothionein" first isolated by Kagi and Vallee (1960), to the "cadmium-binding proteins" (Shaikh and Lucis 1971, 1972) and to other metalloproteins which have been isolated and have properties closely resembling those of the original metallothionein. Thionein will refer to the metal free metallothionein (apo-protein). Reference to a specific metal associated with thionein, will be prefixed by the name of that metal e.g. cadmium-thionein; zinc-thionein. A convention for naming the various metalloproteins, similar to that outlined above, was adopted by Webb and Verschoyle, (1976).

## 5c. Occurrence

Pulido et al (1966) isolated a cadmium-metalloprotein in human kidney which had similar properties to metallothionein and Bűhler and Kagi (1974) reported finding it in human liver. Kagi et al (1974) found metallothionein in horse liver with virtually identical properties to the equine renal protein. The metallothioneins referred to above were from normal animals. Piscator (1964) reported a cadmium-metalloprotein formed in rabbit livers after repeated injections of cadmium which had properties similar to the metallothioneins. Shaikh and Lucis (1970, 1971) reported that a cadmium-binding protein, which possessed properties similar to the metallothioneins, could be

induced in rat liver by injecting cadmium. Webb (1972b) and Davies et al (1973) showed that a zinc metalloprotein, which formed in rat livers in response to zinc injections, had properties similar to the cadmium-metalloproteins (metallothioneins). Piotrowski et al (1974,) found that mercury could also induce metallothionein. These findings were confirmed by Sabbioni and Marafante (1975), who also reported that although silver and tin did not induce metallothionein, they could bind to pre-synthesized protein.

Squibb and Cousins (1974) and Squibb et al (1977) have shown that the metals induce the *de novo* formation of metallothionein at the level of transcription, resulting in the production of a specific mRNA. It has also been shown, so far in the case of zinc, that the messenger is a short lived, poly-(A)-containing RNA which provides for a second control point in the production of thionein, whereby translation of the mRNA is de-repressed in instances of high zinc levels.

Cadmium and zinc metallothioneins have subsequently been discovered in a wide range of animal species and tissues, besides horse kidney. They have been isolated in rabbit liver by Piscator (1964), and Nordberg et al (1972), mouse liver (Nordberg et al 1975), pig kidney (Cousins et al 1973, Webb & Daniel 1975), chickens (Weser et al 1973), fish (Marafante et al 1972), and dog spleen (Amacher and Ewing 1975). The most widely used source of induced metallothionein has been a variety of tissues in the rat, including rat kidney (Webb 1972a, Webb & Magos 1976, Shaikh and Lucis 1972, Shaikh & Crispin Smith 1976), rat testes, (Webb 1972c, Chen & Ganther 1975 a,b), rat spleen (Amacher & Ewing 1975) and rat liver (Shaikh & Lucis 1970, 1971, Webb 1972 a,b, Winge & Rajagopalan 1972, Weser et al 1973, Suzuki & Yoshikawa

1974, Sabbioni & Marafante 1975, Shaikh & Crispin Smith 1976, Squibb et al 1977).

Webb and Daniel (1975) showed that cadmium could induce the synthesis of metallothionein in pig kidney cells *in vitro*. This does not support Piscator's hypothesis (1964) that the metallothionein found in kidney was originally synthesized in the liver and then transported to the kidney in the bloodstream.

The amount of cadmium-thionein produced in response to a given dose of cadmium is dependent upon the route of administration. When cadmium is given orally, by gavage or in the diet, cadmium-thionein tends to accumulate to a higher concentration in the kidney, whereas if the metal is injected intraperitoneally or subcutaneously, the liver is the major target organ. Further, Webb (1975) stated that the pattern of accumulation resulting from the administration of cadmium by one route does not affect the distribution of a subsequent dose of cadmium given by a different route.

Differences between the metalloproteins found between species and between different organs within the same species have been reported. Webb (1975) reported that after induction with cadmium, the metal found accumulating along with cadmium in the protein in rat kidney is mainly copper and not zinc. Kagi et al (1974) stated that human hepatic metallothionein contains predominantly zinc, whereas the renal protein contains more cadmium.

Comparison has also been made between the amino acid composition of the metallothioneins derived from different sources. Nordberg et al

(1972) compared the metallothionein from rabbit liver with horse liver and Winge and Rajagopalan (1972) compared their analysis of rat liver metallothionein with Nordberg's data. Weser et al (1973) compared hepatic metallothioneins obtained from rat and chicken. Kagi et al (1974) compared the amino acid composition between equine hepatic and renal metallothioneins. Only minor differences were found by those researchers; the proteins they investigated all possess the fundamental properties of metallothioneins.

Both human and animal metallothionein have been fractionated further by several workers, resolving the protein into two forms. The two forms are usually called metallothionein 1 and metallothionein 2 and both forms have similar molecular weights. Differences in the amino acid composition have been found between the two forms, however, both are rich in cysteine. Piscator (1964), using rabbit liver as his source, obtained two metallothionein fractions by DEAE-Sephadex-A50 chromatography. Several techniques have been used to isolate and characterize the different forms of metallothionein. These have included: chromatography of rat liver supernatant on DEAE-Sephadex-A-25 (Shaikh and Lucis (1971); isoelectric focussing of rabbit liver proteins (Nordberg et al 1972); and disc-gel electrophoresis of human kidney proteins (Bühler and Kagi 1974). Nordberg et al (1975) isolated two forms of mouse metallothionein, but analysed only one form. Kagi and Vallee (1961) separated equine renal metallothionein into a major peak (75% cadmium) and a minor peak (4.9% cadmium) on DEAE-cellulose, but used only the major peak for their characterization studies.

Human renal metallothionein has only zinc bound to it, whereas the proteins isolated from rat and rabbit, in response to cadmium

injections, contain cadmium as well. The stoichiometric ratio of 3 mercapto groups for 1 metal atom is found in both metallothionein forms. One form of human metallothionein contains more zinc than the other and has also an additional metal binding site which is thought to account for its greater electronegativity. Nordberg et al (1972) reported that one form isolated from rabbit liver has an isoelectric point at 3.9 and the other at 4.5, the latter containing more zinc. The pI for mouse metallothionein investigated by Nordberg et al (1975) was 4.2.

The experimental work dealing with metallothionein in this thesis was conducted on metallothionein obtained after Sephadex G-75 gel chromatography. This was designated as a "crude" preparation by Shaikh and Lucis (1971). The metallothionein was not resolved into its two forms because the quantitation of total metallothionein was of greater relevance. The separation of metallothionein into two separate forms is mentioned here for the sake of completeness in a discussion relating to metallothioneins.

## 5d. Metallothionein in normal tissues

Horse kidneys were chosen for the initial studies on metallothionein because of their relatively high cadmium content (Kagi and Vallee 1960). In other animals e.g. the rat, the amount of thionein or zinc thionein present in non-treated animals, although higher in females than males (Webb 1972a), is extremely small. For this reason, workers have used cadmium to increase the metallothionein content. It has been claimed that metallothionein does not exist in untreated animals, but this assertion rests on two factors. Firstly, the metallothionein present

results from the "normal" metal contact which the animals experience and this is obviously variable. Secondly, if the method of detection is by the absorbance at 250 nm, then this will not detect thionein or zinc-thionein. Vallee (1976) produced and isolated an antibody to thionein and using this antibody, found a high level of thionein in the heart, which is unusual in view of the fact that little cadmium accumulates in that tissue relative to the kidney and liver.

Therefore, it is uncertain whether metallothionein is present because it is normally a protein involved in some biological function or whether it is a result of the normal, low level intake of metals from the environment.

## 5e. Molecular Weight

The reported molecular weight of metallothionein varies in different reports. Kagi and Vallee (1961) estimate the equine renal protein to be 10,000 $\pm$ 260 daltons. Winge and Rajogopalan (1972) found rat liver metallothionein to be 14,000, while Weser et al (1973) reported that both chicken and rat metallothioneins are 12,000 $\pm$ 500. Human metallothionein was estimated to have a molecular weight of 10,500 $\pm$ 1,050 (Pulido et al 1966). Nordberg et al (1972) estimate rabbit liver metallothionein to be 9,000 - 10,000 by Sephadex gel chromatography, whereas the calculated molecular weight from amino acid analysis is 6,000 - 6,600 in agreement with the calculated value for horse metallothionein (Kagi et al 1974) of 6,600. Bühler and Kagi (1974) calculated the molecular weight of human hepatic metallothionein to be 6,100 - 6,200 and 6,600 when bound with metal.

These molecular weights are not erroneous per se, but rather the values are subject to the way in which they were estimated. Indeed, the value reported by Kagi and Vallee (1961) was derived by averaging the values obtained by three different methods.

The higher values (9,000 - 14,000) are obtained if the protein is in its native state, with metal bound to it, and estimated by Sephadex G-75 gel chromatography. Pulido et al (1966) considers these estimates to be accurate to 10%. The lower values (6,000 - 6,600) result when the protein sample is first denatured before being applied to a Sephadex column (Bühler and Kagi 1974 using the method of Fish et al (1969)) or if the molecular weight is estimated by summing the individual molecular weights of the amino acids comprising the protein. The latter method is probably the most accurate.

## 5f. Half-life

Shaikh and Crispin Smith (1976) reported the half-life of metallothionein to be 2.8 days, whereas Chen et al (1975) reported an interval of 4.2 days. Since the latter authors did not take into account the possible reutilization of the $C^{14}$-cysteine, their value could be an overestimation.

## 5g. Function of Thionein and Metallothionein

The association of metals with thionein, including some non-essential and toxic metals, has led to several theories regarding its biological function.

Piscator (1964) proposed that metallothionein (or more correctly thionein) is involved with heavy metal detoxification. Pulido et al (1966) also supports this theory, but suggests that it might also function in either catalysis, transport, storage, or immune phenomena. The detoxification theory has been the most widely accepted however, and was based, as far as information at that time indicated, on the fact that metallothionein was not present in untreated animals but was synthesized, following cadmium administration. A role in the metabolism of metals, e.g. copper, has also been proposed. (Starcher 1969, Evans et al 1970). Weser et al (1973) and Bremner and Marshall 1974) suggested that it was a "metal decontaminating carrier" while Leber and Miya (1976) proposed that thionein was a "cadmium scavenger".

Later, with the knowledge that zinc can also induce thionein and that zinc is associated with cadmium-thionein, suggestions were put forward that thionein played a role in the metabolism of zinc. (Webb 1972b, Bremner et al 1973, Buhler and Kagi 1974, Chen et al 1974, 1975, Richards and Cousins 1975, and Squibb et al 1977).

The latter proposal seems the most likely in view of the presence of zinc-thionein in control animals (Webb 1972a). Secondly, the work of Chen et al (1977), showing that zinc is lost from zinc-thionein three days after the administration of zinc ceases, and the report by Squibb et al (1977) that a labile specific mRNA is produced in response to zinc, together show that a highly refined biological system involving thionein has evolved which could be a part of zinc's homeostatic mechanism.

## 6. Metabolism of Haem

### 6a. Biosynthesis

Figure 1 is a diagrammatic representation of the haem biosynthetic pathway.

In the mitochondrion, succinyl CoA and glycine combine to form δ-aminolaevulinic acid (δ-ALA). The enzyme δ-aminolaevulinic acid synthetase (δ-ALAS) catalyzes this reaction and is the rate limiting step. In the cytosol, δ-aminolaevulinic acid dehydratase (δ-ALAD) condenses two molecules of δ-ALA to form one molecule of porphobilinogen (PBG). Polypyrryl methane, an intermediate structure formed by the combination of several molecules of porphobilinogen, is catalyzed by porphobilinogen deaminase. This molecule forms a tetra-pyrrole ring structure, and under the action of either an isomerase or a deaminase, synthesis of uroporphyrinogen III or I respectively takes place. Next, uroporphyrinogen decarboxylase synthesizes the formation of coproporphyrinogen III and I respectively.

Coproporphyrinogen III enters the mitochondrion, and there coproporphyrinogen oxidase converts it to Protoporphyrin III No. IX (also called protoporphyrin IX). Iron ($Fe^{++}$) is inserted into the protoporphyrin-IX nucleus to form haem in a reaction catalyzed by haem synthetase (also called Ferrochelatase).

### 6b. Biodegradation

Figure 2 is a diagrammatic representation of the haem degradative pathway.

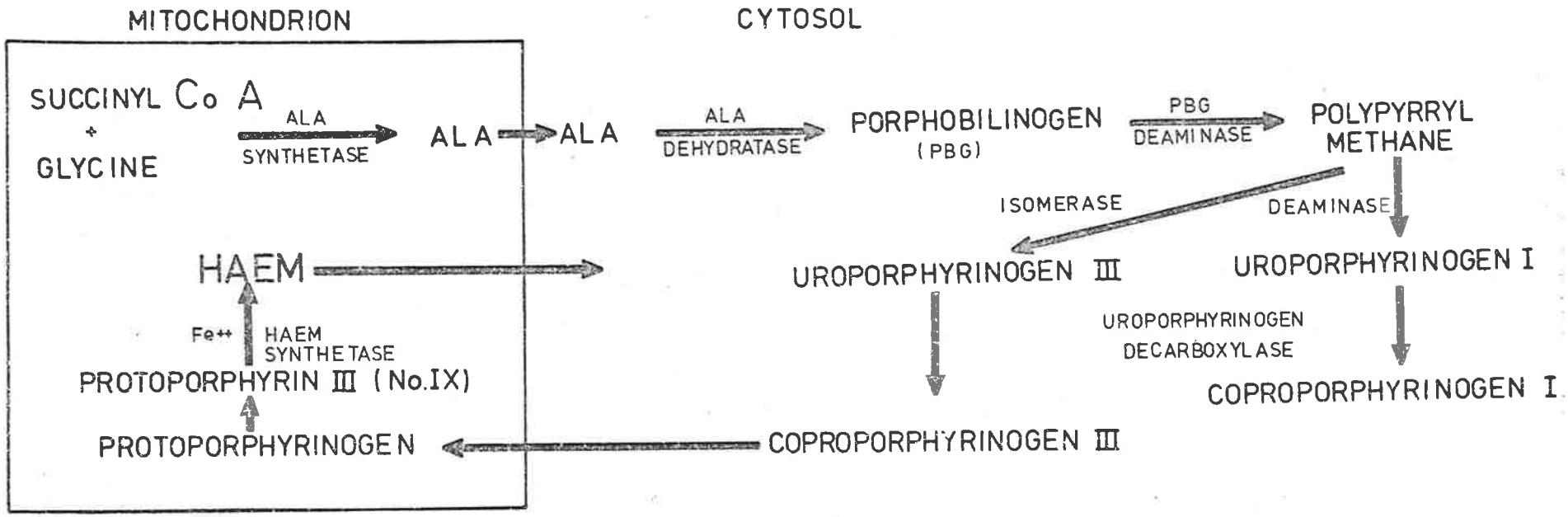


Figure 1. The biosynthesis of haem

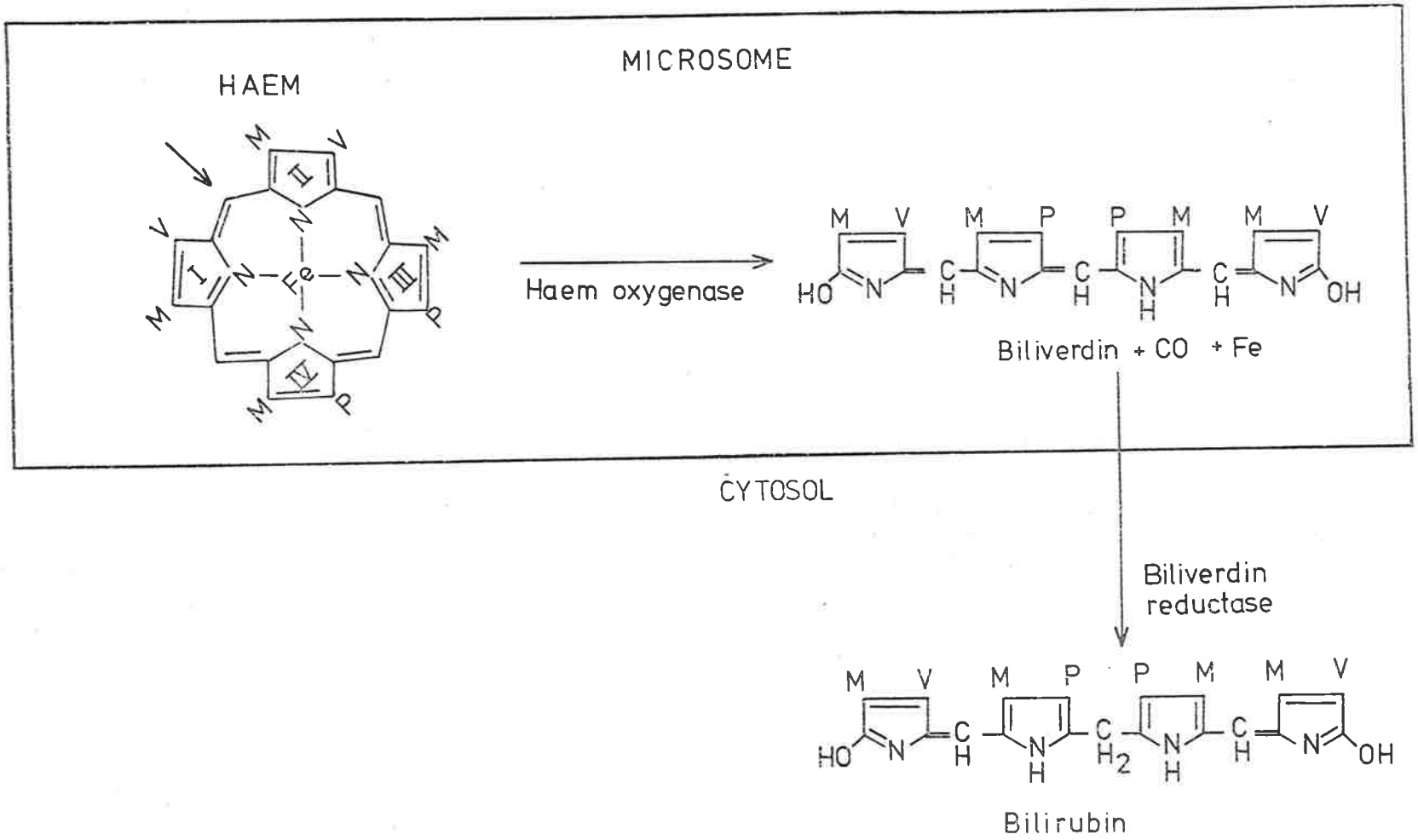


Figure 2. The conversion of haem to bilirubin
M = methyl, P = Propionic acid, V = Vinyl
The arrow denotes the point of cleavage

The first step in the degradation of haem is the rate limiting step and is catalyzed by haem oxygenase, a microsomal enzyme. Haem oxygenase cleaves the tetrapyrrole ring of haem at the α methene bridge and quantitatively converts the meso carbon atom to carbon monoxide. The central iron atom is liberated and biliverdin IXα, a linear tetrapyrrole, is produced. Biliverdin reductase, a cytoplasmic enzyme, converts biliverdin to bilirubin. Bilirubin is either conjugated and excreted or is further metabolized. An outline of the fate of bilirubin is given by White et al (1968) and is mentioned here for the sake of completeness, though the metabolic pathway of bilirubin is not relevant to this discussion.

## 7. Properties of Key Haem Biosynthetic and Degradative Enzymes

### 7a. δ-Aminolaevulinic Acid Synthetase* (δ-ALAS) (EC 2.3.1.37)

δ-Aminolaevulinic acid synthetase is synthesized in an essentially inactive form by ribosomes in the cytoplasm, this being the precursor of the active mitochondrial entity. The cytosol enzyme has a molecular weight of 178,000 and in the process of entering the mitochondrion and being transformed into the active moiety, the molecular weight decreases to 77,000. Reference to δ-ALAS invariably refers to the mitochondrial form. No preformed, inactive, presursor of δ-ALAS can be detected in normal liver. The increase in the activity of the enzyme due to e.g. chemical inducers, can be prevented by glucose, haem and transcriptional and translational inhibitors.

*Summarized from Whiting and Elliot (1972).

## 7b. δ-Aminolaevulinic Acid Dehydratase (δ-ALAD) (EC 4.2.1.24)

High activity of δ-ALAD is found in erythrocytes, liver and kidney where the enzyme resides almost exclusively in the cytosol. A number of workers have examined the enzyme derived from a variety of sources. From the publications of Gibson et al (1955), Del C.Battle et al (1967), Nandi et al (1968), Komai and Neilands (1969), Gibson and Goldberg (1970), Abdulla and Haeger-Aronson (1971), Wilson et al (1972), Cheh and Neilands (1974), Border et al (1976), Haeger-Aronson et al (1976), Thompson et al (1977), and Meredith et al (1977), an introduction to some of the properties of the enzyme can be obtained which are summarized below. δ-ALAD has been reported to behave as an allosteric enzyme, and while the enzyme is thought not to contain a prosthetic group, copper and/or zinc may be required for activity. The purified enzyme can be deaggregated down to dimeric and monomeric forms. This has led tc estimations of its molecular weight in the range from 140,000 to 260,000. The monomeric form of δ-ALAD contains 56 cysteine residues/mole while 11 and 28 thiol groups per molecule has also been reported. For reactivity, the thiol groups of δ-ALAD must be in the reduced state and when the enzyme is partially purified, the presence of glutathione or cysteine is obligatory. As with other thiol containing compounds, δ-ALAD is subject to interference by metals, e.g. p-chloromercuribenzoate and iodoacetimide. Aluminium stimulates δ-ALAD in erythrocytes both *in vivo* and *in vitro*. At low concentrations, zinc is stimulatory but becomes inhibitory at high concentrations, although Abdulla and Haeger-Aronson (1971) have reported the opposite results. Copper, silver and lead are inhibitory which zinc can counteract. Protoporphyrin, haem, oxygen, EDTA and Tris buffer also inhibit activity.

The effect of cadmium is unique. Lauwerys et al (1973) showed that erythrocyte δ-ALAD is not inhibited by cadmium in exposed workers.

Seth et al (1976) found that *in vivo* cadmium did not inhibit erythrocyte δ-ALAD in mice, Yoshida et al (1976) stated that cadmium causes a 20% reduction in ALAD activity in rat liver. Wilson et al (1972) showed that cadmium is inhibitory in the presence of low substrate concentration and stimulatory at high substrate concentration. From this data, Wilson et al (1972) concluded that cadmium can act on the enzyme, both sterically and at its functional site, in such a way that it uncovers more catalytic sites and aids substrate binding. Alternatively, it may stabilize an enzyme conformation which may possess a lower affinity for the substrate but a higher catalytic efficiency.

## 7c. Microsomal Haem Oxygenase (MHO) (EC 1.14.99.3)

Microsomal haem oxygenase is bound to the membranes of the endoplasmic reticulum. It does not require cytochrome P-450 as a terminal oxidase for activity as originally thought (Tenhunen et al 1969, Maines and Kappas 1974a, 1975a). Maines et al (1977) solubilized and partially purified the enzyme and reported its molecular weight to be greater than 68,000 implying that apo-cytochrome P-450 (MW 50,000) is not haem oxygenase as had been suggested. Haem oxygenase is inhibited by carbon monoxide and requires oxygen and NADPH or NADH for activity, suggesting a cytochrome or haemoprotein nature for the enzyme. The proposed mechanism of action, accomodates this possibility. Maines and Kappas (1977a) and Maines et al (1977) suggest that when haem binds to the enzyme, the resulting haemoprotein is capable of acting as a terminal oxygenase of an electron transfer chain which can accept electrons from NADH or NADPH depending on which flavoprotein reductase is catalyzing the reaction. This

results in oxygen activation and haem ring cleavage. The ephemeral nature of the active, haemoprotein state of haem oxygenase is indicated by the fact that following the conversion of haem to biliverdin and the products released from the enzyme, the haemoprotein nature is lost until another molecule of haem binds and forms a complex. Some metals are suitable substrates for haem synthetase, forming metalloporphyrins but not all metalloporphyrins can be degraded. It is suggested that although they can bind to haem oxygenase, only those metalloporphyrins which can bind molecular oxygen are metabolized (Maines and Kappes 1977a). These authors showed that Fe-haem and Co-haem are degraded while Mn-haem and Ni-haem are not.

## 8. Haem

Haem can be used in the production of various haemoproteins such as haemoglobin and cytochromes, while the haem which is not utilized is bound to a carrier protein(s) and constitutes the "free haem pool". The absolute requirement of cytochrome P-450 for haem indicates a fundamental connection between the drug metabolizing system and the haem metabolizing pathways.

In the liver, haem combines with an unknown co-repressor or aporepressor which feed back to repress the activity of δ-ALAS. In instances when the level of haem is lowered, derepression occurs resulting in an increase in the activity of δ-ALAS leading to an increase in haem synthesis. In instances of excess haem, haem stimulates the activity of haem oxygenase thereby increasing the rate of its own destruction. Haem, therefore, is central to the control of both the haem bio-synthetic and catabolic pathways and so factors

which control the level of haem, control the pathways as well.

Theories have been offered on how regulatory haem levels are affected by haemo-compounds, e.g. cytochromes, as well as the nature of the haem source which is catabolized. The following section deals with a discussion of these points as well as some of the proposed theories on the nature of the control of haem biosynthesis and catabolism.

## 9. Haem/Cytochrome-Haem/Haem Oxygenase Interrelationships

It has been observed in the past that the preparation of cultured cells always results initially in losses of cytochrome P-450 and an increase in the activity of haem oxygenase (Bissell et al 1974). Experimental evidence showing a relationship between cytochrome P-450 and haem oxygenase activity has been reported by Bissell et al (1974), Bissell and Hammaker (1976a) and Maines (1977).

Maines and Kappas (1977b) reported that haem in the "free" form is preferentially metabolized *in vivo* compared with haem bound to cytochromes or other functional haemoproteins. This is found when a very low dose of cobalt is injected, but with a larger dose, cytochrome P-450-haem is also catabolized. Bissell and Hammaker (1976a) reported that after a tracer dose of 5-$^{14}$C-ALA, the degradation of intracellular haem proceeded very quickly initially (peaked at 2.5 hours), however, 12 hours after the injection, the loss of haem was log-linear following first-order kinetics.

These workers concluded that the early peak was a result of the catabolism of a rapidly turning over regulatory fraction ("free"

haem pool), while the second phase is attributed to the loss of cytochrome P-450-haem. Alternatively, the biphasic loss of haem can mean that there are two forms of P-450 with different half-lives. The relevance of the two possible forms of cytochrome P-450 will be discussed further in the next section.

Maines (1977) regards the mechanism of the catabolism of cytochrome P-450 as being one in which cytochrome P-450 is first converted to cytochrome P-420 and in this form becomes a substrate for haem oxygenase. Bissell and Hammaker (1976a)suggest however that cytochrome P-450-haem dissociates from its apoprotein and mixes with the regulatory "free" haem pool before degradation. The suggestion by Maines (1977) that the first step in the degredation of cytochrome P-450 is its conversion to P-420, is based on the finding that cytochrome P-420 is a better substrate for haem oxygenase than cytochrome P-450. Indeed, it was reported that cytochrome P-450 is metabolized to a negligible extent, but this may not be absolutely true. De Matteis and Sparks (1973) stated that the aerobic incubation of microsomes with NADPH form many products, one of which is carbon monoxide, suggesting that haem oxygenase is active on cytochrome P-450. McLean and Garner (1974) proposed that cyrochrome P-420 is not a precise entity but rather that cytochrome P-420 preparations are composed of haem non-specifically bound to the apo-protein. This implies therefore, that if a denaturing agent is present *in vivo* which constitutes the first step in the destruction of cytochrome P-450 (Maines 1977), then it may function by dislodging the haem from its normal binding site on the apo-protein. Similarly, it can possibly dissociate some of the haem entirely, in keeping with the idea proposed by Bissell and Hammaker (1976b). Maines et al (1974) showed that cytochrome P-420-haem is more susceptible to dislocation

from its binding site and exchanges readily with albumin, whereas haem from cytochrome P-450 does not. Another aspect of the stimulation of haem oxygenase and the catabolism of haem involves different affinities that haemo-substances have for the enzyme (Maines 1977). This idea enables a marriage between the theory proposed by Bissell and Hammaker (1976b) with that of Maines (1977). The suggestion is that the availability of haem for degradation by haem oxygenase is related to the nature of the apo-protein to which the haem is bound, as the various forms each have a different affinity for the enzyme. When haem is bound to the apo-protein of cytochrome P-450, its attraction for haem is greater than that of the enzyme, whereas when in the denatured form (cytochrome P-420), the affinity by haem oxygenase is greater. Further, when in the "free haem pool" state, the carrier protein could be envisaged to have the least attraction for haem, as compared with the other binding proteins, and so the haem is most readily accessible to degradation.

