SOME HEPATIC AND EXTRAHEPATIC EFFECTS OF
ARSENIC AND SELENIUM ON HEME METABOLISM

A thesis submitted to the University of Surrey
for the degree of

DOCTOR OF PHILOSOPHY

by

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July 1986

ROBENS INSTITUTE OF INDUSTRIAL AND
ENVIRONMENTAL HEALTH AND SAFETY
UNIVERSITY OF SURREY
GUILDFORD
ABSTRACT

The acute administration of AsIII or AsV to rats produced in liver a decrease in the heme saturation of tryptophan pyrrolase (TP) accompanied by dose-related increases in aminolevulinate synthetase (ALAS) and heme oxygenase (HO) activities and corresponding decreases in P-450 concentration. The relationship between heme synthesis and degradation was changed as a result of As treatment. The magnitude of these effects was related to the oxidation state of As, AsIII being more potent than AsV. These results support the contention that the heme saturation of TP is sensitive to treatments that modify liver heme concentration. However, it remains to be established which parameter, heme saturation of TP or ALA synthetase activity reflect more accurately changes in the "free heme" pool size. The increase in heme oxygenase produced by As in liver appears to be mediated by a mechanism largely or entirely independent of heme. There were indications that the inhibition of P-450-dependent monooxygenases reflects a selective effect of As on certain P-450 isoforms. Although only the higher doses of As were able to affect testicular heme metabolism, the general effect was similar to that observed in liver. AsV affected renal ALAS, HO and P-450 at doses which were ineffective in liver and testis.
The main effects of continuous exposure to AsIII were:
1) an initial decrease in the degree of heme saturation of TP which remained constant during the period of treatment and
2) an initial increase in ALA synthetase which after 10 days of exposure was reduced to a 30% increase. No significant effects on heme oxygenase or P-450 concentration were observed. These observations were interpreted as indicative that a new balance between heme synthesis and degradation had been reached and that an adaptive response to the toxic effects of AsIII was taking place.

Se produced effects on TP, ALAS and HO similar to those produced by As, but it was more potent than either AsIII or AsV. Se appears to be unusual in that it increases HO without reducing P-450 concentration. Se also appears to be the first element reported to increase epoxide hydrolase (EH) activity, as yet there is no evidence to explain how or why Se induces EH. It appears that Se has a dual effect on As-induced hepatotoxicity. On the one hand it has additive effects in reducing the heme saturation of TP, and on the other it seems to protect against the decrease in P-450 levels produced by AsIII.
ACKNOWLEDGEMENTS

I am very grateful to my supervisors, Professor James W. Bridges and Dr. John C. Connelly for their encouragement and support throughout this work, and for the useful discussions in the writing of this thesis.

I am indebted to my fellow students and colleagues for their help in many aspects of this work, specially Arnulfo Albores, whose enthusiasm and long-standing friendship have been invaluable.

I am also very grateful to Dr. Manuel V. Ortega and Dr. Saul Villa T. for their advice and support, which made this possible.

Acknowledgement is also due to Consejo del Sistema Nacional de Educacion Tecnologica (S.E.I.T), Centro de Investigacion y Estudios Avanzados del IPN, The British Council and The Robens Institute, which at various stages of this work provided financial support.

Finally, I would like to express my appreciation to Dr. R.F. Crampton, who for many years has been trying to teach me the essentials of Toxicology (which I have yet to grasp), and to Consuelo Agrelo de Crampton for her unfailing encouragement and support.
This thesis is dedicated with love to my family, Lupita, Adren and Mariano, whose patience and understanding have made this work possible.
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CHAPTER ONE

INTRODUCTION
1. INTRODUCTION.

1.1 Arsenic.

1.1.1 Historical aspects.

Although arsenic has acquired since ancient times an unparalleled reputation as a poison, one of its earliest reported uses was in the field of therapeutics. Several arsenic compounds have been used as therapeutic agents and tonics for many centuries. Subsequently, the toxic properties of arsenic compounds came to be fully appreciated and some were put to extensive use by professional poisoners of the middle ages.

The medicinal use of different arsenic compounds has been an important source of knowledge about their toxicity. Nearly two centuries ago, Fowler's solution containing 1% arsenic trioxide, was used in the treatment of chronic myelocytic leukemia, psoriasis and bronchial asthma. Arsenate solutions (Parson's liquor) were prescribed for chronic skin diseases, parasitosis and anemia (Martindale, 1977). Organic arsenicals were used in the treatment of syphilis and other arsenicals, while atoxyl, carbarsone, glycobiarsol, melarsoprol and tryparsamide are still in use as antiparasitic drugs (Martindale, 1977). However, most drugs containing arsenic have now been replaced by more effective
and less toxic compounds in most countries. Although a number of incidents of mass human poisoning have occurred following accidental exposure to arsenic (see Section 1.14), its current toxicological importance is due to environmental and occupational exposure.

1.1.2 Sources and occurrence of arsenic in the environment.

Chemically, arsenic is a period IV metalloid and belongs to the Vb group of the periodic system which also includes nitrogen and phosphorus. Arsenic atomic number is 33 and the atomic weight 74.9. Its valence states are -3, 0, +3 and +5. Arsenic trioxide is only slightly soluble in water while sodium arsenite and sodium arsenate are highly soluble. Interchanges in valence state may occur in water solutions, depending on the pH of the solution as well as the presence of other substances that can be reduced or oxidized (Fowler et al., 1979).

Arsenic ranks twentieth among the elements in abundance in the earth's crust. The abundance of arsenic in the continental crust of the earth is generally given as 1.5 - 2.0 mg/kg. Arsenic is present in all types of soils, the geological history of a particular soil determines its arsenic content. The natural content in virgin soils varies from 0.1 to 40 mg/kg. The average is about 5 - 6, but it varies considerably among geographical regions. For example,
in the Region Lagunera, in Mexico, values between 3 and 9 mg/kg were found at the soil surface and more than 20 mg/kg deep down (Gonzalez, 1977). In addition to their natural arsenic composition, soils and sediments may also become substantially contaminated by arsenic from man-made sources.

Arsenic occurs widely in natural waters as both inorganic and organic compounds (Braman and Foreback, 1973). The main organic arsenic species, methanolsonic acid and dimethylarsinic acid, are generally present in smaller amounts than the inorganic forms, arsenite and arsenate. In the U.S. the EPA has stipulated a maximum allowable concentration of total arsenic in drinking water of 50 ug/l. Much higher values have been reported from some areas of the world: in Antofagasta, Chile, the average As level in a river water supply of drinking water was 0.8 mg/l (Borgono et al., 1977). In Region Lagunera, Mexico, there are several towns which have concentrations ranging from 0.24 to 1.0 mg/l in the drinking water obtained from wells (Cebrian et al., 1983). However, there is little information on the chemical form of arsenic in water; it appears that in anaerobic reservoirs most of it is in trivalent inorganic form, whereas in well-aerated water 90% is in pentavalent inorganic form (Clement and Faust, 1973). In well water samples from an area in Alaska, Harrington et al. (1978) reported that 39% of the arsenic present was trivalent, the rest being pentavalent, while Jauge and Cebrian (unpublished data) found that in well water from Region Lagunera, 70% of arsenic was present in its
pentavalent form.

The total industrial production of arsenic in the world is estimated to be around 60,000 tonnes; the major producing countries are Mexico, Sweden, France and Russia (Stokinger, 1981). The use pattern of arsenic trioxide in the period 1975 - 1978 has been reported as follows: manufacture of agricultural chemicals (pesticides), 82%; glass and glassware, 8%; industrial chemicals, copper and lead alloys, and pharmaceuticals, 10% (W.H.O., 1981). In agriculture, compounds such as lead arsenate, copper acetoarsenite, sodium arsenite, calcium arsenate and organic arsenic compounds are used as pesticides. Methylarsonic acid and dimethylarsinic acid are used as selective herbicides. Chromated copper arsenate, sodium arsenate and zinc arsenate are used as wood preservatives. Some phenylarsenic compounds such as arsenilic acid are used as feed additives for poultry and swine. The war gas lewisite was used in World War I and was highly effective in producing casualties; less toxic arsenic compounds are available for use as riot-control agents (N.A.S., 1977). The semiconductor industry is the youngest market for pure arsenic and its compounds and is rapidly growing in importance (Willardson, 1983).
1.1.3 Metabolism of arsenic.

**Gastrointestinal absorption.** The absorption of inorganic arsenic from the gastrointestinal tract is dependent mainly on the solubility of the arsenic compounds. It can occur following the ingestion of food, water, beverages, or drugs containing arsenic, or as a result of inhalation and subsequent mucociliary clearance. Except for arsenobetain and related compounds, most arsenicals in solution are readily bioavailable. In humans (Tam et al., 1979) and most experimental animals (Vahter and Norin, 1980), more than 90% of trivalent or pentavalent inorganic arsenic given as a solution is absorbed from the gastrointestinal tract. Tsutsumi and Nozaki (1975) found that about 30% of either AsIII or AsV injected into a ligated loop of rabbit intestine was absorbed into the blood over one hour. The mechanism of intestinal absorption of inorganic and organic arsenicals was studied by Hwang and Schanker (1973), their results indicated that in isolated loops of rat small intestine the process was simple diffusion. When undissolved arsenic compounds are ingested the absorption is dependent on particle size. Another factor influencing absorption is the composition of the diet, for example, the absorption of arsenic trioxide in rabbit intestine ligated in the ileocecal portion was inhibited by casein (Nozaki et al., 1975).
Distribution in blood. The absorbed arsenic is transported by the blood to different organs in the body. In humans, dogs, mice and rabbits, the clearance of inorganic AsIII from the blood is fairly well characterized, following a two or possibly three phase exponential curve (Charbonneau et al., 1979; Vahter and Norin, 1980; Bertolero et al., 1981). The major part (>90%) is cleared very rapidly, the half-life being 1 to 2 hours. The rest is cleared at a much lower rate, the half-lives of the second and third phase have been estimated to be about 30 and 200 hours respectively. In contrast to these other species, in the rat the half-time of trivalent or pentavalent inorganic arsenic in the blood is of the order of 60 to 90 days (Ducoff et al., 1948; Klaassen, 1974). This long half-life in rat's blood is probably attributable to tight binding to hemoglobin of the erythrocyte, release only occurring on breakdown of the red blood cells (N.A.S., 1977). The distribution of arsenic in plasma and red cells seems to be dependent on the valence form of the absorbed arsenic. Vahter and Norin (1980) examined the blood distribution in mice 0.5 to 24 hours after a single oral administration of labelled AsV or AsIII in doses of 0.4 and 4 mg/kg. At the high dose level, the ratio between the arsenic concentrations in the erythrocytes and plasma was about 2 to 3 after exposure to AsIII, but close to unity after AsV administration.

Tissue distribution. Experiments by Vahter and Norin (1980) on rabbits and mice have shown that a single dose of
Inorganic arsenic (AsV or AsIII) reaches all studied organs, but produces elevated concentrations in liver, kidney, lung and intestinal mucosa. There was no difference in whole-body retention that could be related to the valence state of the administered arsenic. Some differences were seen at the organ level, these were most marked in liver and bile, where the concentrations of labelled As were up to 10 times higher in the AsIII treated animals than in those treated with AsV. At the higher dose level, the differences were more pronounced, all tissues studied except kidney and bones showing significantly higher levels in the AsIII treated mice. An even more pronounced difference between AsIII and AsV in tissue distribution was seen in mice after intravenous administration (Lindgren et al., 1982). In contrast, using considerably lower doses, Sabbioni et al. (1979) did not find any major differences in tissue distribution in mice after i.p. injection of AsV or AsIII. As concentration decreased rapidly in most organs, 2 to 3 days after injection of AsIII to mice, the highest concentrations were detected in skin, gastrointestinal tract, epididymis and stomach wall. AsV administration produced highest concentrations in skeleton, stomach wall, epididymis, skin and kidney. Four days after administration of AsIII or AsV, the small amount of labelled arsenic remaining in the body was localized primarily in the skin, epididymis, testis, thyroid and lens (Lindgren et al., 1982). There are only a few reports available on accumulation of arsenic in tissues after prolonged exposure. Oral
administration of labelled AsV or AsIII 3 times a week for 12 weeks to mice led to a 60-fold increase in As concentration in hair, an 8-fold increase in skin, but in liver, kidney, lung and intestinal mucose the increase was only 2 to 3-fold. The accumulation was significantly higher after AsIII than AsV exposure (Vahter, 1983). Other studies indicate a more complex accumulation pattern. In mice and rabbits continuously exposed to As via drinking water or inhalation, the tissue levels increased for about two weeks, but then decreased in spite of the continued exposure (Bencko and Symon, 1969). The mechanism for these observations is not known, but presumably these effects partly explain the adaptation or tolerance which has been reported to the toxicity of arsenic.

Elimination. The elimination of As in rats is very slow because of its accumulation in erythrocytes. In animals other than the rat, As is excreted from the body at a much higher rate, mainly via the kidneys. Normally 40 to 70% of a single dose is excreted within 48 hours. In mice, exposure to AsV produces higher elimination rates than AsIII, since it does not bind to tissues to the same extent as the trivalent form. Orally administered As is excreted at a higher rate than As administered by the parenteral route, probably because more complete methylation occurs via the oral route leading to an increased elimination rate (Vahter, 1981).

As a result of an almost complete absorption in the
gastrointestinal tract (>90%) rather little arsenic is eliminated via the feces in experimental animals. Klaasen (1974) has shown that As is excreted into the bile and that this occurs against a large bile/plasma concentration ratio in rats, suggesting excretion by an active transport mechanism. Marked species differences in the biliary excretion were observed, in rats the level was 40 times that in rabbits and 800 times that in dogs.

Studies in rats on the elimination of As via the lungs have indicated that, in contrast to selenium, little is eliminated by this route (Dutkiewicz, 1977).

**Biotransformation.** It has been shown that dimethylarsinic acid (DMA) is the major urinary metabolite following administration of either trivalent or pentavalent inorganic arsenic to rats, hamsters, rabbits and dogs. Methylarsonic acid (MMA) only accounts for a few percent of the urinary As (Charbonneau et al., 1979; Tam et al., 1979; Bertolero et al., 1981). The methylation process is dependent on the valence form of As, the dose level, the route of administration as well as the animal species. In the mice given a low dose of labelled arsenic, the methylation of AsIII and AsV was equally efficient, and about 80% of the dose was excreted as DMA in the 48 hour urine. As the dose increased, the excretion of DMA was reduced, possibly reflecting the saturation of the methylation enzymes. The
decrease in methylation was more pronounced after AsV administration (Vahter, 1981). The excretion of DMA in rat urine was considerably less than in mice and rabbits. In rats the 48-hour excretion of DMA following exposure to AsIII or AsV, corresponded to only about 4% of the given dose (Vahter, 1981). It seems likely that both inorganic arsenic and DMA are retained in the rat erythrocytes. Recently, it has been reported that more than 80% of the total As in the blood of rats one hour after the administration of labelled AsV was in the form of DMA (Rowland and Davies, 1982), indicating that the methylation of inorganic As in the rat is not significantly lower than in other animals. The site of methylation in the body is not yet known, the finding that orally administered As is methylated to greater extent than that given parenterally, suggests that besides occurring in liver, the reduction and methylation by the gastrointestinal microflora plays an important role (Rowland and Davies, 1981). The mechanisms for the methylation of inorganic As in mammals have not been elucidated. It has been suggested that in bacteria, AsV has to be reduced to AsIII before methylation can take place, and that the methylation occurs by non-enzymically mediated nucleophilic or free radical attack by metalloid salts of lower oxidation state on S-adenosylmethionine or methyl-B (Wood et al., 1978). Studies by Vahter and Envall (1983) have shown that in rabbits exposed to AsV, arsenite appeared in the urine before DMA, which may support the proposal that AsV has to be reduced to AsIII before methylation can take place. Furthermore, it has
recently been shown that periodate-oxidized adenosine, a known inhibitor of methyl transferases, significantly decreased the methylation of As in mice, suggesting that AsIII may be a substrate for an enzymically mediated, as well as chemically mediated methylation (Marafante and Vahter, 1983). Which of the methyl transferases is involved remains to be established.

Another important aspect of the in vivo metabolism of inorganic As is the means by which change in the oxidation state of the non-methylated As is brought about. Since AsIII is the more toxic form, reduction of AsV to AsIII is of toxicological interest. Reduction of AsV in vivo has been indicated by certain studies on experimental animals (Ginsburg and Lotspeich, 1963; Ginsburg, 1965). Studies on catheterized rabbits have shown that about 10% of the administered AsV was reduced and then excreted in the urine as AsIII during the first 4 hours after administration. Vahter and Envall (1983) estimated that at least 50% of the AsV given is reduced in the rabbit (40% of the dose excreted as DMA + 10% excreted as AsIII), although only a part of it remains unmethylated. Oxidation of arsenite in vivo has been reported in dogs (Ginsburg, 1965), more recently Vahter and Envall (1983) have shown that it also occurs in mice. There is no information available on the influence of metabolism to methyl analogues on further tissue distribution.
1.1.4 Arsenic toxicity.

The toxicity and LD values observed for arsenic compounds vary greatly depending on the chemical form and oxidation state of the chemical involved: the toxicity of the trivalent compounds is much greater than that in the pentavalent form. For example, the oral LD for AsV in rats and mice has been found to be about 100 mg/kg and that for AsIII about 10 mg/kg. Toxicity is also highly dependent on the mode of administration, and the animal species studied. These differences may be explained, at least partially, by differences in the methylation of the inorganic As by different species of animals (Vahter, 1981). The solubility of an As compound also has a bearing on its oral toxicity as shown by the fact that the more soluble sodium arsenite is approximately 10 times more toxic than arsenic trioxide. Subacute and chronic exposures generally affect the same organ systems as those affected by acute As exposure, however, the changes observed are often more subtle or modified.

**Hepatic effects.** The liver is an organ that accumulates arsenic during exposure to this element (Vahter and Norin, 1980) and lesions have been observed in animals following short and long-term exposure. Ishinishi et al. (1980) administered arsenic trioxide for seven months in the drinking water (the highest level being 62.5 mg/l), the
treatment was then withdrawn and the animals were sacrificed 4 months later. Although no effects were observed in growth and general condition, slight liver injury with dose-dependent proliferation of the bile duct and chronic angitis in the Glisson capsule were found. Shibuya (1971) reported that impaired liver function, including delayed excretion of bromosulphthalein and increased serum transaminases occurred in rabbits given intravenous injections of arsenuous acid in doses of 0.6 mgAs/kg, 3 times a week for 3 months. Mahaffey et al. (1981) observed mild hepatic parenchymal cell swelling and decreased serum levels of alkaline phosphatase and glutamate oxalate transaminase in rats exposed to 50 mg/l of AsV in the drinking water for 10 weeks. Watanabe et al. (1979) reported that after exposure of rats to 340 mg/kg of arsenic trioxide in the diet for 3 weeks, no changes occurred in serum enzymes or in the histology of liver, kidney and spleen. Schroeder et al. (1968) after treating rats with arsenic trioxide for at least two years, did not find any significant sign of toxicity. Liver mitochondria from rats or mice exposed to arsenate in drinking water at concentrations of 0, 20, 40 or 85 mg/l showed mitochondrial swelling in situ, which was accompanied by changes in mitochondrial respiration. Normal conformational (swelling and contraction) behaviour was also depressed in isolated mitochondria, as were a number of mitochondrial enzyme activities, including monoamine oxidase and cytochrome c oxidase (Fowler et al., 1977, 1979). In mice similar alterations in mitochondrial
ultrastructure accompanied by marked time-dependent changes in the inner structure of peroxisomes were reported by Mohelska et al. (1980).

**Renal effects.** The kidney is one of the primary organs in which arsenic accumulates, it also plays a major role in the excretion and metabolism of this metalloid (Ginsburg, 1965; Brown et al., 1976; Vahter, 1981). Indications of impaired renal function, including decreased urea clearance and increased serum creatinine have been reported in rabbits given i.v. injections of arsenious acid 3 times a week for 3 months (Shibuya, 1971). Rats given AsV in the drinking water (40, 85 or 125 mg/l) for 6 weeks, showed increased kidney weights in relation to body weights. Ultrastructural studies in these animals revealed alterations in the proximal tubule cells consisting of swollen mitochondria and increased numbers of dense autophagic lysosome-type bodies. In addition, mitochondrial respiratory function was decreased (Brown et al., 1976). An interesting aspect of Brown’s study was that the changes observed were confined to the proximal tubule cells, which is the major site of As transport in the kidney. The authors suggest that it is possible that the specific effect of *in vivo* AsV exposure on the proximal tubule cell is due to a higher As concentration in these cells, and not to a special sensitivity to the AsV ion. Mitochondrial swelling is a feature of trace metal toxicity and is probably one of the earliest signs of renal toxicity (Squibb and Fowler, 1983). The changes in mitochondrial
Ultrastructure produced by AsV were accompanied by a marked increase (6-fold) in renal copper concentration and by a small (5%), but significant decrease in liver copper concentration. The authors suggested that a functional Cu toxicity plays a role in the observed mitochondrial effects (Mahaffey et al. 1981). In a study by Brazy et al. (1980), AsV was found to inhibit the transport of sodium, phosphate and glucose in the renal tubules. This inhibition was associated with an increased oxygen consumption and a decreased NADH fluorescence (indicative of NAD oxidation) similar to that obtained by addition of ouabain. From these results, the authors proposed that the dominant effect of AsV was an inhibition of the oxidative phosphorylation process.

**Testicular effects.** There is little information regarding the effects of As on testis. Bencko et al. (1968) have exposed hairless mice to arsenic trioxide in the drinking water at doses of 5 or 50 mg/l. No effects were seen at the lower dose, but at the highest level a marked degeneration in the germinal epithelium of testis was observed. Treu et al. (1974) reported an outbreak of poisoning due to ingestion of arsanilic acid in boars. During the acute phase 7 out of 16 died and all showed signs of gastrointestinal and neurological toxicity. However, long after other signs of toxicity disappeared, 3 boars showed adverse effects on sexual behaviour, two developed orchitis, epididymitis, and two showed low sperm counts and
morphological abnormalities. The authors suggested that the
disturbances in sexual behaviour were due to central or
peripheral nerve damage, but that the effects on semen
quality are more likely to be a direct effect on testis or
epididymis.

**Acute toxicity in humans.** The fatal dose of ingested
arsenic trioxide for man has been reported to range from 1 to
2.5 mg/kg (Vallee et al., 1960). The first lesion to appear
is profound gastrointestinal damage resulting in severe
vomiting and diarrhoea. Acute renal damage with microscopic
hematuria accompanied by electrocardiographic disturbances is
another common finding. Peripheral nervous disturbances,
primarily of a sensory type, become manifest between 1 and 2
weeks after ingestion. The hematopoietic system is also
affected, showing pancytopenia (W.H.O., 1981).

**Chronic toxicity in humans.** Skin lesions have been
attributed to chronic exposure to inorganic arsenic. They
include pigmentation changes, hyperkeratosis in palms and
soles, Bowen's Disease and basal and squamous cell
carcinomas. Peripheral vascular lesions consisting in
thromboangiitis obliterans and gangrene were also observed
(Tseng, 1977). It has been reported that after treatment with
Fowler's solution, some cases of portal hypertension without
signs of liver cirrhosis were observed (Martindale, 1977).
Peripheral neuropathy has been reported to occur after long-
term occupational exposure. (W.H.O., 1981). An excess of
deaths due to respiratory cancer and an increased mortality from cardiovascular disease has been observed in epidemiological investigations on smelter workers (Axelson et al., 1978).

1.1.5 *Possible molecular mechanisms of toxicity.*

**Arsenic III.** The most important mechanism by which AsIII is considered to exert its toxic effects is through interaction with active cellular sulfhydryl groups. This was discovered during the early 1900s and still forms the basis of the current understanding of AsIII action (Squibb and Fowler, 1983). Most of the early work was carried out on isolated enzymes, and most effects were inhibitory; Webb (1966) has published a comprehensive list of the enzymes affected. The mechanisms by which arsenicals inhibit enzyme activities varies for different enzyme systems. In some cases the enzyme activities can be restored by addition of excess glutathione, suggesting that the inhibition of activity is due to the reaction of As with a single SH group in the enzyme. However, this simple mechanism clearly does not hold for all enzymes, the activity of some As-inhibited enzymes is not restored by addition of simple monothiols, instead, dithiol compounds are more effective in restoring enzyme activity implying As binding to more than one -SH group.

Although in many cases the effect of arsenic on enzyme
activity appears to be due to direct interaction of As with enzyme sulfhydryl groups, there are other possible means by which it could inhibit enzyme function. Arsenic could for example, react with SH groups of substrates, coenzymes or intermediates. Phelps and Hatefi (1981), based on competition studies, concluded that the arsenical binding to the vicinal dithiol groups of beta-hydroxybutyrate dehydrogenase interfered with coenzyme (NAD/NADH) binding and catalysis, but did not affect substrate/product binding. In an interesting study, Johnson and Rajagopalan (1978) have proposed that the reaction of AsIII with the molybdenum centre of xanthine oxidase causes an inhibition of electron transfer and substrate hydroxylation during the enzyme catalyzed reaction, without any effect on substrate binding. These authors suggested that the site of interaction at the Mo centre was either an SH group liganding to the Mo atom, a persulfide residue associates with the Mo or the Mo atom itself.

**Arsenic V.** Although considerable attention has been paid to AsIII mechanisms of toxicity, direct reaction of inorganic pentavalent arsenic ion within tissues should not be discounted. The arsenate ion is isosteric and isoelectronic with phosphate (Sisler, 1956). A considerable number of in vitro studies have demonstrated that arsenate can substitute for phosphate in enzyme catalyzed reactions. Gresser (1981) has recently demonstrated that ADP-arsenate is indeed synthesized from ADP and arsenate (10 mM) by succinate-
energized submitochondrial particles. The ADP-arsenate formed rapidly hydrolyzes or can react with glucose to form glucose-6-arsenate. The adverse effects of arsenate substitution for phosphate are thought to arise from the fact that arsenical esters are inherently unstable compared to the corresponding phosphate esters (Squibb and Fowler, 1983).
1.2 Selenium.

1.2.1 Historical aspects.

Historically, interest in selenium first arose from its toxic effects, particularly in relation to animal husbandry. Then, for many years the emphasis was placed on its possible carcinogenesis, and eventually, recognition that certain animal diseases could be attributed to a deficiency of Se in the diet, served to focus attention on the beneficial effects of this trace element (Schroeder et al., 1970).

1.2.2 Sources and occurrence of selenium in the environment.

Chemically, Se is a period IV metalloid and belongs to the subgroup VIb of the period system which also includes oxygen, sulphur, tellurium and polonium. The atomic number of Se is 34, its atomic weight 78.96. Its valence states are -2, +4 and +6, it is more toxic in lower valence than in higher valence states. It has three allotropes. Elemental Se is not soluble in water. Selenites and selenates are soluble in water (Glover et al. 1979).

Selenium occurs in the earth's crust, typically in concentrations from 0.05 to 0.09 mg/kg. The occurrence of Se
in soils is a topic of considerable interest particularly in view of its toxic effects on cattle through plants which can accumulate the element from seleniferous soils. On the other hand, there are diseases prevalent in animals on a selenium deficient diet produced by grazing in areas in which the soils are low in selenium (Cooper et al., 1974). As recently shown, Keshan Disease, a cardiomyopathy of children and young women in China, occurs exclusively in regions where Se levels are extremely low. Thus, it is the first human disease shown to be related to Se deficiency. Other factors may be involved in the disease, but Se deficiency plays a major role (Burk, 1983).

Selenium has been identified as a minor constituent in water. The levels in surface and ground waters range from 0.1 to about 400 ug/l. However, depending on geological characteristics they may reach concentrations up to 6 mg/l (Glover et al., 1979).

The main sources of industrial production are the selenides of lead, copper, mercury and nickel. The use pattern of Se in the period 1975 - 1978 has been reported as follows: glass industry, 27%; semiconductor technology and electrical engineering, 23%; chemicals for duplicating machines, 23%; inorganic pigments, 14%. Inorganic salts are used in several countries as feed additives. Selenium sulfide preparations for external application are used in dermatology. Labelled selenomethionine is used for scanning

1.2.3 Metabolism of selenium.

**Gastrointestinal absorption.** Sodium selenite (SeIV) is readily absorbed from the gastrointestinal tract of rats, with most estimates of absorption exceeding 80%. Brown et al. (1972), studied the absorption of labelled selenite from rats that had been fed with Torula yeast diets containing several levels of SeIV; between 95 and 100% of the administered Se was absorbed, this being independent of the different levels of Se in the diet. Cary et al. (1973), reported that the absorption of Se from diets supplemented with selenomethionine, selenite or seleniferous corn averaged 80% and was not affected by the Se source. The site of absorption was investigated by Whanger et al. (1976) using ligated segments of the digestive tract of rats, the absorption of selenite or selenomethionine was greatest in the duodenum, followed by jejunum and ileum. In vitro work with everted intestinal sacs from golden hamsters indicated that selenomethionine, but not selenite or selenocysteine, was actively transported against a concentration gradient (McConnell and Cho, 1965).

**Tissue distribution.** After absorption, a substantial fraction of Se is bound to albumin and globulins. In mice and rats, selenium rapidly enters the erythrocytes where it is
metabolized and then gradually released back into the plasma (McConnell, 1941; Sandholm, 1973). Studies on several animal species indicate that Se in red cells is associated with glutathione peroxidase, a Se containing-enzyme (Hoekstra, 1975). Studies on animals kept on adequate or high Se diets have shown that the highest concentrations of Se are reached in liver and kidney following either single high-level exposure or long-term exposure to physiological levels of Se compounds. Spleen, lung, myocardium, skeletal muscle and brain contained decreasing amounts. (Martin and Gerlach, 1972). Brown and Burk (1973) studied the distribution and retention of Se in the tissues of rats given SeIV after being maintained on a Se-deficient (Torula yeast) diet for one month; female rats consistently retained larger amounts of radioactivity in all tissues except the brain and reproductive organs than did the males. However, a marked uptake and retention of Se was noted in the reproductive organs of males. The testes and epididymis retained about 42% of the total body burden 3 weeks after administration.

