ASPECTS OF THE INTERACTION BETWEEN CADMIUM
AND THE ACUTE INFLAMMATORY RESPONSE

By

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A thesis submitted in accordance
with the requirements of the
University of Surrey for the
Degree of Doctor of Philosophy

October 1988

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SUMMARY

The main aims of this thesis were to establish whether an acute inflammatory response is provoked in rats by the subcutaneous administration of cadmium, and to evaluate the possible role that such a response might play in the alterations in metal homeostasis and the development of anaemia which accompanies the use of this model of cadmium intoxication. An intense local reaction to the subcutaneous administration of cadmium was found. Many of the systemic changes, most notably in haematological parameters and in levels of iron, copper and plasma proteins, mimicked those seen in the acute inflammatory response. Possible causes of the resultant anaemia are discussed and inflammation is implicated as a predominant factor in its development. The results suggest that many of the effects which in previously published work have been attributed to a direct interaction of cadmium with the system under investigation, may in fact be secondary consequences of cadmium-induced inflammation.

Comparison of the effects of subcutaneous administration of cadmium and other selected metal salts with changes occurring in two recognised models of acute inflammation revealed marked differences in the local tissue reaction to different substances as well as in the magnitude of various components of the systemic response. The oedematous, necrotic and extensively destructive nature of the cadmium-induced lesion has been highlighted and shown to be partially alleviated by pre- and simultaneous treatment with zinc. Explanations for this protective phenomenon are offered, based on possible target sites of cadmium, particularly in terms of interaction with zinc-dependent processes.

With a view to understanding the mechanisms involved in acute cadmium toxicity, luminol-amplified chemiluminescence, which is indicative of free radical formation and the production of reactive oxygen species, was measured directly from intact tissue samples. Inflamed tissue sampled from subcutaneous sites of cadmium administration emitted substantially more chemiluminescence than non-inflamed tissue or tissue from sites to which other metals or turpentine was administered. It was demonstrated that intact tissue samples can also be used to assess free radical generation, as detected by chemiluminescence, during in vitro treatment. A pronounced dose-related response was seen with cadmium which could be inhibited by various pretreatment procedures, such as incubation with zinc or certain metal chelators. The significance of these results in relation to the mechanism of toxic action of cadmium as well as to the potential use of this chemiluminescence technique is discussed.
ACKNOWLEDGEMENTS

I should like to express my thanks to all my colleagues in the Department of Biochemistry, past and present, as well as friends elsewhere who have encouraged and helped me in many ways during the course of my studies.

I am particularly grateful to Professor D V Parke for providing me with the opportunity and the facilities to undertake the work for this thesis.

Special thanks are due to my supervisor, Mr D E Hall and to Professor L J King for their guidance, advice and encouragement during the course of this research.

I am especially appreciative of the invaluable technical assistance, support and friendship provided by Mrs Sue Bolwell and Miss Diane Sewry. My thanks for advice on techniques are due to Dr R H Hinton and Dr S Price for protein analysis and Dr E J Dowling for chemiluminescence. The staff of the Animal Unit gave me practical assistance for which I am most grateful.

I also wish to acknowledge Miss Tracey Bakall for the patience and skill displayed during the typing of this manuscript and Mrs Janet Cole for her expertise in the construction of the tables.

Last but by no means least, I offer greatest thanks to my parents for all the encouragement, love and support, both moral and practical, which they have given me throughout my many years of study.
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<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BDS</td>
<td>bathophenanthroline disulphonate</td>
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<tr>
<td>CHE</td>
<td>cholinesterase</td>
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<tr>
<td>CL</td>
<td>chemiluminescence</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
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<tr>
<td>DFX</td>
<td>desferrioxamine mesylate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>DTPA</td>
<td>diethylenetriaminepentaacetic acid</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMH</td>
<td>extreemedullary haemopoiesis</td>
</tr>
<tr>
<td>Ep</td>
<td>erythropoietin</td>
</tr>
<tr>
<td>FCA</td>
<td>Freund's complete adjuvant</td>
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<tr>
<td>H &amp; E</td>
<td>haematoxylin and eosin</td>
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<tr>
<td>i.m.</td>
<td>intramuscular</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>i.v.</td>
<td>intravenous</td>
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<td>LAC</td>
<td>luminol-amplified chemiluminescence</td>
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<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>M:E</td>
<td>myeloid-erythroid</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate, reduced form</td>
</tr>
<tr>
<td>NAP</td>
<td>neutrophil alkaline phosphatase</td>
</tr>
<tr>
<td>PMNL</td>
<td>polymorphonuclear leucocyte</td>
</tr>
<tr>
<td>PPB</td>
<td>Perls' Prussian Blue</td>
</tr>
<tr>
<td>PPD</td>
<td>p-phenylenediamine</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
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<tr>
<td>s.c.</td>
<td>subcutaneous</td>
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<tr>
<td>S.E.M.</td>
<td>standard error of the mean</td>
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<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
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<tr>
<td>TIBC</td>
<td>total iron-binding capacity</td>
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<tr>
<td>X-IEP</td>
<td>crossed immunoelectrophoresis</td>
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## Final Discussion

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CHAPTER 1

GENERAL INTRODUCTION

CADMIUM AND ITS TOXICITY
1.1 Introduction

During the last decade a vast literature has appeared giving details of many environmental and toxicological aspects of cadmium. The subject has been extensively reviewed in books (Webb, 1979; Foulkes, 1986; Friberg et al., 1986) as well as in a number of other articles and government documents (Chmielnicka and Cherian, 1986; DoE, 1980; Fielder and Dale, 1983; Hallenbeck, 1984; MAFF, 1983; Nath et al., 1984; Piscator, 1985). The following introductory overview will briefly summarise the major sources and consequences of the cadmium pollution problem and attempt to give an updated synopsis of some of the factors of current concern relating to the effects of cadmium on health in man. The references to much of the information, if not indicated, are to be found in the above reviews. More details on the background relating to the objectives of this thesis, and in particular, the evidence from animal experiments, will mainly be presented in the relevant experimental Chapters 2, 3 and 4.

1.2 Properties and Natural Occurrence of Cadmium

Zinc, cadmium and mercury, constitute group IIb of the periodic table and despite some similarity in chemical properties (particularly between zinc and cadmium) they have very different biological properties. Zinc is a component of many enzymes and is essential for normal growth, development and functioning of all living matter. Cadmium, in contrast, is a highly toxic heavy metal which is not generally believed to have any known biological function. Schwartz and Spallhotz (1976), however, presented data showing small but positive growth responses in rats with increasing concentration of cadmium at very low dietary levels. From this
they concluded that cadmium fulfilled criteria which are typical for an essential element.

Cadmium metal is of silvery-white appearance and was first identified as an element in 1817. It is a relatively rare element with estimates of its abundance in the earth's crust ranging from 0.1 to 0.2 ppm. Cadmium has been widely mobilised by both natural and anthropogenic processes. The natural input to the biosphere derives from volcanic activity, weathering of rocks and transport by wind, rain and dust fall, forest fires and the uptake and transfer through food chains. The input from these natural processes is relatively insignificant when compared to the much greater environmental contamination which has arisen by anthropogenic processes. There has been an increasing release of cadmium into the environment in more recent times due to changes in man's activity, in particular, the widespread use of cadmium in industry.

1.3 Production and Uses of Cadmium

Zinc and cadmium are usually found together under geological conditions with most zinc ores containing 0.2-0.4% cadmium. Almost all cadmium is obtained as a by-product of zinc mining. World cadmium production, consumption and emission to atmospheric, aquatic and terrestrial environments has increased at an alarming rate throughout this century. It is estimated that global production increased from 14 tonnes in 1900 to 17,000 tonnes in 1974 (DeVoogt et al., 1980). It was calculated that in 1975, 70% of the total world production of cadmium had occurred in the previous 20 years (MAFF, 1983). Cadmium has valuable metallurgical properties and its principal uses are in electroplating and welding, cadmium-nickel batteries, pigments, stabilisers for plastics, alloys and solders. It is used also in neutron absorbers in nuclear reactors, lithographic and photographic materials, ceramics and jewellery and in television, X-ray and image intensifier applications (DoE, 1980).
1.4 Occupational Exposure to Cadmium

Workers employed in the industries described above, as well as those involved in the production of cadmium and its compounds and in demolition work and scrap recycling are at risk of exposure to hazardous levels of cadmium. Cadmium enters the body mainly through the inhalation of particles and fumes which may be absorbed to the extent of 10-50%. Oral intake may also be significant. Although its large scale use dates only from the 1940s it is likely that cadmium constituted a health hazard to some long before its discovery and industrial use since it occurs naturally with zinc, lead and copper which have been used over several thousands of years. Early reports of acute adverse effects date back to the 17th Century although the syndrome of chronic cadmium poisoning was not recognised as an industrial disease until the 1940s. Since then it has been regarded as a serious occupational hazard and a number of examples of both acute and chronic cadmium poisoning have been described in detail by Bernard and Lauwerys (Chapter in Foulkes, 1986), Fielder and Dale (1983), Finkel (1983) and Lauwerys (Chapter in Webb, 1979).

1.5 Environmental Exposure to Cadmium

The various applications of cadmium tend to be dissipative and it has been estimated that less than 5% is recycled (DeVoogt et al., 1980). Dispersion may also occur during the combustion of fossil fuels and during waste disposal. The average total cadmium emission into the global atmosphere per year increased by a factor of 4.3 from the period 1901-1940 to 1971-1979 (Nriagu, 1979). Cadmium has thus become increasingly prominent as an environmental pollutant and with the growing industrial exploitation of both zinc and cadmium the potential for greater human environmental as well as occupational exposure has risen markedly.

A five-fold increase in the total body burden of cadmium since the first part of this century (1897-1914) has been noted in Germany by Drasch (1983). This was
estimated from the measurement of cadmium in historical samples of liver and kidney. It does not seem a great increase in view of the massive elevation in world production of the metal but of more concern was the finding that the concentration of cadmium in the kidney cortex increased nearly 50-fold over this 60-80 years period.

For the general population, not exposed via industry, food is the major source of cadmium, although only about 5% of ingested cadmium is absorbed by the gastrointestinal tract. Food levels of cadmium are generally low but can be increased with contamination of the general environment. The consequences of localised but serious outbreaks of pollution have been seen (reviewed by Bernard and Lauwerys, Chapter in Foulkes, 1986). Greatest concern over cadmium pollution was triggered by recognition in the 1950s and 1960s that chronic cadmium poisoning was not restricted to the cadmium workers but could constitute a health hazard to the general population. The metal has received a great deal of attention because of its high toxicity coupled with its cumulative properties.

In Japan, prolonged discharge of cadmium rich effluent from a copper-zinc mine into river water used for the irrigation of paddy fields and the severe flooding of the Jinzu valley in 1945-1946 led to contamination of soils, the rice crop and drinking water. This subsequently caused an epidemic of cadmium poisoning in the polluted areas known as Itai Itai Byo disease which was particularly prevalent among multiparous post-menopausal women. Renal damage leading to disturbances in calcium and phosphorus metabolism probably combined with dietary deficiencies of vitamin D, calcium and protein were believed responsible for the resulting osteomalacia, skeletal deformities and severe leg and back pain which gave the condition its name which translates as 'Ouch Ouch' disease. Several other areas in Japan have been found to be polluted by cadmium, which has caused health effects (Nogawa, 1984; Foulkes, 1986) and follow-up studies have shown a long-lasting influence of cadmium exposure on the health of inhabitants (Kawana et al., 1986;
In spite of an awareness of the problem and countermeasures taken by various authorities against the pollution, a recent study (Aoshima, 1987) has revealed that many inhabitants of the Jinzu river basin are still exposed to high levels of cadmium through the daily consumption of cadmium-contaminated rice grown for private consumption and they exhibit an associated high prevalence of renal tubular dysfunction even today.

Isolated episodes of excessive environmental pollution are not restricted to Japan. In 1979, a survey revealed substantial contamination of soil by cadmium in Shipham, a Somerset village, part of which was built on spoil heaps arising from extinct calamine workings. Cadmium concentrations in vegetables grown in this area have been shown to be at least 10 times those found in vegetables grown in uncontaminated soil (MAFF, 1983). Despite higher cadmium levels in inhabitants and results of a pilot study suggesting some abnormal renal findings, more extensive follow-up health surveys have reported no evidence that any of the present residents have suffered adverse health effects directly related to exposure to cadmium in this region (reviewed by Bernard and Lauwerys, Chapter in Foulkes, 1986; Philipp, 1983). It cannot be assumed, however, that lack of overt signs of a health hazard is grounds for complacency (Barltrop and Strehlow, 1982).

1.6 Cadmium in Food and its Intake in Man

Attention has recently been diverted to the increased cadmium content of crops from fields fertilised with metal-rich sewage sludge (Davis, 1984; MAFF, 1983). It should also be noted that phosphate fertilisers as well as insecticides and fungicides widely used in agriculture may contain undesirable metals such as cadmium. Furthermore, the acidification of soils by various processes, including the use of certain fertilisers as well as 'acid' rain resulting from high sulphur dioxide emission, can increase the uptake of cadmium in crops (Nordberg et al., 1985). A considerable impact on heavy metal mobility may also result from the increasing use
of synthetic complexing agents, such as nitrilotriacetate in detergents to replace polyphosphate (Forstner, 1984).

Some specific crops which tend to accumulate higher levels of cadmium, especially when grown on polluted soils, include cereals such as wheat and rice, and leafy vegetables such as lettuce and spinach (MAFF, 1983). In addition, the significant levels of cadmium found in tobacco mean that cigarette smoking can provide an additional route of exposure by which the daily cadmium intake of smokers can be doubled and thus contribute markedly to the body burden. Other foods with high cadmium levels are animal offal, in particular liver and kidney, shellfish such as whelks and mussels and the brown meat of crabs and lobsters (MAFF, 1983). The intake of cadmium will of course depend on eating habits but the average daily intake of cadmium in the UK is estimated to be under 20µg (MAFF, 1983). Studies seem to suggest that the amount of cadmium taken up by the general population, even by those who consume large amounts of oysters or crab meat, is unlikely to exceed the Provisional Tolerable Weekly Intake (PTWI) of 400-500µg proposed by the Joint FAO/WHO Expert Committee on Food Additives in 1972. It should not therefore pose any significant health hazard.

The absorption of cadmium can however be influenced not only by unusual dietary patterns but by nutritional factors such as certain metal deficiencies which can lead to a significantly increased absorption (Bernard and Lauwerys, Chapter in Foulkes, 1986). The effects of cigarette smoking and industrial exposure on intake can be pronounced and despite an increasing awareness of the toxicity associated with cadmium a small proportion of people in the UK may exceed the accepted PTWI. Further investigation and surveillance are necessary to identify problem areas and to evaluate possible long term hazards associated with low level exposure, particularly with regard to the interaction of cadmium with endogenous metals.
About 50% of the total body burden of cadmium accumulates in the liver and kidney, about 2/3 of this in the kidney. Most of the cadmium absorbed from the gastrointestinal tract and lungs goes initially to the liver, probably bound mainly to albumin in the blood. In the liver, cadmium induces the synthesis of metallothionein (Mt), a low molecular weight protein, rich in cysteine residues, but deficient in aromatic amino acids, which sequesters cadmium in the cells. There is a slow release of cadmium-metallothionein (Cd-Mt) from the liver to the blood with the complex being redistributed to all organs of the body but in particular, to the kidney. Cd-Mt is filtered through the glomerulus in a fashion similar to other low molecular weight proteins and is subsequently reabsorbed by tubular cells, probably by pinocytosis. In the tubular cells, lysosomes containing digestive enzymes rapidly degrade the Cd-Mt complex and release cadmium into the cytoplasm. The tubular cells have a certain capacity of their own for Mt production thereby preventing the toxic effects from non-Mt-bound cadmium. If the Mt-producing capacity of the tubular cells is exceeded the first signs of kidney toxicity appear. This is the generally accepted view of how renal cadmium toxicity arises (Elinder, 1986; Piscator, Chapter in Foulkes, 1986).

The kidney is thus the critical organ in long-term exposure and renal tubular dysfunction characterised by proteinuria, aminoaciduria, glycosuria and increased excretion of cadmium is estimated to occur when cadmium concentration in the cortex reaches 200µg/g wet weight of tissue (Friberg et al., 1974). This value has been endorsed by the WHO Task Group in 1977 but it has been argued that this figure may in fact be rather low (Ellis et al., 1984; MAFF, 1983). Excretion of cadmium in the absence of renal damage is slow, the biological half-life in humans being in the range of 10-30 years (Friberg et al., 1974). Concentrations of cadmium in the renal cortex appear to peak at about the age of 50 but it is not clear whether this reflects a lower level of exposure in the early part of this century, changes
occurring in the metabolism with old age or some other factor (DoE, 1980; MAFF, 1983).

The principles of controlling cadmium exposure within industry are now well understood and incidents of occupational poisoning should in theory be rare. Nevertheless, reports are still appearing in the literature, giving evidence of renal dysfunction in occupationally exposed workers in the UK (Smith et al., 1986) and in other countries (Christoffersson et al., 1987; Edling et al., 1986; Elinder et al., 1985a; Jakubowski et al., 1987; Shaikh et al., 1987). This may however reflect past exposure since cadmium proteinuria is not reversible and may develop years after exposure has ceased (Friberg et al., 1986).

1.8 Treatment of Cadmium Poisoning

There is no specific treatment for cadmium poisoning. Chelation therapy as soon as possible after acute exposure has some success, with the aim being to eliminate the toxic cation from the body or to nullify its effects (Friberg et al., 1986). The effectiveness of chelation therapy decreases markedly with time after exposure apparently due to the intracellular distribution of cadmium and extracellular distribution of the chelator (Kostial et al., 1987) and not simply because cadmium is bound to metallothionein as previously assumed (Klaassen et al., 1984). A very limited number of competitive, high-affinity chelating agents of low molecular weight have the ability to mobilise cadmium from aged deposits in animals in vivo (Reviewed in Jones et al., 1988). The approach of chelation has the added problem however that some chelating agents can cause a redistribution of cadmium within the body that results in renal accumulation of the metal which potentiates the risk of renal dysfunction (Friberg et al., 1986; Foulkes, 1986).

In most cases of cadmium poisoning the only form of therapy is the symptomatic treatment of the metabolic disturbances in individual cases. Due to the long half-life of cadmium in the critical organ and the irreversibility of critical
effects, primary prevention is essential and this is assisted by environmental and biological monitoring of both the general population and particularly those occupationally at risk.

1.9 **Indicators of Cadmium Exposure and Toxicity**

Biopsy samples of kidney are rarely available for assessment of renal damage and cadmium content. In occupational exposure blood cadmium levels and increased urinary cadmium excretion have been used as indicators of acute and chronic cadmium exposure respectively (Chmielnicka and Cherian, 1986). These methods, together with measurements of cadmium levels in hair tend to be poor indicators of exposure and body burden in the general population for a number of reasons, which include the very low concentrations of cadmium present and consequent analytical difficulties (DoE, 1980). Recently the non-invasive techniques of neutron activation analysis (Ellis et al., 1983a; Ellis et al., 1983b) and more recently X-ray fluorescence analysis (Christoffersson et al., 1987) have been successfully applied to the in vivo measurement of liver and kidney cadmium content in the general population as well as in occupationally exposed workers, however these methods have not yet been perfected.

Cadmium nephropathy is accompanied by low molecular weight proteinuria which is tubular in origin and has been detected by measuring excreted proteins such as \( \beta_2 \) microglobulin, retinol-binding protein and metallothionein as well as enzymes such as alkaline phosphatase (Shaikh and Smith, 1984). A high molecular weight proteinuria has also been described and considered to be due to a selective effect of cadmium on glomerular permeability (Lauwerys, Chapter in Webb, 1979; Lauwerys and Bernard, 1986). It has conversely been suggested that this proteinuria is also secondary to tubular dysfunction (Elinder et al., 1985a; Friberg et al., 1986).

Analysis of urinary proteins can reveal a type of tubular dysfunction fairly characteristic of cadmium toxicity but since other conditions may give
rise to similar effects none of the various markers used can be considered specific indicators of environmental exposure to cadmium. The various biological indicators for cadmium exposure have been critically reviewed by Shaikh and Smith (1984) who suggest the measurement of urinary cadmium, metallothionein and \( \beta_2 \) microglobulin to be the most rational strategy to use in detecting and monitoring cadmium toxicity.

1.10 **Effects of Cadmium on Health**

Exposure to cadmium has resulted in a wide variety of both acute and chronic effects in man which have been described in detail in the reviews quoted at the start of this introduction.

1.10.1 **Acute Effects**

Acute high dose cadmium exposure has usually been observed only in the workplace and fortunately acute cadmium toxicity caused by food consumption is rare. It has however followed the consumption of foods which have been in contact with cadmium-containing materials. For instance, the consumption of fruit juice from a vending machine which incorporated a cadmium-plated reservoir caused acute poisoning in Swedish school children (Nordberg et al., 1973, cited in Friberg et al., 1986). The principal acute manifestations following ingestion are severe gastrointestinal disturbances causing nausea, vomiting, salivation, diarrhoea and severe abdominal pain. Inhalation of excessive cadmium causes severe respiratory symptoms including dyspnea, chest pain and coughing. Intense bronchial and pulmonary irritation and oedema give rise to pneumonitis; nausea and gastrointestinal symptoms can also occur. In fatal cases following uptake by both routes, symptoms are succeeded by death from shock within 24 hours or from renal failure and cardiopulmonary depression within days.
1.10.2 Chronic Effects

Chronic effects may include renal disturbances, anosmia, lung insufficiency and mineral imbalances leading to osteomalacia and anaemia. The literature describing animal experimentation with cadmium has increased considerably in recent years. The effects seen in humans can be reproduced in animals following long-term exposure to low levels of cadmium. Such studies together with others in which relatively large doses of cadmium have been injected into the animal have revealed additional consequences of cadmium intoxication some of which may be relevant to the situation in man. Experimental findings include: testicular necrosis, tumour development, effects on ovaries, destruction of the placenta and teratogenic malformations, possible mutagenic effects, inhibition of hepatic microsomal drug-metabolising enzymes, liver damage, effects on pancreatic function, both suppression and enhancement of the immune response and alterations in susceptibility to infection, cardiovascular effects, defects in the central nervous system and neurobehavioural changes, ulceration of the nasal mucosa and yellow discolouration of teeth.

It has been suggested that in some chronic diseases in man cadmium may be of more significance than was previously suspected. Long-term exposure to cadmium has been implicated as a causative agent in the pathogenesis of such common human conditions as emphysema, hypertension and cancer.

1.10.2.1 Emphysema

It has been a matter of controversy as to whether long-term inhalation of cadmium dust and fumes leads to emphysema in man (reviewed by Bernard and Lauwerys, Chapter in Foulkes, 1986). In experimental studies in rats, cadmium chloride inhalation caused emphysema (Snider et al., 1973; Friberg, 1950, Cited in Friberg et al., 1974)) but conclusions from epidemiological studies are often limited by methodological problems such as the relatively small number of workers examined (reviewed by Davison, 1988). Some studies have given insufficient
consideration to other operative factors such as smoking habits and the presence of other pollutants. Such factors were taken into account in a recent study of 101 men who had manufactured Cu-Cd alloy in the UK and findings were reported to be consistent with the hypothesis that inhaled cadmium fumes cause emphysema (Davison, 1988). Further evidence in favour of cadmium inhalation as a cause of chronic respiratory disease came from a mortality study of 6995 cadmium workers in the UK (Armstrong and Kazantzis, 1983). It revealed a statistically significant excess of deaths due to bronchitis which showed a strong correlation with duration and intensity of exposure, being predominant in the group of men with past heavy exposure to cadmium.

1.10.2.2 Hypertension

Conflicting reports have been published regarding the role of cadmium in the aetiology of cardiovascular disease, in particular, hypertension (reviewed by Kopp, Chapter in Foulkes, 1986; Engvall and Perk, 1985). Increased cadmium levels in tissues of hypertensives have been confirmed by some authors and not by others. Tissue profiles for cadmium appear to be distorted in several diseases including hypertension (MAFF, 1983) and it could be that higher levels are retained in tissues where the metabolism is already abnormal. In spite of some rather convincing animal data confirming a hypertensive action of cadmium, as yet there is no firm evidence of a causal relationship between cadmium, either in acutely toxic amounts or with chronic low level exposure, and the development of human essential hypertension. However, it cannot be discounted that cadmium at low environmental levels does play a role in association with other factors involved in the pathogenesis of this disease and further epidemiological studies are needed.

1.10.2.3 Cancer

The possible carcinogenicity of cadmium has become a controversial issue in recent years. The occurrence of interstitial cell tumours of the testes and sarcomas
at local injection sites of cadmium in rats is considered to constitute unreliable
evidence of a cancer risk to humans exposed to cadmium by ingestion and/or
inhalation (MAFF, 1983). There is no indication that intake of cadmium via food
causes cancer but several epidemiological studies have reported an increased
incidence of prostatic and lung cancer in workers exposed to cadmium (references
cited in Foulkes, 1986; Friberg et al., 1986). Firm conclusions are difficult to draw
because of the difficulty in evaluating the role of other carcinogenic agents such as
tobacco smoke or industrial pollutants.

Though the association between cancer and exposure to cadmium in man
appears tenuous, based on the available human and animal data at the time the
metal was classified in 1976 by the International Agency for Research on Cancer
(IARC) as increasing the risk of prostatic and lung cancer in man following
occupational exposure. Since then a study by Takenaka et al., (1983) has been of great
significance as the first published report of a dose-dependent increase in lung cancer
in rats continuously exposed to low-level cadmium aerosols for 18 months. In
another study in rats an elevated incidence of pancreatic islet cell tumours was
found two years after a subcutaneous dose of cadmium (Poirier et al., 1983).
Updated epidemiological studies with increased numbers of workers (Sorahan, 1987;
Thun et al., 1985) and compilation of all available data gives a clear impression of
an increased risk of lung and prostate cancers among cadmium workers, particularly
at high exposure, compared with the expected figures from the general population
(Elinder, 1985b; Lancet, 1986).

The existence of a relationship between cadmium intake and hypertension,
emphysema and cancer in man remains to be unequivocally demonstrated.
Epidemiological studies are difficult to interpret because of the difficulty in
assessing the contribution of simultaneous exposure to other agents, in particular,
cigarette smoke, which has been implicated as a causative agent in all these
conditions. Nevertheless, this only serves to emphasise that the net result of
multiple risk factors on human health though complex, needs to be evaluated.
1.10.3 Teratogenicity and Neurobehavioural Effects

Teratogenic effects have been caused by cadmium in experimental animals but to date there are no reports directly linking cadmium exposure to human congenital malformation (Friberg et al., 1986). The placenta forms an effective barrier against the transfer of cadmium to the foetus. In women who smoke during pregnancy the placenta contains higher levels of cadmium than in non-smokers (Kuhnert et al., 1982; cited in Kuhnert et al., 1987) and babies born to women cadmium workers tend to be of low birthweight (Cvetkova, 1970, cited in Friberg et al., 1986). Increased maternal whole blood cadmium levels and decreased cord vein red blood cell zinc levels are both significantly related to decreased birthweight in human infants (Kuhnert et al., 1987) suggesting that foetal growth inhibition may be due to cadmium interference of zinc transfer across the placenta.

Harmful effects have been seen on the nervous system of both humans and animals on exposure to cadmium (references cited in Arvidson, 1986). Relatively low oral cadmium exposure of female rats, only moderately higher than that of humans in heavily polluted areas, is sufficient to cause damage to the central nervous system of their offspring which results in behavioural impairment, without any effect on growth or morphology (Baranski et al., 1983). Such observations have serious implications for the offspring of expectant mothers who smoke and/or are occupationally or environmentally exposed to high levels of cadmium during pregnancy.

1.11 Molecular Interactions and Mechanisms of Toxicity;
Interaction of Cadmium with Endogenous Metals

Cadmium interaction with cellular components results from the formation of either ionic or covalent complexes with electron donor atoms present as derivatives of sulphur, nitrogen or oxygen. As a result, cadmium interacts with many cellular constituents including phosphates, cysteine- and histidine-containing
side-chains of proteins, purines, pteridins and porphyrins, the phospholipids, phosphatidylethanolamine and serine. Accumulation of cadmium within mitochondria, microsomes and nuclei has been reported and the disruption of numerous cellular metabolic and biochemical pathways has been well documented (Pool, 1981; Vallee and Ulmer, 1972; Webb, 1979).

Of prime importance to the cellular metabolism of cadmium is the high affinity association with metallothionein (reviewed by Webb, Chapter in Foulkes, 1986) which seems to be responsible for the long biological half-life of cadmium. Metallothionein seems to play a dual role in cadmium toxicity. On the one hand it acts as a detoxifying agent against the acute effects of cadmium whilst on the other hand it seems to be involved in the elicitation of the critical chronic effects of cadmium on the kidney. The primary role of metallothionein however seems to be in the regulation of zinc and copper homeostasis.

Many factors appear to influence the toxicity of cadmium including the route of entry, chemical form of the metal, particle size of inhaled material and in particular, the nutritional status of the animal and dietary intake of several nutrients, especially minerals. Pre-exposure to cadmium or metals such as zinc, selenium or iron has been shown to provide protection against toxic effects of a subsequent exposure to cadmium. Many of these effects can be explained by interactions of cadmium at the subcellular level, in particular with metallothionein, and disturbances of the metabolism of a number of essential metals such as calcium, zinc, selenium, chromium and iron. Deficiencies of these metals, as well as of protein and vitamins, exacerbate cadmium toxicity due to its increased intestinal absorption and greater retention in various organs.

Zinc-cadmium interactions are thought to be of special importance in cadmium toxicity and occur at many sites in the body. Both metals induce metallothionein synthesis which can result in a redistribution of zinc which may affect some of its essential functions by causing local deficiencies. An additional
biochemical explanation for the toxic effects of cadmium has been the possible replacement of zinc by cadmium in zinc-dependent enzymes by virtue of its great affinity for sulphydryl groups and inhibition of a number of enzymes by cadmium has been demonstrated (Vallee and Ulmer, 1972; Pool, 1981). Cadmium also interferes with iron metabolism in some way which may be responsible for the anaemia which often accompanies the other adverse effects of cadmium exposure in humans.

1.12 Cadmium-Induced Anaemia in Man

A mild to moderate anaemia, frequently characterised as hypochromic and microcytic, has been observed in humans following both industrial and environmental chronic exposure to cadmium (Friberg et al., 1986). It appears to represent an early symptom of cadmium toxicity but has only been described in a few cases, probably because blood parameters have not always been monitored after exposure.

Anaemia was diagnosed in industrial cadmium intoxication after chronic exposure via inhalation in the alkaline accumulator industry in Sweden, Germany, France and Poland (Friberg, 1950; Piscator, 1971 - both cited in Friberg et al., 1974; Baader, 1952; Nicaud et al., 1942; Marek et al., 1981) the smelting industry in Japan (Tsuchiya, 1967) and in an electronics workshop, accumulator factory and cadmium-producing plant in Belgium (Lauwerys et al., 1974). Other studies have shown anaemia to be one of the symptoms of the long-term excessive ingestion of cadmium that occurred in Japan (Shinoda et al., 1977 - cited in Foulkes, 1986; Nogawa et al., 1979; Nogawa, 1984).

The diagnosis of anaemia in industrial workers gave an early indication that cadmium may have adverse effects on the metabolism of iron. The interaction between iron and cadmium has since been extensively studied in several species of experimental animal to which cadmium has been administered by a variety of routes (see Introduction, Chapter 2) and it is generally accepted that an interference occurs at the level of intestinal absorption of iron.
In a study on human volunteers, Flanagan et al. (1978) showed that the gastrointestinal absorption of cadmium salts was directly related to serum ferritin levels, i.e., the iron stores in the body, and that absorption of cadmium in people with low body iron stores was on average four times higher than in subjects with normal stores. It was therefore suggested that cadmium may compete directly with iron at binding sites of the iron transfer system in the human gastrointestinal tract. The high gastrointestinal absorption of cadmium in humans with low iron stores was later confirmed by Shaikh and Smith (1980) and it was recognised that cadmium absorption could be generally higher in females than in males because of the lower body iron and higher iron absorption in women.

Metabolic balance studies for cadmium in healthy elderly people with iron status within the normal range also suggested an interaction between iron and cadmium. There was an inverse correlation between cadmium absorption and body iron stores measured by serum iron, percentage saturation of serum with iron and serum ferritin (Bunker et al., 1984). Since the intestinal adaptive response to iron deficiency leads to increased absorption of cadmium, a significant proportion of the population consuming diets limited in iron or other factors important in the absorption or utilisation of iron may show increased susceptibility to the adverse effects of cadmium and be at risk even with very low levels of cadmium exposure.