Agents promoting the degradation of haemoproteins, can be imagined to elicit their actions by lowering the affinity which the apo-proteins have for haem as compared with the haem oxygenase's affinity. Agents which readily convert cytochrome P-450 to cytochrome P-420 have been reported. These include proteases, detergents and mercurials. (Omura and Sato 1964). Cooper et al (1965) reported that p-chloromercuribenzoate converted cytochrome P-450 to cytochrome P-420. Mason et al (1965) observed similar changes with other mercurials, and concluded that a sulphur atom (possibly cysteine) is ligated to haem. Alvares et al (1974) reported that the addition of methylmercuric chloride to microsomal suspensions resulted in the conversion of cytochrome P-450 to cytochrome P-420. The report by Chevion et al (1977) that the axial ligand trans to the sulphur atom is imidazole, means that the two

axial ligands of haem can be attacked by electrophilic substances viz. mercurials. It is conceivable therefore, that cadmium could elicit a similar interaction and change cytochrome P-450 to cytochrome P-420. The denaturation of cytochrome P-450 can also occur if sulphydryl and imidazole groups, being part of the amino-acids constituting the peptide backbone of the apoprotein molecule, are also attacked by mercurial compounds and/or cadmium.

The two theories summarized above and the ideas which have evolved from them are presented to illustrate the intricate relationship which exists between cytochrome P-450 and haem oxygenase.

## 10. Haem/Cytochrome-Haem/δ-ALAS Interrelationships

Evidence indicating that a relationship exists between cytochrome P-450 and δ-ALAS activity is shown by the work of Padmanaban et al (1973), Bissell and Hammaker (1976b) and others.

A theory to account for the control of δ-ALAS activity by cytochrome P-450 has been proposed by Padmanaban et al (1973), who envisaged that a protein, possibly the apo-protein of a haemoprotein or probably apo-cytochrome P-450, serves as a positive control on the translation of the δ-ALAS messenger. However, when haem binds to the apo-protein they form a functional repressor and inhibit translation. Further, the translation of the mRNA provides a trigger for the transcription and formation of more mRNA. This step requires cortisol and is inhibited by actinomycin D.

Drugs affecting the level of cytochrome P-450 cause changes in the activity of δ-ALAS. For example, allylisopropylacetamide (AIA) increases the degradation of cytochrome P-450-haem resulting in an increased rate of synthesis of the enzyme.

Phenobarbitone and Phenylbutazone (Padmanaban et al 1973, De Matteis and Gibbs 1972 and Rajamanickan 1975) increase the production of microsomal proteins, including apo-cytochrome P-450 proteins and this in turn stimulates the production of δ-ALAS, thus ensuring an adequate supply of haem to form the complete holocytochrome P-450. De Matteis and Gibbs (1972) showed that 3,5-diethoxycarbonyldihydrocollidine (DDC) inhibited haem synthetase, resulting in a fall in the level of haem, an increase in δ-ALAS synthesis and in porphyria. Recently, Maines and Kappas (1977b) proposed that the central iron atom of haem is the active regulator of the synthesis of δ-ALAS and not the whole haem entity. The tetrapyrrole molecule serves merely as a carrier of the metal to the regulatory sites in the cells. De Matteis and Sparks (1973) reported that iron is involved in the loss of cytochrome P-450 haem both *in vivo* and *in vitro*. Whiting and Granick (1976a, b) confirmed that chemicals which increase the activity of δ-ALAS (e.g. AIA and DDC) do so by increasing the amount of enzyme in the hepatocytes via changes in the rate of enzyme synthesis. Changes in the rate of transcription and translation of ALAS-mRNA is favoured therefore, rather than activating preformed enzyme as proposed by Patton and Beattie (1973).

The proposition that the repression of δ-ALAS synthesis by haem is mediated by inhibiting the translation of the ALAS-mRNA as

advocated by Sassa and Granick (1970) and Tyrrell and Marks (1972) and partly by Padmanaban et al (1973) was not favoured by Whiting (1976). Whiting (1976) proposed that haem decreases the amount of mRNA *in vivo* rather than causing the accumulation of a specific translational inhibitor. *In vitro* experiments with chick embryo liver cells however, showed that haem did not directly affect the translation of endogenous ALAS-mRNA. Maines and Sinclair (1977) reported that in chick embryo liver cells, the mechanism by which the induction of δ-ALAS was inhibited by cobalt, cobalt-haem diethyl ester and iron-haem was intermediate between that produced by actinomycin D and cycloheximide.

These experiments serve to highlight the fact that the precise mechanism by which haem causes feedback inhibition of the synthesis of δ-ALAS is still not clear, but that it may possibly involve control over the transcription and/or translation of the messenger in a way in which both systems affect each other.

Schmid (1973), in summarizing several publications, concluded that exogenous haem could regulate the biosynthetic and catabolic enzymes in a similar way to that proposed for endogenous haem. However, exogenous haem could not function as a precursor for cytochrome formation. Bissell and Hammaker (1976b) did not support this, as they found suggestions in their work that exogenous haem was incorporated into cytochrome P-450. Consequently, further work is required to clarify whether more than one "free" haem pool can exist.

Another enigma deals with the significance of the multiple forms of cytochrome P-450. The different forms examined differ in several respects, including their electron paramagnetic resonance spectra

(i.e. the spin-state of the haem-iron) and the amino-acid composition of the apo-protein. The latter point in turn reflects the different relative stability and half-life of the many forms. From the work of Levin and Kuntzman (1969), Coon et al (1977) and Dus et al (1977), it is possible to associate these characteristics with contributing to either the fast or slow phase of the biphasic catabolism of cytochrome P-450. If conversion to cytochrome P-420 is the initial step in the degradation of cytochrome P-450, then it is reasonable to speculate on whether the multiple forms of the haemoprotein may differ in their susceptibility.

Further, if multiple forms of cytochrome P-450 have different efficacies with regard to the de-repression of δ-ALAS activity and the induction of haem oxygenase, then it would appear that the multiple forms of cytochrome P-450 will require further investigation.

## 11. Discrepancies in the Cytochrome/Enzyme Interrelationship

The control which cytochrome P-450 exerts over the haem metabolic pathways may not be operable in cells other than in hepatocytes. Maines and Kappas (1974a) reported that cobalt chloride did not affect the haem oxygenase system of the spleen. Correia and Schmid (1975) showed that in the liver, cobalt induced haem oxygenase and benzpyrene hydroxylase activities but decreased cytochrome P-450 levels, while in the intestinal mucosa, haem oxygenase and benzpyrene hydroxylase activities and cytochrome P-450 levels all increased after cobalt treatment. It was suggested that the mechanism of the stimulation of haem oxygenase may differ in extrahepatic tissues, and/or that the level of cytochrome P-450 is not related to enzyme activity.

Equally valid is the possibility that in tissues other than the liver, a putative factor may protect against toxic agents which affect enzymes involved in the haem metabolizing system. Moron et al (1977) reported that the level of reduced glutathione in rat liver was higher than in rat lung. Since glutathione has been found to prevent metal induction of haem oxygenase (Maines and Kappas 1976a), the differences in the effects of cobalt in liver and extrahepatic tissues seem untenable. On the other hand, the requirement by liver for high reduced glutathione levels implies that pertubations in the enzymatic system responsible for maintaining these levels, would cause the level to be more susceptible to toxic effects. In the final analysis however, the reason for the differences is not known.

Besides the drugs already mentioned, heavy metals affect the haem metabolic pathways. Lead inhibits δ-ALAD activity in the blood (Lauwerys et al 1973) and also in the brain, liver, kidney and bone marrow (Gibson and Goldberg 1970). It has also been implicated in inhibiting haem synthetase (Alvares et al (1976). Maines and Kappas (1976a, c) showed that lead stimulates both δ-ALAS and haem oxygenase activities. The inhibition of haem synthetase and the stimulation of haem oxygenase may account therefore for the observed effects of an acute dose of lead, where the levels of haem and cytochrome P-450 fall and this is reflected in a lowered rate of microsomal metabolism both *in vivo* and *in vitro* (Alvares et al, 1976). Further, the elevation of δ-ALAS activity and the reduced δ-ALAD activity result in the accumulation of ALA in the blood but since ALAD is not the rate limiting step and is normally present in large quantities, this enzyme may contribute marginally to the overall fall in the level of haem. Alvares et al (1974) reported a similar fate

for the levels of haem, cytochrome P-450 and microsomal metabolic activity following mercury intoxication, despite the fact that mercury's effects on δ-ALAS differ.

A common feature of all metals is their ability to increase the activity of hepatic microsomal haem oxygenase. The effects of metals on δ-ALAS is varied however, indicating that the ensuing fall in the level of haem, due to the increase in its degradation, does not always result in the expected stimulation of δ-ALAS activity. Cobalt has been the most extensively studied metal, and these findings have been compared with those for other metals.

Yasukochi et al (1977) showed that cobalt decreased the level of cytochrome P-450 but did not affect the level of cytochrome $b_5$. Maines and Kappas (1976a) reported that cobalt, manganese, nickel and lead all increase both δ-ALAS and haem oxygenase activity, while mercury and zinc are inhibitory. Chromium, iron (ferrous and ferric), copper and cadmium increase only haem oxygenase activity without affecting δ-ALAS. Maines and Kappas (1975a) showed that cobalt's effect on δ-ALAS is biphasic in that it causes an early inhibition of δ-ALAS (max 2 hrs), followed by an increase in enzyme synthesis and activity (max 8 hrs). De Matteis and Gibbs (1977) reported that cobalt inhibits the δ-ALAS enzyme itself, rather than inhibiting its formation. These findings were contrary to those of Whiting (1976), who argued that effectors of δ-ALAS mediate their actions via enzyme synthesis rather than directly effecting the enzyme already formed. Cobalt has been implicated in inhibiting haem synthetase, and this may be related to the fact that cobalt-protoporphyrin forms after cobalt injection (De Matteis and Gibbs 1977). Cobalt-protoporphyrin has been speculated

to be the unidentified green bile pigment which forms after cobalt intake, and which De Matteis and Unseld (1976) suggested could be an effective and sustained stimulant to haem oxygenase induction. Maines and Kappas (1975b) report that cobalt-protoporphyrin is a weaker stimulant than ionic copper, but this need not discount the inducer role proposed for cobalt-protoporphyrin.

Guzelian and Bissell (1976) complicated the issue further when they reported that cobalt did not affect haem synthesis but rather inhibited the association of haem with its apoprotein in the formation of holocytochrome-P-450.

Another inconsistency in the reported effects of metals deals with nickel. Maines and Kappas (1976a) showed that nickel increased δ-ALAS activity *in vivo*. In contrast, in a subsequent publication, the same authors (Maines and Kappas, 1977c) reported that nickel (as well as platinum) depressed δ-ALAS activity even though the dose, route of administration and the time after injection at which the animals were examined, was the same. Further, Maines and Kappas (1976b) illustrated clearly that the classical interrelationship of enzyme control does not always apply when they showed that selenium causes an increase in δ-ALAS synthesis, resulting in a transient increase in the "regulatory haem pool" which in turn stimulates the activity of haem oxygenase.

Reports concerning the effects of cadmium on δ-ALAS and haem oxygenase are not as numerous as those for cobalt. Maines and Kappas (1976a) showed that a single subcutaneous injection of cadmium stimulated haem oxygenase activity but did not affect δ-ALAS activity in rat

liver. Maines and Sinclair (1977) reported that in cultures of embryonic chick liver cells cadmium, copper and cobalt all inhibited the induction of δ-ALAS by AIA.

In conclusion, questions relating to the significance of the multiple forms of cytochrome P-450; to how many "free" haem pools exist and which one may be regulatory, along with the other inconsistencies detailed in this section, all suggest that the true nature of the control of haem metabolism and the involvement of cytochrome P-450 appear to be more complex than currently envisaged.

## 12. Summary and Aims of the Project

The General Introduction has attempted to present a synopsis of information dealing with cadmium and the drug metabolizing system, metallothionein and haem metabolism.

At the time that this project was conceived, the information on the interaction of heavy metals with microsomal enzymes was reasonably rudimentary. Furthermore, cadmium was the subject of some concern because it had been clearly established as one of the more toxic of the metals to which Man may be exposed from environmental sources.

The initial aims of the project were to determine whether cadmium induces or inhibits the activity of the microsomal mixed function oxidase system, since the published data at that time were contradictory. The experiments directed towards this aim are reported in Chapter one and they include an investigation of the time-course of the response.

It had been recognized that most of the cadmium found in the liver is bound to metallothionein and there had been some speculation on the possible protective role of binding. The experiments reported in Chapter two deal with the question of whether metallothionein is involved in the recovery of microsomal enzyme activity following inhibition by an acute dose of cadmium.

Finally, the mechanism by which cadmium initiates its effects on microsomal enzyme activity was investigated. Chapter three reports the experiments which were done to determine whether the decline in cytochrome P-450 noted in chapter one, is associated with changes in either the biosynthesis or degradation of haem.

# GENERAL METHODS

The methods for assays common to more than one chapter are presented here. Those assays confined to one chapter only, are detailed in the "Methods" section of that chapter.

## 1. Source of animals and chemicals

Male Porton-derived rats (220-300 grams) were bred at the University of Adelaide Central Animal House. They were allowed food and water ad libitum. Cadmium acetate was obtained from BDH Poole, England and was "Analar Grade". Cadmium acetate was dissolved in normal saline before injection and an equimolar dose of sodium acetate in saline was given to control rats. Biochemical reagents were obtained from the Sigma Chemical Company and Calbiochem. All other reagents were of analytical or laboratory grade.

## 2. Preparation of Microsomes

Rats were stunned and decapitated and their livers, after perfusion in situ with ice-cold saline via the portal vein, were removed and weighed. All subsequent operations were carried out at $4^{o}C$. The livers were minced and then homogenized in 20 ml of 0.25 M sucrose/0.05 M Tris-HCl (pH7.4) using a Potter-Elvehjem homogenizer and a motor driven pestle. The homogenate was centrifuged for 15 minutes at 10,000 xg, after which the supernatant was decanted and centrifuged at 105,000 x g for 1 hour. The microsomal pellet was resuspended in cold 1.15% KCl which was buffered with 5mM phosphate buffer pH7.4 and recentrifuged for 1 hour at 105,000 x g. The washed microsomes were resuspended in 1.15% KCl/phosphate buffer.

## 3. Estimation of Microsomal Protein Content

Protein was assayed by the method of Lowry et al (1951) using Bovine Serum Albumin (B.S.A. Sigma Type V) as standard.

## 4. Estimation of Microsomal Cytochrome P-450

Cytochrome P-450 was determined from the carbon monoxide difference spectrum of dithionite-reduced microsomes assuming a molar extinction coefficient of 91 $cm^{-1}$ $mM^{-1}$ between 450nm and 490nm (Omura and Sato 1964).

## 5. Estimation of Microsomal Cytochrome $b_5$

Cytochrome $b_5$ was determined from the difference spectrum between NADPH reduced and air saturated microsomes. Microsomes were diluted to 2 mg protein/ml with 0.1M phosphate buffer pH7.4 and 3 mls added to each of 2 matched quartz cuvettes. The baseline of equal light absorbance, was determined between 390-450 nm on a Unicam SP1800 split beam spectrophotometer. 20 µl of 2% NADPH were added to the sample cuvette, 20µl distilled water to the reference cuvette and the spectrum between 390-450 nm recorded after thorough mixing. The cytochrome $b_5$ content was determined from the difference spectrum between 424 nm and 409 nm assuming a molar extinction difference of 185$cm^{-1}$ $mM^{-1}$ (Omura and Sato 1964). Results are expressed as nanomoles cytochrome $b_5$ per mg microsomal protein.

## 6. Atomic Absorption Spectrophotometry

The dry-weight content of cadmium and zinc in tissues were estimated using atomic absorption spectrophotometry.

Glassware - all glassware used was washed by soaking in diluted Decon - 90 (Decon Laboratories Ltd, Brighton, England) for 24 hours and rinsed in distilled-deionized water. This was followed by immersion for a minimum of 24 hours in nitric acid/water 1:1. A further rinse using copious amounts of running distilled-deionized water was followed by drying in a heated cabinet.

Reagents - Merck Perchloric Acid (70%)

"Analar" Nitric Acid, glass distilled

"Analar" cadmium acetate was used as a source for cadmium in the preparation of the stock solution, from which the standards were prepared. The concentration of the cadmium stock solution was 1 mg cadmium metal/ml in 5% nitric acid.

Pure metallic zinc was dissolved in hydrochloric acid and the standard stock solution prepared as described in Varian's Technical Manuals.

Analysis - all tissues were dried at about 90-100$^{o}$C for approximately 8 hours in an electric oven before digestion. Up to 250 mgms of dry tissue was weighed and placed in a graduated 50 ml test tube then 1 ml perchloric acid (70%) and 2 ml distilled nitric acid (concentrated) were added and the tubes placed in holes drilled in an electrically heated aluminium block. The temperature of the block was gradually

raised to 180°C and the tissues allowed to digest. Digestion time was about 3 - 5 hours and was terminated when about 1 ml of clear liquid remained and no trace of yellow vapours could be seen. The tubes were allowed to cool and the volume made up to 25 mls with distilled-deionized water. Blanks were treated in the same way. After thorough vortexing, the solution was aspirated directly into the flame of a Varian Techtron 1200 Atomic Absorption Spectrophotometer. The photomultiplier was set to 228.8 nm for the estimation of cadmium and 213.9 nm for zinc and the absorption noted from the digital readout after integration over a 3 second period. The metal content was quantitated by interpolation from a standard curve. It was established that this method did not suffer from non-atomic absorption and so matrix matching of the standards was not necessary.

## 7. Statistics

The effect of experimental treatment was evaluated by applying the unpaired Student's t-test (two tailed) to values obtained from treated and control group of animals. A significance level of $p < 0.05$ was used as a basis for rejection of the null hypothesis.

# CHAPTER ONE
# THE DRUG METABOLIZING SYSTEM

## INTRODUCTION

The acute and the chronic effects of cadmium on the rat hepatic drug metabolizing system are examined in this chapter. These experiments were performed to clarify the apparent discrepancy between the enzyme inductive effect reported by Wagstaff (1973) and the inhibition reported by Unger and Clausen (1973) and Hadley et al (1974). Furthermore, it was intended that these experiments form the basis for an evaluation of the mechanism of the interaction between cadmium and microsomal enzymes (see chapter 3).

To satisfy both objectives, the time-course of the effects of a single, intraperitoneal injection of 5 mg cadmium acetate/Kg. (equivalent to 2.1 mg Cd/Kg) was examined, by assaying the activity of the rat microsomal drug metabolizing system *in vitro* at 12 hours and 1,2,3,4,6,9 and 12 days after injection. Aminopyrine was used as a representative of a type I substrate and aniline for type II. Further, hepatic microsomal cytochrome P-450, protein yield, liver weights, the metal contents of some of the microsomal preparations and the cadmium and zinc contents of the livers and kidneys were estimated.

The chronic experiments, as compared with the acute, represent a situation in which the animals receive a small amount of cadmium continuously over a long period of time.

The chronic studies were undertaken by housing rats in community cages, from the time they weighed 150 grams, during which time they were allowed food and water ad libitum. The treatment group had cadmium (100 ppm ) added to their drinking water. Based upon

estimates of water consumption, this represented an intake of approximately 1-3 mg cadmium metal/day. The animals lived under these conditions for 222 days, after which they were killed and microsomes prepared from their livers. The microsomal cytochrome P-450 and $b_5$ contents were estimated as were aniline-para-hydroxylase and aminopyrine - N - demethylase activities. Microsomal protein yield, liver weights and the cadmium and zinc contents of the livers and left kidneys were also determined.

The drug metabolizing system can be altered in many ways. In order to evaluate whether the acute effects of cadmium were relatively non-specific, the following parameters were assayed. Microsomal glucose-6-phosphatase activity was determined as an index of the functional integrity of the microsomal membranes (Feuer et al 1965). The effect of food deprivation was also examined, as it was noted that the cadmium treated rats eat less food than the controls. This observation was also reported and investigated by Hadley et al (1974).

The control rats of the acute experiments were injected with saline as this was the solvent used for the cadmium acetate, however, rats treated with an equimolar dose of sodium acetate were also examined, to establish that any changes seen in the cadmium dosed group were not due to the acetate molecule.

## METHODS

### 2a. Estimation of Hepatic *in vitro* Drug Metabolism

Aminopyrine-N-demethylase and aniline parahydroxylase activities were determined in incubation media as described by Schenkman et al

(1967), except that semicarbazide (4.1 mM) was added to trap the formaldehyde produced during the demethylation of aminopyrine. The incubation media contained 5mM substrate and were incubated with shaking (120 oscillations/minute) for 20 minutes at 37°C. Washed microsomes (3 mg microsomal protein/3 ml incubate) was used as an enzyme source. Formaldehyde was estimated by the method of Nash (1953) and para-aminophenol, the metabolic product of aniline parahydroxylation, by a modified method of Imai et al (1966).

## 2b. Hepatic Microsomal Glucose-6-Phosphatase Activity

The protocol for the estimation of hepatic microsomal glucose-6-phosphatase activity was based on the method of Harper (1965). The colorimetric determinations of the phosphate content was based on the method of Fiske and Subbarow (1925).

Microsomes were diluted to 2 mg protein/ml with 0.1 M citrate buffer pH6.8. 0.1 ml microsomes were incubated with 0.1 ml 0.08 M glucose-6-phosphate for 15 minutes. 2 ml 10% TCA was used to stop the reaction and the precipitate removed by centrifugation. 1 ml supernatant was mixed with 1 ml 10% TCA and then 1 ml 15 mM ammonium molybdate dissolved in 5 M sulphuric acid was added and mixed thoroughly. When this was completed for all tubes, 1 ml 1% metol in 3% sodium sulphite was added and the blue colour allowed to develop for 15 minutes and then read at 680 nm against the reagent blank.

Standards and both reagent and incubation blanks were prepared and carried through the same procedure.

The enzyme activity was quantitated on the basis of inorganic phosphate released and this was calculated from the following formula.

$$\frac{ODexp - ODcont}{ODstand} \times \frac{(P)}{1} \times \frac{2.2}{1} \times \frac{4}{1} \times \frac{5}{1}$$

where ODexp = Average optical density of the duplicate experimental tubes

OD cont = Average optical density of the duplicate control tubes

ODstand = Average optical density of the duplicate standard tubes

(P) = µmoles phosphate in standard tube (0.3228 µmoles)

4 = converts 15 minute incubation time to 1 hour

5 = converts 0.2 mg. microsomal protein to 1.0 mg

2.2 = volume of enzymatic reaction mixture after addition of TCA.

## RESULTS

## I ACUTE STUDIES

### (a) The Acute Effects of Cadmium on the Microsomal Drug Metabolizing System

The means (with the standard errors) of both the control and cadmium treated animals' aniline-p-hydroxylase and aminopyrine-N-demethylase activities as well as their cytochrome P-450 content for each time of cadmium treatment, is shown in Table 1. By expressing the mean of each cadmium treated group's data as a percentage of the mean of its respective control and plotting this against the time of cadmium treatment, the graph shown in figure 3 is derived. These data show the time-course of the effects of cadmium on the drug metabolizing system of the male rat.

TABLE 1 TIME-RESPONSE RELATIONSHIP OF CADMIUM ACETATE (5 mg/Kg i.p.) IN THE MALE RAT

| Time after cadmium | Aminopyrine Demethylase nmoles product/mg microsomal protein/minute | | Aniline hydroxylase nmoles product/mg microsomal protein/minute | | Cytochrome P-450 nmoles/mg microsomal protein | |
|---|---|---|---|---|---|---|
| | Control | Treated | Control | Treated | Control | Treated |
| 12 hours | 5.14±0.17 (4) | 3.6±0.1 (4) | 0.42±0.02 (4) | 0.43±0.02 (4)* | 0.98±0.05 (4) | 0.7±0.01 (4) |
| 1 day | 6.85±0.32 (8) | 5.14±0.21 (5) | 0.65±0.03 (8) | 0.54±0.03 (5) | 1.04±0.05 (8) | 0.65±0.03 (5) |
| 2 days | 6.92±0.33 (8) | 2.57±0.16 (8) | 0.71±0.02 (8) | 0.32±0.02 (8) | 0.99±0.02 (8) | 0.44±0.02 (8) |
| 3 days | 7.00±0.25 (4) | 2.99±0.15 (4) | 0.84±0.03 (4) | 0.44±0.02 (4) | 1.04±0.06 (4) | 0.57±0.05 (4) |
| 4 days | 7.59±0.44 (6) | 3.68±0.18 (5) | 0.8±0.02 (6) | 0.46±0.01 (5) | 0.99±0.03 (6) | 0.63±0.02 (5) |
| 6 days | 5.06±0.39 (9) | 2.96±0.25 (8) | 0.75±0.02 (9) | 0.55±0.03 (8) | 1.07±0.04 (9) | 0.90±0.07 (8) |
| 9 days | 5.62±0.18 (6) | 3.82±0.12 (5) | 0.60±0.02 (6) | 0.54±0.02 (5)* | 0.84±0.04 (6) | 0.73±0.05 (5)* |
| 12 days | 5.74±0.29 (6) | 4.53±0.18 (6) | 0.70±0.02 (6) | 0.61±0.02 (6) | 0.96±0.06 (6) | 0.83±0.05 (6)* |

All cadmium treated groups differ significantly ($p < 0.05$) from their respective controls, except those marked with an asterisk. Values are means± S.E.M. for (n) determinations.