Elimination. Selenium may be eliminated from the body by the three major excretory pathways urine, feces and expired air, however, under most dietary conditions and at most dose levels, urinary excretion is the most important route of elimination. Burk et al. (1972) reported that the urinary excretion of Se is reduced at high doses, possibly due to an increase of Se in the expired air. The oxidation state of the administered Se also affects the
extent of the urinary excretion, Hirooka and Galambos (1966) compared the disposition of selenite and selenate administered intraperitoneally. Approximately 51% of the selenate was excreted in the urine, while only 37% of selenite was eliminated in the 24 hour period. Se from selenomethionine is probably less readily excreted in the urine than that from selenite. The trimethylselenonium ion accounted for about 40% of the total dose of selenite given parenterally, but increased to 56% when the element was given orally to animals that had previously been ingesting large amounts of Se in the diet (Palmer et al., 1970).

In some of the above mentioned studies, fecal excretion accounted for about 10% of the intraperitoneally administered Se. However, other studies indicated that as much as 30% of a subcutaneous dose of selenate or selenite was present in the feces and gastrointestinal contents of rats at 24 hours (Ganther and Baumann, 1962). It appears that biliary excretion amounts to only 2% of the administered dose (Levander and Baumann, 1966). However, the amount of Se excreted in the bile is greatly increased when arsenic compounds are administered concurrently with Se (Levander and Baumann, 1966). In addition, under conditions of chronic oral exposure, fecal elimination plays a more prominent role in the disposition of Se.

In contrast to arsenic, the respiratory excretion of
volatile Se (dimethylselenide) can be an important elimination route, and depending on the dose it can reach up to 30% of the administered dose. It appears that after doses below 0.1 mg/kg respiratory excretion is minimal (1%), and at doses of 1 mg/kg it is about 10% (Olson et al., 1963).

**Biotransformation.** Methylation yielding dimethylselenide, dimethyldiselenide and trimethylselenonium ion, appears to be the major pathway of Se metabolism. The highest methylating activity has been found in liver and kidney (Hsieh and Ganther, 1977). The formation of dimethylselenide involves a reduction from the +4 oxidation state of selenite to the -2 level followed by methylation of the selenide. Both the 105,000 g supernatant fluid and the washed microsomes were able to form dimethylselenide, but neither was as active as the 9,000 g supernatant fraction. Glutathione was required for the formation of dimethylselenide and could not be replaced by other thiols. Hsieh and Ganther (1977) found that the soluble fraction of homogenates contained glutathione reductase and another NADPH-dependent disulfide reductase, both presumably involved in the reduction of selenite to selenide, and a methyltransferase with a molecular weight of about 30,000. In contrast, the microsomal fraction had only methyltransferase activity and apparently methylated hydrogen selenide nonenzymatically in the presence of high GSH levels. The microsomal methyltransferase was exceedingly sensitive to inhibition by arsenite, which is known to inhibit the
formation of dimethylselenide in vivo; the nature of this inhibition was not stated in the publication. It remains to be established whether the soluble and microsomal Se-methyltransferases are unique enzymes specific for Se or if they are similar to the known S- or O-methyltransferases. The reduction reaction selenite to selenide has been shown to operate in red blood cells, for example, Gasiewicz and Smith (1978) presented evidence that hydrogen selenide was the final product of selenite metabolism by rat erythrocytes. The biochemical mechanisms involved in the formation of the urinary metabolite trimethylselenonium have not been investigated thoroughly. It would seem reasonable to assume that trimethylselenonium is formed from dimethylselenide by the addition of a third methyl group, however, Obermeyer et al. (1971) found that less than 0.5% of i.p administered labelled dimethylselenide was excreted in rat urine within 24 hours. On the other hand, following the administration of trimethylselenonium 9% of the dose was excreted in the expired air of the rats within 6 hours. This findings seem incompatible with a straightforward precursor-product relationship.

1.2.4 Toxicity of selenium.

A few minutes after subcutaneous or peroral administration of high doses of selenium to dogs, the signs observed include vomiting, garlic odor of the breath, tetanic
and death from respiratory failure (Franke and Moxon, 1937). Pathological changes include congestion of the liver with areas of focal necrosis, congestion of the kidney, endocarditis, myocarditis, petechial hemorrhages of the epicardium and atony of smooth muscles. The organ most affected by Se is the liver, which initially undergoes a reversible fatty degeneration, but if the exposure continues over a long period, cirrhosis is likely to occur. In the kidney there is commonly mild tubular degeneration. The spleen becomes enlarged and the stomach and intestinal tract show hemorrhages. Smith et al. (1973) reported that the minimum lethal dose of Se as sodium selenite or selenate in rabbits, rats and cats was between 1.5 and 3.0 mg/kg, regardless of whether the compounds were administered orally, subcutaneously, intraperitoneally or intravenously. This effect probably reflects the rapid and complete absorption of soluble Se compounds either from the site of injection or from the gastrointestinal tract. Halverson et al. (1966) found that the addition of Se (6.4 mg/kg) to the diet of rats caused significant growth depression, liver cirrhosis and splenomegaly, whereas diets containing 8 mg/kg or more caused additional effects such as anemia, pancreatic enlargement, elevated serum bilirubin levels and after 4 weeks death. In contrast, levels of 4.8 mg/kg or less had no apparent effects on rats after 6 weeks. Jacobs and Forst (1981), using selected serum enzyme levels and histopathology as indicators, suggested that rats can be exposed to sodium selenite via drinking water (1 to 4 mg/l) for up to one year.
without apparent signs of toxicity.

1.2.5 **Carcinogenic and anticarcinogenic effects of selenium.**

The first report concerning the carcinogenic effects of selenium was that of Nelson et al. (1943) who fed rats with diets containing Se as seleniferous corn or wheat. From 126 exposed rats, 73 survived less than 18 months and showed no evidence of liver tumours. From the remaining 53 that survived 18 to 24 months, 11 developed liver-cell adenoma or low grade carcinoma without metastasis in cirrhotic livers. No tumours developed in livers that were not cirrhotic. Seifter et al. (1946) found multiple thyroid adenomas and liver hyperplasia in rats fed bis-4-acetylaminophenyl selenium dehydroxide (0.05%) for 105 days. Based largely upon these studies, Se was included as a carcinogen in the Delaney Clause of the Food Additive amendment of 1958. However, two studies that specifically looked for carcinogenic effects of Se on mice (Schroeder and Mitchener, 1972) and rats (Harr et al., 1967) failed to observe significant differences in the number of malignancies between Se-treated groups and controls.

In contrast, Shamberger (1970) reported that topical administration of Se solution (0.0005%) reduced the number of tumours produced by the concomitant application of croton oil
and 7,12-dimethylbenz(a)anthracene (DMBA) or 3-
methylcholanthrene (MC). Harr et al. (1972) reported that
mammary adenocarcinomas and/or hepatic carcinomas developed
more slowly in groups of animals treated with 150 ppm of 2-
acetylaminofluorene (AAF) and 0.5 - 2.5 ppm of sodium
selenite than in those treated with AAF plus 0 - 0.1 ppm of
Se; the reduction was 85 - 95% after 200 days of exposure to
the AAF, but only 20 to 30% after 320 days of exposure. Harr
et al. (1973) in a corollary to the above study also reported
that the concentration of Se in the liver of rats fed
selenite-supplemented diets was inversely correlated with the
incidence of hepatic and mammary tumours. Dietary sodium
selenite was also reported to reduce the number and the size
of liver tumours induced by repeated small doses of aflatoxin
B (Grant et al., 1977). Selenium supplementation inhibited
1 DMBA-induced mammary gland tumorigenesis in mice and rat
studied the preventive effect of Se on the initiation and
promotion of mammary carcinogenesis, by supplementing the
diets of rats with Se before, during or after DMBA exposure.
He concluded that while Se can inhibit both the initiation
and promotion phases of carcinogenesis, a continuous intake
of Se was necessary to achieve maximal tumorigenesis
inhibition. Its efficacy was attenuated when it was given
long after carcinogenic injury. The intraperitoneal injection
of various chemical forms of Se at several times after
inoculation of mice with Erlich ascites tumour cells has also
been shown to prevent the development of tumours, but one
week delay after the inoculation reduced the effectiveness of this Se effect (Greeder and Milner, 1980). It is clear that administration of Se compounds in the diet or in water is an effective inhibitor of carcinogenesis produced by a number of chemical carcinogens; however, despite extensive study, the mechanism by which selenium prevents tumour formation in the above studies has not yet been elucidated. This subject is further discussed in Chapter 4.
1.3 Heme metabolism.

1.3.1 Outline of the pathway.

The synthesis of heme in mammalian cells involves cooperation between mitochondrial and cytoplasmic compartments. The synthesis of delta-aminolevulinic acid (ALA) is a mitochondrial process, but its subsequent conversion into coproporphyrinogen occurs in the cytoplasm. The last steps (from coproporphyrinogen to heme) again take place within the mitochondria. The stages in the heme metabolism pathway are outlined in Fig. 1.1 (Connelly, 1983).

The initial step is the condensation of glycine and succinyl coenzyme A by the enzyme ALA synthetase to form ALA in the mitochondria. The ALA then passes into the cytosol where ALA dehydratase (ALAD) catalyzes the condensation of two molecules of ALA to form the pyrrole porphobilinogen (PBG). Four molecules of this monopyrrole are condensed by the concerted activity of two cytoplasmic enzymes: uroporphyrinogen I synthetase (URO-I-S) and uroporphyrinogen III cosynthetase (URO-III-CoS), forming the first porphyrin ring in the pathway, uroporphyrinogen III. Another cytoplasmic enzyme, uroporphyrinogen decarboxylase (UROD), converts the four acetic acid side chains of the uroporphyrinogen III to methyl groups to yield coproporphyrinogen III. The coproporphyrinogen III enters the mitochondria where coproporphyrinogen oxidase oxidatively
decarboxylates the two propionic acid side chains on rings A and B to vinyl groups forming protoporphyrinogen IX. Protoporphyrinogen oxidase is then responsible for removing six hydrogen atoms to yield protoporphyrin IX. The final step in heme biosynthesis is the insertion of ferrous iron into protoporphyrin IX to form protoheme (heme) by the mitochondrial enzyme heme synthetase (ferrochelatase). Heme is then incorporated into various apoproteins of the cell, to form biologically active hemoproteins.

Hemoprotein degradation takes place in a number of tissues, the heme is released and it is cleaved at the alphamethene bridge carbon atom by microsomal heme oxygenase to produce biliverdin IX-alpha and carbon monoxide. The biliverdin is then reduced to bilirubin IX-alpha by the cytoplasmic enzyme biliverdin reductase. Bilirubin is transported from its sites of formation to the liver where the carboxyl groups on the propionic acid side chains are conjugated with glucuronic acid, glucose, xylose etc. The conjugated compounds are then excreted in the bile and thence into the gut where additional modifications are performed by gut bacteria. Recent reviews on this subject are those of Sassa and Kappas (1981), Ibrahim et al. (1983) and Maines (1984a).
Fig. 1.1 Heme synthesis and degradation (taken from Connelly, 1983).
1.3.2 Delta-aminolevulinic acid synthetase.

Some characteristic of the enzyme. All the evidence to date is consistent with the formation of ALA, catalyzed by ALAS, being the rate-limiting step in heme biosynthesis. ALAS is synthesized on the ribosomes in the cytoplasm and is then transported to the mitochondria. Normally, the activity of the enzyme in mitochondria is very low and in the cytosol is barely detectable. It appears that ALAS is either free in the mitochondrial matrix or loosely bound to the inner mitochondrial membrane. However, in animals treated with porphyrinogenic drugs, where ALAS activity is much higher than normal, a significant proportion (35%) of the total activity is present in the cytoplasm, much more than can be accounted for by mitochondrial damage (Hayashi et al., 1969). This cytosolic enzyme may represent newly synthesized ALAS on the way to being incorporated into the mitochondria (Kikuchi and Hayashi, 1981). Both mitochondrial and cytoplasmic enzymes have been shown to be synthesized on cytoplasmic ribosomes (Tait, 1978).

ALAS has one of the shortest half-lives yet reported for any mammalian liver enzyme. In adult rat liver, the half-lives of both the mitochondrial enzyme and its mRNA has been found to be about 1 hour (Tsuchudy et al., 1965); those of fetal rat liver are about 34 minutes (Woods, 1974). Adult rat liver cytoplasmic enzyme has a half life of 20
minutes in the absence of hemin, and 120 minutes in its presence (Hayashi et al. 1972). This short life permits regulation of the enzyme activity by control of its synthetic mechanism.

Nakakuki et al. (1980) have reported that rat liver cytosolic ALAS consists of a dimer of identical subunits having a molecular weight of 51,000 plus two catalytically inactive subunits having molecular weights of 79,000 and 120,000.

Catalytic mechanism. The formation of ALA from glycine and succinyl-CoA is accompanied by loss of the carboxyl group of glycine as carbon dioxide and the liberation of CoA. The participation of pyridoxal phosphate in ALA synthesis is well established and the first stage in the catalytic reaction may be envisaged as a transimination between enzyme-pyridoxal phosphate and glycine to form an enzyme-pyridoxal phosphate-glycine Schiff base complex, thus forming a stable carbanion with loss of a proton. According to Jordan and Shemin (1972), this is followed by condensation in which the electrophilic acyl-C atom of succinyl CoA acts as an electron acceptor to form alpha-amino-beta-oxoadipic acid. The decarboxylation occurs simultaneously or shortly thereafter.
Induction of ALAS. It has been postulated that the control of hepatic ALAS activity by heme repression requires the existence of a regulatory heme pool and that most, if not all, of the chemicals which cause increased ALAS activity do so by primarily or secondarily decreasing the regulatory heme pool (Meyer, 1982). Some mechanisms by which chemicals do this are:

1) Increase in heme breakdown: In the case of 2-allyl-2-isopropylacetamide (AIA) and other allyl-containing compounds, the loss of liver-heme is due to increased destruction and conversion into abnormal porphyrins or green pigments (De Matteis et al. 1982). The endoplasmic reticulum is the most important site of heme destruction, and the effect seems to be confined to the cytochrome P-450 heme without affecting the apoprotein, which is therefore able to accept newly formed heme. The new heme is also diverted into this degradative pathway and hence the "free heme" pool is lowered producing a marked increase in ALAS activity (De Matteis, 1978).

2) Inhibition of heme synthesis: In the case of 3,5-diethoxy carbonyl-1,4-dihydrocollidine (DDC) and griseofulvin there is some degree of liver heme destruction, but inhibition of heme synthesis is probably more important in causing the loss of heme and the stimulation of ALAS activity. This was shown in experiments where a rapid loss
in heme synthetase activity was caused by DDC before any increase in either ALAS activity or in liver porphyrin concentration had occurred (Abbritti and De Matteis, 1983).

3) Induction of apoproteins: Among the reactions that compete for heme in the cell are the syntheses of apoproteins, among which synthesis of the apoproteins of cytochrome P-450 account for the major part of the total hepatic heme synthesized. The concentration of P-450 increases in response to various lipophilic drugs, and is regularly accompanied by increased ALAS activity. The secondary rise of ALAS activity following apocytochrome P-450 induction by these compounds may be seen as a coordinated adaptive response providing the additional heme for hemoprotein synthesis, although the precise manner by which induction of apocytochrome and heme synthesis are coordinated remains to be elucidated (Meyer, 1982).

4) Combined mechanisms: In contrast to a large number of drugs and chemicals that induce cytochrome P-450 and secondarily lead to a controlled and coordinated induction of ALAS, a small number (e.g. DDC) affect heme synthesis in such a way as to produce porphyria characterized by a massive increase in ALAS and marked hepatic overproduction, accumulation and excretion of heme precursors.
1.3.3 Heme oxygenase.

The mechanisms by which heme and hemoglobin are converted to bile pigments in vivo remained controversial until 1968, when Tenhunen et al. (1968, 1969) showed that the microsomal fraction of rat liver, kidney and bone marrow possesses a mixed-function oxidase enzyme, heme oxygenase, which cleaves the alpha-methene bridge of heme. This heme oxygenase system catalyses the degradation of heme to yield biliverdin IX-alpha and consumes NADPH and molecular oxygen.

Mechanism of heme degradation via the heme oxygenase system. This system consists of heme oxygenase and NADPH-cytochrome P-450 reductase, the latter serving as the electron donor for the reaction. The mechanism is similar to that of the cytochrome P-450 dependent hydroxylation reactions in microsomes, the heme oxygenase reaction requires NADPH and molecular oxygen, indeed it was thought at one time that the system involved a cytochrome P-450 as a terminal oxidase (Tenhunen et al. 1968). However, purification of the heme oxygenase by Yoshida and Kikuchi (1979) demonstrated that it is independent of any type of cytochrome P-450.

The purified heme oxygenase from rat liver has a minimum molecular weight of 32,000 (Yoshida and Kikuchi, 1979). It is not a hemoprotein by nature, but it binds heme to form a 1:1 complex (Yoshida and Kikuchi, 1978a). In the
heme oxygenase reaction, heme acts as both the substrate and coenzyme and the heme oxygenase protein provides a suitable site for the rapid autocatalytic oxidation of the bound heme. The heme binding environment of the heme oxygenase protein may be similar to those of hemoglobin and myoglobin with heme binding to heme oxygenase through a coordination linkage with an aminoacid residue of the protein (Kikuchi and Yoshida, 1980).

The possible chemical and reaction sequence of heme degradation, according to Kikuchi and Yoshida (1983), is summarized as follows: as a preliminary step, protoheme binds to the HO protein to form a ferric heme-HO complex. Then the heme is reduced to ferrous heme by the NADPH-cytochrome P-450 reductase system, followed by binding with molecular oxygen to form the oxygenated heme-HO complex which initiates the subsequent heme degradation. For the onset of the reaction, a reducing equivalent is indispensable (Yoshida et al., 1980a), and the first step of heme oxidation is assumed to be hydroxylation at the protoheme alpha-methene carbon producing alpha-hydroxyheme. The molecular oxygen which is bound to heme on HO is probably reduced to a 'peroxo' form of dioxygen and this activated form of dioxygen is then utilized directly to oxidize the heme moiety. Although protoheme has four methene carbons which link four pyrrole rings in the porphyrin moiety, the activated oxygen attacks specifically the alpha-methene of heme.
The most difficult unsolved problem in the HO reaction is the nature of the chemical sequence from hydroxyheme to iron-biliverdin complex involving carbon monoxide liberation. It is thought that alpha-hydroxyheme is further oxidized to the so called 688 nm substance thereby consuming a reducing equivalent. This substance shows an absorption maximum at 688 nm and is presumed to be an intermediate whose chemical structure has not been fully elucidated (Yoshida et al., 1980b). The alpha-methene carbon of the porphyrin is liberated as carbon monoxide at this step. Conversion of the 688 nm substance to the biliverdin-iron complex, which still remains attached to the HO protein, also requires oxygen and reducing equivalents suggesting that another hydroxylation reaction is involved in this step. The release of iron from the biliverdin-iron complex is facilitated in vitro by the addition of either desferrioxamine, a ferric iron chelator, or the NADPH-cytochrome P-450 reductase system, indicating that for the release of iron from the biliverdin-iron chelate in vivo, the iron may have to be reduced to the ferrous state by the NADPH-cytochrome P-450 reductase system (Yoshida and Kikuchi, 1978c).

Induction of heme oxygenase activity by xenobiotics. Sibahara et al. (1979) and Kikuchi and Yoshida (1983), studied the cell-free synthesis of HO directed by polysomes isolated from pig alveolar macrophages or rat liver
obtained from animals treated with cadmium and bromobenzene. A combination of \(^{14}C\) or \(^{3}H\)-labeled leucine and antibodies (IgG) specific to pig spleen HO and rat liver HO was used. In both, macrophages and rat liver, free polysomes were the major site of HO synthesis. The ability to conduct HO synthesis was greatly increased in the induced systems and it was proportional to the HO activities observed in the livers from which the polysomes were prepared. This was taken as an indication that all these inducers enhanced the synthesis of HO mRNA, which in turn increased HO synthesis.

Stimulation of hepatic HO by hemin and non-heme substances was first demonstrated by Tenhunen et al. (1970b). Since then a number of reports have appeared dealing with HO stimulation by hemin or various non-heme substances in liver. The mechanism of induction, however, has eluded convincing explanation and different hypotheses have emerged. One hypothesis expresses the concept that the induction of HO reflects the intermediate action of endogenous heme, specifically that of derived from P-450 degradation. Evidence in favour of this hypothesis is provided by the finding that stimulation of liver HO follows administration of exogenous heme (Tenhunen et al., 1970), and also by the finding that drugs like AIA, which preferentially destroy rapidly turning over liver heme, cause a 50\% decrease in liver HO activity (Maines and Kappas, 1975). A question that still remains to be answered is whether the stimulation of the enzyme produced
by several non-heme chemicals is also mediated by heme (De Matteis, 1982). Bissell and Hammaker (1976a, b; 1977) reported that endotoxin administration produced an increase in the heme saturation of TP, and that the loss of radioactivity from prelabelled P-450 heme and the exhalation of CO were noted before the stimulation of HO. In all these respects, endotoxin resembled administration of exogenous heme. On this basis these authors concluded that HO-inducing agents act by primarily producing a release of heme from P-450 which in turn induces HO. Similarly, CS₂ administration caused a rapid release of heme from the microsomes and significant conversion of 5-amino (4-C) levulinate into bile bilirubin, as well as HO stimulation. These results suggested that the main target of CS was the protein moiety, rather than the heme moiety, of the cytochrome. A lesion of the apoprotein may be expected to reduce the affinity for heme, with some of the P-450 heme being released and also with less of the newly formed heme being accepted and stabilized in a hemoprotein structure. The net result being a substrate-mediated HO induction (Jarvisalo et al., 1978).

According to Maines (1984), the possibility of P-450 heme regulating both the activity of ALAS and HO is an attractive concept which fits both the experimental data on the response of these enzymes to exogenously administered heme, as well as, providing an uncomplicated explanation for how homeostatic control of ALAS and HO is maintained. The
difficulty arises when attempts are made to explain the induction of HO by hormones, starvation and metals, and to rationalise HO induction in extrahepatic systems, which possess low concentrations of P-450.

It has been reported that insulin and epinephrine administration moderately induced HO in rat liver. At the same time they caused a transient maximal saturation of TP with heme. Insulin produced a small transient increase in ALAS activity, whereas epinephrine transiently reduced it to about 50% of control. However, the content of P-450 was not appreciably decreased (Kikuchi and Yoshida, 1983). The question thus remains, as to whether the source of heme which appeared to have entered into the "regulatory heme" pool was from P-450 or not. It has been reported that a single subcutaneous injection of bromobenzene caused a progressive and extensive increase in HO activity, with TP heme saturation reduced to nearly zero at 12 h or later after injection. ALAS activity was transiently reduced to 60% of control and afterwards it returned to control values. The P-450 content was also gradually decreased. However, the activity of HO still continued to increase linearly even 12 hours after injection (Guzelian and Elshourbagy, 1979; Kikuchi and Yoshida, 1983). It is also known that a number of transition elements and heavy metals, including Co, Mn, Fe, Ni, Cu, Zn, Cd, Hg, Pb, Sn, Pt, Sb, Se and some organometallic compounds, are powerful inducers of HO.
Besides producing an initial inhibition in ALAS activity, followed by a rebound increase, these elements decrease the heme saturation of TP and the cytochrome P-450 concentration (Maines and Kappas, 1975, 1976a, 1976b, 1977a; Eaton et al., 1980; Sunderman et al., 1983; Rosenberg et al., 1984). The above mentioned reports led Kikuchi and Yoshida (1983) to suggest that the release of P-450 heme and the subsequent increase in the "free heme" pool size is not a common underlying mechanism for the induction of HO. According to Maines (1984a), these results attest to the unlikelihood that the most effective inducers of HO, metal ions and bromobenzene, mediate their action on HO via their effect on the cellular "free heme" pool.

The following discussion attempts to summarize the current hypotheses on the mechanisms of induction in the above mentioned cases. De Matteis (1982) has suggested the possibility that HO stimulation caused by starvation, iron and metals could be substrate-mediated via a microsomal lesion produced through the action of peroxides. There is evidence that in vitro, starvation and iron increase the peroxidative damage of the microsomal membranes and that sulfhydryl reagents, such as heavy metals, can convert P-450 to P-420, resulting in less heme being retained or accepted due to a loss of a stable apoprotein structure, which in turn would lead to an increase in HO activity. Attention is drawn by De Matteis (1982) to the work of Bakken et al. (1972), Penning and Scoppa (1977), and Burk and Masters (1975), which
respectively explored the influence of cyclic AMP, lipid peroxidation and selenium deficiency on the relationships between P-450 and HO induction, providing indirect evidence in favour of this attractive hypothesis.

Another hypothesis, particularly as it pertains to the action of metal ions, has been suggested by Maines and Kappas (1976b). They have hypothesized that the irreversible interaction of metal ions with specific receptor sites, containing key sulfhydryl groups, may trigger cellular events leading to HO induction. They also suggest that a direct relationship exists between the cellular content of SH and the ability of metals to affect enzyme synthesis. If the endogenous content of SH groups is increased by the oral administration of cysteine, before the injection of metals, the HO inductive action is blocked. Conversely, the HO effect can be magnified by depleting the cellular content of SH groups prior to the metal administration (Maines and Kappas, 1977b). Recent studies indicate that not only metal ions, but other agents which increase HO activity, also alter the cellular levels of GSH suggesting that perturbations in the ratio of GSSG/GSH constitute a common basis by which agents of great diversity induce HO activity (Chung and Maines, 1981; Chung et al., 1982). An -SH-containing protein which would appear to be a good candidate for functioning as an HO activity regulator is biliverdin reductase (BR) which is highly sensitive to SH reagents
The possibility that metal ions act via the formation of metallophyrin complexes has also been suggested. There is evidence supporting the formation of Zn- and Co-protoporphyrins in vivo (Sinclair et al. 1982). However, it has been suggested that no other metal ion forms a stable protoporphyrin chelate complex in biological systems (Maines, 1984a).

In summary, the induction of hepatic HO involves two apparently different mechanisms; one, heme mediated and other possibly independent of heme mediation. The moderate increases in HO activity produced by the administration of CS₂, endotoxin, insulin and epinephrine are likely to be mediated by heme in view of the observations that the administration of these agents produced an increase in the heme saturation of TP. On the other hand, the extensive increase in HO activity observed after the administration of metal ions and bromobenzene, seem to be independent of mediation by heme, since there was no indication of increases in the "free heme" pool as judged from the heme saturation of TP (Kikuchi and Yoshida, 1983). However, the precise mechanism of HO induction by these inducers is not yet clear.
1.3.4 Tryptophan pyrrolase

Tryptophan pyrrolase also known as tryptophan oxygenase (TP) is the heme-dependent liver cytosol enzyme that catalyzes the oxidative cleavage of the pyrrole ring of L-tryptophan to produce N*-formylkynurenine in the first and rate-limiting step of the kynurenine-nicotinic acid pathway of tryptophan degradation (Badawy, 1979).

Some characteristics of the enzyme: Liver is the only mammalian organ known to contain TP activity, and this enzyme may be considered as a specific marker for hepatic tissue (Knox and Mehler, 1950; Rose, 1972). It has been reported that TP consists of four subunits (43,000 molecular weight) which are nonidentical and separable into two distinct species on polyacrylamide gel; the molecular weight of TP is about 167,000, comprising 2 moles of heme and 2 moles of copper per mole of tetrameric enzyme (Schutz and Feigelson, 1972; Brady et al., 1972).

In the liver of man, rat and certain, but not all, other animal species (Badawy and Evans, 1974), the enzyme exists in at least two forms. The already active reduced holoenzyme does not require the addition of hematin for demonstration of its activity in vitro, whereas the heme-free predominant form (apoenzyme) does (Feigelson and Greengard, 1961a). The activation of the rat liver enzyme in vitro
consists of two steps, both of which require the participation of tryptophan. The inactive apoenzyme is first conjugated with hematin, this reaction requires a small concentration of L-tryptophan, although some of its congeners can replace it. The oxidized holoenzyme formed by conjugation is then reduced by ascorbic acid, this reaction also requires tryptophan which can not be replaced by its congeners. The reduced holoenzyme that is formed is active in the catalytic reaction, this reaction also specifically requires L-tryptophan as its substrate (Knox, 1966).

**Catalytic mechanism.** The preponderant existent evidence suggest that at the active site one substrate, tryptophan, binds to the cuprous moiety and the other substrate, oxygen, binds to the heme iron. It seems possible that in the activated transition complex either or both substrates may serve as bridging ligands between the heme and copper moieties, bringing the system into the appropriate electronic conformation. This generates enzyme-bound electrophilic tryptophan and nucleophilic $\text{O}_2$ species which then interact to form the reaction product, formylkynurenine (Feigelson and Brady, 1974).