Anaemia was suggested to be an early symptom of cadmium exposure since Tsuchiya (1967) found that all of the 13 workers occupationally exposed to cadmium for up to 12 years exhibited somewhat lower haemoglobin levels and blood gravity. They included three with less than one year exposure, whose proteinuria and cadmium excretion remained within normal limits. In contrast, significant correlation coefficients were observed between erythrocyte counts and parameters in the blood and urine used as markers of renal function in cadmium-exposed groups. These were comprised of 40 Itai Itai patients and 61 suspected patients, who displayed moderate to severe anaemia with significantly reduced erythrocyte
counts, haemoglobin and haematocrit and higher mean cell haemoglobin, mean cell volume and mean cell haemoglobin concentration, compared to non-cadmium-exposed controls (Nogawa et al., 1979). No significant difference in liver iron levels of cadmium-exposed groups compared to non-exposed was found (Nogawa et al., 1984) and it was concluded that anaemia in patients with or suspected of having Itai Itai disease was not hypochromic and microcytic nor due to iron deficiency and that renal damage caused by cadmium exposure may be one of the factors in its development.

Marek et al., (1981) similarly found that iron deficiency was not evident in workers in an accumulator factory who had significantly lowered haemoglobin, haematocrit and erythrocyte counts. These investigators suggested that disturbances of haem biosynthesis were involved on the basis of increased δ-ALA dehydratase activity and reduced erythrocyte protoporphyrin concentration proportional to the concentration of cadmium in the air to which workers were exposed.

1.13 Animal Models of Cadmium Intoxication

Much of the information regarding cadmium toxicity has been derived from studies using laboratory animals, such as the rat, as experimental models because of the constraints laid on studies in humans by the relatively small numbers affected by cadmium poisoning and the difficulties of assessing their body burden of cadmium, particularly prior to the advent of more recent non-invasive techniques of diagnosis.

In order to study the adverse effects of cadmium in experimental animals and produce measurable responses within a reasonable period of time, ie, within the lifetime of relatively short-lived rodents, higher levels than the corresponding dietary cadmium intake for man have generally been used. Cadmium is accumulated slowly via the gastrointestinal trace, so despite the importance of the oral route in man, many controlled experiments in cadmium toxicology have presented the metal
by subcutaneous (s.c.), intraperitoneal (i.p.), intramuscular (i.m.) or intravenous (i.v.) injection. This model of parenteral administration thereby overcomes the low uptake of cadmium achieved by oral presentation and produces a high organ and body burden of cadmium within a short time. The occupational exposure to cadmium, mainly through inhalation, can result in substantially greater accumulation of cadmium in both liver and kidney than after environmental exposure when cadmium is ingested. Such a situation can thus be created by repeated parenteral administration to animals (Shaikh, 1982). On the whole, however, these procedures do not fully model human exposure to cadmium, although much valuable information on the metabolic effects of cadmium has been provided by their use.

Observations made in this Department and by a very limited number of the many workers who have used a parenteral route of administration have indicated that it is accompanied by a local tissue reaction to cadmium. The occurrence of any local inflammatory lesions and consequent systemic inflammatory responses could have some bearing on the flux of metals which has previously been ascribed to a direct effect of cadmium. Thus, many of the early changes of cadmium toxicity could conceivably be attributed to acute inflammation and this particularly concerns the relationship between iron and the observed anaemia following parenteral dosing (see Chapter 2, Introduction).

1.14 Aims of the Study

This study has three aims:

1. It became apparent from the literature that the aetiology of the anaemia which develops following exposure to cadmium is not fully understood. An attempt to elucidate the mechanism involved by using an animal model of cadmium exposure therefore forms the subject of the first part of this thesis. The aim was to establish the extent of any local and systemic
inflammatory response to the subcutaneous administration of cadmium and to evaluate the possible role that it might play in the disturbances in metal homeostasis and the development of anaemia that occur in experimental cadmium intoxication (see Chapter 2).

2. The objective of the second part of the study was to compare the inflammatory changes provoked by cadmium with those provoked by the metals, copper, zinc and aluminium, administered in the same way and with characteristic changes in the turpentine and food pad oedema models of acute inflammation. An investigation into whether the reaction could be alleviated by zinc pretreatment was also carried out with a view to understanding the nature of the response to cadmium.

3. The possible role of free radicals and reactive oxygen species in the development of the local reaction to cadmium was investigated by measuring luminol-amplified chemiluminescence directly from inflamed tissue (see Introduction, Chapter 4). In an attempt to determine the contribution made by reactive oxygen species released from infiltrating inflammatory cells to the observed effects and to understand the mechanism of acute action of cadmium, an _in vitro_ system was developed to allow chemiluminescence to be detected from samples of normal skin and subcutaneous tissue during various treatments.
CHAPTER 2

INVESTIGATION INTO THE ACUTE INFLAMMATORY RESPONSE PROVOKED BY
THE SUBCUTANEOUS ADMINISTRATION OF CADMIUM TO RATS AND ITS POSSIBLE
ROLE IN DISTURBANCES IN METAL HOMEOSTASIS AND THE
DEVELOPMENT OF ANAEMIA
2.1 Introduction

2.1.1 General Introduction

The anaemia which accompanies occupational or environmental exposure to cadmium in humans (Chapter 1) has been reproduced in a number of species of experimental animal following exposure to the metal in a number of forms and by a variety of routes. Various suggestions have been made as to the cause, but the problem has by no means been resolved.

2.1.2 Anaemia in Experimental Animals Following Oral Exposure to Cadmium

Haematological changes, most commonly reduced haematocrit and/or haemoglobin levels, but also reduced red cell counts and changes in the morphology of red cells, as well as disturbances in iron metabolism, have been observed following both long-term and relatively short-term oral exposure to cadmium. Concentrations of cadmium in the diet or drinking water have ranged from 1 to 1350ppm and anaemia has been described in a number of species including rats (Banis et al., 1969; Bunn and Matrone, 1966; Cousins et al., 1977; Decker et al., 1958; Fitzhugh and Meiller, 1941; Ginn and Volker, 1944; Itokawa et al., 1974, 1978; Lawford, 1961a; Pindborg et al., 1946; Pond and Walker, 1972; Prigge et al., 1977, Schafer and Forth, 1984; Suzuki and Yoshida, 1977, 1978; Thawley et al., 1977; Wilson et al., 1941), mice (Bunn and Matrone, 1966; Flanagan et al., 1978; Hays and Margaretten, 1985; Siewicki et al., 1983; Webster, 1979), rabbits (Nomiyama et al., 1975; Stowe et al., 1972, 1974), pigs (Cousins et al., 1973; Pond and Yen, 1983; Pond et al., 1973), calves (Powell et al., 1964), lambs (Doyle et al., 1974), monkeys (Nomiyama et al., 1979), Japanese quail (Fox et al., 1971; Jacobs et al., 1969; Richardson et al., 1974), chickens (Freeland and Cousins, 1973; Hill et al., 1963) and fish (Gill and Pant, 1986; Lowe-Jinde and Niime, 1986).
Anaemia appears to be one of the most sensitive signs of toxicity to cadmium ingestion since reduced haematocrit and haemoglobin values have been detected when as little as 25ppm was added to diet given to rats for 7 and 14 weeks (Prigge et al., 1977; Cousins et al., 1977) and were evident after only two weeks in rats given drinking water containing 50ppm cadmium (Decker et al., 1958) and four weeks in quail given 75ppm dietary cadmium (Fox and Fry, 1970). Even lower levels of 5 and 20ppm cadmium in the diet given for two weeks to mice maintained on a slightly iron-deficient diet also caused anaemia (Siewicki et al., 1983), and Flanagan et al., (1978) found that only 1ppm cadmium added to drinking water (equivalent to 2.5ppm in diet) (Cousins et al., 1977) of mice for 18 weeks increased the severity of the anaemia effected by an iron-deficient diet. This suggested that the low level iron deficiency and anaemia common in many parts of the world could be similarly exacerbated in a large number of people exposed not only to relatively high levels such as the 1ppm cadmium in contaminated rice which formed a staple part of the diet in polluted areas of Japan (Lauwerys, Chapter in Webb, 1979) but also possibly to much lower levels of cadmium.

The severe anaemic response which often results from cadmium poisoning in experimental animals in comparison to the mild to moderate anaemia seen in humans may primarily be related to the levels of cadmium which are frequently much greater in animal studies. Additionally, the rapid growth rate of young laboratory animals and hence their iron status at the time of dosing is probably an important factor in determining their susceptibility to cadmium-induced iron deficiency, and hence the severity of the anaemia. This may explain why low levels of dietary cadmium have been found to cause anaemia in some studies whilst much higher levels in other studies have not.

2.1.3 Effect of Oral Exposure to Cadmium on Iron Metabolism

The anaemia which develops as a result of oral cadmium intoxication is generally of the microcytic hypochromic type and as seen in iron deficiency
anaemia, is associated with depleted iron stores (Banis et al., 1969; Bunn and Matrone, 1966; Cousins et al., 1974; Doyle et al., 1975; Fox et al., 1971; Freeland and Cousins, 1973; Pindborg et al., 1946; Pond and Walker, 1972; Pond et al., 1973; Stowe et al., 1974; Suzuki and Yoshida, 1978). Depleted stores of iron in liver and other organs have also been a feature of oral cadmium intoxication in the absence of anaemia in mice (Sugawara et al., 1984) and rats (Bianusa et al., 1983; Stonard and Webb, 1976 Weigel et al., 1984, 1987) when presumably the degree of iron deficiency was not of sufficient severity to be manifest as anaemia. The reduction in body iron and haematocrit have been directly related to cadmium ingestion and not secondary to reduced diet consumption (Cousins et al., 1977).

The anaemia thus appears to be a manifestation of impaired iron metabolism and explanations of the cadmium-induced iron deficiency state have been proposed which take into account the interaction between the metals at the intestinal level. It has been confirmed by direct measurement of iron uptake from in situ isolated duodenal loops and whole body counting techniques that cadmium inhibits iron absorption in chicks and mice (Freeland and Cousins, 1973; Hamilton and Valberg, 1974). A close inverse relationship apparently exists between the cadmium and iron contents of duodenal mucosa after cadmium administration suggesting that the primary interaction between iron and cadmium may involve competition for mucosal binding sites (Jacobs et al., 1974, cited Bremner, 1974). Further evidence of the possible existence of a common mucosal uptake step for iron and cadmium was provided by studies which showed that the gastrointestinal uptake of orally administered cadmium was greater in iron-deficient mice (Valberg et al., 1976) and rats (Ragan, 1977). Additionally, dietary supplements of iron and ascorbic acid tended to reduce the tissue cadmium content in rats (Maji and Yoshida, 1974).

The nature of the common cadmium-iron binding site in the intestine is not yet clear. Cadmium has been purported to bind to ferritin in the liver (Stonard and Webb, 1976; Cochen and Greener, 1975) and transferrin is known to bind a wide
range of transition metals in addition to iron (Aisen et al., 1974). Both of these proteins are present in the intestinal mucosa and appear to be intimately involved in the control of mucosal uptake and transfer of iron and it seems likely also of cadmium (Schafer and Elsenhans, 1985; Chmielnicka and Cherian, 1986; Bremner, 1978). Since cadmium does not impair the iron-transporting system in the mucosal epithelium of the intestine, metal absorption increases with the iron deficiency caused by competition for sites by cadmium. This leads to increased assimilation of cadmium thus accentuating the sensitivity of the animal to dietary cadmium levels and enhancing the iron deficiency (Schafer and Strugala, 1986).

The suggestion of a competitive inhibition of intestinal iron uptake by cadmium was further supported by studies in which depressed iron content of tissues and the anaemia could be prevented or reversed by levels of dietary iron which exceed normal requirements in rats (Banis et al., 1969; Pond and Walker, 1972; Suzuki and Yoshida (1978), mice (Webster, 1979), pigs (Pond et al., 1973) and quail (Fox et al., 1971). Additionally, protection could be offered by supplements of ascorbic acid which is thought to improve the utilisation of iron (Fox et al., 1971). Furthermore, supplements of iron administered parenterally also overcame the anaemia in rats and pigs (Pond and Walker, 1972; Pond et al., 1973) suggesting that there were no systemic effects of cadmium on iron metabolism and that cadmium was primarily inhibiting iron absorption. However, Pond and Walker stressed the necessity of establishing the relative importance of gastrointestinal effects of cadmium versus its effects on tissue utilisation of iron. Since supplements of iron or ascorbic acid have not been found to offer complete protection against iron deficiency and anaemia (Webster, 1979; Fox et al., 1971) this has been interpreted as indicating that factors other than inhibited iron absorption must play a part.

A decrease in the iron content in multiple organs and an increase in urinary iron excretion whilst no change in the concentration of iron in the faeces of rats following addition of cadmium to the diet were taken to be indicative of a depletion of body iron stores (Julshamn et al., 1977). Stonard and Webb (1976) also found an
effect of cadmium on iron metabolism which was independent of that on iron absorption. In the rat, ingestion of cadmium rapidly depleted iron stores in liver and kidney by inhibition of both ferritin synthesis and iron incorporation into the protein with the result that organs contained reduced amounts of iron-deficient ferritin. The effect of cadmium may be due at least in part to the small amounts of the metal that are incorporated into ferritin being bound at sulphhydryl groups that are essential for iron uptake (Kochen and Greener, 1975).

The finding by Richardson et al., (1974) that the circulating nucleated erythrocytes of quail fed cadmium had larger and more immature nuclei and less cytoplasm as compared to those from quail fed iron-deficient diets also suggested that factors other than a deficiency of iron contribute to cadmium-induced anaemia; a direct toxic effect of cadmium on developing erythrocytes was proposed. There is some controversy about whether an oral route of exposure is required to produce anaemia. Anaemia was demonstrated following dietary cadmium exposure of rats but not after long-term inhalation of cadmium in an aerosol form despite higher levels of cadmium in the liver and kidneys of the inhalation group (Prigge et al., 1977). This supported the hypothesis that anaemia seen with cadmium ingestion is the result of inhibition of iron absorption and led Prigge and co-workers to conclude that haemolytic effects do not contribute to the anaemia. These results in rats were in contrast to the finding of anaemia in humans inhaling cadmium at places of work (see Chapter 1). Perhaps in man there is a greater component of ingestion and ultimate intestinal absorption of originally inhaled particles than in animals. It has proved difficult to assess the involvement of transfer of cadmium via hand to mouth in man. However, in contrast to the study by Prigge et al., (1977), Friberg (1950), cited in Friberg et al., (1974) showed a slight decrease in haemoglobin and red cell counts following exposure of rabbits to cadmium-iron oxide dust by inhalation.
2.1.4 Anaemia in Experimental Animals Following Parenteral Exposure to Cadmium

Although results indicate that dietary cadmium influences intestinal iron absorption and this may be a primary factor in the development of the anaemia following dietary exposure, it is interesting that experimental studies in which relatively large doses of cadmium have been injected parenterally into animals have also produced anaemia. This has generally been characterised as hypochromic and microcytic with reduced haematocrit and haemoglobin. In the majority of cases cited in the following literature survey the cadmium has been administered parenterally as a soluble salt, most commonly the chloride in a saline solution.

One of the earliest reports of haematological change in experimental animals parenterally exposed to cadmium refers to intravasal haemolysis and reduced erythrocyte resistance following i.v. injection of cadmium in dogs (Athanasiu and Langlois, 1896). Since then, anaemia has been demonstrated following s.c. administration of cadmium to rats (Dudley et al., 1985; Faeder et al., 1977; Komsta-Szumksa and Czuba, 1986; Kunimoto and Miura, 1986; Suzuki et al., 1984; Howarth and Hall, 1980, 1981) and rabbits (Axelsson and Piscator, 1966; Berlin and Friberg, 1960; Berlin and Piscator, 1961; Berlin et al., 1961; Friberg, 1955; Swensson, 1957) as well as following i.m. injection in rats (Der et al., 1976) and i.p. injection in rats (Morgan et al., 1984) and chickens (Sturkie, 1973).

2.1.5 Effect of Parenteral Administration of Cadmium on Iron Metabolism

It might be presumed that much of the parenterally administered cadmium would by-pass the intestine and therefore have no effect on iron absorption but it was significant that more cadmium accumulated in the duodenum of iron-deficient mice compared to mice of normal iron status when both were given cadmium by the subcutaneous route (Valberg et al., 1976). This confirms the high affinity of the duodenum for cadmium and suggests that an interference with iron metabolism at the intestinal level may also be involved in the development of anaemia following parenteral administration of cadmium.
As with dietary cadmium exposure, the anaemia could be prevented by iron supplements given by i.m. or i.v. injection following s.c. administration of cadmium to rabbits (Berlin and Friberg, 1960; Berlin et al., 1961; Friberg, 1955). Friberg (1955) found that the hypochromic anaemia was only partially alleviated by i.v. administration of iron in large amounts and noted that other factors must play a part besides iron deficiency. The possibility remains therefore that the alteration in iron metabolism caused by cadmium may be an indirect systemic effect not involving the site of iron absorption.

2.1.6 Cadmium-Induced Haemolysis

A haemolytic action of cadmium on red blood cells has been described. Erythrocytes from rabbits s.c. exposed to cadmium displayed low osmotic resistance (Swensson, 1957) and increased destruction (Berlin and Friberg, 1960) as was seen for dog erythrocytes following i.v. injection of cadmium (Athanasiu and Langlois, 1896). An ahaptoglobinaemia (which is usually a sign of haemolysis) developed after 11 weeks of repeated s.c. administration of cadmium to rabbits (Axelsson and Piscator, 1966) and reduced haptoglobin levels have also been observed in cadmium workers (Friberg et al., 1986). A direct effect of cadmium on red blood cells cannot be ruled out.

2.1.7 Effect of Cadmium on Bone Marrow

Signs of atrophy or toxic damage in the bone marrow cells have seldom been reported in cadmium poisoning. A mucoid type of connective tissue degeneration was seen in s.c. cadmium-poisoned rabbits but no significant difference in the number of erythropoietic cells was found (Berlin et al., 1961). Marrow hypoplasia was described in mice given cadmium in drinking water for over one year characterised by significant reductions of the pluripotent stem cells, granulocyte monocyte progenitor cells and erythroid progenitor cells (Hays and Margaretten, 1985). These marrow alterations were reflected by an anaemia with
reticulocytopenia and neutropenia. Iron deficiency was also demonstrated by hypochromia of peripheral red cells and diminished marrow iron stores. Anaemia was thought to be the result of bone marrow hypoplasia with reduced erythroid progenitors and an added component of iron deficiency, although it was thought possible that iron deficiency might be responsible for the marrow hypoplasia. Contrary to these findings several authors have described hyperplasia of the bone marrow and reticulocytosis in rats (Pindborg et al., 1946; Wilson et al., 1941; Decker et al., 1958; Fitzhugh and Meiller, 1941) and quail (Richardson et al., 1974) after oral cadmium exposure and in rabbits (Swensson, 1957) after s.c. administration.

2.1.8 Transport of Cadmium in the Blood

Although the total amount of cadmium in the blood may be small, exposure of blood to cadmium absorbed from ingested food is continuous and it comes into contact with high levels of cadmium following parenteral administration. The interaction of cadmium with components of the blood is of considerable interest since the fluid serves a transport function for delivery of cadmium to the various target organs of the body. It is pertinent therefore to summarise the current knowledge on the interaction of cadmium with the red cell compartment of the blood with a view to understanding whether a direct toxic interaction might occur and/or whether there is incorporation of cadmium into newly formed cells in the marrow.

There has been a certain amount of controversy in the literature over the mechanism(s) responsible for redistribution of cadmium in the blood particularly with regard to the time intervals involved and the intra- and extracellular proteins to which cadmium is bound. This probably arises from the different dose levels, routes of administration and animal species used. Reports are in general agreement about the phenomenon of a rapid removal of cadmium from plasma within the first few hours and a slower removal from erythrocytes followed by a surprising increase in blood cadmium concentration 24 to 96 hours after the initial elimination phase,
mainly due to an increased amount associated with red blood cells (references cited in review chapter by Bremner in Webb, 1979).

Carlson and Friberg (1957) found that cadmium was present bound to haemoglobin. Axelsson and Piscator (1966) proposed that intravascular haemolysis resulting from the deposition of cadmium in erythrocytes liberated haemoglobin. Pronounced haemolysis would result in ahaptoglobinaemia, and an excess of haemoglobin would then be excreted via glomeruli and reabsorbed by the tubules of the kidney. It was suggested that this could be one way in which cadmium reaches the tubules. This may well be one of the mechanisms of cadmium accumulation and development of anaemia in rabbits exposed to high levels of cadmium since this species is particularly vulnerable to haemolysis. However, it is generally accepted now that cadmium reaches the kidney primarily as cadmium-thionein (Chapter 1).

Further attempts to identify the form in which cadmium occurs in red blood cells found that most of the cadmium in haemolysates of erythrocytes collected 24 hours or more after injection of the cation was bound to a low molecular weight fraction similar to metallothionein which also binds cadmium in plasma (Nordberg et al., 1971). Hildebrand and Cram (1979) studied blood cells incubated in culture with cadmium and showed that metallothionein was induced only in lymphocytes and not in mature non-nucleated erythrocytes whilst Garty et al., (1981) found concentrations of cadmium in leucocytes to be 10 times higher than in red cells on a per cell basis. However, the biphasic variation in concentration was not apparent in leucocytes.

Garty et al., (1981) also showed cadmium in red blood cells to be increased to the same degree in the ghost (primarily membrane) as in the cytosol. It was bound mainly to high molecular weight proteins (not haemoglobin) in the cytosol at both early and later time points whereas at later times cadmium was also bound to a low molecular weight protein similar to metallothionein but differing in some characteristics. It was suggested that cadmium may induce metal-binding proteins in the ghost as well as in the cytosol of immature nucleated erythrocytes in the bone
marrow and that a lag time of 60 hours was needed for induction of these proteins and the release of the mature erythrocyte into the bloodstream. Tanaka et al., (1985) also reported that the synthesis of cadmium-binding protein metallothionein in erythroblasts of mice might account for the increased cadmium concentration in red blood cells.

The metallothionein in erythrocytes has recently been shown to undergo degradation along with the erythrocyte in the liver and spleen (Tanaka et al., 1986) but the toxic effect of cadmium on the erythrocyte is still at present unclear and the significance of metallothionein uncertain. Much of the work on the separation and identification of protein fractions has utilised electrophoretic techniques but Rabenstein et al.,(1983) used proton nuclear magnetic resonance to study the interaction of cadmium with human erythrocytes and obtained direct evidence for the binding of cadmium by intracellular glutathione and haemoglobin.

Recently Garty et al., (1986) published data indicating that cadmium uptake into mature rat blood cells occurs by passive transport and that alterations of sulphhydrals of red cell membranes may modulate the process. Vincent and Blackburn (1958) reported changes in erythrocyte metabolism in the form of an efflux of potassium ions from human erythrocytes incubated in vitro with low concentrations of cadmium. Cadmium or zinc was found to increase the accumulation of calcium in human red cells in vitro by increasing passive influx without enhancing permeability to other ions (Plishker, 1984). It was suggested that the cadmium effect involves a disulphide bond between cysteinyl residues and the possible interaction between cadmium and zinc and the mechanism of uptake by red cells are discussed further by Garty et al., (1986).

2.1.9 Inconsistencies in the Theory that Iron Deficiency is the Primary Cause of Anaemia Following Parenteral Administration of Cadmium

Anaemia appears to be a common early observation in cadmium toxicity regardless of the route of administration although basis for the anaemia and altered
Iron metabolism appears from the literature to be controversial. Iron concentrations in organs are influenced differentially by oral and parenteral routes of cadmium administration. The theory of cadmium-induced anaemia being due to a deficiency of iron absorption is not supported by the finding that the anaemia following parenteral administration is not generally accompanied by depleted iron stores. Indeed, hepatic iron stores have been found to increase following s.c. administration of cadmium to mice (Sugawara et al., 1984) and rats (Bonner, 1980; Komsta-Szumska and Czuba, 1986; Howarth and Hall, 1980, 1981). There appears to be conflicting results with respect to serum iron concentrations which have been reported to be both elevated (Sugawara and Sugawara, 1984a), decreased (Bonner et al., 1979; Ashby et al., 1980; Howarth and Hall, 1980, 1981) and to remain unchanged (Sugawara et al., 1984; Komsta-Szumska and Czuba, 1986) following s.c. cadmium administration. An enhancement of iron absorption following cadmium injection is unlikely to account for increased hepatic iron since firstly it cannot adequately explain the decreased iron seen in some tissues such as kidney (Sugawara et al., 1986; Komsta-Szumska and Czuba, 1984; Bonner, 1980) and serum and secondly the presence of cadmium in the duodenum has generally been reported to reduce iron absorption.

It seems possible that altered mobilisation of iron may be involved although depressed mobilisation of stored iron from organs to the blood circulation was not thought to be consistent with the finding of increased activity of serum caeruloplasmin by Sugawara et al. (1984) since this protein has a role in the mobilisation of iron. More stainable iron was shown in the reticular cells of the marrow of rabbits s.c. dosed with cadmium and the development of a hypochromic anaemia despite the apparent presence of iron was thought to indicate that some form of inhibition in the transfer of iron to haemoglobin-synthesising cells is induced by cadmium (Berlin et al., 1961). Conversely, Berlin and Friberg (1960) reported less iron in the bone marrow of similarly treated rabbits, however, no data appeared to be presented to substantiate this.
From the differential alterations in iron levels in various tissues, following parenteral administration of cadmium it seems likely that the metal interferes with iron homeostasis possibly at more than one site, which may or may not include the intestine but ultimately results in withholding of iron from erythropoietic cells. The degree of involvement of iron deficiency and other mechanisms in the aetiology of the anaemia may depend entirely on the route of exposure to cadmium.

Many investigations have concentrated on the action of cadmium on the homeostasis of various essential metals, particularly in conjunction with the induction of metallothionein. Iron metabolism is influenced by levels of other endogenous metals, for example, copper, which in turn have been shown to be influenced by cadmium levels and metallothionein synthesis thus strengthening the possibility that the interaction between cadmium and iron could occur at sites other than the intestinal mucosa.

2.1.10 The Parenteral Route of Cadmium Administration and Inflammation

Observations made in this department (Bonner et al., 1979, 1980) of local abscesses developing at sites of s.c. administration of cadmium are supported by only a limited number of similar observations (see Discussion) considering the wealth of published results derived from the use of parenteral routes of administration. The occurrence of any local inflammatory lesions and consequent systemic inflammatory responses could have some bearing on the flux of metals which has previously been ascribed to a direct effect of cadmium. Thus, many of the early changes of cadmium toxicity could conceivably be attributed to acute inflammation and this particularly concerns the relationship between iron and the observed anaemia following parenteral administration of cadmium for which no rational explanation has been forthcoming.
2.1.11 **Aim of the Study**

Since scant attention appears to have been paid by workers in this field to the early and possibly inflammatory effects of parenteral dosing it was decided to investigate how the temporal characteristics of such a response might relate to perturbations in metal homeostasis and the development of anaemia.

2.1.12 **Outline of the Investigation**

The subcutaneous administration of cadmium at a dose level of 1.5mg Cd\(^{2+}\)/kg/day was chosen in order to maintain comparability with previous studies carried out in this Department (Bonner et al., 1979, 1980; Howarth and Hall, 1980, 1981) and by a number of other workers. It was expected that the toxic effects such as renal and testicular damage would be reproduced. However, growth rate has been shown to be severely depressed at this dose level and studies in which suppression of growth is extreme raise concern about the secondary effects that might be more directly related to decreased food intake than to the direct effects of cadmium. Since the severe adverse effects of cadmium on food consumption, body weight gain, kidneys and testes might complicate an interpretation of inflammatory effects of cadmium it was decided to compare the results at 1.5mg Cd\(^{2+}\) with those of a preliminary study fortuitously carried out at half the dose. Unfortunately, a full range of tests were not performed at 0.75mg which should, with hindsight, have been included, however, the results are presented since crucial observations were made.

In order to understand the pathogenesis of the anaemia it was deemed necessary to study the alterations in iron homeostasis and red cell production or destruction during the early stages of its development. Therefore the experiment was designed to take into account the sequence of events taking place over a period of 20 days s.c. dosing. Since particular attention was paid to a closely spaced series of early time-points animals were dosed for 7 days per week in contrast to the frequently used 5 days per week regime to avoid any anomalies that intermittent dosing might cause.
Histopathological examination of a comprehensive array of organs, including skin injection sites, was carried out to assess the level of tissue damage after increasing numbers of injections and its relation to haematological changes. Effects on peripheral blood elements were considered since these changes reflect events in the entire haemopoietic system. Additionally, bone marrow preparations were examined at the high dose since the effects of cadmium on bone marrow have been controversial. A number of markers of inflammation which display characteristic changes in an inflammatory response were also investigated in order to relate these to alterations of essential metals in a number of tissues and to the development of anaemia.

In the following pages, the Methods and Results sections of the two dose level studies are presented separately for clarity, the higher dose first, followed by the low dose; the results of which are compared to the main study. This is followed by a combined Discussion of both sets of results.
2.2 Effect of Repeated Administration of Cadmium at 1.5mg/kg/day

2.2.1 Materials and Methods

2.2.1.1 Materials

Unless otherwise stated, all chemicals used were of analytical grade and supplied by BDH Chemical Company, Poole. All suppliers were located in the UK. A commercially available control serum (Precinorm® U, Boehringer Corporation Limited, Lewes) was used to monitor the reproducibility of plasma assays. Spectrophotometric techniques were performed on a CE292 digital ultraviolet spectrophotometer (Cecil Instruments Limited, Cambridge).

2.2.1.2 Animals and Husbandry

Male Wistar albino rats (University of Surrey) were housed in groups of 5 in solid polycarbonate cages with wood shavings as bedding. Standard pelleted rodent breeding diet (Labsure Limited, Poole) and water were provided ad libitum. The manufacturers stated content of minerals in the diet was iron (100mg/kg), zinc (40mg/kg) and copper (20mg/kg). The animals were housed at a temperature of 22 ± 2°C and relative humidity of 50 ± 3% in an artificially controlled 12h light:dark cycle (0700-1900h). Food and water consumption and general condition of the animals was monitored daily. The weight range from which animals were selected was 175-275g.

After an acclimatisation period, groups of 5 animals were given cadmium, 1.5mg Cd²⁺/kg/day (13.35μmol Cd²⁺/kg/day) as CdCl₂.2½H₂O in 0.9%w/v (0.154M) sodium chloride solution (saline) by daily subcutaneous injection in the abdominal flank for either 1, 3, 6, 10, 15 or 20 days. Control animals received 0.9%w/v saline only, at the same volume of 1ml/kg. The solutions were initially sterilized by autoclaving at 15lb/in² for 15min and sterile hypodermic needles and syringes were used (Sabre International Products Limited, Reading).
Groups of animals were dosed for periods of either 1, 3, 6 or 10 days (Study A) and either 10, 15 or 20 days (Study B). For logistical reasons the studies were carried out on different occasions; to verify the reproducibility of effects, the 10 day dose interval was chosen as a common link point. A further investigation over the 10 day period (Study C) provided additional confirmation and allowed measurement of other indices. Each experiment was terminated 24h after the final injection. A further 2 groups of 3 animals each (in Study B), which had received 20 injections of either cadmium or saline (as the controls), were maintained for an additional 9 days after the final injection and are referred to here as the 'recovery group' (Figure 2.1).

2.2.1.3 Autopsy

The animals were anaesthetised with sodium pentobarbitone, 60mg in 1ml (Sagatal\textsuperscript{R}, May and Baker, Dagenham), administered as an intraperitoneal injection (1ml/kg). Observations on the condition of animals and descriptions of any lesions seen at injection sites were recorded. The abdominal cavity was opened and blood collected into a dry plastic syringe (Sabre International Products Limited, Reading) via the posterior vena cava. The sample was dispensed into collection tubes for whole blood for haematology (tube containing potassium salt of ethylenediamine tetraacetic acid, EDTA, 2mg/ml as anticoagulant), for plasma (tube containing lithium heparin), for osmotic fragility tests (0.5ml blood in a 2ml heparinised tube), and for serum (glass tube, no anticoagulant). EDTA tubes were obtained from Teklab Medical Laboratories Limited, Durham and heparinised tubes from Sterilin Limited, Feltham. Blood for serum was allowed to clot for a minimum of 3h, centrifuged at 2500rpm for 15min and the serum obtained used on the same day for protein analysis. Plasma was separated by centrifugation at 2500rpm for 15min and stored at -20\textdegree C.
Study A and Study C

1.5 mg Cd\(^{2+}\)/kg/dy

No. of Daily Doses

Study B

0.75 mg Cd\(^{2+}\)/kg/dy

No. of Daily Doses

Figure 2.1
Schematic Plan of Dosing Schedules
Each horizontal bar represents one group of male Wistar albino rats and shows the number of daily subcutaneous injections of saline or CdCl\(_2\cdot2\frac{1}{2}\) H\(_2\)O in saline which were given at the dose levels indicated in the right hand margin. Each experiment was terminated 24h after the final dose (\(\square\) = day of autopsy) except for a 20-day group in Study B which was allowed a 9-day 'recovery period'.
The gross appearance of all major organs was recorded. Liver, spleen, kidneys and testes were weighed and samples of liver, spleen, right kidney and intestine (a portion of jejunum taken 10cm from the pyloric sphincter and washed in 0.9% saline to remove the contents) were stored at -20°C for subsequent metal analysis. Samples of liver, spleen, left kidney, intestine, lung, lymph nodes, sternum and skin (injection sites) were fixed in 10%v/v neutral buffered formalin for histological examination. The left femur was removed, split longitudinally and the appearance of the marrow noted. Samples from 3 and 10 day groups were frozen for metal analysis. Bone marrow smears were prepared at each time point by dipping a squirrel hair brush previously wetted with albumin/EDTA solution (1ml of 6%w/v bovine serum albumin added to a 2.5ml EDTA tube) into the femoral cavity to take up a portion of marrow. This was then 'painted' onto a glass slide, rapidly air dried and fixed in methanol for 20min. Blood films were prepared from freshly collected EDTA anticoagulated blood, rapidly air dried and fixed in methanol for 10min.