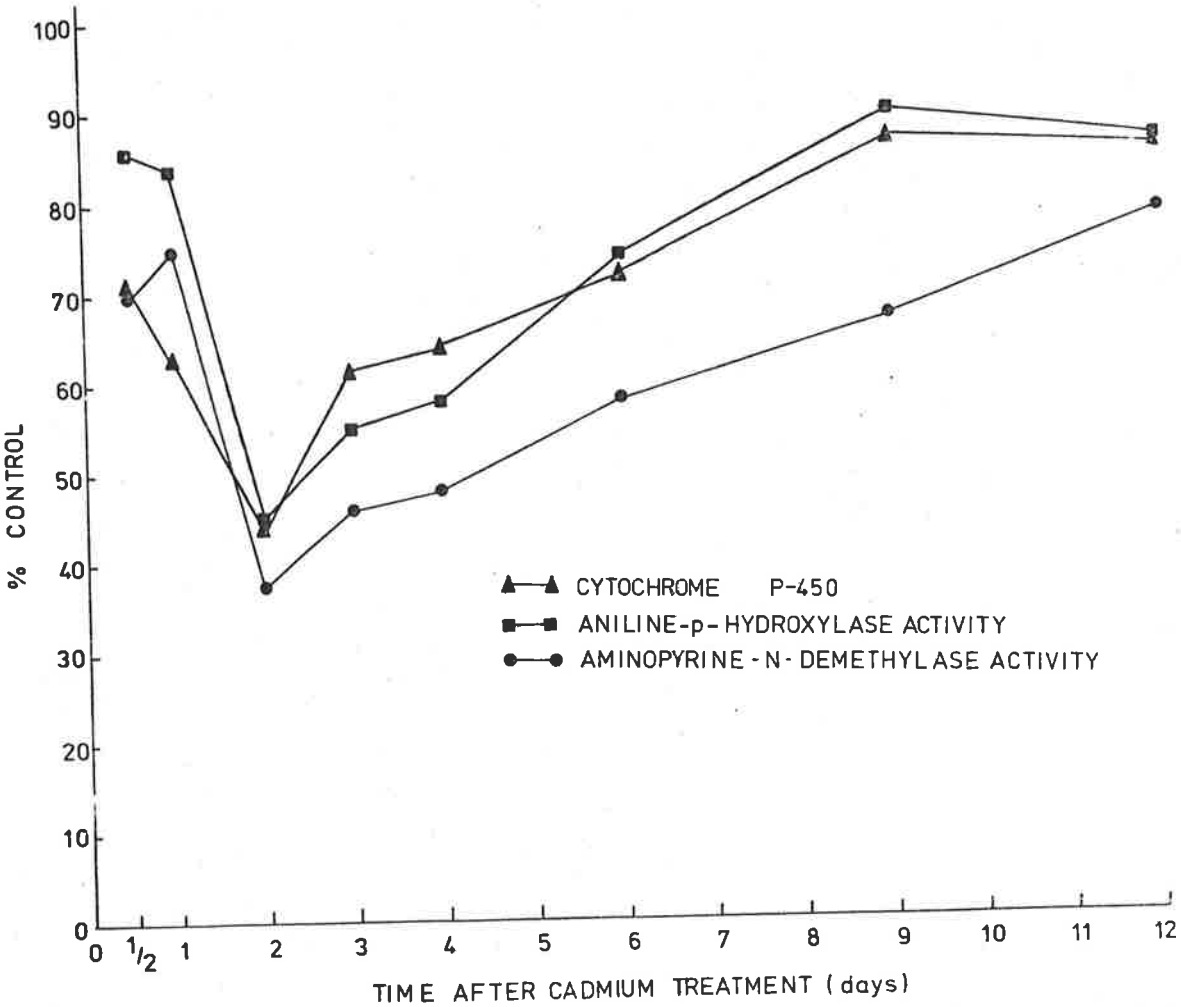


Figure 3. Time-response relationship of cadmium acetate (5mg/Kg i.p.) in the male rat.
Each point is the mean of the experimental values expressed as a percentage of the mean of their respective controls.

The changes in the metabolism of aminopyrine and aniline and the level of cytochrome P-450 occurred quickly following the injection of cadmium. At 12 hours, the three parameters had decreased to 70% 86% and 71% of control, respectively, while the greatest effect of cadmium occurred at the 48th hour, resulting in the maximum loss of type I and II metabolic activity and in the level of cytochrome P-450. At this time, the metabolism of aminopyrine and aniline was 37% and 45% of the respective controls and also had 44% of the control's cytochrome P-450 content. From between 48-72 hours, the trend observed in the 3 parameters during the first 48 hours reversed. At the 72nd hour, the metabolic activity of aminopyrine demethylase and aniline hydroxylase in the cadmium treated group was 43% and 53% of their respective control and the cytochrome P-450 content was 55% of control. The recovery to control levels continued, but by comparison with the rate with which the enzyme system's activity was initially decreased, this was slow, as control values had not been reached by the 12th day. On the 12th day, the type I and II metabolic activities and cytochrome P-450 contents were 79%, 88% and 86% of their respective controls. The demethylation of aminopyrine was the most sensitive to the effects of cadmium, while the metabolism of aniline and the level of cytochrome P-450 paralleled each other throughout the 12 day investigation.

(b) Microsomal Protein Yield

Table 2 shows that from 12 hours through to 12 days after cadmium treatment, there was no significant change in microsomal protein yield per gram of liver.

TABLE 2 MICROSOMAL PROTEIN YIELD (mg/gm liver)

| TIME AFTER CADMIUM | CONTROL | | CADMIUM | |
|---|---|---|---|---|
| 12 Hours | 9.55 ± 0.46 | (4) | 9.73 ± 0.20 | (4) |
| 1 Day | 10.14 ± 0.53 | (8) | 9.74 ± 0.59 | (5) |
| 2 Days | 12.68 ± 0.79 | (8) | 12.91 ± 0.52 | (8) |
| 3 Days | 7.83 ± 0.76 | (4) | 8.42 ± 0.57 | (4) |
| 4 Days | 11.62 ± 0.19 | (6) | 11.08 ± 0.58 | (6) |
| 6 Days | 13.30 ± 0.99 | (5) | 11.47 ± 0.44 | (5) |
| 9 Days | 10.24 ± 1.02 | (6) | 10.74 ± 1.01 | (5) |
| 12 Days | 10.78 ± 0.73 | (6) | 11.42 ± 0.82 | (6) |

The mean microsomal protein yield (± S.E.M.) of each group is shown and the figures in the parentheses represent the number of rats in each group.

No values differed significantly from controls ($p < 0.05$)

(c) Liver weights

Liver weight (in gm/100 gm body weight) of cadmium treated rats, and their respective controls are shown in Table 3. At most sampling times, there were no significant differences ($p > 0.05$), but on days 1 and 2, a significant decrease was observed ($0.02 < p < 0.05$) and on day 12, significant increase was observed ($0.001 < p < 0.01$).

(d) The Levels of Cadmium and Zinc in the Livers and Left Kidneys

Figure 4 shows the cadmium content in the liver and left kidney after cadmium administration. The cadmium content in controls was less than the quantitation threshold (1 µg Cd/gm dry liver). The highest amount of cadmium was detected at day 2 where it was $123 \pm 9$ µg cadmium/gram dry liver. Subsequently, the mean level decreased and remained approximately 80µg cadmium/gm dry liver for the remainder of the observation period. Cadmium accumulated slowly in the kidney throughout the course of the experiment and it was consistently lower than corresponding hepatic concentrations.

Control liver and kidneys had a zinc content averaging $100 \pm 4$ µg zinc/gm dry liver and $98 \pm 3$µg zinc/gm dry kidney. Figure 5 shows that after cadmium administration, the zinc content increased above control levels in both liver and kidney, although there was some difference in the rate of accumulation. On day 1, the zinc content in the liver had nearly doubled but in the kidney, no significant increase was seen until day 2.

TABLE 3 LIVER WEIGHT (gm liver/100 gm body weight)

| TIME AFTER CADMIUM | CONTROL | | CADMIUM | |
|---|---|---|---|---|
| 12 Hours | 4.05 ± 0.18 | (4) | 3.79 ± 0.12 | (4) |
| 1 Day | 5.10 ± 0.21 | (8) | 4.31 ± 0.20 | (5)* |
| 2 Days | 4.67 ± 0.11 | (8) | 4.37 ± 0.07 | (8)* |
| 3 Days | 4.50 ± 0.11 | (4) | 4.29 ± 0.21 | (3) |
| 4 Days | 4.54 ± 0.09 | (6) | 4.72 ± 0.14 | (6) |
| 6 Days | 4.39 ± 0.10 | (5) | 4.69 ± 0.29 | (5) |
| 9 Days | 4.53 ± 0.11 | (6) | 4.72 ± 0.08 | (5) |
| 12 Days | 3.84 ± 0.10 | (6) | 4.29 ± 0.08 | (6)* |

The mean liver weights (± S.E.M.) per 100 gm body weight of each group is shown and the figures in the parantheses represent the number of rats in each group.

*Significantly different from control $p < 0.05$.

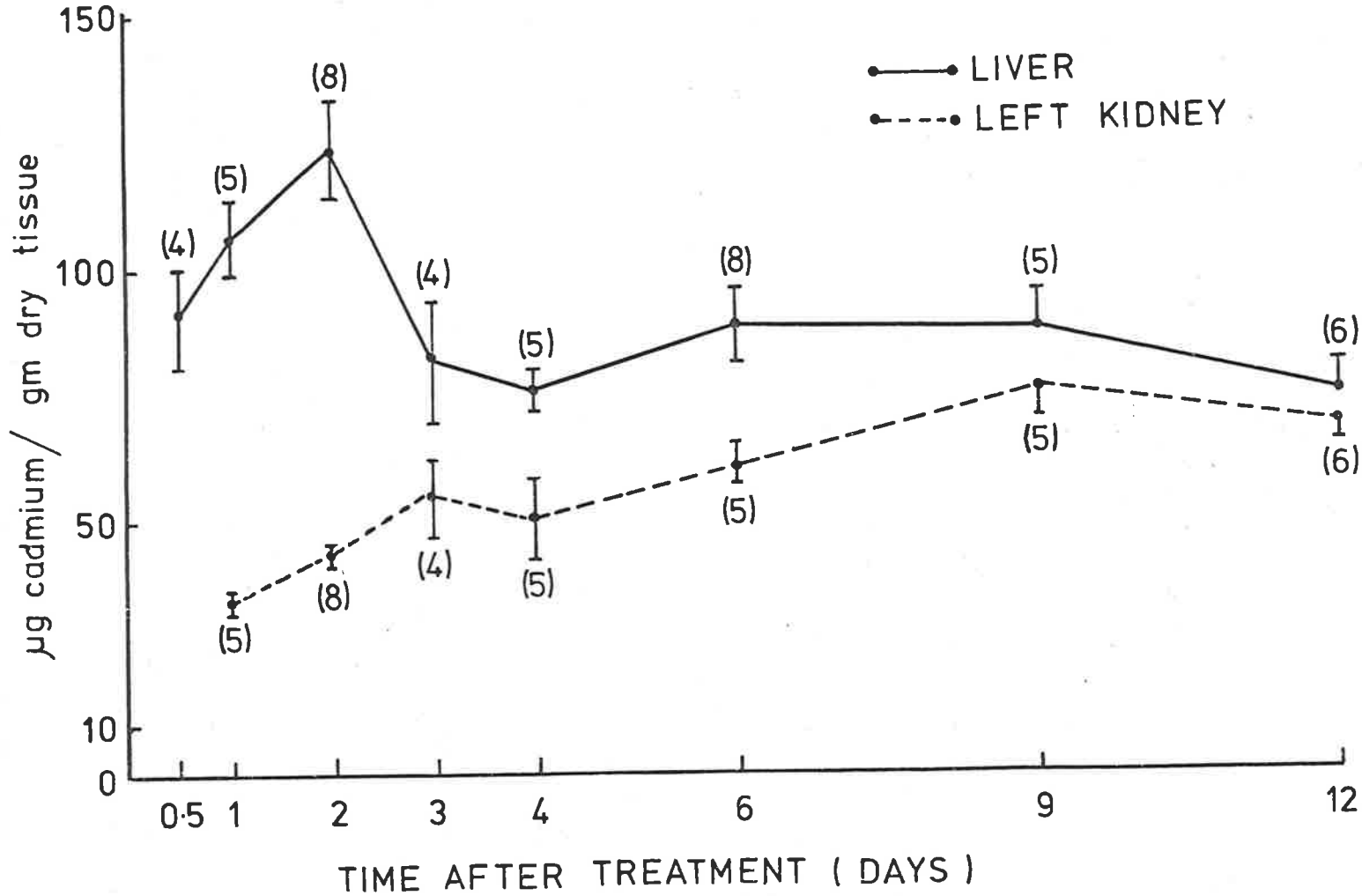


Figure 4. Cadmium content (µg/gm dry weight) of liver and left kidney of rats injected with 5 mg/Kg i.p. cadmium acetate. Value of controls less than detection threshold (1µg/gm dry weight)

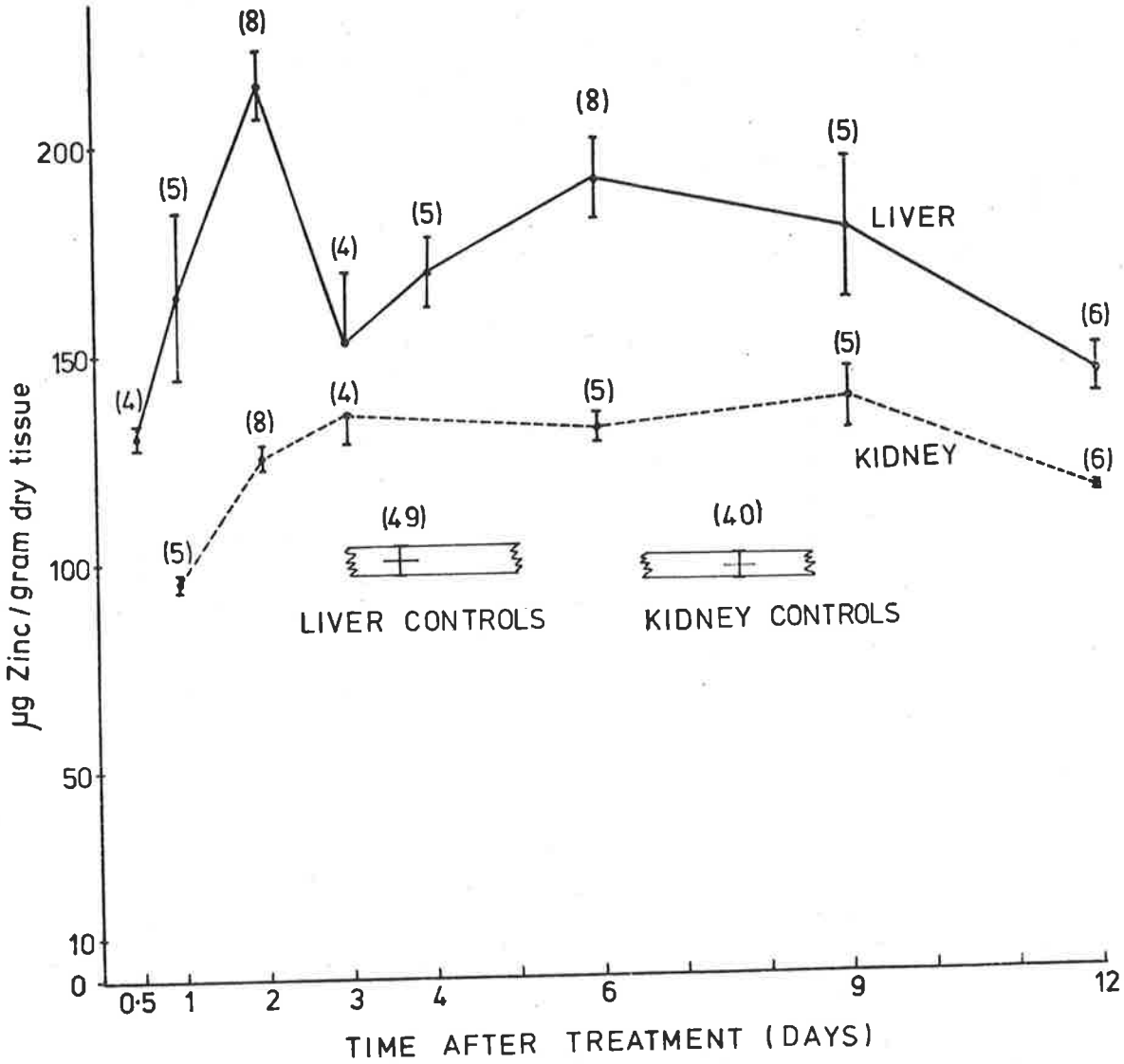


Figure 5. Zinc content (µg/gm dry weight) of liver and left kidney of rats injected with 5 mg/Kg i.p. cadmium acetate.

(e) Cadmium and Zinc Content of Microsomes

Microsomes prepared from rats 1 or 4 days after treatment with cadmium acetate were each assayed for their cadmium and zinc content. The data are shown in table 4, and expressed both as nanogram metal/3mg. microsomal protein in order to show how much of each metal was introduced to the incubation medium by the microsomes, and as cadmium concentration (nanomolar) after preparation of a microsomal incubate containing 1 mg microsomal protein/ml.

At both 1 and 4 days, these values are well below the cadmium or zinc concentrations reported by Hadley et al 1974, and Chvapil et al 1975, 1976 to inhibit microsomal metabolism.

(f) Sodium Chloride and Sodium Acetate Treated Controls

Table 5 shows the results obtained from 4 rats treated with saline and 4 rats dosed with an equi-volume, equimolar dose (relative to cadmium acetate) of sodium acetate.

The data indicate that microsomal protein yield in the sodium acetate treated rats was slightly less than that obtained in the saline group ($0.1 < p < 0.2$). There was no significant difference in any parameters ($p < 0.05$) indicating that either sodium chloride or sodium acetate could be used as an appropriate control treatment.

(g) Hepatic Microsomal Glucose-6-Phosphatase Activity

Figure 6 shows the hepatic microsomal glucose-6-phosphatase activities found in control animals, as well as rats treated with

TABLE 4 THE CADMIUM AND ZINC CONTENT OF MICROSOMES

| | TIME AFTER TREATMENT | | | |
|---|---|---|---|---|
| | 1 DAY | | 4 DAYS | |
| nanograms cadmium/3 mg microsomal protein | 31<br>37<br>40 | 36 ± 3 | 72<br>58<br>58<br>149 | 84 ± 22 |
| nM cadmium final solution of incubate | 92<br>110<br>119 | 107 ± 8 | 214<br>172<br>172<br>442 | 250 ± 65 |
| nanograms zinc/3 mg microsomal protein | 294<br>304<br>394 | 331 ± 32 | 275<br>252<br>260<br>278 | 266 ± 6 |
| nM zinc final solution of incubate | 1497<br>1550<br>2009 | 1685 ± 163 | 1402<br>1285<br>1326<br>1417 | 1358 ± 31 |

Data shown both as values from individual rats, and as mean ± S.E.M.

TABLE 5 MICROSOMAL PARAMETERS OF CONTROL PREPARATIONS

| | SODIUM CHLORIDE (n = 4) | SODIUM ACETATE (n = 4) |
|---|---|---|
| Aniline-p-hydroxylase [a] | 0.51 ± 0.01 | 0.43 ± 0.02 |
| Aminopyrine-N-demethylase [a] | 6.78 ± 0.05 | 6.63 ± 0.36 |
| Cytochrome P-450 [b] | 1.18 ± 0.02 | 1.13 ± 0.05 |
| Microsomal protein yield [c] | 9.45 ± 0.40 | 8.45 ± 0.38 * |
| Liver weight [d] | 4.53 ± 0.03 | 4.53 ± 0.08 |

a nmoles product formed/mg microsomal protein/minute

b nmoles/mg microsomal protein

c mg/gm liver

d gm/100 gm body weight

* significantly different from control ($p < 0.05$)

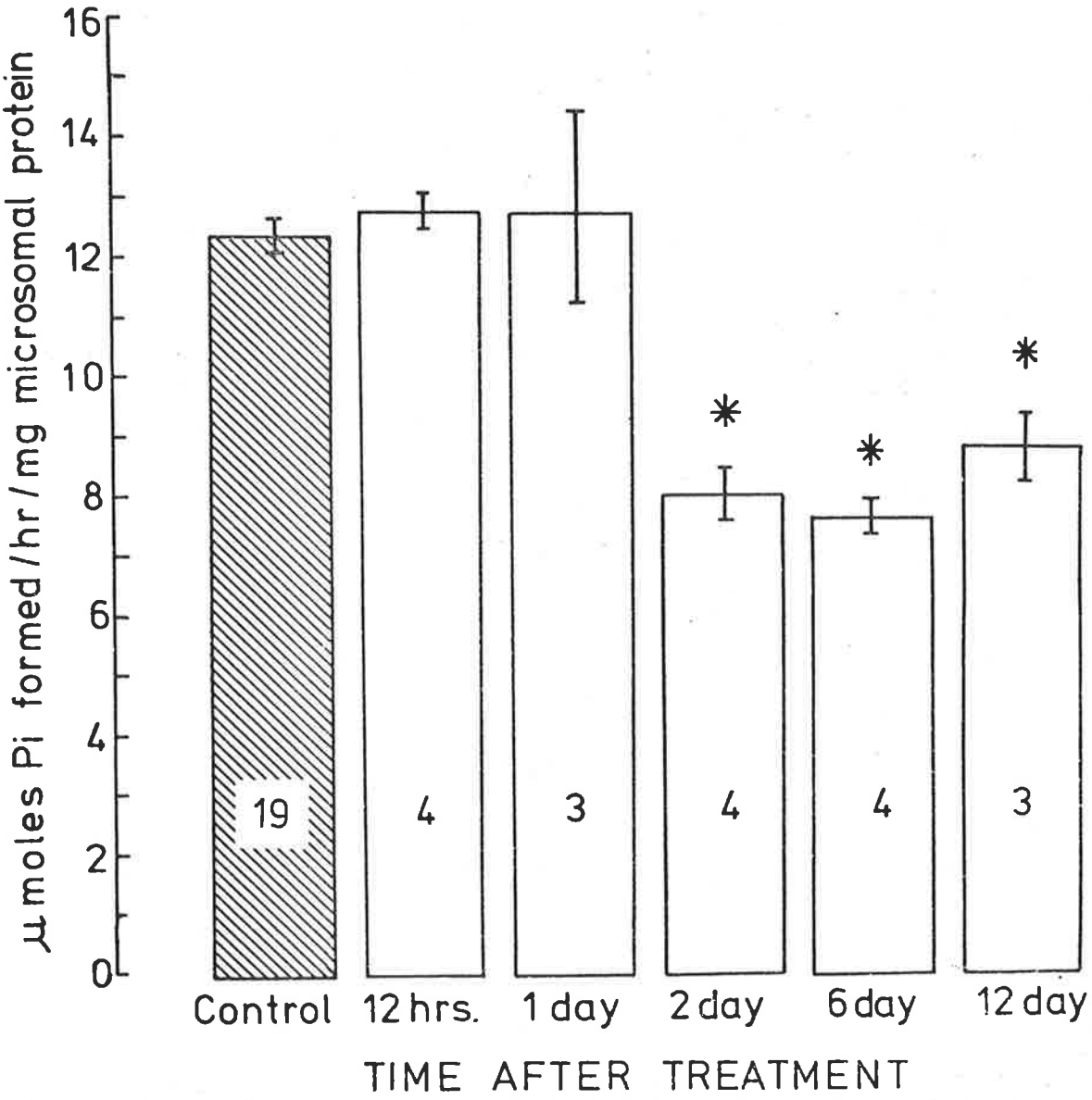


Figure 6. The effects of cadmium on hepatic microsomal glucose-6-phosphatas activity. Number enclosed within columns represent the number of observations.
*Significantly different from pooled controls ($p < 0.05$)

cadmium for 12 hours, 1 day, 2 days, 6 days and 12 days. The data indicates that there was no change in activity relative to the pooled control valves, ($p > 0.6$) during the first 24 hours after the injection of cadmium. From 48 hours and extending to the 12th day, enzyme activity was significantly lower than the control. ($p < 0.001$).

(h) The Effects of Starvation on the Microsomal Drug Metabolizing System

A consistent feature seen in all the cadmium treated rats was a drop in the amount of food they eat each day. By 24 hours it was common to see a fall of about 10 grams in body weight and 20 grams by 48 hours. The intake of food usually resumed by the third or fourth day, but a gain in body weight often did not occur until after the fifth day. Also, the microsomal pellet in these animals was less "sticky" than the controls, probably due to a lower glycogen content. The nutritional status of a rat can effect its drug metabolizing system (Campbell and Hayes (1974)), and so the effects of food deprivation was examined. A starvation period of 48 hours was chosen as it was at this time that the microsomes were maximumly inhibited after a single injection of cadmium (Figure 3).

The results are presented in table 6, and they show that starvation caused an increase in the rate of aniline-p-hydroxylation ($p < 0.001$) and a decrease in aminopyrine N-demethylation ($p < 0.001$), but no change in the amount of cytochrome P-450. ($0.6 < p < 0.7$). The microsomal protein yield per gram of liver also did not change ($0.5 < p < 0.6$) but, as expected, there was a marked stimulation in hepatic microsomal glucose-6-photphatase activity. ($p < 0.001$).

TABLE 6 THE EFFECT OF FOOD-DEPRIVATION (48 Hours) ON HEPATIC MICROSOMES

| | Ad-libitum fed control rats | | Food-deprived rats | |
|---|---|---|---|---|
| Aniline-p-hydroxylase[a] | 0.71 ± 0.02 | (8) | 0.94 ± 0.03 | (8)* |
| Aminopyrine-N- demethylase[a] | 6.39 ± 0.26 | (12) | 4.47 ± 0.28 | (12)* |
| Cytochrome P-450[b] | 0.92 ± 0.05 | (12) | 0.88 ± 0.06 | (12) |
| Glucose-6-phosphatase[c] | 15.79 ± 0.46 | (12) | 29.77 ± 1.44 | (12)* |
| Microsomal protein[d] yield | 11.80 ± 0.50 | (12) | 12.28 ± 0.64 | (12) |
| Liver weight[e] | 4.24 ± 0.08 | (12) | 3.36 ± 0.08 | (12)* |

*significantly different from controls ($p < 0.05$)

a. nmoles product formed/mg microsomal protein/minute

b. nmoles cytochrome P-450/mg microsomal protein

c. nmoles inorganic phsophate released/mgm microsomal protein/hour

d. mg/gm liver weight

e. gm/100 gm body weight

## II CHRONIC STUDIES

Table 7 shows parameters of microsomal enzyme activity in rats which had 100 ppm cadmium in their drinking water for 222 days. In contrast to rats treated with an acute dose of cadmium, there was no evidence of microsomal enzyme dysfunction.

Hepatic and renal cadmium concentrations in rats chronically exposed to cadmium (table 8) were higher than those achieved in the acute cadmium experiments, although zinc levels were not markedly different. The main difference in metal distribution in this experiment was that renal cadmium content was substantially greater than the hepatic content, whereas, the reverse was found after an acute dose (figure 4).

TABLE 7 THE EFFECTS OF CHRONIC CADMIUM INGESTION ON MICROSOMAL PARAMETERS

| | CONTROL (n=4) | CADMIUM INGESTION (n=4) |
|---|---|---|
| Aniline-p-hydroxylase [a] | 0.32 ± 0.01 | 0.32 ± 0.03 |
| Aminopyrine-N-demethylase [a] | 3.49 ± 0.13 | 3.22 ± 0.19 |
| Cytochrome P-450 [b] | 0.89 ± 0.02 | 0.87 ± 0.04 |
| Cytochrome $b_5$ [b] | 0.49 ± 0.01 | 0.46 ± 0.01 |
| Liver weight [c] | 3.25 ± 0.13 | 3.12 ± 0.20 |
| Microsomal protein yield [d] | 11.66 ± 0.66 | 9.68 ± 1.30 |

Rats were maintained for 222 days on drinking water containing 100 ppm cadmium

a nmoles product formed/mg microsomal protein/minute

b nmoles/mg microsomal protein

c gm/100 gm body weight

d mg/gm liver weight

No values differed significantly from controls ($p < 0.1$)

TABLE 8 HEPATIC AND RENAL METAL CONTENTS (μg/gm dry weight)

| | CONTROL RATS (n=4) | CHRONIC CADMIUM INGESTION (n=4) |
|---|---|---|
| CADMIUM | | |
| Liver | a | 155 ± 27 |
| L. Kidney | a | 391 ± 19 |
| ZINC | | |
| Liver | 97 ± 3 | 150 ± 14 |
| L. Kidney | 102 ± 6 | 106 ± 3 |

a The cadmium content of the control rats was below the quantitation threshold of 1μg cadmium/gm dry liver

Values are mean ± S.E.M. of 4 rats

L. Kidney denotes the metal content of the rats' left kidney

## DISCUSSION

The only significant effect of cadmium on cytochrome P-450-dependent drug oxidation was an inhibition observed after acute cadmium administration. These results are therefore consistent with those of Unger and Clausen (1973), Hadley et al (1974) and Yoshida et al (1976) and they are at variance with those of Wagstaff (1973).