**Regulation of tryptophan pyrrolase activity.** Knox (1951, 1955) was the first to demonstrate the enhancement of rat TP activity by both corticosteroids and tryptophan. Glucocorticoid hormones act directly upon the isolated
perfused liver, as well as upon fetal liver cells in organ culture to increase the levels of TP, thus indicating that the hepatocyte is a direct target cell for these hormones (Feigelson and Brady 1974). The increased production of enzyme protein occurs in a process distinct from substrate induction and does not involve prior saturation of apo-TP with its heme cofactor. The hormonal mechanism involves the induction of apoenzyme synthesis, probably by increasing the amount of TP mRNA (De Lap and Feigelson, 1978). The substrate mechanism consists of decreased degradation of pre-existing apo-TP in the presence of a normal rate of synthesis. It is thought that tryptophan stabilizes the enzyme (Badawy and Evans, 1975). There is also evidence to suggest that tryptophan may in addition enhance the synthesis of liver heme (Badawy and Evans, 1973a). However, further work is clearly required to elucidate the mechanism(s) by which tryptophan exerts its effects. The existence of a cofactor mechanism in the regulation of TP activity has been suggested by Badawy (1978) on the basis that the administration of 5-aminolevulinate or hematin increases the saturation of apo-TP with its heme activator (Badawy and Evans, 1973b, 1975), in a way resembling tryptophan administration, however, neither compound stabilizes TP. It has been suggested that reciprocal control mechanisms between hemoprotein and ALAS are involved in the coordinate synthesis of the heme and apoenzyme moieties of TP (Marver et al., 1966). It has also been shown (Badawy, 1977a,b), that saturation of the apoenzyme is sensitive to treatments that
affect the heme biosynthetic pathway.

**Relationships between TP and the "free heme" pool.**

The existence of an interrelation between heme concentration and ALAS and HO activities at the cellular level has led to the concept of a regulatory "free heme pool" (Granick et al., 1975). Although, it has not been possible to define the identity and intracellular localization of this pool, it could be visualized as a small concentration of free or loosely-bound heme with a rapid rate of turnover into which newly synthesized heme is fed and out of which heme is drawn for either the synthesis of hemoproteins or for degradation (De Matteis, 1975).

The theoretical size of the "free heme" pool comes from the work of Granick et al. (1975). In his study, avian embryo liver cells in culture were treated with AIA, after which several concentrations of hemin were added. The hemin concentration that inhibited the increase in the protoporphyrin yield by 50% was 0.05 to 0.1 uM and this was taken as an indication of the "free heme" pool size.

According to Granick's hypothesis, this minute amount of free heme (0.1% of the total hemoprotein-heme of the hepatic parenchyma cell) would be most suitable to control the rate of heme metabolism. If the heme generated in the mitochondria caused the "free heme" to increase beyond 100
nM, the formation of ALAS would be repressed, the increased heme would be degraded more rapidly by HO and, more slowly, it would induce HO. In contrast, when "free heme" decreased below 50 nM the synthesis of ALAS would not be repressed, more of this enzyme would be made, and therefore, more heme would be produced. It was also suggested that the heme combines with some apoprotein to modulate the translation of ALAS. As discussed previously, there is evidence suggesting that TP activity is subject to regulation by its cofactor heme (Druyan and Kelly, 1972), and that the heme saturation is also affected by changes in heme metabolism; the heme saturation of apo-TP is decreased by agents that destroy heme or inhibit its synthesis, whereas the opposite is true for treatments increasing the utilization or the synthesis of this pigment (Badawy and Evans, 1973a). It has been calculated (Badawy, 1978) that during the early depletion of TP by DDC plus phenylbutazone, there is a heme loss of 0.093 uM from the cytosol. This loss represents the largest depletion of TP under conditions of potentiated experimental porphyria, and is similar to the concentration (0.1 uM) suggested by Granick et al. (1975) for the readily exchangeable "free heme". The above considerations have led Badawy (1978) to suggest that TP utilizes the "free heme" pool involved in the regulation of heme biosynthesis, and that this enzyme may play an important role in the regulation of heme biosynthesis.
1.3.5 Epoxide Hydrolase.

Epoxide hydrolase (EH) is the microsomal enzyme that catalyzes the hydration of epoxides to trans-1, 2-dihydrodiols. Its role with regard to normal mammalian function is presently unclear although a number of endogenous steroids and lipids have been found to serve as substrates. Epoxides of cholesterol and fatty acids may be formed during lipid peroxidation and EH could conceivably protect the cell against possible deleterious effects of these lipid epoxides (Watabe et al., 1980). Considerably more attention has been given to the role of EH in metabolizing xenobiotic compounds of toxicological potential. Many chemical substances are aromatic or olefinic compounds which can be metabolized by the microcrosomal P-450 system producing reactive epoxides and arene oxides. Since certain of these compounds have toxic, mutagenic and carcinogenic properties, the ability of epoxide hydrolase (EH) to convert epoxides to chemically less reactive trans-dihydrodiols is an important detoxication mechanism. However, it has also been shown that P-450 and EH convert certain polycyclic aromatic hydrocarbons to more reactive bay region diol epoxide derivatives (Lu and Miwa, 1980). Thus, depending on the particular metabolic pathway, EH can either play the role of activator or inactivator of the mutagenicity of polycyclic aromatic hydrocarbon derivatives.
Some characteristics of the enzyme. The purified microsomal enzyme contains a single polypeptide with a molecular weight of 50,000 in the presence of sodium dodecylsulfate; the aminoacid analysis indicate that it has a relatively high content of tryptophan and tyrosine, and that it does not contain a prosthetic group such as heme or flavin (Lu et al., 1975).

Although the substrate specificity of EH is very broad, it is highly regiospecific with monosubstituted 1, 1- and cis-1, 2- disubstituted epoxides which contain large lipophilic groups being the most readily hydrated (Oesch, 1973). The available evidence indicates that EH-catalyzed hydration occurs by nucleophilic attack at the least hindered epoxide carbon atom by an incoming hydroxyl ion resulting in ring opening away from this ion and the formation of trans-dihydrodiol product (Lu and Miwa, 1980).

Induction of the enzyme. EH is induced by the administration of certain xenobiotics, traditionally the most popular inducing agent has been phenobarbitone (Guengerich, 1982). In earlier studies, 3- methyl- cholanethrene has been reported to be more potent than phenobarbitone (Oesch, 1973), but the effect seems to be variable and in some cases non-existent (Jerina et al., 1977). EH is also induced by polyhalogenated biphenyls (Oesch et al., 1977). In general, compounds that induce EH also appear to induce P-450 and
other drug metabolizing enzymes, but it is thought that these enzymes are under separate genetic control (Oesch et al., 1973).

Epoxides which are good substrates of EH are not good inducers, possibly due to their fast hydration by the enzyme (Schmassmann et al. 1978). Schmassmann and Oesch (1978) studied trans-stilbene oxide as an example of a substance that can induce EH without inducing P-450 enzymes. Although it strongly induced EH, the activity of four monooxygenase parameters was not changed, but the activity of 7-ethoxycoumarin O-deethylation was increased by a similar degree to EH, and the benzo[a]pyrene metabolite pattern was drastically changed (Bucker et al., 1979). Thus, at this time there appears to be no xenobiotic that will specifically alter EH activity.
1.4 Effects of arsenic and selenium on heme metabolism.

1.4.1 Effects of arsenic on heme metabolism.

Woods and Fowler (1977b, 1978) exposed rats and mice to (AsV) in concentrations of 20, 40 and 85 mg/l in drinking water. At the end of the exposure period, a non dose-related decrease in ALAS activity was observed. A marked dose-related decrease in heme synthetase activity was also observed. H0 activity was not determined. In rats, the activities of ALA dehydratase and uroporphyrinogen synthetase were not significantly altered. In contrast, the activity of uroporphyrinogen synthetase was increased in a dose-related manner in mice. Dose-related increases in urinary porphyrins, without significant changes in urinary excretion of ALA or porphobilinogen, were also observed. Cytochrome P-450 concentration and aminopyrine demethylase activity were not significantly altered. Subsequent studies (Fowler and Mahaffey, 1978) showed that the combination of lead plus AsV produced an additive effect on coproporphyrin, but not uroporphyrin excretion. Woods et al. (1981) reported that the addition of HgCl₂, AsV, AsIII and other metals (10 M), -3 reduced the activity of uroporphyrinogen decarboxylase in vitro; further addition of reduced glutathione or dithiothreitol completely prevented the effect.
Martinez et al. (1983) administered AsIII via drinking water in concentrations of 5, 50 and 100 ppm for up to 7 weeks to female rats. A dose-related increase in urinary uroporphyrin excretion was observed after one week of treatment. As the treatment continued, a gradual decrease in the response was observed, such that at the end of treatment only the rats exposed to the highest level showed significant uroporphyrinuria. These changes in the magnitude of the response were interpreted as part of an adaptive process which may limit the usefulness of uroporphyrin excretion as a biological index of As toxicity.

Sardana et al. (1981) reported that AsIII (25 to 100 umol/kg) produced dose-related increases in hepatic ALAS and HO activities accompanied by corresponding reductions in P-450 content. AsV (25 to 100 umol/kg) did not increase hepatic HO, but a small increase in renal HO was observed. Administration of the parasiticidal agents melarsoprol (organic AsIII) and tryparsamide (organic AsV) also increased hepatic HO activity. However, Sardana et al. (1982) reported that in chick embryo liver cells, both AsIII and melarsoprol were powerful HO inducers while AsV and tryparsamide failed to do so.

These effects and their implications are discussed in detail in the following Chapters.
1.4.2 Effects of selenium on heme metabolism.

Maines and Kappas (1976a) reported that sodium selenite administered subcutaneously to rats (10 to 100 umol/kg), produced non dose-related increases in ALAS activity and dose-related increases in HO activity. A dose-related inhibition in ethylmorphine N-demethylase activity was also observed. No significant reductions in P-450 content or changes in hepatic porphyrin concentration were noted. These findings were observed 14.5 hours after treatment. Eaton et al. (1980) reported that doses of 13 and 25 umol/kg increased HO activity. However, only the highest dose reduced P-450 concentration in 25%. Sardana et al. (1982) reported that addition of sodium selenite to cultured chick embryo cells (concentration not stated) did not increase HO activity.

Schnell et al. (1983) reported that 72 hours after i.p. administration of 2.4 mg/kg of sodium selenite there was a significant decrease in ethylmorphine N-demethylase activity (28%) and cytochrome P-450 content (18%). No change in aniline hydroxylase or NADPH cytochrome c reductase was observed. Exposure to selenite in the drinking water (1, 2 or 4 mg/l) for 30 days produced no alterations in any of the parameters measured. The addition of selenite to microsomes (final concentration 10^-3 M) obtained from untreated rats resulted in 40% inhibition of aniline hydroxylase and
ethylmorphine N-demethylase activities. However, no change was observed in P-450 concentration. Reiter and Wendel (1985) reported that exposure to SeIV in the diet (10 ppm) for 6 months produced no significant effects on HO activity or in aminopyrine-N-demethylase. However, a 50% reduction in P-450 concentration and ethoxycoumarin O-deethylase activity was observed.

These effects and their implications are discussed in detail in the following Chapters.

**Aims of this study** This work was undertaken to study some effects of arsenic and selenium on heme metabolism with reference to: 1) the time-course and dose relationships of As effects on the hepatic "free heme" pool, as measured by the heme saturation of tryptophan pyrrolase; 2) some aspects of the target organ selectivity of AsIII and AsV, with special reference to testicular effects; 3) The comparison between the effects of As and Se on cytochrome P-450 and P-450-dependent monooxygenases. The overall objective being to gain some insight on the effects of these metalloids on heme metabolism which might be related to the genesis of overt toxic effects.
CHAPTER TWO

MATERIALS AND METHODS
2.1 CHEMICALS.

Sodium arsenite and sodium arsenate, Suprapur grade, were supplied by Merck GmbH (Darmstadt, West Germany). Sodium selenite and cadmium chloride were obtained from British Drug Houses Ltd. (Poole, Dorset).

NADH, NADPH, glycine, ATP, sucrose, EDTA, sodium succinate, biliverdin, pyridoal-5'-phosphate, bilirubin and Tris (Trizma) were all purchased from Sigma London Chemical Co. Ltd. (Poole, Dorset).

\[ ^{14} \text{[2,3-C]} \text{ succinic acid, } ^{2} \text{[7-H]} \text{ styrene oxide and } \begin{array}{c} ^{14} \text{C} \ 2 \\
^{14} \text{C} \ 3 \\
\end{array} \text{ amino } ^{4} \text{clevulnic acid hydrochloride were supplied by Amersham International plc (Amersham, Buckinghamshire).} \]

Hemin, ALA and L-tryptophan were supplied by British Drug Houses Ltd. (Poole, Dorset).

Sodium dithionite and Folin-Ciocalteu phenol reagent were obtained from Fisons Scientific Equipment Ltd. (Loughborough, Leicestershire).

Styrene oxide was purchased from Aldrich Chemical Co. Ltd. (Poole, Dorset).
Ethoxyresorufin (50 μM in methanol) and resorufin (10μM in ethanol) were purchased from Pierce and Warriner Ltd. (Chester, Cheshire).

The Dowex AG 50W-X8 resin in the sodium form, 200 - 400 mesh, was obtained from Bio-Rad Co. (Richmond, California).

Carbon monoxide and oxygen were supplied by British Oxygen Co. (London).

All other reagents were of Analar grade or equivalent.
2.2 ANIMALS AND TREATMENTS.

2.2.1 Animals.

Male Wistar Albino rats, of the University of Surrey strain (180-210g), were obtained from the University of Surrey Animal Unit. The rats were housed in cages with sawdust bedding (Lee and Co., Chertsey, Surrey), and were allowed food (LAD-1, K + K, Croydon) and water ad libitum, except when indicated. A 12 h light/dark cycle was operated (0700-1900 light), at a temperature of 22 °C and 50% humidity.

2.2.2 Treatments.

Food deprivation

In the acute studies, animals were allowed access to food and water ad libitum for 24 hours, then deprived of food for the following 24 hours and subsequently treated. Food deprivation was continued until the animals were sacrificed. In the subchronic study, food was withheld for 24 hours before sacrifice.

Control animals

Control animals received 0.1 ml of sodium chloride 0.9% (w/v) by subcutaneous injection in the loose tissues of
the abdominal region. Animals were killed by cervical dislocation 16 h after the injection, except where indicated.

**Treated animals**

The elements were administered as a solution in distilled water and injected subcutaneously in the loose tissues of the abdominal region, the cervical region was also used when two different metalloids were injected. The doses of sodium arsenite (AsIII) were 12.5, 25, 50, 75 and 100 umol/kg. Those of sodium arsenate (AsV) were 25, 50, 100, 150 and 200 umol/kg. Those of sodium selenite (SeIV) were 2.5, 5, 10, 20 and 40 umol/kg. Those of cadmium chloride were 7 and 20 umol/kg. Animals were sacrificed by cervical dislocation 16 h after the injection. Alternatively, for time course studies, animals were killed at various time intervals. In the subchronic study, the animals were exposed to AsIII in the drinking water at a concentration of 50 mg/l for a period of 5, 10, 20 and 30 days, and sacrificed as described above.
2.3 PREPARATION OF SUBCELLULAR FRACTIONS FROM LIVER, KIDNEY AND TESTIS.

This method is based on that of de Duve et al. (1959).

**Principle.** The differential centrifugation of tissue homogenates separates the components of interest into fractions which can then be analysed for their enzyme content, revealing the association of particular enzymes with cellular structures.

**Reagents**

- 20 mM Tris-HCl buffer, pH 7.4, containing 250 mM sucrose.
- 20 mM Tris-HCl buffer, pH 7.4, containing 250 mM sucrose and 5.4 mM EDTA.

**Method**

Livers were perfused in situ through the portal vein with ice-cold 0.9% NaCl until bleached of hemoglobin. Liver and other organs excised at sacrifice were blotted dry and weighed. After scissor-mincing they were homogenised in 3 volumes of ice-cold 20 mM Tris-HCl buffer, pH 7.4, containing sucrose (250 mM), using three strokes of a Potter-Elvejhem glass-teflon homogeniser, with a pestle rotating at 2400
A sample of the homogenate was retained for further analysis, and the remainder was centrifuged in 50 ml polycarbonate tubes at 10,000 x g for 20 minutes, using an 8 x 50 ml aluminium head rotor in an MSE "High Speed 18" refrigerated centrifuge at 4 °C. The supernatant was decanted and recentrifuged at 40,000 rpm (105,000 x g) for one hour, using an 8 x 35 titanium angle head rotor in an MSE "PrepSpin 50" refrigerated centrifuge.

The supernatant obtained (cytosol) was retained for further analysis, and the pellet was resuspended in ice-cold 20 mM Tris-HCl, pH 7.4, containing sucrose (250 mM), using a Potter-Elvehjem homogeniser. The resulting suspension was recentrifuged at 40,000 rpm for 1 hour. The washed microsomal pellet was finally resuspended in ice-cold 20 mM Tris-HCl buffer, pH 7.4, containing sucrose (250 mM) and EDTA (5.4 mM), to a concentration equivalent to 1.0 g wet weight of tissue per ml. The microsomal suspension was either assayed immediately or stored in 1.5 ml aliquots in polycarbonate sample cups at -20°C.
2.4 ASSAY OF SOLUBLE ENZYMES.

2.4.1 Tryptophan Pyrrolase.

This method is based on that of Badawy and Evans (1975).

Principle. The product of tryptophan oxidation by TP, formylkynurenine, is hydrolyzed by formylase normally present in excess in liver. The kynurenine formed is determined by its absorption at 365 nm. It can be measured either in the absence (holoenzyme activity) or in the presence ("total enzyme" activity) of added hematin. The apoenzyme activity is calculated by difference.

Reagents

0.1 M Potassium phosphate buffer, pH 7.4, containing sucrose (250 mM). In the subchronic study, this buffer was substituted by 20 mM Tris-HCl buffer at the same pH and sucrose concentration.

0.03 M L-tryptophan.
0.2 M Sodium phosphate buffer, pH 7.0.
0.6 M Sodium hydroxide.
0.9 M Trichloroacetic acid.
0.36 mM Hematin hydrochloride in 0.1 M sodium
hydroxide (1.17 mg/5 ml).

Method.

Samples of the homogenate (4.5 ml) were diluted with 4.5 ml of the homogenising buffer. They were added to the incubation mixture containing 3 ml of tryptophan (0.03 M), 9 ml of 0.2 M sodium phosphate buffer pH 7.0 and 15 ml of distilled water. The mixture was divided into two aliquots, and 0.1 ml of the hematin solution was added to one aliquot, to give a final concentration of 2 uM. Samples (3 ml) of the assay mixture where incubated at 37 C for 0, 15, 30, 40, 50 and 60 minutes with shaking in stoppered tubes in an atmosphere of 0. The reaction was stopped at each of the above time-intervals by the addition of 2 ml of 0.9 M trichloroacetic acid; the tubes and contents were shaken for a further 15 min and then centrifuged at 2,000 rpm for 20 min. To 2.5 ml of the supernatant, 1.5 ml of 0.6 M were added. The kynurenine present was determined at 365 nm in a Perkin-Elmer Lambda 5 spectrophotometer, and the concentration was calculated using an extinction coefficient of 4540 M cm⁻¹. TP activity was calculated from the increase in the E with time during the linear phase and expressed as umol of kynurenine formed/g (wet weight) of liver.
2.4.2 ALA Synthetase.

This method is based on that of Condie and Tephly (1978), as modified by De Matteis (1981).

**Principle.** ALAS catalyzes the condensation of glycine and succinyl-CoA to form ALA. Its activity is determined in rat tissues by measuring the incorporation of $^{14}$C]succinate into ALA and by isolating the $^{14}$C]ALA on Dowex 50 ion-exchange columns. This is essentially the method described by Ebert et al. (1970), but modified by the authors mentioned above.

**Reagents.**

Reaction Cocktail (Tris-glycine-EDTA):
1 M Tris-HCl, pH 7.2 (90 ml).
1 M Glycine (120 ml).
0.1 M EDTA (90 ml).
1.6 mM Pyridoxal phosphate.

The pH was adjusted to 7.5 with 1 M NaOH. This solution was prepared fresh each week and stored at 4°C. Pyridoxal phosphate (7.9 mg/20 ml) was added to the cocktail on the day of the experiment.

4 mM Sodium succinate.

$^{14}$C]succinic acid was dissolved in 4 mM succinate to achieve a specific activity of 2.73 uCi/μmol.
5-amino \([\text{C}^-\text{C}]\) levulinic acid hydrochloride was dissolved in 10 mM HCl to achieve a specific activity of 1 uCi/ml.

Carrier ALA: 16.3 mg/10 ml 0.1 M HCl.

1.8 mM ATP (5 mg/ml).

25\% (w/v) Trichloroacetic acid.

1\% (w/v) Trichloroacetic acid.

1 M Acetate buffer, pH 4.6.

0.1 M Acetate buffer pH 3.9.

Methanol – 0.1 M acetate buffer pH 3.9 mixture (2:1, v/v).

1 M HCl.

10 mM HCl.

1 M Sodium acetate.

Equilibrated ethylacetate: Equal volumes of 1 M acetate buffer (pH 4.6) and ethylacetate were mixed. The layers were allowed to separate and the top layer was collected.

**Preparation of cation exchange resin.**

Dowex 50 resin (AG 50W-X8, H form) 200-400 mesh, was converted to the sodium form by heating 2-3 times in 2 M NaOH at 50°C until no colour was observed in the wash. The resin was then washed extensively with water and equilibrated with 0.1 M acetate buffer, pH 3.9. The resin was added to chromatographic columns (20 cm x 1 cm). A resin column of 2
cm in height was used.

**Method.**

An aliquot (0.5 ml) of a 10% (w/v) whole organ homogenate was added to the incubation mixture containing 250 ul of the reaction cocktail, 100 ul sodium [2,3 C] succinate, 150 ul ATP. The combined reagents were incubated without shaking for 1 h. At the end of the incubation period, 250 ul of trichloroacetic acid (25%) were added to stop the reaction, followed by 50 ul of carrier ALA.

**Isolation and counting of ALA.**

Following centrifugation, supernatant separation and pellet resuspension in 1.5% TCA, the supernatants were pooled and added to the chromatographic column. The sample was allowed to flow through and the columns were washed successively with 20 ml of 0.1 M acetate buffer (pH 3.9), 20 ml methanol-0.1 M acetate buffer, pH 3.9 (2:1 v/v), and 10 ml of 0.01 M HCl. The ALA was then eluted with 5 ml of 1 M sodium acetate, pH 8.5. As a standard, samples containing 5-14 amino [4- C] levulinic acid instead of [2,3- C ] succinic acid were run through the same process. Over 90% of the ALA applied to the columns was recovered by these isolation procedures.

An aliquot (0.5 ml) of the eluate was taken for
counting. In a stoppered tube, 4.5 ml 1 M acetate buffer (pH 4.6), 1.5 ml 1M HCl and 100 ul of acetyl acetone were added to the remaining 4.5 ml of eluate. The tubes were stoppered tightly and boiled for 20 minutes, allowed to cool and added with 4.5 ml of equilibrated ethyl acetate. The mixture was shaken and allowed to separate. 2 ml of the ethyl acetate phase were counted.

Enzyme activity was expressed as pmol of ALA formed per mg of homogenate protein per hour.

2.4.3 Biliverdin reductase.

This method is based on that of Krasny and Holbrook (1977).

Principle. Biliverdin reductase is the cytosolic enzyme that catalyzes the reduction of the central alpha-methene bridge of biliverdin to bilirubin. The enzyme activity is measured spectrophotometrically by following the rate of bilirubin formation at 468 nm.

Reagents.

20 mM Tris-HCl buffer, pH 7.4, containing sucrose (250 mM) and EDTA (5.4 mM).

60 uM Biliverdin.
27 mM NADPH.

Cytosol ~ 20 mg protein/ml.

Method.

To an appropriate amount of cytosol in a test tube (usually 1 ml for liver, 1.5 ml for kidney and 3 ml for testis), were added 100 ul of biliverdin solution, and corresponding amounts of Tris-HCl/sucrose buffer to make a total volume of 3 ml. The suspension was gently agitated to mix the contents and then divided equally between two 1.5 ml optical cuvettes having a 1 cm light path. The cuvettes were preincubated for 5 minutes at 37 °C in a constant temperature cuvette chamber. The reaction was then initiated by addition of 30 ul of NADPH to the sample cuvette and the same volume of buffer to the reference cuvette. The rate of bilirubin formation was determined by continuously recording for 5 minutes the increase in optical density at 468 nm. Enzyme activity was calculated using a millimolar extinction coefficient of 30 mM cm⁻¹ and expressed as nmol of bilirubin formed per hour, per mg of cytosolic protein.
2.5 ASSAY OF MICROsomAL ENZYMES.

2.5.1 Heme oxygenase.

This method is based on that of Tenhunen et al. (1969).

Principle. The microsomal heme oxygenase catalyzes the oxidative cleavage of the heme (ferriprotoporphyrin) ring at the alpha-methene bridge, to form the linear tetrapyrrole biliverdin IX. The initial product formed from HO (biliverdin) has a broad absorption peak with a maximum at 670 nm with a low extinction coefficient and is not an optimal reaction product to assay spectrophotometrically. For this reason, a source of NADPH-dependent biliverdin reductase is added in excess to the incubation mixture to quantitatively convert the biliverdin formed during the reaction to bilirubin, which is then measured spectrophotometrically, by following the rate of formation of bilirubin at 468 nm.

Reagents.

20 mM Tris-HCl buffer, pH 7.4, containing sucrose (250 mM) and EDTA (5.4 mM).

1.7 mM Hematin hydrochloride dissolved in 0.1 M sodium hydroxide.
27 mM NADPH.
Microsomal suspension, ~ 20 mg protein/ml.
Cytosol ~ 20 mg protein/ml.

Method.

To an appropriate amount of microsomal suspension in a test tube (usually 0.5 ml for liver and testis, and 1 ml for kidney) were added respectively 2.5 or 2.0 ml of TrisHCl buffer, pH 7.4, containing sucrose (250 mM) and EDTA (5.4 mM), 0.5 or 1 ml of cytosol and 44 ul of hemin hydrochloride, to make a total volume of 3.5 ml. The suspension was gently agitated to mix the contents and then divided equally between two optical cuvettes having a 1 cm light path. The cuvettes were preincubated for 5 min at 37 C in a constant temperature cuvette chamber. The reaction was then initiated by addition of 20 ul of NADPH to the sample cuvette and the same volume of buffer to the reference cuvette. The rate of bilirubin formation was determined by continuously recording (5 to 10 min) the increase in optical density at 468 nm. Enzyme activity was calculated using a millimolar extinction coefficient of 30 mM cm \(^{-1}\) and expressed as nmol of bilirubin formed per hour per mg of microsomal protein.
2.5.2 Epoxide Hydrolase.

This method is based on that of Oesch et al. (1971).

**Principle.** Microsomal epoxide hydrolase catalyses the hydration of both alkene and arene oxides to the corresponding trans-dihydrodiols. The activity was determined by measuring the conversion of \([\text{-H}^3\text{-H}]\)-styrene oxide to \([\text{-H}^3\text{-H}]\)-styrene glycol.

**Reagents.**

- 0.5 M Tris-HCl buffer, pH 9.0.
- 40 mM \([\text{-3-H}\text{-H}]\)-styrene oxide (156.7 uCi/mmol) in acetonitrile.

**Method.**

To an appropriate amount of hepatic microsomal suspension in a Sovirel tube (usually 100 ul) were added 100 ul of 0.5 M Tris-HCl buffer (pH 9.0) and distilled water to give a final volume of 380 ul. The tubes were preincubated in a shaking water bath (120 cycles/min) at 37°C for 5 minutes. The reaction was started by adding 20 ul of \([\text{-H}^3\text{-H}]\)-styrene oxide working solution (2.0 mM, 125 nCi). After 7 minutes of incubation at 37°C, the reaction was terminated by the addition of 9 ml of petroleum ether (boiling range 40-60°C).
The incubates were extracted on a rotary mixer, then centrifuged for 3 minutes at 1000 rpm. The aqueous and organic layers were separated by freezing in a cardice/ethanol bath and the upper organic layer was discarded. To the remaining aqueous phase, 9 ml of petroleum ether were added and the process of tumble mixing and layer separation was repeated. Two ml of ethyl acetate were added to the remaining aqueous phase and tumble mixed for 10 minutes in order to extract the reaction product, [7- H]-styrene dihydrodiol. Aqueous and organic phases were separated by centrifugation at 2000 rpm for 5 minutes and 0.5 ml aliquots of the ethyl acetate layer were removed for scintillation counting. Blanks containing boiled microsomes, 3 and [7- H]-styrene oxide standards were also prepared.

2.5.3 Hepatic cytochrome P-450.

This method is based on that of Omura and Sato (1964).

**Principle.** Sodium dithionite when added to a microsomal suspension reduces the cytochrome P-450. The subsequent gassing of the sample with CO results in a CO-complex of reduced cytochrome P-450, which has a maximal spectral absorption at 450 nm.
Reagents.

Carbon monoxide.
Sodium dithionite, solid.
66 mM Tris-HCl buffer, pH 7.4.

Method.

Aliquots of microsomal suspensions were diluted to about 2 mg protein/ml with 66 mM Tris-HCl buffer, pH 7.4. A few crystals of sodium dithionite were added, mixed and the solution divided equally between two cuvettes. Following the establishment of a baseline of equal light absorbance between 400 and 500 nm, carbon monoxide was bubbled through the sample cuvette for 30 seconds, and the resulting difference spectrum was recorded. The amount of cytochrome P-450 present in the sample was calculated using the peak absorbance at 450 nm minus the absorbance at 490 nm, using an extinction coefficient of 91 mM cm$^{-1}$.

2.5.4 Hepatic cytochrome b.

This method is due to Omura and Sato (1964).

Principle. The addition of a source of reducing equivalents, NADH, to the contents of the sample cuvette containing a catalytic amount of NADH-cytochrome b reductase, results in
the reduction of cytochrome b with an associated change in spectra between 409 and 424 nm.

Reagents.

- 66 mM Tris-HCl buffer, pH 7.4.
- 11.5 mM NADH.

Method.

Aliquots of microsomal suspensions were diluted to about 2 mg protein/ml with 66 mM Tris-HCl buffer pH 7.4, and were divided equally between two cuvettes. Following the establishment of a baseline of equal light absorbance between 400 and 500 nm, NADH was added to the sample cuvette to give a final concentration of 0.45 mM and the resulting difference spectrum was recorded. The concentration of cytochrome b was calculated by measuring the change in absorbance between 424 and 409 nm using an extinction coefficient of 185 mM cm\(^{-1}\).