**Haematology**

Unless otherwise indicated, standard techniques which are all described in full in Dacie and Lewis (1984) were used for the following haematological methods.

### 2.2.1.4 Haematocrit (Packed Red Cell Volume)

The haematocrit was determined by means of a microhaematocrit centrifuge (MSE Limited, Crawley) and results expressed as a percentage of unit volume.

### 2.2.1.5 Haemoglobin Concentration

The haemoglobin content of whole blood was measured by the cyanmethaemoglobin method using Drabkin's cyanide-ferricyanide reagent and suitable reference and quality control standards (Diagnostic Reagents Limited, Thame). Spectrophotometric absorbance was measured at 540nm and results
expressed as g/dl blood. Mean cell haemoglobin concentration (MCHC) was calculated from the formula:

\[
\frac{\text{Haemoglobin Concentration (g/dl)}}{\text{Haematocrit (%)}} \times 100
\]

2.2.1.6 Red Cell Count

Red cells from Group C (10 days maximum) were counted visually using an improved Neubauer counting chamber by making a 1 in 300 dilution of blood in formal-citrate solution (1% v/v formalin in 31.3g/l trisodium citrate).

2.2.1.7 Reticulocyte Count

The percentage of reticulocytes was determined by examination of unfixed films prepared from blood stained with 1% w/v Brilliant Cresyl Blue (Cl No. 51010, RA Lambs, London) in citrate-saline solution (1 volume of 3% w/v trisodium citrate to 4 volumes of 0.9% w/v sodium chloride). The absolute numbers of reticulocytes were calculated from the total red cell counts.

2.2.1.8 Red Cell Osmotic Fragility

The method for measurement of red cell osmotic fragility was based on that of Parpart et al., (1947) as described in Dacie and Lewis (1984). A stock solution of buffered sodium chloride, osmotically equivalent to 100g/l (1.71mol/l) NaCl containing 90g/l NaCl, 13.95g/l Na\textsubscript{2}HPO\textsubscript{4} and 2.43g Na\textsubscript{2}H\textsubscript{2}PO\textsubscript{4} \cdot 2H\textsubscript{2}O was used to make dilutions equivalent to 1.0, 3.0, 3.25, 3.5, 3.75, 4.0, 4.25, 4.5, 5.0, 5.5, 9.0 and 12g/l NaCl and of pH 7.4. 20μl of heparinised blood was added to 5ml of each solution and after mixing the tubes were allowed to stand at room temperature for 30min. They were re-mixed and centrifuged at 1200-1500g for 5min. The absorbance of each supernatant was measured by spectrophotometry at a wavelength setting of 540nm and the highest recorded absorbance taken as 100%
haemolysis. The amount of haemolysis in each solution was compared to this value and an osmotic fragility curve drawn by plotting the percentage lysis against the corresponding concentration of salt solution.

2.2.1.9 **Total White Cell Count**

White cells were counted visually using an improved Neubauer counting chamber by making a 1 in 20 dilution of blood in 2% v/v acetic acid coloured pale violet with Gentian Violet (Cl No. 42535).

2.2.1.10 **Differential White Cell Count and Red Cell Morphology**

Blood films were stained by the May-Grunwald-Giemsa technique and a differential white cell count of 200 cells carried out. Absolute values were calculated from total white cell counts. The films were scrutinized for the presence of any abnormalities in red cell morphology.

2.2.1.11 **Neutrophil Alkaline Phosphatase**

Alkaline phosphatase activity was demonstrated in neutrophils by the azo-dye coupling technique of Rutenburg et al., (1965) which depends on the hydrolysis of a substrate containing naphthol AS phosphate (Sigma Chemical Company, Poole). As hydrolysis occurs, the liberated naphthol couples to a diazotised amine, Fast Blue BB salt (CI No. 37175, RA Lamb Limited, London) and forms an insoluble blue precipitate. The intensity of the precipitate is a rough measure of the enzyme content of individual cells and was rated as follows:

0 = negative - no granules
1 = positive but very few granules
2 = positive with few to moderate granules
3 = strong positive with numerous granules
4 = very strong positive with cytoplasm crowded with granules
The score in a film consists of the sum of scores of 100 consecutive neutrophils.

2.2.1.12 Differential Bone Marrow Examination

Haematoxylin and eosin stained sections of decalcified sternum were assessed for general cellularity of the marrow. Bone marrow smears were stained by May-Grunwald-Giemsa technique and a differential cell count carried out. Myeloid:erythroid ratios were based on a count of 1000 cells which were further categorised as adult neutrophil, metamyelocyte, myelocyte, normoblast and erythroblast to give an indication of maturity. The use of a grid graticule in the microscope eyepiece facilitated a simultaneous count of the other cell types, namely, eosinophilic, monocytic, basophilic and plasma cells, lymphocytes and megakaryocytes. The incidence of different cells was expressed as a percentage of total cells counted and the percentage which were in mitosis was also recorded.

Biochemistry

2.2.1.13 Total Plasma Protein

Total plasma protein concentration was determined by the protein-dye binding assay according to Read and Northcote (1981) based on the method of Bradford (1976). It involves binding of Coomassie Brilliant Blue G (CI No. 42655, Sigma Chemical Company Limited, Poole), to protein which causes a shift in the absorption maximum of the dye from 465 to 595nm. The increase in absorption at 595nm is then monitored by spectrophotometry and compared to a standard curve of bovine serum albumin (Sigma Chemical Company Limited, Poole) dissolved in 0.9% w/v sodium chloride solution in the range of 0-600μg/ml.

2.2.1.14 Serum Protein Analysis

Crossed immunoelectrophoresis (X-IEP) is a combination of the electrophoretic separation of proteins in agarose gel followed by electrophoresis
perpendicular to those in an antibody-containing gel. Serum proteins were characterised by X-IEP as fully described by Axelsen et al., (1973) and outlined as follows:

Initially 5μl of a 1 in 20 dilution of serum in 0.9%w/v NaCl was applied to a well punched in gel (10ml of 1%w/v Agarose A, Pharmacia Fine Chemicals, Milton Keynes, in barbitone-acetate buffer) coating one side of an 8cm² glass plate. Proteins were separated by electrophoresis at 200V until a bromophenol blue tracker dye reached the far end of the plate (about 1.5h). This was followed by electrophoresis in a perpendicular direction into gel containing 5%v/v rabbit anti-(rat serum) obtained from Dako Limited, High Wycombe, at 45V overnight (16h). The separated antigens produced multiple precipitant peaks which were visualised by staining with Coomassie Brilliant Blue R (Cl No. 42660, Sigma Chemical Company Limited, Poole).

Identification of individual precipitates may be most accurately achieved by the use of specific antisera or lectins or by applying specific staining techniques to replicate plates. The high cost of antisera and limited financial resources made identification by these procedures impossible and precluded X-IEP being performed on all samples. Therefore one sample from each treatment group was examined after 1 or 3 injections and the resulting protein patterns compared with reference profiles obtained for the Wistar albino rat (University of Surrey) by Hinton et al., (1985), the Wistar albino rat by Abd-El-Fattah et al., (1976) and Scherer et al., (1977), the laboratory rat by Schade and Burger (1979) and the Sprague-Dawley rat by Emmett et al., (1984). A tentative identification of proteins was made by correlating precipitation peaks displaying similar electrophoretic mobility (relative to albumin) and characteristic appearance and for which the identity has been verified by the use of specific antisera, staining methods, biochemical functions or physico-chemical parameters by Abd-El-Fattah et al., (1976), Schade and Burger (1979) and Emmett et al., (1984). Plates were first assessed by visual comparison with controls and any apparent differences noted. Quantitation of individual
antigens was performed by determining the area enclosed by the precipitate (as estimated by height x width at 1/2 height) and expressing this value as a percentage of the area of the corresponding peak in the control.

2.2.1.15 Plasma Haptoglobin

Plasma haptoglobin levels were determined by rocket immunoelectrophoresis as described in full by Axelsen et al., (1973). 5μl of a 1 in 8 dilution of each plasma sample in NaCl/azide solution was applied to wells in agarose gel containing 2%v/v rabbit anti-(human haptoglobin) (Dako Limited, High Wycombe) and electrophoresis was carried out for 2h at 8V/cm plate. Marked cross-reactivity between rat and human haptoglobin has been shown (Hinton et al., 1980). Standards were not available so the determination of absolute values for haptoglobin concentration was not possible. However, the heights of the precipitates formed by rocket immunoelectrophoresis are proportional to the concentration of protein so comparisons were made between control and cadmium-exposed animals.

2.2.1.16 Plasma Caeruloplasmin

Caeruloplasmin in plasma from rats given 3 and 10 doses of either cadmium or saline was measured by its p-phenylenediamine (PPD) oxidase activity using the method of Sunderman and Nomoto (1970). At pH 5.2 caeruloplasmin catalyses the oxidation of p-phenylenediamine dihydrochloride (Sigma Chemical Company Limited, Poole) to yield a purple-coloured product the absorbance of which was measured at a wavelength of 530nm. The rate of formation of the coloured oxidation product is proportional to the concentration of caeruloplasmin if correction is made for the non-enzymic oxidation of PPD. Therefore simultaneous assays are carried out with and without sodium azide which inhibits enzymic oxidation. The difference between the results of the two assays is proportional to the caeruloplasmin concentration. The PPD oxidase reaction is subject to a lag phase owing to the oxidation of plasma ascorbic acid. To avoid this potential source
of error the timing of the reaction is delayed until after the lag phase. The concentration of caeruloplasmin in g/l was calculated as 0.752 (A_R - A_B) where A_R is the absorbance in the assay without azide and A_B is the absorbance in the assay with azide.

2.2.1.17 **Plasma Iron and Total Iron-Binding Capacity (TIBC)**

Plasma iron concentration (μmol/l) was determined by the ferrozine assay of Ceriotti and Ceriotti (1980) and Mori et al., (1981) adapted to the Cobas Bio centrifugal analyser (Roche Products Limited, Welwyn Garden City) by Schlosnagle et al., (1982). Iron is released from transferrin and reduced at acid pH by treating with ascorbic acid in hydrochloric acid. On addition of the sodium salt of 3-(2-pyridyl)-5,6-bis(4-phenylsulphonic acid)-1,2,4-triazine (ferrozine, Sigma Chemical Company Limited, Poole) a magenta complex with iron is formed, the absorbance of which is measured at 562nm by spectrophotometry. The iron-binding capacity of plasma is saturated by mixing a sample with ferric chloride solution. Excess iron is adsorbed onto magnesium carbonate and removed by centrifugation. TIBC is then determined by the ferrozine method.

2.2.1.18 **Tissue Metal Analysis**

Tissues were prepared for metal analysis by the wet-ashing procedure described by Delves et al., (1971) and Thompson and Blanchflower (1971). It requires the digestion of 0.5g of tissue (except in the case of marrow, where only 0.040-0.075g was obtained) in a mixture made up of 5ml nitric acid (1.412-1.417g/ml, Aristar grade) and 1ml perchloric acid (specific gravity - 1.54, Analar grade) by heating and taking to dryness. The ash was re-dissolved in double distilled, deionised water and the concentrations of cadmium, copper and iron in the liver and cadmium and iron in the kidney, spleen, intestine and marrow were determined using an SP9 flame atomic absorption spectrophotometer (Pye Unicam, Cambridge). This method gives recoveries of better than 98% for cadmium, copper and iron at a concentration
of 40µg/100ml and a coefficient of variation of less than ±8% at a concentration of 10µg/100ml (Bonner et al., 1979). The detection limits for flame atomic absorption spectrophotometry obtained from Berman (1975) are stated to be cadmium 1 x 10^{-3} µg/ml (9 nmol/l), iron 1 x 10^{-2} µg/ml (179 nmol/l), copper 5 x 10^{-3} µg/ml (79 nmol/l).

2.2.1.19 Histology

Samples of formalin-fixed tissues were routinely wax-embedded, sectioned and stained with either Haematoxylin and Eosin (RA Lamb, London) or by Perl's Prussian Blue reaction for ferric iron. Skin sections were also stained by Van Gieson method for connective tissue and sections of testes and skin with Von Kossa technique for calcium. All the methods used are to be found in Culling (1974).

2.2.1.20 Presentation of Results and Statistical Analysis

The data obtained from replicate time point groups were pooled (see note in Results section) and all results expressed as the mean (x) ± standard error of the mean (SEM). Some measurements were not carried out on samples from all three studies and there was unavoidable loss of an occasional blood sample due to clotting. Therefore, the number of samples (n) per treatment group at each time point is included in the Figures and Tables and where appropriate, the identification of the study from which samples originated.

All data were subjected to statistical analysis by the Student's t-test using the formula:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\text{SEM}_1^2 + \text{SEM}_2^2}}$$

with (n_1 + n_2 -2) degrees of freedom.

Levels of significance are indicated as:

* p<0.05, ** p<0.01, *** p<0.001
2.2.2 Results

2.2.2.1 General Condition of Animals

Control animals remained in good condition throughout the study. Rats receiving cadmium became subdued and displayed piloerection and lacklustre fur after 2 to 3 injections. It was apparent on handling the rats that their extremities were cold. Intense swelling developed at injection sites and for this reason further injections were given at different sites on both flanks to avoid repeated administration into areas of swelling. At later time points, superficial lesions developed at some injection sites. More detailed observations on the macroscopic appearance of organs at autopsy are given in the histopathology section.

2.2.2.2 Body Weight Gain, Food and Water Consumption

Figure 2.2 shows the mean daily body weight with daily food and water consumption for animals which were dosed for 20 days. There was a complete cessation of body weight gain in cadmium-treated animals which became apparent during the 24 to 48h period after the first injection. This coincided with a marked reduction in food consumption from about 8.5 to 4.0g per 100g body weight per day. Food consumption per 100g body weight in the control group declined gradually over the course of the study whereas in the cadmium group it remained at the lower level. The difference in mean body weights of control and cadmium-treated groups became statistically significant after the fifth injection and the body weights of the cadmium-treated rats reached only 72% of those of controls by the end of the study. Water intake rose slightly during the 24h period after the first injection, then fell sharply and followed a similar pattern of reduced intake to that seen for food consumption. Figure 2.3 shows that withdrawal from cadmium administration resulted in the eventual restoration of the rate of body weight gain to control levels.
Figure 2.2

Body Weight Gain (c) and Daily Food (b) and Water (a) Consumption of Rats During Daily Subcutaneous Administration of Cadmium for 20 Days (Study B)

The figures presented are (c) the means ± SEM (shown as bars) and (a + b) the means for saline-(●) and cadmium-(○), 1.5mg Cd²⁺/kg/day, treated animals. There were five animals per group and statistically significant differences between test and control groups are shown as *p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 2.3
Body Weight Gain of Rats During Daily Subcutaneous Administration of Cadmium for 20 Days Followed by a Recovery Period of Nine Days (Study B)
The figures presented are the means ± SEM (shown as bars) for saline-( ○ ) and cadmium-( O ), 1.5mg Cd²⁺/kg/day, treated animals. There were three animals per group and statistically significant differences between test and control groups are shown as * p < 0.05, ** p < 0.01.

Figure 2.4
Body Weight Gain (b) and Food Consumption (a) of Rats During Daily Subcutaneous Administration of Cadmium for 10 Days (Study C)
The figures presented are (b) means ± SEM (shown as bars) and (a) means for saline-( ○ ) and cadmium-( O ), 1.5mg Cd²⁺/kg/day, treated animals where the number of animals per group = 5.
A similar response was seen in other groups in Studies A and B but not in Study C. The daily body weight and food consumption for the 10 day group of the latter study are shown in Figure 2.4. By comparison with Study B (Figure 2.2) it can be seen that similarly there was an initial marked drop in food consumption after the first injection of cadmium which coincided with a slight fall in mean body weight. However, there followed a gradual return to normal food consumption and an immediate return to almost normal body weight gain. This is in sharp contrast to the prolonged cessation of body weight gain seen in Study B. By comparing the growth rates of the animals in the two studies it can be seen that controls in Study B (Figure 2.2) grew at a faster rate than those in Study C (Figure 2.4), an average of 7g/day compared with 4g/day. This was despite the food consumption of the two groups being approximately the same and can only be attributed to biological factors of unknown aetiology.

It should be pointed out, however, that Study C, with the slower growing rats, was carried out in January, whereas Studies A and B were undertaken in June and August respectively. There is some evidence (J Arendt, personal communication) that even animals maintained in constant conditions of light and temperature still retain some aspects of seasonal periodicity, although the signal entraining the internal 'clock' is not clear. It is predicted that one of the consequences might be alterations in growth rate. This may explain the contrasting effect that exposure to cadmium had on growth rate in the studies.

NB Such temporal variations in growth rate are commonly encountered in the strain of rat used in this laboratory. It is considered a valid exercise therefore to pool the data accumulated over a 2 year period in these experiments, and Figure 2.5 shows the pooled mean terminal body weights for each time point. However, it is recognized that variations in growth rate might have a marked effect on the expression of cadmium toxicity and that by pooling results substantial group variation could be masked.
Figure 2.5
Terminal Body Weights of Rats Following Daily Subcutaneous Administration of Cadmium

The figures presented are the means ± SEM (shown as bars) for saline- (∙) and cadmium- (○), 1.5mg Cd²⁺/kg/day, treated animals. The number of rats per treatment group at each time point is indicated and statistically significant differences between test and control groups are shown as ** p < 0.01, *** p < 0.001.
Therefore, individual results were carefully scrutinised and since this did not reveal any intergroup variation only pooled results are presented.

2.2.2.3 Necropsy Data - Organ Weights

Figure 2.6 shows effects of cadmium administration on the mean weights of liver, spleen, testes and kidneys relative to body weight, and Table 2.1 gives the mean absolute organ weights.

Liver: The relative liver weight was significantly increased in cadmium-treated animals at 10 days (p < 0.001) (Figure 2.6d) and reached 26% and 36% above control values 24h and 9 days respectively after administration of 20 doses. However, absolute liver weights were unchanged so the increase in relative weights may be partly attributable to the lower body weights of cadmium-treated animals.

Spleen: Relative spleen weight (Figure 2.6c) was significantly raised after 6 injections of cadmium and steadily increased in comparison to the gradual reduction observed in controls. By day 20 the control spleens were on average 0.23% of body weight compared to 0.44% in test animals. In terms of absolute weight cadmium caused the spleens to reach a peak of 1.8 times the weight of control spleens by 9 days after the 20th injection.

Kidneys: In controls, relative kidney weight fell slightly with time (Figure 2.6b) whereas cadmium administration resulted in a significant increase from day 6 (p < 0.01). There were no differences between groups in absolute kidney weights.

Testes: The relative weights of control testes decreased gradually with time (Figure 2.6a). There was a slight increase in both relative and absolute weights (p < 0.05) 24h after the first injection of cadmium which was followed by a marked reduction.
Figure 2.6
Relative Weights of Rat Testes (a), Kidneys (b), Spleen (c) and Liver (d) Following Daily Subcutaneous Administration of Cadmium

The figures presented are the means ± SEM (shown as bars) for saline- (●) and cadmium- (○), 1.5mg Cd²⁺/kg/day, treated animals. The number of samples per group is given in Table 2.1 and statistically significant differences between test and control groups are shown as * p < 0.05, ** p < 0.01, *** p < 0.001.
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<th>No. of Daily Doses</th>
<th>Dose of Cadmium (mg/kg/dy)</th>
<th>n</th>
<th>Liver (g)</th>
<th>Spleen (g)</th>
<th>Kidneys (g)</th>
<th>Testes (g)</th>
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<td>3.50 ± 0.24*</td>
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Table 2.1 The Effect of Daily Subcutaneous Administration of Cadmium on Absolute Weights of Rat Liver, Spleen, Kidneys and Testes

All values are means ± standard error of the mean (SEM) where n = the number of samples per group (number in brackets refers to n for testes). Statistically significant differences between test and control groups are shown as *p<0.05; **p<0.01; ***p<0.001.
By day 15 relative testes weight had fallen to 48% of control values \( (p < 0.001) \) and 9 days after dosing had ceased, the absolute weights of testes from cadmium-treated rats were less than half those of controls.

**Haematology**

2.2.2.4 **Haematocrit, Haemoglobin Concentration and Mean Cell Haemoglobin Concentration**

The mean haematocrit of control animals increased gradually from 41.6 to 46.0% over the course of the study (Figure 2.7b). A transient increase in both haematocrit \( (p < 0.05) \) and haemoglobin concentration (Figure 2.7a) was observed one day after the first injection of cadmium. After 6 injections there was a significantly reduced haematocrit \( (p < 0.001) \) and the lowest mean value of 36.3% was recorded after 10 injections. The mean control value at this time point was 41.6% and this represents a 13% drop provoked by cadmium administration. The haematocrit remained lowered at subsequent time points including 9 days after dosing had ceased.

Temporal variations in haemoglobin concentration of control blood followed a different pattern to haematocrit with a transient decrease after 3 and 6 injections of saline. A similar drop occurred in the blood of cadmium-treated animals but haemoglobin concentrations were significantly lower than controls from 6 days. This was consistent with the reduced haematocrit. Mean cell haemoglobin concentrations (Figure 2.8) reflected variations in the haemoglobin concentration but there were no statistically significant differences between treatment groups.

2.2.2.5 **Red Cell Count**

Red cell counts were only carried out on samples from animals dosed for up to 10 days (Study C) (Figure 2.9a). Individual counts ranged from 4.635 to 8.025 \( \times 10^{12} \) cells per litre blood for both control and cadmium-treated animals. There were no statistically significant differences between treatment groups at days 1, 3
Figure 2.7
Haematocrit (b) and Haemoglobin Concentration (a) of Rat Blood Following Daily Subcutaneous Administration of Cadmium

The figures presented are the means ± SEM (shown as bars) for saline- (●) and cadmium- (○), 1.5mg Cd²⁺/kg/day, treated animals. The number of samples per treatment group at each time point is indicated and statistically significant differences between test and control groups are shown as * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 2.8
Mean Cell Haemoglobin Concentration of Rat Blood Following Daily Subcutaneous Administration of Cadmium

The figures presented are means ± SEM (shown as bars) for saline- (●) and cadmium-(○), 1.5mg Cd²⁺/kg/day, treated animals. The number of samples per treatment group at each time point is as in Figure 2.7. There were no statistically significant differences between test and control groups at any time point.
Figure 2.9
Red Cell (a) and Reticulocyte (b + c) Counts of Rats Following Daily Subcutaneous Administration of Cadmium

The figures presented are means ± SEM (shown as bars) for saline- (●) and cadmium- (○), 1.5mg Cd^{2+}/kg/day, treated animals. The results for (a) and (b) were obtained from Study C. The number of samples per treatment group at each time point is indicated and statistically significant differences between test and control groups are shown as * p < 0.05.
or 6 but red cell counts from animals given 10 injections of cadmium were lower than those from controls \( (p < 0.05) \). Figure 2.9a shows that the control value at this time point was raised and although red cells in rats are known to increase in number with age, in view of the wide range of counts recorded and since the number of samples in this control group was only 4, the significance of this result is questionable.

2.2.2.6 Reticulocyte Count

Reticulocytes were counted in blood samples from Studies B and C and Figure 2.9c shows that the percentage of reticulocytes in controls declined with time. No significant differences were observed between treatment groups at any time point except 9 days after dosing was discontinued. At this time a marked reticulocytosis was seen in cadmium-treated animals which had a mean reticulocyte count of 4.61% compared to 2.03% in controls \( (p < 0.05) \). Absolute numbers of reticulocytes were calculated for Study C (Figure 2.9b) and there were no statistically significant differences between groups.

2.2.2.7 Red Cell Morphology

Whilst manual red cell counts were being undertaken, it was noticed that cells with irregularly shaped outlines were present in the dilutions of blood obtained from animals given cadmium, particularly for 6 and 10 days. This feature was also seen in reticulocyte films from 6 days onwards.

Examination of May-Grünewald-Giemsa stained blood films confirmed the presence of abnormalities in red cell morphology following cadmium treatment. Blood films prepared from control animals had a fairly uniform population of cells with some polychromasia and slight anisocytosis, which is not uncommon in rat blood (Figure 2.10). After one injection of cadmium red cells had a similar appearance to controls but from day 3 onwards there were increasing numbers of poikilocytes and
Figure 2.10

Red Cell Morphology in Films Prepared From Rat Blood After s.c. Administration of (a) Saline, (b) Cadmium (1.5mg Cd^{2+}/kg/day) for 6 Days and (c) Saline (b) Cadmium (1.5mg Cd^{2+}/kg/day) for 20 Days

Note: Lymphocyte, L; neutrophil, N; hypersegmented neutrophil, HN; examples of burr cells, B; polychromasia, P; poikilocytosis, Pk; and hypochromia, H.

Stain: May-Grunwald/Giemsa. Magnification: x1000 (a, b), x500 (c, d).
enhanced anisocytosis. Poikilocytes took the form of 'burr' cells especially at the earlier time points and there were also large numbers of cells of a variety of irregular shapes and cell fragments. The most marked poikilocytosis was seen 9 days after cessation of dosing for 20 days. Another feature seen in the films was hypochromasia of the cells and the presence of 'target' cells which became apparent after 6 doses of cadmium. Interestingly, the red cells of the 'recovery' group appeared to be less hypochromic than those in samples taken 24h after the 20th dose. There appeared to be slightly increased polychromasia in some cadmium-treated animals but this was not marked in the recovery group. There were no consistent trends but films from some cadmium-treated animals appeared to have more platelets some of which were of a larger size. Photographs of representative blood films displaying the features described are shown in Figure 2.10.

2.2.2.8 Red Cell Osmotic Fragility

Figure 2.11a, b and c show the mean osmotic fragility of blood after 3, 10 and 20 doses respectively. Similar curves to the 10 and 20 day time points were seen at the 6, 15 and 20 day+ 'recovery' groups and the percentage of lysis at each concentration of saline for all time points is given in Table 2.2. Figure 2.11 shows that cadmium administration caused the osmotic fragility curve to shift to the left. This was evident at day 3, and statistically significant from day 6 and indicates a reduced osmotic fragility, i.e., the cells are more resistant to lysis. The control values for Mean Corpuscular Fragility (Table 2.2), (i.e., the concentration of saline causing 50% lysis) were between 4.0 and 4.2g/l, and fall within the range of 4.0 to 4.45g/l quoted by Dacie and Lewis (1984) for human blood. Cadmium treatment resulted in a maximal reduction of Mean Corpuscular Fragility to 3.6g/l.

The significance of osmotic fragility tests (Dacie and Lewis, 1984) is as follows: The osmotic fragility of freshly taken red cells reflects their ability to take up water without lysis. This is determined by their volume to surface area.
Figure 2.11
(a) Red Cell Osmotic Fragility Curve of Rats Following Daily Subcutaneous Administration of Cadmium for Three Days
The figures presented are means ± SEM (shown as bars) for saline-(●) and cadmium-(○), 1.5mg Cd^{2+}/kg/day, treated animals. The number of samples per group was five and statistically significant differences between test and control groups are shown as * p < 0.05.
Figure 2.11
(b)  Red Cell Osmotic Fragility Curve of Rats Following
Daily Subcutaneous Administration of Cadmium for
10 Days

The figures presented are means ± SEM (shown as bars) for saline-(●) and
cadmium-(○), 1.5mg Cd\(^{2+}\)/kg/day, treated animals. The number of samples
per group was five and statistically significant differences between test and
control groups are shown as * p < 0.05, ** p < 0.01.
Figure 2.11
(c) Red Cell Osmotic Fragility Curve of Rats Following Daily Subcutaneous Administration of Cadmium for 20 Days

The figures presented are means ± SEM (shown as bars) for saline-(●) and cadmium-(○), 1.5mg Cd²⁺/kg/day, treated animals. The number of samples per group was five and statistically significant differences between test and control groups are shown as * p < 0.05, ** p < 0.01, *** p < 0.001.
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Table 2.2 The effect of Daily Subcutaneous Administration of Cadmium on the Osmotic Fragility of Rat Red Blood Cells

Results are expressed as % Lysis of red blood cells following the addition of blood to various concentrations of saline and Mean Corpuscular Fragility (MCF) i.e. the concentration of saline causing 50% lysis (g/L). All values are means ± standard error of the mean (SEM) where n = the number of samples per group. 1-10 day groups were from Study A. Statistically significant differences between test and control groups are shown as *p<0.05; **p<0.01; ***p<0.001.
The ability of the normal red cell to withstand hypotonicity results from its biconcave shape which allows the cell to increase its volume by some 70% before the surface membrane is stretched and once this limit is reached, lysis occurs. Spherocytes have an increased volume to surface area ratio. Their ability to take in water before stretching the surface membrane is thus more limited than normal and they are particularly susceptible to osmotic lysis. This results in a 'tail' of very fragile cells to the right of the curve.

Decreased osmotic fragility indicates the presence of unusually flattened red cells (leptocytes) in which the volume to surface area ratio is decreased. Such a change occurs in iron deficiency anaemia and the cells are unusually resistant to osmotic lysis. Reticulocytes also tend to have a greater amount of membrane compared with normal cells and are also osmotically resistant. The reduced osmotic fragility following exposure to cadmium is consistent with the raised numbers of hypochromic cells and poikilocytes which were observed in blood films and the presence of a population of more fragile cells is supported by the finding of cell fragments in blood films.

2.2.2.9 Total White Cell Count

Figure 2.12 shows that the mean total white cell counts of control animals increased slightly during the course of the study. A significant rise in the number of circulating white cells was evident 24h after administration of the first dose of cadmium ($p < 0.01$). After 3 injections a peak level of $13.20 \pm 1.05 \times 10^9/\text{l}$ blood was reached which represented a 236% increase over control values which were $5.6 \pm 0.73 \times 10^9/\text{l} (p < 0.001)$. The numbers of white cells then declined gradually but remained higher than controls throughout the dosing period. Nine days after the 20th injection of cadmium, although still slightly higher, there was no statistically significant difference in total white cell count from controls.
Figure 2.12
Total White Cell Count of Rats Following Daily Subcutaneous Administration of Cadmium

The figures presented are the means ± SEM (shown as bars) for saline-(●) and cadmium-(○), 1.5mg Cd²⁺/kg/day, treated animals. The number of samples per treatment group at each time point is indicated and statistically significant differences between test and control groups are shown as * p < 0.05, ** p < 0.01, *** p < 0.001.
2.2.2.10 Differential White Cell Count

The predominant white blood cell in the rat is the lymphocyte and Figure 2.13 and Table 2.3 shows that it constituted 71-79% of cells in control animals in this study with the percentage increasing marginally with time. This is in sharp contrast to humans where the neutrophil is the predominant cell. Mean neutrophil counts were between 17-24% in control rats and showed a slight decrease with time. A neutrophilia was invoked by cadmium administration which was sufficiently pronounced to completely reverse the usual lymphocyte:neutrophil ratio. This reversal persisted during the period of cadmium administration.

Figure 2.14 and Table 2.3 shows the counts expressed as absolute numbers of cells per litre blood. The numbers of lymphocytes increased quite markedly in both control and test groups towards the end of the study (Figure 2.14b). After 6, 10 and 15 injections of cadmium, lymphocytes were slightly reduced in numbers (p < 0.05) as compared to controls. Neutrophil numbers in controls fell slightly during the course of the study so the gradual rise in total white cells in these animals was attributable solely to elevated numbers of lymphocytes.

The higher total white cell counts of cadmium-treated animals was due to an intense neutrophilia which reached a peak at 3 days when there was a 7-fold increase in the numbers of circulating neutrophils over control values (p < 0.001). The neutrophil population remained appreciably higher over the course of the study but declined gradually, particularly during the recovery period. The increase in numbers of neutrophils was accompanied by hypersegmentation of their nuclei (Figure 2.10).