Differences between the findings of various investigations may be due to a combination of factors, including the species, enzyme substrates, doses and routes of administration. However, the fact that the metabolic activities of the drug metabolizing system and the cytochrome P-450 content differed each day after cadmium treatment, implies that time should now be an important factor guiding further investigations into cadmium's effect on the system.

The reports by Schnell et al (1974) and Hadley et al (1974) showed that the cadmium-induced potentiation of hexobarbital sleeping time is the result of the inhibition of the metabolism of hexobarbital and that the maximum potentiation of sleeping time occurs on day 2. Statistically, this inhibition was shown to extend through to day 5. These results imply that the time-course of cadmium's effect on hexobarbital sleeping time could be a result of the time-course of cadmium's effect on the drug metabolizing system. The results of this chapter confirm and illustrate this implication of their work for the first time. Later, a report by Yoshida et al (1976), showed that the maximum inhibition of aminopyrine-N-demethylase activity in mouse 9,000 x g supernatant also occurred 48 hours after cadmium injection (3mg/kg), but by 72 hours, the enzyme activity was nearly back to normal.

The cadmium contents in the liver are a pertinent consideration, especially in view of the high content obtained by 24 hours. Hadley et al (1974), showed that cadmium added to microsomes *in vitro* in a concentration range of 0.5 μM-0.5 mM inhibited microsomal metabolism. Although high hepatic levels of cadmium were recorded (figure 4), very little of it was localized within the microsomal fraction. The quantities of cadmium found in microsomes prepared from cadmium-treated rats (table 4) were less than the concentrations found by Hadley et al (1974) and Priestly (unpublished observations) to inhibit metabolic activity *in vitro*. It may therefore be concluded that the inhibitory effect of cadmium on the microsomal enzyme system, is probably an *in vivo* and not an *in vitro* phenomenon.

Despite the large amounts of cadmium retained in the liver and kidney, the chronic administration of the metal did not result in any changes in the hepatic drug metabolizing system. This suggests firstly, that metallothionein may have been synthesized and secondly that, tissue metal content alone is not a significant factor in the aetiology of enzyme inhibition. The possibility that metallothionein acted as a detoxicant is discussed in chapter 2.

The rate of metal intake was lower in the chronically dosed animals compared with the injected group, as it took 222 days to reach a hepatic cadmium content of $155 \pm 27$ μg cadmium/gm dry liver and only 48 hours after a single, 5 mg cadmium acetate/Kg. intraperitoneal dose, to reach $123 \pm 9$ μg cadmium/gm dry liver. The difference in the rate of cadmium accumulation is probably due to the differing bioavailability between oral and parenteral routes of administration. It appears, therefore, that the inhibitory effects of cadmium on the system can be initiated

only if the amount of cadmium presented to the liver, soon after administration, exceeds some "toxic-threshold". The "toxic-threshold" for the rat hepatic drug metabolizing system is not known, but Schnell et al (1974) reported that injected cadmium doses, less than 2.0 mg cadmium/Kg, did not significantly alter rat hexobarbital sleeping time. This dose threshold is consistent with data obtained in our laboratory as well (Hawke et al 1977).

Glucose-6-phosphatase activity was not affected during the first 24 hours, during which time cytochrome P-450 dependent activity declined sharply. This suggests that the integrity of the microsomal membranes were not affected and so the actions of cadmium, at this stage, are direct and relatively specific, for cytochrome P-450. The fall in the activity of glucose-6-phosphatase from 48 hours onwards, suggests cadmium-induced damage to the microsomal membranes which presumably has an inhibitory effect on drug metabolic activity. Consequently, after 48 hours, it appears that cadmium has also an indirect, inhibitory effect on the activity of the microsomal drug metabolizing system.

However, from the 3rd to the 12th day after cadmium, drug metabolic activity returns towards control rates, while glucose-6-phosphatase's activity is markedly inhibited and shows no signs of returning to control levels. Indeed, there is no significant difference between the activities observed on the 2nd, 6th and 12th days after cadmium (figure 6). This indicates that the time-course of microsomal glucose-6-phosphatase activity after cadmium is different to the time-course of cadmium's effect on the drug metabolizing system. It seems therefore, that the effects of cadmium on the drug metabolizing system are due mainly

to the initial, direct actions of cadmium on the system, while the effects resulting from the proposed membrane damage, contribute only minimally, if at all, to the inhibition in the rate of drug metabolism.

The data from the experiments with complete food deprivation, indicate that reduced food intake alone could not have been responsible for the inhibitory effects on metabolism. A similar conclusion was reported by Hadley et al (1974). It was stated in the Results section, (part h), that the cadmium-injected rats eat a little food (estimated at 5-10% of control intake) and so the data obtained from the food deprived rats represents an extreme case. Figure 6 indicates that there was no fall in glucose-6-phosphatase activity during the first 48 hours after cadmium and it was concluded that membrane damage did not occur during this time (Feuer et al 1965). However, it is possible that damage to the endoplasmic reticulum did occur but was not detected because of the tendency for glucose-6-phosphatase activity to increase under conditions of reduced food intake. It follows, therefore, that the decline phase could result from both the indirect effects of microsomal membrane damage plus the direct effect of cadmium on the microsomal mixed-function oxidases.

The data obtained in the acute and chronic studies do not provide a conclusive answer to cadmium's mechanism of action, however, they elucidate much information. The fact that the initial acute effects seen are a result of a direct, *in vivo* action of cadmium on the microsomal constituents and together with the fact that the metabolism of type I and II substrates follow a similar pattern to the level of cytochrome P-450, suggests strongly that the initial inhibition of metabolism could be a result of the fall in the level of cytochrome P-450.

Further, the apparent reversibility of the metabolic inhibition after day 2, parallels the reversibility of the prevailing levels of cytochrome P-450 and adds further support to this theory.

# CHAPTER TWO
# METALLOTHIONEIN

## INTRODUCTION

The time-course of the inhibition of the drug metabolizing system induced by cadmium, was discussed in chapter one. The observation that the inhibition reversed after the 48th hour, following the injection of cadmium, indicated that cadmium may have ceased to have any effect on cytochrome P-450. Further, the failure to induce a toxic reaction following the chronic administration of the metal and the continued high level of cadmium in the liver, merited investigation. If cadmium was bound to metallothionein then firstly, did this protein play a role in reversing the acute inhibitory effects of cadmium and secondly, did it protect the chronically treated animals? This chapter attempts to answer this.

The possibility that a detoxification mechanism operates within the liver was one of the suggestions put forward by Pulido (1966) as a role for metallothionein and he cited as supporting evidence, the work of Piscator (1964). Since then other papers have appeared claiming a similar role for metallothionein and these have been discussed in the General Introduction.

If the effects of cadmium on microsomal enzymes are related to "free" cadmium concentration; i.e. simple sequestration of cadmium is the mechanism of detoxification, then one might expect that the recovery from the inhibitory effects on drug metabolism should follow a similar time-course to the changes in metallothionein content in the liver. That is, the level of metallothionein should increase after the injection of cadmium, with the maximum content occurring on or after the 48th hour, to coincide with the beginning of the return to normal levels of cytochrome P-450 and the metabolic

activity of the enzyme system.

To investigate this, the hepatic metallothionein content was estimated in rats ½, 1, 2, 6 and 12 days after administration of the same dose of cadmium acetate as that used for the metabolic studies.

## 2. Methods

### 2a. Preparation of Metallothionein

Two or three rats, dosed with 5 mg cadmium acetate/Kg i.p. were killed at the times indicated and their livers perfused with ice-cold saline via the hepatic vein, excised, blotted dry and weighed. The livers were homogenized in 20 mls of cold 0.1M ammonium acetate buffer pH7.5 using a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 10,000 x g for 15 minutes and the supernatants centrifuged at 105,000 x g for one hour. The resultant supernatants were combined and clarified by filtering through Whatman No. 542 filter paper (see Appendix A). The filtrate was assayed for protein by the method of Lowry et al (1951) and 400 mgms of protein, (see Appendix B), in a final volume of 15 mls, was applied to a Sephadex G-75 superfine column (85 x 5) cm with a flow rate of 0.91 mls/minute which was maintained by an auto-analyzer peristaltic pump using grey shouldered pump tubing with an I.D. of 0.051 inches. The eluting buffer was 0.1M ammonium acetate pH7.5. The eluate was continuously monitered at 254 nm by a Uvicord II spectrophotometer and 15 minute fractions were collected. The spectrophotometer was connected to a 2 channel Rikadenki recorder; one channel tracing the 254 nm absorbance/transmittance output, while the second channel was arranged

to mark the chart paper at the change of every fraction tube. The absorbance at 254 nm was used to monitor the eluates because cadmium-thionein absorbs strongly near this wavelength. A peak found at about Ve/Vo = 2, with an estimated molecular weight of 10,500 daltons (see Appendix C) and which was not present in the control livers, was initially accepted as being that of cadmium-thionein. Subsequent assays on the fractions provided evidence confirming this. The criterion used to identify cadmium-thionein was based on the properties described by Kagi and Vallee (1960) for the equine renal metallothionein. These properties have been discussed in the General Introduction.

## 2b. Assays

The eluates which were thought to contain metallothionein were each assayed for cadmium and zinc, directly, by atomic absorption spectrophotometry. The cadmium and zinc content of each metallothionein peak was quantitated by summing the cadmium and zinc content of each fraction comprising the peak. The metal content of each tube is presented in figures 7-12 while the total metal content of each cadmium-thionein peak is listed in table 9, columns 5 and 8. The 250 nm and 280 nm absorbances of each of these tubes were read on a Unicam SP1800 split-beam spectrophotometer and the values plotted as shown in figures 7-12. Cadmium-thionein was quantitated by weighing a cut out of the peak defined by the plotted 250 nm points. The value of the areas thus obtained are presented in table 9 under column 1 and are expressed in arbitary units.

2.7 mls of eluate from each of these fractions was placed in each of 2 cuvettes and the spectrophotometer was adjusted to zero absorbance if necessary. 10-20 µl of cadmium acetate (2.5 mg salt/ml) was added to the sample cuvette, and ammonium acetate

buffer to the reference cuvette, each was mixed thoroughly and the absorbance reading noted. An extra 10 µl was added to ensure that there was no further increase in O.D. thus establishing that the metallothionein was fully saturated with cadmium. The resultant increase in absorbance due to the excess cadmium was plotted against the eluted fractions and was quantitated by weighing as before. The values obtained are presented in table 9, under column 2. Cadmium binds by displacing zinc from its binding site (Kagi and Vallee 1961), therefore, the values obtained are taken to be an estimate of the zinc-thionein content for each time of treatment.

## 3. Results

Figures 7-12 enable comparisons to be made between each eluted fraction comprising each cadmium-thionein peak. They show clearly that the tube with the highest cadmium concentration had the largest absorbance at 250 nm, in keeping with the properties reported by Kagi and Vallee (1960) for metallothionein. Table 9 presents relevant data obtained from the whole of each cadmium-thionein peak. They enable comparisons to be made between the time after treatment at which cadmium-thionein was examined.

### 3a. Metal contents

(i) ZINC (columns 7, 8, 9 of table 9).

In the control animals, a small amount of zinc was detected in the eluates at Ve/Vo=2 (6 µg zinc). After treatment with cadmium, the percentage of zinc found in the metallothionein peak increased almost

13-fold to an average of 57.6% of the total zinc. The remainder was bound to other proteins indicating that zinc was widely distributed in the eluates. 12 hours after injection, an additional 50 µg zinc was present in the 400 mg sample of protein applied to the column. An additional 112 µg of zinc appeared in the metallothionein peak, indicating that with the *de novo* synthesis of thionein protein, a transfer of zinc occurred from other proteins in the supernatant to thionein and also that the uptake of zinc by the liver had increased. The level of zinc in metallothionein increased until a plateau was reached at day 6. (columns 7 and 8).

(ii) CADMIUM (columns 4, 5 and 6 of table 9)

The level of cadmium in the eluates from control rats was found to be so small as not to be detectable by the instrument in use. In the treated animals the levels found were markedly increased. Column 5 shows that the level of cadmium in metallothionein from 12 hours through to 12 days was of the same order. This is reflected in the data in columns 4 and 6, where it was found that there was always approximately 120 µg cadmium applied to the column and about 90% of this was recovered bound to metallothionein.

3b. Metallothionein contents (columns 1, 2 and 3 of table 9)

(Special note to clarify the data of table 9)

Figures 8-12 show that a contaminating protein was present in the first 5 - 6 tubes of each cadmium-thionein peak. The contaminating protein always peaked in the third fraction (41 mls) before the peak of the cadmium-thionein protein. The result of this is that the beginning of the cadmium-thionein peak is ill-defined as the absorbance at 250 nm

due to cadmium-thionein is overwhelmed by the much higher 250 nm absorbance (part of the shoulder of the 280 nm peak) of the contaminating protein. Therefore, the data of table 9 cannot be regarded as being absolute; table 9 however, is of value, because it can show the trends of the various parameters over the course of the experiment.

The detection of metallothionein depends upon the use of indirect methods. The Lowry method for protein estimation (Lowry et al 1951) is unsuitable as it depends upon the presence of phenylalanine and tyrosine, both of which are absent or in extremely small quantities in metallothionein. The absorptive behaviour of metallothionein at 250 nm and 280 nm is however, highly specific. Zinc-thionein does not have an absorption maxima at 250 nm, but is reported to absorb strongly at 215 nm (Kagi and Vallee 1961).

In the control rats, no cadmium-thionein could be detected as there was an absence of both cadmium and a distinct absorption at 250 nm in the eluted fractions which would normally contain metallothionein. The addition of cadmium to the eluates produced a small increase in optical density. Figure 7 indicates that this forms a peak in an identical manner to those observed in the treated groups in that the maximum absorption appeared in the third fraction after the 280 nm absorbance peak. This suggests that a thionein is present in the control animals, but in a small quantity and is probably zinc-thionein. The pattern of zinc levels in these same fractions, support most strongly the proposition that it is zinc-thionein. The zinc-thionein content, as estimated by the increased 250 nm absorbance after cadmium addition to the eluates, is listed in table 9, column 2 and it is evident that this increased with time.

The trend in the values are that the levels at 6 and 12 days are about the same, but are a little greater than at any time before.

Column 1, table 9, lists the cadmium-thionein content in the treated rats and shows that the amount found at 12 hours did not change during the remainder of the observation period.

The method used to quantitate the level of metallothionein (cadmium-thionein) depends upon the presence of cadmium and so the relative amounts of cadmium found at each time after treatment, should be the same as the relative amounts of metallothionein found. This was indeed the case, however, the important implication is that the cadmium present in the metallothionein peak is bound specifically to thionein and not to other 10,000 M.W. proteins.

Column 3, which is the sum of columns 1 and 2 at each time of treatment, reveals the total metallothionein content. This data illustrate that throughout the observation period, there was a continuing production of thionein with the greatest amount appearing on the 6th day after treatment, which is in agreement with the results of Chen et al (1975).

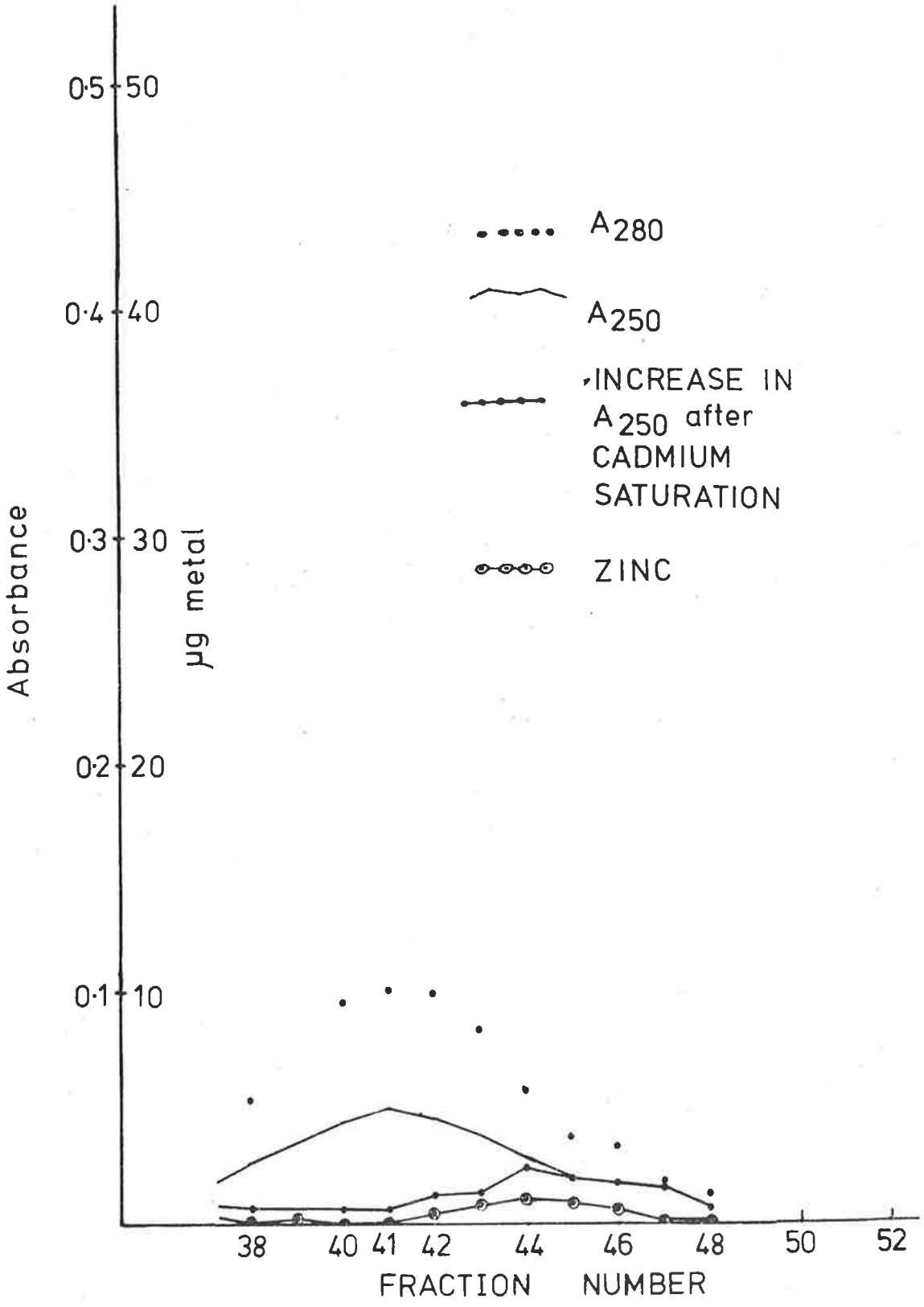


Figure 7. Control

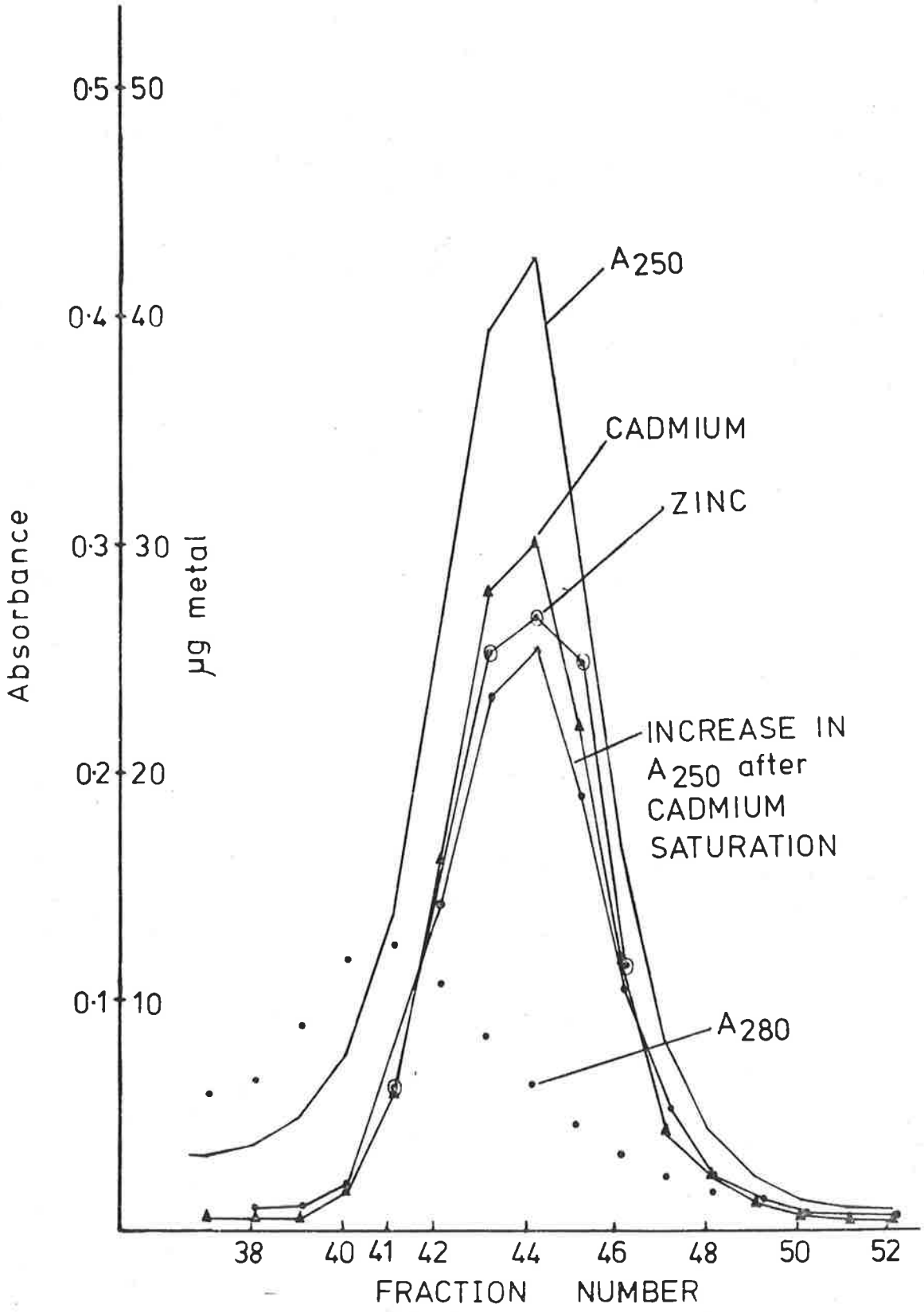


Figure 8. 12 hours after cadmium (5mg/Kg i.p. cadmium acetate)

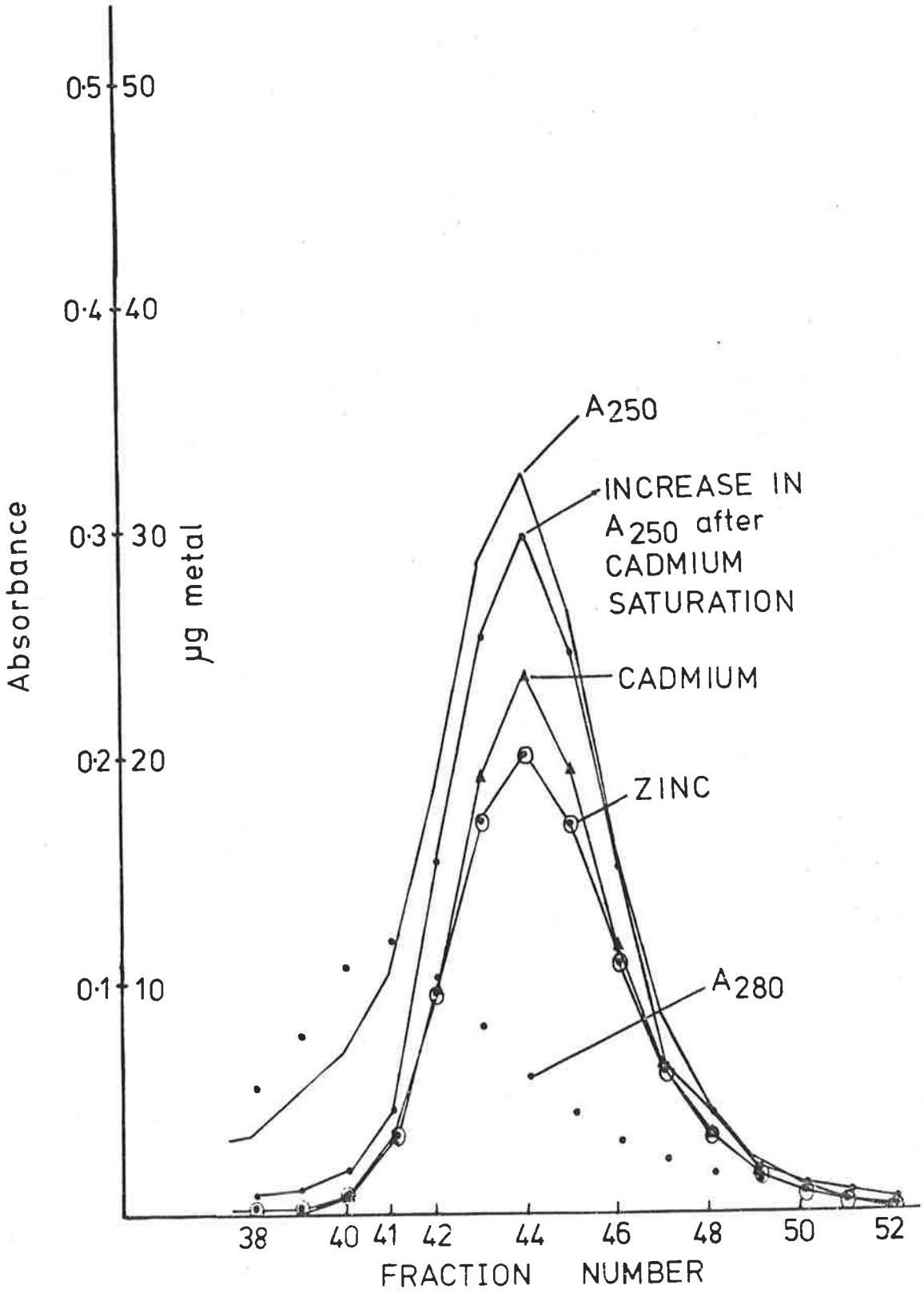


Figure 9. 1 day after cadmium (5mg/Kg i.p. cadmium acetate)