2.5.5. Renal cytochromes P-450 and b.

This method is based on that of Orrenius et al. (1973).

Principle. According to Orrenius et al. (1973), the classical method for estimation of cytochromes P-450 and b...
gives inaccurate results if hemoproteins which have CO-induced absorbance bands in the 450-490 nm wavelength region are present in great quantities in the microsomal suspension. At least two such proteins, hemoglobin and mitochondrial cytochrome oxidase, are present in kidney microsomes.

Reagents.

20 mM Tris-HCl buffer, pH 7.4, containing sucrose (250 mM).
300 mM Sodium succinate.
16.7 mM NADH
Carbon monoxide.
Sodium dithionite, solid.

Method.

Aliquots of microsomal suspensions were diluted to about 2 mg protein/ml with 200 mM Tris-HCl buffer, pH 7.4 containing 250 mM sucrose. 100 ul of 300 mM sodium succinate were added to 3 ml of microsomal suspension, mixed and bubbled with CO for 30 seconds. The mixture was incubated at room temperature until maximal mitochondrial pigment reduction was achieved. Following the establishment of a baseline of equal light absorbance between 400 and 500 nm, 20 ul of 16.6 mM NADH were added to the sample cuvette and the resulting difference spectrum was recorded. A few crystals of sodium dithionite were added to the sample cuvette and the
resulting difference spectrum was again recorded. The amounts of cytochromes P-450 and b present in the sample were calculated using extinction coefficients of 91 and 185 mM cm$^{-1}$ respectively.

2.5.6 Ethoxycoumarin O-deethylase.

This method is based on that of Ullrich and Weber (1972).

**Principle.** 7-ethoxycoumarin is metabolised by hepatic microsomes to a single major fluorescent product, 7-hydroxycoumarin (umbelliferone). The rate of reaction may be measured by monitoring the linear increase in fluorescence with time.

**Reagents.**

- 66 mM Tris-HCl buffer, pH 7.4.
- 1 mM 7-Ethoxycoumarin.
- 1 mM 7-Hydroxycoumarin.
- 6.2 mM NADPH.

**Method.**

100 ul of microsomal suspension (about 20 mg protein/ml), 250 ul of 7-ethoxycoumarin (1 mM) and 2.15 ml of
66 mM Tris-HCl buffer, pH 7.4 were mixed in a fluorescence cuvette and allowed to equilibrate to 37°C for 5 minutes in the thermostatted cuvette holder of a Perkin-Elmer LS-5 fluorescence spectrophotometer. The baseline was recorded and 20 µl of 6.2 mM NADPH was added to the cuvette and mixed. The increase in fluorescence at 450 nm was monitored for at least 3 minutes, using an excitation wavelength of 370 nm. Standards of 7-hydroxycoumarin were added to calibrate the reaction.

2.5.7 Ethoxyresorufin O-deethylase.

This method is based on that of Burke and Meyer (1975).

Principle. Hepatic microsomes metabolise 7-ethoxyresorufin to a single fluorescent product, resorufin. The rate of the reaction may be measured by monitoring the linear increase in fluorescence with time.

Reagents.

- 66 mM Tris-HCl buffer, pH 7.4.
- 50 µM 7-ethoxyresorufin in methanol.
- 10 µM resorufin in ethanol.
- 31 mM NADPH.
Method.

50 ul of microsomal suspension (about 20 mg protein/ml), 10 ul of 50 uM 7-ethoxyresorufin and 2.45 ml of 66 mM Tris-HCl buffer, pH 7.4, were mixed in a fluorescence cuvette and allowed to equilibrate to 37 C for 5 minutes in the thermostatted cuvette holder of a Perkin-Elmer LS-5 fluorescence spectrophotometer. The base line was recorded and 20 ul of 31 mM NADPH were added to the cuvette and mixed. The increase in fluorescence at 586 nm was monitored for at least 3 minutes, using an excitation wavelength of 510 nm. Standards of resorufin were added to calibrate the reaction.
2.6 PROTEIN ASSAY IN TISSUE SUBFRACTIONS.

This method is based on a modification of that of Lowry et al. (1951).

**Principle.** Two reactions are involved in this method: 
a) an initial interaction of protein and Cu(II) in alkali; b) 
a reduction of the phosphotungstic and phosphomolybdic acids 
to molybdenum blue and tungsten blue, both by the Cu-protein 
complex and by the tyrosine and tryptophan of the protein. 
The latter two aminoacids give colour in the absence of 
Cu(II), but the rest of the protein gives no colour without 
Cu(II). About 75% of the colour is dependent on the Cu(II).

**Reagents.**

- 0.5 M NaOH.
- 1% (w/v) Copper sulphate pentahydrate.
- 2% (w/v) Sodium potassium tartrate.
- 0.05 M NaOH, containing 2% (w/v) sodium carbonate.
- Folin-Ciocalteu phenol reagent, diluted with water 
  (1:2, v/v).
- Bovine serum albumin in 0.05 M NaOH (250 ug/ml).

**Method.**

Samples of the homogenates were predigested with 0.5 M
NaOH. The samples of tissue subfractions were diluted with 50 mM NaOH to give appropriate protein concentrations. The protein standard was diluted with 50 mM NaOH to give concentrations of 50, 100, 150, 200 and 250 ug/ml. Copper sulphate, sodium potassium tartrate and sodium carbonate in NaOH were mixed in a ratio 1:1:100 (v/v) immediately before use. The protein standards, samples and blanks (0.5 ml) were mixed with the sodium carbonate reagent (5 ml) and allowed to stand for 10 minutes at room temperature. Diluted Folin-Ciocalteau phenol reagent (0.5 ml) was added, and the solutions were mixed immediately. After standing for a minimum of 30 minutes at room temperature, the absorbance at 750 nm was recorded using a Perkin-Elmer Lambda 5 spectrophotometer.

2.7 STATISTICS.

The results obtained were reported as the mean plus/minus one standard deviation. Statistically significant differences between individual treatments and control were determined by the standard "t" test. The value of p < 0.05 was regarded as denoting significance.
CHAPTER THREE

SOME EFFECTS OF ARSENIC AND
SELENIUM ON HEPATIC HEME METABOLISM
3.1 INTRODUCTION.

Arsenic and selenium are common environmental agents which have been shown to produce hepatotoxic effects in various species following acute or subacute exposure (Fowler et al., 1979; Fishbein, 1977). Exposure to these metalloids is also known to alter the activities of some enzymes in the heme synthesis pathway (Maines and Kappas, 1976a; Woods and Fowler, 1978; Sardana et al., 1981).

This Chapter describes the acute effects of these metalloids on the heme saturation of TP, a sensitive indicator of "free heme" pool changes (Badawy, 1979). They are discussed in relation to the concomitant effects on the activities of ALAS and HO, respectively the rate limiting enzymes of heme synthesis and catabolism. The differences in potency between two different oxidation states of arsenic, AsIII and AsV, are also studied. A comparison between the effects of single and continuous administration of AsIII is made and the possible appearance of tolerance to its toxic effects is examined.
3.2 RESULTS.

3.2.1 Effects of sodium arsenite (AsIII).

a) Tryptophan pyrrolase.

The effects of 5 different dose levels ranging from 12.5 to 100 umol/kg at 24 hours are shown in table 3.1, and the effects of a single dose at different times (2 to 24 hours) are shown in table 3.2. The AsIII was administered by subcutaneous injection.

Dose response study. At dose levels above 12.5 umol/kg, there was a progressive decrease in the heme saturation of TP (Table 3.1). At the lowest dose (12.5 umol/kg) both calculated apoenzyme values and holoenzyme activity were increased. Above 50 umol/kg a progressive decrease in holoenzyme activity was observed and the calculated values of apoenzyme were increased. Although the holoenzyme/apoenzyme ratio changes were more pronounced, they closely paralleled those observed in the heme saturation percentage.

Time course study: during the first 4 hours after 100 umol/kg treatment with AsIII, a small but significant increase in the degree of heme saturation was observed. This was followed by a gradual decrease which was still falling
Table 3.1 Effects of sodium arsenite (AsIII) on rat liver tryptophan pyrrolase.

<table>
<thead>
<tr>
<th>AsIII (umol/kg)</th>
<th>Apoenzyme</th>
<th>Holoenzyme</th>
<th>Total</th>
<th>Saturation %</th>
<th>Holo/Apo ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.8 ± 0.9</td>
<td>2.8 ± 0.6</td>
<td>7.6 ± 1.4</td>
<td>37.3 ± 3.9</td>
<td>0.60 ± 0.11</td>
</tr>
<tr>
<td>12.5</td>
<td>6.9 ± 0.6</td>
<td>4.1 ± 0.4</td>
<td>11.0 ± 1.0</td>
<td>37.1 ± 0.4</td>
<td>0.59 ± 0.01</td>
</tr>
<tr>
<td>25.0</td>
<td>7.3 ± 0.3</td>
<td>2.9 ± 0.4</td>
<td>10.1 ± 0.4</td>
<td>28.2 ± 2.8</td>
<td>0.39 ± 0.05</td>
</tr>
<tr>
<td>50.0</td>
<td>6.3 ± 1.1</td>
<td>1.9 ± 0.4</td>
<td>8.2 ± 1.4</td>
<td>23.1 ± 2.5</td>
<td>0.30 ± 0.04</td>
</tr>
<tr>
<td>75.0</td>
<td>7.0 ± 1.7</td>
<td>1.6 ± 0.4</td>
<td>8.6 ± 2.0</td>
<td>18.1 ± 2.6</td>
<td>0.22 ± 0.04</td>
</tr>
<tr>
<td>100.0</td>
<td>6.4 ± 0.5</td>
<td>1.1 ± 0.1</td>
<td>7.5 ± 0.5</td>
<td>15.2 ± 2.2</td>
<td>0.18 ± 0.03</td>
</tr>
</tbody>
</table>

1 Activities are expressed as umol/h of kynurenine formed per g (wet weight) of liver: \( \bar{X} \pm S.D. \) (n = 24 for controls, n = 4 for other data). These effects were studied at 24 hours after AsIII administration.

* Differs from control data p < 0.05.
### Table 3.2 Time-course of sodium arsenite (100 umol/kg) effects on rat liver tryptophan pyrrolase.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Apoenzyme</th>
<th>Holoenzyme</th>
<th>Total</th>
<th>Saturation %</th>
<th>Holo/Apo ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.8 ± 0.9</td>
<td>2.8 ± 0.6</td>
<td>7.6 ± 1.4</td>
<td>37.3 ± 3.9</td>
<td>0.60 ± 0.11</td>
</tr>
<tr>
<td>2</td>
<td>1.5 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>2.7 ± 0.3</td>
<td>42.8 ± 3.0</td>
<td>0.75 ± 0.10</td>
</tr>
<tr>
<td>4</td>
<td>0.8 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>1.5 ± 0.2</td>
<td>43.0 ± 3.3</td>
<td>0.76 ± 0.10</td>
</tr>
<tr>
<td>8</td>
<td>2.0 ± 0.3</td>
<td>1.0 ± 0.1</td>
<td>3.0 ± 0.3</td>
<td>32.9 ± 3.2</td>
<td>0.50 ± 0.07</td>
</tr>
<tr>
<td>16</td>
<td>4.4 ± 0.9</td>
<td>1.3 ± 0.3</td>
<td>5.7 ± 1.3</td>
<td>22.4 ± 1.3</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>24</td>
<td>6.4 ± 0.5</td>
<td>1.1 ± 0.1</td>
<td>7.5 ± 0.5</td>
<td>15.2 ± 2.2</td>
<td>0.18 ± 0.03</td>
</tr>
</tbody>
</table>

1 Activities are expressed as umol/h of kynurenine formed per g (wet weight) of liver: mean ± S.D. (n = 24 for controls, n = 4 for other data). AsIII was administered by subcutaneous injection.

*Differs from control data p < 0.05.
when the last observation was made at 24 hours (Table 3.2). During the first 4 hours, "total enzyme", holoenzyme activities and apoenzyme values, were markedly reduced by amounts which resulted in the transient increases in heme saturation shown in table 3.2. The holoenzyme activity continued to be depressed for the remaining period, whereas "total enzyme" activity and apoenzyme values returned to near control values.

b) ALA synthetase.

The dose response data are shown in table 3.3. The enzyme activity showed a dose-dependent increase, being significant at doses of 50 and 75 umol/kg of AsIII. At a dose of 75 umol/kg, the enzyme activity was about twice that of the control level.

c) Heme oxygenase.

The increase in this enzyme activity was progressive and pronounced at all dose levels above 12.5 umol/kg (Table 3.3). The increment in activity appeared to follow the increase in dosage. At the highest dose (100 umol/kg) a 12-fold increase over control values was observed.

d) Biliverdin reductase.

No change of activity was observed at any dose level.
Table 3.3 Effects of sodium arsenite (AsIII) on hepatic ALA synthetase, heme oxygenase and biliverdin reductase.

<table>
<thead>
<tr>
<th>AsIII (umol/kg)</th>
<th>ALA Synthetase(^1)</th>
<th>Heme Oxygenase(^2)</th>
<th>Biliverdin Reductase(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>134.8 ± 28.2</td>
<td>1.70 ± 0.25</td>
<td>20.5 ± 3.7</td>
</tr>
<tr>
<td>12.5</td>
<td>134.6 ± 23.4</td>
<td>1.71 ± 0.31</td>
<td>19.2 ± 2.4</td>
</tr>
<tr>
<td>25</td>
<td>153.4 ± 26.2</td>
<td>3.90 ± 0.42</td>
<td>19.3 ± 4.4</td>
</tr>
<tr>
<td>50</td>
<td>187.6 ± 20.0</td>
<td>9.28 ± 0.12</td>
<td>21.9 ± 5.8</td>
</tr>
<tr>
<td>75</td>
<td>265.4 ± 16.6</td>
<td>14.09 ± 0.62</td>
<td>20.2 ± 5.4</td>
</tr>
<tr>
<td>100</td>
<td>N.A.</td>
<td>20.67 ± 0.88</td>
<td>18.8 ± 3.2</td>
</tr>
</tbody>
</table>

1 pmol ALA/mg homogenate protein/h.
2 nmol bilirubin/mg microsomal protein/h.
3 nmol bilirubin/mg cytosolic protein/h.

All above given as \( \bar{x} \pm S.D. \) (n = 17 for controls, n = 3 for other data). These effects were studied at 16 hours after subcutaneous administration of AsIII.

* Differs from control data, p < 0.05.

N.A. Data not obtained.
c) Cytochromes P-450 and b$_5$

There was a progressive decrease in P-450 concentration at all dose levels above 25 umol/kg. At the highest dose (100 umol/kg) the concentration was reduced to 44% of the control level (Table 3.4). No effect on cytochrome b$_5$ concentration at any dose level was observed (Table 3.4).

Figure 3.1 summarizes the effects of AsIII on some indicators of heme status.
Table 3.4 Effects of sodium arsenite (AsIII) on hepatic cytochromes P-450 and b5.

<table>
<thead>
<tr>
<th>AsIII (umol/kg)</th>
<th>Cytochrome P-450</th>
<th>Cytochrome b5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.63 ± 0.08</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td>12.5</td>
<td>0.58 ± 0.05</td>
<td>0.27 ± 0.01</td>
</tr>
<tr>
<td>25</td>
<td>0.51 ± 0.09</td>
<td>0.28 ± 0.04</td>
</tr>
<tr>
<td>50</td>
<td>0.42 ± 0.07</td>
<td>0.26 ± 0.04</td>
</tr>
<tr>
<td>75</td>
<td>0.37 ± 0.07</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td>100</td>
<td>0.28 ± 0.06</td>
<td>0.26 ± 0.03</td>
</tr>
</tbody>
</table>

1 nmol/mg microsomal protein.

All above given as $\bar{x} \pm$ S.D. (n = 17 for controls, n = at least 3 for other data). These effects were studied at 16 hours after AsIII administration.

* Differs from control data, p < 0.05.

N.A. Data not obtained.
Fig. 3.1 Effects of sodium arsenite (AsIII) on some indicators of cellular heme status. The effects were studied at 16 hours after subcutaneous administration of AsIII.
3.2.2 Effects of sodium arsenate (AsV).

a) Tryptophan pyrrolase.

Dose response study. The effects of 5 different dose levels ranging from 25 to 200 umol/kg at 24 hours are shown in table 3.5. AsV was administered by subcutaneous injection. At dose levels above 25 umol/kg, there was a progressive decrease in the heme saturation of TP (Table 3.5), accompanied by corresponding increases in apoenzyme values. The holoenzyme/apoenzyme ratio followed closely the changes in the extent of heme saturation.

Time course study. Four hours after treatment the heme saturation of TP was reduced to 67% of its control value, and remained at about this level for the rest of the observation period (Table 3.6). A small increase in "total enzyme" activity was observed.

b) ALA synthetase.

A dose-dependent increase in this enzyme activity was observed (Table 3.7), but only at dose levels above 100 umol/kg of AsV was the increase significant. The highest dose (200 umol/kg) increased the activity to almost twice the control value.
Table 3.5 Effects of sodium arsenate (AsV) on rat liver tryptophan pyrrolase.

<table>
<thead>
<tr>
<th>AsV (umol/Kg)</th>
<th>Apoenzyme</th>
<th>Holoenzyme</th>
<th>Total</th>
<th>Saturation %</th>
<th>Holo/Apo ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.8 ± 0.9</td>
<td>2.8 ± 0.6</td>
<td>7.6 ± 1.4</td>
<td>37.3 ± 3.9</td>
<td>0.60 ± 0.11</td>
</tr>
<tr>
<td>25</td>
<td>5.4 ± 0.9</td>
<td>3.0 ± 0.3</td>
<td>8.4 ± 1.0</td>
<td>36.3 ± 3.7</td>
<td>0.57 ± 0.07</td>
</tr>
<tr>
<td>50</td>
<td>4.7 ± 0.9</td>
<td>2.4 ± 0.3</td>
<td>7.2 ± 1.1</td>
<td>34.3 ± 2.1</td>
<td>0.53 ± 0.05</td>
</tr>
<tr>
<td>100</td>
<td>6.5 ± 1.0</td>
<td>2.7 ± 0.4</td>
<td>9.2 ± 1.3</td>
<td>28.9 ± 2.4</td>
<td>0.41 ± 0.05</td>
</tr>
<tr>
<td>150</td>
<td>8.2 ± 1.4</td>
<td>2.7 ± 0.2</td>
<td>10.8 ± 1.6</td>
<td>24.8 ± 1.9</td>
<td>0.33 ± 0.03</td>
</tr>
<tr>
<td>200</td>
<td>6.9 ± 1.3</td>
<td>1.7 ± 0.4</td>
<td>8.6 ± 1.6</td>
<td>19.8 ± 2.5</td>
<td>0.25 ± 0.04</td>
</tr>
</tbody>
</table>

1 Activities are expressed as umol/h of kynurenine formed per g (wet weight) of liver: $\bar{X}$ ± S.D. (n = 24 for controls, n = 4 for other data). These effects were studied at 24 hours after subcutaneous administration of AsV.

*Differs from control data $p < 0.05$ ; ** $0.05 > p < 0.10$. 

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Table 3.6 Time-course of the effects of sodium arserate (AsV) (100 umol/kg) on rat liver tryptophan pyrrolase1.

<table>
<thead>
<tr>
<th>AsV (umol/Kg)</th>
<th>Apoenzyme</th>
<th>Holoenzyme</th>
<th>Total</th>
<th>Saturation %</th>
<th>Holo/Apo ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.8 ± 0.9</td>
<td>2.8 ± 0.6</td>
<td>7.6 ± 1.4</td>
<td>37.3 ± 3.9</td>
<td>0.60 ± 0.11</td>
</tr>
<tr>
<td>4 h</td>
<td>7.0 ± 2.2</td>
<td>*</td>
<td></td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>8 h</td>
<td>7.0 ± 0.8</td>
<td>2.5 ± 0.2</td>
<td>9.5 ± 0.7</td>
<td>26.2 ± 3.9</td>
<td>0.36 ± 0.07</td>
</tr>
<tr>
<td>16 h</td>
<td>6.8 ± 0.9</td>
<td>2.5 ± 0.4</td>
<td>9.3 ± 1.2</td>
<td>27.0 ± 2.5</td>
<td>0.37 ± 0.05</td>
</tr>
<tr>
<td>24 h</td>
<td>6.5 ± 1.0</td>
<td>2.7 ± 0.4</td>
<td>9.2 ± 1.3</td>
<td>28.9 ± 2.4</td>
<td>0.41 ± 0.05</td>
</tr>
</tbody>
</table>

1 Activities are expressed as umol/h of kynurenine formed per g (wet weight) of liver: \( \bar{X} \pm S.D. \) (n = 24 for controls, n = 4 for other data). These effects were studied after subcutaneous administration of AsV.

* Differs from control data, p < 0.05 ; ** 0.05 > p < 0.10
Table 3.7  Effects of sodium arsenate (AsV) on hepatic ALA synthetase, heme oxygenase and biliverdin reductase.

<table>
<thead>
<tr>
<th>AsV (umol/kg)</th>
<th>ALA Synthetase&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Heme Oxygenase&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Biliverdin Reductase&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>134.8 ± 28.2</td>
<td>1.70 ± 0.25</td>
<td>20.5 ± 3.7</td>
</tr>
<tr>
<td>25</td>
<td>N.A.</td>
<td>1.68 ± 0.13</td>
<td>18.2 ± 1.9</td>
</tr>
<tr>
<td>50</td>
<td>142.8 ± 22.4</td>
<td>1.73 ± 0.10</td>
<td>20.6 ± 4.3</td>
</tr>
<tr>
<td>100</td>
<td>133.4 ± 13.0</td>
<td>1.64 ± 0.16</td>
<td>20.8 ± 3.7</td>
</tr>
<tr>
<td>150</td>
<td>190.2 ± 20.0</td>
<td>2.68 ± 0.11</td>
<td>19.2 ± 2.6</td>
</tr>
<tr>
<td>200</td>
<td>259.6 ± 62.6</td>
<td>5.05 ± 0.55</td>
<td>20.0 ± 4.8</td>
</tr>
</tbody>
</table>

1 pmol ALA/mg homogenate protein/h.<br>2 nmol bilirubin/mg microsomal protein/h.<br>3 nmol bilirubin/mg cytosolic protein/h.<br><br>All above given as $\bar{X} \pm$ S.D. ($n = 17$ for controls, $n = \text{at least 3 for other data}$). These effects were studied at 16 hours after subcutaneous administration of AsV.<br><br>* Differs from control data, $p < 0.05$.<br>N.A. Data not obtained.
c) Heme oxygenase.

A dose-related increase in HO activity was observed (Table 3.7). No effects were observed at doses below 150 umol/kg of AsV; the highest dose (200 umol/kg) produced a 3-fold increase in activity.

d) Biliverdin reductase.

No change of activity was observed at any dose level (Table 3.7).

e) Cytochrome P-450 and b.

No effects were observed on P-450 concentration at doses below 150 umol/kg (Table 3.8). However, the highest dose reduced the concentration to 73% of control values. There was no change of cytochrome b concentration at any dose level (Table 3.8).

Figure 3.2 summarizes the effects of AsV on some indicators of heme status.
Table 3.8 Effects of sodium arsenate (AsV) on hepatic cytochromes P-450 and b\textsubscript{5}.

<table>
<thead>
<tr>
<th>AsV (umol/kg)</th>
<th>Cytochrome P-450\textsuperscript{1}</th>
<th>Cytochrome b\textsubscript{5}\textsuperscript{1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.63 ± 0.08</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td>25</td>
<td>0.65 ± 0.05</td>
<td>0.30 ± 0.04</td>
</tr>
<tr>
<td>50</td>
<td>0.62 ± 0.06</td>
<td>0.28 ± 0.05</td>
</tr>
<tr>
<td>100</td>
<td>0.60 ± 0.05</td>
<td>0.31 ± 0.04</td>
</tr>
<tr>
<td>150</td>
<td>0.57 ± 0.04</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>200</td>
<td>0.46 ± 0.05</td>
<td>0.29 ± 0.02</td>
</tr>
</tbody>
</table>

1 nmol/mg microsomal protein.

All above given as $\bar{X} \pm$ S.D. (n = 17 for controls, n = at least 3 for other data). These effects were studied at 16 hours after subcutaneous administration of AsV.

* Differs from control data, p < 0.05.
Fig. 3.2. Effects of sodium arsenate (AsV) on some indicators of cellular heme status. The effects were studied at 16 hours after subcutaneous administration of AsV.

- □ P-450 concentration (% of control).
- □ Heme saturation of TP (% of control).
- ○ ALAS activity (% of control).
- ● HO activity (% of control).

Dose (umol/kg body wt.)
3.2.3 Effects of sodium selenite.

a) Tryptophan pyrrolase.

Dose-response study. The effects of 5 different dose levels administered subcutaneously and ranging from 1.25 to 20 umol/kg at 16 hours are shown in table 3.9. At dose levels above 2.5 umol/kg, there was a progressive decrease in the heme saturation of TP, with the highest dose (20 umol/kg) decreasing the heme saturation to half the control value (Table 3.9). Although the holoenzyme/apoenzyme ratio changes were more pronounced, they closely followed those observed in the degree of heme saturation. Doses of 40 umol/kg and higher were lethal to all animals within 12 hours.

b) ALA synthetase.

A dose-related increase in the activity of this enzyme was observed (Table 3.10); the highest dose (20 umol/kg) produced a 2-fold increase.

c) Heme oxygenase.

A marked dose-dependent increase in HO activity was also observed (Table 3.10), the highest dose produced an increase of almost 4 times the control values.
Table 3.9 Effects of sodium selenite (Se IV) on rat liver tryptophan pyrrolase.

<table>
<thead>
<tr>
<th>SeIV (umol/kg)</th>
<th>Apoenzyme</th>
<th>Holoenzyme</th>
<th>Total</th>
<th>Saturation %</th>
<th>Holo/Apo ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.8 ± 0.9</td>
<td>2.8 ± 0.6</td>
<td>7.6 ± 1.4</td>
<td>37.3 ± 3.9</td>
<td>0.60 ± 0.11</td>
</tr>
<tr>
<td>1.25</td>
<td>5.8 ± 0.4</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>0.66 ± 0.11</td>
</tr>
<tr>
<td>2.5</td>
<td>5.4 ± 1.1</td>
<td>2.8 ± 0.3</td>
<td>8.2 ± 1.5</td>
<td>34.8 ± 2.3</td>
<td>0.54 ± 0.05</td>
</tr>
<tr>
<td>5.0</td>
<td>3.5 ± 0.4</td>
<td>1.6 ± 0.2</td>
<td>5.1 ± 0.6</td>
<td>31.1 ± 0.9</td>
<td>0.45 ± 0.02</td>
</tr>
<tr>
<td>10.0</td>
<td>8.1 ± 0.9</td>
<td>2.6 ± 0.2</td>
<td>10.7 ± 1.1</td>
<td>24.1 ± 0.3</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>20.0</td>
<td>5.4 ± 0.9</td>
<td>1.3 ± 0.2</td>
<td>6.7 ± 1.1</td>
<td>19.7 ± 1.2</td>
<td>0.25 ± 0.02</td>
</tr>
</tbody>
</table>

1 Activities are expressed as umol/h of kynurenine formed per g (wet weight) of liver: X ± S.D. (n = 24 for controls, n = 4 for other data). These effects were studied at 16 hours after subcutaneous administration of Se IV.

* Differs from control data, p < 0.05.
Table 3.10 Effects of sodium selenite (SeIV) on hepatic ALA synthetase, heme oxygenase and biliverdin reductase.

<table>
<thead>
<tr>
<th>SeIV (umol/kg)</th>
<th>ALA Synthetase¹</th>
<th>Heme Oxygenase²</th>
<th>Biliverdin Reductase³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>134.8 ± 28.2</td>
<td>1.70 ± 0.25</td>
<td>20.5 ± 3.7</td>
</tr>
<tr>
<td>5</td>
<td>194.8 ± 22.2</td>
<td>2.74 ± 0.25</td>
<td>19.1 ± 1.1</td>
</tr>
<tr>
<td>10</td>
<td>231.4 ± 38.6</td>
<td>4.19 ± 0.38</td>
<td>21.4 ± 5.0</td>
</tr>
<tr>
<td>20</td>
<td>296.8 ± 23.8</td>
<td>6.92 ± 0.59</td>
<td>19.1 ± 2.7</td>
</tr>
</tbody>
</table>

1 pmol ALA/mg protein/h.
2 nmol bilirubin/mg microsomal protein/h.
3 nmol bilirubin/mg cytosolic protein/h.

All above given as X ± S.D. (n = 17 for controls, n = at least 3 for other data). These effects were studied 16 hours after subcutaneous administration of SeIV.

* Differs from control data, p < 0.05.
d) Biliverdin reductase.

No change of activity was observed at any dose level (Table 3.10).

e) Cytochromes P-450 and b.

In marked contrast with the effects seen after treatments with AsIII and AsV, no significant effects on P-450 concentration were observed at any dose level. Also no effect on b was noted (Table 3.11).

Figure 3.3 summarizes the effects of SeIV on some indicators of heme status.
Table 3.11 Effects of sodium selenite on hepatic cytochromes P-450 and b₅.

<table>
<thead>
<tr>
<th>SeIV (umol/kg)</th>
<th>Cytochrome P-450¹</th>
<th>Cytochrome b₅¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.63 ± 0.08</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td>5</td>
<td>0.55 ± 0.09</td>
<td>0.32 ± 0.05</td>
</tr>
<tr>
<td>10</td>
<td>0.57 ± 0.10</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>20</td>
<td>0.56 ± 0.05</td>
<td>0.32 ± 0.05</td>
</tr>
</tbody>
</table>

¹ 1 nmol/mg microsomal protein.