No significant differences were observed in numbers of eosinophils (Table 2.3 but, as shown in Figure 2.14a, monocytes rose in numbers with a marked peak at 3 days when again a 7-fold increase in number was seen as compared to control values (p < 0.001). Many of the cells which constituted this rise displayed multiply indented, atypically-shaped nuclei and a more basophilic cytoplasm characteristic of immature cells.
Figure 2.13
Percentage of Neutrophils and Lymphocytes in Peripheral Blood
White Cell Counts of Rats Following Daily Subcutaneous Administration of Cadmium

The figures presented are means ± SEM (shown as bars) for saline-(●) and cadmium-(○), 1.5mg Cd^{2+}/kg/day, treated animals. The number of samples per treatment group at each time point is indicated and statistically significant differences between test and control groups are shown as * p < 0.05, ** p < 0.01, *** p < 0.001.
| No. of Daily Doses | Dose of Cadmium (mg/kg/dy) | Neutrophils | | | Lymphocytes | | | Eosinophils | | | Monocytes | | |
|--------------------|---------------------------|-------------|---|---|-------------|---|---|-------------|---|---|-------------|---|
|                    |                            | % | Abs. No. | % | Abs. No. | % | Abs. No. | % | Abs. No. | % | Abs. No. |
| 1                  | 0                          | 24.3 ± 1.7 | 1.658 ± 0.167 | 70.9 ± 2.1 | 3.707 ± 0.345 | 1.0 ± 0.3 | 0.049 ± 0.014 | 3.8 ± 0.4 | 0.187 ± 0.021 |
|                    | 1.5                        | 50.8 ± 5.3*** | 6.153 ± 0.744*** | 46.0 ± 5.5*** | 3.536 ± 0.356 | 0.6 ± 0.1 | 0.059 ± 0.018 | 2.7 ± 0.4 | 0.255 ± 0.057 |
| 3                  | 0                          | 22.3 ± 1.5 | 1.234 ± 0.176 | 72.9 ± 1.7 | 4.103 ± 0.574 | 1.1 ± 0.4 | 0.057 ± 0.020 | 3.8 ± 0.5 | 0.126 ± 0.024 |
|                    | 1.5                        | 66.0 ± 1.7*** | 8.731 ± 0.783*** | 27.0 ± 1.8*** | 3.495 ± 0.312 | 0.6 ± 0.2 | 0.070 ± 0.025 | 6.5 ± 0.9* | 0.904 ± 0.181** |
| 6                  | 0                          | 23.6 ± 2.5 | 1.494 ± 0.166 | 70.9 ± 2.8 | 4.479 ± 0.413 | 1.2 ± 0.3 | 0.068 ± 0.017 | 4.3 ± 0.6 | 0.290 ± 0.058 |
|                    | 1.5                        | 69.1 ± 2.0*** | 8.234 ± 0.692*** | 27.4 ± 2.1*** | 3.190 ± 0.263* | 0.6 ± 0.2 | 0.062 ± 0.022 | 3.1 ± 0.6 | 0.378 ± 0.084 |
| 10                 | 0                          | 21.5 ± 1.3 | 1.405 ± 0.173 | 73.5 ± 1.4 | 3.676 ± 0.237 | 1.1 ± 0.2 | 0.055 ± 0.012 | 4.0 ± 0.3 | 0.198 ± 0.022 |
|                    | 1.5                        | 71.2 ± 1.3*** | 8.069 ± 0.515*** | 24.5 ± 1.4*** | 2.794 ± 0.253* | 0.7 ± 0.2 | 0.061 ± 0.014 | 3.6 ± 0.5 | 0.420 ± 0.058** |
| 15                 | 0                          | 18.2 ± 1.5 | 1.099 ± 0.087 | 77.2 ± 1.5 | 4.777 ± 0.513 | 1.8 ± 0.7 | 0.100 ± 0.033 | 2.8 ± 0.6 | 0.184 ± 0.055 |
|                    | 1.5                        | 71.6 ± 3.7*** | 7.926 ± 1.168*** | 23.8 ± 2.7*** | 2.582 ± 0.407* | 1.0 ± 0.6 | 0.124 ± 0.080 | 3.6 ± 0.8 | 0.398 ± 0.105 |
| 20                 | 0                          | 16.8 ± 3.9 | 1.168 ± 0.444 | 78.6 ± 3.5 | 5.078 ± 0.783 | 1.6 ± 0.4 | 0.092 ± 0.014 | 3.0 ± 0.6 | 0.203 ± 0.054 |
|                    | 1.5                        | 64.2 ± 1.9*** | 6.583 ± 0.386*** | 29.4 ± 2.7*** | 3.098 ± 0.525 | 2.0 ± 0.7 | 0.209 ± 0.080 | 4.4 ± 1.2 | 0.430 ± 0.127 |
| 20+ Recovery Period | 0                          | 18.7 ± 3.3 | 1.668 ± 0.165 | 77.0 ± 3.5 | 7.652 ± 2.025 | 1.3 ± 0.9 | 0.148 ± 0.122 | 3.0 ± 0.6 | 0.267 ± 0.033 |
|                    | 1.5                        | 37.7 ± 2.9 * | 4.609 ± 0.371** | 56.0 ± 2.1** | 6.974 ± 0.790 | 2.0 ± 1.2 | 0.217 ± 0.121 | 4.3 ± 1.5 | 0.567 ± 0.248 |

Table 2.3 The Effect of Daily Subcutaneous Administration of Cadmium on Differential White Cell Counts of Rats

Results are expressed as % of cells counted and as absolute numbers (Abs. No.) of cells x 10⁷/L blood. All values are means ± standard error of the mean (SEM) where n = the number of samples per group. Statistically significant differences between test and control groups are shown as *p<0.05; **p<0.01; ***p<0.001.
Figure 2.14
Absolute Monocyte (a) and Neutrophil, Lymphocyte (b) Counts in
Peripheral Blood of Rats Following Subcutaneous Administration
of Cadmium
The figures presented are means ± SEM (shown as bars) for saline-(•) and
cadmium-(○), 1.5mg Cd^{2+}/kg/day, treated animals. The number of samples
per group is shown in Table 2.3 and statistically significant differences
between test and control groups are shown as * p < 0.05, ** p < 0.01, *** p <
0.001.
2.2.2.11 Neutrophil Alkaline Phosphatase

It was discovered that the blue precipitate which formed at the site of enzyme activity rapidly faded with time and in retrospect examination of the films to score the activity should have been carried out as soon as possible, within 1-2 days. As a result, films prepared from 3 day samples were not readable and the variability in mean cell scores of control samples (Figure 2.15) may have been attributable to differential fading. Any conclusions derived from these results can therefore only be tentative but since the pattern of variation was similar in both treatment groups, a comparison was made. The results presented in Figure 2.15 indicate that cadmium administration appears to increase neutrophil alkaline phosphatase activity and the difference between controls and test groups was statistically significant at 10, 15 (p < 0.01) and 20 days (p < 0.05).

2.2.2.12 Differential Bone Marrow Examination

On splitting open the femur to prepare bone marrow smears it was noticed that the marrow from cadmium-treated animals had a brown colouration, particularly noticeable after 10, 15 and 20 injections, as compared to the normal deep red colour of control marrows. After the recovery period, the colour had returned to normal. Examination of H + E sections of sterna at low magnification revealed that marrow occupied the whole of the sternal cavity, with only an occasional fat space and no obvious difference in the cellularity was seen between groups. At higher magnification however, it was clear that cadmium treatment resulted in a predominance of myelopoietic cells. In some of these marrows the medullary pulp sinususes were dilated and appeared to be more numerous. This is probably a function of increased blood flow although sinuses seemed to contain fewer red cells, but often large numbers of polymorphonuclear cells. After the 'recovery' period increased numbers of erythropoietic cells were present.

These observations were verified by a quantitative assessment of cell types in bone marrow smears. The results presented in Figures 2.16 to 2.18 and Tables 2.4
Figure 2.15

Neutrophil Alkaline Phosphatase Activity in Rat Blood Following Daily Subcutaneous Administration of Cadmium

The figures presented are the means ± SEM (shown as bars) for saline-(●) and cadmium-(○), 1.5mg Cd²⁺/kg/day, treated animals (Studies A and B). The number of samples per treatment group at each time point is indicated and statistically significant differences between test and control groups are shown as * p < 0.05, ** p < 0.01.
and 2.5 fall within acceptable ranges obtained by reputable techniques (D. E. Hall, personal communication, 1987). The M:E ratio of myeloid (neutrophilic to nucleated erythroid cells in control marrows remained relatively unchanged with mean values between 0.5 to 1.0:1.0. The ratio was increased to a maximum of $2.8 \pm 0.1:1.0$ after six injections of cadmium compared with $0.6 \pm 0.3:1.0$ for controls ($p<0.001$) (Figure 2.16d). The significance of raised M:E ratios is difficult to evaluate since it may reflect either increased numbers of myeloid cells or reduced numbers of erythroid cells or a combination of both. However, taking into account the elevated numbers of circulating neutrophils together with differential changes in the numbers of immature and adult myeloid cells in the marrow with time (Figure 2.17c and d) and the increased myeloid mitotic index (Figure 2.16b), it can be deduced that the raised ratio is primarily due to enhanced myeloid activity.

The M:E ratio was slightly reduced after one day due to a non-significant reduction in the percentage of adult neutrophils. These cells remained slightly reduced in number after three days whilst the M:E ratio was marginally above control levels due to a significant rise in the percentage of myelocytes ($p<0.01$) (Figure 2.17c). By day 6 the high M:E ratio was mainly attributable to raised numbers of neutrophils and the ratio remained elevated during the dosing period due to increases in all stages of myeloid maturation. The raised mitotic index (Figure 2.16c) during the period of cadmium administration was due to myeloid mitotic activity (Figure 2.16b) which showed a maximum of $1.7 \pm 0.1\%$ at 3 days compared to $0.8 \pm 0.1\%$ in controls. Nine days after the cessation of dosing enhanced erythroid activity contributed to the mitotic index (Figure 2.16a). The percentages of both erythroblasts and normoblasts were consistently lower than controls after 6 to 20 injections of cadmium but returned to slightly above control levels after the 'recovery' period.

The percentage of lymphocytes increased gradually in control marrows towards the end of the study (Figure 2.18b). Cadmium treatment caused a reduction at 3 days which was significant after 6 days ($p<0.01$). In contrast, the eosinophilic cells were increased after 15 and 20 injections of cadmium ($p<0.01$) (Figure 2.18a). No
Figure 2.16
Erythroid (a), Myeloid (b) and Total (c) Mitotic Index and Ratio of Myeloid to Erythroid Cells (d) in Rat Bone Marrow Following Daily Subcutaneous Administration of Cadmium.

The figures presented are the means ± SEM (shown as bars) for saline-(●) and cadmium-(○), 1.5mg Cd²⁺/kg/day, treated animals (Studies A and B). The number of samples per treatment group at each time point is indicated and statistically significant differences between test and control groups are shown as * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 2.17

Erythroid Cell Counts:- Erythroblasts (a), Normoblasts (b) and
Myeloid Cell Counts:- Myelocytes (c), Metamyelocytes (d) and
Neutrophils (e) in Rat Bone Marrow Following Daily Subcutaneous
Administration of Cadmium

The figures presented are the means ± SEM (shown as bars) for saline-( ● ) and cadmium-( ○ ), 1.5mg Cd²⁺/kg/day, treated animals (Studies A and B). The number of samples per group is shown in Table 2.4 and statistically significant differences between test and control groups are shown as * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 2.18
Eosinophilic (a) and Lymphocytic (b) Cell Counts in Rat Bone Marrow Following Daily Subcutaneous Administration of Cadmium

The figures presented are the means ± SEM (shown as bars) for saline-(●) and cadmium-(○), 1.5mg Cd²⁺/kg/day, treated animals (Studies A and B). The numbers of samples per group at each time point are indicated and statistically significant differences between test and control groups are shown as *p < 0.05, **p < 0.01.
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</table>

Table 2.4 The Effect of Daily Subcutaneous Administration of Cadmium on Differential Bone Marrow Cell Counts of Rats

Results are expressed as % of cells counted except M:E (1.0) which is the ratio of myeloid to unit erythroid cells. All values are means ± standard error of the mean (SEM) where n = the number of samples per group. Statistically significant differences between test and control groups are shown as *p<0.05; **p<0.01; ***p<0.001.
<table>
<thead>
<tr>
<th>No. of Daily Doses</th>
<th>Dose of Cadmium mg/kg/dy</th>
<th>n</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
<th>Basophils</th>
<th>Eosinophils</th>
<th>Plasma Cells</th>
<th>Unclassified Stem Cells</th>
<th>Total</th>
<th>Myeloid</th>
<th>Erythroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.5</td>
<td>5</td>
<td>2.0 ± 0.6</td>
<td>3.0 ± 0.4</td>
<td>1.4 ± 0.2</td>
<td>4.0 ± 0.4</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>1</td>
<td>1.5</td>
<td>5</td>
<td>2.0 ± 0.6</td>
<td>2.8 ± 0.4</td>
<td>1.1 ± 0.2</td>
<td>3.7 ± 0.3</td>
<td>0.3 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>0</td>
<td>1.5</td>
<td>5</td>
<td>2.9 ± 0.7</td>
<td>3.4 ± 0.6</td>
<td>0.9 ± 0.1</td>
<td>3.3 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>0.8 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>5</td>
<td>1.6 ± 0.4</td>
<td>3.6 ± 0.5</td>
<td>1.1 ± 0.2</td>
<td>3.7 ± 0.5</td>
<td>0.3 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>2.3 ± 0.1**</td>
<td>1.7 ± 0.1***</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>6</td>
<td>1.5</td>
<td>5</td>
<td>2.4 ± 0.3</td>
<td>3.1 ± 0.2</td>
<td>0.9 ± 0.4</td>
<td>4.6 ± 0.4</td>
<td>0.2 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>10</td>
<td>1.5</td>
<td>10</td>
<td>2.7 ± 0.5</td>
<td>3.4 ± 0.3</td>
<td>0.8 ± 0.1</td>
<td>3.6 ± 0.3</td>
<td>0.4 ± 0.0</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>15</td>
<td>1.5</td>
<td>5</td>
<td>2.0 ± 0.3</td>
<td>2.6 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>3.9 ± 0.4</td>
<td>0.3 ± 0.4</td>
<td>0.6 ± 0.0</td>
<td>0.8 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>20</td>
<td>1.5</td>
<td>5</td>
<td>2.8 ± 0.5</td>
<td>3.2 ± 0.2</td>
<td>1.1 ± 0.1</td>
<td>3.1 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>20+ Recovery Period</td>
<td>1.5</td>
<td>3</td>
<td>4.9 ± 0.7</td>
<td>3.6 ± 1.1</td>
<td>1.1 ± 0.1</td>
<td>2.8 ± 0.6</td>
<td>0.2 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>0.3 ± 0.0</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>3</td>
<td>3.0 ± 0.8</td>
<td>3.7 ± 0.5</td>
<td>1.1 ± 0.3</td>
<td>3.9 ± 0.8</td>
<td>0.5 ± 0.1</td>
<td>0.8 ± 0.2</td>
<td>1.6 ± 0.2</td>
<td>0.2 ± 0.0</td>
<td>1.2 ± 0.2</td>
</tr>
</tbody>
</table>

Table 2.5 The effect of Daily Subcutaneous Administration of Cadmium on Differential Bone Marrow Cell Counts of Rats

Results are expressed as % of cells counted. All values are means ± standard error of the mean (SEM) where n = the number of samples per group. Statistically significant differences between test and control groups are shown as *p<0.05; **p<0.01; ***p<0.001
changes were seen either with time or with treatment in the numbers of plasma cells, basophils, monocytes or megakaryocytes (Table 2.5).

**Biochemistry**

2.2.2.13 **Total Plasma Protein**

Total plasma protein results are shown in Figure 2.19 and it can be seen that the SEM's were high for many of the mean values, both in control and treated groups. It is not known whether this reflects interindividual variations in plasma protein concentration or experimental error, a possible source of error being the effect of storage of samples at -20°C.

The results suggest a slight transient, though not statistically significant, increase in concentration of plasma protein 24h after the first injection, followed by a decrease which became significant at 6 (p < 0.01) and 10 (p < 0.05) days. There was little difference between treatment groups 9 days after the 20th injection.

2.2.2.14 **Serum Protein Analysis**

Figures 2.20 and 2.21 show the patterns obtained when rat serum proteins were fractionated by X-IEP. Precipitation peaks were numbered according to the arbitrarily designated code adopted by Hinton et al., (1985) which takes into account the approximate positions of \( \alpha_1, \alpha_2 \) and \( \beta \) globulins. \( P1 \) and \( P2 \) are proteins with more mobility than albumin. The correlation of the peaks with those produced by other workers (see Methods Section) was limited by variations in the electrophoretic mobilities of some proteins between different rat strains and individuals of rat populations. Additionally, quantitative variations in the titre of antibody to different proteins between different batches of antisera caused some difficulty in correlating some of the minor proteins. Nevertheless, the characteristic appearance of the precipitation outlines formed by some proteins made them readily distinguishable; for example, the assymetrical shape of \( \alpha_1.7 \). Therefore a number of the major proteins could be identified.
Figure 2.19
Total Plasma Protein Concentration of Rats Following Daily Subcutaneous Administration of Cadmium

The figures presented are means ± SEM (shown as bars) for saline-(•) and cadmium-(○), 1.5mg Cd²⁺/kg/day, treated animals. The number of samples per group at each time point is indicated and statistically significant differences between test and control groups are shown as * p < 0.05, ** p < 0.01.
Figure 2.20
Pattern Obtained After Crossed Immunelectrophoresis of Rat Serum
24h After a Single Subcutaneous Administration of (a) Saline and
(b) Cadmium, 1.5mg Cd^{2+}/Kg
Serum proteins were fractionated by X-IEP of 5μl of a 1 in 20 dilution of rat
serum (in 0.9% NaCl) into a 1% agarose gel containing 5% rabbit anti-rat
serum.
Figure 2.21
Pattern Obtained After Crossed Immunoelectrophoresis of Rat Serum Following Daily Subcutaneous Administration of (a) Saline and (b) Cadmium, 1.5mg Cd^{2+}/kg/Day for Three Days
Samples were taken 24h after the final dose. Serum proteins were fractionated by X-IEP of 5μl of a 1 in 20 dilution of rat serum (in 0.9% NaCl) into a 1% agarose gel containing 5% rabbit anti-rat serum.
In control serum, it was possible to detect 24 distinct proteins and an additional six proteins were detected in sera from cadmium-treated animals. These were designated \( \alpha_1 A \); \( \alpha_2 A \) to D and \( \beta A \) and made correlation of some of the minor proteins difficult. For instance, \( \alpha_2.8 \) could not be readily recognised in serum from cadmium-treated animals due to the presence of two proteins with similar electrophoretic mobility, \( \alpha_2C \) and \( \alpha_2D \).

Statistical analysis could not be applied to the results due to the small number of samples but a visual comparison of the patterns obtained revealed marked treatment-related changes in the concentrations of a large number of the proteins detected which were quantified by expressing the area enclosed by the precipitate as a percentage of the appropriate control values (Figure 2.22, Table 2.6). In total, 17 proteins reacted with an increase in concentration whilst nine were decreased. The most dramatic increases which were exhibited at both time points were by \( \alpha_1.7 \) (\( \alpha_1 \) acid glycoprotein) and \( \alpha_2.4 \) which, after three injections of cadmium, reached 749% and 529% of control concentrations respectively. \( \alpha_2B \), which was not detected in control serum, also increased markedly. Less pronounced increases of up to about 2-fold and not always apparent after the first injection of cadmium were seen with proteins \( \alpha_1.1 \), \( \alpha_1.4 \) (caeruloplasmin), \( \alpha_1.5 \), \( \alpha_1.8 \), \( \alpha_2.2 \) and \( \alpha_2.5 \) (haptoglobin), \( \alpha_2.6 \), \( \alpha_2.7 \) (haemopexin), \( \beta_2 \) and \( \beta_3^1 \). In contrast, the concentrations of P2, Alb, \( \alpha_1.3 \), \( \alpha_1.6 \), \( \alpha_1.9 \) and \( \alpha_2.1 \) were all reduced at both time points whilst \( \alpha_2.3 \) was slightly reduced only at day 3 and \( \beta_3^2 \) and \( \beta_7 \) (transferrin) only at day 1. \( \beta_5 \) was not readily measurable due to its flattened profile but appeared to be reduced after three injections.

The serum concentration of only two proteins P1 (high density lipoprotein) and \( \alpha_1.2 \) (\( \alpha_1 \) macroglobulin), remained relatively unchanged. Proteins may exhibit qualitative as well as quantitative variations under pathological conditions giving rise to subtle changes in precipitate shape or substantial shifts in electrophoretic mobility (Emmett and Crowle, 1982). P1 displayed an altered shape after cadmium treatment characterised by a reduced anodic form. A reaction of partial identity gives the characteristic double loop of \( \beta_3 \) which was differentially altered in size following
Figure 2.22

Changes in the Concentration of Rat Serum Proteins Following Daily Subcutaneous Administration of Cadmium for One or Three Days

The concentrations were estimated from the areas under the precipitation peaks formed by X-IEP and expressed as a percentage of control (saline treated animal) values. (n = 1 per treatment group per time point).
Table 2.6 Quantitative Changes in Rat Serum Protein Concentrations following Daily Subcutaneous Administration of Cadmium for 1 or 3 Days

(a) Protein numbers shown on figures 2.20 and 2.21 (code according to Hinton et. al., 1985)

(b) Protein numbers according to Abd-el-Fattah et. al. (1976)

(c) By reference to published protein patterns, a tentative identification of proteins was made (as described in methods and results section). Ref (i) R.H. Hinton (person. commun.); (ii) Abd-el-Fattah et. al. (1976) and Scherer et. al. (1977); (iii) Schade & Burger (1979); (iv) Emmett et. al. (1984)

(d) The concentration of each serum protein from cadmium (1.5 mg Cd^{2+}/kg/dy) treated rats (estimated from the area under each precipitate) was expressed as percentage of the concentration of the same protein in the corresponding control sample (n=1 per treatment group per time point).

(e) Refs: Billingham & Gordon (1976); Gauldie et. al. (1985); Whicher (1984).

(f) Precipitation peaks numbered α1A, α2A to D and βA were detected only in sera from cadmium treated animals (Either α2C or α2D may correspond to α2.8 which would therefore have shown an increase (↑) in concentration)
<table>
<thead>
<tr>
<th>Protein No. (a)</th>
<th>Protein No. (b)</th>
<th>Identity (c) Refs. (i-iv)</th>
<th>% of Control (d) 1 day 3 days</th>
<th>Function (e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>1</td>
<td>High density Lipoprotein(i)</td>
<td>109 96 Transport</td>
<td></td>
</tr>
<tr>
<td>A1b</td>
<td>2</td>
<td>Albumin (i-iv)</td>
<td>75 64 General transport</td>
<td></td>
</tr>
<tr>
<td>α1.1</td>
<td>3</td>
<td></td>
<td>91 130</td>
<td></td>
</tr>
<tr>
<td>α1.2</td>
<td>5</td>
<td>α1-macroglobulin(ii,iii)</td>
<td>97 109</td>
<td></td>
</tr>
<tr>
<td>α1.3</td>
<td></td>
<td></td>
<td>80 58</td>
<td></td>
</tr>
<tr>
<td>(α1A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α1.4</td>
<td>11</td>
<td>Caeruloplasmin (ii)</td>
<td>116 189 (\text{O}_2)-scavenger; (\text{Cu}) transport ferroxidase activity</td>
<td></td>
</tr>
<tr>
<td>α1.5</td>
<td>8</td>
<td></td>
<td>87 184</td>
<td></td>
</tr>
<tr>
<td>α1.6</td>
<td>12</td>
<td></td>
<td>64 74</td>
<td></td>
</tr>
<tr>
<td>α1.7</td>
<td>7</td>
<td>α1-acid-glycoprotein (ii)</td>
<td>209 749 Immune regulation; repair and resolution</td>
<td></td>
</tr>
<tr>
<td>α1.8</td>
<td>14</td>
<td></td>
<td>96 131</td>
<td></td>
</tr>
<tr>
<td>α1.9</td>
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<td></td>
<td>71 40</td>
<td></td>
</tr>
<tr>
<td>α2.1</td>
<td>16</td>
<td></td>
<td>81 76</td>
<td></td>
</tr>
<tr>
<td>α2.2</td>
<td>19</td>
<td></td>
<td>98 144</td>
<td></td>
</tr>
<tr>
<td>α2.3</td>
<td></td>
<td></td>
<td>103 80</td>
<td></td>
</tr>
<tr>
<td>(α2A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α2.4</td>
<td>15</td>
<td>Haptoglobin (i,ii)</td>
<td>206 529 Binds and conserves haemoglobin</td>
<td></td>
</tr>
<tr>
<td>α2.5</td>
<td>17</td>
<td></td>
<td>133 182</td>
<td></td>
</tr>
<tr>
<td>α2.6</td>
<td></td>
<td></td>
<td>125 160</td>
<td></td>
</tr>
<tr>
<td>α2.7</td>
<td>20</td>
<td>Haemopexin (ii,iii,iv)</td>
<td>107 172 Binds and conserves haem</td>
<td></td>
</tr>
<tr>
<td>α2.8</td>
<td>22</td>
<td>Fibronectin (iv)</td>
<td>↑ (see f) Inhibitor of exudation and PMNL chemotaxis</td>
<td></td>
</tr>
<tr>
<td>(α2B)</td>
<td></td>
<td>α2-macro(feto)globulin(iii)</td>
<td>↑ (see f)</td>
<td></td>
</tr>
<tr>
<td>(α2C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(α2D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β2</td>
<td>24</td>
<td></td>
<td>111 218</td>
<td></td>
</tr>
<tr>
<td>β3(^1)</td>
<td>23β1A</td>
<td>C3 Complement component (ii,iv)</td>
<td>133 175 Opsonisation</td>
<td></td>
</tr>
<tr>
<td>β3(^2)</td>
<td>27β1C</td>
<td>C3 Complement component (ii,iv)</td>
<td>86 108 chemotaxis</td>
<td></td>
</tr>
<tr>
<td>β5</td>
<td></td>
<td>IgG (iii,iv)</td>
<td>70 101 Antibodies</td>
<td></td>
</tr>
<tr>
<td>β7</td>
<td>26</td>
<td>Transferrin (ii,iii)</td>
<td>70 101 Iron transport</td>
<td></td>
</tr>
<tr>
<td>(βA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
cadmium treatment. This probably represents conversion of $B_1^C$ to $B_1^A$ which can be used to monitor C3 complement activation (Scherer et al., 1977).

2.2.2.15 Plasma Haptoglobin

Figure 2.3a shows some of the rockets formed by immunoelectrophoresis of both individual and pooled plasma samples from each treatment group. The height of the rockets produced from pooled samples was equal to the mean height of rockets from individual samples. Therefore samples from Study B were pooled since only a limited quantity of antisera was available. Figure 2.3a also shows that haptoglobin from control samples precipitated with antibody in the gel to form well peaked rockets with sharp outlines. In contrast, the concentration of haptoglobin in plasma from cadmium-treated rats appeared to be high enough to prevent complete formation of precipitate. Antigen excess may result in a certain amount of diffusion into the gel and this may have been the cause of the wider and less distinct rockets. Provided sufficient antibody was present in the gel the rockets may have become more distinct with continued electrophoresis.

The concentrations of haptoglobin in control samples were similar throughout the study except for a higher value obtained for pooled controls at day 15 (Figure 2.23b). It can only be postulated that this maveric result was due to either an unusually high concentration in one of the samples or an error in sampling. Cadmium treatment resulted in a significant rise in haptoglobin concentration after 24h ($p < 0.01$) and by three days concentrations were 168% of those seen in controls ($p < 0.001$) (Table 2.7). Higher concentrations persisted throughout the study and although they declined to 77% of maximal levels after the 'recovery' period they were still appreciably higher than in controls.

2.2.2.16 Plasma Caeruloplasmin

Plasma derived from cadmium-treated animals had a blue colouration as compared to the normal straw colour of control plasma and this is indicative of the
(a) Example of rockets formed by immunoelectrophoresis of individual and pooled (n = 5) plasma samples from rats given saline (S) or cadmium (Cd), 1.5mg Cd^{2+}/kg/day, for six days (Study A).

(b) The figures presented are means ± SEM (shown as bars) for animals given saline-(●) or cadmium-(○), 1.5mg Cd^{2+}/kg/day for up to 10 days (Study A). From 15 days the points show the height of rockets formed from pooled plasma samples (Study B). The number of samples per group at each time point is indicated and statistically significant differences between test and control groups are shown as ** p < 0.01, *** p < 0.001.
<table>
<thead>
<tr>
<th>No. of Daily Doses</th>
<th>Dose of Cadmium mg/kg/dy</th>
<th>Plasma Haptoglobin</th>
<th>Plasma Caeruloplasmin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rocket Height (cm)</td>
<td>n</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>2.74 ± 0.14</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>3.48 ± 0.12**</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>2.85 ± 0.12</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>4.80 ± 0.15***</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>2.87 ± 0.19</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>4.41 ± 0.06***</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>2.90 ± 0.07</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>4.64 ± 0.09***</td>
<td>10</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>4.40</td>
<td>5 pooled</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>5.20</td>
<td>5 pooled</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
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<td>5 pooled</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>5.00</td>
<td>5 pooled</td>
</tr>
<tr>
<td>20+ Recovery Period</td>
<td>0</td>
<td>3.25</td>
<td>3 pooled</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>4.00</td>
<td>3 pooled</td>
</tr>
</tbody>
</table>

Table 2.7 The Effect of Daily Subcutaneous Administration of Cadmium on Concentrations of Haptoglobin and Caeruloplasmin in Rat Plasma

Results are expressed as the height in cm of rockets formed by immunoelectrophoresis for haptoglobin and the concentration of caeruloplasmin in g/L. All values are means ± standard error of the mean (SEM) except for the haptoglobin results following 15 and 20 doses where plasma samples were pooled prior to analysis. In all cases n = the number of samples per group. Statistically significant differences between test and control groups are shown as **p<0.01; ***p<0.001. Results are also presented as percentage of control values at each time point.
Caeruloplasmin concentration in plasma from animals dosed for 3 and 10 days is shown in Table 2.7. The mean concentration after 3 days exposure to cadmium was double that in control plasma \( (p < 0.001) \) and by day 10 levels of caeruloplasmin were elevated 4-fold \( (p < 0.001) \).

### 2.2.2.17 Plasma Iron and Total Iron-Binding Capacity

Results of the measurement of plasma iron concentration and total iron-binding capacity (TIBC) are shown in Figure 2.24b and percentage saturation of TIBC with iron in Figure 2.24a. Control animals displayed a gradual decline in plasma iron concentration with time from \( 46.12 \pm 4.84 \mu \text{mol/l} \) at day 1 to \( 33.17 \pm 0.70 \mu \text{mol/l} \) by the end of the study. This was paralleled by a slight rise in TIBC from \( 62.00 \pm 2.40 \mu \text{mol/l} \) to a maximum of \( 76.34 \pm 1.80 \mu \text{mol/l} \) at day 20. This represented a steady decrease in the percentage saturation of TIBC with iron from 74 to 45%. Cadmium treatment produced an immediate and marked drop in plasma iron concentration with a mean value of \( 30.36 \pm 3.45 \mu \text{mol/l} \) which was 34% lower than controls \( (p < 0.05) \) 24h after the first injection. By 3 days, the levels had fallen to 44% of control values \( (p < 0.001) \) and this was accompanied by a slightly reduced TIBC. Plasma iron concentration continued to decrease to a minimum mean value of \( 16.18 \pm 1.17 \mu \text{mol/l} \) at 10 days which was only 40% of controls and equivalent to only 26% saturation of the TIBC with iron compared to 66% seen in controls. By day 20 iron concentration in the plasma was on the increase and following the recovery period had returned to control levels. This rise coincided with enhanced TIBC which became significantly higher than controls \( (p < 0.05) \) 9 days after the last injection of cadmium.

### 2.2.2.18 Tissue Metal Analysis

#### Cadmium

Cadmium concentration in the liver and spleen was determined at all time points, in the kidney and intestine after 10 to 20 injections and in femoral marrow at 3 and 10 days. Results were expressed both as concentrations of cadmium in \( \mu \text{mol/g wet} \)
Figure 2.24

**Plasma Iron Concentration, Total Iron-Binding Capacity, TIBC (b) and Percentage Saturation of TIBC with Iron (a) of Rats Following Daily Subcutaneous Administration of Cadmium**

The figures presented are means ± SEM (shown as bars) for plasma iron concentration (●, ○) and TIBC (■, □). The mean value of each of these results was used to calculate the percentage saturation of TIBC with Fe (▲, △) for saline-(●, △) and cadmium-(■, □) 1.5 mg Cd²⁺/kg/day, treated animals. The number of samples per group at each time point is indicated (number in brackets refer to n for plasma iron concentration). Statistically significant differences between test and control groups are shown as * p < 0.05, ** p < 0.01, *** p < 0.001.
weight of tissue (Figure 2.25) and as tissue content in μmol (Table 2.8). Table 2.8 also shows the cadmium content of tissues expressed as a percentage of the total administered dose including estimates for the intestine and marrow based on the relative weights indicated in the Legend.

In all tissues from control animals cadmium levels were below the limits of detection. Repeated administration of cadmium caused the accumulation of considerable amounts of the metal in the liver and kidney with the liver containing 34 times as much cadmium as did both kidneys together after 15 injections. This represented 68.7% of the total administered dose compared to 2.0% in the kidneys.

The spleen and intestine similarly accumulated cadmium with time but by day 15 the spleen retained only 0.3% of the total dose at a concentration which was 4.8% of that in the liver. The concentration of cadmium in the small intestine (jejunum) was about half that in the spleen but an estimate of the total content from the pyloric junction to the ileal-caecal junction suggests that nearly 1% of the total administered dose may be present in this organ. This therefore represents about half the amount retained in the kidneys. However, the estimate assumes an even distribution of cadmium along the length of the small intestine which may be an oversimplification of the situation. Cadmium was also detectable in the femoral marrow at a concentration comparable to that found in the spleen after 3 injections, and slightly raised after 10 doses. Again, an estimate of total body marrow cadmium, assuming an even distribution of the metal, suggests that between 1 and 2% of the total dose is present in this tissue.