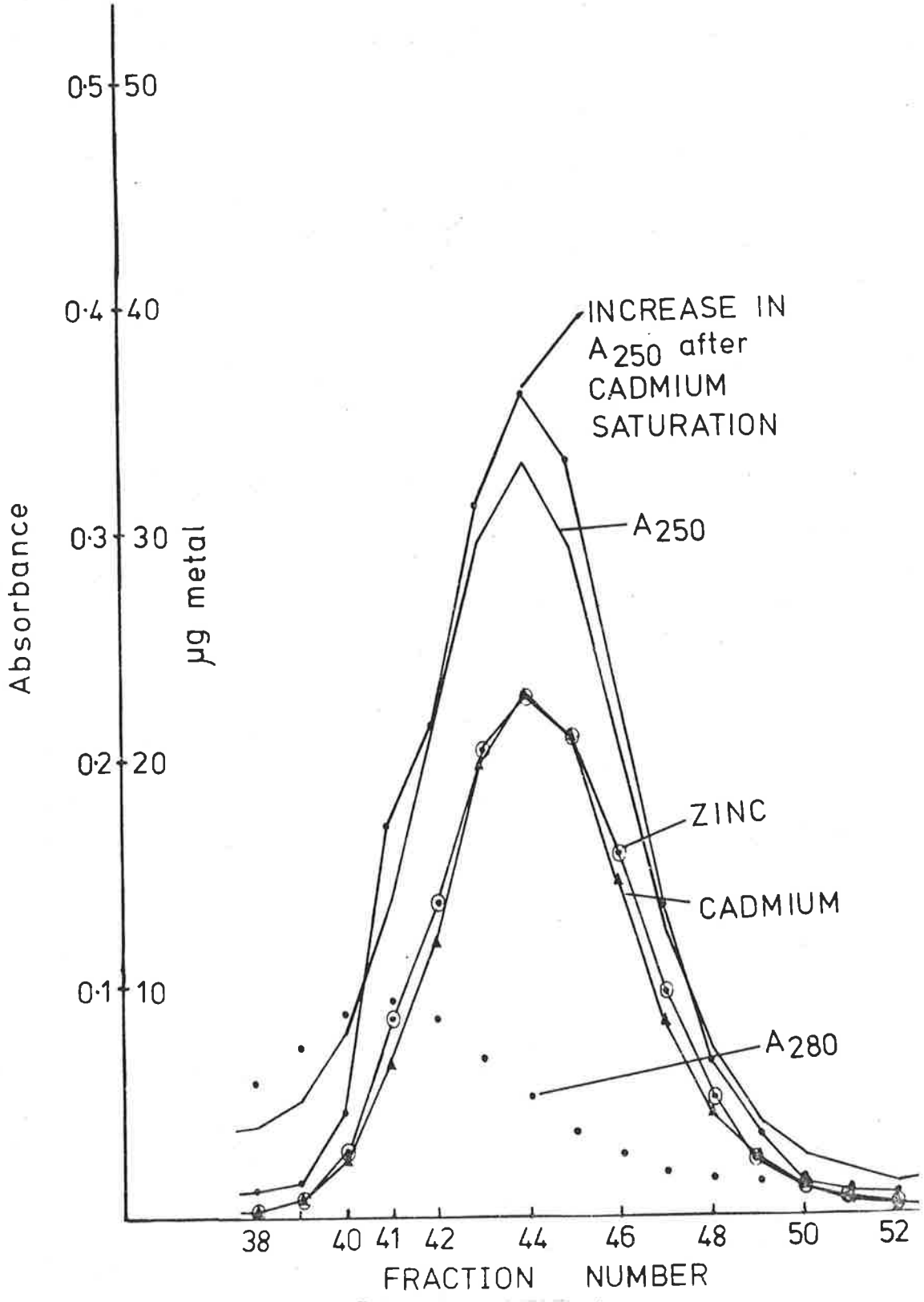


Figure 10. 2 days after cadmium (5mg/Kg i.p. cadmium acetate)

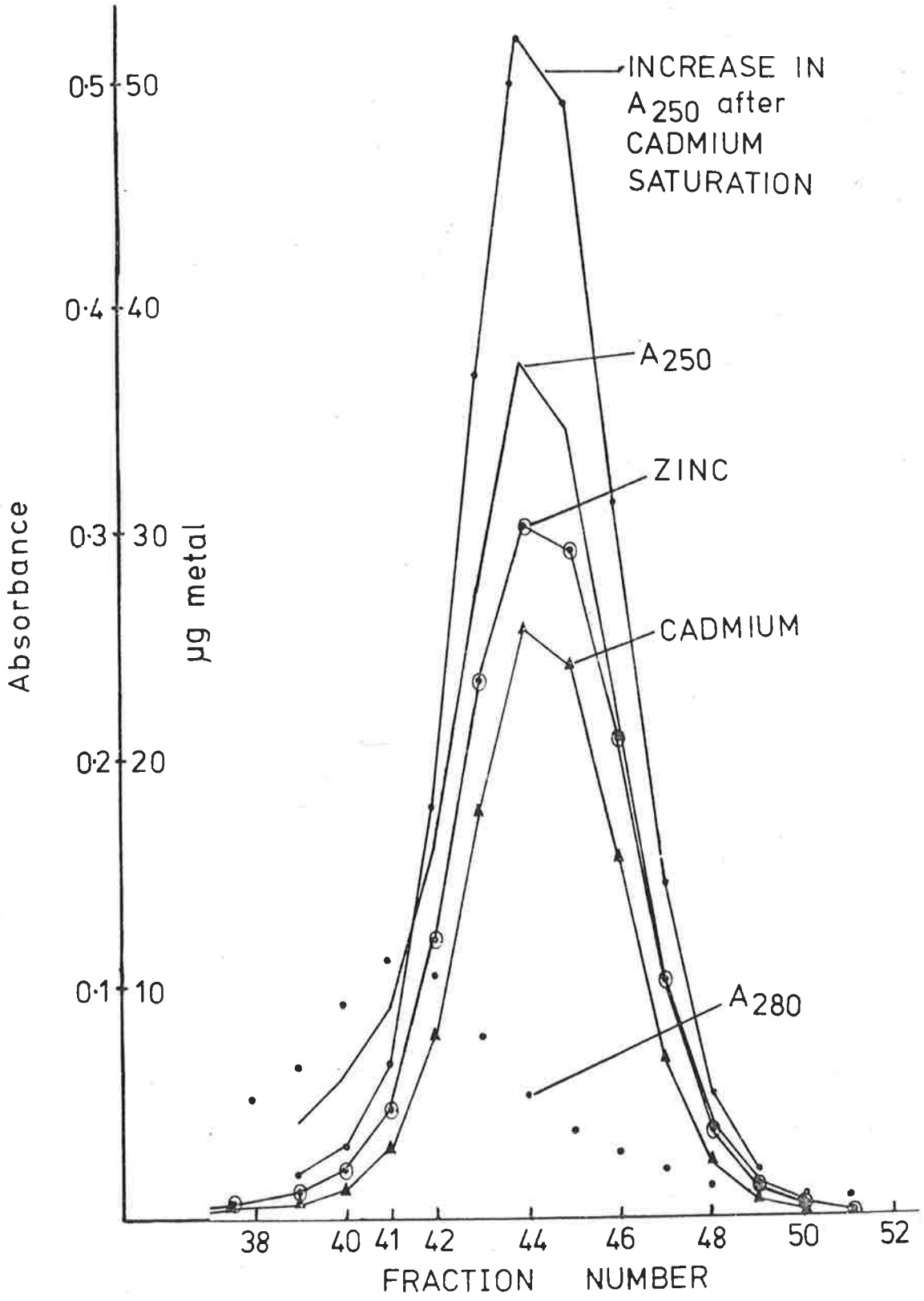


Figure 11. 6 days after cadmium (5mg/Kg i.p. cadmium acetate)

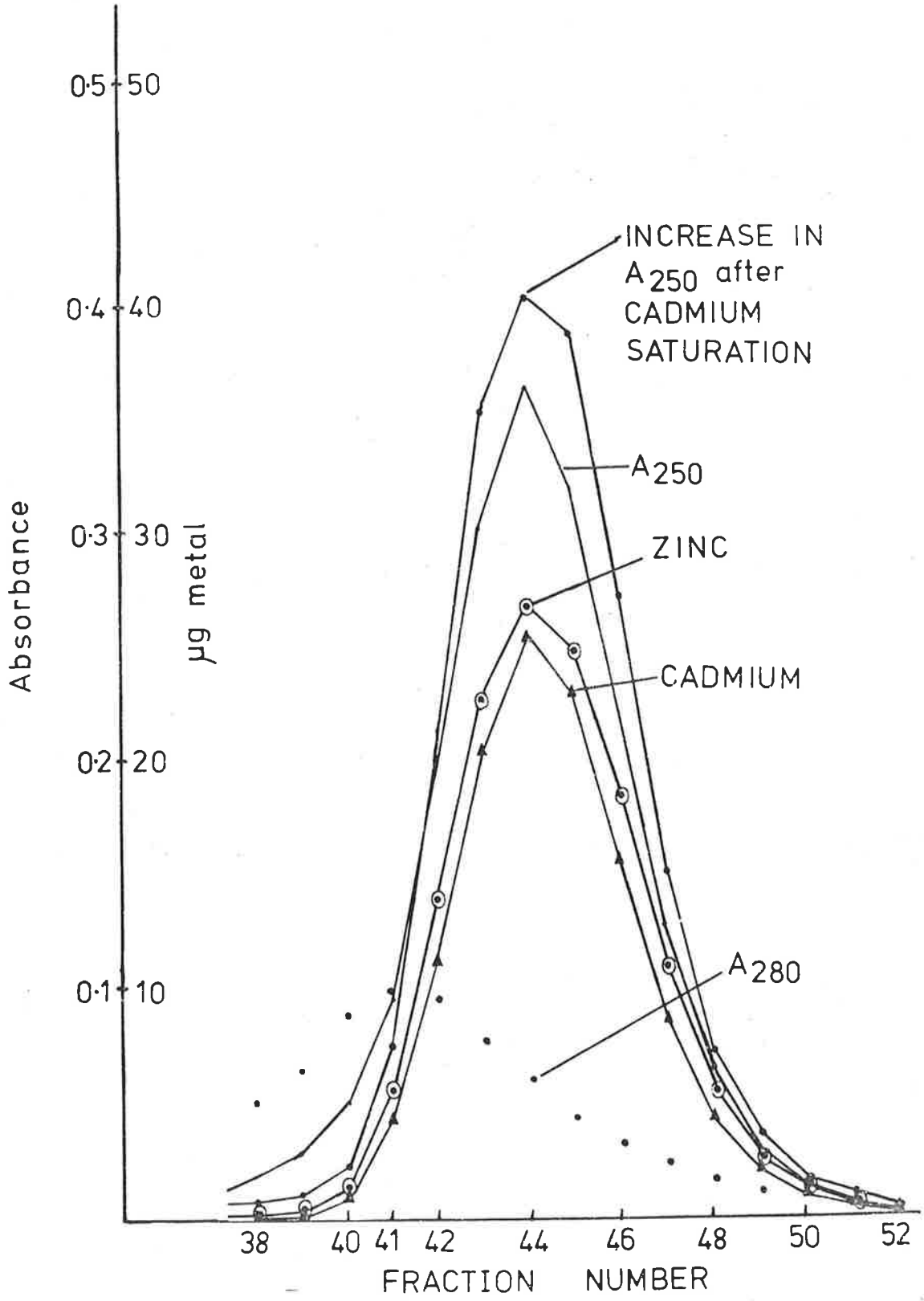


Figure 12. 12 days after cadmium (5mg/Kg i.p. cadmium acetate)

TABLE 9 METALLOTHIONEIN CONTENT AFTER CADMIUM ACETATE INJECTION 5 mg/Kg i.p.

| COLUMN NO | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| PARAMETERS / TIME AFTER CADMIUM | CADMIUM-THIONEIN CONTENT (Area under curve defined by $A_{250}$) | ZINC-THIONEIN CONTENT (Area increase after cadmium saturation) | TOTAL METALLO-THIONEIN CONTENT (Sum of columns (1) & (2) | CADMIUM CONTENT APPLIED TO COLUMN (µg CADMIUM) | CADMIUM CONTENT OF CADMIUM-THIONEIN PEAK (µg CADMIUM) | PERCENT OF CADMIUM APPLIED FOUND IN PEAK (%) | ZINC APPLIED TO COLUMN (µg ZINC) | ZINC CONTENT OF CADMIUM-THIONEIN PEAK (µg ZINC) | PERCENT OF ZINC APPLIED FOUND IN PEAK (%) |
| Control | N.D.[a] | 300 | 300 | < 0.15[a] | < 2.0[a] | N.D.[a] | 124 | 6 | 4.8 |
| 12 Hours | 7554 | 4743 | 12297 | 129 | 122 | 94.6 | 174 | 118 | 67.8 |
| 1 Day | 6528 | 5585 | 12113 | 111 | 99 | 89.2 | 189 | 92 | 48.7 |
| 2 Days | 7031 | 8650 | 15681 | 124 | 115 | 92.7 | 224 | 124 | 55.4 |
| 6 Days | 7345 | 10094 | 17439 | 111 | 107 | 96.4 | 239 | 142 | 59.4 |
| 12 Days | 7752 | 9204 | 16956 | 128 | 117 | 91.4 | 239 | 135 | 56.5 |

The lower limit of sensitivity was 10 nanogram cadmium/ml above blank levels using the most sensitive wavelength of 228.8 nm and so, the eluates would have contained 0-10 ngm cadmium/ml. Since 15 mls of supernatant was fractionated, then the total cadmium content was less than 0.15 µg.

On average, the metallothionein peak was collected in 15 tubes of 13.6 mls each. Therefore, the control rats would have had no more than 2.0 µg cadmium in their comparable fractions.

## DISCUSSION

It is possible to derive the following conclusions from the data shown in table 9, columns 1, 2 and 8.

After the injection of cadmium, thionein is produced and together with the zinc-thionein normally present, bind some 90% of the cadmium in the soluble cytoplasm. The sequestration is completed by the 12th hour and the cadmium-thionein content thus produced does not increase thereafter. However, the production of thionein continues and this incorporates zinc into its binding sites. This last point has been subsequently confirmed by Leber and Miya (1976), whose experimental evidence showed that both cadmium-thionein and non-cadmium bound thionein had been produced in their cadmium treated rats.

It is unlikely that the newly synthesized metallothionein was responsible for reversing the inhibitory effects of cadmium on metabolism at 48 hours, because the cadmium-thionein content was already maximal at 12 hours, at which time the activity of the microsomal enzyme system continued in its decline phase. Therefore, the act of sequestering cadmium, does not mark the point where a reverse in its effects begin. Instead, the reversal may be the result of normal, healing mechanisms coming into operation. It is probable that the sequestration of cadmium prevents the induction of further toxic effects.

The explanation consistent with the relative time-course of cytochrome P-450 dysfunction and metallothionein synthesis is that, once cadmium initiates its toxic effects, then these subsequently

progress without further requirement for cadmium. It seems then that the initiation of the effects of cadmium on metabolism precedes the induction of thionein. Therefore, an examination of the time-course of metallothionein production should indicate how soon after injection do cadmium's toxic actions begin. A number of workers have published papers which provide such information, many of which were published after the completion of these experiments.

Cempel and Webb (1976) followed the production of metallothionein from 1-48 hours after an intravenous injection of 1.6 mg cadmium/kg in 10 week old rats. They detected metallothionein by assaying their Sephadex eluates for cadmium and assumed that metallothionein appeared at Ve/Vo = 2. From their data, they reported that the uptake of cadmium by the liver was complete by 1 hour after administration. At this stage, in the male rats, the cadmium was bound to high molecular weight proteins and there was no metallothionein detected. They did not find metallothionein until the 4th hour which coincided with the data reported by Squibb and Cousins (1974), who injected 4.5 µmoles cadmium intraperitoneally and used $^{3}$H-cysteine as an index of protein synthesis. They found that it took 2-3 hours for cadmium to induce the transcription and to begin translation, of the specific mRNA for thionein. Once produced, a rapid transfer of cadmium from the high molecular weight proteins to the nascent thionein began and the formation of metallothionein covered the 4th to the 8th hour, when production was complete. Sabbioni and Marafante (1975) reported supporting evidence when they found that in rats, injected with 1.0 mg cadmium/Kg i.p., the synthesis of metallothionein was incomplete at 6 hours but was complete by 12 hours. Shaikh and Lucis (1971) reported that the incorporation of $^{14}$C-cysteine into their cadmium

binding protein increased continuously from 5 to 24 hours after a single subcutaneous dose of cadmium chloride (0.03 mM/Kg).

Bryan and Hidalgo (1976) examined the association of cadmium with the nucleus. Their information expands the data reported above. They found that not only did cadmium bind to the high molecular weight proteins, but it also entered the nucleus and bound firmly to nuclear material. The transcription of the mRNA for thionein began after a lag phase of about one hour and the cadmium which transferred to the thionein protein came from both the high molecular weight proteins and from the nucleus.

In contrast, Nordberg et al (1971) reported a lag phase of 24 hours between the injection of cadmium (3.0 mg cadmium/Kg s.c.) and the onset of synthesis of metallothionein. Webb (1975) however, regards this as being due to Nordberg's high dose of cadmium which can inhibit protein synthesis in the liver.

Despite the different doses of cadmium and methods of quantitating metallothionein used in all of these studies, a sequence of events defining the synthesis of metallothionein can be compiled from the data. It is clear that in the first hour after injection, cadmium is taken up by the liver. A lag phase of perhaps 1 - 2 hours is followed by the beginning of transcription of mRNA and metallothicnein appears at about the 4th hour. Cadmium-thionein production continues until it is completed by the 8th hour. Squibb et al (1977) examined the time-course of zinc-induced synthesis of thionein and their data showed that it was similar to the events seen with cadmium. Consequently, it appears that the initiation of cadmium-induced changes in

microsomal enzyme activity occurs during the first 1-2 hours after injection.

The discussion so far has rested on the premise that thionein is a cadmium detoxicant. This was not observed in the experiments presented here and it was argued that this was principally because the rate of thionein synthesis did not exceed the rate of development of the toxic actions of cadmium. It follows therefore, that with the formation of more binding sites after a single dose of cadmium, as seen by the formation of zinc-thionein, the animals should be able to tolerate second and subsequent doses of cadmium with less adverse reaction. This has been the argument behind the many papers which have examined the role of metallothionein and a short resumé of these papers will be given. However, the simple sequestration of cadmium has not been accepted by all, and exceptions have also been noted. This evidence will also be included.

The recent investigations into the protective role of metallothionein has been based on inducing metallothionein with either zinc or cadmium several hours before a larger challenge dose of cadmium is administered. The protection afforded by the induced metallothionein against the challenge dose(s), has been substantiated by reports that it has prevented testicular damage (e.g. Parizek 1957, 1960, Gun & Gould 1966), decreased cadmium-induced mortality, in male rats (Leber and Miya 1966, Yoshikawa 1970), in female rats, (Webb 1972) and inhibited cadmium-induced lesions in sensory ganglia (Gabbiani et al 1967).

Leber and Miya (1976) and Probst et al (1977) showed that the

degree of protection, as illustrated by reduced mortality and a fall in LD50s respectively, was related to the amount of pre-inducing cadmium injected. The dose presumably influences how much thionein is synthesized and what proportion of this has cadmium bound to it.

Protection against the effects of cadmium requires its quick immobilization, perhaps by exchange with zinc at binding sites on preinduced metallothionein. This exchange has been shown to occur both *in vivo* and *in vitro* (Suzuki and Yoshikawa 1976, Leber and Miya 1976). However, an alternative hypothesis is favoured by Webb (1972). He suggested that the exchange of zinc for cadmium was a transitory and minor pathway, while protection was afforded via the preinducing levels of cadmium or zinc "priming" the thionein synthesizing system. That is, the inducing dose of cadmium or zinc gives the liver a capacity to synthesize metallothionein at a much greater rate and with a minimal lag phase. This mechanism is also supported by the published data of Squibb et al (1977) and Bryan and Hidalgo (1976). Furthermore, Stoll et al (1976) and Weser and Hubner (1970) showed that, *in vivo*, cadmium and zinc could increase the rate of mRNA synthesis as well as RNA-directed amino-acid incorporation. Squibb et al (1977) reported that the poly-(A)-containing mRNA for thionein was labile, so, the extent of protection due to preinduction, may be related to either the life of the messenger or, whether it is continually synthesized and/or, for how long the ability lasts to derepress the translation of the messenger.

Chen et al (1977) reported that after the induction of zinc-thionein with zinc, the zinc content decreased 3 days after the treatment ceased. This means that the translation of the messenger for thionein

is repressed in the manner described by Squibb et al (1977) and since the messenger is labile, the mRNA content can decrease with the absence of further stimulus. This may explain the 3 day limit of protection against cadmium-induced mortality (Yoshikawa (1970) and Webb and Verschoyle (1976)).

The situation with cadmium therefore, can be that transcription may not be affected and proceeds quickly upon the administration of a second dose of cadmium but translation of the preformed mRNA, which will provide the immediate *de novo* binding sites for cadmium, is delayed. During this delay , mRNA is derepressed, and despite the subsequent incorporation of cadmium into thionein, the toxic reactions leading to death occur.

Therefore, protection is attained when there is sufficient messenger to be translated immediately in response to the second and subsequent doses of cadmium and to bind cadmium quickly, before the toxic reactions can begin. This process occurs concomittantly with the incorporation of cadmium into preformed thionein, presumably zinc-thionein.

There are however, doubts in the theory for a protective role for metallothionein. The protective effects of a previous dose of metal against cadmium-induced mortality last for only 3 days. Although this has been used above, to support the hypothesis for a labile "priming" mechanism, Webb & Verschoyle (1976) have themselves argued against this hypothesis on the grounds that the increased capacity to synthesize metallothionein persists for 10 days after injection of cadmium (Webb 1976). Shaikh & Crispin Smith (1976) have reported that this increased

capacity may be maintained for at least 3 weeks. Also, Yoshikawa (1970) reported that there are metals such as indium and manganese, which protect against cadmium-induced mortality, but do not induce the synthesis of metallothionein.

In summary, there is evidence supporting both mechanisms for cadmium detoxification. It is likely that both cadmium-zinc exchange on preinduced metallothionein and priming of the synthesizing system play a role, with the degree of protection afforded by the pretreatment, being a function of the total sequestering capacity of these two mechanisms. Furthermore, the rapidity with which cadmium may be sequestered is an important factor in the protective mechanism. The importance lies in keeping the unbound cadmium below the "toxic threshold". In instances where the challenge dose overwhelms the total chelating capacity of the liver, then it may still be possible for a toxic reaction to occur. Such cases could have occurred in the data presented by Webb and Verschoyle (1976) where the zinc-thionein induced by starvation had no effect on the LD 50's in female rats. Starvation produced a very small amount of zinc-thionein (3.8 µg Zn/gm wet wt tissue) and so its capacity for cadmium could have been limited. Also, since the zinc-thionein synthesis was not via an earlier encounter with cadmium or zinc, then there would have been a lag phase before further thionein was synthesized.

The conclusions of this chapter are supported by the findings from the chronic administration of cadmium (see Chapter 1). This data showed that despite the continued intake of the metal and also that the hepatic and renal cadmium contents exceeded the values obtained in the injected group of animals, no inhibition (or

stimulation c.f. Wagstaff 1973) of the drug metabolizing systems was found. This phenomenon can now be explained on the basis of the rate of sequestration by metallothionein.

The amount of cadmium presented to the liver after the initial drinking of water containing cadmium, may not have been sufficient to induce any effects on the drug metabolizing system, but it probably initiated the synthesis of thionein. Subsequently, after each intake of cadmium, there would have been binding sites available to sequester the cadmium and hence no inhibition or stimulation of the enzyme system could have occurred. The behaviour of thionein therefore, was to bind and store the cadmium, thereby preventing the free ionic form from accumulating to an amount capable of inducing a toxic reaction.

It is concluded therfore, that the major limitation to cadmium's bioavailability is the sequestering action of thionein and that it is the factors which control thionein synthesis which control the capacity for protection.

The theory, while based on findings on work done with zinc and thionein synthesis, could be applicable to cadmium, as the two metals induce the synthesis of a thionein with similar properties and with a similar time-course of production.

# CHAPTER THREE
# HAEM METABOLISM

## INTRODUCTION

In Chapter 1, it was postulated that the reduced rate with which both type I and II substrates were metabolized was a result of the decreased cytochrome P-450 content. An attempt is made in this chapter to define cadmium's mechanism of action. To undertake such an investigation requires a more detailed examination of the decline phase of the microsomal enzyme system's activity and so the first 48 hours after the injection of cadmium is of interest.

A fall in the level of cytochrome P-450 can be due to the following possibilities.

Cadmium could (1) inhibit the biosynthesis of haem.

(2) stimulate the catabolism of haem.

(3) inhibit the biosynthesis of cytochrome P-450-apoprotein.

(4) stimulate the catabolism of cytochrome P-450-apoprotein.

(5) disrupt the interaction of the haem and apoprotein moiety in cytochrome P-450 already formed.

(6) prevent the formation of nascent cytochrome P-450 by interfering with the combination of haem and the apoprotein moiety.

It was not possible to examine all of these possibilities with the facilities available, but several of them appear to be less likely than others. For example, if the mechanism involves changes in the amount of the apo-protein, then one might have expected to see a significant difference in the microsomal protein yield between control

and cadmium treated rats. This was not found (Chapter 1, I part b). A change in the turnover of haem is more likely and extensive work on heavy metal interactions with haem oxygenase by Maines and Kappas (1976) and De Matteis and Unseld (1976) has emphasised the importance of this factor. In order to investigate the relationship between haem turnover and the cadmium-induced impairment of microsomal enzyme activity, the time-course of the cadmium-induced decline in cytochrome P-450 was studied in relation to changes in enzymes involved in the biosynthesis and degradation of haem.

The experimental protocol used, was to assay the activities of δ-aminolaevulinic acid synthetase at 1, 2, 22 and 46 hours and haem oxygenase at 1, 2, 12, 22 32 and 46 hours after the injection of 5 mg cadmium acetate/Kg in male rats, as these enzymes catalyze the rate-limiting steps in the biosynthesis and catabolism of haem, respectively. δ-aminolaevulinic acid dehydratase was also assayed at 1, 2, 22 and 46 hours after injection. At the times of 1, 2, 12, 22, 32 and 46 hours after cadmium, the amount of cytochrome P-450 $b_5$ and total microsomal haem were estimated.

As in the previous chapters, hepatic cadmium and zinc contents were estimated to provide an insight into the accumulation of these two metals over the first two days after the injection of cadmium.

METHODS

(a) Haem

The haem content of microsomes was estimated by the difference spectrum of the oxidized/reduced pyridine-haemochromogen between 441-457 nm using an extinction coefficient of 20.7 $mM^{-1}$ $cm^{-1}$ as described by Falk (1964).

1 ml of microsomes was added to 3.5 ml of water. 1 ml pyridine and 0.5 ml 1N sodium hydroxide were added consecutively and gently mixed in a capped tube. 3 mls of the resultant haemochromogen was pipetted into each of 2 matched quartz cuvettes, the reference cuvette containing 50 µl of 3mM potassium ferricyanide while the sample cuvette contained a few crystals of sodium dithionite. Complete reduction was assumed when no increase in optical density occurred with additional dithionite.

(b) Evaluation of hepatic microsomal haem oxygenase activity *in vitro*

Haem oxygenase was assayed by the method described by Maines and Kappas (1975) with two minor modifications.

These were (1) The NADPH generating system used isocitric dehydrogenase (1.65 units of activity) and isocitric acid (48 mM) in place of glucose-6-phosphate/glucose-6-phosphate dehydrogenase (3 units activity/6ml incubate)

(2) Individual reference and sample incubates were prepared instead of dividing a common 6 ml incubate.

(c) *In vitro* determination of δ-aminolaevulinic acid dehydratase activity

Rats were decapitated and their livers perfused *in situ* with ice-cold saline, then rapidly excised and weighed. They were then homogenized in 0.1 M phosphate buffer pH6.8 and 10,000g supernatants prepared and adjusted so that 1 ml contained 250 mg liver.

Incubations were carried out under nitrogen in Packard scintillation vials, sealed with rubber stoppers through which 3 syringe needles were passed. These provided for a nitrogen inlet and outlet and for the introduction of substrate. Supernatant (0.75 ml) and 0.1M phosphate buffer pH 6.8 (0.75 ml) were pipetted into the vials, which were then sealed and gassed with nitrogen for 1 hour to exclude oxygen. The substrate, 0.5 ml of 30mM δ-aminolaevulinic acid, neutralized to pH6.8 with 1N sodium hydroxide was added using a 1 ml syringe attached to one of the needles. Incubation proceeded for 10 minutes, with shaking at 37°C and the reaction was stopped with 1 ml 25% (w/v) TCA containing 0.1M mercuric chloride.