All above given as $\bar{X} \pm S.D. \ (n = 17 \ for \ controls, \ n = \ at \ least \ 3 \ for \ other \ data). \ These \ effects \ were \ studied \ at \ 16 \ hours \ after \ subcutaneous \ administration \ of \ SeIV.$

* Differs from control data, $p < 0.05.$
Fig. 3.3 Effects of sodium selenite (SeIV) on some indicators of cellular heme status. These effects were studied at 16 hours after subcutaneous administration of SeIV.
3.2.4. Effects of the combined administration of AsIII and SeIV

a) Tryptophan pyrrolase.

The effects of 4 different doses of AsIII, ranging from 12.5 to 75 umol/kg, plus 5 umol/kg of SeIV at 16 hours are shown in table 3.12. AsIII was injected subcutaneously in the abdominal region and SeIV immediately afterwards in the cervical region.

At all dose levels of AsIII plus 5 umol/kg of SeIV there was a progressive decrease in the heme saturation of TP (Table 3.12). The decrease produced was more pronounced (p < 0.05) than that observed with AsIII alone (Table 3.1). The holoenzyme/apoenzyme ratio followed closely the heme saturation percentage changes. The administration of 12.5 umol/kg of AsIII plus 20 umol/kg of SeIV resulted in a response similar to that observed after the administration of SeIV alone (20 umol/kg).

b) Heme oxygenase.

The increase in this enzyme activity was greater (p < 0.05) than that observed after the administration of either AsIII or SeIV alone (Tables 3.3 and 3.13). At the lowest dose a 2-fold increase over control values was observed,
Table 3.12 Effects of the combined administration of sodium arsenite (AsIII) and sodium selenite (SeIV) on rat liver tryptophan pyrrolase1.

<table>
<thead>
<tr>
<th>AsIII + SeIV (umol/kg)</th>
<th>Apoenzyme</th>
<th>Holoenzyme</th>
<th>Total</th>
<th>Saturation %</th>
<th>Holo/Apo ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.8 ± 0.9</td>
<td>2.8 ± 0.6</td>
<td>7.6 ± 1.4</td>
<td>37.3 ± 3.9</td>
<td>0.60 ± 0.11</td>
</tr>
<tr>
<td>12.5 + 5</td>
<td>6.8 ± 0.9</td>
<td>2.7 ± 0.7</td>
<td>9.5 ± 1.5</td>
<td>27.7 ± 3.2</td>
<td>0.39 ± 0.06</td>
</tr>
<tr>
<td>25.0 + 5</td>
<td>8.3 ± 1.6</td>
<td>2.5 ± 0.3</td>
<td>10.6 ± 2.0</td>
<td>23.4 ± 1.3</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>50.0 + 5</td>
<td>5.6 ± 1.0</td>
<td>1.3 ± 0.1</td>
<td>6.8 ± 1.1</td>
<td>19.0 ± 2.1</td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td>75.0 + 5</td>
<td>9.6 ± 1.5</td>
<td>1.0 ± 0.2</td>
<td>10.6 ± 1.6</td>
<td>9.8 ± 1.8</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>12.5 + 20</td>
<td>5.7 ± 0.8</td>
<td>1.4 ± 0.2</td>
<td>7.1 ± 1.0</td>
<td>19.9 ± 0.2</td>
<td>0.25 ± 0.01</td>
</tr>
</tbody>
</table>

1 Activities are expressed as umol/h of kynurenine formed per g (wet weight) of liver: X ± S.D. (n = 24 for controls, n = 4 for other data). These effects were studied 16 hours after subcutaneous administration of these metalloids.

* Differs from control data, p < 0.05.
while a 9-fold increase was produced by the highest dose. The increase in activity produced by an AsIII dose of 12.5 plus 20 umol/kg of SeIV (Table 3.13) was similar to that produced by 20 umol/kg of Se alone (Table 3.10).

d) Biliverdin reductase.

No change of activity was observed at any of the 3 dose levels tested.

e) Cytochromes P-450 and b.

No change in P.450 concentration was observed when doses of AsIII (12.5 umol/kg) plus SeIV (5umol/kg) or AsIII (12.5 umol/kg) plus SeIV (20 umol/kg) were administered (Table 3.13). However, a reduction to 71% of control values was produced by the highest dose (AsIII 75 ug/kg plus SeIV 5 umol/kg); This reduction was less than that observed after the administration of AsIII alone (Table 3.4). No effect on cytochrome b concentration at any dose level was observed.
Table 3.13 Effects of the combined administration of sodium arsenite (AsIII) and sodium selenite (SeIV) on hepatic biliverdin reductase, heme oxygenase, cytochromes P-450 and b5.

<table>
<thead>
<tr>
<th>AsIII + SeIV (μmol/kg)</th>
<th>Biliverdin Reductase¹</th>
<th>Heme Oxygenase²</th>
<th>Cytochrome P-450³</th>
<th>Cytochrome b5³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20.5 ± 3.7</td>
<td>1.70 ± 0.25</td>
<td>0.63 ± 0.08</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td>12.5 + 5</td>
<td>19.8 ± 4.0</td>
<td>3.52 ± 0.63</td>
<td>0.56 ± 0.05</td>
<td>0.27 ± 0.05</td>
</tr>
<tr>
<td>75.0 + 5</td>
<td>22.9 ± 2.8</td>
<td>15.75 ± 1.99</td>
<td>0.45 ± 0.04</td>
<td>0.28 ± 0.04</td>
</tr>
<tr>
<td>12.5 + 20</td>
<td>24.0 ± 2.9</td>
<td>7.06 ± 0.58</td>
<td>0.57 ± 0.04</td>
<td>0.30 ± 0.05</td>
</tr>
</tbody>
</table>

1 nmol bilirubin/mg cytosolic protein/h.
2 nmol bilirubin/mg microsomal protein/h.
3 nmol/mg microsomal protein.

All above given as $\bar{x} \pm S.D.$ (n = 17 for controls, n = at least 3 for other data). These effects were studied at 16 hours after subcutaneous administration of these metalloids.

* Differs from control data, $p < 0.05$. 


3.2.5 Effects of cadmium chloride.

a) Heme oxigenase.

The effects of two dose levels, 7 and 20 umol/kg, at 16 hours were examined. Cd was administered by subcutaneous injection. A 7-fold increase in HO activity was observed at the higher dose (20 umol/kg), no significant change was observed at the lower dose (Table 3.14).

b) Biliverdin reductase.

No significant change in the activity of this enzyme was observed at any dose level (Table 3.14).

c) Cytochromes P-450 and b.

At the highest dose, P-450 content was reduced to 80% of control values. No significant change was observed at the lower dose (Table 3.14). No effect on cytochrome b concentration at any dose level was observed (Table 3.14).
Table 3.14 Effects of cadmium chloride on hepatic biliverdin reductase, heme oxygenase and cytochromes P-450 and b5.

<table>
<thead>
<tr>
<th>CdCl₂ (µmol/kg)</th>
<th>Biliverdin Reductase¹</th>
<th>Heme Oxygenase²</th>
<th>Cytochrome P-450³</th>
<th>Cytochrome b5³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20.5 ± 3.7</td>
<td>1.70 ± 0.25</td>
<td>0.63 ± 0.08</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td>7</td>
<td>24.3 ± 2.9</td>
<td>1.87 ± 0.19</td>
<td>0.62 ± 0.04</td>
<td>0.30 ± 0.04</td>
</tr>
<tr>
<td>20</td>
<td>25.9 ± 4.4</td>
<td>12.93 ± 0.76</td>
<td>0.50 ± 0.07</td>
<td>0.30 ± 0.04</td>
</tr>
</tbody>
</table>

1 nmol bilirubin/mg cytosolic protein/h.
2 nmol bilirubin/mg microsomal protein/h.
3 nmol/mg microsomal protein.

All above given as X ± S.D. (n = 17 for controls, n = at least 3 for other data). These effects were studied at 16 hours after subcutaneous administration of CdCl₂.

* Differs from control data, p < 0.05.
3.2.6 Effects of 30 day administration of sodium arsenite (AsIII) in drinking water (50 ppm).

Water consumption and estimated AsIII intake. A decrease in water consumption was observed in treated animals. The reduction was from approximately 130 ml/kg/day (range 110 - 150) in the control group, to approximately 62 ml/kg/day (range 55 - 69) in treated animals. From these data, the daily AsIII intake in the treated group was estimated to be about 24 umol/kg/day (range 21 - 27 umol/kg/day).

a) Tryptophan pyrrolase.

The control values showed no significant changes during the 30 day period of study. After 5 days of treatment, the degree of heme saturation of TP was reduced to 77% of control values. This reduction was maintained without significant change when the treatment was continued for 10, 20 and 30 days (Table 3.15). The holoenzyme/apoenzyme ratio followed closely the changes in heme saturation percentage. There was a progressive increase in "total enzyme" activity and in calculated apoenzyme values, which was significant (p < 0.05) after 10 days of treatment; the maximum increase was observed after 30 days treatment (30 and 50%, respectively).
Table 3.15 Effects of 30 day administration of sodium arsenite (AsIII) in drinking water (50 ppm) on rat hepatic tryptophan pyrrolasel.

<table>
<thead>
<tr>
<th>Days of Treatment</th>
<th>Apoenzyme</th>
<th>Holoenzyme</th>
<th>Total</th>
<th>Saturation %</th>
<th>Holo/Apo ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.6 ± 0.3</td>
<td>1.8 ± 0.2</td>
<td>4.3 ± 0.4</td>
<td>40.5 ± 1.4</td>
<td>0.68 ± 0.04</td>
</tr>
<tr>
<td>5</td>
<td>3.1 ± 0.5</td>
<td>1.4 ± 0.4</td>
<td>4.5 ± 0.9</td>
<td>31.3 ± 2.4</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>10</td>
<td>3.5 ± 0.6</td>
<td>1.6 ± 0.2</td>
<td>5.1 ± 0.8</td>
<td>31.9 ± 0.7</td>
<td>0.47 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>20</td>
<td>3.6 ± 0.1</td>
<td>1.6 ± 0.2</td>
<td>5.2 ± 0.2</td>
<td>30.5 ± 3.8</td>
<td>0.44 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>30</td>
<td>3.9 ± 0.6</td>
<td>1.7 ± 0.3</td>
<td>5.7 ± 0.9</td>
<td>30.2 ± 2.5</td>
<td>0.43 ± 0.05</td>
</tr>
</tbody>
</table>

1 Activities are expressed as umol/h of kynurenine formed per g (wet weight) of liver: X ± S.D. (n = 6 for controls, n = at least 4 for other data).

* Differs from control data, p < 0.05.
b) ALA Synthetase.

ALA Synthetase activity showed an increase of 60% over control values at day 5 and day 10 of treatment, after which a small decrease was observed; at day 20 and 30 the activity was still 30-40% above control values (Table 3.16).

c) Heme oxygenase and biliverdin reductase.

No change of activity was observed for either enzyme at any of the treatment periods studied (Table 3.16).

d) Cytochromes P-450 and b.

No change in either of these parameters at any exposure period was observed (Table 3.17).
Table 3.16 Effects of 30 day administration of sodium arsenite (AsIII) in drinking water (50 ppm) on rat ALA synthetase, heme oxygenase and biliverdin reductase.

<table>
<thead>
<tr>
<th>Days of Treatment</th>
<th>ALA Synthetase&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Heme Oxygenase&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Biliverdin Reductase&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>134.8 ± 28.2</td>
<td>1.70 ± 0.25</td>
<td>20.5 ± 3.7</td>
</tr>
<tr>
<td>5</td>
<td>221.4 ± 44.0</td>
<td>1.80 ± 0.14</td>
<td>19.3 ± 1.8</td>
</tr>
<tr>
<td>10</td>
<td>220.8 ± 19.8</td>
<td>1.86 ± 0.23</td>
<td>18.2 ± 3.0</td>
</tr>
<tr>
<td>20</td>
<td>175.0 ± 36.0</td>
<td>1.73 ± 0.30</td>
<td>19.9 ± 4.8</td>
</tr>
<tr>
<td>30</td>
<td>192.8 ± 41.8</td>
<td>1.79 ± 0.46</td>
<td>17.5 ± 4.4</td>
</tr>
</tbody>
</table>

1 pmol ALA/mg homogenate protein/h.
2 nmol bilirubin/mg microsomal protein/h.
3 nmol bilirubin/mg cytosolic protein/h.

All above given as $\bar{x} \pm $ S.D. (n = 17 for controls, n = at least 4 other data).

* Differs from control data, $p < 0.05$; ** $0.05 < p < 0.1$. 
Table 3.17 Effects of 30 day administration of sodium arsenite (AsIII) in drinking water (50 ppm) on rat hepatic cytochromes P-450 and b^.

<table>
<thead>
<tr>
<th>AsIII (Days of treatment)</th>
<th>Cytochrome P-450$^1$</th>
<th>Cytochrome b$^5$ $^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.63 ± 0.08</td>
<td>0.31 ± 0.03</td>
</tr>
<tr>
<td>5</td>
<td>0.68 ± 0.09</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>10</td>
<td>0.65 ± 0.07</td>
<td>0.29 ± 0.05</td>
</tr>
<tr>
<td>20</td>
<td>0.59 ± 0.04</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>30</td>
<td>0.69 ± 0.07</td>
<td>0.34 ± 0.03</td>
</tr>
</tbody>
</table>

$^1$nmol/mg microsomal protein.

All above given as $\bar{X} \pm$ S.D. (n = 17 for control, n = at least 4 for other data).

* Differs from control data, p < 0.05.
3.3 DISCUSSION.

3.3.1 Assessment of acute effects of As and Se on cytosolic heme status, using tryptophan pyrrolase as an index.

TP is a heme-dependent enzyme that in most animal species is normally 35 to 45% saturated with heme (Marver et al., 1966; Seglen and Jervel 1969). Circumstances which increase cellular "free heme" produce rapid changes in the enzyme activity by converting the remaining inactive heme-free apoenzyme to the active heme-containing holoenzyme (Wetterberg et al., 1969). Conversely, reductions in cellular heme decrease the heme saturation of TP (Feigelson and Greengard, 1961b; Badawy, 1977a). In the studies reported here, the responsiveness of TP was employed as an indicator of changes in heme metabolism/availability produced by As and Se administration.

Effects on the degree of heme saturation. The results of this study showed that the overall response to AsIII, AsV and SeIV acute treatments was a dose-related decrease in the degree of heme saturation of TP, thus clearly indicating that these elements decreased the content of cytosolic heme in liver cells. The ability of As and Se to markedly reduce the proportion of TP saturated with heme is consistent with their abilities to rapidly and potently induce HO as shown in the present study and previously reported by Maines and Kappas.
(1976a) for acute doses of SeIV, and by Sardana et al. (1981) for acute doses of As. The rapidity with which TP responds to the changes in heme metabolism produced by As and Se is in agreement with the suggestion of Badawy (1977b) that TP utilizes a pool of "free heme" which has a rapid turnover rate.

AsIII, AsV and SeIV all produce a similar effect on TP i.e. a decrease in the heme saturation. However, there are marked differences in the potencies of these elements; on a molar basis, SeIV was 10 times as potent as AsV, and 4 times as potent as AsIII. AsIII was about 2.5 times more potent than AsV (Fig. 3.4). Badawy (1977a) has reported that the subcutaneous injection of only 2.5 umol/kg of CdCl or 12.52 umol/kg of CuCl reduced the heme saturation of TP to about 2 50% of control values, whereas 250 umol/kg of Fe, Ni, Mn, Li and Co salts were required to achieve that effect. In the present study, 20 umol/kg of SeIV reduced the heme saturation to 50% of control values indicating that Se is among the more potent elements that reduce the heme saturation of TP.

The difference in potency between AsIII and AsV is not surprising since AsIII is generally considered to be more toxic than AsV (Byron et al., 1967; Nakamuro and Sayato, 1981). Several factors may account for this: 1) the retention of As in the body has been shown to be dependent on the valence form, AsIII being retained more effectively than AsV (Vahter, 1981); 2) the binding of AsIII to hepatic
Fig. 3.4 Relative potencies of AsIII (○), AsV (▲) and SeIV (■). Effects on the heme saturation of tryptophan pyrrolase studied at 16 hours after subcutaneous administration.
cellular constituents is more pronounced than that of AsV (Vahter and Marafante, 1983). 3) it has been shown that AsV is reduced to AsIII before being methylated (a detoxication mechanism) and it has been suggested that AsV toxicity is due to its conversion to AsIII (Mc Bride et al., 1978; Vahter and Envall, 1983). The latter suggestion is still the subject of debate. The possibility that the two valence forms may have different mechanisms of toxicity has also been suggested (Thompson, 1948; Mitchell et al., 1971). AsIII is thought to exert its toxic effects through interactions with active cellular sulfhydryl groups, whereas arsenate could act by substituting for phosphate in enzyme catalyzed reactions. The difference in potencies between SeIV and AsIII is more difficult to explain, because Se is not considered to be a porphyrinogenous element (Maines and Kappas, 1976a). The difference in porphyrinogenous potential of AsIII and SeIV could affect their different abilities to induce HO (Fig. 3.10). This possibility is further discussed in Section 3.3.3.

Possible mechanisms of As and Se effects on cellular heme status. It is known that acute and subchronic As exposure alter the activities of some enzymes in the heme synthesis pathway. Sardana et al. (1981) have reported that acute administration of AsIII produces a biphasic effect on ALAS activity, namely, an initial decrease followed by a rebound increase. Woods and Fowler (1977b, 1978) have reported that subchronic exposure (6 weeks) to AsV in the
drinking water resulted in decreases in ALAS and heme synthetase activities in rats and mice. Accompanying these alterations were dose-related increases in urinary porphyrin excretion. Similar increases in urinary porphyrins were observed after 7 days exposure to AsIII (Martinez et al., 1983). Furthermore, both AsIII and AsV produced in vitro a significant inhibition of uroporphyrinogen decarboxylase (Woods et al., 1981). The results reported here show that an acute dose of As produced a marked increase in HO activity. Thus, cellular heme status is a major target by virtue of the inhibitory effect of As on two enzymes involved in heme synthesis and its inducing effect on HO, which increases heme degradation. A result of the effects on ALAS, uroporphyrinogen decarboxylase, and heme synthetase is the excess production of heme intermediates leading to porphyria.

The mechanisms underlying the acute effects of Se are less clear. Although it seems to have the same biphasic effect as AsIII on ALAS described above, it has no effect on the cellular content of porphyrins (Maines and Kappas, 1976a). However, there is no available information on the effects of Se on uroporphyrinogen synthetase and uroporphyrinogen decarboxylase. Se is a potent inducer of HO, but it has been suggested that is not a direct inducer, and that it mediates HO induction through increased production and availability of cellular "free heme" (Maines and Kappas, 1976a). However, if this were the case, either no effect or
an increased heme saturation of TP should have been found in the present study. Thus, the increase in HO activity (Table 3.10) is a possible cause of the observed decrease in the heme saturation of TP, although the mechanism proposed by these authors to explain HO induction may be open to alternative interpretation.

**Early effects of As III.** The time-course study showed a small but significant \( p < 0.05 \) increase in the degree of heme saturation of TP during the first four hours after 100 umol/kg AsIII treatment (Table 3.2 and Fig. 3.5). This was in contrast to the pronounced decrease observed 24 hours after the same treatment. This rapid effect is in agreement with previous reports (Feigelson et al., 1959; Badawy and Evans, 1975) indicating that TP-apoenzyme is one of the most rapidly turning over and, hence, one of the most responsive of hepatic proteins. The holoenzyme has a half life of 7.4 hours, whereas that of the apoenzyme has been reported to be 2.3 hours (Correia and Burk, 1978). This transient response to AsIII, if the effects on heme saturation are considered in isolation, could be interpreted simply as indicative of an increase in the "free heme" pool size, however, if we consider the marked decrease in "total enzyme", holoenzyme and calculated apoenzyme activities at these early times (Fig. 3.5), it is clear that the increase in heme saturation is mainly due to drastic reductions in "total enzyme" activity. Since such changes are generally considered to reflect changes in apoenzyme synthesis, the use of the heme
Fig. 3.5 Time-course of sodium arsenite (AsIII) effects on tryptophan pyrrolase activities. AsIII was administered subcutaneously.
saturation of TP alone as an index of increase in "free heme pool size is misleading. Interestingly, the subsequent decrease in heme saturation is concurrent with the recovery from the effects on holoenzyme and "total enzyme" activities (Fig. 3.5).

A possible explanation of the transient increase in the degree of heme saturation is that AsIII, either by direct inhibition of apoenzyme synthesis, or via an allopurinol-type of inhibition (see below), reduces the number of apo-TP molecules saturated with heme, thereby increasing the "free heme" pool size. This increase would explain the early inhibition of ALAS activity produced by AsIII (100 umol/kg) reported by Sardana et al. (1981), for it is generally held that the size of the "free heme" pool regulates ALAS activity (Fig. 3.9; Section 3.3.2). There have been other instances in which metals, early on, increase the heme saturation of TP. Badawy (1977a) reported that one hour after an i.p. dose of aluminium (150 mg/kg; 240 umol/kg), the heme saturation reached 80%, apparently due to a two fold increase in holoenzyme activity without changes in "total enzyme" activity. Indications of a similar "early" effect of cobalt, without further explanation, were also reported by Kikuchi et al. (1982).

How could interference of AsIII with TP occur? The inhibition of "total enzyme" activity produced by AsIII
appears to be similar to that observed two hours after the administration of allopurinol. This agent has been shown to interfere with the conjugation of apo-TP with its heme prosthetic group; the interference being reversed by excess hematin (Beking and Johnson, 1967; Badawy and Evans, 1973b). It would be desirable to have some experimental evidence regarding this point. If AsIII produced an allopurinol type of inhibition it would be dependent on the As concentration reached in the cytosol. Thus, the addition of excess hematin (in vitro) should be able to restore the "total enzyme" activity to control values and could also prevent the increase in both holoenzyme and "total enzyme" activities produced by tryptophan. Alternatively, it has been suggested that arsenicals affect protein and aminoacid metabolism leading to depressed protein synthesis through interference with the availability of certain aminoacids, and possibly by direct actions on the aminoacid's synthetic pathways (Webb, 1966). Therefore, it is possible that the above changes could have resulted from AsIII inhibition of the synthesis of the apoprotein moiety.

The time-course of AsV effects showed that in contrast to the early inhibition of "total enzyme" activity produced by AsIII, AsV produced a small but significant (p < 0.05) increase in total enzyme activity accompanied by a decrease in holoenzyme activity, which resulted in a reduction of the heme saturation to 70% of its control values (Fig. 3.6); these changes remained without significant variations between
4 and 16 hours after AsV administration. Possible explanations for this difference include: 1) Most enzyme inhibitions produced by As are presumably due to the arsenical reacting with SH groups at or near the active site of the enzyme; pentavalent arsenicals generally produce much less inhibition than the corresponding trivalent compounds, as would be expected from the fact that they do not react readily with SH groups (Webb, 1966). 2) the dose of AsV used in this part of the study was comparatively low (100 umol/kg) as witnessed by the fact that it failed to produce significant effects on other enzymes measured, whereas that of AsIII was comparatively high (100 umol/kg), approaching lethal doses and producing large effects on other enzymes.

Further considerations of the effects on "total enzyme" activity. So far, most experimental work devoted to studying the relationships between TP activity and heme metabolism alterations has been focused on the heme saturation value, and has attached much less importance to the other parameters measured. However, the possibility that some metals could affect the apoenzyme moiety of TP is apparent from the above discussion. Data reported in this study on the effects of AsIII and AsV at 24 hours (Tables 3.1 and 3.5; Figs. 3.5 and 3.6), and also by Badawy (1977a, 1978), on the effects of AIA and DDC show that "total enzyme" activity is increased under some conditions. Thus, it is clear that heme saturation can be reduced either by
Fig. 3.6 Time-course of sodium arsenate (AsV) effects on tryptophan pyrrolase activities. AsV was administered subcutaneously.
decreasing the holoenzyme activity, or by enhancing that of the "total enzyme", or by exerting both effects. A likely explanation for the effects on "total enzyme" activity is provided by the work of Knox (1951) and Knox and Auerbach (1955) who studied the role of the adrenals. These authors showed that TP activity is enhanced by the administration of corticosteroids and stress-producing agents. Schimke et al. (1965) and De Lap and Feigelson (1978) have shown that this increase involves induction of apoenzyme synthesis, due to increases in the amount of TP mRNA. Furthermore, acute and subacute administration of AsIII and CdCl₂ have been shown to alter carbohydrate metabolism, probably via effects on the adrenal glands causing the release of epinephrine and corticosteroids (Ghafghazi and Mennear, 1973; Ghafghazi et al., 1980). Thus, it is possible that some metals increase "total enzyme" activity via an effect on the adrenals, a mechanism which has also been put forward to explain AIA-induced TP activity (Feigelson and Greengard, 1961b).

In view of the above effect, how valuable is the measurement of heme saturation as an index of "free heme pool" size? One problem in the study of the regulation of hemoprotein synthesis is lack of knowledge of the means of integration between apoprotein synthesis and that of its prosthetic group heme. It has been claimed that changes in one constituent lead to adjustments in the synthesis of the other (Tait, 1978). To assess the heme saturation as the sole variable, it is obviously necessary to prevent changes other
than those caused by an altered heme availability. Prior starvation of experimental animals has been used to raise optimally the "total enzyme" activity by an hormonal mechanism, thus forestalling any enhancement by agents which act by this mechanism (Badawy, 1978 and 1981). However, in some experiments with metallic and other compounds (Badawy, 1977a), it seems that either starvation is not enough or that the enhancement is not solely due to the hormonal mechanism discussed previously. It has been suggested that corticoids induce apo-TP synthesis and increase the holoenzyme activity in proportion to the rise in total activity, so that the heme saturation remains essentially constant (Feigelson and Brady, 1974; Morgan and Badawy, 1980). This consideration is not applicable to substrate or cofactor-type enhancement of TP activity, which produces a large increase in holoenzyme activity with little or no synthesis of apoenzyme and a rise in the heme saturation. Therefore, if an effect on "total enzyme" activity is observed, it is important to identify the mechanism which is responsible, in order to interpret the heme saturation data.

The degree of heme saturation of TP has been expressed in the literature either as holoenzyme/apoenzyme ratio or as percentage of the holoenzyme activity against that of "total enzyme". Badawy (1978) prefers to use the concept of holoenzyme/apoenzyme ratio rather than the percentage because higher doses of compounds are required to produce a 50%
decrease in the latter, a finding that the present study confirms. However, the possible effects of AsIII and other metals on apoenzyme synthesis, indicates the importance of expressing the degree of heme saturation of TP as percentage instead of holoenzyme/apoenzyme ratio. This gives separate consideration of the effects of chemicals on parts of the TP system other than heme availability.
3.3.2 Effects of acute administration of As and Se on ALA Synthetase activity.

ALAS is thought to play a key role in the regulation of heme synthesis for two main reasons: 1) its activity appears to be rate limiting in the pathway; 2) it is the site where heme exercises a negative feedback control on its own synthesis (De Matteis, 1975).

The results of this study show that the overall response to acute administration of AsIII, AsV and SeIV was a dose-related increase in hepatic ALAS activity (Tables 3.3, 3.7 and 3.10). A comparison with literature data, indicate that after Fe-dextran (Ibraham et al., 1979) and Co (Maines and Kappas, 1976b), Se and As are among the most potent inducers of ALAS. However, there were marked differences between As and Se potencies; on a molar basis, SeIV was 8 times as potent as AsV, and about 3.5 times as potent as AsIII. AsIII was about 2.5 times more potent than AsV (Fig. 3.7).

Possible mechanisms of As and Se effects on ALA Synthetase activity. The main regulatory aspects of heme synthesis, utilization and degradation were brought together in a model centered on the feedback control exercised by heme at the ALAS level (De Matteis, 1975; Granick et al., 1975). Currently the chemically mediated increase of ALAS in liver
Fig. 3.7 Relative potencies of AsIII (○), AsV (▲) and SeIV (□). Effects on ALA synthetase activity studied at 16 hours after subcutaneous administration.
is supposed to be mainly due to a reduction in the "free heme" pool (see Section 1.3.2), which can be elicited by one or more of the following mechanisms: 1) inhibition of heme synthesis; 2) increase in the rate of heme utilization; or 3) increase in the rate of heme degradation. As discussed in Section 3.3.1, there is evidence that arsenic affects mechanisms 1 and 3, inhibiting heme synthetase activity, producing porphyria and increasing HO activity. Se, however, has no effect on the cellular content of porphyrins, but it is a potent HO inducer.

**Effects of As and Se on the relationships between the heme saturation of TP and ALA Synthetase activity.** Welch and Badawy (1980), have reported that ALAS activity is enhanced when the heme saturation of TP is reduced by treatments that decrease cellular heme content. On the other hand, ALAS activity has been reported to be decreased when the heme saturation of TP is increased after hematin administration (Welch and Badawy, 1980; Yamamoto et al., 1981). These findings led these authors to suggest that an inverse relationship exists between ALAS activity and the degree of heme saturation of TP. In the present study the relative potencies of As and Se on ALAS activity were similar to those on the heme saturation of TP, suggesting that the mechanism of both effects is similar. In order to explore the effects of the acute administration of AsIII, AsV and SeIV on the relationships between the "free heme pool" and ALAS activity, two factors were investigated: 1) a dose-response study with
the effects measured 16 hours after treatment; 2) the time-
course of AsIII effects.