The cadmium concentration declined in liver, spleen and intestine after dosing ceased, but Table 2.8 emphasises the importance of calculating organ content since it shows that the metal continued to accumulate in the liver, spleen and kidneys. In contrast, the figures presented in Table 2.8 suggest that cadmium was gradually lost from the intestine.
Figure 2.25
Cadmium Concentration in Rat Liver, Kidney, Spleen, Small Intestine and Bone Marrow Following Daily Subcutaneous Administration of Cadmium
(a) Liver (△), kidney (▲) and Spleen (■)
(b) Spleen (■), Intestine (□) and Bone Marrow (○)
The figures presented are means ± SEM (shown as bars) for cadmium, 1.5mg Cd²⁺/kg/day treated animals only, since the metal was not detected in tissues from control rats. The number of samples per group is shown in Table 2.8.
<table>
<thead>
<tr>
<th>No of Daily Doses</th>
<th>Total Administered Dose of Cadmium (μmol)</th>
<th>n</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidneys</th>
<th>Intestine (estimate)</th>
<th>Bone Marrow (estimate)</th>
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<td></td>
<td></td>
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<td>μmol</td>
<td>μmol</td>
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<td>1.36 ± 0.08</td>
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<td>0.008 ± 0.001</td>
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<td>3</td>
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<td>45.6</td>
<td>0.023 ± 0.003</td>
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<td>-</td>
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<td>0.051 ± 0.007</td>
<td>0.26</td>
<td>-</td>
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<td>10</td>
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<td>5 (10)</td>
<td>22.96 ± 1.01</td>
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<td>20+ Recovery Period</td>
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<td>58.1</td>
<td>0.233 ± 0.016</td>
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Table 2.8 The Effect of Daily Subcutaneous Administration of Cadmium on the Content of Cadmium in Rat Liver, Spleen, Kidney, Small Intestine and Bone Marrow

Results are expressed as content in μmol and as % of the total administered dose and are presented for cadmium-treated animals only since cadmium was not detected in organs from saline-treated animals.

(a) The cadmium content of the small intestine was estimated from the concentration by using a value of 2.6% as the relative weight of an empty intestine from the pyloric to the ileo-caecal junction.

(b) The estimate of total marrow cadmium was based on a value of 3% as the percentage of bodyweight that marrow constitutes (Cochin & Roe, 1967).

Content values (μmol) are means ± standard error of the mean (SEM) where n = the number of samples per group (number in brackets refers to n for liver and spleen).
Copper

Copper levels were only determined in the liver and Figure 2.26 shows the results expressed both as concentration (μmol/g) and content (μmol). Significantly elevated copper concentrations were seen after 6 (p < 0.01) 10 and 15 (p < 0.001) doses of cadmium and statistical significance was reached at 10 days in the copper content of the organ. The liver copper content returned to control levels 24h after the last dose but interestingly there was a marked elevation 8 days later when cadmium treated animals were found to have twice as much liver copper as compared to controls.

Iron

Figure 2.27 shows the effect of cadmium administration on iron concentration in liver and spleen at all time points, in kidney and intestine from 10 days and the results for marrow after 3 and 10 doses are included in Table 2.9. The results for liver, spleen and kidney iron content are also shown in Table 2.9.

The administration of cadmium had no statistically significant effect on the concentration of iron in intestine, marrow or spleen although intestinal iron concentration was consistently lower in cadmium treated animals as was marrow iron, especially after 10 days. However, marrow, liver and particularly spleen showed marked individual variations in concentrations of iron probably attributable to the high blood content and iron storage capacities of these tissues. Less variable results may have been attained by perfusion of organs prior to sampling.

The kidney displayed a marked drop in iron concentration and content following exposure to cadmium in comparison to the increase with time shown by control kidneys. These changes persisted into the 'recovery' period. By day 20 the cadmium-treated animals had kidney iron contents which were 52% of those seen in controls (p < 0.001). In contrast, after 20 doses of cadmium the liver had 57% higher concentrations of iron than controls (p < 0.01).
Figure 2.26
Copper Content (a) and Concentration (b) in Rat Liver Following Daily Subcutaneous Administration of Cadmium

The figures presented are means ± SEM (shown as bars) for saline-(•) and cadmium-(○), 1.5mg Cd²⁺/kg/day, treated animals (Studies C and B). The number of samples per treatment group at each time point is indicated and statistically significant differences between test and control groups are shown as ** p < 0.01, *** p < 0.001.
Figure 2.27
Iron Concentration in Rat Liver, Kidney, Small Intestine and Spleen Following Daily Subcutaneous Administration of Cadmium
(a) Liver (○, ○), Kidney (■, ○), Intestine (▲, △)
(b) Spleen (▼, ○)
The figures presented are means ± SEM (shown as bars) for saline-(○, ■, ▼, △) and cadmium-(○, ■, ▼, △) 1.5mg Cd²⁺/kg/day, treated animals. The number of samples per group is shown in Table 2.9 and statistically significant differences between test and control groups are shown as ** p < 0.01.
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<th>No. of Daily Doses</th>
<th>Dose of Cadmium mg/kg/dy</th>
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<th>Liver $\mu$mol/g</th>
<th>Spleen $\mu$mol</th>
<th>Kidneys $\mu$mol/g</th>
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<th>Bone Marrow $\mu$mol/g</th>
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<td></td>
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<td>4.95±0.98</td>
<td>0.64±0.02**</td>
<td>1.32±0.00**</td>
</tr>
</tbody>
</table>

Table 2.9  The Effect of Daily Subcutaneous Administration of Cadmium on the Concentration and Content of Iron in Rat Liver, Spleen, Kidney, Intestine and Bone Marrow

Results are expressed as concentration in $\mu$mol/g wet weight of tissue and as content in $\mu$mol. All values are means ± standard error of the mean (SEM) where n = the number of samples per group (number in brackets refer to n for (a) liver (b) spleen). Statistically significant differences between test and control groups are shown as *p<0.05; **p<0.01; ***p<0.001
Histopathology

2.2.2.19 Skin Injection Sites

Figure 2.28 is a diagrammatic representation of a section through rodent skin showing the various layers which are referred to in this report. The photograph in Figure 2.29 shows a comparison of a section of control skin with sections taken at various intervals after cadmium administration.

The skin sampled from injection sites of control animals showed no gross abnormalities and microscopic examination revealed minimal capillary dilatation and an occasional small focus of inflammatory cells, predominantly mononuclear, confined to the subcutaneous connective tissue and probably corresponding to the site(s) of transitory saline retention (Figure 2.30a). In contrast, all cadmium-treated animals developed swelling at the site of injection within hours and by 24h after administration there was substantial swelling measuring between 3 and 5cm diameter (Figure 2.29). Microscopic examination (Figure 2.30b and c) revealed an intense acute local inflammatory response characterised by a marked oedema, primarily of the subcutaneous tissue but also extending into the dermis and accompanied by extensive necrosis. Numerous inflammatory cells were scattered throughout the area although somewhat absent in the very oedematous central part. The presence of pyknotic and karyorrhexic nuclei (Figure 3.8a) showed that large numbers of these cells were clearly necrotic which made identification of cell types very difficult. However, there tended to be a predominance of mononuclear cells in the dermis and more polymorphonuclear cells subcutaneously. The panniculus carnosus was very oedematous and there was apparent thinning in some areas, presumably due to stretching by the underlying oedema although at later time points this muscle was totally absent due to necrosis. Blood vessels within the lesion were severely dilated and congested and contained large numbers of inflammatory cells, predominantly polymorphonuclear and which frequently displayed margination. The endothelial cells of the vessels were often pyknotic and the damage resulted in surrounding areas of haemorrhage.
In rodents, an extensive subcutaneous layer of striated muscle called the panniculus carnosus is present. The subcutaneous tissue is thus an anatomical entity which separates the skin from the underlying deep muscle. The subcutaneous adipose and connective tissue layers are richly supplied with blood vessels and lymphatics as well as nerves. The adipose layer in the ventral abdominal flank in the male rat additionally contains vestigial mammary gland tissue.

The vertical sections, stained with haematoxylin and eosin, show the appearance of control skin compared with skin taken from injection sites 24 hours after 1, 6 and 15 doses of cadmium (1.5mg Cd²⁺/kg/day) and 9 days after administration of 20 doses of cadmium. The intensity of oedema and extent of superficial lesions is evident.
Figure 2.30
Skin and Subcutaneous Tissue at Injection Sites 24 Hours After Administration of
(a) Saline, (b) Cadmium (1.5mg Cd^{2+}/kg)
Dermis, D; panniculus carnosus, PC; adipose tissue, AT. Note: minimal
inflammatory cell infiltrate, I, in (a) and oedema, particularly of the subpannicular
connective tissue, SCT, in (b).

Stain: haematoxylin and eosin. Magnification: x40.
Figure 2.30(c)

Subcutaneous Tissue at Injection Site 24 Hours After Administration of Cadmium (1.5mg Cd$^{2+}$/kg)

Note: oedema, O; haemorrhage, H; and necrotic inflammatory cells, NI.

Stain: haematoxylin and eosin. Magnification: x250.

Figure 2.30(d)

Subcutaneous Tissue at Injection Site After Administration of Cadmium (1.5mg Cd$^{2+}$/kg/day) for 6 Days

Note: oedematous and necrotic appearance of panniculus carnosus, PC; and subpannicular connective tissue, SCT; and general absence of inflammatory cells.

Stain: haematoxylin and eosin. Magnification: x40.
The picture at 48h was similar to that seen after 24h except the oedema was more pronounced, affecting more of the dermis and also the abdominal musculature. Measurements taken microscopically revealed a 10-fold increase in the thickness of the subcutaneous tissue alone, as compared to controls. The fresh tissue could be sliced with a scalpel without loss of fluid since the oedematous tissue had a firm jelly-like consistency and there was adhesion between layers to form a firm cohesive mass. Necrosis was more obvious at 48h and inflammatory cells were noticeably absent from the central necrotic regions and formed a diffuse margin at the circumference of the lesion. It was also noticed macroscopically at early stages that a sharply defined thin red ring, varying up to about 1cm diameter, was visible superficially which was thought to encircle the point of entry of the dosing needle. It was visualised microscopically to be a margin of erythema within which the stratum spinosum of the epidermis was necrosed although a thin layer of stratum corneum was still present.

After several injections, the age of individual lesions could not be determined since they had coalesced into each other so the major features of the development of the inflammatory reaction are described. Oedema persisted with time and was accompanied by a progressive, extensive coagulative necrosis that frequently extended from the epidermis down through the dermis and subcutaneous tissue and through the abdominal musculature to the connective tissue lining the abdominal cavity (Figure 2.30d). By day 6, although the extent of the necrosis was still marked, there were small areas of fibrosis in the connective tissue between the layers of abdominal musculature and around some of the larger blood vessels. By 10 days there was more evidence of fibroblastic activity and these areas seemed to be associated with small foci of dystrophic mineralization (Figure 2.30e). As time progressed the superficial lesions resulting from epidermal necrosis became plugged with thick deposits of fibrin and surrounded by dense aggregates of necrotic polymorphonuclear cells (Figure 2.30f). However, fibrosis and the presence of inflammatory cells in general was still limited due to the persistence of extensive necrosis and oedema.
Figure 2.30(e)
Subcutaneous Tissue at Injection Site After Administration of Cadmium (1.5mg Cd^{2+}/kg/day) for 10 Days
Note: Calcification, C; and fibrosis, F, in panniculus carnosus, PC, and subpannicular connective tissue, SCT.

Stain: haematoxylin and eosin. Magnification: x100.

Figure 2.30(f)
Dermis and Epidermis at Injection Site After Administration of Cadmium (1.5mg Cd^{2+}/kg/day) for 15 Days
Note: superficial lesion, SL; calcification, C; oedematous and necrotic appearance of underlying dermis, D; and panniculus carnosus, PC.

Stain: haematoxylin and eosin. Magnification: x40.
Nine days after dosing had ceased there was a moderate regression of the reaction with a reduction of oedema and more fibrosis. In addition, there were quite dense infiltrates of inflammatory cells which were more mononuclear in character in some areas but polymorphonuclear at the healing superficial lesions. Also large vacuoles had formed predominantly in the subcutaneous tissue but also in the dermis.

As mentioned earlier, the inflammation frequently extended through the abdominal musculature and at autopsy it was sometimes found to have penetrated into the abdominal cavity. The visceral organs in the adjacent area displayed regions of superficial fibrosis, resulting in adhesion of the organs to each other and to the cavity wall. Very occasionally testes were also found to be adherent to the scrotal sac.

2.2.2.20 Testes

Figure 2.31e shows a photograph of whole section mounts of testes stained with haematoxylin and eosin to illustrate the marked changes in size and appearance of organs at various intervals after cadmium administration. On gross examination, testes from control animals were seen to maintain a normal light-pink colour and no abnormalities were detected microscopically throughout the study Figure 2.31a). In sharp contrast, testes appeared pinker and swollen only 24h after the first injection of cadmium. Histological examination showed this to be due to interstitial oedema with clear spaces resulting from the detachment of the seminiferous tubules from the interstitial tissue (Figure 2.31b). This explains the increased weight recorded at this time. There was some interindividual variation in the time course of changes with some testes having oedema only at the circumference of the organ near the blood vessels whilst in others the whole organ was affected. In addition, blood vessels were dilated and the endothelial cells of some of the capillaries were necrosed which resulted in interstitial haemorrhage. Necrosis of the tubular cells started with the germinal epithelium and outer spermatogonia but as the injury progressed all cells were involved, including the Sertoli cells. There was also early interstitial cell
Figure 2.31
Testis After Subcutaneous Administration of (a) Saline and (b) 1, (c) 3, (d) 10 Daily Subcutaneous Injections of Cadmium (1.5mg Cd\textsuperscript{2+}/kg/day)

Note: (a) normal appearance, (b) haemorrhage, H; and oedema, O, of interstitial tissue, IT, and detachment of seminiferous tubules, ST, (c) dense infiltrate of polymorphs, PMNL, and tubular necrosis, TN, (d) complete tubular necrosis, TN; interstitial fibrosis, F, and calcification, C.

Stain: haematoxylin and eosin. Magnification: x250
Figure 2.31(e)
Whole Section Mounts (TS) of Rat Testes
Stained with haematoxylin and eosin to illustrate the difference in appearance and size of control testis and testes at intervals (1, 3, 6, 10, 15 and 20 days) during daily s.c. administration of cadmium. Interstitial oedema is evident at day 1; eosinophilia from day 3 is associated with areas of necrosis; deep basophilia from day 10 is caused by calcification.

Figure 2.31(f)
Testis After s.c. Administration of Cadmium (1.5mg Cd²⁺/kg/day) for 20 Days
Note: golden-yellow pigment, YP, and fibrosis, F, in interstitial tissue.

Stain: haematoxylin and eosin. Magnification: x630.
necrosis at day 1 but inflammatory cell infiltrates were minimal except where they accompanied haemorrhage.

By day 3, the testes no longer appeared swollen but were often deep red in colour with thrombosed veins. Interstitial haemorrhage was extensive in some areas with large numbers of polymorphonuclear leukocytes, many of them necrotic and especially abundant around the major blood vessels (Figure 2.31c). Tubular necrosis was also more pronounced and most contained only masses of eosinophilic material interspersed with scanty basophilic nuclear residue.

By day 6 gross examination showed the testes to be markedly shrunken, a dull yellow colour and firm in consistency. Microscopically, the interstitial tissue was severely necrosed resulting in almost total obliteration of the architecture. There remained a rim of necrotic polymorphs at the circumference of the organ. However, in some testes there was evidence of fibroblastic activity (mitotic figures were numerous) particularly in the zone of blood vessels at the circumference and occasionally within the centre of the organ (Figure 2.31d). Scattered foci of mineralization, especially in areas of fibrosis, were shown by Von Kossa reaction to be calcium and polymorphs present in fibrotic tissue appeared more viable. Numerous small granules of yellow pigment were readily detectable in sections particularly at the junctions of necrotic and fibrotic tissue (Figure 2.31f) and clumps of clear birefringent material was also present within areas of fibrosis.

At later time points, testes were severely atrophied with a considerably thickened tunica albuginea. Fibrosis became more marked until by 15 to 20 days the interstitial tissue was completely replaced by compact and vascularised fibrous tissue in most animals. There was also widespread calcification and these features show up in Figure 2.31e as areas of deep basophilia.

Nine days after dosing had ceased, fibrosed interstitial tissue had a more eosinophilic appearance and also seemed somewhat oedematous. It was not readily discernable which interstitial cells besides fibroblasts were regenerating but there was no sign of regenerating spermatogenic cells.
Throughout the study the proximal end of the caput epididymis displayed macroscopically similar changes to those observed in the testes and there was an associated marked reduction in size of seminal vesicles.

2.2.2.21 Liver

Evidence of persistent low-grade chronic infections was manifested by the presence of small isolated and randomly distributed foci of mononuclear cells with occasional flares of polymorphonuclear cells customarily associated with recent focal hepatic necrosis. This was apparent in both treatment groups and cadmium exposure did not appear to have any influence on the incidence. The hepatocytes from control livers, particularly periportally and at early time points in the study, displayed marked 'plant cell' appearance, believed to be a function of glycogen storage (Figure 2.32a). A determination of mitotic index was not undertaken but 24h after the first injection of cadmium there were noticeably increased numbers of mitotic figures (Figure 2.32b), especially in the mid-zonal to periportal regions, as compared to controls where mitotic figures were only occasionally seen. Sinusoids tended to be dilated and moderately congested with blood which contained raised numbers of polymorphonuclear leucocytes.

After 3 injections of cadmium, low magnification examination of the liver showed marked changes in the appearance of the cytoplasm which emphasized the lobular zoning. There was an overall increase in basophilia, particularly notable periportally and an absence of the 'plant cell' appearance characteristic of the control livers (Figure 2.32c). Periportal hepatocytes occasionally contained numerous vacuoles indicative of fatty degeneration but special stains are required to confirm the changes in fat and glycogen deposition implied in haemotoxylin and eosin stained sections. Mitotic figures were seldom seen in 3 day cadmium-treated animals although scattered 'dark cells' were frequently present in the mid-zonal region previously occupied by the mitotic figures. These 'dark' cells are commonly
Figure 2.32
Liver After Administration of (a) Saline, and (b) 1, (c) 3, (d) 6 Daily Injections of Cadmium (1.5mg Cd\(^{2+}\)/kg/day)

Note: portal tract, PT; central vein, CV; and in (a) 'plant cell' appearance, PC, (b) mitotic figure, \(\rightarrow\), (c) periportal basophilia, B, and dark cells (DC), (d) isolated necrotic cell, N.

Stain: haematoxylin and eosin. Magnification: x100 (a, c), x250 (b), x630 (d)
regarded as being damaged during division. Sinusoidal dilatation was marked, especially centrilobularly, where some hepatocytes had a pale foamy appearance.

By 6 days there were few 'dark cells' and less periportal vacuolar degeneration but the other features described persisted. Additionally there was scattered and isolated necrosis of what were thought to be Kupffer cells. Occasionally liver lobes were enveloped in a thick fibrous capsule which was involved in the adhesions observed macroscopically. With time the features described became less pronounced and livers from cadmium-treated animals assumed a similar appearance to controls.

2.2.2.22 Kidneys

All kidneys from control animals had a normal appearance as shown in Figure 2.33a. After 6 injections of cadmium the proximal convoluted tubules of some animals showed early degenerative changes manifested by slight hydropic degeneration and occasional darkly staining cells. Increased numbers of polymorphonuclear leucocytes were frequently visible in the glomeruli and blood vessels. Although there was interindividual variation in the extent of damage, by 10 days the hydropic degeneration was in general more pronounced. Additionally, there were more necrotic cells, particularly of the first segment of the proximal tubules and evidence of regenerative activity in these damaged areas. After 15 and 20 injections of cadmium all kidneys displayed extensive tubular degeneration and necrosis accompanied by widespread regeneration (2.33b and c). Nine days after the 20th injection, the severity of the damage had subsided although the replacement of cellular debris by regenerating tubules was still evident. Throughout the study there was little evidence of tubular dilatation nor interstitial fibrosis and pathological changes were not detected in the glomeruli nor in the medulla.
Figure 2.33
Kidney After s.c. Administration of (a) Saline, (b and c) Cadmium (1.5mg Cd^{2+}/kg/day) for 15 Days
Note: (a) normal glomerulus, G, and proximal convoluted tubule, PCT, (b and c) degenerate and necrotic tubular cells, N, and regenerating proximal tubules, R, and mitotic figures, MF.

Stain: haematoxylin and eosin. Magnification: x400
2.2.2.23 Spleen

The spleens from control animals showed a normal appearance both macroscopically and microscopically (Figure 2.34a) and animals from both treatment groups displayed marked variability in the amounts of extramedullary haemopoiesis (EMH) as indicated by numerous foci of erythroblasts in the red pulp. This is a normal feature of rat spleen especially of young or stressed animals and the incidence of EMH tended to decline during the course of the study. There was no obvious treatment-related effect on EMH except in the recovery group where animals which had received cadmium displayed substantially enhanced EMH as compared to controls at this time.

Large numbers of polymorphonuclear leucocytes were apparent in the red pulp particularly after 3 to 20 doses of cadmium and to a lesser extent, in the 'recovery' group. They were mostly adult forms but large numbers of metamyelocytes with their large ring-shaped nuclei were also present (Figure 2.34c and d). Additionally moderate numbers of karyorrheic and pyknotic nuclei were scattered throughout the red pulp. Spaces were also apparent which may have been due to dilated sinuses; macrophages frequently had a clearer foamy appearance to their cytoplasm. Cadmium caused a marked splenomegaly and stained sections from the organs were increasingly more basophilic than those of controls (Figure 2.34b). Microscopic examination revealed that this was probably due to the increased myelopoietic activity combined with a reduced sequestration of blood. The latter observation may in part have been an illusion created by the reduced density of red cells resulting from the increased size of the organ, which seemed to be mainly due to a higher ratio of red to white pulp. There was seldom thickening of the splenic capsule but in one animal fibrous thickening of the capsule on one side probably associated with the adhesions described earlier, resulted in distortion of the shape of the organ.
Figure 2.34

Spleen After s.c. Administration of (a) Saline, and (b) Cadmium (1.5mg Cd\(^{2+}\)/kg/day) for 15 Days and (c and d) Cadmium for 10 Days

Note: (a) normal appearance of red pulp, (b) increased basophilia of red pulp, (c and d) large numbers of polymorphs, PMNL; metamyelocytes, Mm; macrophages containing cellular debris, Mac.

Stain: haematoxylin and eosin. Magnification: x40 (a, b), x630 (c, d)
2.2.2.24 Lung

Mild symptoms of respiratory distress were present in some of the animals from both treatment groups, namely, snuffling and rhonchi (wheezing and rasping). Lungs were examined for signs of infection since the disease status of an animal could affect the parameters under investigation, particularly haematological indices. Macroscopic and microscopic examination revealed low grade chronic lung disease (Innes et al., 1967).

2.2.2.25 Lymph Nodes

All lymph nodes examined, which included subcutaneous, mesenteric, dorso-lumbar and perirenal, were greatly enlarged following cadmium administration, and were frequently up to twice the size of those in controls. Microscopic examination showed that this was primarily caused by marked reactive changes in the medulla. Lymphatic sinuses were dilated most having a clear lumen but some containing eosinophilic lymph and large numbers of degenerate polymorphs and other cell debris. Macrophages containing phagocytosed degenerate cells were evident both in cortical lymphoid tissue and medulla.

2.2.2.26 Other Tissues

No abnormalities were detected in sections of intestine, pancreas or adrenal gland.

2.2.2.27 Perls Prussian Blue (PPB) Reaction for Ferric Iron

Skin, testes, marrow (sternal and femoral) and intestine were all negative for PPB reaction whilst livers and kidneys from both treatment groups displayed only trace amounts of positive material. Spleens showed a positive reaction varying from trace to minimal amounts of deposit mainly in the macrophages of the red pulp. There was a tendency for stainable iron to increase with time but no discernable difference in spleens was caused by treatment. Lymph nodes were generally negative or with trace deposits in macrophages except for a non-treatment related marked positive reaction in an occasional peri-renal lymph node.
2.3.1 Materials and Methods

Cadmium or saline was administered by daily subcutaneous injection for 1, 3, 5, 10 or 15 days exactly as previously described in Section 2.2.1.2 (Figure 2.1) except that the dose was $0.75 \text{mg} \frac{\text{Cd}^{2+}}{\text{kg/day}}$ and the weight range from which animals were selected was lower; 135-175g.

Identical methods were employed throughout and the following parameters were determined: daily body weight and food consumption; liver, spleen, kidneys and testes weights; haematocrit; haemoglobin concentration; reticulocyte counts; differential white cell count; cadmium and iron concentration in liver, kidney, spleen and intestine and copper in liver on day 15 samples only; plasma iron and total iron-binding capacity; plasma haptoglobin; histopathology of liver, spleen, intestine, lymph nodes, kidney, sternum and injection sites in skin.

2.3.2 Results

2.3.2.1 General Condition of Animals

Control animals remained in good condition throughout the study. Cadmium-treated animals exhibited reduced activity with piloerection and lacklustre fur after about 8 injections; subcutaneous swelling also developed at the injection sites.

2.3.2.2 Body Weight Gain and Food Consumption

Figures 2.35a and b show the mean daily body weight and food consumption respectively for groups of animals dosed for 10 and 15 days. Cadmium treatment caused a slight depression in body weight gain after 6 to 7 injections, however, there was no statistically significant difference between treatment groups in mean terminal body weight at any time (Figure 2.35c). All groups of cadmium-treated animals exhibited a moderate reduction in food consumption, particularly
Figure 2.35

Body Weight Gain (b) and Food Consumption (a) of Rats During Daily Subcutaneous Administration of Cadmium for 10 or 15 Days and Terminal Body Weights (c) Following Administration for up to 15 Days

The figures presented for (b) and (c) are means ± SEM (shown as bars) for saline- (○) and cadmium- (●), 0.75mg Cd²⁺/kg/day, treated animals (five per group).
during 24-48h period after the first injection and it remained somewhat lower than in control animals for the duration of the study.

2.3.2.3 Necropsy Data: Organ Weights

Figure 2.36 shows the effect of cadmium administration on the mean weights of liver, spleen, kidneys and testes relative to body weight and the absolute organ weights are included in Table 2.10. There was no significant effect on the absolute or relative weights of kidneys and testes.

Liver: The relative liver weight was significantly decreased as compared to controls after 3 (p < 0.001) and 5 (p < 0.05) injections of cadmium but slightly higher after 10 (p < 0.05). The absolute liver weights were reduced only after 15 days of cadmium administration (p < 0.05).

Spleen: In controls the relative spleen weight declined gradually with time whereas cadmium administration brought about a splenomegaly with significantly increased relative spleen weights at 10 (p < 0.05) and 15 days (p < 0.001). After 15 injections the control spleens were on average 0.27% of body weight compared to 0.40% in test animals.

Haematology

2.3.2.4 Haematocrit, Haemoglobin Concentration and Mean Cell Haemoglobin Concentration

Figure 2.37a and b show that there was a significantly reduced haematocrit (p < 0.001) and haemoglobin concentration (p < 0.05) in cadmium-treated animals as compared to controls 24h after the first injection. Differences between treatment groups at subsequent time points did not reach statistical significance but cadmium-treated animals had slightly lower haematocrit and
Figure 2.36
Relative Weights of Rat Testes (a), Kidney (b), Spleen (c) and Liver (d) Following Daily Subcutaneous Administration of Cadmium

The figures presented are means ± SEM (shown as bars) for saline-(●) and cadmium-(○), 0.75mg Cd²⁺/kg/day, treated animals. The number of rats per group was five and statistically significant differences between test and control groups are shown as * p < 0.03, ** p < 0.001.

<table>
<thead>
<tr>
<th>No. of Daily Doses</th>
<th>Dose of Cadmium mg/kg/dy</th>
<th>Organ Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>10.71 ± 0.45</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>9.60 ± 0.20</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>10.08 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>9.45 ± 0.51</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>11.13 ± 0.39</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>9.94 ± 0.62</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>11.24 ± 0.48</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>11.26 ± 0.36</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>12.41 ± 0.59 *</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>10.73 ± 0.38 *</td>
</tr>
</tbody>
</table>

Table 2.10 The Effect of Daily Subcutaneous Administration of Cadmium on Absolute Weights of Rat Liver, Spleen, Kidneys and Testes

All values are means ± standard error of the mean (SEM) where the number of animals per group = 5. Statistically significant differences between test and control groups are shown as follows *p<0.05
Figure 2.37
Haematocrit (b), Haemoglobin Concentration (a), Mean Cell Haemoglobin Concentration (d) and Reticulocyte Counts (c) of Rats Following Daily Subcutaneous Administration of Cadmium

The figures presented are means ± SEM (shown as bars) for saline-(•) and cadmium-(○), 0.75mg Cd²⁺/kg/day, treated animals. There were five samples per group (except day 1 Cd group, n = 4) and statistically significant differences between test and control groups are shown as * p < 0.05, *** p < 0.001.
haemoglobin concentration after 10 and 15 days. Cadmium treatment had no effect on mean cell haemoglobin concentration at any time point (Figure 2.37d).

2.3.2.5 Reticulocyte Count

Both control and cadmium-treated animals displayed time-dependent reduction in the percentage of reticulocytes (Figure 2.37c). No significant differences were seen between groups although after 15 injections cadmium-treated animals had slightly higher reticulocyte counts than controls.

2.3.2.6 Red Cell Morphology

Examination of reticulocyte films and May-Grunwald-Giemsa stained films revealed no obvious irregularities in red cell morphology.

2.3.2.7 Differential White Cell Count

Since total white cell counts were not carried out the absolute numbers of different white cell types are not available. However, a comparison of differential white cell counts expressed as percentage of white cells counted revealed a significantly increased percentage of neutrophils and concomitant decrease in lymphocytes following cadmium treatment (Figure 2.38). After 10 injections of cadmium the mean neutrophil count was 46.4 ± 2.8% compared to 17.0 ± 2.4% (p < 0.001) in controls whilst the lymphocyte count had dropped to 52.0 ± 2.4% from 80.2 ± 2.7% (p < 0.001). No significant differences between treatment groups in percentage of eosinophils or monocytes was recorded at any time (Table 2.11).

Biochemistry

2.3.2.8 Plasma Haptoglobin

The justification for the measurement of haptoglobin in pooled plasma samples and the method of assessment of concentration have been explained in Section 2.2.2.16. The heights of rockets obtained by immunoelectrophoresis of
Figure 2.38

Percentage of Neutrophils and Lymphocytes in Peripheral Blood White Cell Counts of Rats Following Daily Subcutaneous Administration of Cadmium

The figures presented are means ± SEM (shown as bars) for saline-(●) and cadmium-(○), 0.75mg Cd²⁺/kg/day, treated animals. The number of samples per group was five (except day 1 Cd group, n = 4) and statistically significant differences between test and control groups are shown as * p < 0.05, ** p < 0.01, *** p < 0.001.

<table>
<thead>
<tr>
<th>No. of Daily Doses</th>
<th>Dose of cadmium (mg/kg/dy)</th>
<th>Neutrophils (%)</th>
<th>Lymphocytes (%)</th>
<th>Eosinophils (%)</th>
<th>Monocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>20.0 ± 1.2</td>
<td>78.8 ± 1.2</td>
<td>0.0</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>34.0 ± 4.5*</td>
<td>64.6 ± 4.4*</td>
<td>0.6 ± 0.2</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>20.2 ± 3.6</td>
<td>76.0 ± 3.6</td>
<td>0.8 ± 0.4</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>40.0 ± 2.4**</td>
<td>57.0 ± 2.8**</td>
<td>0.6 ± 0.2</td>
<td>2.4 ± 0.7</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>15.4 ± 1.8</td>
<td>81.0 ± 1.4</td>
<td>0.6 ± 0.2</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>39.4 ± 3.2***</td>
<td>58.0 ± 3.1***</td>
<td>0.8 ± 0.4</td>
<td>1.8 ± 0.7</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>17.0 ± 2.4</td>
<td>80.2 ± 2.7</td>
<td>0.4 ± 0.2</td>
<td>2.8 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>46.4 ± 2.8***</td>
<td>52.0 ± 2.4***</td>
<td>0.4 ± 0.2</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>21.4 ± 3.2</td>
<td>75.2 ± 3.1</td>
<td>1.0 ± 0.3</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>45.8 ± 5.7**</td>
<td>51.8 ± 5.8**</td>
<td>0.8 ± 0.4</td>
<td>1.6 ± 0.4</td>
</tr>
</tbody>
</table>

Table 2.11 The Effect of Daily Subcutaneous Administration of Cadmium on Differential White Cells Counts of Rats

Results are expressed as % of cells counted. All values are means ± standard error of the mean (SEM) where the number in each group = 5 (except 1 day Cd group, n = 4). Statistically significant differences between test and control groups are shown as * p<0.05; ** p<0.01; *** p<0.001.
plasma pooled from samples taken at intervals up to 10 days after commencement of dosing are shown in Table 2.12. Cadmium treatment resulted in elevated concentrations of haptoglobin from 24h after the first injection and by 5 days the levels were double those seen in controls.