The precipitate was removed by centrifugation and 1.5 ml of the supernatant was mixed with 1.5 ml of modified Ehrlich reagent, made 2N with respect to perchloric acid. The resultant chromagen was read at 553 nm after 15 minutes. The porphobilinogen content was quantitated assuming a molar extinction coefficient of 61 $mM^{-1}cm^{-1}$. (Mauzerall and Granick 1956).

(d) *In vitro* determination of δ-aminolaevulinic acid synthetase activity

δ-aminolaevulinic acid synthetase activity was assayed in whole liver homogenate (250 mg liver/ml in 0.1 M phosphate buffer pH 6.8) by the method of Marver et al (1966). The δ-aminolaevulinic acid (ALA) produced was quantitated colorimetrically by the acetylacetone method of Poland and Glover (1973).

Incubates contained: 0.5 ml of the homogenate, 0.5 ml 40 mM EDTA, neutralized to pH 7.0 with sodium hydroxide, 0.5 ml Tris-HCl buffer pH 7.2 and 0.5 ml 30 mM glycine. Incubation proceeded for 1 hour with vigorous shaking at 37°C in 25 ml Erlenmeyer flasks and the reaction was stopped with 0.5 ml 25% TCA containing 0.1M mercuric chloride and then centrifuged. A 1.8 ml aliquot of the supernatant was mixed with 0.9 ml 1N sodium acetate/acetylacetone (10:1 V/V) at 60°C, and the mixture heated at 80°C for 10 minutes. The tubes were then cooled and the pH adjusted to 7 with 0.6 ml of a solution containing 0.5M $Na_2HPO_4$/1N NaOH, 1:3 V/V. The aminoketone-pyrroles were extracted by adding 2.5 ml of the neutralized solution with 10 ml dichloromethane. Following this, 1.5 ml of the aqueous phase was mixed with 1.5 ml of modified Ehrlich reagent and the absorbance of the chromogen was measured after 15 minutes at 553 nm. The ALA was quantitated assuming a molar extinction coefficient of the ALA-pyrroles to be $58mM^{-1}cm^{-1}$. (Whiting, M. personal communication).

(e) The *in vitro* addition of cadmium to microsomes

3 mls of a microsomal suspension (2mg protein/ml) buffered with 1.15% KCl/Tris-HCl pH 7.4 and reduced with sodium dithionite were

placed into each of 2 matched cuvettes. One cuvette (sample) was bubbled with CO enabling the quantification of cytochrome P-450. Following this, cumulative amounts of cadmium 0, 60, 120, 240, 720, 960, 1440 and 2160 nanomoles, from a cadmium acetate solution (6mM) dissolved in KCl/Tris, were added to the sample cuvette and a equivolume of buffer added to the reference cuvette. After each addition, a difference spectrum from 390-510nm was recorded to evaluate the cytochrome P-450 and cytochrome P-420 contents.

In another pair of clean, matched cuvettes, the cumulative additions of cadmium was repeated to microsomes which had been neither reduced nor bubbled with CO. A difference spectrum was recorded between 330-590 nm.

In a third pair of clean, matched cuvettes, containing 3 mls of unreduced microsomes, 2160 nmoles cadmium (360 nmoles/mg microsomal protein) was added to one cuvette (sample) and then both were reduced and carbon monoxide bubbled into the sample cuvette. A difference spectrum between 350-550 nm was then recorded.

(f) Hepatic cadmium and zinc determinations

The hepatic metal content from rats dosed with cadmium (5 mg cadmium acetate/Kg i.p.) 1 hour, 2 hours, 12 hours, 22 hours, 32 hours and 46 hours after injection, was determined using atomic absorption spectrophotometry, as described in the General Methods section.

(g) Estimation of cytochrome P-420

Cytochrome P-420 was determined in hepatic microsomes from the carbon monoxide difference spectrum of dithionite reduced microsomes assuming a molar extinction coefficient of 110 $mM^{-1}cm^{-1}$ between 420-490 nm (Omura and Sato 1964).

RESULTS

(a) The *in vivo* effect of cadmium on the haem biosynthetic and catabolic enzymes

Figure 13 shows the activities of haem oxygenase (upper portion of figure) and δ-aminolaevulinic acid synthetase and dehydratase (lower section of figure) during the first 46 hours after the administration of cadmium. The activities of δ-aminolaevulinic acid synthetase and dehydratase did not alter relative to the pooled control values throughout the 46 hour period.

The activity of haem oxygenase did not alter during the first hour after cadmium but increased by the second hour to 234% of control and continued to increase until it reached a maximum at the 22nd hour, where it was 719% of control. It then declined gradually to 300% of control at the 46th hour.

(b) The *in vivo* effect of cadmium on hepatic microsomal haemoproteins

Figure 14 shows the cytochrome P-450, cytochrome $b_5$ and total microsomal haem contents in rat hepatic microsomes during the first

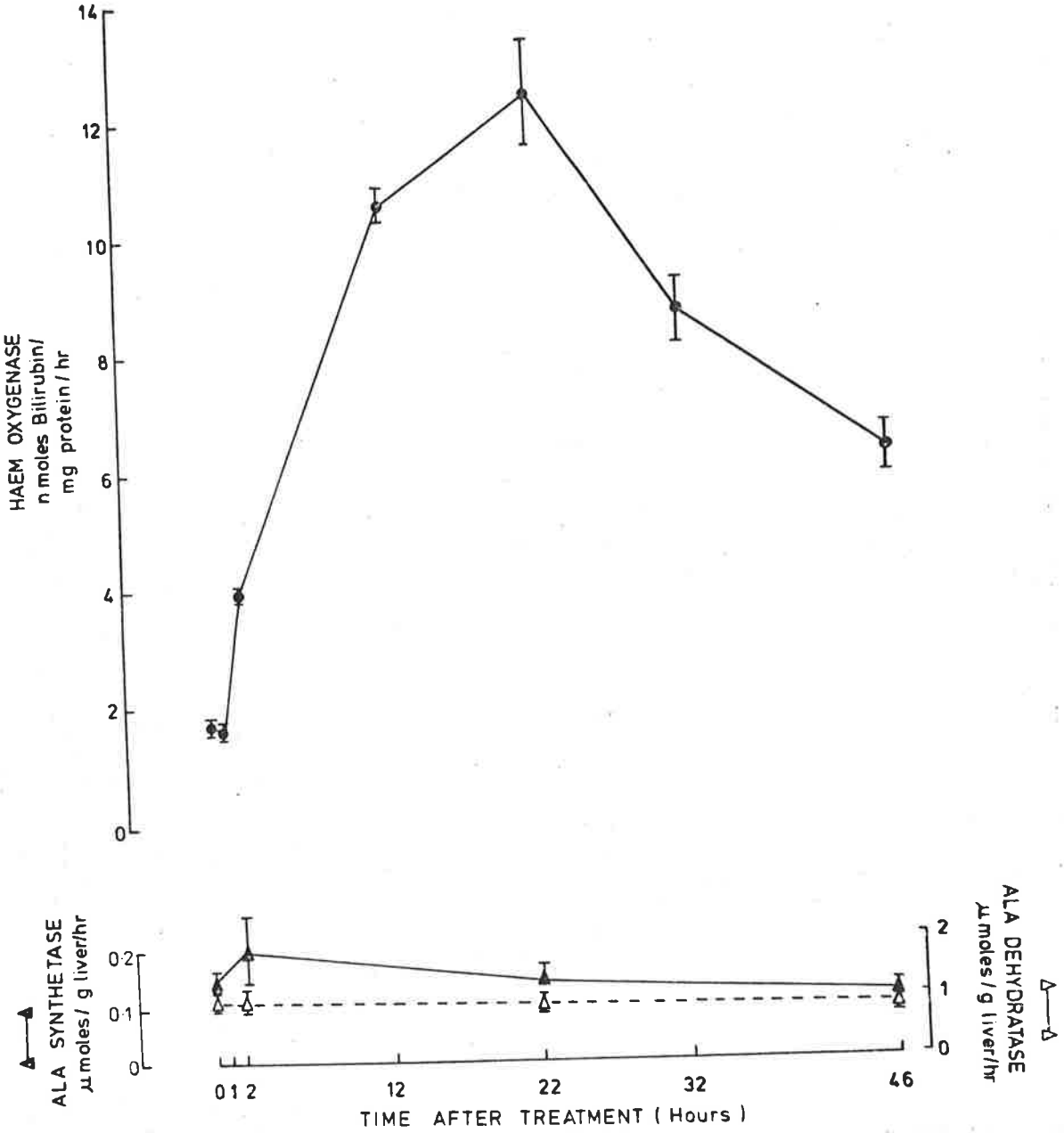


Figure 13. Time-course of activity of haem biosynthetic enzymes, δ-aminolaevulinic acid synthetase and δ-aminolaevulinic acid dehydratase and the haem degradative enzyme, microsomal haem oxygenase after administration of cadmium (5mg/Kg i.p. cadmium acetate).

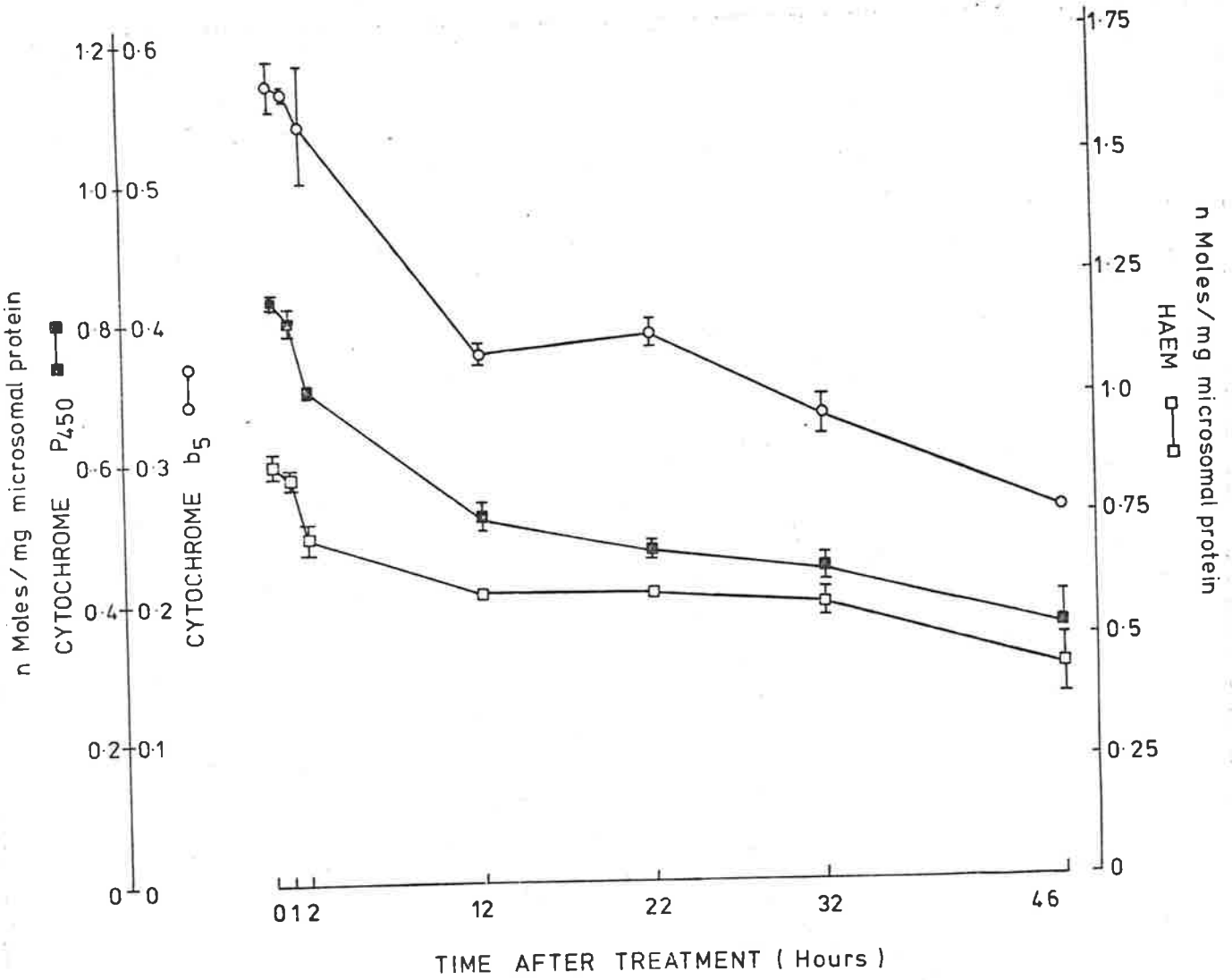


Figure 14. Time-course of degradation of cytochrome P-450 and $b_5$ and total microsomal haem after cadmium (5mg/Kg i.p. cadmium acetate).

46 hours after the administration of cadmium.

All three parameters declined in a biphasic manner, with the slow phase occurring after approximately 12 hours. None of the changes achieved statistical significance (p=0.05) during the first hour.

(c) The cadmium and zinc contents

Figure 15 shows the amount of cadmium and zinc in the livers after cadmium administration. The hepatic cadmium content of the control rats was less than the quantitation threshold (1µg cadmium/gm dry liver). The cadmium content increased after injection, being 24±8 µg cadmium/gm dry liver at the first hour and reaching a plateau at the 12th-22nd hour with about 80 µg cadmium/gm dry liver.

The control animals had a hepatic zinc content of 86 ± 2µg zinc/gm dry liver. One hour after injection, the amount detected was 90 ± 1 µg zinc/gm dry liver and at two hours it was 80 ± 2 µg zinc/gm dry liver. The content then increased continuously throughout the observation period, reaching 182 ± 8 µg zinc/gm dry liver at the 46th hour.

(d) The *in vitro* effects of cadmium on microsomes

The difference spectra obtained between dithionite reduced-carbon monoxide saturated microsomes and reduced microsomes after cumulative additions of cadmium to the carbon monoxide bubbled cuvette, are presented in figure 16. The cytochrome P-450 and cytochrome P-420 content found after each cadmium addition is presented

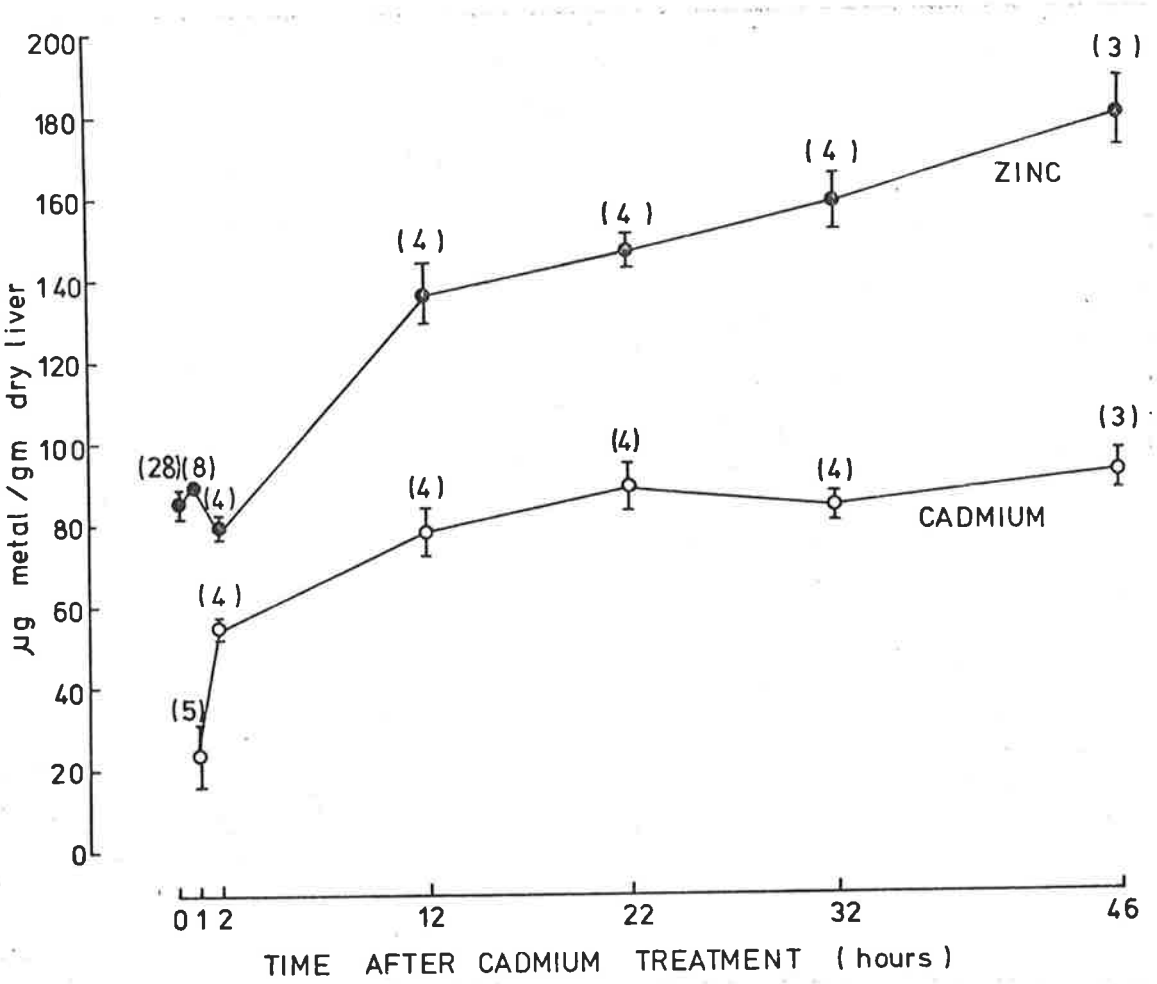


Figure 15. Cadmium and zinc content of liver during the first 2 days after cadmium (5mg/Kg i.p. cadmium acetate). The zinc content of the pooled controls was 86±2 µg zinc/gm dry tissue, n = 28, while cadmium was below detection threshold. (1µg cadmium/gm dry tissue).

in table 10. This shows that cadmium caused the quantitative conversion of cytochrome P-450 to cytochrome P-420, as the sum of the two cytochromes after each estimation, closely approximates the amount of cytochrome P-450 found initially.

Figure 17 shows the difference spectra obtained between cadmium added, non-reduced microsomes and non-reduced microsomes. The first tracing was obtained when 160 nmoles cadmium/mg microsomal protein was added and shows 2 peaks with maxima at 406 nm and 430 nm. As the concentration of cadmium increased, the 430 nm peak decreased while the 406 nm peak increased.

Figure 18 shows the difference spectrum obtained when cadmium (360 nmoles/mg microsomal protein) was added to microsomes followed by sodium dithionite reduction and carbon monoxide and normal, non-reduced microsomes (B). The tracing obtained after the addition of the same amount of cadmium but without further treatment (A) (as in figure 17) is included as a comparison.

The difference spectrum, B, has 2 peaks with maxima at 420 nm and 450 nm indicating that the microsomes now contain both cytochrome P-450 and cytochrome P-420.

TABLE 10 THE EFFECTS OF CADMIUM ADDED TO MICROSOMES *IN VITRO*

| AMOUNT OF CADMIUM a | CYTOCHROME P-420 CONTENT b | CYTOCHROME P-450 CONTENT b | SUM OF P-450 + P-420 CONTENTS b |
|---|---|---|---|
| 0 | 0 | 0.9890 | 0.9890 |
| 10 | 0.0455 | 0.9753 | 1.0208 |
| 20 | 0.0568 | 0.9615 | 1.0183 |
| 40 | 0.0341 | 0.9341 | 0.9682 |
| 120 | 0.0728 | 0.8571 | 0.9299 |
| 160 | 0.0796 | 0.8516 | 0.9312 |
| 240 | 0.1364 | 0.8242 | 0.9606 |
| 360 | 0.2614 | 0.7280 | 0.9894 |

Two matched quartz cuvettes each containing 3 mls microsomes (2mg protein/ml) were reduced and CO bubbled into one cuvette. The cytochrome P-450 content was determined. Cumulative amounts of cadmium acetate (aliquots from 3 mM cadmium acetate in TRIS-HCl pH 7.4) was added to the CO containing cuvette and the cytochrome P-450 and P-420 content estimated after each addition.

a nmoles of cadmium added/mg microsomal protein

b µmoles of cytochrome/mg microsomal protein

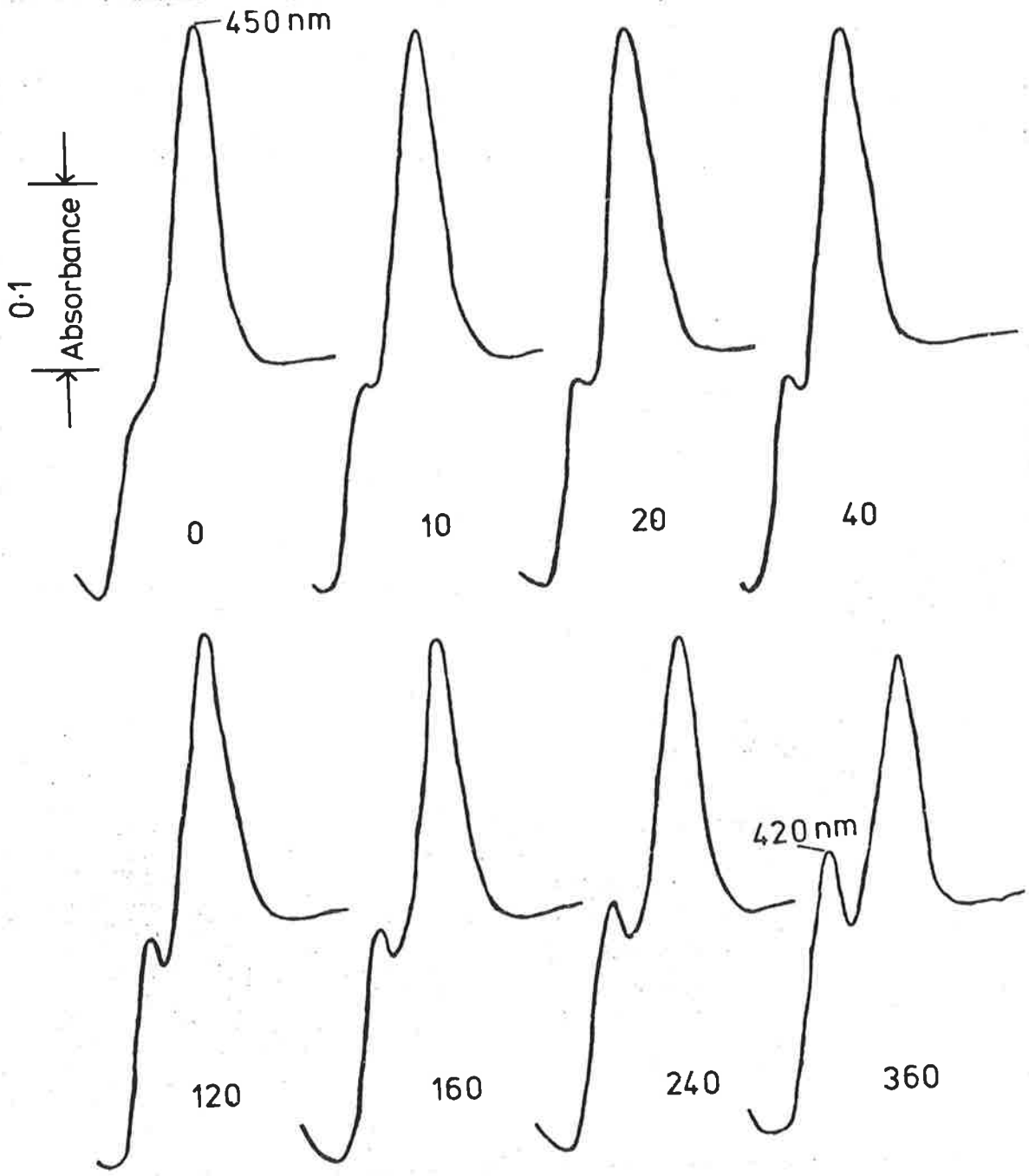


Figure 16. Sequential addition of cadmium (0-360 nmoles/mg microsomal protein) on the CO difference spectrum of dithionite reduced microsomes.

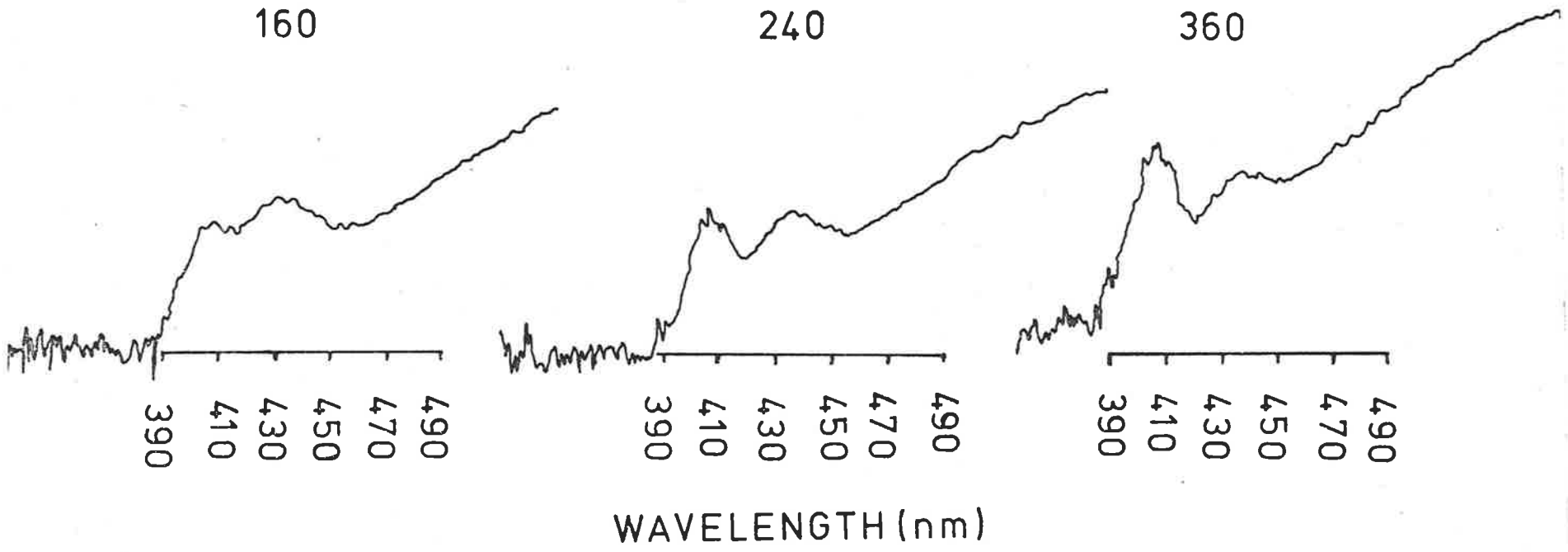


Figure 17. Difference spectrum after sequential addition of cadmium (160-360 nmoles/mg microsomal protein) to non-reduced microsomes.