As and Se produced a dose-related decrease in the
percentage of TP saturated with heme, accompanied by dose-
related increases in ALAS activity; the correlation
coefficient (r) between these effects was better than -0.94
for each metalloid indicating that ALAS activity was
inversely proportional to the TP heme saturation. These "r"
values (see also figure 3.8) suggested that if ALAS activity
were dependent on the "free heme" pool size, as measured by
the heme saturation of TP, a good correlation would be
expected between these parameters, regardless of the
metalloid used to produce the effects. The combined
correlation coefficient obtained for these data, when the
effects of the 3 salts were considered, was -0.86,
supporting this proposition. Two models may be used to
explain these data, a linear model (y = 406.2 - 7.88x; r =
-0.86) or an exponential model (lny = ln 546.9 - 0.040x; r
= -0.86). Unfortunately there are insufficient data,
especially at the lower TP heme saturation values, to decide
between these models. The findings indicate: 1) that As and
Se reduce the size of the regulatory "free heme" pool,
causing a positive feedback control of ALAS activity. 2)
that most chemicals which increase ALAS activity, may do so
by decreasing the regulatory "free heme" pool (Meyer, 1982).
3) that if the exponential model is the one which represents
Fig. 3.8 Relationships between the heme saturation of tryptophan pyrrolase and ALA synthetase activity. The effects were studied 16 hours after subcutaneous administration of the metalloids.
this relationship, there may be circumstances in which no further depletion of the heme saturation of TP could be achieved while maintaining a concurrent increase in ALAS activity.

The time-course studies reported by Sardana et al. (1981) of the effects of a single dose of AsIII (100 umol/kg), showed that this metalloid produces a decrease in ALAS activity 1 hour after treatment, followed by a rebound increase which reached a peak at 8 hours before returning to control levels at 24 hours (Fig. 3.9). This biphasic effect is characteristic of the administration of a number of transition and heavy metals (De Matteis and Gibbs, 1976; Maines and Kappas, 1977b). The time-course of the effects on the heme saturation of TP reported in the present study showed a small but significant increase during the first four hours after treatment (AsIII; 100 umol/kg), followed by a gradual decrease which at the end of the observation period (24 hours) corresponded to a heme saturation of 40% of control values (Fig. 3.9). Kikuchi et al. (1982), have reported similar time-courses for the effects of Co on ALAS and TP administration i.e. an initially increased ALAS activity declining rapidly after 12 hours, while the heme saturation of TP continued to decrease; however, in their publication the values of "total enzyme" and holoenzyme activity were not given. The effects observed between 1 and 8 hours after AsIII administration support the suggestion that an inverse relationships exist between ALAS activity and heme
Fig. 3.9 Time-course of sodium arsenite (AsIII) effects (100 umol/kg body wt.) on ALA synthetase activity (○) and the heme saturation of tryptophan pyrrolase (●). The data on ALA synthetase were taken from Sardana et al. (1981).
saturation of TP. Although ALAS activity was still above control values, its declining trend in the presence of a parallel decrease in the heme saturation of TP is difficult to explain (Fig. 3.9). This indicates that the relationship between these parameters is not a simple inverse one, and that there are conditions in which it does not hold. Further experiments are needed to: 1) investigate the time-course of the production of porphyria and the inhibition of heme synthetase activity produced by AsIII, to assess the likely causes of reduction in the "free heme" pool size which result in changes in ALAS activity and heme saturation of TP. The information obtained in this proposed experiment may allow the reconciliation of the information from the dose-response studies. It is possible that ALAS activity may reflect more accurately the changes in the "free heme" pool than the heme saturation of TP, or alternatively, ALAS activity may be controlled by a heme pool not measured by TP; 2) study the possibility of TP playing a more active role in the regulation of ALAS, rather than being a passive indicator of heme availability, and 3) explain some other circumstances in which the above relationship does not hold. In particular an assessment is needed of: a) the effects and time-course of other potent ALAS inducers on TP e.g. administration of Fe-dextran, which according to Bonkowsky et al. (1981) produces a 4-fold increase in ALAS activity; b) the mitochondrial and cytosolic ALAS activities and their corresponding relationships to TP heme saturation after metal treatment.
Although most experimental evidence indicates that ALAS activity is regulated by heme availability, alternative mechanisms unrelated to heme repression, have also been proposed to explain ALAS induction. They include direct effects on the nucleus to increase induction-specific RNA (Tyrrell and Marks, 1972), effects on ALAS translation and alterations in the enzyme's half life (Sassa and Granick, 1970).
3.3.3 Effects of the acute administration of As and Se on heme oxygenase activity.

Heme oxygenase plays a key role in mammalian heme catabolism and it is widely accepted that in physiological conditions its activity is controlled by the amount of heme available for catabolism (Pimstone et al., 1971).

The results of this study show that the overall response to acute administration of AsIII, AsV and SeIV was a dose-related increase in hepatic HO activity (Tables 3.3, 3.7 and 3.10), thereby, enhancing the heme degradation rate. This confirm earlier reports of Sardana et al. (1981) and Maines and Kappas (1976a). AsIII has also been reported to increase HO activity in cultured avian liver cells, whereas SeIV failed to elicit this response (Sardana et al., 1982). There are marked differences in the potencies of these metalloids; on a molar basis, SeIV was marginally (1.3 times) more potent than AsIII, and 15 times as potent as AsV. AsIII was 12 times more potent than AsV (Fig. 3.10). These relative potencies were markedly different from those described for the effects on ALAS and TP (Section 3.3.2). The main change was a marked increase in the ability of AsIII to induce HO, suggesting the possibility that more than one mechanism may be involved in the induction of heme oxygenase.
Fig. 3.10 Relative potencies of AsIII (●), AsV (○) and SeIV (■). Effects on heme oxygenase activity, studied 16 hours after subcutaneous administration.
Possible mechanisms of As and Se effects on HO activity. Although the ability of hemin and some xenobiotics to induce HO activity has been known since 1970 (Tenhunen et al., 1970), the mechanisms of induction have eluded explanation and different hypotheses have emerged. In general terms, it could be said that these hypotheses are based on the effects that a particular inducing agent has on enzymes, such as heme saturation of TP and cytochrome P-450 concentration, which are recognized as indicators of cellular heme status. According to Kikuchi and Yoshida (1983), hepatic HO induction may occur by two apparently different mechanisms. The first type of mechanism is epitomised by the induction caused by carbon disulphide, endotoxin, insulin and epinephrine. This induction is considered to be mediated by heme because these agents each produce a significant increase in the heme saturation of TP, and a moderate increase in HO activity, comparable to that caused by small doses of hemin (3 umol/kg). The second type of mechanism is characteristic of the induction produced by cadmium, cobalt and bromobenzene and seems to be independent of heme mediation, since these agents reduce the heme saturation of TP and cause a far more extensive HO induction than that observed after the administration of 30 umol/kg of hemin.

In the case of As and Se, the dose-response data obtained 16 hours after treatment provided indirect evidence to suggest that the increase in HO cannot be explained in
terms of the first mechanism i.e. the regulatory "free heme" pool model because: 1) There were no indications of increases in "free heme pool" size, as measured by the heme saturation of TP, which could trigger a positive feedback on HO. On the contrary, it appears that one reason for the reduction in heme saturation was the increased HO activity (Fig. 3.11). 2) the concomitant increase in ALAS activity is further indication of cellular heme depletion.

A particular point of interest arises in the case of AsIII, where data on the time-course of the effects on ALAS and TP are available; namely if the early increase in the degree of heme saturation of TP observed in the present study [together with the inhibition of ALAS activity observed 2 hours after AsIII treatment (Sardana et al. 1981)] could be interpreted as indicative of an increased "free heme" pool size (Section 3.1.1; Fig. 3.5), then this transient increase would stimulate the synthesis of HO-specific mRNA, which according to Kikuchi and Yoshida (1983) has a long half-life (4 hours). Because HO has a 6 hours' half-life a sustained increase in HO activity could occur, which in turn would reduce the heme saturation of TP. Thus, at the end of the observation period (16 hours), the picture would be that of an increased HO activity accompanied by a reduction in the heme saturation of TP. However, the increase in heme saturation values was far below the saturation produced by a 3 umol/kg hemin injection which only produced a 3-fold increase in HO activity in the experiments of Kikuchi and
Fig. 3.11 Relationships between the heme saturation of tryptophan pyrrolase and heme oxygenase activity. AsIII (●), AsV (○) and SeIV (□). The effects were studied 16 hours after subcutaneous administration.
Alternatively, it may be postulated that it is heme utilization by TP and not the degree of heme saturation that is related to HO induction. If this is the case, then the early increase in the heme saturation of TP is due to the reduction in the "total enzyme" and holoenzyme activities, the net effect being to reduce the number of apo-TP molecules saturated with heme, thus allowing the unused heme to increase the "free heme" pool size. Further experiments are needed to explore this possibility.

A comparison between doses of AsIII, AsV and SeIV which were equally effective in producing a 50% decrease in the heme saturation of TP at 16 hours after treatment showed that the AsIII dose increased HO activity almost twice as much as that of SeIV or AsV (Fig. 3.11). This suggests that the increase in HO activity produced by these metalloids is a property partly or totally independent of the "free heme" pool size, as measured by the heme saturation of TP. It also raises a question: where does the heme metabolized by excess AsIII-induced HO comes from? It remains to be established whether HO activity is directly affected by these metalloids, or indirectly through degradation of P-450. Possibly, P-450 serves to influence the size of a specific subcompartment of the heme pool.
A comparison between the increase in ALAS and HO activity produced by AsIII at 16 hours after treatment (Fig. 3.2 and 3.12) showed that the increase (with respect to control values) in HO activity was always greater than that produced in ALAS activity. The highest dose (75 umol/kg) produced a 2-fold increase in ALAS activity, while HO activity was increased 8-fold. This disparity between the effects on ALAS and HO activities and the fact that 8 molecules of ALA are required to form one molecule of heme clearly illustrates the lack of parallelism between heme synthesis and degradation.

It is known that between 15 and 20 transition elements, heavy and organometallic compounds are HO inducers (Maines and Kappas, 1977a; Eaton et al., 1980; Rosenberg et al., 1984). From data reviewed by these authors and those reported here, it could be said that besides increasing HO activity, all elements tested so far that increase ALAS activity, decrease TP heme saturation and reduce P-450 concentration. Se is notable because it does not seem to reduce P-450 content. The possible role of P-450 in HO induction is briefly discussed in the following Section.
Fig. 3.12 Effects of sodium arsenite (AsIII) on the relationships between the heme saturation of tryptophan pyrrolase and ALA synthetase and heme oxygenase activities. The effects were studied 16 hours after subcutaneous administration of AsIII.
3.3.4 Effects of As and Se on cytochrome P-450 concentration.

It has been suggested that HO-inducing agents might act by first labilizing the heme moiety of P-450, the released heme then acting as the inducer for HO (Bissell and Hammaker, 1976 a, b; Lim et al. 1980). The acute administration of AsIII and AsV produced a dose-related decrease in P-450 concentration, AsIII being 3 times more potent than AsV. This difference in relative potency was similar to that observed in the effects on TP and ALAS.

Possible mechanisms of As effects on cytochrome P-450.
De Matteis (1982), has reviewed evidence indicating that in vitro, metals could produce peroxidative damage to microsomal membranes leading to decreased affinity of the P-450 apoprotein for heme, and suggested that if these agents produced similar effects in vivo, this would lead to a substrate mediated HO induction. Recently, Klimczac et al. (1984) showed that lipid peroxidation produced by Cd in vivo is concurrent with the reduction of P-450 content, and increases in ALAS and HO activities. Although no direct information is available regarding the effects of As on lipid peroxidation, Squibb and Fowler (1983) have reviewed information indicating that As administration reduces catalase activity. Thus, it is possible that P-450 loss produced by As could be explained by this mechanism. Further work is necessary to explore this possibility.
Although published literature favours an association between lipid peroxidation and P-450 loss, no evidence is available to support the suggestion that an increase in "free heme" pool due to P-450 breakdown had taken place before the increase in HO activity. Furthermore, Drummond et al. (1982) reported that SKF 525A protects the integrity of P-450 throughout the immediate period (0 - 16 h) after the administration of Co, Sb, AsIII and other metals, without affecting the extent of HO or ALAS induction; thus, providing evidence that P-450 degradation is not an essential event for metals to initiate HO induction. This raises the question: is heme the stimulus for HO induction? and if so, where does it come from when P-450 is protected by SKF 525A? Does it come from an isoform of P-450 not protected by SKF 525A or from another heme pool(s), microsomal or cytosolic? It would be helpful to know the effect of SKF 525A on the heme saturation of TP in As treated rats.

The administration of Se produced a dose-related decrease in TP heme saturation and also resulted in dose-related increases in ALAS and HO activities. However, it did not seem to significantly affect the microsomal P-450 content (Tables 3.9, 3.10 and 3.11). These results confirm those reported by Maines and Kappas (1976a). They have shown that hepatic total P-450 levels were refractory to a subcutaneous dose of 50 umol/kg at 14.5 hours after treatment, but found that the activity of ethylmorphine N-demethylase was reduced
by 50%. This finding was difficult to repeat in our hands, since in our strain of rats such doses were lethal after a short period. According to Luckey and Venugopal (1978) the LD for sodium selenite in rats is about 43 umol/kg. Eaton et al. (1983) have reported that in two groups of animals treated with two consecutive i.p. injections of 13 umol/kg or 25 umol/kg (at 12 and 36 hours before sacrifice), a 3-fold increase in HO activity was observed, while only the highest dose significantly reduced P-450 concentration; No effects on P-450 dependent enzymes were measured in this study. However, Schnell et al. (1983) reported that 72 hours after treatment with a single i.p. dose (23 umol/kg) of SeIV, a 20% reduction in P-450 content and a 30% decrease in ethylmorphine N-demethylase activity were produced, but no effect on aniline hydroxylase activity was observed. The effects on HO activity were not determined by these authors. The above data suggest that there are doses of SeIV which induce HO, but have no effect on P-450 concentration, and that this occurs between 14.5 and 24 hours after treatment. This would support the contention of Drummond et al. (1982) that P-450 heme degradation is not essential to initiate HO induction. However, it would be interesting to know what changes, if any, occur in HO activity at later times when the P-450 concentration is reduced. Time-course studies on the effects of Se on these parameters are needed to resolve this.

Why does SeIV administration not reduce P-450 content
at times up to 24 hours? Se has been shown to be necessary to maintain the normal function of P-450 (Burk and Correia, 1981; Burk, 1983). Se also increases the levels of Se-glutathione peroxidase which protects against lipid peroxidation (Tappel, 1980), a possible factor in P-450 losses (De Matteis, 1982). Furthermore, in vitro, and in contrast to most metals, Se in concentrations ranging from $10^{-6}$ to $10^{-3}$ M produced no effect on total P-450 content; however, a 25% decrease in aniline hydroxylase was observed in the same experiment (Schnell et al., 1983). Another possibility is that SeIV acts in a manner analogous to that of SKF 525A, complexing with heme, thereby preventing the degradatory effects of some compounds on P-450. However, Se does not impair the ability of P-450 to bind CO, as SKF 525A does (Buening and Franklin, 1976), which makes this possibility unlikely.
3.3.5 *Is biliverdin reductase an important factor in the regulation of heme oxygenase activity?*

It has been suggested by Maines and Kappas (1977) that the interaction of metal ions with specific receptor sites, containing key sulphydryl groups, may trigger cellular events leading to HO induction. According to Maines (1984), an SH-containing protein which appears to be a good candidate for functioning as the regulator of HO activity is biliverdin reductase (BR). The hypothesis proposed was that the activity of BR is inhibited upon exposure to agents with a high affinity for SH groups. As a result, the enzyme would be unable to remove the biliverdin formed by HO. Thus, HO activity would be inhibited as the result of the hindered dissociation of biliverdin from its heme-binding site. This could trigger cellular events to accommodate the inhibited HO activity, leading to increased HO production. The evidence supporting this hypothesis comes from experiments in which rats were treated with biliverdin (Kutty and Maines, 1984). The response was an initial (within 3 h) inhibition followed by a late (48 h) rebound increase in BR and HO activities. However, there is little published information on the effects of metals on this enzyme. Frydman et al. (1979) mentioned that in rats dosed with 200 mg/kg (730 umol/kg) of CoCl$_2$ an increase in BR activity was observed, but no quantitative data were given; Kutty and Maines (1983) could not confirm these findings.
The results of the present study showed no detectable effects of AsIII, AsV or SeIV, at any dose level, on BR activity. These negative results argue against the hypothesis mentioned above, because As and Se are powerful HO inducers and markedly increased biliverdin production without significant changes in BR activity. Under physiological conditions biliverdin reduction is not the rate limiting step in heme catabolism and it appears that neither is this so under the altered conditions following As and Se poisoning. Therefore, it is highly unlikely that BR plays an important role in the HO induction produced by As and Se.
3.3.6 **Effects of the combined administration of AsIII and SelIV.**

The simultaneous administration of AsIII and SelIV resulted in a dose-dependent decrease in the heme saturation of TP, as described by Albores (1984). However, the effect was significantly ($p < 0.05$) more pronounced than that observed with AsIII alone (Tables 3.1, 3.9 and Fig. 3.12). Only the effects of 3 combined doses on HO activity were examined, however it was clear that this activity was significantly higher ($p < 0.05$) than that produced by AsIII alone (Tables 3.3 and 3.13). The additive effects of AsIII and SelIV on HO activity, plus the ability of As to inhibit heme synthesis at other steps not affected by Se, might explain the additive effects of these metalloids in decreasing the degree of heme saturation of TP. As in the treatment with either AsIII or SelIV alone, the combined administration of these metalloids did not produce any significant effect on biliverdin reductase activity.

There were indications that SelIV administration may protect against the P-450 degradation produced by AsIII (Tables 3.4 and 3.13). The levels of P-450 after AsIII treatment (75 umol/kg) were reduced to 59% of control values while those of AsIII (75 umol/kg) plus SelIV (5 umol/kg) were reduced to 71% of control ($0.1 > P > 0.05$). Early and Schnell (1981) have reported that i.p. administration of SelIV
Fig. 3.13 Effects of the combined administration of AsIII and SeIV on tryptophan pyrrolase. AsIII (■), SeIV (○), AsIII + 5 umol/kg SeIV (○).
(9.3 umol/kg) blocked the Cd-induced (3.8 umol/kg) reduction in P-450 and P-450 dependent monooxygenases. These data suggest that Se can prevent some manifestations of hepatotoxicity associated with the activity of the P-450 dependent enzyme system; however, further experiments are needed to find the range of Se doses able to protect against this type of As hepatotoxicity. It is possible that the protective effect of Se against As-induced destruction of P-450 may involve the role of Se as a component of glutathione peroxidase (Hoekstra, 1975), which is particularly important for maintaining the integrity of cellular and subcellular membranes from the oxidative damage thought to be caused by metals (De Matteis, 1982). As selenium deficiency results in reduction of Se-dependent glutathione peroxidase activity (Burk, 1983), it would be interesting to know if Se administration increases its activity above control levels. The data from the present study indicate that Se may have a dual effect in As-induced hepatotoxicity. On one hand, it protects against the decrease in P-450 concentration, and on the other, it has additive effects increasing HO activity and further reducing the heme saturation of TP.

There is evidence that Se changes the biological effects of a number of heavy metals and render them less toxic (Nordberg, 1976). With respect to As and Se, it has been reported that they ameliorate each other's toxicities (Frost, 1983). The protective effects of As have been known since 1945 when Moxon et al. showed that As could prevent Se
poisoning when compounds of both elements were injected subcutaneously. Holmberg and Ferm (1969) showed that SeIV decreased the teratogenicity of AsV in hamsters when injected simultaneously. However, there are some situations in which As increases Se toxicity. Obermeyer et al. (1971) have shown that poisoning by trimethylselenonium chloride, a compound of relatively low toxicity compared to many other Se compounds, is markedly increased by simultaneous injection with AsIII. Cabe et al. (1979) showed that in rats exposed to AsV (50 ppm) plus SeIV (10 ppm) via drinking water for 13 weeks the mortality rate was 70% compared to less than 10% in the rats exposed to AsV or SeIV alone. As if this picture were not confused enough already, Levander, (1977) and Alexander (1980) have reported that AsIII potentiates the beneficial effect of SeIV in protecting against methylmercury toxicity. A general mechanism has been proposed to explain the mutually antagonistic abilities of As and Se. Levander and Baumann (1966) have suggested that Se and As react in the liver to form a detoxication conjugate which is then excreted into bile; the chemical nature of this conjugate has not yet been established. This is consistent with the fact that As and Se each increase the biliary excretion of the other. However, this explanation would not account for the additive effects observed in this study.
3.3.7 Effects of 30 day exposure to AsIII.

Although the control values did not change significantly during the 30 day period, TP "total enzyme" activity and apoenzyme values were lower (Table 3.15) than those obtained in the acute experiment series (Table 3.1). However, the degree of heme saturation and the holoenzyme/apoenzyme ratio did not significantly change. A possible explanation for this reduction in TP activity is that the incubation of homogenate samples in the 30 day study was not done immediately after homogenization, as it was in the acute experiment series, but was kept in the incubation mixture at 4°C for four hours before starting the incubation period. Badawy and Evans (1974) have suggested that delays in sample incubation may decrease enzyme activity. The main effects of continuous exposure to AsIII on TP were:

1) a progressive increase in "total enzyme" activity and in calculated apoenzyme values (Table 3.15). Although there is little published information which directly relates to the effects of As on TP apoenzyme synthesis, Ghafghazi and Mennear (1973) and Ghafghazi et al. (1980) have shown that the acute and subacute administration of Cd and AsIII affect the adrenal glands, releasing epinephrine and corticoids. This in turn leads to an increase in TP-apoenzyme synthesis as discussed in Section 3.3.1. Indirect support to this suggestion was given by the report (Cebrian et al., 1984)
that administration of AsIII in drinking water (5, 50 and 100 ppm) produced a dose-related weight increase in the adrenal glands of female rats at the end of 8 weeks of treatment.

2) an initial decrease in the heme saturation of TP, which did not subsequently change significantly during the period of treatment (Table 3.15). This decrease from control values indicates that continuous treatment with AsIII has reduced the availability of heme in liver cells. This effect was similar to that observed after the acute administration of 25 umol/kg. It also seems to indicate that a new equilibrium, at a lower level, between synthesis and degradation of heme has been reached during the 30 days of treatment.

There was a 60% increase in ALAS activity, during the first 10 days of exposure and subsequently this level was reduced to a 30% increase over control values (Table 3.16). This appears to be in response to the decreased heme availability shown by reduction in the heme saturation of TP. Although the increases were modest, they are within the range observed after acute treatment with doses higher than 50 umol/kg of AsIII.

In marked contrast to the dose-related increases in HO activity and corresponding decreases in cytochrome P-450 concentrations observed after the acute administration of
AsIII (Table 3.3 and 3.4), no detectable effects on these parameters were observed at any of the interim time points during treatment (Table 3.16). A possible explanation for this is that at the exposure levels selected for this experiment (50 ppm; 24 umol/kg/day) AsIII does not reach the endoplasmic reticulum in sufficient concentrations to produce a significant effect. It should be noted that the binding of As to microsomes is fairly weak in several animal species, the exception being the marmoset monkey in which 50% of the As present in the liver cell is bound to the microsomal fraction (Vahter et al., 1982). The discrepancy between the effects of acute and chronic treatments are not exclusive to As. It is known that acute treatment with Co, Cd, Zn, Ni or Hg markedly increase ALAS and HO activities and reduce P-450 contents. However, daily administration of Co(NO\textsubscript{3})\textsubscript{2} (20mg/kg; 69 umol/kg), CdSO\textsubscript{4} (10 mg/kg; 13umol/kg) and ZnSO\textsubscript{4} (20 mg/kg; 111 umol/kg) in drinking water for 30 days produced only moderate increases in ALAS activity (30-40%), decreases in HO activity (30-40%) and increased P-450 concentration (20-30%). Similar treatment with NiSO\textsubscript{4} (20 mg/kg; 71 umol/kg) or HgCl\textsubscript{2} (5mg/kg; 18.4 umol/kg) produced no detectable effects on any of these parameters (Kadiiska et al., 1985). Se, which is a powerful HO inducer in acute experiments, when administered for 6 months in the diet (10 ppm) did not induce HO; also in contrast to the absence of effects after acute treatment, subchronic exposure produced a 20% decrease in P-450 concentration (Reiter and Wendel, 1985).
In the present study, the acute subcutaneous administration of AsIII produced a marked increase in HO activity, which was considered to be a major contributing factor to the observed decrease in heme availability (see Section 3.1.1). However, in the 30 day study, where the dose of AsIII (50 ppm in the drinking water) was equivalent to 3.1 mg/kg/day (24 umol/kg/day), the combination of increased ALAS activity, diminished heme availability and apparently normal rate of heme degradation, strongly suggests that an alteration in the heme synthesis pathway is produced after a relatively short time of exposure. Woods and Fowler (1978) and Martinez et al. (1983), showed that AsV and AsIII produce porphyria in rats, this would be expected to be associated with a reduction in the production of heme and hence, a decrease in heme availability. This pattern was observed in the present study.

Woods and Fowler (1978), have reported that exposure for 6 weeks to AsV at 20, 40 and 85 ppm in the drinking water resulted in non-dose-related effects on ALAS activity; while no alteration occurred in rats exposed to 20 ppm, a 20% reduction in activity was observed at the 40 ppm level, but only a 13% reduction was produced by the highest concentration. No effect was observed in mice at 20 and 85 ppm exposure, but a 13% reduction was observed at the 40 ppm level. No effects on ALA dehydratase were observed in rats
or in mice. The activity of uroporphyrinogen I synthetase, was not affected in rats, but there was a dose-related increase in mice. A dose-related inhibition of heme synthetase was also found, the maximum decrease (40%) was observed at the 85 ppm level. Accompanying these alterations in enzyme activity, there were substantial dose-related elevations in the concentrations of urinary porphyrins. Martinez et al. (1983) have shown that AsIII administration in drinking water at 5, 50 and 100 ppm produced a dose-related increase in uroporphyrin excretion after one week of treatment. Although there is agreement that As produces porphyria, there is little information on the organs affected; the report that in AsV-induced porphyria a hepatic reduction in ALAS activity was present (Woods and Fowler, 1978) is suggestive that other organs, specially kidney, may be affected and could contribute to the total excretion of porphyrins (see Chapter 5).

In the study of Martinez et al. (1983), the initial marked increase in uroporphyrin excretion was followed by a gradual decrease towards control levels, as the period of exposure continued (Fig. 3.14), this was interpreted as a sign of adaptation. In the present study, the response of ALAS followed a similar pattern, giving support to the idea that an adaptive response to the toxic effects of AsIII on heme synthesis takes place. Other biochemical studies of As toxicity have also suggested the existence of an adaptation/compensation phenomenon. Schiller et al. (1978)
Fig. 3.14. Urinary uroporphyrin excretion in rats treated with sodium arsenite (AsIII). Percentages of control. Control values were 0.38 ± 0.09 (X ± S.E.).
reported that an inhibition of pyruvate dehydrogenase induced by exposure to As for 3 weeks was not present after 6 weeks of continuous treatment. The exact mechanisms by which adaptation to As toxicity develops are not known; however, the following possibilities have been mentioned: decreased gastrointestinal absorption, changes in excretion rates, or alterations in the rate at which AsIII is converted to AsV in vivo (Squibb and Fowler, 1983).
CHAPTER FOUR

SOME EFFECTS OF ARSENIC AND SELENIUM
ON HEPATIC XENOBIOTIC METABOLISM.
4.1 INTRODUCTION

Some effects of arsenic and selenium on hepatic heme metabolism were described and discussed in Chapter 3. A prominent effect of the acute administration of these metalloids was an increase in the rate of heme degradation, which in turn affected either the specific activity or the absolute amounts of several heme-dependent enzymes. Among these enzymes the concentration of cytochrome P-450 was markedly reduced by both pentavalent and trivalent arsenic, whereas selenium did not seem to affect this enzyme system. Thus, it was considered of interest to study the effects of these metalloids on hepatic cytochrome P-450-dependent metabolism, since this enzyme system is involved in the biotransformation of many endogenous and exogenous substances.

It was also decided to study the effects of As and Se on epoxide hydrolase (EH), a microsomal enzyme concerned with the conversion of highly reactive epoxides and arene oxides to chemically less reactive dihydrodiols. This reaction has been recognized as an important metabolic step in the detoxication of these compounds (Lu and Miwa, 1980). It is generally considered that compounds that induce EH will also induce P-450 and other P-450 dependent metabolizing enzymes (Guengerich, 1982). However, there are several furan derivatives that are good EH inducers, but either have no
significant effects on P-450 concentration (e.g., dibenzofuran and dihydrobenzofuran) or decrease it (e.g., furan and benzofuran), Connelly (1983). From Connelly's data it is also apparent that some compounds (e.g., isosafrole, benzofuran, dihydrobenzofuran and trans-stilbene oxide) that induce EH, induce concurrently heme oxygenase (HO). Although there is very little available information on the in vivo effects of inorganic compounds on EH activity, or on the possibility of As or Se or their metabolites forming epoxides, arsenic shares with furan and benzofuran HO inducing and P-450 depleting properties, and Se shares with dibenzofuran and dihydrobenzofuran the ability to induce HO without affecting P-450 content. On this basis it was decided to study the effects of AsIII and SeIV on EH activity.
4.2 RESULTS.

4.2.1 Effects of Arsenic.

The effects of 4 different dose levels of AsIII (ranging from 12.5 to 75 umol/kg) and 5 different levels of AsV (ranging from 25 to 200 umol/kg) at 16 hours were studied. Both salts of As were administered by subcutaneous injection. In the subchronic study, AsIII was administered via drinking water at a concentration of 50 mg/l.

a) Ethoxycoumarin and ethoxyresorufin O-deethylases.

(i) Acute effects of AsIII. The activity of both these P-450 dependent enzymes was decreased in a dose-related manner (Table 4.1). However, the effect was slightly greater for ERD; the reduction in activity was significant even at a lower dose; the highest dose (75 umol/kg) reduced the activity to 47% of control. For ECD the activity was reduced to 66% of control by 75 umol/kg of AsIII.