2.3.2.9 Plasma Iron and Total Iron-Binding Capacity

The effect of cadmium administration on plasma iron and total iron-binding capacity (TIBC) is shown in Figure 2.39b and the percentage saturation of TIBC with iron in Figure 2.39a. A sufficient volume of plasma was not available to measure TIBC at day 10 and only sufficient was available to give group sizes of 3 at day 15. Control animals displayed a time-dependent decrease in plasma iron concentration and the percentage saturation of TIBC with iron. Administration of cadmium produced an immediate and marked fall in plasma iron to 56% of the concentration in control plasma by 24h. TIBC was unchanged so this represented a drop in the percentage saturation of TIBC with iron from 71% to 40%. The reduced plasma iron concentration persisted in cadmium-treated animals until after 15 injections when it had returned to control levels as had the percentage saturation of TIBC with iron. Meanwhile the plasma TIBC was enhanced and by day 15 cadmium-treated animals had significantly higher TIBC than controls (p < 0.01).

2.3.2.10 Tissue Metal Analysis

Cadmium: The concentration of cadmium in liver, spleen, kidney and intestine was determined after 15 injections of the metal. The retention of cadmium in the tissues (expressed as concentration, content and percentage of the total administered dose) is given in Table 2.13. Also shown are the figures which were obtained after administration of cadmium for 15 days at the higher dose level of 1.5mg/kg/day for comparison.
<table>
<thead>
<tr>
<th>No. of Daily Doses</th>
<th>Dose of cadmium mg/kg/day</th>
<th>Plasma Haptoglobin</th>
<th>Rocket Height (cm)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>2.5</td>
<td>3.8</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>2.8</td>
<td>4.6</td>
<td>164</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>2.3</td>
<td>4.7</td>
<td>204</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>2.9</td>
<td>4.6</td>
<td>159</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.12 The Effect of Daily Subcutaneous Administration of Cadmium on the Concentration of Haptoglobin in Rat Plasma

Results are expressed as the height in cm of rockets formed by immunoelectrophoresis of pooled plasma from 5 samples per group. Each test result is presented as the percentage of the appropriate control.
Figure 2.39
Plasma Iron Concentration, Total Iron-Binding Capacity, TIBC (b) and Percentage Saturation of TIBC with Iron (a) of Rats Following Daily Subcutaneous Administration of Cadmium

The figures presented are means ± SEM (shown as bars) for plasma iron concentration (●,○) and TIBC (■,□). The mean value of each of these results was used to calculate the percentage saturation of TIBC with Fe (▲,▲) for saline-(●,■,▲) and cadmium-(○,□,△), 0.75mg Cd²⁺/kg/day, treated animals. The number of samples per group was five (except day 1 Cd group, n = 4 and TIBC at day 15, where n = 3) and statistically significance differences between test and control groups is shown as * p < 0.05, ** p < 0.01.
<table>
<thead>
<tr>
<th>Dose of Cadmium mg/kg/dy</th>
<th>Cadmium ( \mu \text{mol/g} )</th>
<th>( \mu \text{mol} )</th>
<th>% of Total Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total dose administered</td>
<td>0.75</td>
<td>-</td>
<td>22.2</td>
</tr>
<tr>
<td></td>
<td>1.50</td>
<td></td>
<td>52.3</td>
</tr>
<tr>
<td>Liver</td>
<td>0.75</td>
<td>1.07 ± 0.04</td>
<td>11.49 ± 0.42</td>
</tr>
<tr>
<td></td>
<td>1.50</td>
<td>2.07 ± 0.09</td>
<td>35.94 ± 1.32</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.75</td>
<td>0.69 ± 0.055</td>
<td>1.075 ± 0.059</td>
</tr>
<tr>
<td></td>
<td>1.50</td>
<td>0.576 ± 0.067</td>
<td>1.066 ± 0.132</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.75</td>
<td>0.075 ± 0.005</td>
<td>0.073 ± 0.006</td>
</tr>
<tr>
<td></td>
<td>1.50</td>
<td>0.138 ± 0.003</td>
<td>0.158 ± 0.006</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.75</td>
<td>0.027 ± 0.003</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1.50</td>
<td>0.071 ± 0.004</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.13 Total Cadmium Administered and retained in the Liver, Kidneys, Spleen and Intestine following Daily Subcutaneous Administration of 0.75 or 1.5mg Cd\(^{2+}\)/kg/dy to Rats for 15 days

Results are expressed as concentration in \( \mu \text{mol/g} \) wet weight of tissue, as content in \( \mu \text{mol} \) and as % of total administered dose. Values are means ± standard error of the mean (SEM) where the number per group = 5.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Dose of Cadmium mg/kg/dy</th>
<th>Iron ( \mu \text{mol/g} )</th>
<th>( \mu \text{mol} )</th>
<th>Copper ( \mu \text{mol/g} )</th>
<th>( \mu \text{mol} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0</td>
<td>1.64±0.07</td>
<td>20.49±1.77</td>
<td>0.064±0.001</td>
<td>0.800±0.045</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>1.79±0.18</td>
<td>19.71±2.56</td>
<td>0.097±0.002***</td>
<td>1.045±0.044**</td>
</tr>
<tr>
<td>Kidney</td>
<td>0</td>
<td>1.56±0.11</td>
<td>2.75±0.27</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>1.56±0.14</td>
<td>2.42±0.21</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Spleen</td>
<td>0</td>
<td>5.14±0.53</td>
<td>3.86±0.35</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>4.83±0.52</td>
<td>4.69±0.52</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Intestine</td>
<td>0</td>
<td>0.32±0.02</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>0.32±0.03</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.14 The Effect of Daily Subcutaneous Administration of Cadmium for 15 days on the Concentration and Content of Copper in the Liver and of Iron in the Liver, Kidney, Spleen and Intestine

Results are expressed as concentration in \( \mu \text{mol/g} \) wet weight of tissue and as content in \( \mu \text{mol} \). All values are means ± standard error of the mean (SEM) where the number of samples per group is 5. Statistically significant differences between test and control groups are shown as **p<0.01; ***p<0.001.
In all tissues from control animals the levels of cadmium were below the limits of detection. As might be expected, the spleen and intestine accumulated about twice as much cadmium when the metal was administered at double the dose and over 3 times as much cadmium was retained in the livers of the high dose group as compared to the livers of the low dose group. In the kidneys however, a higher concentration of cadmium (p < 0.01) was reached after administration of the lower dose than the higher dose and resulted in a slightly higher kidney content which represented 4.8% of the total dose administered as compared to 2% when cadmium was given at 1.5mg/kg/day.

**Copper:** Table 2.14 shows that administration of cadmium resulted in a significantly higher concentration (p < 0.001) and content (p < 0.01) of copper in the liver as compared to controls at day 15.

**Iron:** No significant differences were found between groups for iron concentration or content in liver, kidneys, spleen or intestine (Table 2.14).

**Histopathology**

2.3.2.11 Skin - Injection Sites

The injection sites from cadmium-treated animals had a similar appearance after 0.75mg/kg/day to that already described after administration of 1.5mg/kg/day. Approximately the same area of tissue was involved with massive oedema and inflammatory cell infiltration of all layers as before. In contrast, although still extensive, the resulting tissue and inflammatory cell necrosis was not quite so severe and fewer areas of haemorrhage was seen. More evidence of resolving inflammation, as shown by fibroblastic activity, was present earlier and towards the end of the study several areas with dense calcification were seen.
2.3.2.12 Liver: The histopathological appearance and time sequence of changes in the liver was similar after both doses of cadmium except that scattered necrotic cells were seldom seen after the lower dose. There was an impression of elevated numbers of mitotic figures after 1 injection, but controls displayed a higher mitotic index than in the previous study, presumably due to the younger age of the animals, so the regenerative response did not appear as pronounced.

2.3.2.13 Spleen, Lymph Nodes and Sternal Marrow

The changes described for these tissues after administration of 1.5mg Cd²/kg/day were observed after 0.75mg/kg/day although were less dramatic in intensity. Spleens from all animals displayed more extramedullary haemopoiesis than was present in the previous study, and this is probably an age-related effect.

2.3.2.14 Testes, Kidneys and Intestine

No abnormalities were detected in these organs in any of the animals.
2.4 Discussion

The tissue distribution of cadmium and toxic effects arising from administration of the metal to animals by a variety of routes are well documented (see Fielder and Dale, 1983 for review) and many of the results obtained in the present study are consistent with previous observations which have been reported following the use of a subcutaneous (s.c.) mode of administration. Several of the results also indicated the presence of a severe acute inflammatory reaction, and this discussion will concentrate on those aspects which are of relevance to this response and to its possible effect on metal homeostasis and the development of anaemia in the rat.

The dose-related growth retardation which was apparent throughout the period of cadmium administration is in accordance with observations by Bonner et al., (1979) and many others. This is likely to be primarily the result of reduced food consumption which is generally accepted as an undesirable manifestation of the toxic effect of cadmium reflecting the inhibitory effects of the metal on many aspects of metabolism. The altered physiological status of the animals exhibited by reduced growth and activity would diminish the food requirement. However, a common manifestation of acute infectious illness is a decrease in food appetite and McCarthy et al., (1985) have shown that suppression of food intake is in part mediated by the release of interleukin-1 so it would be an expected response in acute inflammation triggered by any stimulus. The reduced food intake and hence growth rate of rats given s.c. cadmium may therefore be a component of the acute phase response. The adaptive value of such a response seems paradoxical at a time when metabolic needs are increased to sustain the reaction and Murray and Murray (1979) concluded that it may serve to reduce the availability of nutrients essential to the growth of pathogenic organisms.
The major burden of cadmium was taken up by the liver with the high dose resulting in three times the cadmium load seen following the low dose. Interestingly, a higher concentration of cadmium accumulated in the kidneys of the low dose group after 15 injections, than in the high dose group. This is probably explained by the observation that the animals given the higher dose developed extensive proximal tubular necrosis whereas no injury was detected microscopically in kidneys of those receiving the low dose. The tubular dysfunction would be expected to result in urinary loss of cadmium and probably also explains the lower iron concentration seen in these kidneys. The kidneys exposed to cadmium at the lower dose were presumably able to synthesise metallothionein at a sufficient rate to bind all the metal being presented to them and thus prevent damage occurring, whereas cadmium uptake in the kidneys at the higher dose must have exceeded the metallothionein-synthesising capacity. The hydropic degeneration and regenerating tissue probably accounts for the higher weight of these organs.

Despite the severe proximal tubular damage caused by the high dose of cadmium, the kidney appeared to have a marked regenerative capacity and ability to accumulate further cadmium. Nine days after the cessation of dosing the amount of damage seen microscopically was markedly reduced in spite of the kidneys having accumulated more cadmium, so presumably the regenerating tubules were synthesising sufficient metallothionein to minimise further damage.

The testes from animals given the higher dose of cadmium presented the typical changes which have been described by many authors (for review see Samarawickrama, 1983). Two theories are generally offered for the mechanism: a circulatory failure due to vascular damage resulting in ischaemic necrosis of the tubular cells and a direct action of cadmium on the spermatogenic cells possibly by interaction with zinc-dependent enzymes. In this investigation, the acute inflammatory nature of the injury as indicated by the marked initial oedema and subsequent infiltration by large number of polymorphonuclear leucocytes is emphasised.
The s.c. administration of cadmium at both dose levels provoked an intense inflammatory reaction at injection sites which was characterised by massive oedema, extensive necrosis and the presence of rather limited numbers of inflammatory cells, particularly after the high dose. Despite minimal fibroblastic activity, which was apparent at later stages, there appeared to be only limited containment of the lesions, although this may in part be due to the close proximity of the injection sites to each other.

Despite the frequent use of this mode of cadmium administration involving a number of species similar reactions have seldom been reported and descriptions of local effects, when included, have been varied. Interestingly, Der et al., (1977) noted that female and not male (Der et al., 1976) rats developed 'sores on the sites' of daily intramuscular (i.m.) injections of cadmium. This presumably referred to superficial lesions since Der et al., (1976) did acknowledge a 'loss of muscle mass due to the trauma of repeated injections'. The strains and weights of rats used in the two studies differed and it is not clear what dose of cadmium was given, however, no further reference was made to the possible effect of such a local response on the investigation being undertaken.

Sobocinski et al., (1981) described in detail severe inflammatory lesions induced 24 hours after s.c. administration of cadmium (6mg/kg) to male rats and compared the reaction to that produced by the s.c. administration of turpentine. Indeed, cadmium was used to induce inflammation in order to study the role of the pituitary and adrenal glands in mediating hypozincemia and hepatic synthesis of metallothionein (Sobocinski et al., 1979, 1981). The predominant features in the lesions of marked oedema and necrosis were identical to those seen in this study. In contrast, only small fibrous granulomata appeared in the s.c. tissue around injection sites in mice given several s.c. injections of cadmium (0.14µmol/inj) in comparison to a marked inflammatory reaction produced by the s.c. administration of mercury (Nicholson et al., 1984).
Abscess formation at s.c. injection sites necessitated the termination of a study in which rats were repeatedly administered 3.0mg Cd\(^{2+}\)/kg/day (Bonner et al., 1979). An investigation into the toxic effects of cadmium on canine testes found CdCl\(_2\) administration s.c. or i.m. caused severe inflammation, oedema, haemorrhage and necrosis at sites of injection (Donnelly and Monty, 1977). More recently, Bomhard et al. (1987) described transient inflammatory reactions with ulceration at s.c. injection sites during similar studies in rats.

During a study into the induction of sarcoma at sites of s.c. cadmium injection in rats an initial reaction after 24 hours was described by Gunn et al. (1964) as 'localised oedema which subsided in a few days to leave only a small scarred area'. During the same type of study Kazantzis and Hanbury (1966) gave suspensions of CdS and found an acute inflammatory reaction characterised by a neutrophil response close to the deposit which persisted for several days and developed into a chronic inflammatory reaction. When cadmium was given as a metallic powder by i.m. injection to rats severe inflammation followed but not until after 3 days (Heath et al., 1962). The i.p. administration of 1mg Cd\(^{2+}\)/kg to rats induced peritonitis with extensive adhesions among visceral organs. Large numbers of inflammatory cells, predominantly neutrophils, and various chemical mediators of the inflammatory process were found in the peritoneal exudate (Giri et al., 1979).

The systemic response which accompanied the local reaction in the present study was characteristic of the changes which are well recognised to take place in acute inflammation. There was an intense and persistent neutrophilia, the magnitude of which was dose-dependent with neutrophils accounting for 72% of the circulating white cells after 15 injections of the high dose (representing an 8-fold increase in the absolute numbers of neutrophils) as compared with only 46% at the lower dose. This is in accordance with the less intense local reaction and the absence of testicular injury at the low dose. The neutrophilia was prolonged presumably as a result of a persistent stimulus due to repeated injections of cadmium. Morgan et al. (1984) similarly found raised numbers of total white cells.
and percentage of neutrophils with a concomitant reduction in lymphocytes after repeated i.p. administration of 1.0 or 2.5mg Cd\(^{2+}\)/kg to rats. Incorrectly, it was stated that the change in numbers of lymphocytes was larger and that these cells were therefore more sensitive to cadmium treatment than granulocytes. If the percentage counts had been converted to absolute numbers, a neutrophilic leucocytosis would have become apparent with little change in the numbers of circulating lymphocytes. Local inflammatory reactions were not reported and no explanation for the white cell changes was suggested.

During the initial stages of an inflammatory reaction the predominant cell at the site of inflammation is the neutrophil and its role in the phagocytosis and killing of organisms by both oxygen-dependent and independent mechanisms is well recognised. The intense neutrophilia undoubtedly reflects mobilisation of neutrophils into the blood from the bone marrow and other sites of sequestration. Examination of sections of spleen and marrow showed that it was sustained by increased myelopoietic activity in these tissues. Previous studies of acute cadmium toxicity in animals have seldom focussed on effects on the bone marrow. Sections of marrow are unreliable for cytological identification and this appears to be the first report in which changes in the cellularity of rat bone marrow following the induction of s.c. lesions by cadmium administration have been demonstrated by cytological preparation. Proper interpretation of these marrow results was hampered by the absence of total marrow cell counts. However, recognising these limitations, speculative inferences could be made and the results indicated shifts in cell populations which followed a predictable time course and on the whole reflected changes seen in the peripheral blood.

The mobilisation of sequestered neutrophils from the marrow resulted in a slight reduction in numbers of adult neutrophils in the marrow after one and three injections of cadmium. The loss of neutrophils was compensated for by myeloid hyperplasia evident at day 3 as raised numbers of myelocytes and significantly increased numbers of myeloid cells in mitosis. Maturation of these cells gave rise to
raised numbers of neutrophils in the marrow by day 6. This accords with the known sequential changes observed in the maturation of granulocytes as measured by radioisotope techniques (Boggs, 1967). Enhanced myeloid activity continued during dosing and then returned to control levels by the end of the recovery period when erythroid activity was raised as evidenced by a slight rise in erythroid mitoses.

Stelzer and Pazdenik (1983) showed substantial cadmium-induced shifts in bone marrow cell size distribution profile to larger diameters after both i.p. and s.c. injections to mice and similar slight shifts in spleen cell size. Identification of the particular cell types exhibiting the shifts were not made but such changes would be consistent with a higher incidence of larger myeloid precursors as was observed in this study. However, no evidence of an inflammatory response was presented; the study concentrated on the effect of cadmium on the immune response and the changes were suggested to be indicative of perturbations in haemopoiesis. Hypercellular marrow resulted from the s.c. administration of cadmium to rabbits for 2-4 months (Swensson, 1957; Berlin et al., 1961). Swensson additionally stated that the marrow displayed erythro- and leucocytopoiesis but no further details on the cell types responsible were given nor any reference to an inflammatory response if present.

Increased numbers of circulating monocytes, many immature in appearance, were evident after the appearance of neutrophils. These cells are widely regarded as the immature transient form of the adult macrophage so the raised numbers were consistent with the well established scavenging/phagocytic role of these cells at the sites of inflammation as well as their secretion of a wide range of products. Raised numbers of blood monocytes were not reflected by an increased count in the marrow. Either the time of sampling missed the event or the evaluation of differential marrow cell counts in the absence of absolute numbers of marrow cells was not sensitive enough to detect a change.

The significantly increased numbers of eosinophilic cells in the marrow at 15 and 20 days were consistent with the slight, though non-significant rise in the
numbers of circulating eosinophils seen at 20 days and after the recovery period. This delayed eosinophilia may represent a sympathetic collateral response to degradation products such as fibrin.

The observations of nuclear remnants in the spleen and generalised lymph node enlargement suggest an enhanced phagocytic role of the organs with the retention of damaged and degenerate polymorphonuclear leucocytes. It was concluded that the splenomegaly induced by both doses of cadmium was primarily a consequence of the increased myelopoietic activity combined with an enhanced phagocytic role. Splenomegaly was similarly observed in mice after i.p. administration of carrageenan, a potent stimulator of inflammatory response (Maitani and Suzuki, 1981).

Splenomegaly has been reported in a number of animal species following cadmium exposure and although the conclusions as to the cause have been somewhat varied, the change has not been attributed to part of an acute inflammatory response. A marked splenomegaly followed s.c. injection of 3.0mg Cd²⁺/kg to rats four times per week for one to six weeks. However, no appreciably histological alterations were found and no local reactions at injection sites were reported and it was concluded that despite severe enlargement of the spleen the organ was not susceptible to cadmium loading (Suzuki et al., 1983). This observation contrasted with those made by Yamada et al., (1981) who similarly found splenomegaly after a single i.p. injection of 1.8mg Cd²⁺/kg to mice but noted that the white pulp tended to decrease in size whilst the red pulp enlarged and contained increased numbers of polymorphonuclear leucocytes and metamyelocytes. Changes in circulating white cells and any observations of adverse reactions at the i.p. site of administration were not reported. Bozelka et al., (1978) concluded that raised numbers of polymorphs and macrophages seen in the red pulp after i.p. administration of cadmium to mice did not contribute significantly to the splenic hyperplasia observed. They further noted that the marked splenomegaly was paradoxical in view
of the suppressed immune response which they demonstrated; again, no mention was made of any adverse local reaction to the i.p. injection.

In contrast to observations of myelopoietic activity, Der et al., (1977) reported increased sequestration of red blood cells in the spleens of rats given repeated i.m. injections of cadmium and concluded that this was a factor causing the observed splenomegaly. Spleen enlargement was also seen after repeated daily s.c. administration of 0.5mg Cd\textsuperscript{2+}/kg to rats and microscopic examination revealed follicular hyperplasia, hyperaemia of the red pulp and foci of erythropoietic haematopoiesis associated with a microcytic hypochromic anaemia (Dudley et al., 1985). A normal splenic PPB reaction and absence of congestion by effete red cells throughout the present study was surprising in view of the large numbers of abnormal red cells appearing in the circulation. The splenic changes described by Der et al., (1977) and Dudley et al., (1985) were seen after a longer period of dosing, 54 days and 14 weeks respectively, and although the results are therefore not readily comparable with the present study they suggest that such features might have developed with continued dosing. Since the rat spleen functions as a haemopoietic organ as well as a functional part of both the reticulo-endothelial and immune systems clearly the factors which contribute to splenomegaly are likely to depend on the timing of examination. In addition, the response of the spleen seems to depend upon the physical nature of the cadmium. Maitani and Suzuki (1982) found no splenomegaly after a single i.v. dose of a solution of cadmium whereas an identical quantity of cadmium suspended as cadmium carbonate in sodium bicarbonate solution resulted in increased spleen weight four and seven days after its i.v. administration.

There was evidence of low grade infection in the livers and lungs of rats probably attributable to the transfer of animals from an SPF breeding unit to a mixed animal unit prior to the start of the study with the resulting risk of exposure to infection. A mild lymphocytosis accounted for the steady rise in circulating white cells seen in control animals of the high dose study and might reasonably be
attributed to an increased burden of recurrent infection. This probably accounts for the slightly larger bone marrow population of lymphocytes seen in control animals. Cadmium treatment resulted in a reduced population of lymphocytes in the marrow at six days which was accompanied and followed by peripheral lymphopenia. Clearly the triggering of multiphysiological responses in acute inflammation which are known to result in redistribution of lymphocytes will affect the compartmentalisation of cells. Cadmium-induced toxicity to the immune system has been extensively studied and there have been conflicting reports of both immunosuppression and immune enhancement (references cited in Ohsawa et al., 1986). This is not unexpected in view of the complex cellular interactions governing the system and will clearly be dependent on the route of exposure to cadmium as well as the dose, length of exposure and function examined.

The mild lymphopenia with cadmium treatment was consistent with the finding by Ohsawa et al., (1983) after repeated s.c. administration of 1.0mg Cd^{2+}/kg for five days to mice. In addition Ohsawa and co-workers showed a differential reduction in blood B lymphocytes accompanied by a corresponding increase in splenic B lymphocytes and splenomegaly. Mice given cadmium in drinking water displayed no splenomegaly despite a similar splenic cadmium content and a reduction in blood T lymphocytes associated with an increase in the number of splenic T lymphocytes. Again, no mention was made of any local inflammatory response to injected cadmium although blood taken three days after the final injection showed no leucocytosis. Interestingly, higher levels of plasma corticosterone were found in cadmium-injected mice than in controls. Glucocorticoid hormone promotes the redistribution of lymphocytes causing mild lymphopenia (Cohen, 1972) and it was suggested by Ohsawa et al., (1983) that this may be the cause of the differential modification of lymphocyte distribution. Elevated levels of glucocorticoids accompany various stresses including acute inflammatory responses (Kushner, 1982; Suzuki et al., 1981), so this may well have been the underlying cause of the changes in lymphocyte distribution.
Koller et al., (1976) proposed that the altered immune response of mice depended on the route of exposure since cadmium given orally decreased IgG antibody synthesis in mice whereas it was increased following i.p. administration of the metal. This was proposed to be due to the higher retention of cadmium by the i.p. route but again may be related to the presence of an acute inflammatory response. The parenteral mode of cadmium administration has been extensively utilised in studies of immune competence and the usefulness of such a model is questionable in view of observations implicating the involvement of lymphocytes in the development of acute non-immune inflammation (Sannomiya et al., 1985) and the evident involvement of the spleen and probably other parts of the immune system in the response, as implicated by this study. Further studies into the role of the spleen in cadmium toxicity may elucidate its possible correlation with the manifestation of haemotologic or immunologic anomalies.

Neutrophils from cadmium-treated rats exhibited nuclear hypersegmentation and enhanced alkaline phosphatase activity which was typical of the neutrophilia of infections in humans (Wachstein, 1946). Neutrophil alkaline phosphatase (NAP) activity is also reported to be increased in man in a variety of stress conditions (Valentine et al., 1954) and accompanying tissue necrosis regardless of aetiology (Kaplow, 1968). The role of NAP has been stated to be unknown (Kaplow, 1968; Okun and Tanaka, 1978) but the enzyme is generally implicated in the processes of absorption and active transport across membranes. Since it is localised in the specific granules of the neutrophil (Spicer and Hardin, 1969) it is presumably involved in the process of intracellular lysosomal digestion of phagocytosed material. Wachstein (1946) proposed that increased NAP activity under inflammatory stimuli probably signified increased cell metabolism. Spiers et al., (1975) postulated that NAP activity decreases with cell age so the increased levels in circulating neutrophils may be due to the release of young strongly NAP positive cells from the bone marrow and spleen.
Alkaline phosphatase is a zinc metalloenzyme so it is noteworthy that cadmium treatment did not appear to reduce NAP activity as has been demonstrated for alkaline phosphatase in bone (Bonner et al., 1980) and in bone and intestine (Kobayashi and Kimura, 1985) after long-term dietary exposure of rats to cadmium, in rat lung following inhalation of cadmium (Boudreau et al., 1988) and after exposure of bone homogenates to cadmium in vitro (Sugawara et al., 1983). Enzyme activities may be altered by cadmium as a consequence of tissue damage and this was proposed as the process by which enzyme activity was reduced in the intestine.

That displacement of the essential zinc cation by cadmium occurs in vivo is speculative. More likely is a cadmium-induced redistribution of zinc resulting in localised zinc deficiencies and Bonner et al., (1980) attributed the reduced bone alkaline phosphatase activity to derangement of zinc homeostasis. Rosner and Lee (1972) showed that human NAP can vary in activity without direct relationship to zinc levels probably because two isoenzymes are present, one requiring zinc and the other, magnesium (Trubowitz et al., 1957). Results obtained by Trubowitz et al., suggested that the increased activity during infection in man was due primarily to increased Zn-NAP rather than magnesium. Rabbit NAP however, was not found to be so dependent on zinc and magnesium. It is not known which cation is present in rat NAP but it is postulated that the enzyme may be non-zinc dependent since antagonism by cadmium was not apparent.

The use of the technique of crossed immunoelectrophoresis (X-IEP) to detect multiple quantitative serum protein changes in response to the parenteral administration of cadmium to rats is believed to be novel. Although further analysis is required to establish the identity of the 29 proteins detected, marked treatment-related alterations in the concentrations of a large number of proteins were evident. Acute phase proteins have been defined as those whose plasma concentration rises 25% or more following stimulus (Kushner, 1982). Seventeen proteins showed such increases after cadmium administration including \( \alpha_1 \) acid glycoprotein, haptoglobin and caeruloplasmin, which are all recognised acute phase proteins in the rat (Kushner and Mackiewicz,
1987; Gauldie et al., 1985). The percentage increase in the concentrations of haptoglobin and caeruloplasmin as measured by X-IEP were closely comparable with those obtained after measurement of haptoglobin by rocket immunoelectrophoresis and of caeruloplasmin by its PPD oxidase activity. Haptoglobin levels were raised by a similar amount following administration of cadmium at both dose levels and remained elevated throughout both studies.

There were concomitant reductions in the concentrations of nine proteins, most notably, albumin. Such reductions during an acute phase response are believed to be a counter-regulatory phenomenon caused in some instances by a selective decrease in hepatic synthesis (Billingham and Gordon, 1976). Such proteins have been termed negative acute phase reactants and are an essential part of the response allowing the positive acute phase proteins to rise in concentration without producing an osmotic imbalance (Billingham and Gordon, 1976). In other instances the decrease in amounts of circulating proteins might also be due to a redistribution between plasma and other body fluids, for instance, local binding at the site of inflammation or accelerated catabolism. Cadmium interferes with the tubular reabsorption of low molecular weight proteins and the glomerular filtration of high molecular weight proteins in the kidney resulting in proteinuria (Lauwerys and Bernard, 1986). Therefore some reduction in plasma proteins might be expected to result from urinary loss but this seems unlikely to have played a part at the early time point investigated here since renal damage was not histologically evident until after six days. The differential alterations in protein concentration exclude haemoconcentration as the major cause of the increases. The reduced albumin probably accounts for the minimal reduction in total plasma protein seen after three days, in the face of high concentrations of so many other proteins.

The X-IEP pattern of serum protein changes observed after s.c. cadmium exposure showed marked similarity to the patterns obtained after X-IEP of serum following an acute inflammatory response provoked by i.p. administration of lipid A component (Abd-El-Fattah et al., 1976), subplantar carrageenan (Scherer et al.,
Changes in plasma protein concentrations after cadmium administration to animals have been reported previously. An initial reaction to repeated s.c. administration of 0.25mg Cd/kg to rabbits was a reduction in albumin and higher levels of α and β globulins including haptoglobin (Axelsson and Piscator, 1966) and it was stated that such an acute response would be expected in animals exposed to toxic substances. Zak and Dubin (1978) found similar changes with increased synthesis of fibrinogen, seromucoid fraction and glycoprotein and reduced albumin resulting in a slightly reduced total plasma protein 48 hours after i.p. injection of 1mg Cd/kg to rats. This indicates the occurrence of a typical acute phase response, however, no local effect of the i.p. injection was reported and it was suggested that cadmium accumulated in the liver as a cadmium-metallothionein complex and that this lead to enhanced synthesis of plasma proteins. Although this study does not provide direct evidence, it seems more likely that the synthesis of proteins is altered in response to locally derived chemical mediators, the presence of which were demonstrated in the peritoneal exudate of rats after i.p. cadmium administration (Giri et al., 1979). In contrast to these effects seen after parenteral administration of cadmium, the predominant change in serum protein following chronic exposure to cadmium via the diet (Jacobs et al., 1969) or drinking water (Lawford, 1961a) was enhanced transferrin associated with anaemia.

In the present study additional unidentified proteins were demonstrated in the serum from cadmium-treated animals which may have been present in control sera but in amounts too small to be detected by X-IEP. The most marked rise was shown by α₂B and this protein may correspond to α₂ macroglobulin otherwise referred to as α macrofetoglobulin (Van Gool et al., 1982) which is a major rat acute phase protein increasing 100-fold or more in concentration during inflammation.
(Kushner and Mackiewicz, 1987; Gauldie et al., 1985). However, it was surprising to find that the commercially available antiserum, raised to normal rat serum, contained a sufficient titre of antibody to precipitate large amounts of this protein since the protein is usually absent or present only in very small amounts in normal adults. This may account for the faint nature of the precipitate produced by protein α₂B. Sobocinski et al., (1981) measured the increase in plasma α macrofetoprotein as a marker in order to follow the inflammatory response provoked by the s.c. administration of 0.6mg Cd/kg or turpentine to rats.

The appearance of serum protein fractions not normally detectable in adult rats has been described during pathological conditions induced by administration of a number of agents, including cadmium. Lawford (1961b) described the appearance of an abnormal serum component amongst other minor changes detected by starch gel electrophoresis after i.p. administration of cadmium to rats. The same mode of administration of cadmium amongst other agents was used to study the effect of liver damage on serum levels of the same protein, termed embryonic α₂ glycoprotein. The most effective increases were produced by cadmium but were believed to be related to liver damage and no mention was made of inflammation (Stanslawski-Birencwaj et al., 1967). What was also believed to be the same protein was called α₂ acute phase protein by Weimer and Benjamin (1965). They found its presence to be accompanied by increases in haptoglobin, fibrinogen and seromucoid with reduction in albumin and total protein in a wide variety of pathological conditions including that produced 24 hours after i.p. administration of 1.0mg Cd/kg to rats. Interestingly, they also found α₂ acute phase protein to be increased after s.c. administration of sterile saline. It should be emphasised that minimal cellular infiltrates were evident at injection sites in control animals in the present study consistent with the administration of saline and which might well be expected to be accompanied by minor systemic changes.

The major changes in circulating acute phase proteins result from alterations in their synthesis which occurs primarily in the liver (Kushner and
Mackiewicz, 1987). Cadmium causes hepatic damage resulting in altered structure and function, especially when a large dose is administered via the parenteral route (Dudley et al., 1982). Histological examination of the livers in the present study however, revealed only minimal damage despite the large burden of cadmium and this was in sharp contrast to the extensive injury observed in the kidneys, testes and injection sites. This is presumably due to the high capacity of the liver to synthesise metallothionein which binds cadmium thus preventing its interaction with cellular organelles and subsequent disruption of biochemical processes. At the high dose of 2mg/kg i.p. the abnormal serum component of Lawford (1961b) was not produced and this was attributed to abnormal biosynthesis due to cadmium-induced cytotoxicity, probably because the cadmium-binding capacity of the liver had been overwhelmed. At the doses used in this study, the ability of the liver to produce appropriate quantities of the range of typical rat acute phase proteins seemed to be unimpaired. These proteins play essential roles in restoring the homeostatic balance disturbed by tissue injury (Gauldie et al., 1985).