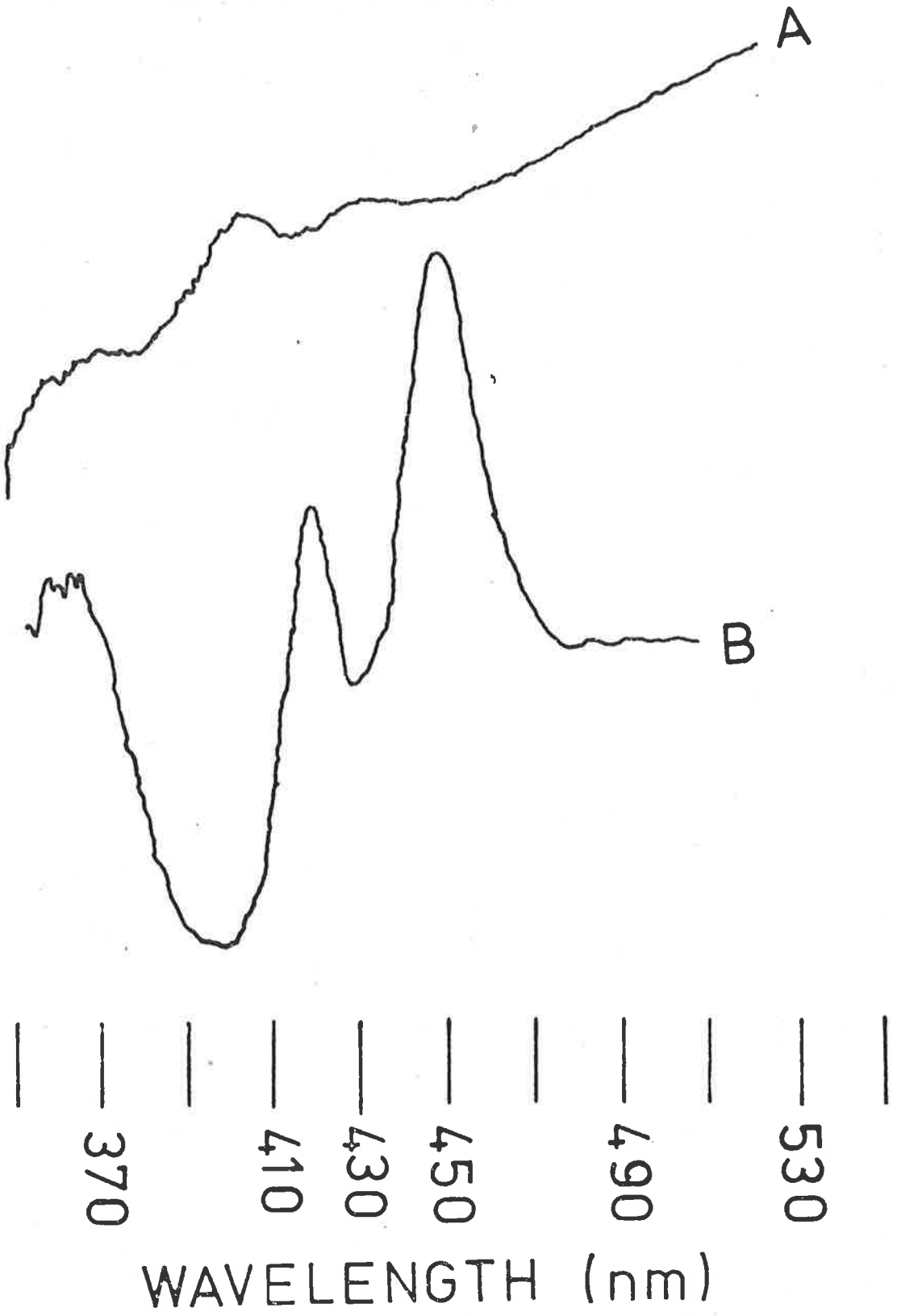


Figure 18. Difference spectrum after cadmium (360 nmoles/mg microsomal protein) added to non-reduced microsomes (A) followed by dithionite reduction and CO bubbled to cadmium side (B).

## DISCUSSION

The fact that the activity of δ-aminolaevulinic acid dehydratase was not inhibited or stimulated is in agreement with the findings of Lauwerys et al (1973) who studied blood δ-ALAD. The 20% inhibition of hepatic δ-ALAD reported by Yoshida et al (1976) was not found, at least during the first 46 hours after the cadmium dose and in any case, Haeger-Aronson et al (1971) reported that the enzyme's activity may be reduced to a third of the normal value before haem synthesis is disturbed. Subsequent enzymes in the haem biosynthetic pathway were not assayed and it cannot be ruled out that they were inhibited. Tephly et al (1978) showed that cadmium inhibits haem synthetase but is not as potent as manganese and cobalt. Nevertheless, since δ-ALAS, the rate-limiting step, was not affected it is unlikely that the overall rate of haem biosynthesis had been altered to such an extent as to affect the total microsomal haem content and the cytochromes.

The data in figures 13 and 14 therefore, suggest a correlation between the loss of both microsomal haemoproteins and haem with the increase in the activity of haem oxygenase.

Krasny and Holbrook (1977) have published qualitatively similar findings, however they found peak microsomal haem oxygenase activity occurred at the 48th hour, in contrast to the peak at 22 hours reported here. Further, the earliest time at which they examined this enzyme's activity and the cytochrome P-450 and $b_5$ contents, was 12 hours. Figure 4 shows clearly that the first significant effect of cadmium on these parameters was observed 2 hours after injection.

It is not known why the fall in the level of haem did not stimulate the activity of δ-aminolaevulinate synthetase. Such behaviour, contrary to the accepted theories, has been observed for nickel, platinum, iron and copper (Maines and Kappas 1976a, 1977c). It can be concluded that the metals may interfere with the mechanism of δ-ALAS derepression directly or aid and augment the action of the *de novo* repressor unit on the δ-ALAS operon.

A most important issue yet to be resolved, is whether cadmium initiates the destruction of cytochrome P-450 resulting in the stimulation of microsomal haem oxygenase activity, or whether cadmium stimulates microsomal haem oxygenase activity which in turn causes the decrease in the cytochromes' and total microsomal haem contents. Maines and Kappas (1975) favour the idea that heavy metals (e.g. cobalt) induce microsomal haem oxygenase first, while Bissell et al (1974), Bissell and Hammaker (1976a, b) and Maines (1977) support the breakdown of cytochrome P-450 as the initial step. These hypotheses have been discussed in the General Introduction.

The data obtained with sequential additions of cadmium to microsomes provide the following information. Table 10 shows that cadmium can attack and denature cytochrome P-450 converting it to cytochrome P-420. The spectra in Figure 18 show that cadmium denatures the cytochrome molecule whether it is in the reduced or oxidized state and causes alterations to the ligands about the iron. This is indicated by the changes in the absorbance of 2 peaks at 406 nm and 430 nm; these are similar to a modified type II spectra. It suggests that cadmium attacks the cytochrome molecule wherever a suitable site exists. It can't be claimed from this data alone that cadmium attacks only the ligands, which are believed to be cysteine and histidine (Chevion et al 1977).

If one conceives that cadmium attacks cysteine, histidine and other susceptible residues and that these residues are situated throughout the molecule, then conformational changes could indirectly effect the nature of the ligands about the iron.

The information obtained in figure 18 shows that even though cadmium interacts with the cytochrome molecule to change its spectral characteristics, the resulting cytochromes can still be reduced and bind CO. Since CO binds to the 6th ligand, then it would appear that this ligand is not adversely affected by cadmium. Also, it can be seen that the denatured microsomes, which give the modified type II spectra, are comprised largely of cytochrome P-450 and P-420.

Consequently, the belief that the catabolism of cytochrome P-450 begins with its conversion to cytochrome P-420 or the liberation of haem (Bissell and Hammaker 1976a, Maines 1977) gains support from these findings, although they do not provide proof. Although the ability of cadmium to initiate such actions is shown, the extrapolation from *in vitro* behaviour to *in vivo* mechanisms should be made reservedly, particularly in view of the fact that the cadmium concentrations added *in vitro* exceeded those found *in vivo* (see tables 4 and 10).

Maines (1977) has postulated the existance of an endogenous denaturant *in vivo* which initiates cytochrome-haem catabolism by denaturing cytochrome P-450. Therefore, an alternative to the hypothesis of direct denaturation, is that cadmium stimulates or potentiates this endogenous denaturant. Whatever the mechanism, it is unlikely that direct denaturation can account for the total loss of cytochrome P-450 after *in vivo* cadmium administration. The

more important factor is presumably the marked increase in microsomal haem oxygenase activity.

There is other evidence which suggests that degradation of cytochrome P-450 precedes the induction of haem oxygenase. Krasny and Holbrook (1977) point out that non-haem inducers of haem oxygenase cause a fall in cytochrome P-450 before the induction phase. They also report that actinomycin D or cycloheximide block most of the induction of haem oxygenase in cadmium treated rats, but neither prevents the fall in cytochrome P-450 and $b_5$. It is not clear whether the extent of the fall in cytochrome P-450 is as great under these conditions. Therefore, although it appears likely that cytochrome P-450 destruction is responsible for the induction of haem oxygenase, the precise sequence and mechanism of cadmium action on microsomes and cytochrome P-450 is still uncertain.

The data of figure 15 provide more information about the rate of cadmium and zinc accumulation in the liver during the first 48 hours after the injection of cadmium compared with figure 4 in chapter 1. Furthermore, it was stated in the second chapter that cadmium-thionein production was complete by about the 8th-12th hour after the injection of cadmium, whereas zinc-thionein synthesis continued beyond this time. The findings (figure 15) that the cadmium content increased until it reached a plateau at about the 12th hour and that the trend of the zinc content was to increase at every time of estimation, confirm these conclusions reached in chapter two.

# GENERAL DISCUSSION

The investigations in this thesis have been aimed at characterizing cadmium's effects on the rat hepatic drug metabolizing system, by relating all the observations with the time at which they occurred after cadmium administration. The importance of considering time is made clear in chapter one where the mode by which cadmium affects the drug metabolizing system was resolved into one and possibly two actions. While the decline in glucose-6-phosphatase activity and hence microsomal membrane damage was manifested at the 48th hour, it is possible that the initiation of these effects occurred earlier. The half-life of the endoplasmic reticulum membrane proteins is also 48 hours (Arias et al 1969) and so, it is conceivable that cadmium's interaction with the membrane constituents begins soon after injection but takes 48 hours for it to be resolved into a decline of glucose-6-phosphatase activity. A possible reason for the lack of resolution was suggested in chapter one. This aspect of cadmium-induced membrane damage was not investigated further in this thesis, but for continuing studies, considerable knowledge will be obtained by examining more specific tracers of membrane integrity, e.g. lipid peroxidation, changes in glutathione peroxidase activity and turnover studies of membrane constituents.

The results of chapters 1 and 3 enable one to conclude that following the administration of cadmium, the catabolism of cytochrome P-450 increases. The conclusion reached that the decreased content of cytochrome P-450 was responsible for the fall in type I and II metabolic activity, is further strengthened by the fact that the time of the greatest rate of haem and cytochrome catabolism, preceded

the observed time of maximum loss of type I and II metabolic activity.

The mechanism of cadmium-induced catabolism of cytochrome-haem is still not clear. The various theories put forward for the breakdown of cytochromes have been discussed in the General Introduction. If the theory proposed by Maines (1977) is correct, in that an endogenous denaturant may be present which converts cytochrome P-450 to P-420, then the *in vitro* evidence of Chapter 3 supports such a mechanism for cadmium. However, cytochrome P-420 has not been detected in microsomes derived from cadmium treated animals. Indeed, cytochrome P-420 seems to have only ever been recorded in *in vitro* experiments, so, perhaps its recording is an *in vitro* phenomenon. Alternatively, any cytochrome P-420 formed *in vivo*, may have been rapidly metabolized. Further, the denaturant and/or cadmium could dislodge the haem completely from the apo-protein *in vivo*; this could account for the inability to detect cytochrome P-420 which is a haemoprotein, and the resultant free haem could induce haem oxygenase.

In trying to rationalize the data into an acceptable theory, it must be borne in mind that the premise that cytochrome destruction precedes and consequently initiates the increase in the activity of haem oxygenase, may be incorrect. Just as it seems likely that cadmium induces the production of thionein at the transcriptional level, then equally acceptable is the idea that cadmium can induce transcription leading to an increase in haem oxygenase synthesis.

Another factor to be taken into consideration is that cadmium may attack other key components of the microsmal drug metabolizing system, as well. As an example, suppose NADPH-cytochrome-P-450

reductase was inhibited by cadmium, then this could also contribute to the fall in the metabolic rate.

This possibility has been investigated by other workers in relation to the inhibition of microsomal enzyme activity by other metals. Alvares et al (1974) suggested that inhibition of NADPH-cytochrome P-450 reductase may be a significant factor in the effects of methyl mercuric chloride on microsomes. However, Tephly and Hibbeln (1971) and Maines and Kappas (1975) reported that cobalt chloride (60 mg/Kg) did not affect NADPH cytochrome c reductase activity.

There has not been a great deal of consideration given to whether changes in the cytochrome $b_5$ content contribute to the results observed. Other workers have generally considered that any effect resulting from a fall in the cytochrome content is attributable to a fall in cytochrome P-450, as its half-life, (reported by Levin and Kuntzman 1969 to be 7-9 hours fast phase, 46-48 hours slow phase) is less than cytochrome $b_5$'s half-life (5 days Omura et al 1967, 4 and 4.5 days Kuriyama et al 1967). However, since cytochrome $b_5$ is involved with the delivery of the second electron to the substrate-cytochrome P-450 complex, then the prevailing level of cytochrome $b_5$ could contribute, even in a small way, to the overall rate of metabolism observed at any time after cadmium administration.

Cobalt does not alter the content of cytochrome $b_5$ (Tephly and Hibbeln 1971, Yasukochi et al 1977), whereas the results presented here show clearly that cadmium decreases the content of cytochrome $b_5$. This illustrates an example of where some of cadmium's actions differ to those of other heavy metals, and also suggests that cadmium-

induced inhibition of drug metabolism may involve, to some extent, ramifications from the loss of cytochrome $b_5$.

The lack of feedback stimulation of δ-ALAS activity following cadmium-mediated reduction of microsomal haem has been mentioned in Chapter 3. This enigma is one more inconsistency in the observed behaviour of cadmium compared with the interaction of heavy metals with the rate limiting enzymes of the haem biosynthetic and catabolic pathways. Aspects of this interaction were discussed in the General Introduction.

While the current state of knowledge is such that it is not known how δ-ALAS activity is actually modified by either induction or feedback inhibition, then the situation becomes even more complex when trying to explain mechanisms by which cadmium and other heavy metals interact with those systems. To understand this interrelationship will require extensive knowledge of how heavy metals affect DNA, transcription, translation and other nuclear processes. When such systems are fully understood, it may then also be possible to evaluate whether cadmium can induce directly the transcription of haem oxygenase.

The chronic administration of cadmium to rats, described in chapter one, carried implications concerning the function of metallothionein. The acceptance in the past that metallothionein was a cadmium detoxicant was based largely on the observation that this metalloprotein increased in amount after cadmium administration. The experimental work in this thesis showed that with chronic, low level exposure, the administration of cadmium, although resulting in the accumulation of a high concentration of cadmium in the liver,

was nevertheless not associated with abnormal metabolic function.

It was concluded in chapter two, that the rate of metal uptake was important in that for cadmium to be toxic, the amount of cadmium absorbed must exceed the "toxic threshold". From the data derived in chapter two and from information obtained from other workers it was hypothesised that it is factors controlling thionein mRNA synthesis which controls whether or not an animal is protected from the effects of cadmium. Consequently, it is now concluded that a manifestation of cadmium toxicity, like inhibiton of drug metabolism, results from perturbations in the balance of 3 factors. These are the rate with which cadmium is delivered to the liver, the prevailing sequestration capacity of the liver, and the rate with which this capacity can increase after cadmium. Therefore, the acute dose of cadmium (5mg cadmium acetate/Kg i.p.) used throughout the experimental work of this thesis, is toxic because a large quantity was presented to the liver quickly, and since there was no previous encounter with metals capable of inducing metallothionein, other than those levels of metal encountered in the rats' environment, then the rate of cadmium-induced inhibiton of drug metabolism exceeded the rate of metallothionein synthesis and cadmium sequestration.

Further, it can be said that since environmental levels of cadmium at the moment are below the relatively large acute doses administered, the ingestion of cadmium by Man from the environment resembles closely the chronic administration of the metal. The implication is that there is no significant hazard to the mixed function oxidase system from prevailing environmental levels of cadmium.

# APPENDICES

# APPENDIX A

## The use of Whatman Filter Paper No. 542

John and Van Laerhoven (1976) reported that when their aqueous cadmium solutions (1-10 μg/ml) were filtered through Whatman No. 42 filter paper, 40-100% of the cadmium was lost from the solution due to its absorption to the filter.

Whatman filter paper No. 542 was used in the present study to clarify the 105,000 x g supernatants before they were fractionated on the Sephadex column. Since the results pertaining to metallothionein quantitation depend upon the amount of cadmium bound to the apoprotein (thionein), it was important to determine whether any losses occurred as a result of the filtration. Consequently, several 105,000 x g supernatants were prepared and the cadmium and zinc content determined by atomic absorption spectrophotometry before and after filtration.

| | CADMIUM (μg/ml supernatant) | | ZINC (μg/ml supernatant) | |
|---|---|---|---|---|
| | BEFORE | AFTER | BEFORE | AFTER |
| TRIAL 1 | 9 | 9 | 13 | 13 |
| TRIAL 2 | 5 | 6 | 8 | 8 |
| TRIAL 3 | 6 | 6 | 8 | 8 |
| TRIAL 4 | 5 | 6 | 8 | 8 |
| TRIAL 5 | 6 | 6 | 8 | 8 |

It was concluded that there was no loss of cadmium or zinc from the 105,000 x g supernatants as a result of filtration through No. 542, Whatman filter paper.

# APPENDIX B

## The use of 400 mg Protein for the Preparation of Metallothionein

To ensure that the supernatant applied to the Sephadex column and from which metallothionein was extracted, came from a constant amount of liver, groups of animals were injected with cadmium (5 mg cadmium acetate/Kg) and the protein content of their 105,000 xg supernatants yielded from 1 gram liver was quantitated. The data shows that the protein yield did not alter with the time after cadmium administration and so, applying 400 mg protein rather than the soluble fraction from a fixed weight of liver, had no affect on the results. The table below shows the data.

| TIME AFTER CADMIUM | n | MG PROTEIN IN 105,000 XG SUPERNATANTS DERIVED FROM 1 GM LIVER |
|---|---|---|
| CONTROL | 2 | 44.39 ± 3.16 |
| 12 Hours | 4 | 48.59 ± 1.58 |
| 1 day | 4 | 45.92 ± 0.85 |
| 2 days | 4 | 44.89 ± 1.33 |
| 6 days | 4 | 45.39 ± 1.22 |
| 12 days | 3 | 47.32 ± 0.39 |

# APPENDIX C

## Estimation of Metallothionein's Molecular Weight

The Sephadex column was calibrated using Bovine Serum Albumin (B.S.A.) Sigma Type V, (MW = 69,000), Cytochrome C, Sigma Type III, (MW = 12,384), and Myoglobin, from Whale Skeletal Muscle, Grade A, Calbiochem, (MW = 17,000).

Figure 19 shows the graph of Elution Volume vs Molecular Weight for the three compounds. The elution volume of the metallothionein was routinely found to be 1,156 mls and by extrapolation, the molecular weight was found to be 10,500 daltons. This is in good agreement with other published values which range from 6,000 - 12,000. (See section 5e of the General Introduction).

Blue Dextran (Pharmacia) was used to calculate the void volume of the column (570 mls), and the Ve/Vo of metallothionein was 2.03, which is in excellent agreement with other workers (e.g. Shaikh and Lucis, 1971).

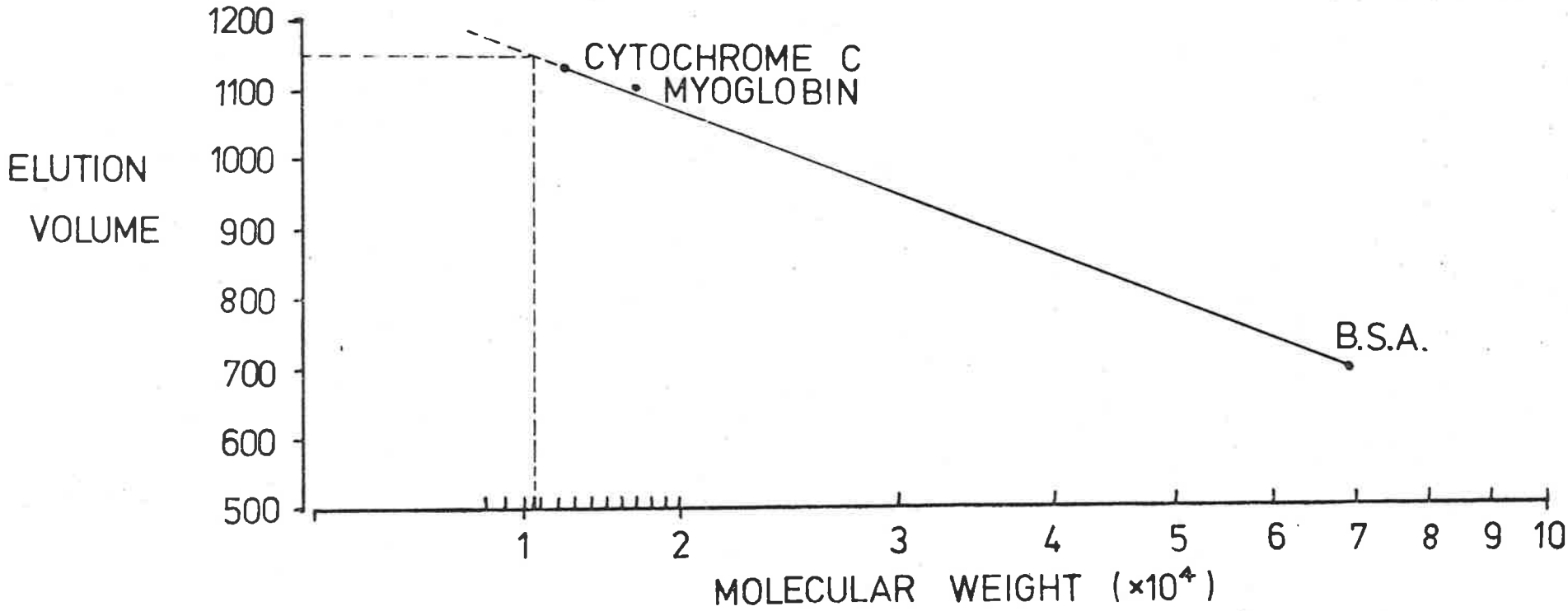


Figure 19. Estimation of Metallothionein's Molecular Weight.

# BIBLIOGRAPHY

ABDULLA, M. and HAEGER-ARONSEN, B. (1971). ALA-dehydratase activation by zinc. Enzume 12, 708-710.

ALVARES, A.P., COHN, J. and KAPPAS, A. (1974). Studies on the effects of methylmercury on ethylmorphine N-demethylase and aniline hydroxylase activities on the conversion of cytochrome P-450 to cytochrome P-420. Drug Metabolism and Disposition 2, 259-266.

ALVARES, A.P., FISHBEIN, A., SASSA. S., ANDERSON, K.E., and KAPPAS, A.(1976) Lead intoxication: Effects on cytochrome P-450-mediated hepatic oxidations. Clin. Pharm. and Therap. 19, 183-190.

AMACHER, D.E. and EWING, K.L. (1975). A soluble cadmium-binding component in rat and dog spleen. Arch. Environ. Health 30, 510-513.

ANDREUZZI, P. and ODESCALCHI, C.P. (1958). Experimental acute intoxication from cadmium chloride in the rabbit. I. Changes in the GOT-activity in the serum. Bull. Soc. Ital. Biol. Sper. 34, 1376.

ARIAS, I.M., DOYLE, D., and SCHIMIKE, R.T. (1969). Studies on the synthesis and degradation of proteins of the endoplasmic reticulum of rat liver. J. Biol. Chem. 244, 3303-3315.

BISSELL, D.M. GUZELIAN, P.S., HAMMAKER, L.E. and SCHMID, R. (1974). Stimulation of hepatic heme oxygenase and turnover of cytochrome P-450 may be related. Fed. Proc. 33, 1246, (Abst. 123).

BISSELL, D. AND HAMMAKER, L.E. (1976a). Cytochrome P-450 heme and the regulation of hepatic heme oxygenase activity. Arch. Biochem. & Biophys 176, 91-102.

BISSELL, D., and HAMMAKER, L.E. (1976b). Cytochrome P-450 heme and the regulation of δ-aminolevulinic acid synthetase in the liver. Arch. Biochem & Biophys. 176, 103-112.

BORDER, A.E., CANTRELL, A.C. and KILROE-SMITH, T.A. (1976). The *in vitro* effect of zinc and other metal ions on the activity of human erythrocyte aminolaevulinic acid dehydratase. Environ. Res. 11, 319-325.

BREMNER, I., DAVIES, N.T., and MILLS, C.F. (1973). The effect of zinc deficiency and food restriction on hepatic zinc proteins in the rat. Biochem. Soc. Trans. 1, 982, 539th Meeting, Uxbridge.

BREMNER, I. and MARSHALL, R.B. (1974). Hepatic copper-and zinc-binding proteins in ruminants. 2. Relationship between Cu and Zn concentrations and the occurence of a metallothionein-like fraction. Br.J. Nutri. 32 293.

BRYAN, S.E. and HIDALGO, H.A. (1976). Nuclear $^{115}$ cadmium: uptake and disappearance correlated with cadmium-binding protein synthesis. Biochem & Biophys. Res. Comm. 68, 858-866.

BÜHLER, R.H.O. and KAGI, J.H.R. (1974). Human hepatic metallothioneins. F.E.B.S. Letters. 39, 229-234.

CAMPBELL, T.C. and HAYES, J.R. (1974). Role of nutrition in the drug-metabolizing enzyme system. Pharmacological Reviews. 26, 171-197.

CEMPEL, M. and WEBB, M. (1976). The time-course of cadmium-thionein synthesis in the rat. Biochem. Pharmacol. 25, 2067-2071.

CHAPLIN, M.D., and MANNERING, G.J. (1969). Role of phospholipids in the drug metabolizing system of rat liver microsomes. Fed. Proc. 28, 484.

CHAPLIN, M.D., and MANNERING, G.J. (1970). Role of phospholipids in the drug metabolizing system of rat liver microsomes. Mol. Pharmacol. 6, 631-640.

CHEH, A.M. and NEILANDS, J.B. (1974). Role of zinc ion in beef liver δ-aminolevulinate dehydratase. Fed. Proc. 33, 1245, Abs. 120.

CHEN, R.W., EAKIN, K.J. and WHANGER, P.D. (1974). Biological function of metallothionein. II. Its role in zinc metabolism in the rat. Nutr. Rep. Int. 4, 195-200.

CHEN R.W. and GANTHER, H.E. (1975a). Some properties of a unique cadmium-binding moiety in the soluble fraction of rat testes. Environ. Physiol. Biochem. 5, 235-243.

CHEN, R.W. and GANTHER, H.E. (1975b). Relative cadmium-binding capacity of metallothionein and other cytosolic fractions in various tissues of the rat. Environ. Physiol. Biochem. 5, 378-388.

CHEN, R.W., VASEY, E.J. and WHANGER, P.D. (1977). Accumulation and depletion of zinc in rat liver and kidney metallothionein. J. Nutr. 107: 805-813.

CHEN, R.W. WHANGER, P.D. and WESWIG, P.H. (1975). Biological function of metallothionein. I. Synthesis and degradation of rat liver metallothionein. Biochemical Medicine. 12, 95-105.

CHEVION, M., PEISACH, J. and BLUMBERG, W.D. (1977). Imidazole, the ligand trans to mercaptide in ferric cytochrome P-450. An epr study of proteins and metal compounds. J. Biol. Chem. 252, 3637-3645.

CHVAPIL, M. SIPES, I.G., LUDWIG, J.C. and HALLADAY, S.C. (1975). Inhibition of NADPH oxidation and oxidative metabolism of drugs in liver microsomes by zinc. Short communications. Biochem. Pharm. 24, 917-919.