(ii) Acute effects of AsV. The observed effects were similar to those produced by AsIII administration. the activities of both enzymes were reduced, but only at doses higher than 100 umol/kg (Table 4.2). Again, this reduction was slightly greater for ERD; the highest dose (200 umol/kg) reduced the ERD activity to 49% of control, while ECD activity was
Table 4.1 Effects of sodium arsenite (AsIII) on hepatic epoxide hydrolase, heme oxygenase, ethoxycoumarin and ethoxyresorufin O-deethylases, and cytochrome P-450.

<table>
<thead>
<tr>
<th>AsIII (umol/kg)</th>
<th>Epoxide Hydrolase&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Heme Oxygenase&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Ethoxycoumarin O-deethylase&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Ethoxyresorufin O-deethylase&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Cytochrome P-450&lt;sup&gt;4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.9 ± 2.0</td>
<td>1.70 ± 0.25</td>
<td>0.38 ± 0.08</td>
<td>18.2 ± 3.0</td>
<td>0.63 ± 0.08</td>
</tr>
<tr>
<td>12.5</td>
<td>11.7 ± 1.4</td>
<td>1.71 ± 0.31</td>
<td>0.40 ± 0.04</td>
<td>16.1 ± 2.2</td>
<td>0.56 ± 0.08</td>
</tr>
<tr>
<td>25</td>
<td>10.5 ± 0.9</td>
<td>3.90 ± 0.42</td>
<td>0.36 ± 0.06</td>
<td>14.2 ± 1.5</td>
<td>0.51 ± 0.09</td>
</tr>
<tr>
<td>50</td>
<td>14.0 ± 1.8</td>
<td>9.28 ± 0.12</td>
<td>0.30 ± 0.01</td>
<td>10.1 ± 0.6</td>
<td>0.42 ± 0.07</td>
</tr>
<tr>
<td>75</td>
<td>12.6 ± 2.0</td>
<td>14.09 ± 0.62</td>
<td>0.25 ± 0.02</td>
<td>8.5 ± 1.0</td>
<td>0.37 ± 0.07</td>
</tr>
</tbody>
</table>

1 nmol/min/mg microsomal protein.
2 nmol/h/mg microsomal protein.
3 pmol/min/mg microsomal protein
4 nmol/mg microsomal protein.

All above given as \( \bar{x} \pm S.D. \) (n = 6 for control, n = at least 4 for other data). These effects were studied at 16 hours after subcutaneous administration of AsIII.

*Differs from control data \( p < 0.05 \).
Table 4.2 Effects of sodium arsenate (AsV) on hepatic cytochrome P-450, ethoxycoumarin and ethoxyresorufin O-deethylases.

<table>
<thead>
<tr>
<th>AsV (umol/kg)</th>
<th>Cytochrome P-450&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Ethoxycoumarin O-deethylase&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Ethoxyresorufin O-deethylase&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.63 ± 0.08</td>
<td>0.38 ± 0.08</td>
<td>18.2 ± 3.0</td>
</tr>
<tr>
<td>25</td>
<td>0.65 ± 0.05</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>50</td>
<td>0.62 ± 0.06</td>
<td>0.36 ± 0.07</td>
<td>19.6 ± 2.5</td>
</tr>
<tr>
<td>100</td>
<td>0.60 ± 0.05</td>
<td>0.34 ± 0.08</td>
<td>20.7 ± 3.2</td>
</tr>
<tr>
<td>150</td>
<td>0.57 ± 0.04</td>
<td>0.31 ± 0.02</td>
<td>14.3 ± 2.3</td>
</tr>
<tr>
<td>200</td>
<td>0.46 ± 0.05</td>
<td>0.23 ± 0.04</td>
<td>8.9 ± 1.0</td>
</tr>
</tbody>
</table>

1 nmol/mg microsomal protein.
2 nmol/mg microsomal protein/min.
3 pmol/mg microsomal protein/min.

All above given as X ± S.D. (n = 17 for controls, n = at least 3 for other data). These effects were studied at 16 hours after subcutaneous administration of AsV.

* Differs from control data, p < 0.05

N.A. Data not obtained.
reduced to 60% of control.

(iii) Effects of administration of AsIII daily for 30 days. No significant effects on either ERD or ECD were observed at 5, 10, 20 or 30 days of exposure (Table 4.3).

b) Epoxide Hydrolase.

No significant effects of acute administration of AsIII were observed at any dose level (Table 4.1).

c) Heme oxygenase and Cytochrome P-450.

The effects of AsIII and AsV on these parameters have been described and discussed in Chapter 3 i.e, acute As treatment resulted in dose-related increases in HO and dose-related decreases in P-450 concentration (Table 4.1), while exposure for 30 days to AsIII produces no significant changes in these parameters.
Table 4.3 Effects of 30 day administration of sodium arsenite (AsIII) in drinking water (50 ppm) on hepatic cytochrome P-450, ethoxycoumarin and ethoxyresorufin O-deethylases.

<table>
<thead>
<tr>
<th>Days of treatment</th>
<th>Cytochrome P-450$^1$</th>
<th>Ethoxycoumarin O-deethylase$^2$</th>
<th>Ethoxyresorufin O-deethylase$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.63 ± 0.08</td>
<td>0.38 ± 0.08</td>
<td>18.2 ± 3.0</td>
</tr>
<tr>
<td>5</td>
<td>0.68 ± 0.09</td>
<td>0.43 ± 0.01</td>
<td>20.4 ± 2.6</td>
</tr>
<tr>
<td>10</td>
<td>0.65 ± 0.07</td>
<td>0.41 ± 0.08</td>
<td>15.9 ± 3.5</td>
</tr>
<tr>
<td>20</td>
<td>0.59 ± 0.04</td>
<td>0.37 ± 0.05</td>
<td>15.7 ± 3.8</td>
</tr>
<tr>
<td>30</td>
<td>0.69 ± 0.07</td>
<td>0.39 ± 0.05</td>
<td>14.4 ± 1.5</td>
</tr>
</tbody>
</table>

1 nmol/mg microsomal protein.

2 nmol/mg microsomal protein.

3 pmol/mg microsomal protein.

All above given as $\overline{X} \pm $ S.D. (n = 6 for control, n = at least 4 for other data).

*Differs from control data, p < 0.05.
4.2.2 Effects of SelV.

The effects of 3 different doses of SelV at 16 hours, ranging from 5 to 20 umol/kg were studied. Se was administered by subcutaneous injection.

a) Ethoxycoumarin and ethoxyresorufin O-deethylases.

No significant effects of acute administration of SelV on the activity of either ERD or ECD was observed (Table 4.4).

b) Epoxide Hydrolase.

A dose related increase in this enzyme activity was produced by SelV (Table 4.4). Doses below 10 umol/kg produced no significant changes, while the highest dose (20 umol/kg) produced a 2-fold increase over control values.

c) Heme oxygenase and cytochrome P-450.

After acute SelV treatment a dose-related increase in HO activity was observed, but P-450 content was unchanged (Table 4.4). These effects in relation to heme catabolism were described and discussed in Chapter 3.
Table 4.4 Effects of sodium selenite (SeIV) on hepatic epoxide hydrolase, heme oxygenase, ethoxycoumarin and ethoxyresorufin O-deethylases and cytochrome P-450.

<table>
<thead>
<tr>
<th>SeIV (μmol/kg)</th>
<th>Epoxide Hydrolase^1</th>
<th>Heme Oxygenase^2</th>
<th>Ethoxycoumarin O-deethylase^1</th>
<th>Ethoxyresorufin O-deethylase^3</th>
<th>Cytochrome P-450^4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.9 ± 2.0</td>
<td>1.85 ± 0.30</td>
<td>0.38 ± 0.08</td>
<td>18.2 ± 3.0</td>
<td>0.61 ± 0.06</td>
</tr>
<tr>
<td>5</td>
<td>12.1 ± 0.4</td>
<td>2.74 ± 0.25</td>
<td>0.40 ± 0.06</td>
<td>16.2 ± 1.3</td>
<td>0.55 ± 0.09</td>
</tr>
<tr>
<td>10</td>
<td>15.4 ± 1.9</td>
<td>4.19 ± 0.38</td>
<td>0.44 ± 0.02</td>
<td>15.9 ± 1.4</td>
<td>0.54 ± 0.10</td>
</tr>
<tr>
<td>20</td>
<td>22.7 ± 4.0</td>
<td>6.92 ± 0.59</td>
<td>0.35 ± 0.04</td>
<td>18.9 ± 2.5</td>
<td>0.64 ± 0.07</td>
</tr>
</tbody>
</table>

1 nmol/min/mg microsomal protein.
2 nmol/h/mg microsomal protein.
3 pmol/min/mg microsomal protein.
4 nmol/mg microsomal protein.

All above given as X ± S.D. (n = 6 for control, n = at least 4 for other data). These effects were studied at 16 hours after subcutaneous administration of SeIV.

* Differs from control data, p < 0.05.
4.3 DISCUSSION.

4.3.1 Effects on mixed function oxidase activity.

There is some evidence that, under certain conditions, arsenic stimulates detoxicating enzyme activities in microorganisms (Legge, 1974), but little is known about this effect in mammals. Ribeiro (1971, as cited by Wagstaff, 1978) has reported that hexobarbitone sleeping time was reduced in mice pretreated with arsenic trioxide, but that rates of hexobarbitone oxidation or N-demethylation of aminopyrine were not altered in liver tissue from these same mice. These observations contrast with the results obtained in the present study in which the acute administration of AsIII and AsV resulted in a dose-dependent reduction in P-450 concentration accompanied by dose-related decreases in the activity of ethoxycoumarin (ECD) and ethoxyresorufin (ERD) O-deethylases. The O-deethylation of 7-ethoxycoumarin has been shown to be inducible by both 3-methylcholanthrene (MC) and phenobarbitone (PB) in rat hepatic tissues (Ullrich et al., 1973; Sundheimer et al., 1983). In contrast, ethoxyresorufin O-deethylase activity is low in liver microsomes from control or phenobarbitone-treated rats, but it is induced 50 to 60-fold upon pretreatment with MC (Burke and Mayer, 1974, 1975). ERD activity is considered a specific indicator of MC-inducible cytochrome P-450.
There are indications that the inhibitory effect of AsIII could be substrate dependent: the effect on ERD occurred at a lower dose, and the reduction in activity (with respect to control values of both enzymes) was 20% more pronounced than that observed for ECD activity (Table 4.1). Although the difference is quantitatively small, it is reproducible and suggests that AsIII has a differential effect on cytochrome P-450 composition in hepatic microsomes preferentially affecting the MC-inducible P-450 form (P-448). Further experiments are needed to determine fully whether these observations represent a direct effect of AsIII on P-448 or are due to an effect on hemoprotein synthesis/degradation. In the first instance, it would be useful to study the in vitro effects of AsIII on the rates of benzo[a]pyrene hydroxylation, benzphetamine N-demethylation and acetanilide hydroxylation, followed by SDS-gel electrophoresis, these are well established procedures for identifying effects on particular forms of P-450 (Lu and West, 1979; Sundheimer et al.).

The acute administration of AsV also resulted in reduction of P-450 concentration and decreased ECD and ERD activities (Table 4.2). As observed with AsIII, the effect on ERD was greater than that on ECD. However, AsIII was 2.5 times more potent than AsV in altering the activities of both demethylases (Fig. 4.1), this parallels the effects on cytochrome P-450 concentration.
Fig. 4.1 Effects of Arsenic on ethoxycoumarin (ECD) and ethoxyresorufin (ERD) O-deethylases. The effects were studied 16 hours after subcutaneous administration.

Effects of AsIII: ECD (○) ERD (●). Effects of AsV: ECD (□) ERD (■).
The acute administration of SeIV produced no significant changes in P-450 concentration or in ECD and ERD activities (Table 4.3). These results are consistent with those reported by Maines and Kappas (1976a), who found that high doses of SeIV (50 umol/kg and higher) did not significantly reduce P-450 concentration 14.5 hours after treatment. However, these results contrast with those reported by Schnell et al. (1983) who found that an i.p. dose of 2.4 mg/kg (23 umol/kg) produced a 20% reduction in P-450 concentration, accompanied by a 30% decrease in ethylmorphine N-demethylase activity, without changes the rate of aniline hydroxylation. This discrepancy may be related to differences in the period of Se administration, since Schnell's measurements were made at 72 hours after administering SeIV, whereas in the present study and in that of Maines and Kappas (1976a) the measurements of P-450 and P-450 related monooxygenases were made much earlier (16 and 14.5 hours respectively after treatment). Time course studies on the effects of SeIV on selected monooxygenases could contribute to explaining the differences mentioned above. A brief discussion on the absence of effects on P-450 concentration and on the effects on HO activity was presented in Chapter 3.

In the present study, the administration of AsIII (50 ppm) in the drinking water for 30 days produced no
significant changes in P-450 levels or in ECD and ERD activity at any of the intervals studied (after 5, 10, 20 and 30 days of treatment (Table 4.2). Although non-significant, there was a tendency towards an exposure-related decrease in ERD activity, suggesting that if the exposure had been continued, or higher levels used, a significant reduction could have been observed. Nonetheless, this contrasts with the marked reductions in P-450 content and in hepatic drug metabolism observed after the acute administration of AsIII. Our findings on the effects of continuous administration are in agreement with data reported by Woods and Fowler (1978), who found that 6 weeks of AsV treatment in drinking water (20, 40 and 85 ppm) produced no significant changes in P-450 concentration or in aminopyrine demethylase activity. However, the absence of effects mentioned above contrasts with the data reported by Wagstaff (1978), who found that administration of arsenic trioxide (100 and 1000 ppm) in a vitamin A supplemented diet to female rats produced a 20% reduction in hexobarbitone sleeping time; the highest level (1000 ppm; ~ 500 umol/kg of rat/day) also produced an increase in O-ethyl-O-p-nitrophenyl phenylphosphoethionate detoxication and in the O-demethylation of p-nitroanisole, both measured in the 9000 g supernatant. Besides the difference in sex and the vitamin A supplemented diet, no other explanation is at hand to account for the differences in the response to AsIII (present study), AsV (Woods and Fowler, 1978) and arsenic trioxide. Schnell et al. (1978) have reported that cadmium, which is known to markedly reduce
P-450 concentration and inhibit drug metabolism upon acute treatment (3 umol/kg, i.p.), did not produce changes in these parameters when it was subchronically administered via drinking water (100 and 200 mg/l) for up to 12 weeks. These authors were unable to satisfactorily explain their observations since they considered that the dose of Cd in the subchronic study was not large enough to induce sufficient metallothionein to inactivate the free Cd. Some considerations on the adaptation/compensation phenomena observed with As were made in Chapter 3.

4.3.2 Effects on epoxide hydrolase.

The data presented in tables 4.1 and 4.4 indicate that As and Se share HO inducing properties, but differ in their ability to increase microsomal EH activity and in their effects on P-450 concentration and related monooxygenase activities. Thus, AsIII has no significant effect on EH activity but reduces P-450 content and the activity of P-450-related monoxygenases, whereas SeIV induce EH but has no effect on P-450 and related enzymes. There is very little information on the effects of elements on EH that could be used as a reference for these effects. In vitro addition of metals (Cd, Zn and Hg) and organometallic compounds to microsomes has been shown to inhibit different drug metabolizing enzyme systems, including EH activity towards different substrates (Parkki, 1980, 1981). The hydration of
styrene 7, 8-oxide was inhibited in an apparently noncompetitive manner, whereas the inhibition of benzo[a]pyrene 4,5-oxide hydration with Cd was competitive (Parkki et al., 1980). The in vitro addition of AsIII in concentrations up to 10 mM did not produce significant changes in EH activity (Parkki, 1981). However, it is difficult to extrapolate the significance of these results to studies made in vivo.

As yet we have no evidence to explain how Se induces EH, one might hypothesize a direct effect of Se on the EH receptor, in a way similar to that described for HO induction by metals (Kikuchi and Yoshida, 1983). Similarly, the reasons for the induction are uncertain. Is it possible that Se could form an epoxide which is a substrate for epoxide hydrolase? To date, a separate EH receptor has not been described, and judging from the limited information available on the biotransformation of Se, it seems improbable that it could be transformed into an epoxide. The induction process may be complex for there have been several studies on the structure-activity relationships of EH inducers (Oesch, 1979; Seidegard, 1981; Connelly 1983) which have not produced conclusive evidence regarding the structural requirements for EH induction. It is of course possible that EH is coinduced in no more than fortuitous association with other enzymes that are affected during a toxic process, or that EH induction is a particular response to certain forms of toxic or carcinogenic damage.
Is there evidence to suggest that Se is an hepatic carcinogen? Several studies concerning the alleged carcinogenicity of Se to laboratory animals are available: Nelson et al. (1943) fed diets containing 5, 7 or 10 ppm of Se to rats for a life-time period and found that the liver suffered fatty degeneration and necrosis after a short period of exposure. However, when the exposure continued over a long period (1.5 years), cirrhosis in some animals was produced. Eleven out of 53 animals developed liver cell adenoma or low grade carcinoma without metastasis in cirrhotic livers, whereas the control rats had neither liver tumours nor cirrhosis. Two studies that specifically looked for carcinogenic effects of Se in mice (Schroeder and Michener, 1972) and rats (Harr et al., 1967, as cited by Glover et al., 1979) failed to demonstrate any such effects. The overall conclusion could be that the neoplastic lesions, if any, are not present in the absence of severe hepatotoxic phenomena.

Is it possible that the Se-mediated increase in epoxide hydrolase could result from elevations in the concentration of bile components in liver? It has been hypothesized that as the increases in EH occur very shortly after exposure to hepatocarcinogens, such increase could be due more to the toxic effects of the compounds than to their carcinogenic properties (Dent and Graichen, 1982). Support for this hypothesis comes from acute studies with alpha and
beta-naphthylisothiocyanates (ANIT and BNIT) (Leonard et al., 1981 a, b) which showed that ANIT increased EH activity that correlated well with the extent of bile duct proliferation and elevated serum bilirubin. Conversely, BNIT which shares many toxicological properties with ANIT, did not produce bile duct proliferation and did not alter EH activity. Furthermore, bile duct ligation caused bile duct proliferation and increases in EH similar to those caused by ANIT. There is some evidence to suggest that Se treatment alters bilirubin metabolism. It has been reported (Halverson et al., 1966, 1970) that diets containing 6-8 ppm of sodium selenite produced liver cirrhosis, splenomegaly, hemolytic anemia and elevated serum bilirubin levels in rats. Furthermore, the increase in HO activity with the consequent increase in bilirubin production observed at 16 hours after a single injection of sodium selenite reported in the present study, give some support to the assumption that Se treated animals have elevated concentrations of bile components, which in turn could induce EH activity. However, the mechanism by which bile components could induce EH has not been established.

Could epoxide hydrolase induction contribute to explaining the anticarcinogenic properties of selenium? It is now generally accepted that Se, in addition to its possible carcinogenic properties, also has anticarcinogenic properties. This is supported by epidemiological evidence, animal experiments, and indirectly by bacterial mutagenicity
tests (Reddy and Massaro, 1983). In animal experiments, Se has been shown to counteract the induction of liver tumours by 2-acetylamino-1-fluorene (Harr et al., 1972; Marshall et al., 1979), by aflatoxins (Grant et al., 1977), and by 3-methyl-4-dimethylaminoazobenzene (Griffin and Jacobs, 1977). Supplementation of the diet with Se also inhibited mammary gland tumours induced by 7,12-dimethylbenz(a)anthracene (DMBA) in mice and rats (Medina and Sheperd, 1981; Welsch et al., 1981), while Se deficiency enhanced DMBA-induced mammary tumorigenesis in rats having a high polyunsaturated fat intake (Ip and Sinha, 1981). It has also been shown that Se reduces skin tumours due to 3-methylcholanthrene and benzo(a)pyrene (Shamberger, 1970). Se, however, may not be effective against all types of carcinogens, since it had no effect on the induction of tracheal cancer by 1-methyl-1-nitrosourea (Thompson and Becci, 1979), and it does not appear to be as effective against N-methyl-N-nitrosourea induced mammary tumours as it is against those induced by DMBA (Thompson and Becci, 1980).

Although the mechanism by which selenium functions as an anticarcinogen is not clear, it is known that compounds like aflatoxins, DMBA, and benzo(a)pyrene are transformed to epoxides in order to exert their carcinogenic effects (Heidelberger, 1975). Therefore, it is possible that the induction of EH produced by the acute administration of Se could play a role in the protection afforded by Se against
some chemical carcinogens, increasing the rate of metabolism of these highly reactive epoxides and reducing the possibility of interactions with genetic material. Further experiments are needed to determine if during supplementation of the diet with Se, EH is maintained in an induced state. However, such a mechanism would not explain the protective effect of Se on 2-acetylaminofluorene or 3-methyl-4-dimethylaminoazobenzene tumours.

Other possible mechanisms to explain the anticarcinogenic properties of Se include: 1) The antioxidant properties attributed to Se. As an antioxidant, Se could inhibit oxidative reactions required for transforming procarcinogens into ultimate carcinogens or, more likely, autooxidative damage. However, by itself Se is not a good antioxidant, but it is a constituent of Se-dependent glutathione peroxidase which reduces hydroperoxides, which otherwise might undergo autooxidation by metal catalyzed reactions to generate highly reactive alkyl peroxy, alkoxy and hydroxyl radicals (Combs et al., 1975; Reddy and Massaro, 1983); 2) Alterations in the metabolic fate of chemical carcinogens. It has been reported that Se inhibits aryl hydrocarbon hydroxylase activity of human lymphocytes in culture (Griffin, 1979). It also appears that Se treatment protects against 2-acetylaminofluorene carcinogenesis by increasing the ring hydroxylation along with a decrease in the N-hydroxylation (Marshal et al., 1979); 3) There have been reports that Se
inhibits events occurring during the promotion stage of carcinogenesis, as in the case of N-methyl-N-nitrosurea (Thompson and Becci, 1980), but little is known about these events or the possible mechanism(s) of Se effects. The increasing number of animal tumour model systems responsive to Se-mediated inhibition should allow the elucidation of the particular significance of these modes of action to be identified.
CHAPTER FIVE
SOME EFFECTS OF ARSENIC ON TESTICULAR
AND RENAL HEME METABOLISM
5.1 INTRODUCTION.

All tissues are susceptible to the toxic effects of chemicals, but the majority of compounds that cause systemic toxicity do not affect all organs equally, rather they exhibit toxicity in specific organs (Cohen, 1983). The kidney plays a major role in As excretion and it is one of the primary organs in which As accumulates and exerts its toxicity (Brown et al., 1976). There is little information regarding the effects of As in testis, but it has been reported that As also accumulates in this organ (Gunn et al., 1967) and that it produces marked degeneration in the germinal epithelium (Bencko et al., 1968).

This Chapter describes the effects of arsenic on some enzymes of the heme metabolism pathway in kidney and testis, and studies the differences in organ susceptibility to the administration of As in two different oxidation states, AsIII and AsV. A comparison is also made between the response patterns of liver and testis to the acute administration of arsenic and cadmium.
5.2 TESTICULAR EFFECTS.

5.2.1 Effects of sodium arsenite (AsIII).

The effects of 5 different dose levels at 16 hours, ranging from 12.5 to 100 umol/kg are shown in table 5.1. AsIII was administered by subcutaneous injection.

a) ALA synthetase.

A 67% increase in activity above control levels was observed at the highest dose (75 umol/kg). Lower doses produced no significant effects (Table 5.1).

b) Biliverdin reductase.

No change of activity was observed at any dose level (Table 5.1).

c) Heme oxygenase.

An increase of about 60 - 70% over control values was observed after treatment with 75 or 100 umol/kg of AsIII, lower doses produced no significant effects (Table 5.1).
Table 5.1 Effects of sodium arsenite (AsIII) on testicular ALA synthetase, biliverdin reductase, heme oxygenase and cytochrome P-450.

<table>
<thead>
<tr>
<th>AsIII (umol/kg)</th>
<th>ALA Synthetase</th>
<th>Biliverdin Reductase</th>
<th>Heme Oxygenase</th>
<th>Cytochrome P-450</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>71.6 ± 9.6</td>
<td>2.7 ± 0.2</td>
<td>6.5 ± 0.8</td>
<td>34.0 ± 6.0</td>
</tr>
<tr>
<td>12.5</td>
<td>72.0 ± 9.2</td>
<td>2.8 ± 0.1</td>
<td>6.4 ± 0.9</td>
<td>29.0 ± 2.0</td>
</tr>
<tr>
<td>25</td>
<td>76.4 ± 11.4</td>
<td>2.5 ± 0.2</td>
<td>6.6 ± 0.9</td>
<td>26.0 ± 3.0</td>
</tr>
<tr>
<td>50</td>
<td>83.3 ± 12.6</td>
<td>2.5 ± 0.2</td>
<td>7.0 ± 0.3</td>
<td>23.0 ± 2.0</td>
</tr>
<tr>
<td>75</td>
<td>117.0 ± 16.2</td>
<td>2.8 ± 0.3</td>
<td>10.6 ± 0.8</td>
<td>14.0 ± 3.0</td>
</tr>
<tr>
<td>100</td>
<td>N.A.</td>
<td>2.8 ± 0.1</td>
<td>11.0 ± 0.8</td>
<td>9.0 ± 2.0</td>
</tr>
</tbody>
</table>

1 pmol ALA/mg homogenate protein/h.
2 nmol bilirubin/mg cytosolic protein/h.
3 nmol bilirubin/mg microsomal protein/h.
4 pmol/mg microsomal protein.

All above given as $\bar{x} \pm S.D.$ (n = 14 for controls, n = at least 3 for other data). These effects were studied at 16 hours after subcutaneous administration. * Differs from control data, p < 0.05.
N.A. Data not obtained.
d) Cytochrome P-450.

There was a progressive decrease in P-450 concentration at all dose levels above 25 umol/kg. At the highest dose (100 umol/kg) the concentration was reduced to 27% of the control level (Table 5.1).

5.2.2 Effects of sodium arsenate (AsV).

The effects of AsV injected subcutaneously in doses ranging from 25 to 200 umol/kg were studied at 16 hours after treatment.

a) ALA synthetase.

A small but significant increase (13%) in the activity of this enzyme was observed at the highest dose (200 umol/kg). No significant effects were observed at the lower dose (Table 5.2).

b) Biliverdin reductase.

No changes in the activity of this enzyme were observed at any dose level (Table 5.2).
Table 5.2 Effects of sodium arsenate (AsV) on testicular ALA synthetase, biliverdin reductase, heme oxygenase and cytochrome P-450.

<table>
<thead>
<tr>
<th>AsV (umol/kg)</th>
<th>ALA Synthetase 1</th>
<th>Biliverdin Reductase 2</th>
<th>Heme Oxygenase 3</th>
<th>Cytochrome P-450 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>71.6 ± 9.6</td>
<td>2.7 ± 0.2</td>
<td>6.5 ± 0.8</td>
<td>34.0 ± 6.0</td>
</tr>
<tr>
<td>25</td>
<td>N.A.</td>
<td>2.7 ± 0.3</td>
<td>6.6 ± 0.6</td>
<td>35.0 ± 7.0</td>
</tr>
<tr>
<td>50</td>
<td>N.A.</td>
<td>2.5 ± 0.3</td>
<td>6.7 ± 0.5</td>
<td>31.0 ± 6.0</td>
</tr>
<tr>
<td>100</td>
<td>73.3 ± 7.0</td>
<td>2.8 ± 0.4</td>
<td>6.6 ± 0.5</td>
<td>29.0 ± 7.0</td>
</tr>
<tr>
<td>150</td>
<td>76.7 ± 6.6</td>
<td>2.8 ± 0.4</td>
<td>6.7 ± 0.5</td>
<td>33.0 ± 6.0</td>
</tr>
<tr>
<td>200</td>
<td>80.6 ± 6.0</td>
<td>2.6 ± 0.3</td>
<td>7.1 ± 0.7</td>
<td>24.0 ± 4.0</td>
</tr>
</tbody>
</table>

1 pmol ALA/mg homogenate protein/h.
2 nmol bilirubin/mg cytosolic protein/h.
3 nmol bilirubin/mg microsomal protein.
4 pmol/mg microsomal protein.

All above given as \( \overline{x} \pm S.D. \) (n = 14 for controls, n = at least 3 for other data).

These effects were studied at 16 hours after subcutaneous administration of AsV.

* Differs from control data, p < 0.05.

N.A. Data not obtained.
c) Heme oxygenase.

No significant effects of AsV on this enzyme activity were observed (Table 5.2).

d) Cytochrome P-450.

At the highest dose (200 umol/kg) the content of P-450 was reduced to 71% of the control level. No significant changes were observed at lower doses (Table 5.2).

5.2.3 Effects of sodium selenite (SeIV).

The effects at 16 hours of 3 different dose levels, ranging from 5 to 20 umol/kg and administered by subcutaneous injection are shown in table 5.3.

a) ALA Synthetase.

A small increase (18%) in the activity of this enzyme was observed at the highest dose (20 umol/kg). No significant effects were observed at lower doses (Table 5.3). SeIV was administered by subcutaneous injection.

b) Biliverdin reductase.

No changes in the activity of this enzyme were observed at any dose level (table 5.3).
c) Heme oxygenase.

No significant effects of SeIV on this enzyme activity were observed (Table 5.3).

d) Cytochrome P-450.

There were no detectable effects on P-450 concentration at any dose level (Table 5.3).

5.2.4 Effects of cadmium chloride.

The effects at 16 hours of 7 and 20 umol/kg administered by subcutaneous injection are shown in table 5.4.

a) Biliverdin reductase.

No change of activity was noted at any dose level (Table 5.4).

b) Heme oxygenase.

At the highest dose (20 umol/kg) the activity of HO was decreased to 47% of control values by Cd. No significant change was observed at lower doses (Table 5.4).
Table 5.3 Effects of sodium selenite (SeIV) on testicular ALA synthetase, biliverdin reductase, heme oxygenase and cytochrome P-450.