Suzuki et al., (1985a) and Uehara et al., (1985) measured serum enzymes as markers of hepatic injury after a single s.c. injection of 1.0, 1.5 or 2.0mg Cd^{2+}/kg to rats. They were surprised to find no elevation thus indicating the limited extent of hepatic damage. The amount of serum cholinesterase (CHE), a secreted enzyme from the liver, was markedly reduced to minimum levels two to three days after the injection. Suzuki et al., (1985a) additionally showed decreased total serum protein largely attributable to reduced albumin, as demonstrated in the present study. Lowest levels were seen on day 2 which corresponds to the interval to maximum acute phase response. However, no inflammation was reported and the results were taken to suggest that cadmium inhibits the synthesis of secretory proteins and/or secretory processes in the liver (Mitane et al., 1987). Serum CHE was therefore stated to be a sensitive marker of cadmium toxicity in the liver since reduction occurred before the onset of serum increases in leaked enzymes from the liver.
However, reduced levels of rat serum CHE have also followed parenteral administration of carrageenan or lipid A component, both irritants which induce a systemic acute phase response (Scherer et al., 1977). Rather than as a direct effect of cadmium on the liver it seems more likely that changes in protein synthesis and secretion are mediated as part of a systemic response to cadmium-provoked inflammation.

Repeated administration of the high dose of cadmium resulted in increased liver weight relative to body weight which confirms the findings of Bonner et al., (1979) who stated that this probably reflected accumulation of the metal in the organ. More likely, the hepatomegaly is associated with enhanced synthesis of cadmium-induced metallothionein and acute phase proteins, the latter of which are known to cause an increase in liver weight (Weinberg, 1986; Little, 1978). The observed cytoplasmic basophilia of the hepatocytes which is indicative of protein synthesis would seem to support this particularly since the basophilia was predominantly in the periportal area where plasma protein synthesis is more marked (Rapaport, 1975).

It is appropriate here to point out that liver metallothionein synthesis, and hence alterations in zinc homeostasis, characterised by pronounced hypozincaemia and changed tissue levels of zinc occur not only in response to cadmium accumulation but also to inflammation (Maitani and Suzuki, 1981; Sobocinski et al., 1978) and as a result of food restriction (Bremner and Davies, 1975). Such coincidental responses will complicate any interpretation of quantitative studies into the induction of metallothionein by cadmium. Hence, the validity of using a parenteral mode of administration which not only causes inflammation but reduces food consumption in such studies must be questioned. Such responses also raise doubt about the purported induction of metallothionein by certain other metals since it may depend on the route of administration. For instance, Maitani et al., 1986) recognised the possible misinterpretation of results when they found that more hepatic metallothionein was induced after i.p. administration of lead than after i.v.
injection despite the higher hepatic lead concentration after the latter route. They suggested the possible role of mediators produced by peritoneal leucocytes after the i.p. route in the induction of metallothionein and hence some of the resulting changes in essential metals that were observed.

The rapid mitotic response of hepatocytes observed 24 hours after cadmium administration is an unusual finding. Hoffman et al., 1975) observed increased mitosis accompanying degenerative changes in neighbouring hepatocytes after i.v. administration of cadmium and attributed it to a regenerative response to cadmium-induced damage. Since damage to hepatocytes was not histologically evident in the present study, it is proposed that mitosis may be part of the inflammatory response. An alternative explanation was proposed by Dudley et al., 1982) who found increased mitosis 1 hour after a high i.v. dose of cadmium and which declined before the appearance of severe damage. They suggested that division was stimulated by a mechanism involving the interaction of cadmium with nuclear DNA in hepatocytes. The reduced liver weights seen after administration of cadmium at the low dose is in sharp contrast to the hepatomegaly associated with the higher dose and the reason for this is not clear, especially since cadmium appeared to initially induce mitotic division at both dose levels. Control animals in the low dose study displayed higher hepatic mitotic index initially than controls in the high dose study probably associated with the more rapid growth rate of younger animals. The effects of the cadmium-induced reduction in food consumption might be more readily manifest in rapidly growing rats with high metabolic requirements and result in reduced liver weight. This is supported by the observation (data not presented) that a 24 hour food restriction in normal rats causes a fall in body weight and a 25% reduction in absolute and relative liver weights. Suzuki et al., 1981) similarly found a transient reduction in liver weight after the i.p. administration of cadmium to mice. This clearly emphasises the differing responses that might result from the use of animals of slightly different ages and the problems of comparing such data.
It is tempting to conclude that all the changes in plasma protein concentrations induced by the s.c. administration of cadmium were attributable to the acute phase response, however, the synthesis of some of the proteins and metallothionein is certainly one, may have been primarily induced or suppressed as a direct response to cadmium. Nevertheless, it is likely that the marked alterations in the relative concentrations of plasma proteins together with the state of metabolic stimulation of the liver and other organs in response to acute inflammation may considerably alter the binding, transport and ultimate distribution and retention of cadmium from that which would occur after a mode of administration which does not provoke inflammation. For example, the $\alpha_2$ macroglobulin zinc-binding protein of humans has been shown to be a major cadmium binding protein in vitro (Watkins et al., 1977; Carson, 1984). Whilst $\alpha_2$ macroglobulin in man does not normally behave like an acute phase protein, an homologous protein in the rat, $\alpha$ macrofetoprotein or $\alpha_2$ macroglobulin (Kushner, 1982; Gauldie et al., 1985) is a major acute phase reactant, increasing over 100-fold in inflammation (Kushner and Mackiewicz, 1987). If this rat protein shows a similar avidity for cadmium in vivo, the raised levels present could bind substantial amounts of the metal and thus have a marked effect on its ultimate distribution and retention. Sites of catabolism of proteins, such as the liver, might then be expected to accumulate a considerable quantity of cadmium. In contrast, albumin which is a major human protein to which cadmium is loosely bound (Carson, 1984) falls in concentration during an acute phase response and this would reduce the amount of cadmium transported. In addition to quantitative changes, most of the acute phase glycoproteins undergo qualitative alterations manifest by changes in glycosylation patterns (Kushner and Mackiewicz, 1987) which might result in different cadmium-binding properties to those of normal serum proteins.

The measurement of plasma TIBC is a crude assessment of transferrin levels since this is the major iron-binding protein in plasma. The measurement of plasma iron therefore represents iron originating predominantly from transferrin
since the small amount that might be present in haemoglobin causes little interference in the method used in this study. Plasma ferritin, normally present in small amounts which increase slightly with inflammation (Birgegard and Caro, 1984) differs from intracellular ferritin in containing virtually no iron (Finch and Huebers, 1986). Transferrin and in particular, its level of saturation with iron are of particular relevance in this investigation into the effects of cadmium on iron homeostasis. Transferrin is considered as a negative acute phase protein in the rat (Gauldie et al., 1985) and TIBC has been shown to be decreased in concentration or unchanged during inflammation (O'Shea et al., 1973). Transferrin was shown by X-IEP to be reduced after one injection of cadmium but not after three, however, these results are inconclusive since they represent only one animal at each time-point. TIBC fell but not significantly until after six doses of 1.5mg Cd\(^{2+}\)/kg. In contrast, TIBC appeared to be higher after administration of cadmium at the low dose although not significantly until after 15 days. The reason for these discrepancies is not clear but may be an age and hence growth-related effect.

Cadmium administration caused profound changes in iron homeostasis in the rats, the most dramatic of which was a significant and prolonged hypoferraemia apparent 24 hours after the first injection and of similar magnitude after both dose levels. Decreased plasma iron was similarly observed 24 hours after a single s.c. injection of cadmium by Bonner (1980) and after two daily s.c. injections by Ashby et al., (1980). The diminished plasma iron levels could not be accounted for by reduced transferrin and the percentage saturation of TIBC with iron was greatly decreased. Plasma iron represents a multi-compartmental source of iron made up of a balance between iron delivered to plasma from haemoglobin breakdown, intestinal absorption and iron stores, and that removed for haem biosynthesis, cell metabolism, excretion and deposition into stores. In order to understand how cadmium was causing hypoferraemia it was necessary to assess which transport element of plasma iron was being affected.
The rat has a high dietary iron requirement with growing animals reported to absorb 52 times as much iron as man (Cook et al., 1973) whilst Dallman (1986) reported a peak iron requirement in the growing rat six times that seen in human infants. The transport of absorbed iron therefore represents a large proportion of rat plasma iron and any reduction in food consumption would quickly cause a deficiency in iron intake and be reflected immediately in the plasma iron concentration. Indeed, it has been demonstrated by Cook et al., (1973) that a 24 hour food restriction in rats caused a 25-50% decrease in plasma iron and these figures were confirmed for the University of Surrey rats during a separate study (data not presented). Although a marked reduction in food consumption was observed in all cadmium-treated rats it did not take place until the 24-48 hour period after dosing commenced by which time the plasma iron concentration had already fallen. Therefore, food restriction was not the primary cause of hypoferraemia although it may have been a contributing factor in keeping the plasma iron levels low at later time points.

It would be tempting to assume that decreased plasma iron after s.c. administration of cadmium is attributable to a direct interaction of the metal with iron metabolism but hypoferraemia is a well recognised component of the acute phase response observed after injection of a variety of agents which cause inflammation. It is believed to be primarily the result of impaired release of reticuloendothelial iron, derived from red cell breakdown, into plasma transferrin and this dramatic response has been regarded as a defence mechanism to withhold essential iron from bacterial pathogens (Weinberg, 1984; Letendre, 1985). The rapidity and magnitude of the response after the s.c. administration of cadmium suggests that hypoferraemia is probably a secondary effect of cadmium-induced inflammation. That Sugawara and Sugawara (1984a) found raised plasma iron 24 hours after two daily s.c. injections of a similar dose of cadmium to mice was surprising since hypoferraemia is a consistent finding in inflammation. Although no
inflammatory response was reported by Sugawara and Sugawara (1984a) it is suggested by the raised plasma caeruloplasmin which they observed.

Interestingly, the restoration of plasma iron levels towards normal at the end of the study was achieved primarily by increasing TIBC and not by raising the percentage saturation of TIBC with iron. The observed return of plasma iron concentration to normal may be associated with remission of inflammation but surprisingly occurred at a time before dosing at both levels of cadmium had ceased when presumably the episode of acute inflammation was continuing. Eosinophils play a part in the destruction of activators and mediators released from mast cells, and thus have a role in dampening the response. Their appearance might therefore account for the above observation as well as for the partial return of circulating neutrophils to normal levels despite the continued stimulus of injection. Compensation for low iron levels by increasing TIBC is an expected response in iron deficiency anaemia and therefore the raised TIBC seen after repeated administration of 1.5mg Cd^{2+}/kg may have been a response to the anaemia. Although anaemia was not manifested after the low dose of cadmium, hypoferraemia may have caused a sufficient deficiency of iron supply to the marrow to induce compensatory higher levels of TIBC.

De novo synthesis of ferritin in reticuloendothelial tissue is a rapid response in inflammation (Konijn and Hershko, 1977; Birgegard and Caro, 1984) and may create a driving force for the preferential incorporation of haem-derived iron into that pool (Letendre, 1985). It might be expected that this would be immediately reflected by a higher iron content in the liver and spleen. There was indeed a slight though non-significant and transient rise in iron content of the liver and spleen following administration of cadmium at 1.5mg/kg and a similar unexplained rise in hepatic iron was observed 24 hours after i.p. administration of 1.8mg Cd^{2+}/kg to mice (Suzuki et al., 1981). However, the total loss of plasma iron was only about 0.25μmol (estimated from a plasma volume of 10ml for a 250g rat (Ringler and Dabich, 1979) and a drop in iron concentration of 25μmol/l) which is
negligible in comparison to 15 μmol of iron present in the liver and 3 μmol in the spleen. The methods used in this study were of insufficient sensitivity to detect small changes in iron concentration of reticuloendothelial cells distributed throughout the body. More sensitive techniques which could be employed are to measure plasma iron turnover by the use of radiolabelled iron tracers or the measurement of ferritin synthesis. The higher iron content of the liver at later time points may have reflected continued accumulation of iron derived from senescent red cells due to impeded iron recycling and since intravascular haemolysis might have been expected in view of the presence of poikilocytes in peripheral blood smears, some of the iron may have derived from removal of haemoglobin-haptoglobin complexes by hepatocytes.

This study confirms the results of Ashby et al., (1980) and Bonner et al., (1979) that liver copper concentration is largely unaffected by the repeated s.c. administration of cadmium at early time-points but later considerable increases were seen after both dose levels. Also, in agreement with Ashby et al., (1980) is the observation that plasma caeruloplasmin increases in response to cadmium exposure, which consequently raises plasma levels of copper since caeruloplasmin accounts for 70-80% of plasma copper in the rat (Frieden, 1986). Ashby et al., (1980) additionally found that cadmium administration resulted in reduced biliary excretion of copper which was thought to be the result of cadmium-induced hepatic metallothionein synthesis with subsequent binding of copper which in turn stimulated the synthesis of caeruloplasmin. Sugawara and Sugawara (1984a) and Sugawara et al., (1984) also observed increased liver copper and plasma copper and caeruloplasmin following subcutaneous administration of cadmium to mice and similarly concluded that caeruloplasmin synthesis may be stimulated by high hepatic copper levels caused by induction of metallothionein by cadmium. However, they failed to explain why the high levels of copper seen in the liver following ingestion of cadmium were not also accompanied by high plasma caeruloplasmin; again, no mention was made of any inflammation. Several experiments have demonstrated that plasma caeruloplasmin
becomes elevated in response to increased hepatic copper levels (reviewed by Evans, 1973) although Holtzman and Graummitz (1970) suggested that copper does not stimulate the release of newly-synthesised caeruloplasmin. Recently, Sugawara and Sugawara (1987) showed that administration of copper to mice had no effect on caeruloplasmin activity, despite marked increases in hepatic copper and copper-metallothionein induction. Rat caeruloplasmin is an acute phase protein (Gauldie et al., 1985) and the results of this study suggest that its raised plasma concentration, especially during the early part of the study, may be primarily due to increased synthesis as a systemic response to local inflammation rather than in response to high levels of hepatic copper which were not anyway seen until well after the rise in caeruloplasmin. It is not known by the author but seems likely that biliary excretion of copper may be reduced as part of the inflammatory response via inflammation-induced synthesis of hepatic metallothionein, in order to provide a pool of copper required for caeruloplasmin synthesis.

The cause of the 2-fold rise in liver copper following the nine day period without dosing is not clear. It would be of interest to determine whether plasma caeruloplasmin fell to control levels by this time in a similar way to haptoglobin. Then the reduced utilisation of copper for caeruloplasmin synthesis might account for the accumulation of very high levels of hepatic copper presumably bound to the increased amount of metallothionein. This would also provide further evidence that caeruloplasmin was not being induced by high levels of copper. In contrast, Komska-Szumska and Czuba (1986) found a gradual reduction in liver copper concentration up to 35 days after cessation of repeated administration of 3mg Cd²⁺/kg to rats for four weeks. The reason for the discrepancy is not known but may be related to the higher dose of cadmium used.

The role of inflammation in disturbances of copper homeostasis might therefore explain some of the equivocal effects of different routes of cadmium exposure in rats. Namely, the increased plasma copper and caeruloplasmin and hepatic copper frequently seen after parenteral administration (Ashby et al., 1980;
Suzuki et al., 1981; Sugawara et al., 1984; Chmielnicka et al., 1985) in contrast to unchanged (Stonard and Webb, 1976) or lowered hepatic copper (Banis et al., 1969; Bunn and Matrone, 1966; Campbell and Mill, 1974) and decreased plasma copper and caeruloplasmin (Whanger and Weswig, 1970; Campbell and Mills, 1974; Sowa and Steibert, 1985) after dietary cadmium.

Ashby et al., (1980) showed that increasing doses of cadmium progressively inhibited biliary copper excretion whether cadmium was given by s.c. administration or by gastric intubation. That the effect was dose-related does not exclude inflammation from playing a part since there was some indication in the present study that the magnitude of the systemic response was closely associated with the severity of the induced local inflammation which was dependent on the dose of cadmium. The local effects of a high i.g. dose of cadmium on the gastrointestinal tract have not been investigated so comment cannot be made on the possible involvement of inflammatory processes in copper homeostasis following this mode of administration.

The proposed multifunctional role of caeruloplasmin has been well reviewed by Gutteridge and Stocks (1981) and more recently by Frieden (1986). The importance of caeruloplasmin during inflammation may be in its role in the supply of copper to essential copper-requiring proteins and its antioxidant activity may offer protection against the potential damaging effects of reactive oxygen species released by white blood cells during phagocytosis. However, a major role of caeruloplasmin depends upon its ferroxidase activity to facilitate the incorporation of iron into transferrin. Despite the low ferroxidase activity reported for rat caeruloplasmin it is considered to be adequate and indeed, essential for the mobilisation of iron from reticuloendothelial cells to transferrin in rat plasma (Frieden and Hsieh, 1976). Elevated levels of the protein would therefore seem to be inconsistent with the hypoferraemia of inflammation and increased hepatic iron. Sugawara et al., (1984) concluded that for this reason hepatic iron stores may not be related to caeruloplasmin activity. However, Piercy (1979) proposed that
caeruloplasmin may be synthesised in order to stimulate the fresh release of iron for restoration of plasma iron levels at the end of an inflammatory response. If this is the case, the highest levels of plasma caeruloplasmin in the present study might be expected at the later time-points when hypoferraemia was resolving. The number of measurements made were limited but certainly by 10 days there was a 4-fold increase in caeruloplasmin as compared to only 2-fold at three days.

Repeated s.c. administration of 1.5mg Cd\(^{2+}\)/kg provoked a moderate non-progressive anaemia as shown by reduced haematocrit and haemoglobin concentration and characterised by hypochromic cells and poikilocytes in peripheral blood films. The initial transient haemoconcentration may have been the result of acute fluid loss into the sites of oedema in the skin and testes. Dehydration due to loss of fluid from the blood would be expected to be rectified by increased water intake and decreased urinary output and a slightly raised water consumption was observed initially. Water consumption at the low dose was not measured and the cause of the apparent haemodilution is not clear.

The anaemia may have been the result of one or more events, the possible role of each of which will be discussed in turn. The first possibility is the loss of red cells by either haemolysis or haemorrhage. Haemolysis seems unlikely to have played a major role during the development of the anaemia since three features which are usually indicative of the process were not present. Firstly, haemolysis results in the accumulation and phagocytosis of abnormal red cells in the spleen and despite the splenomegaly microscopic examination showed no increased congestion by red blood cells. Additionally, an increase in Perls Prussian blue stainable iron derived from the breakdown of haemoglobin from phagocytosed cells would be expected and this was not observed throughout the study. Secondly, plasma haptoglobin levels usually provide a good indication of the amount of ongoing intravascular haemolysis since its function is to bind haemoglobin and remove it from the circulation. An ahaptoglobinaemia would result when an appreciable number of cells had haemolysed. However, with the large amounts of haptoglobin
which were induced in response to inflammation any reduction might well have been masked. The lower concentrations of plasma haptoglobin seen after the recovery period may have been the consequence of resolving inflammation or be associated with the increasing numbers of red cell fragments resulting in haemolysis. Axelsson and Piscator (1966) reported an initial increase in haptoglobin after s.c. administration of cadmium to rabbits followed by ahaptoglobin aemia after 11 weeks due to intravasal haemolysis.

A third event which would be expected to occur in response to haemolysis would be a compensatory reticulocytosis provided red cell production was normal, but this was not apparent during the period of dosing. The suppressing effect of cadmium on growth and activity, either directly or indirectly, via inflammation, might be expected to result in a lowered tissue oxygen requirement and therefore numbers of circulating red cells. This is not supported by the unchanged red cell count nor would it account for the presence of hypochromic and poikilocytic cells. However, the effect of cadmium on numbers of circulating red cells cannot be conclusive from these results since counts were only carried out up to 10 day and although both erythroblasts and normoblasts in the marrow showed a consistent reduction in numbers during the dosing period the results were only relative and may therefore simply mirror the increase in myeloid activity.

Withdrawal from cadmium treatment failed to restore the haematocrit and haemoglobin concentration to normal after nine days, despite a marked reticulocytosis at this time. This was achieved by increased erythropoiesis both in the marrow and spleen and may have been a response to mild haemolysis and/or removal of the suppressing effects of cadmium on growth and activity. Had the recovery period been extended the anaemia may have resolved although Suzuki et al., (1984) found a similar effect of cadmium loading, by repeated s.c. administration of 3.0mg/kg/day to rats, on haematocrit was still significant even 16 weeks after cessation of dosing.
Haemorrhage was observed in both subcutaneous and testicular tissue which would undoubtedly mean a loss of red cells from the circulation so the finding of unchanged red cell and reticulocyte counts early on was surprising unless compensatory re-entry into the circulation of red cells occurs from areas of sequestration, such as the spleen. Microscopic examination of this organ revealed apparently less congestion with red cells which would seem to support this. The anaemia resulting from s.c. administration of cadmium to rabbits (Berlin and Piscator, 1961) was in part attributed to an increase in plasma volume, ie, blood dilution, but this seems unlikely to be playing a major part here in view of the continual fluid loss into the skin and since it would not account for the changes in red cell morphology.

The evidence does not support an appreciable loss of red cells from the circulation and the possibility remains that the anaemia may be due to impaired red cell production. Erythropoietin (Ep) a glycoprotein hormone primarily of renal origin, stimulates red cell proliferation and differentiation in mammals in response to hypoxia (reviewed by Erslev et al., 1980). In addition Ep is thought to initiate and govern the rate of synthesis of haemoglobin in erythroblasts and early normoblasts (Harris and Kellermeyer, 1970). The production of Ep is decreased by a variety of disorders impairing renal function and which are often accompanied by anaemia (Erslev et al., 1980). The phagocytic mesangial cells of the glomerulus are proposed to have a role in Ep production (Paul et al., 1984). Although cadmium-induced kidney injury appeared histologically to be confined to the proximal convoluted tubules, alterations to glomerular function have been reported (Lauwerys and Bernard, 1986). Furthermore cadmium has been shown to impair the function and viability of rodent phagocytic cells (Amoruso et al., 1982; Hilbertz et al., 1986; Coin and Stevens, 1986) so it is possible that cadmium-induced kidney damage may result in an inappropriate Ep response. This might account for the anaemia, the onset of which coincided with the appearance of kidney damage. Measurement of plasma Ep levels might indicate whether this was a factor. However, extra renal production of
the hormone has been confirmed and the liver Kupffer cells have been implicated as an important source (Paul et al., 1984) so the animals may have been capable of maintaining an appropriate Ep response despite the kidney damage.

The inflammation induced by cadmium might also affect Ep levels since a lowered Ep response has been demonstrated during starvation, inflammatory and infectious disorders in humans (Erslev et al., 1980) and endotoxin-induced inflammation in mice with a relative reduction in cell division by early marrow normoblasts (Schade and Fried, 1976). The Ep producing apparatus may be intact but output reduced after cadmium administration because less oxygen is required for the reduced energy expenditure in food consumption, growth and activity. Conversely, a high metabolic turnover might be expected in order to maintain the inflammatory response. It is speculated that an inadequate Ep response might function to permit recruitment of bone marrow precursors into the myeloid proliferative compartment at the expense of erythroid cells.

Another hormone involved in erythropoiesis is testosterone and its major mode of action is the enhanced production of Ep through a renotrophic effect influencing kidney mass and augmenting the production of Ep (Rencricca et al., 1969, cited in Harris and Kellermeyer, 1970; Mann et al., 1968) although a more direct action of the hormone on erythropoietic marrow cells has been suggested (Reisner, 1966). Whatever its mode of action, levels of testosterone could conceivably have been reduced by high doses of cadmium due to the necrosis of the hormone-secreting Leydig cells of the testis and this too could be postulated to have an effect on erythropoiesis.

In addition to indirect hormonal effects on erythropoiesis tissue damage may have a more direct action on blood cells. The abnormal erythrocyte morphology which was seen after high cadmium doses was strikingly similar to that observed in the microangiopathic haemolytic anaemia which accompanies a number of human disease states including renal cortical necrosis (Harris and Kellermeyer, 1970). Mechanical factors appear to play a prominent role as the red cells pass...
through altered microvasculature in the formation of 'burr cells' 'triangle cells' 'helmet cells' schistocytes and spherocytes. It is proposed that mechanical trauma together with excessive exposure to toxic metabolites (and cadmium itself cannot be excluded) as the erythrocytes passed through the kidneys, testes and subcutaneous injection sites, which were all organs displaying extensive damage, may have contributed to the abnormal morphology. A consequence of altered shape might be enhanced packing of the cells during centrifugation which might account for the reduced haematocrit in the face of unchanged red cell counts. The absence of injury to kidneys and testes after the low dose of cadmium could in part explain why anaemia did not develop.

The anaemia accompanying cadmium intoxication has frequently been attributed to an interference of the metal with iron absorption resulting in a classic iron deficiency anaemia (see Introduction). The results of this study demonstrated a considerable accumulation of cadmium in the intestine considering the metal was not administered by an oral route. Presumably the intestine retains loosely bound cadmium from the blood passing through it. Nicholson et al., (1984) concluded that cadmium in the duodenum following parenteral administration of cadmium originated from either biliary excretion or pancreatic secretion. They observed a parallel pattern of accumulation and loss of cadmium by the intestine and spleen with a drop at about the same time after dosing by an i.p. route had ceased. It was suggested that cohorts of red blood cells binding cadmium were being eliminated from the blood by the spleen and the breakdown products excreted in the bile via the liver and reabsorbed by the duodenal mucosa.

El-Shobaki and Rummel (1985) reported a decrease in iron absorption during endotoxin-induced inflammation in the rat which was associated with a change of iron distribution between transferrin and ferritin in the jejunal mucosal cytosol in favour of ferritin. This may be an additional factor contributing to the hypoferraemia of inflammation. Ferritin also binds cadmium (Kochen and Greener, 1975) so if substantial amounts of this metal were bound to ferritin in place of iron,
the absorption of iron might become permanently blocked and eventually a true iron deficiency would arise. Iron concentration measured in the intestine during the present study remained unchanged but the method used was insufficiently sensitive to detect small changes in the distribution of iron within different iron pools. Nevertheless, altered intestinal absorption of iron seems unlikely to have played a major role in the development of anaemia in the short-term in view of the rapid onset of anaemia and since body iron stores were not depleted.

Anaemias that are hypochromic and microcytic generally result from defects in haemoglobin synthesis (Lee et al., 1976). It was reported back in 1947 by Wintrobe et al., that turpentine- or endotoxin-induced inflammation caused impaired haemoglobin synthesis and evidence suggests that the so-called anaemia of chronic disease (Lee, 1983) in which low haemoglobin levels are coupled with greater than normal levels of storage of iron may be caused by failure of mononuclear phagocytes involved in erythrocyte catabolism to release iron normally to the circulation (Harris and Kellermeyer, 1970; Roeser, 1980). If the situation of inadequate delivery of iron to developing red cells in the marrow persists long enough, as seems to be the case in the prolonged acute inflammatory response provoked by repeated s.c. administration of cadmium, then a significant hypochromia and microcytosis becomes evident in the peripheral blood whilst iron stores remain adequate or even increased. The time scale of the changes observed after cadmium administration make this mechanism a prime candidate for the cause of the anaemia. The osmotic fragility tests on red cells revealed the presence of increased numbers of cells with more resistance to lysis as would be expected of hypochromic cells plus a small population of cells with increased fragility which is consistent with the observed poikilocytes and cell fragments.

Quastel and Ross (1966) observed a drop in haemoglobin values without any apparent change in haematocrit following an initial haemoconcentration stage after i.m. administration of turpentine to rats and this mimics the situation seen between three and six days of treatment with saline in the present study although
the cause is not clear. The effect of injections of saline on haemoglobin was surprising in view of the absence of any apparent hypoferraemic response, however, it might reflect a temporary diversion of iron away from haemoglobin synthesis. Iron is required not only for haemoglobin but for other functional iron-containing compounds such as myoglobin, cytochromes and a variety of other iron-containing enzymes including those of the haem-biosynthetic pathway and any of these might be expected to be adversely affected by a restricted supply of iron.

The results confirm autoradiographic studies on mice (Berlin and Ullberg, 1963; Nordberg and Nishiyama, 1972; Samarawickrama, Chapter in Webb, 1979) in showing a considerable accumulation of cadmium in the marrow of rats after s.c. administration of cadmium. Berlin et al., (1961) reported a mucoid type of degenerative change in the fat and connective tissue stroma in marrows of rabbits given s.c. cadmium. Such changes were not apparent microscopically in this study although a brown discolouration of the marrow was evident macroscopically. It is speculated that reduced haemoglobin synthesis might be sufficient to cause a loss of red colouration. Since the blockade on the iron recirculation would be lifted with subsidence of inflammation, thus allowing a sufficient supply of iron for haemoglobin synthesis, this would account for the return to normal marrow colour after the recovery period. No difference in marrow iron concentration was apparent after three and ten injections of cadmium but the withholding of iron from erythropoietic cells does not necessarily imply reduced iron in the marrow since the phagocytic cells present in this tissue are also involved in iron recycling. No evidence of increased sequestration of iron as PPB stainable iron was seen in the marrow nor any of the other organs examined. This is in contrast to the PPB stainable iron found in marrow cells of cadmium treated rabbits by Berlin et al., (1961) however this may represent a species difference.

It is tempting to conclude that a restricted supply of iron to erythropoietic tissue due to impaired reticuloendothelial cell iron release as part of an inflammatory response is the fundamental cause of the anaemia following
parenteral administration of cadmium, however it should not be ruled out that cadmium may exert a more direct toxic influence on erythropoietic cells. Lutton et al., (1984) have demonstrated toxic effects of cadmium on in vitro erythropoiesis as shown by inhibitory effects on rat bone marrow erythroid colony forming unit (CFU_E) growth. The exact mechanism is not clear but could involve altered activities of enzymes of the haem biosynthetic and degradative pathways. A possible candidate is the zinc-dependent enzyme δ-aminolevulinic acid (ALA) dehydratase and preliminary unpublished studies in this department have suggested that the activity of this enzyme may be inhibited in the marrow of rats following oral administration of cadmium (Chantrell, 1981). Blood ALA dehydratase activity has been shown to be reduced in chick embryos (Prasad et al., 1985) and in rats (Kesten et al., 1980) exposed to cadmium.

Edwards et al., (1986) found that the release of iron from transferrin within iron-deficient reticulocytes in vitro is impaired by the presence of millimolar concentrations of cadmium resulting in decreased iron accumulation in the cells. It was proposed that cadmium might compete with calcium or magnesium in a metal-dependent enzyme system concerned with the release of iron from transferrin. The term 'polychromasia' attributed to stained blood films, refers to the presence of cells with a blue-grey appearance because they lack a full complement of haemoglobin and is usually associated with reticulocytes. However, there appeared to be raised number of these basophilic cells in films from cadmium-treated animals despite the absence of increased reticuloocyte counts. The appearance of such cells was also described by Stowe et al., (1974) in blood from rats given dietary cadmium and similarly found to be unassociated with reticulocytosis. It was considered to represent a failure of red blood cells to lose their initial quantities of RNA and acquire normal haemoglobin and was thus believed to be a manifestation of impaired iron metabolism.
Other trace metals are involved in haematopoiesis. Copper deficiency leads to a depletion of the mitochondrial copper protein cytochrome oxidase resulting in impaired intracellular handling of iron and reduced haemoglobin synthesis (Lee et al., 1976; Deur et al., 1981). Since cadmium alters copper homeostasis, both directly by inducing metallothionein and indirectly through inflammation, another possible mechanism by which haemoglobin synthesis might be altered following cadmium administration is by depleted availability of copper in the marrow resulting in ineffective erythrocyte handling of iron.

A further way in which cadmium might affect red cells is by a direct interaction with components of the cell membranes, possibly through reaction with sulphhydryl groups, thus modifying membrane properties (Suzuki et al., 1985b; Plishker, 1984). Results of in vitro incubation studies of rat red blood cells with cadmium (Kunimoto et al., 1986) suggest that the organisation of cell membranes, especially cytoskeletal networks is altered by cadmium accumulation in the cytoskeletal fraction, which results in acceleration of age-related changes such as shape change. Echinocytes were demonstrated which were very similar in appearance to the 'burr' cells described in this study. That similar changes occur in vivo was suggested by anaemia as demonstrated by reduced haematocrit, following a single s.c. administration of 1.0mg Cd/kg to rats (Kunimoto and Miura, 1986). Increasing red cell density was again demonstrated along with accelerated clearance of red blood cells. This was thought to result in accelerated red cell sequestration presumed to take place in the spleen since this organ displayed a marked increase in weight. However, since the spleen enlarged prior to the decrease in the haematocrit it seems unlikely that red cell sequestration was the primary cause of splenomegaly; no inflammatory effects were reported.