CHVAPIL, M. LUDWIG, J.C. SIPES, I.G. and MISIOROWSKI, R.L. (1976). Inhibition of NADPH oxidation and related drug oxidation in liver microsomes by zinc. Biochem. Pharm. 25, 1787-1791.

COON, M.J., BALLOU, D.P., HAUGEN, D.A., KREZOSKI, S.O., NORDBLOM, G.D. and WHITE, R.E. (1977). Purification of membrane-bound oxygenases: Isolation of two electrophoretically homogenous forms of liver microsomal cytochrome P-450. Microsomes and Drug Oxidations P82-94, Permagon Press. (Proceedings of the 3rd International symposium, Berlin, July 1976).

COOPER, D.Y., NARASIMHULU, S., ROSENTHAL, O., and ESTABROOK, R.W. (1965). Oxidases and related redox systems. ed. by T. KING, H.S. MASON and M. MORRISON. New York & Wiley, P. 838.

CORREIA, M.A. and SCHMID. R. (1975). Effect of cobalt on cytochrome P-450: Differences between liver and intestinal mucosa. Biochem & Biophys. Res. Comm. 65, 1378-1384.

COUSINS, R.J., BARBER, A.K. and TROUT, J.R. (1973). Cadmium toxicity in growing sevine. J. Nutri. 103, 964.

DAVIES, N.T., BREMNER, I., and MILLS, C.F. (1973). Studies on the induction of a low molecular weight zinc binding protein in rat liver. Biochem. Soc. Trans. 1, 985. 539th Meeting, Uxbridge.

DEL C. BATTLE, A.M. FERRAMOLA, A.M. and GRINSTEIN, M. (1967). Purification and general properties of δ-aminolaevulinate dehydratase from cow liver. Biochem. J. 104, 244-249.

DE MATTEIS, F. and GIBBS, A.H. (1977). Inhibition of Haem synthesis caused by cobalt in rat liver. Evidence for 2 different sites of action. Biochem. J. 162, 213-216.

DE MATTEIS, F., and SPARKS. (1973). Iron-dependent loss of liver cytochrome P-450 haem *in vivo* and *in vitro*. FEBS Letters, 29, 141-144.

DE MATTEIS, F. and UNSELD, A. (1976). Increased liver haem degradation caused by foreign chemicals: A comparison of the effects of 2-Allyl-2-isopropylacetamide and cobaltons chloride. Biochem. Soc. Trans. 4, 205-209.

DUS, K., CAREY, D. GOEWERT, R. and SWANSON, R.A. (1977). Microsomal hemeproteins P-450$_{LM-2}$ and P-450$_{LM-4}$: Comparative structural studies. Microsomes and Drug Oxidations p 95-102, Permagon Press (Proceedings of the 3rd International Symposium, Berlin, July 1976).

ESTABROOK, R.W., HILDEBRANDT, A.G., BARON, J., NETTER, K.J., and LIEBMAN, K. (1971). A new spectral intermediate associated with cytochrome P-450 function in liver microsomes. Biochem. Biophys. Res. Comm. 42, 132-139.

EVANS, G.W., MAJORS, P.F. and CORNATZER, W.E. (1970). Mechanism for cadmium and zinc antagonism of copper metabolism. Biochem. Biophys. Res. Comm. 40, 1142.

FALK, J.E. (1964). Porphyrins and metalloporphyrins. Vol. 2, B.B.A. Library. Elsevier, London. P.182-241.

FAVINO, A. and NAZARI, G., (1967). Renal Lesions induced by a single subcutaneous Cd $Cl_2$ injection in rat. Lav. Um., 19, 367.

FEUER, G., GOLDBERG, L. and LE PELLEY, J.R. (1965). Liver response tests. I. Exploratory studies on glucose-6-phosphatase and other liver enzymes. Food Cosmet. Toxicol. 3, 235-249.

FISH, W.W., MANN, K.G. and TANFORD, C. (1969). The estimation of polypeptide chain molecular weights by gel filtration in 6M Guanidine Hydrochloride. J. Biol. Chem 244, 4989-4994.

FISKE, C.H. and SUBBAROW, Y. (1925). The Colorimetric determination of phosphorous. J. Biol. Chem. 66, 375-400.

FOSTER, C.L. and CAMERON, E. (1963). Observations on the histological effects of sub-lethal doses of cadmium chloride in the rabbit. II: The effect on kidney cortex, J. Anat., 97, 281.

FRIBERG, L., PISCATOR, M., NORDBERG, G.F., and KJELLSTROM, T. (1974). Cadmium in the Environment. Cleveland, CRC-Press, Second Ed.

GIBSON, S.M. and GOLDBERG, A. (1970). Defects in haem synthesis in mammalian tissues in experimental lead poisoning and experimental porphyria. Clin. Sci. 38, 63-72.

GISBON, K.D., NEUBERGER, A., and SCOTT, J.J. (1955). The purification and properties of δ-aminolaevulinic acid dehydrase. Biochem. J. 61, 618-629.

GREIM, J., SCHENKMAN, J.B., BLOTZBÜCHER, M. and REMMER, H. (1970). The influence of phenobarbital on the turnover of hepatic microsomal cytochrome $b_5$ and cytochrome P-450 hemes in the rat. Biochemica et Biophysica Acta. 201, 20-25.

GUNN, S.A., GOULD, T.C., ANDERSON, W.A.D., (1961). Zinc portection against cadmium injury to rat testis. Arch. Pathol., 71, 274.

GUNN, S.A., GOULD, T.C., ANDERSON, W.A.D. (1963). The selective injurious response of testicular and epididymal blood vessels to cadmium and its prevention by zinc. Am. J. Pathol. 42, 685.

GUZELIAN, P.S. and BISSELL, D.M. (1976). Effect of cobalt on synthesis of heme and cytochrome P-450 in the liver. Studies of adult rat hepatocytes in primary mono-layer culture *in vivo*. J. Biol. Chem. 251, 4421-4427.

HADLEY, W.M., MIYA, T.S., and BOUSQUET, (1974). Cadmium inhibition of hepatic drug metabolism in the rat. Tox. and Appl. Pharm. 28, 284.

HAEGER-ARONSEN, B., ABDULLA, M., and FRISTEDT, B.I. (1971). Effect of lead on δ-aminolevulinic acid dehydrase activity in red blood cells. Arch. Env. Health. 23, 440-445.

HAEGER-ARONSEN, B., SCHÜTZ, A. and ABDULLA, M. (1976). Antagonistic effect *in vivo* of zinc on inhibition of δ-aminolevulinic acid dehydratase by lead. Arch. of Environ. Health 31, (4) 215-219.

HARPER, A.E., (1965). Glucose-6-phosphatase. In Methods of Enzymatic Analysis Pp 788-792 (Ed) H.V. Bergmeyer, Academic Press, N.Y.

HAWKE, D., PETERS, P.G. and PRIESTLY, B.G. (1977). Dose-, Route-and Sex-Related differences in the effects of cadmium on hepatic cytochrome P-450-dependent metabolism in the rat. Presented at the 11th Annual Meeting of the Australasian Soc. of Clin. & Exp. Pharmacologists. Nov. 23-25. New South Wales, Sydney, Australia.

IMAI, Y., ITO, A., and SATO, R. (1966). Evidence for biochemically different types of vesicles in the hepatic microsomal fraction. J. Biochem. 60, 417-428.

JOHN, M.K. and VAN LAERHOVEN C.J. (1976). Errors in cadmium determinations due to absorption by filter papers. Bull. of Environ. Contam & Toxicol. 15, 211-213.

JOHNSTON, R.E., SCHNELL, R.C. and MIYA, T.S. (1974). Cadmium potentiation of drug response: Lack of change in brain sensitivity. Tox. and Appl. Pharm. 30, 90-95.

JOHNSTON, R.E., MIYA, T.S. and SCHNELL, R.C. (1975). Cadmium potentiation of drug response - Role of the liver. Biochem. Pharm. 24, 877.

KÄGI, J.H.R., HIMMELHOCH, S.R., WHAUGER, P.D., BETHUNE, J.L. and VALLEE, B.L. (1974). Equine hepatic and renal metallothioneins. Biochemistry. 1249, 3537.

KÄGI, J.H.R. and VALLEE, B.L. (1961). Metallothionein: a cadmium and zinc-containing protein from equine renal cortex. J. Biol. Chem. 235, 3460.

KÄGI, J.H.R. and VALLEE, B.L. (1961). Metallothionein: a cadmium and zinc-containing protein from equine renal cortex, II. Physiochemical properties. J. Biol. Chem. 236, 2435.

KOMAI, N. and NEILANDS, J.B. (1969). The metalloprotein nature of Ustilago δ-aminolevulinic dehydratase. Biochemica et Biophysica Acts. 171, 311-3

KRASNY, H.C. and HOLBROOK. Jr., D.J. (1977). Effects of cadmium on microsomal hemoproteins and heme oxygenase in rat liver. Molec. Pharmacol. 13, 759-765.

KURIYAMA, Y, OMURA, T., SIEKEVITZ, P. and PALADE, G.E. (1969). Effects of phenobarbital on the synthesis and degradation of the protein components of rat liver microsomal membranes. J. Biol. Chem. 244, 2017-2026.

LAUWERYS, R.R., BUCHET, J.P. and ROELS, H.A. (1973). Comparative study of effect of inorganic lead and cadmium on blood δ-aminolaevulinate dehydratase in man. Br. J. Ind. Medicine. 30, 359-364.

LEBER, A.P., and MIYA, T.S. (1976). A mechanism for cadmium - and zinc - induced tolerance to cadmium toxicity: Involvement of Metallothionein. Tox. and Appl. Pharmacol. 37, 403-414.

LEVIN, W. and KUNTZMAN, R. (1969). Biphasic decrease of radioactive hemoprotein from liver microsomal carbon monoxide-binding particles. Effect of Phenobarbital and Chlordane. Molec. Pharmacol. 5, 499-506.

LOWRY, O.H., ROSENBROUGH, N.J., FARR, A.L. and RANDALL. R.J. (1951). Protein measurement with Folin Phamol reagent. J. Biol. Chem. 193, 265.

MCLEAN, A.E.M. and GARNER, R.C. (1974). Does cytochrome P-420 exist? Haem reductase activity in liver microsomes. Biochemical Pharmacology. 23, 475-476.

MAINES, M.D. (1977). The mechanism of degradation of endogenous heme and cytochrome P-450 by heme oxygenase. Microsomes and Drug Oxidations, Permagon Press, Ed. by ULLRICH, V. P.543-550.

MAINES, M.D., ANDERS, M.W. and MULLER-EBERHARD, U. (1974). Studies on heme transfer from microsomal hemoproteins to heme-binding plasma proteins. Mol. Pharmacol. 10, 204-213.

MAINES, M.D., IBRAHIM, N.G. and KAPPAS, A. (1977). Solubilization and partial purification of heme oxygenase from rat liver. J. Biol. Chem. 252, 5900-5904.

MAINES, M.D. and KAPPAS, A. (1974a). Cobalt induction of hepatic heme oxygenase; with evidence that cytochrome P-450 is not essential for this enzyme activity. Proc. Natl. Acad. Sci. USA. 71, 4293-4297.

MAINES, M.D. and KAPPAS, A. (1975a). Cobalt stimulation of heme degradation in the liver. Dissociation of microsomal oxidation of heme from cytochrome P-450. J. Biol. Chem. 250, 4171-4178.

MAINES, M.D. and KAPPAS, A. (1975b). The degradative effects of porphyrins and heme compounds on components of the microsomal mixed function oxidase system. J. Biol. Chem 250, 2363-2369.

MAINES, M.D. and KAPPAS, A. (1976a). Studies on the mechanism of induction of haem oxygenase by cobalt and other metal ions. Biochem J. 154, 124-131.

MAINES, M.D. and KAPPAS, A. (1976b). Selenium regulation of hepatic heme metabolism: Induction of δ-aminolevulinate synthase and heme oxygenase. Proc. Natl. Acad. Sci. USA 73, 4428-4431.

MAINES, M.D. and KAPPAS, A. (1976c). The induction of heme oxidation in various tissues by trace metals: Evidence for the catabolism of endogenous heme by hepatic heme oxygenase. Annals of Clin. Res. 8 Suppl. 17, 39-45.

MAINES, M.D. and KAPPAS, A. (1977a). Enzymatic oxidation of cobalt protoporphyrin IX: Observations on the mechanism of heme oxygenase action. Biochemistry, 16, 419-423.

MAINES, M.D. and KAPPAS, A. (1977b). Regulation of cytochrome P-450 dependent microsomal drug metabolizing enzymes by nickel, cobalt and iron. Clin. Pharmacol. & Therapeutics. 22, 780-790.

MAINES, M.D. and KAPPAS, A. (1977c). Regulation of heme pathway enzymes and cellular glutathione content by metals that do not chelate tetrapyrroles: Blockade of metal effects by thiols. Proc. Natl. Acad. Sci. 74, 1875-1878.

MAINES, M., and SINCLAIR, P. (1977). Cobalt regulation of heme synthesis and degradation in avian embryo liver cell culture. J. Biol. Chem. 252, 219-223.

MANNERING, G.J. (1971). Properties of cytochrome P-450 as affected by environmental factors: Qualitative changes due to administration of polycyclic hydrocarbons. Metabolism 20, 228-245.

MANNERING, G.J., KUWAHARA, S., OMURA, T. (1974). Immunochemical evidence for the particiaption of cytochrome $b_5$ in the NADH synergism of the NADPH-dependent mono-oxygenase system of hepatic microsomes. Biochem. Biophys. Res. Comm. 57, 476-81.

MARAFANTE, E., POZZI, G. and SCOPPA, P. (1972). Heavy metals detoxification in fish: isolation of metallothionein from liver of Carassius auratus. (abs). Boll. Soc. ital. Biol. Sper. 48, 109.

MARGOSHES, M. and VALLEE, B.L. (1957). A cadmium protein from equine kidney cortex. J. Am. Chem. Soc. 79, 4813.

MARSELOS, M., and LAITINEN, M. (1975). Starvation and Phenobarbital Treatment. Effects on drug hydroxylation and glucuronidation in the rat liver and small intestinal mucosa. Biochem. Pharm. 24, 1529-1535.

MARVER, H.S. TSCHUDY, D.P., PERLROTH, M.G. and COLLINS, A. (1966). δ-aminolevulinic acid synthetase. J. Biol. Chem. 241, 2803-2809.

MASON, H.S., NORTH, J.C. and VANNESTE, M. (1965). Microsomal mixed function oxidations: the metabolism of xenobiotics. Fed. Proc. 24, 1172-1180.

MAUZERALL, D. and GRANICK, S. (1956). The occurrence and determination of δ-aminolevulinic acid and prophobilinogen in urine. J. Biol. Chem. 219, 435-446.

MEREDITH, P.A., MOORE, M.R. and GOLDBERG, A. (1977). Effects of Aluminium, lead, zinc on δ-aminolaevulinic acid dehydratase. Enzyme 22, 22-27.

MORON, M.S., DePIERRE, J.W. JACOBSON, K. and MANNERVIK, B. (1977). Levels of glutathione and glutathione - metabolizing enzymes. Microsomes and Drug Oxidations p.447. Permagon Press (Proceedings of the 3rd International Sumposium, Berlin, July, 1976). Ed. by Ullrich, V., ROOTS, V, Hildebrandt, A., Estabrook, R.W. and Conney, A.H.

NANDI, D.L., BAKER-COHEN, K.F. and SHEMIN, D. (1968). δ-Aminolevulinic acid dehydratase of Rhodopseudomonas spheriods. I. Isolation and properties. J. Biol. Chem. 243, 1224-1230.

NASH, T. (1953). The colorimetric estimation of formaldehyde by means of the Hantzch reaction. Biochem. J. 55, 416-421.

NORDBERG, G.F., PISCATOR, M., and LIND, B. (1971). Distribution of cadmium among protein fractions of mouse liver. Acta Pharmacol. Toxicol. 29, 456.

NORDBERG, G.F., NORDBERG, M., PISCATOR, M., and VESTERBERG, O. (1972). Separation of components of rabbit metallothionein by isoelectric focusing. Biochem. J. 126, 491.

NORDBERG, M., NORDBERG, G.F. and PISCATOR, M. (1975). Isolation and characterization of a hepatic metallothionein from mice. Environ. Physiol. Biochem. 5, 396-403.

OMURA, T. and SATO, R. (1964). The carbon monoxide-binding pigment of liver microsomes I: Evidence for its hemoprotein nature. J. Biol. Chem. 239, 2370-2378.

OMURA, T., SIEKEVITZ, P., and PALADE, G.E. (1967). Turnover of constituent of the endoplasmic reticulum membranes of rat hepatocytes. J. Biol. Chem. 242, 2389-2396.

PADMANABAN, G., SATYANARAYANA RAO, M.R. and MALATHI, K. (1973). A model for the regulation of δ-aminolaevulinate synthetase induction in rat liver. Biochem. J. 134, 847-857.

PARIZEK, J. (1956). Effect of cadmium salts on testicular tissue. Nature, 177, 1036.

PARIZEK, J. (1957). The destructive effect of cadmium ion on testicular tissue and its prevention by zinc. J. Endocrinal. 15, 56.

PARIZEK, J. (1960). Sterilization of the male by cadmium salts. J. Reprod. Fertil. 1, 294.

PATTON, G.M. and BEATTIE, D.S. (1973). Studies on hepatic δ-amino-levulinic acid synthetase. J. Biol. Chem. 248, 4467-4474.

PISCATOR, M. (1964). Cadmium in the kidneys of normal human beings and the isolation of metallothionein from liver of rabbits exposed to cadmium. Nord. Hyg. Tidskr. 45, 76.

PIOTROWSKI, J.K., TROJANOWSKA, B., WISNIEWSKA-KNYPL, J.M. and BOLANOWSKA, W. (1974). Mercury binding in the kidney and liver of rats repeatedly exposed to $HgCl_2$: Induction of Metallothionein by Hg & Cd. Toxicol. & Appl. Pharm. 27, 11-19.

POLAND, A. and GLOVER, E. (1973). 2,3,7,8 - Tetrachlorodibenzo-p-dioxin: A potent inducer of δ-ALA synthetase. Science 179, 476-477.

PULIDO, P., KAGI, J.H.R., VALLEE, B.L. (1966). Isolation and some properties of human metallothionein. Biochemistry. 5, 1768-1777.

RAJAMANICKAM C., SATYANARAYANA RAO, M.R., and PADMANABAN, G. (1975). On the sequence of reactions leading to cytochrome P-450 synthesis - Effect of drugs. J. Biol. Chem. 250, 2305-2310.

REMMER, H. (1972). Induction of drug metabolizing enzyme system in the liver. Europ. J. Clin. Pharmacol. 5, 116-136.

RICHARDS, M.P. and COUSINS, R.J. (1975). Mammalian zinc homeostasis: Requirement for RNA and metallothionein synthesis. Biochem. and Biophys. Res. Comm. 64, 1215-1223.

SABBIONI, E. and MARAFANTE, E. (1975). Heavy metals in rat liver cadmium binding protein. Environ. Physiol. Biochem. 5, 132-141.

SASSA, S. and GRANICK, S. (1970). Induction of δ-aminolevulinic acid synthetase in chick embryo liver cells in culture. Proc. Nat. Acad. Sci. USA. 67, 517-522.

SCHENKMAN, J.B., REMMER, H., and ESTABROOK, R.W. (1967). Spectral studies of drug interaction with hepatic microsomal cytochrome. Mol. Pharmacol. 3, 113-123.

SCHNELL, R.C., PROSSER, T.D., MIYA, T.S. (1974). Cadmium induced potentiation of hexobarbital sleep in rats. Experentia 30, 528.

SCHMID, R. (1973). Synthesis and degradation of microsomal hemoproteins. Drug Metab. and Disp. 1, 256-258.

SCHROEDER, H.A. (1956). Trace metals and chronic diseases. Adv. Internal. Med. 8, 259-303.

SETH, T.D., AGARWAL, L.N., SATIJA, N.K. and HASAN, M.Z. (1976). The effect of lead and cadmium on liver, kidney, and brain levels of cadmium, copper, lead, manganese and zinc, and on erythrocyte ALA-D activity in mice. Bull. of Environ. Contam.& Toxicol. 16, 190-196.

SHAIKH, Z.A. and CRISPIN SMITH (1976). The biosynthesis of metallothionein in rat liver and kidney after administration of cadmium. Chem. Biol. Interactions, 15, 327-336.

SHAIKH, Z.A. and LUCIS, O.J. (1970). Induction of cadmium binding protein. Fed. Proc. Abstr. 29. 298.

SHAIKH, Z.A. and LUCIS, O.J. (1971). The nature and biosynthesis of cadmium binding proteins. Fed. Proc. Abstr. 30, 238.

SHAIKH, Z.A. and LUCIS, O.J. (1972). Cadmium and zinc binding in mammalian liver and kidneys. Arch. Environ. Health. 24, 419.

SINGHAL, R.L., MERALI, Z., HRDINA, P.D. (1976). Aspects of the biochemical toxicology of cadmium. Fed. Proc. 35, 75-80.

SQUIBB, K.S. and COUSINS, R.J. (1974). Control of cadmium binding protein synthesis in rat liver. Environ. Physiol. Biochem. 4, 24-30.

SQUIBB, K.S., COUSINS, R.J. and FELDMAN, S.L. (1977). Control of zinc-thionein synthesis in rat liver. Biochem. J. 164, 223-228.

STARCHER, B.C. (1969). Studies on the mechanism of copper absorption in the chick. J. Nutri. 97, 321.

STOLL, R.E., WHITE, J.F., MIYA, T.S., and BOUSQUET, W.F. (1976). Effect of cadmium on nucleuc acid and protein synthesis in rat liver. Toxicol. and Appl. Pharmacol. 37, 61-74.

SUZUKI, Y. and YOSHIKAWA, H. (1974). Role of metallothionein in the liver in protection against cadmium toxicity. Ind. Health. 12, 141-154.

TEARE, F.W., JASANSKY, P., RENAUD, L., and READ, P.R. (1977). Acute effect of cadmium on hepatic drug metabolizing enzymes in the rat. Tox. and Appl. Pharm. 41, 57.

TEPHLY, T.R. and HIBBELN, P. (1971). The effect of cobalt chloride administration on the synthesis of hepatic microsomal cytochrome P-450. Biochem. and Biophys. Res. Comm. 42, 589-595.

TEPHLY, T.R., WAGNER, G., SEDMAN, R. and PIPER, W. (1978). Effects of metals on heme biosynthesis and metabolism. Federation Proceedings 37, 35-39.

TENHUNEN, R., MARVER, H.S. and SCHMID, R. (1969). Microsomal heme oxygenase, characterization of the enzyme. J. Biol. Chem. 244, 6388-6394.

THOMPSON, J., JONES, D.D. and BEASLEY, W.H. (1977). The effect of metal ions on the activity of aminolevulinic acid dehydratase. British Journal of Industrial Medicine, 34, 32-36.

TYRRELL, D.L. and MARKS, G.S. (1972). Drug-induced porphyrin biosynthesis V. Effect of protoheme on the transcriptional and post-transcriptional phases of δ-aminolevulinic acid synthetase induction. Biochem. Pharm. 21, 2077-2093.

ULLRICH, V. (1969). On the hydroxylation of cyclohexane in rat liver microsomes. Hoppe-Seyler's Z. Physiol. Chem. 350, 357-365.

ULLRICH, V. (1977). The Mechanism of cytochrome-P-450 action. Proc. 3rd Int. Symp. Berlin July 1976. in Microsomes & Drug Oxidations. Ed. ULLRICH, V. P.192-201.

UNGER, M., and CLAUSEN, J. (1973). Liver cytochrome P-450 activity after intraperitoneal administration of cadmium salts in the mouse. Environ. Physiol. Biochem. 3, 236-242.

VALLEE, B.L. (1976). Address presented at 15th annual meeting of the Society of Toxicology. March 14-18, Atlanta, Georgia, USA.

WAGSTAFF, D.D. (1973). Stimulation of Liver Detoxification enzymes by dietary cadmium acetate. Bull. Environ. Contam. and Tox. 10, 328.

WEBB, M. (1972a). Binding of cadmium ions by rat liver and kidney. Biochem. Pharmacol. 21, 2751.

WEBB, M. (1972b). Protection by zinc against cadmium toxicity. Biochem. Pharmacol. 21, 2767-2771.

WEBB, M. (1972c). Biochemical effects of $Cd^{++}$ injury in the rat and mouse testis. J. Reprod. Fertil. 30, 83.

WEBB, M. (1975). The Metallothioneins. Biochem. Soc. Trans. 3, 632, 557th Meeting, Liverpool.

WEBB, M. and DANIEL, M. (1975). Induced synthesis of metallothionein by pig kidney cells *in vitro* in response to cadmium. Chem. Biol. Interactions. 10, 269.

WEBB, M. and ETIENNE, A.T. (1977). Studies on the toxicity and metabolism of cadmium-thionein. Biochem. Pharmacol. 26, 25-30.

WEBB, M. and MAGOS, L. (1976). Cadmium-thionein and the protection by cadmium against the nephro-toxicity of mercury. Chem. Biol. Interactions 14, 357.

WEBB, M. and VERSCHOYLE, R.D. (1976). An investigation of the role of metallothioneins in protection against the acute toxicity of the cadmium ion. Biochem. Pharmacol. 25, 673-679.

WESER, U., DONAY, F. and RUPP, H. (1973). Cadmium-induced synthesis of hepatic metallothionein in chicken and rats. FEBS. Letters 32, 171.

WESER, U. and HÜBNER, L. (1970). $Cd^{2+}$, $Mn^{2+}$ and $Zn^{2+}$ induced synthesis of nuclear RNA in the livers of normal and adrenalectomized rats. FEBS Letters. 10, 169-174.

WHITE, A., HANDLER, P. and SMITH, E.L. (1968). Principles of Biochemistry. 4th edition, McGraw-Hill Book Company P.744.

WHITING, M. (1976). Synthesis of δ-aminolaevulinate synthase by isolated liver polyribosomes. Biochem. J. 158, 391-400.

WHITING, M.J. and ELLIOTT, W.H. (1972). Purification and properties of solubilized mitochondrial-δ-Aminolevulinic acid synthetase and and comparison with the cytosol enzyme. J. Biol. Chem. 247, 6818-6826.

WHITING, M.J. and GRANICK, S. (1976a). δ-Aminolevulinic acid synthase from chick embryo liver mitochondria. I. Purification and some properties. J. Biol. Chem. 251, 1340-1346.

WHITING, M.J. and GRANICK, S. (1976b). δ-Aminolevulinic acid synthase from chick embryo liver mitochondria. II. Immunochemical correlation between synthesis and activity in induction and repression. J. Biol. Chem. 251, 1347-1353.

WICKRAMASINGHE, R.H. (1975). Biological aspects of cytochrome P-450 and associated hydroxylation reactions. Enz. 19, 348-376.

WILSON, E.L., BURGER, P.E. and DOWDLE, E.B. (1972). Beef-liver δ-aminolevulinic acid dehydratase. Eur. J. Biochem. 29, 563-571.

WINGE, D.R. and RAJAGOPALAN, K.U. (1972). Purification and some properties of cadmium-binding protein from rat liver. Arch. Biochem. Biophys. 153, 755.

YASUKOCHI, Y.I., NAKAMURA, M. and MINAKAMI, S. (1977). Effect of cobalt on the synthesis of liver microsomal cytochromes. J. Biochem. 81, 1005-1009.

YOSHIDA, T., ITO, Y., and SUZUKI, Y. (1976). Inhibition of hepatic drug metabolizing enzyme by cadmium in mice. Bull. Environ. Contam. and Tox. 15, 402.

YOSHIKAWA, H. (1970). Preventive effect of pretreatment with low dose of metals on the acute toxicity of metals in mice. Ind. Health 8, 184-191.