<table>
<thead>
<tr>
<th>SeIV (μmol/kg)</th>
<th>ALA Synthetase$^1$</th>
<th>Biliverdin Reductase$^2$</th>
<th>Heme Oxygenase$^3$</th>
<th>Cytochrome P-450$^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>71.6 ± 9.6</td>
<td>2.7 ± 0.2</td>
<td>6.5 ± 0.8</td>
<td>34.0 ± 6.0</td>
</tr>
<tr>
<td>5</td>
<td>68.8 ± 4.4</td>
<td>2.8 ± 0.3</td>
<td>6.7 ± 0.8</td>
<td>28.0 ± 5.0</td>
</tr>
<tr>
<td>10</td>
<td>75.8 ± 12.1</td>
<td>2.7 ± 0.2</td>
<td>6.9 ± 0.8</td>
<td>29.0 ± 2.0</td>
</tr>
<tr>
<td>20</td>
<td>84.1 ± 10.0</td>
<td>2.8 ± 0.3</td>
<td>7.1 ± 0.8</td>
<td>34.0 ± 4.0</td>
</tr>
</tbody>
</table>

$^1$ pmol/ALA/mg homogenate protein/h.
$^2$ nmol/bilirubin/mg cytosolic protein/h.
$^3$ nmol bilirubin/mg microsomal protein.
$^4$ pmol/mg microsomal protein.

All above given as $\bar{x} \pm \text{S.D.}$ (n = 14 for controls, n = at least 3 for other data). These effects were studied at 16 hours after subcutaneous administration of SeIV. * Differs from control data, p < 0.05.
c) Cytochrome P-450.

At the highest dose, the P-450 content was reduced to 74% of the control level. No significant reductions were observed at the lowest dose (Table 5.4).
Table 5.4 Effects of cadmium chloride on testicular biliverdin reductase, heme oxygenase and cytochrome P-450.

<table>
<thead>
<tr>
<th>CdCl₂ (μmol/kg)</th>
<th>Biliverdin Reductase¹</th>
<th>Heme Oxygenase ²</th>
<th>Cytochrome P-450³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.70 ± 0.18</td>
<td>6.48 ± 0.79</td>
<td>34.0 ± 6.0</td>
</tr>
<tr>
<td>7</td>
<td>3.06 ± 1.20</td>
<td>6.08 ± 0.71</td>
<td>31.0 ± 5.0</td>
</tr>
<tr>
<td>20</td>
<td>2.22 ± 0.60</td>
<td>3.05 ± 0.14</td>
<td>25.0 ± 2.0</td>
</tr>
</tbody>
</table>

¹ nmol bilirubin/mg cytosolic protein/h.
² nmol bilirubin/mg microsomal protein.
³ pmol/mg microsomal protein.

All above given as $\bar{X} \pm S.D.$ (n = 14 for controls, n = at least 3 for other data). These data were studied at 16 hours after subcutaneous administration of CdCl₂.

* Differs from control data, p < 0.05.
5.3 DISCUSSION ON TESTICULAR EFFECTS.

There have been suggestions that the regulation of heme metabolism in the testes differs from that of liver. Tofilon and Piper (1980), reported that ALAS activity in rat testes is refractory to treatment with the known hepatic ALAS inducers allylisopropylacetamide (AIA) and 3, 5-dicarbethoxy 1, 4-dihydrocollidine (DDC). Maines et al. (1982), reported that CdCl₂ is a very effective inhibitor of testicular HO, and suggested that the regulatory mechanisms for HO activity and the relationships of the enzyme with microsomal heme content are distinctly different from that of liver. The results of the present study do not support the above suggestions; testicular ALAS and HO activities, and P-450 content responded to AsIII administration in a similar way to that of liver. However, only the highest doses (75 and 100 umol/kg) were able to elicit a response. The highest dose of AsV (200 umol/kg) resulted in increased ALAS activity and reduced P-450 concentration without significant effects in HO. SeIV (20 umol/kg) increased ALAS activity without apparent effects on the rest of enzymes.

There are two possibilities that may help to explain the difference between the effects of As or Se on testicular ALAS and those observed after AIA or DDC treatment: 1) it is
known that administration of As or Se produce high concentrations in most rat and mice organs a short time after exposure. The clearance from those organs is relatively fast, except in the testis-epididymis complex where As and Se accumulate and are retained (Gunn et al., 1967; Vahter et al., 1982; Lindgreen et al., 1982). It is possible that AIA and DDC, inducers of hepatic ALAS, failed to alter testicular ALAS activity because they were unable to reach the testicular cells in sufficient concentrations. This is known to occur for a number of substances, like salicylic acid or sulfoguanidine, which are prevented from passing the testicular capillary barrier (Gunn and Gould, 1970). It is known that AIA or DDC effects are mediated by their active metabolites, therefore, it is possible that these active metabolites cannot be formed in sufficient concentrations in the testis (Tofilon and Piper, 1980).

An interesting observation regarding testicular HO is that under physiological conditions, the control rate of heme degradation is particularly high. In the present study, HO activity in testis of control animals was found to be 3.5 times higher than that of liver (Tables 3.3 and 5.1), and 7-fold that of kidney (table 5.5). Spleen is the only organ which has been reported (Maines and Kappas, 1974) to have a higher HO activity than the testicular level reported in the present study. HO is most active in organs normally engaged in the sequestration and breakdown of senescent red cells; in view of this, the biological significance of high
Fig. 5.1 Effects of arsenic on heme oxygenase activity in liver, kidney and testis. The effects were studied 16 hours after subcutaneous administration.
Effects of AsIII on: liver (●), kidney (■) and testis (▲).
Effects of AsV on: liver (○), kidney (●) and testis (▲).
HO activity in testis remains to be elucidated. The activities of ALAS, ALAD, uroporphyrinogen I synthetase and heme synthetase in testis seem to be lower than those reported for liver (Tait, 1978; Maines and Kutty, 1983), as it would be expected for an organ apparently much less involved in xenobiotic metabolism.

AsIII has a pronounced effect on testicular P-450 concentration: a significant reduction was observed at doses which were ineffective in altering ALAS and HO activities, and the highest dose (100 umol/kg) reduced P-450 concentration to 26% of control levels. This suggests that the degenerative changes in germinal epithelium reported to occur after As treatment (Bencko et al., 1968) are caused by reductions in steroidogenic activity subsequent to effects on P-450 content. Little is known about the content of hemoproteins other than cytochromes P-450 and b.

In marked contrast to the effects of AsIII on ALAS and HO activities, and to its own effects in liver (Table 3.14), CdCl₂ (20 umol/kg) decreased HO activity to 50% of control values. A reduction of 25% in P-450 concentration was also observed (Table 5.4), confirming results reported earlier (Maines et al., 1982). Cadmium, like As and Se, reaches the testis shortly after administration, but in contrast to these two metalloids, less is retained there than in any other organ (Gunn and Gould, 1970). Surprisingly therefore, in
rodents, the testis is the organ most susceptible to Cd toxicity: an accumulation in the testis of only 0.13% of an administered dose (20 umol/kg) produces hemorrhagic necrosis followed by atrophy and permanent sterility. Liver and kidneys, which may attain Cd concentrations 20 to 60-fold greater (Gunn and Gould, 1970), showed in this experiment no macroscopic evidence of damage. In contrast, As and Se do not seem to cause testicular necrosis or other macroscopic alterations despite their ability to accumulate in this organs, even after administration of doses which are in the lethal range. The question thus arises: is there a genuine difference between the biochemical effects of As and Cd on rodent testis, or are the specific effects on heme catabolism of Cd masked by the massive injury it produces? Further experiments are required to answer this question. In addition, other metal ions such as Cu, Sn, Pb and Hg, known for their ability to increase HO activity in liver and other organs, have been shown to be ineffective in altering the enzyme activity in the testis (Maines and Kutty, 1983); there is no information about their effects on ALAS activity. The difference in tissue responsiveness to metal induction of HO may reflect one or more of a number of factors including: bioavailability to the testis, differences in binding affinities in the testis, cellular contents of functional groups which complex and block metal actions. In order to adequately interpret these data, it is important to obtain more information on intratissue and/or intracellular distribution of these elements.
5.4 RENAL EFFECTS.

5.4.1 Effects of sodium arsenite (AsIII).

The effects of 5 different dose levels at 16 hours, ranging from 12.5 to 100 umol/kg are shown in table 5.5. AsIII was administered by subcutaneous injection.

a) ALA synthetase.

The enzyme activity showed a dose-dependent increase, being significant at doses above 25 umol/kg (Table 5.5). At the highest dose, the enzyme activity was about 3.5 times that of the control level.

b) Heme oxygenase.

The increase in this enzyme produced by AsIII was progressive and pronounced at all dose levels above 12.5 umol/kg (Table 5.5). The increase in activity was directly related to the increase in dosage. At the highest dose (100 umol/kg) an 11-fold increase over control values was observed.

c) Biliverdin reductase.

No change of activity was observed at any dose-level
Table 5.6 Effects of sodium arsenate (AsV) on renal ALA synthetase, biliverdin reductase, heme oxygenase and cytochromes P-450 and b.

<table>
<thead>
<tr>
<th>AsV (μmol/kg)</th>
<th>ALA Synthetase</th>
<th>Biliverdin Reductase</th>
<th>Heme Oxygenase 3</th>
<th>Cytochrome P-450</th>
<th>Cytochrome b5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>71.1 ± 10.4</td>
<td>26.9 ± 5.1</td>
<td>0.9 ± 0.1</td>
<td>91.0 ± 12.0</td>
<td>77.0 ± 5.0</td>
</tr>
<tr>
<td>25</td>
<td>N.A.</td>
<td>2.9 ± 0.4</td>
<td>*</td>
<td>82.0 ± 5.0</td>
<td>*</td>
</tr>
<tr>
<td>50</td>
<td>N.A.</td>
<td>3.5 ± 0.2</td>
<td>*</td>
<td>68.0 ± 3.0</td>
<td>*</td>
</tr>
<tr>
<td>100</td>
<td>138.2 ± 36.8</td>
<td>27.6 ± 3.9</td>
<td>*</td>
<td>61.0 ± 4.0</td>
<td>*</td>
</tr>
<tr>
<td>150</td>
<td>185.8 ± 16.8</td>
<td>30.1 ± 1.8</td>
<td>*</td>
<td>47.0 ± 10.0</td>
<td>*</td>
</tr>
<tr>
<td>200</td>
<td>241.8 ± 45.2</td>
<td>30.0 ± 2.4</td>
<td>*</td>
<td>37.0 ± 5.0</td>
<td>*</td>
</tr>
</tbody>
</table>

1 pmol ALA/mg homogenate protein.
2 nmol bilirubin/mg cytosolic protein.
3 nmol bilirubin/mg microsomal protein.
4 pmol/mg microsomal protein.

All above given as X ± S.D. (n = 12 for controls, n = at least 3 for other data).

* Differ from control data, p < 0.05.
N.A. data not obtained.
(Table 5.5).

d) Cytochromes P-450 and b

There was a progressive decrease in P-450 concentration at dose levels above 12.5 umol/kg (Table 5.5). At the highest dose (100 umol/kg) the concentration was reduced to 42% of the control level. No effect on cytochrome b5 concentration at any dose level was observed (Table 5.5).

5.4.2 Effects of sodium arsenate (AsV).

The effects of AsV injected subcutaneously in doses ranging from 25 to 200 umol/kg and studied at 16 hours after treatment are shown in table 5.6.

a) ALA synthetase.

There was a dose-related increase in enzyme activity, being significant at doses above 100 umol/kg (Table 5.6). At the highest dose (200 umol/kg), the enzyme activity was about 3.5 times that of control level.

b) Heme oxygenase.

The enzyme activity showed a dose-dependent increase, being significant at all doses given (Table 5.6). At the
Table 5.5 Effects of sodium arsenite (AsIII) on renal ALA synthetase, biliverdin reductase, heme oxygenase and cytochromes P-450 and b5.

<table>
<thead>
<tr>
<th>AsIII (umol/kg)</th>
<th>ALA Synthetase¹</th>
<th>Biliverdin Reductase²</th>
<th>Heme Oxygenase³</th>
<th>Cytochrome P-450⁴</th>
<th>Cytochrome b₅⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>71.1 ± 10.4</td>
<td>26.9 ± 5.1</td>
<td>0.9 ± 0.1</td>
<td>91.0 ± 12.0</td>
<td>77.0 ± 9.0</td>
</tr>
<tr>
<td>12.5</td>
<td>N.A.</td>
<td>23.4 ± 1.5</td>
<td>0.9 ± 0.2</td>
<td>85.0 ± 6.0</td>
<td>76.0 ± 8.0</td>
</tr>
<tr>
<td>25.0</td>
<td>* 156.1 ± 12.4</td>
<td>24.3 ± 3.6</td>
<td>* 2.2 ± 0.3</td>
<td>77.0 ± 8.0</td>
<td>* 73.0 ± 6.0</td>
</tr>
<tr>
<td>50.0</td>
<td>* 171.7 ± 31.7</td>
<td>23.6 ± 2.0</td>
<td>* 4.7 ± 0.3</td>
<td>66.0 ± 4.0</td>
<td>* 72.0 ± 5.0</td>
</tr>
<tr>
<td>75.0</td>
<td>* 246.7 ± 51.6</td>
<td>21.6 ± 3.0</td>
<td>* 9.1 ± 0.4</td>
<td>43.0 ± 2.0</td>
<td>* 74.0 ± 9.0</td>
</tr>
<tr>
<td>100.0</td>
<td>N.A.</td>
<td>N.A.</td>
<td>9.9 ± 0.4</td>
<td>38.0 ± 2.0</td>
<td>69.0 ± 6.0</td>
</tr>
</tbody>
</table>

¹ pmoles ALA/mg homogenate protein.
² nmol bilirubin/mg cytosolic protein.
³ nmol bilirubin/mg microsomal protein.
⁴ pmoles bilirubin/mg microsomal protein.

All above given as X ± S.D. (n = 12 for controls, n = at least 3 for other data). These effects were studied at 16 hours after subcutaneous administration of AsIII. * Differs from control data, p < 0.05.
N.A. Data not obtained.
highest dose (200 umol/kg) a 6-fold increase over control values was observed.

c) Biliverdin reductase.

No change of activity was observed at any dose level (Table 5.6).

d) Cytochromes P-450 and b.  

There was a progressive decrease in P-450 concentration at dose levels above 25 umol/kg of AsV (Table 5.6). At the highest dose (200 umol/kg) the concentration was reduced to 40% of the control level. No effect on cytochrome b at any dose level was observed (Table 5.6).
5.5 DISCUSSION ON RENAL EFFECTS.

The results of this study indicate that As administration produces alterations in renal heme metabolism similar to those observed in liver, i.e. it increases ALAS and HO activities and decreases P-450 concentration (Tables 5.5 and 5.6). As in liver, the effects of AsIII on kidney were more pronounced than those of AsV: on a molar basis, AsIII increased HO activity 5.5 times as much as AsV, confirming results reported earlier by Sardana et al (1981); this difference in potency was less apparent for the effects on ALAS and P-450, where AsIII was twice as potent as AsV (Tables 5.5 and 5.6). Although AsIII was more potent than AsV in both liver and kidney, AsV affected the above mentioned enzymes in kidney at doses which were ineffective in liver and testis (Tables 3.7, 5.2 and 5.6; Fig. 5.1). There is some indirect evidence which may explain this difference in the relative potency of AsIII and AsV between liver and kidney: 1) AsIII administration produced higher concentrations than equimolar doses of AsV in most tissues due to a more pronounced binding to cellular organelles (Brown et al., 1976), the exceptions being kidney and skeleton (Vahter, 1981; Vahter and Marafante, 1983); 2) hepatocytes have a relatively low AsV uptake, as compared to that of AsIII (Lerman et al., 1983) and 3) in dogs, the fast initial clearance of unmetabolized AsV from other tissues gives rise to high concentrations in kidney, where part of it
before being eliminated undergoes tubular reabsorption (Ginsburg and Lotspeich, 1963) followed by intracellular reduction to AsIII, producing net secretion of AsIII (Ginsburg, 1965). It has been suggested that a similar mechanism operates in rabbits and mice (Vahter and Marafante, 1983). From above, it is clear that AsV selectively accumulates in kidney, but there is still an open question: are the renal effects of AsV produced by the high concentrations reached, or are they due to the effectiveness of its transformation to AsIII? It would be interesting to administer probenecid to AsV treated animals in order to investigate if it could block the tubular reabsorption of AsV, thus decreasing the amount available for intracellular reduction.

Although the regulation of heme metabolism in kidney is less well studied than that in liver, and the attempts in the present study to assess changes in renal "free heme" pool (measuring TP activity) were unsuccessful, it seems reasonable to assume that renal ALAS induction is a response to heme depletion. It appears that the degree of ALAS induction (relative to control values) in kidney is higher than in liver; the highest doses of AsIII and AsV were able to increase renal ALAS 3-fold, while in liver the maximum increase produced by AsIII was about 2 fold. This effect suggests that kidney could be an important contributor to the porphyria produced by As, however, the relative difference in
heme turnover between liver and kidney needs to be considered. There are few reports dealing with renal porphyria: Woods and Fowler (1977a) have reported that methylmercury produces renal porphyria (without hepatic involvement) in rats. It would be interesting to investigate the effects of As on uroporphyrinogen synthetase, uroporphyrinogen decarboxylase and heme synthetase and on the renal content of porphyrins to elucidate this point.

As observed in liver, no effects of AsIII or AsV on biliverdin reductase activity were found in kidney. This absence of effects is not surprising, since the only metal that has been reported to alter this enzyme activity in vivo is mercury, which at high doses (30 umol/kg) decreased the activity by 50% (Kutty and Maines, 1983); interestingly, Hg does not affect the enzyme activity in liver. As discussed previously (Section 3.3.5), it is highly unlikely that biliverdin reductase plays an important role in the regulation of heme catabolism.

Under physiological conditions, renal HO activity is very low, but Pimstone et al. (1971a) have reported that following a single intravenous injection of hemoglobin a 36-fold increase in activity was observed, while in liver only a 2-fold increase was seen. Although there is little information published on the regulation of heme catabolism to interpret these findings, they suggest the possibility that renal HO induction could be related not only to the cellular
level of heme, but also be extended to the degradation of hemoglobin heme reabsorbed by the tubular cells. Arsenic shares the ability to induce renal HO with several ions like Sn (Maines and Kappas, 1976d), Hg, Pb, Cu, Cd (Maines and Kappas, 1976c), Sb (Drummond and Kappas, 1981b) and Ni (Sunderman et al., 1983); it appears that AsIII is one of the more potent of these elements. At the moment, no clear evidence is available as to whether HO induction in kidney is mediated entirely by a heme-dependent mechanism, or by one that is partly or totally independent of "free heme" levels. Further experiments are needed to clarify this point.

The kidney appears to be less concerned than liver with P-450-dependent oxidative metabolism of xenobiotics and more with the metabolism of endogenous compounds (Connelly and Bridges, 1980). The marked decrease in P-450 content produced by both AsIII and AsV (Tables 5.5 and 5.6), suggests that renal functions mediated by the P-450 system could be primary targets of acute As toxicity. The ω-hydroxylation of fatty acids seems to be one of the most important functions of P-450 in kidney, if it is impaired (by a reduction in P-450 content, for example), the consequent excess of saturated fatty acids is known to produce mitochondrial swelling and respiratory uncoupling, while the excess of ω-hydroxylated products caused neither swelling nor respiratory uncoupling (Ellin and Orrenius, 1975). It is possible that the mitochondrial swelling and respiratory uncoupling observed in
kidney after As administration (Brown et al., 1976), could be produced by damage to this form of P-450. This possibility could be followed by investigating whether As produces a selective effect on this P-450 species and also by comparing As effects with the effects of other metals.
CHAPTER SIX

SUMMARY AND FINAL DISCUSSION
6.1 EFFECTS OF ARSENIC.

In liver, arsenic produced a dose-related decrease in the percentage of TP saturated with heme. Dose-related increases in ALAS and HO activities accompanied by dose-related decreases in P-450 content were also observed. The cytosolic heme status is a major target by virtue of the inhibitory effects of As on uroporphyrinogen synthetase and heme synthetase, and its inducing effect on HO. The magnitude of these effects was related to the oxidation state of the As salts used. AsIII was more potent than AsV. However, the differences in relative potency varied between enzymes. Nonetheless, the results are in agreement with the general concept that AsIII is more toxic than AsV, and that AsV may require to be converted to AsIII to exert its toxic action.

So far, most experimental work devoted to studying the relationships between TP activity and heme metabolism alterations has been focused on the heme saturation value, and has attached much less importance to the effects on the apoenzyme. However, the study of both effects is necessary in order to understand the effects on ALAS and HO. The time-course study of AsIII effects showed a drastic reduction in TP "total enzyme" activity during the first 4 hours after treatment. This reduction may have occurred as a result of AsIII interfering with the conjugation of apo-Tp with heme or
by inhibiting apoprotein synthesis. These effects suggested that the heme utilization by TP, as opposed to the heme saturation, could serve as an alternative explanation for the effects on ALAS and HO: The net effect of the reduction in "total enzyme" values would be a reduction in the number of apo-TP molecules saturated with heme, thus allowing the unused heme to increase the "free heme" pool size. This could result initially in inhibition of ALAS activity and an increase in HO activity. Further experiments are needed to explore in detail this possibility. The results from this study are in agreement with Badawy's suggestion that the heme saturation of the TP-apoenzyme is sensitive to treatments that modify liver heme concentration. The chemically mediated increase in ALAS activity is thought to be caused by a reduction in the "free heme" pool (Meyer, 1982), the latter effect is produced by As inhibiting the synthesis of heme and increasing its degradation. However, it remains to be shown which parameter, heme saturation of TP or ALAS, reflect more accurately changes in the "free heme" pool size.

The increase in HO produced by As in liver 16 hours after treatment, appears to be mediated by a mechanism largely or entirely independent of heme, since the observed changes could not be explained by the classical regulatory "free heme" pool model i.e. there were no indications of increases in the "free heme" pool, as measured by the heme saturation of TP, that could trigger a positive feedback on HO. On the contrary, it seems that one of the reasons for the
"free heme" pool depletion was the increased HO activity. The disparity between the effects of AsIII on ALAS (2-fold) and HO (8-fold) plus the fact that 8 molecules of ALA are needed to form one molecule of heme, suggests that the relationship between heme synthesis and degradation is out of balance as a result of As exposure. The extrapolation of these effects to the in vivo situation is difficult because substrate accessibility could limit the increase in HO activity. Further studies are required to identify the role of alterations in heme catabolism in the genesis of more overt toxic effects of As and other metals.

It is possible that the dose-dependent reduction in hepatic P-450 concentration produced by AsIII and AsV could have been produced by peroxidative damage to microsomal membranes, supporting this possibility is the suggestion that As reduces catalase activity (Squibb and Fowler (1983). The reduction in P-450 was accompanied by dose-related decreases in the activity of ethoxycoumarin and ethoxyresorufin O-deethylases. There were indications that the inhibitory effect of As was substrate dependent, the effect on ERD being more pronounced than that on ECD. This might imply a selective effect of As on certain P-450 isoforms. Further experiments are needed to determine if these observations represent a direct specific effect on a particular isoform e.g. P-448, or are due to more general effect of As on hemoprotein synthesis/degradation. Not enough is known about
the relative susceptibility of cytochrome P-450 to chemicals which alter heme metabolism and the relative turnover rate of specific forms of the cytochrome to answer this question. These effects on P-450 isoforms may be reflected in a diminished ability of cells to carry out oxidations of drug and other chemicals that depend on P-450 and P-448. If so, this would in some causes lead to the toxicity of a compound being increased and in others to a decrease in toxicity.

Although only the higher doses of AsIII (75 and 100 umol/kg) were able to elicit a testicular response, the general effect was similar to that of liver i.e. increases in ALAS and HO activities accompanied by reductions in P-450 content. Maines et al. (1982) based on their finding that cadmium is a potent inhibitor of testicular HO have suggested that the HO regulatory mechanism(s) and the relationship between this enzyme's activity and microsomal heme content are distinctly different from that of liver. The results of the present study do not support this suggestion. The massive testicular injury produced by Cd is considered as an alternative explanation for the inhibitory effect of Cd on HO activity. Under physiological conditions testicular heme oxygenase activity is higher than in most organs. Although the physiological significance of this observation has not yet been elucidated, it is more likely to be associated with the high P-450 dependent steroid biosynthesis than to the process of spermatogenesis per se, because HO activity is 10-fold higher in microsomes derived from Leydig cells than in
those from Sertoli cells (Maines, 1984b). If HO does indeed regulate steroidogenic activity, then perhaps the resultant changes in steroid levels produced by As treatment play a role in the degenerative effects it causes in the germinal epithelium (Bencko et al., 1968). Judging from the similar increases in HO and ALAS activities, it appears that the deficit in the heme synthesis/degradation produced by As in testis, is not as marked as in liver.

AsV and SeIV acute treatment produced a small increase in ALAS activity without significant effects on HO activity, suggesting that testicular ALAS is more sensitive than HO to the effects of these salts.

Although AsIII was more potent than AsV in both liver and kidney, AsV affected renal ALAS, HO and P-450 at doses which were ineffective in liver and testis. It also appears that the kidney is able to respond with increases in ALAS activity greater than those observed in liver (as compared to control values), if the effectiveness of uroporphyrinogen decarboxylase inhibition is similar between liver and kidney then the kidney could be an important contributor to the porphyria produced by As. However, there is an open question: is the particular response of kidney to AsV produced by the high concentrations of AsV reached in this organ (Vahter and Marafante, 1983), or more likely, is it due to the processes of tubular re-absorption and subsequent intracellular
reduction to AsIII? Experiments administering inhibitors of renal tubular transport may contribute to answer this question. Further experiments are also required to study the regulation of heme metabolism and its alterations in extrahepatic organs and the possible relationship between these effects and the subsequent genesis of overt toxic effects in target organs.

The main effects of continuous exposure to AsIII were: 1) a progressive increase in TP "total enzyme activity"; 2) an initial decrease in the degree of heme saturation of TP, which remained more or less constant during the period of treatment; 3) an initial increase in ALAS activity (60%) which after 10 days of exposure was reduced to a 30% increase. In marked contrast to the dose-related increases in HO activity and cytochrome P-450 reductions observed after the acute administration of AsIII, no significant effects on these parameters were observed after continuous exposure. These observations were interpreted as indicative that a new equilibrium, at a lower level, between synthesis and degradation of heme had been reached during the continuous treatment. It was also considered that an adaptive response to the toxic effects of AsIII on heme metabolism was taking place. Further studies in experimental animals and humans are needed to investigate this process. AsIII produces porphyria in rats, but there are no reports of this effect occurring in human populations exposed to As, and there is a need to investigate if the skin lesions observed in individuals
chronically exposed to As are related to alterations in porphyrin metabolism.

Arsenic is an important environmental contaminant in several countries, including Mexico, with a proven risk to the human population. The explanation of As effects in man depend, in large part, on relating the results of experimental work, both in vitro and in vivo, with the clinical findings in exposed populations. The overall aims for future studies in this field are: 1) identify the earliest detectable biochemical and clinical effects of As in man, using the knowledge of As effects on body tissues of the rat, to develop test(s) that could reflect tissue changes resulting from As exposure; 2) identify possible ways of reducing chronic As toxicity once it has been established.
6.2 EFFECTS OF SELENIUM.

Se produced effects on TP, ALAS and HO similar to those produced by AsIII; however, Se was more potent in this regard than either AsIII or AsV. It was also more toxic, doses of 40 umol/kg and higher producing death in all animals in less than 12 hours. The differences between the induction of ALAS and HO were not as marked as those observed with AsIII, indicating that the balance between hepatic heme synthesis and degradation resulting from Se and As exposure are distinctly different.

All the elements whose effects on these enzymes have been reported, induce HO and concomitantly reduce P-450 concentration together with the activity of P-450 dependent monooxygenases, Se is a notable exception to this. The effect of Se to increase HO activity, without significantly affecting P-450 concentration, supporting the idea that P-450 destruction is not a prerequisite for HO induction. It also suggests that Se could be a useful tool for further studying the role of P-450 in the regulation of heme metabolism. It should provide a useful adjunction to methods such as pretreatment with classical P-450 inhibitors e.g. SKF 525A, which are thought to bind to the active sites of P-450 and thereby stabilise it.
Se appears to be the first element reported to increase epoxide hydrolase activity. However, in comparison with 2-acetamidofluorene or aflatoxin which respectively produced 10 and 5-fold induction (Sharma et al., 1981), its potency is relatively low, being 2-fold at the maximum dose. This effect might contribute to the explanation of the protective effect of Se against such compounds as aflatoxins or DMBA, which have to be transformed to epoxides in order to exert their carcinogenic effects (Grant et al., 1977; Welsch et al., 1977). As yet we have no evidence to explain how or why Se induces EH, but it is possible that EH is coinduced with other enzymes affected during a toxic process or that it is a particular response to certain forms of toxic or carcinogenic damage.

It appears that selenium has a dual effect on As-induced hepatotoxicity. On the one hand, it has additive effects in reducing the heme saturation of TP, and on the other, it seems to protect against the decrease in P-450 levels produced by AsIII. The additive effects of AsIII and SeIV on HO activity, plus the ability of As to inhibit heme synthesis at other steps not affected by Se, might explain the additive effects of these metalloids. Possible explanations for the P-450 protective effect include: the role of Se, as a component of glutathione peroxidase, in maintaining the integrity of cellular and subcellular membranes from the oxidative damage caused by metals, or less
likely, that Se complexes with P-450 heme preventing the degradatory effects of AsIII.

Further studies are needed to determine if Se protects against other manifestations of As toxicity and/or against similar manifestations produced by other toxic agents. While it is important to delineate the protective effects, it is equally important to study the mechanisms underlying such effects. Regarding studies on human populations, it would be interesting to investigate the possibility of interactions between these metalloids, for example, the effects of high As intake on the Se status of the exposed individuals, and vice-versa, since Se levels might modify As toxicity.
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