Clearly a complex interaction of events lead to normal erythropoiesis. A concept of the disturbances which might give rise to anaemia following the s.c. administration of cadmium is represented by the flow diagram in Figure 2.40 and it seems likely that a combination of factors may be involved. Evidence is lacking in
Figure 2.40
Possible Factors Involved in the Development of Anaemia Following
Repeated Subcutaneous Administration of Cadmium at 1.5 Cd\(^{2+}\)/kg/Day to Rats
Abbreviations Used: Cd = cadmium, Cu = copper, Fe = iron, Ep = erythropoietin, Hb = haemoglobin, Lf = lactoferrin, RE = reticuloendothelial, RBC = red blood cells.
this study to substantiate the involvement of each pathway depicted in the flow pattern but despite this limitation it is evident that the alterations caused by cadmium-induced inflammation particularly in relation to iron metabolism occupy a central position in the overall pattern of development of the anaemia. There is certainly scope for further investigation to elucidate the mechanisms involved in the effects of cadmium on erythropoiesis.

In conclusion, trace element interactions can be significant in the aetiology and consequences of cadmium intoxication. Much information on these interactions obtained from animal models using a parenteral mode of administration may require re-evaluation in view of the results in this Chapter indicating that many of the changes may be directly attributable to the inflammatory response.
CHAPTER 3

COMPARATIVE ASPECTS OF THE ACUTE INFLAMMATORY RESPONSE RESULTING FROM VARIOUS SUBCUTANEOUS TREATMENTS, INCLUDING CADMIUM, AND THE INFLUENCE OF ZINC PRETREATMENT ON ACUTE INFLAMMATION PROVOKED BY CADMIUM
Introduction

In view of the severity of inflammatory lesions induced at injection sites following s.c. administration of cadmium at two dose levels (Chapter 2) and the infrequency of reports of similar changes by other workers, it was decided to investigate whether this was a specific effect of cadmium or would be a feature reproduced by, for example, the administration of other metal salts. Endogenous metals such as iron, copper and zinc have been used experimentally, given not only as supplements in the diet but also frequently by a parenteral route, in order to load the body quickly and to alleviate the toxic effects of other metals such as cadmium. If an inflammatory response is inadvertently provoked by the parenteral administration of these metals, then, as discussed in Chapter 2, this would itself markedly alter metal homeostasis, amongst a number of other factors, possibly resulting in erroneous conclusions being drawn from such experimental investigations. Trace metal supplementation in humans is also commonly achieved by a parenteral route. Administration in this manner, rather than supplying additional iron, for instance, to sites where it is required might, on the contrary, lead to withholding iron as a consequence of the hypoferraemic response of inflammation, if present. Another route of entry of metals into the human body which makes an understanding of the mechanisms of metal-tissue interaction important is the use of metal implants in the form of prosthetic devices made of alloys.

A three-day multiple dosing schedule of metal administration was selected since this was found to induce the maximum inflammatory response with cadmium (Chapter 2). Comparisons were made between responses to equimolar concentrations of the physiological metals copper and zinc, and another non-physiological metal with no known biological function, aluminium. A further group was given copper at an equivalent weight (1.5mg/kg/day) to which cadmium was administered. Different chemical forms of cadmium may be absorbed and retained
at varying rates (Moore et al., 1973). To eliminate the possibility that the response to s.c. cadmium chloride was related to the presence of the chloride anions, three different chemical forms of cadmium were administered, as well as two different forms of copper and aluminium. If the type of anion influences the uptake of cadmium from the injection site, it would be expected to be reflected in the amount of metal reaching the target organs, hence, it was decided to measure liver and kidney cadmium concentration as an indication. Iron salts were excluded from the experimental protocol since it was not possible to maintain the solubility of the two chosen salts, ferrous sulphate and ferric chloride, in saline.

Additionally, for comparison, turpentine-induced s.c. inflammation in the rat was selected as a classical model of acute sterile inflammation whilst the 'foot pad oedema' model was used as an example of acute inflammation via a cell-mediated hypersensitivity response.

Particular attention was paid to the comparative aspects of local inflammation. The results of Chapter 2 gave some indication that the magnitude of the systemic response was appropriate to the intensity of the local reaction. Neutrophil counts and spleen weights showed dose-dependent increases whereas plasma iron and haptoglobin displayed a similar magnitude of response independent of cadmium dose. These parameters seem to be very sensitive indicators of the systemic inflammatory response in the rat and were therefore selected to compare the effects of administration of the agents. Since diminished appetite is also a component of the acute inflammatory response (McCarthy et al., 1985) food intake and body weight gain were also monitored.

A number of studies with rodents have indicated that pretreatment or simultaneous administration of zinc can provide protection against the consequences of acute cadmium toxicity such as testicular damage (Gunn et al., 1961, 1963a, 1968a; Parizek, 1957; Webb, 1972), lethality (Gunn et al., 1968a; Leber and Miya, 1976; Shippee et al., 1983) hepatotoxicity (Goering and Klaassen, 1984) inhibition of
hepatic microsomal drug metabolism (Early and Schnell, 1978) and altered pancreatic and hepatic carbohydrate metabolism (Merali and Singhal, 1976). The protective properties exhibited by zinc in vivo have also been demonstrated for cadmium-induced cytotoxicity under in vitro conditions for fibroblasts (Borenfreund and Puerner, 1986; Frenial et al., 1986) hepatocytes (Stacey and Klaassen, 1981) rat bone marrow erythropoietic cells (Lutton et al., 1984) and isolated seminiferous tubules (Kar et al., 1966). Furthermore, the development of cadmium-induced tumours both locally at s.c. injection sites and systemically in the interstitial tissue of testes in rats and mice is inhibited by the s.c. administration of zinc acetate at different sites (Gunn et al., 1963b; Gunn et al., 1964). There is a significant correlation between the development of local cancers and the presence of infectious or non-infectious inflammatory processes (Templeton, 1975), so since the s.c. trauma and subsequent tissue reaction caused by the injection of cadmium might be a prerequisite for tumour formation, it was decided to investigate whether the acute inflammatory response provoked by the s.c. administration of cadmium could also be alleviated by zinc.

High levels of zinc exceeding those of cadmium in terms of molarity by a factor of between five and 100 have been used in previous studies with the higher doses being administered at distant sites. A dose level of 5mg Zn^{2+}/kg, as used by Ashby et al., (1980) and Bonner (1980) was chosen and given as a pretreatment and simultaneously with cadmium (1.5mg/kg) at the same site. This amounted to a total dose exceeding the molarity of cadmium by a factor of 12. The severity of inflammation was determined by assessment of local oedema, microscopic examination of injection sites and testes as well as the magnitude of selected systemic changes.
3.2 Comparison of the Acute Inflammatory Responses Provoked by Various Subcutaneous Treatments

3.2.1 Materials and Methods

3.2.1.1 Animals and Treatment Schedule

Male Wistar albino rats were maintained as previously described in Chapter 2. After an acclimatisation period, groups of three animals were given equimolar amounts of cadmium, copper, aluminium or zinc as solutions of various salts of the metals made up in 0.9% w/v saline, as follows:

<table>
<thead>
<tr>
<th>Metal Salt</th>
<th>Formula</th>
<th>Molecular Weight</th>
<th>Weight of Metal mg/kg/day</th>
<th>pH of Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>NaCl</td>
<td>-</td>
<td>(0.9%)</td>
<td>8.22</td>
</tr>
<tr>
<td>Cadmium chloride</td>
<td>CdCl₂₂H₂O</td>
<td>288.31</td>
<td>1.5</td>
<td>6.16</td>
</tr>
<tr>
<td>Cadmium sulphate</td>
<td>3CdSO₄.8H₂O</td>
<td>769.51</td>
<td>1.5</td>
<td>6.54</td>
</tr>
<tr>
<td>Cadmium acetate</td>
<td>(CH₃COO)₂Cd.2H₂O</td>
<td>266.53</td>
<td>1.5</td>
<td>7.36</td>
</tr>
<tr>
<td>Copper chloride</td>
<td>CuCl₂.2H₂O</td>
<td>170.49</td>
<td>0.85</td>
<td>4.95</td>
</tr>
<tr>
<td>Copper chloride</td>
<td>CuCl₂.2H₂O</td>
<td>170.49</td>
<td>1.5</td>
<td>4.78</td>
</tr>
<tr>
<td>Aluminium chloride</td>
<td>AlCl₃.6H₂O</td>
<td>241.43</td>
<td>0.36</td>
<td>3.77</td>
</tr>
<tr>
<td>Aluminium sulphate</td>
<td>Al₂(SO₄)₃.16H₂O</td>
<td>630.38</td>
<td>0.36</td>
<td>3.99</td>
</tr>
<tr>
<td>Zinc sulphate</td>
<td>ZnSO₄·7H₂O</td>
<td>287.55</td>
<td>0.87</td>
<td>6.11</td>
</tr>
</tbody>
</table>

All compounds were of analytical grade and obtained from BDH Chemical Company, Poole. The pH of the solutions was recorded. For all groups except (a), the dose was 13.35μmol metal/kg/day (as given to the high dose cadmium group in Chapter 2) and administered by subcutaneous injection in alternate abdominal flanks for a total of three days. Control animals received an equal volume (1ml/kg) of 0.9% saline. A further group of five rats (a) were given copper chloride solution at the higher dose level of 23.6μmol Cu²⁺/kg/day.
A single injection of turpentine (0.5ml) obtained from Banks and Son, Walton on Thames, was administered subcutaneously in the flanks of an additional five animals. In all cases injection sites were encircled with a marker pen. Food consumption was recorded daily and the general condition of the animals including the development of any local reactions at injection sites, was monitored at regular intervals after dosing.

For the foot pad oedema model, heat killed *Mycobacterium tuberculosis*, human strain C, DT and PN mixed (Central Veterinary Laboratories, MAFF, Weybridge) was finely ground and freshly suspended (10mg/ml) in liquid paraffin BP. The mixture, widely referred to as Freund's complete adjuvant (FCA), was used for both the immunisation and challenge procedures. On day 0, rats were immunised by inoculation subcutaneously in the scruff with FCA (75μl). On Day 6, the rats were lightly anaesthetised with ether, inoculated (challenged) s.c. in the right hind paw with FCA (50μl) and then allowed to recover and maintained for either one day or three days (n = 5). The control group comprised animals that were not challenged with FCA.

3.2.1.2 Autopsy and Analytical Procedures

Autopsy procedures and subsequent measurements were carried out as previously described in Chapter 2.

EDTA-anticoagulated blood was used to determine haematocrit, haemoglobin concentration, total and differential white cell counts. Plasma separated from heparinised blood was frozen for subsequent measurement of caeruloplasmin, haptoglobin, iron and total iron-binding capacity. Liver and kidney samples from cadmium-treated animals were frozen for later analysis of cadmium content. Spleens were weighed and samples fixed for histology together with skin and underlying subcutaneous tissue taken from injection sites after shaving the fur with Oesterclippers. Oedema at injection sites was assessed by measuring the thickness
of the skin and subcutaneous tissue in haematoxylin and eosin stained sections using a microscope eyepiece micrometer graticule which was calibrated against a stage micrometer. Sections of injection sites from aluminium-treated animals were also stained histochemically by the fluorescent Morin method to demonstrate aluminium (Pearse, 1972, p1407).

3.2.2 Results

3.2.2.1 Body Weight Gain, Food Consumption and General Condition of Animals

All cadmium-treated groups exhibited a marked reduction in food consumption during the 24 to 48-hour period after the first injection (as reported in Chapter 2) accompanied by a slight reduction in body weight gain. Other metals had no effect on body weight gain despite a transient drop in food consumption 24 to 48-hours after the initial dose of copper chloride and aluminium sulphate (results not presented). Mean terminal body weights for metal-treated animals were between 267-281g and showed no statistically significant difference from controls. The mean terminal body weight for the turpentine group was 240 ± 8g and for the 0, 1 and 3 day food pad oedema (FCA) groups between 219 and 231g. The general condition of all animals remained good. Animals dosed with copper and aluminium salts (ie, solution of pH less than 5, see Table 3.1) flinched initially as the solution made contact with the subcutaneous tissue but otherwise no general adverse reactions were observed.

3.2.2.2 Injection Sites - Macroscopic Appearance

Administration of solutions gave rise initially to a small palpable s.c. mass corresponding to the input of fluid. This disappeared within four hours in saline-and aluminium-treated animals, but in the case of cadmium-treated groups was replaced by marked swelling which increased in size with time (as described in Chapter 2). Copper and zinc salts also produced swelling but this took longer to develop and was
minimal in comparison to that produced by cadmium. The adhesion between the skin and underlying layers characteristic of the cadmium lesion was not apparent in copper and zinc groups. Only cadmium-treated animals displayed the sharply defined red circles surrounding injection sites although superficial lesions developed following some of the copper injections.

Turpentine did not cause any apparent initial irritant response but gave rise to an intense swelling which was more extensive in area than that produced by cadmium. On dissection, pockets of residual turpentine were evident by the odour and these were surrounded by margins of white tissue. There was some adhesion of the skin to underlying layers. A marked swelling of the paws was apparent one to three days after challenge with FCA in the foot pad oedema groups.

3.2.2.3 Injection Sites - Assessment of Oedema

Figure 3.1 shows the results of the measurement of skin thickness at injection sites following the administration of saline, metal salts or turpentine. The most intense oedema (maximal after 48 hours) was seen in the presence of cadmium and also 24 hours after turpentine administration. A significant oedema was also caused by copper and zinc salts but reached less than half the increase in skin thickness over control values that was seen with cadmium. Only a slight increase in skin thickness was seen three days after administration of the aluminium salts.

3.2.2.4 Injection Sites - Histopathology

Histological examination of injection sites from saline and all cadmium-treated animals revealed the same picture as already described in full in Chapter 2. In short, cadmium produced an intense acute inflammatory response characterised by marked oedema and necrosis and extending from the epidermis through to the underlying abdominal musculature with little sign of any repair or resolution.
Figure 3.1
Skin Thickness at Injection Sites as a Measure of Oedema Following Various Subcutaneous Treatments

The measurement includes epidermis, dermis and subcutaneous tissue but excludes the underlying abdominal musculature. Metal salts were administered at a dose level of 13.35 μmol metal/kg/day for 3 days except copper chloride (a) which was given at 23.6 μmol Cu²⁺/kg/day. Turpentine (0.5 ml) was administered once, 24 hours prior to sampling. All values are means ± SEM where n = number of samples per group. Statistically significant differences between the test groups and the mean of all the saline treated samples are shown as * p < 0.05, ** p < 0.01, *** p < 0.001.
In contrast, inflammatory reactions were minimal in aluminium injection sites and consisted of an initial influx of white cells, chiefly mononuclear in character and confined mainly to the subcutaneous tissue (Figure 3.2a). There was little sign of necrosis or oedema and the local inflammation subsided by three days into small granulomas containing material which was shown by the Morin fluorescent staining technique to be aluminium.

Zinc caused moderately extensive subcutaneous inflammation, especially by day 3, as shown by oedema and infiltrating inflammatory cells of both polymorphonuclear and mononuclear type (Figure 3.2b). It frequently involved the panniculus carnosus and in one case, the subjacent layer of the deep abdominal musculature also. However, significant numbers of inflammatory cells were present in the dermis of only one of the sections examined. There was only minimal necrosis, no superficial lesions were evident and the lesion appeared to be well contained with repair processes at work.

Copper caused similar changes to zinc but they were more intense and apparent earlier. There were quite large superficial lesions (up to 9mm in diameter) in several of the sections which were plugged with fibrin and collagen and undergoing active repair as shown by the presence of fibroblasts. Subcutaneous capillaries were noticeably dilated and in contrast to cadmium lesions, large numbers of intact polymorphonuclear and mononuclear inflammatory cells were scattered throughout the lesion. Inflammation was quite marked in the panniculus carnosus and first layer of the abdominal muscle. However, the lesion seldom penetrated further and on the whole appeared to be well contained with areas of fibrosis in the muscle layers. The characteristics were the same when copper was administered at the higher dose except that inflammation affected a slightly larger area and penetrated through the muscle layers marginally more. Again, superficial lesions were present but repair processes were clearly active (Figure 3.2c,d).
Figure 3.2(a)
Subcutaneous Tissue at Injection Site 2 Days After Administration of Aluminium Chloride (13.35μmol Al\(^{3+}\)/kg/day)
Note: granuloma confined to subpannicular connective tissue, boundaries indicated by arrows.
Stain: haematoxylin and eosin. Magnification: x40

Figure 3.2(b)
Subcutaneous Tissue at Injection Site, 24 Hours After Administration of Zinc Sulphate (13.35μmol Zn\(^{2+}\)/kg)
Note: moderate oedema of s.c. tissue; predominantly mononuclear inflammatory cells, I, infiltrating panniculus carnosus, PC.
Stain: haematoxylin and eosin. Magnification: x40
Figure 3.2(c)
Subcutaneous Tissue at Injection Site 24 Hours After Administration of Copper Chloride (23.6μmol Cu²⁺/kg)
Note: marked oedema, O, of subpannicular connective tissue, SCT, predominantly mononuclear inflammatory cells, I, infiltrating panniculus carnosus, PC.

Stain: haematoxylin and eosin. Magnification: x40

Figure 3.2(d)
Panniculus Carnosus at Injection Site 24 Hours After Administration of Copper Chloride (23.6μmol Cu²⁺/kg)
Note: large number of mononuclear inflammatory cells, MN, and fibrosis, F, in place of muscle fibres.

Stain: haematoxylin and eosin. Magnification: x400
Turpentine caused the formation of large subcutaneous abscesses with broad rims of intense suppuration around residual pockets of turpentine (Figure 3.2e). These abscesses also penetrated the dermal tissue in some areas and peripherally there was extensive oedema of the surrounding subcutis, overlying dermis and subjacent deep musculature with diffuse infiltration of these tissues by large numbers of polymorphonuclear and mononuclear cells.

One day after FCA challenge an intense acute inflammatory response was provoked in the foot pad with oedema, early necrosis and large numbers of polymorphs (Figure 3.2f). Small vessels were congested with some showing margination of polymorphs. By three days there was still an acute response in the centre of the lesion together with some haemorrhage but the reaction had become more mononuclear in character with some fibroblast activity around the periphery of the lesion.

3.2.2.5 Spleen Weights and Histopathology

There were no significant differences in the relative spleen weights between the treatment groups and controls (Range of means 0.28 to 0.36%) except for the three day foot pad oedema group where the mean relative spleen weight had increased to 0.50 ±0.03% compared to 0.40 ± 0.03% (p < 0.05) for the control group.

Similar pathological changes were seen in the spleens from cadmium-treated groups as described previously (Chapter 2) and increased myeloid activity was also evident in the three-day foot pad oedema group. No abnormalities were detected in any other group.

Haematology

3.2.2.6 Haematocrit and Haemoglobin Concentration

No significant differences in haematocrit were seen between groups (results not presented). Haemoglobin concentrations remained unchanged except for a
Figure 3.2(e)

Subcutaneous Tissue at Injection Site After Administration of Turpentine

Note: abscess with rim of intense suppuration, S, round residual pocket of turpentine, T, and surrounding oedema, O, of subcutaneous tissue.

Stain: haematoxylin and eosin. Magnification: x40.

Figure 3.2(f)

Tissue at Injection Site 24 Hours After Challenge with Freund's Complete Adjuvant

Note: intense acute inflammatory response with marked oedema and dense infiltrate of polymorphs, PMNL.

Stain: haematoxylin and eosin. Magnification: x40
significant reduction in the low dose copper chloride group (12.16 ± 0.15g/dl; p < 0.05) and copper sulphate group (11.85 ± 0.09g/dl; p < 0.01) as compared to the control group (13.35 ±0.28g/dl).

3.2.2.7 Total White Cell Count

All cadmium-treated groups displayed markedly elevated total white cell counts (p < 0.05) as compared to controls (Figure 3.3). Zinc sulphate administration also induced moderately higher numbers of circulating white cells (p < 0.05) as did FCA challenge by both one and three days (p < 0.001) whilst no statistically significant differences were seen with other groups.

3.2.2.8 Differential White Cell Count

As seen in Chapter 2, cadmium administration caused a complete reversal of the usual lymphocyte: neutrophil ratio (Figure 3.4a) (cadmium chloride p < 0.01; cadmium sulphate p < 0.001; cadmium acetate p < 0.01). This was also the case one day after FCA challenge in the foot pad oedema group (p < 0.001) and a significant shift in the ratio was still apparent three days after challenge (p < 0.01). The percentage of monocytes was significantly increased in the cadmium acetate group (p < 0.05) and three day FCA group (p < 0.001) but reduced in the one day FCA group (p < 0.01).

Calculated as absolute numbers of cells (Figure 3.4b) all cadmium groups as well as the one day FCA group exhibited an intense neutrophilia with the cadmium sulphate group reaching a mean 10-fold increase in the numbers of circulating neutrophils over control values. Neutrophil counts were still significantly elevated three days after FCA challenge (p < 0.001) although they were lower than after one day. No significant differences from controls were observed in numbers of neutrophils in other groups.
Figure 3.3

Total White Cell Count Following Various Subcutaneous Treatments

Metal salts were administered at a dose level of 13.35μmol metal/kg/day for 3 days except copper chloride (a) which was given at 23.6μmol Cu²⁺/kg/day. All values are means ± SEM where n = number of samples per group. Statistically significant differences between test and the appropriate control groups are shown as * p < 0.5, ** p < 0.001.
Figure 3.4

Differential White Cell Counts Following Various Subcutaneous Treatments: (a) Percentage of Cells Counted, (b) Absolute Numbers of Cells

Metal salts were administered at a dose level of 13.35 μmol metal/kg/day for 3 days except copper chloride (a) which was given at 23.6 μmol Cu²⁺/kg/day. In (b) all values are means ± SEM where n = the number of samples per group. Statistically significant differences between test and the appropriate control group are shown as * p < 0.05, ** p < 0.01, *** p < 0.001.
Lymphocyte numbers remained unchanged with the exception of slightly raised numbers in the zinc sulphate groups ($p < 0.05$) and reduced numbers in the turpentine ($p < 0.05$) and one day FCA ($p < 0.01$) groups. Numbers of eosinophils were not affected by treatment but monocytes were elevated in numbers in the cadmium sulphate ($p < 0.05$) cadmium acetate ($p < 0.001$) copper sulphate ($p < 0.05$) and three day FCA ($p < 0.001$) groups. Neutrophils with hypersegmented nuclei and monocytes with atypical morphology as described in Chapter 2 were seen following cadmium and FCA treatment.

**Biochemistry**

3.2.2.9 Plasma Haptoglobin and Caeruloplasmin

Figure 3.5 shows the mean rocket height obtained by immunoelectrophoresis and clearly indicates the higher concentrations of haptoglobin (about 2-fold) present in the plasma of cadmium, copper, turpentine and FCA-treated animals ($p < 0.001$). Haptoglobin levels were also moderately raised in aluminium chloride ($p < 0.01$) and zinc sulphate ($p < 0.05$) groups. Visual inspection of the rockets formed revealed that samples from all cadmium-, turpentine- and FCA-treated animals produced wider, more rounded and less well-defined rockets in comparison to the well-peaked rockets with sharp outlines from all other samples. This suggests that the rockets might have become taller with continued electrophoresis and that the relative amount of haptoglobin in plasma from cadmium, turpentine and FCA groups may well have been more than indicated by the measurement of peak height.

Plasma from cadmium, copper, turpentine and FCA-treated animals displayed a blue colouration indicative of the presence of copper. All groups showed a significant rise in caeruloplasmin concentration above control values except aluminium sulphate (Figure 3.5). The highest (3-fold) increases were seen one day after turpentine administration and three days after FCA challenge.
Figure 3.5
Plasma Haptoglobin and Caeruloplasmin Following Various Subcutaneous Treatments

Metal salts were administered at a dose level of 13.35μmol metal/kg/day for 3 days except copper chloride (a) which was given at 23.6μmol Cu^{2+}/kg/day. All values are means ± SEM where n = the number of samples per group. Statistically significant differences between test and the appropriate control groups are shown as * p < 0.05, ** p < 0.01, *** p < 0.001.
3.2.2.10 Plasma Iron and Total Iron-Binding Capacity (TIBC)

Figure 3.6 shows that all cadmium-treated animals experienced a significant drop in plasma iron concentration (p < 0.01) whilst TIBC remained unchanged, as was seen in Chapter 2. This represented a drop in the percent saturation of TIBC with iron from 83% to 27% in the case of cadmium acetate. The most dramatic fall in plasma iron concentration occurred one day after administration of turpentine or FCA with values of less than $10 \mu\text{mol/l}$ compared to $35-38 \mu\text{mol/l}$ in controls. However, three days after FCA challenge plasma iron levels had returned to normal. Copper sulphate and copper chloride (high dose) groups displayed a slight decrease in plasma iron (p < 0.05) and the latter group also showed a significant reduction in TIBC (p < 0.05).

3.2.2.11 Liver and Kidney Cadmium

Cadmium concentration (µmol/g) and content (µmol) was determined in the liver and kidneys of cadmium-treated animals:

<table>
<thead>
<tr>
<th>Table 3.2</th>
<th>Liver Cadmium Concentration and Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organ</td>
<td>Group</td>
</tr>
<tr>
<td>Liver</td>
<td>Chloride</td>
</tr>
<tr>
<td></td>
<td>Sulphate</td>
</tr>
<tr>
<td></td>
<td>Acetate</td>
</tr>
<tr>
<td>Kidneys</td>
<td>Chloride</td>
</tr>
<tr>
<td></td>
<td>Sulphate</td>
</tr>
<tr>
<td></td>
<td>Acetate</td>
</tr>
</tbody>
</table>

All values are means $\pm$ SEM. * p < 0.05

A significantly higher concentration of cadmium was present in the livers of cadmium acetate-treated rats than in the livers of chloride or sulphate groups (p < 0.05). There was also a tendency towards higher liver cadmium content and kidney concentration and content of cadmium in the acetate group although the difference
Figure 3.6

Plasma Iron Concentration, Total Iron-Binding Capacity, TIBC (b) and Percentage Saturation of TIBC with Iron (a), Following Various Subcutaneous Treatments

Metal salts were administered at a dose level of 13.35 μmol metal/kg/day for 3 days except copper chloride (a) which was given at 23.6 μmol Cu²⁺/kg/day. All values in (b) are means ± SEM where n = the number of samples per group. Statistically significant differences between test and the appropriate control groups are shown as * p < 0.05, ** p < 0.01, *** p < 0.001.
was not statistically significant. This trend was mainly attributable to one animal which had accumulated nearly twice as much cadmium in the liver and kidneys as other individuals and a second animal that had accumulated more cadmium in its liver.

3.3 The Effect of Zinc Pretreatment on the Acute Inflammatory Response Provoked by the Subcutaneous Administration of Cadmium

3.3.1 Materials and Methods

3.3.1.1 Animals and Treatment Schedule

Dosage solutions were prepared as shown in Table 3.3. The pH of solutions was recorded and they were sterilised prior to use.

Table 3.3

<table>
<thead>
<tr>
<th>Dosage Solutions</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Saline</td>
<td>6.16</td>
</tr>
<tr>
<td>2. Zinc</td>
<td>4.95</td>
</tr>
<tr>
<td>3. Cadmium</td>
<td>5.82</td>
</tr>
<tr>
<td>4. Zinc and Cadmium</td>
<td>5.14</td>
</tr>
</tbody>
</table>

Twenty male Wistar albino rats were sorted into groups of five and maintained as previously described in Chapter 2. Dosing was a two stage procedure consisting of pretreatment with zinc or saline solution and treatment with saline, zinc, cadmium or zinc plus cadmium 24 hours after pretreatment according to the schedule in Table 3.4.
Table 3.4  

<table>
<thead>
<tr>
<th>Group</th>
<th>Pretreatment</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Saline</td>
<td>Saline</td>
</tr>
<tr>
<td>Zinc only</td>
<td>Zinc</td>
<td>Zinc</td>
</tr>
<tr>
<td>Cadmium only</td>
<td>Saline</td>
<td>Cadmium</td>
</tr>
<tr>
<td>Zinc &amp; Cadmium</td>
<td>Zinc</td>
<td>Zinc &amp; Cadmium</td>
</tr>
</tbody>
</table>

Doses were administered subcutaneously to the left flank and the injection site was then circled with a marker pen. The second injection was given into the same site. Injection sites were inspected at regular intervals after dosing.

3.3.1.2 Autopsy and Analytical Procedures

Autopsy procedures and subsequent measurements were carried out as previously described in Chapter 2. The experiment was terminated one day after treatment. EDTA-anticoagulated blood was used to determine haematocrit and total and differential white cell counts. Plasma was separated from heparinised blood for the measurement of iron and caeruloplasmin concentrations. Testes were examined, weighed and fixed for histology.

The injection site was shaved and examined for any signs of inflammation. A 4.5 x 4.5cm square of skin and underlying subcutaneous tissue encompassing the dose site was dissected from the deep musculature. This area was chosen by taking a cadmium-treated animal first, visually assessing the extent of the oedema and determining the square that included the lesion. Hence, a fixed area of skin from each animal was obtained for comparison of oedema which was assessed by determining (a) the weight of the sample, and (b) the thickness at point of maximum oedema. The latter was measured by placing the sample flat on a solid surface and
using a Vernier height gauge. The skin was then fixed for histology, sections prepared and stained with haematoxylin and eosin and by Von Kossa technique for calcium.

3.3.2 Results

3.3.2.1 Body Weight Gain and General Condition of Animals

Treatment had no effect on body weight gain or the general condition of the animals. The mean terminal weights were between 231-238g.

3.3.2.2 Injection Sites - Macroscopic Appearance

Administration of the pretreatment saline or zinc solutions gave rise initially to a small palpable subcutaneous mass corresponding to the input of fluid. This had dissipated completely by four hours after dosing in saline-treated animals whilst zinc pretreatment gave rise to an area of swelling of approximately 1-2cm diameter.

Treatment doses administered one day after the first injection again produced a palpable mass, temporary with respect to the saline group, but giving rise to swelling in all other groups. This was apparent by two hours and at four and six hours seemed more pronounced in the cadmium only group, this group alone also displayed a tendency towards testicular reddening and swelling.

At autopsy, the bulk of the cadmium-induced oedema was generally encompassed by the chosen 4.5 x 4.5cm sample area although in some cases the oedema had spread ventrally and was not therefore all included in the sample. The oedematous lesions at injection sites of animals given cadmium only were clearly larger than those of animals given either zinc only or zinc plus cadmium and which produced lesions of comparable size. Superficial erythematous rings were present in all groups except controls whilst zinc-treated animals also displayed subcutaneous tissue which was white in appearance.
3.3.2.3 Injection Sites - Assessment of Oedema

The weights of skin samples and thickness of skin and subcutaneous tissue are shown in Figure 3.7 a and b. All three groups given zinc and/or cadmium showed a significant increase in mean weight and thickness of the skin samples as compared to the saline-treated group with the difference being more marked in the cadmium only group. There were no significant differences in the mean weights or thicknesses between the zinc only group and the zinc plus cadmium group but samples from the cadmium only group were significantly heavier (p < 0.01) and thicker (p < 0.01) than the zinc only group and also the zinc-pretreated cadmium group (weight p < 0.001; thickness p < 0.01).

3.3.2.4 Injection Sites - Histopathology

Both saline and cadmium only injection sites displayed the same histological picture as already described in Chapter 2. As with cadmium, zinc treatment produced a marked inflammatory reaction affecting both the subcutaneous and dermal tissue with necrosis of the panniculus carnosus. Sections from two zinc injection sites had areas of epidermal hyperplasia overlying the worst of the damage. Oedema was evident although it did not seem as extensive as following cadmium treatment. Numerous inflammatory cells were present in all layers, both mononuclear and polymorphonuclear, but far fewer appeared necrotic than was the case with cadmium. In the subcutis and lower part of the dermis there were numerous basophilic and eosinophilic foci of a mineralised appearance which stained positive by the Von Kossa technique. The injection sites from the zinc-pretreated cadmium animals showed similar features to the zinc only group including the mineralised foci (Figure 3.8). Pretreatment with zinc seemed to prevent the cadmium causing necrosis of the inflammatory cells and also to minimise the oedema.
Figure 3.7
Effect of Pretreatment with Zinc on Oedema at Injection Sites
(a and b), Total (c) and Differential (d) White Cell Counts,
Plasma Caeruloplasmin (e) and Plasma Iron (f), Following the
Subcutaneous Administration of Cadmium
Details of treatments are given in the Methods section. All values are means ±SEM with five samples per group. Statistically significant differences between test and saline-treated control groups are shown as * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 3.8(a)
Subcutaneous Tissue at the Centre of Injection Site 24 Hours After Administration of Cadmium (1.5mg Cd²⁺/kg)
Note: necrotic appearance of inflammatory cells, predominantly PMNLs.
Stain: haematoxylin and eosin. Magnification: x630

Figure 3.8(b)
Subcutaneous Tissue at Centre of Injection Site 24 Hours After the Administration of Cadmium (1.5mg Cd²⁺/kg) Plus Zinc (5mg Zn²⁺/kg) Following Zinc Pretreatment
Note: mineralised focus, MF, and intact appearance of polymorphs, PMNL, and mononuclear cells, MN.
Stain: haematoxylin and eosin. Magnification: x630
3.3.2.5 Testes Weights and Histopathology

Macroscopically, testes from the cadmium only group had a haemorrhagic and swollen appearance and the mean weight was slightly higher than other groups.

Table 3.5 Absolute and Relative Testes Weights

<table>
<thead>
<tr>
<th>Group</th>
<th>Absolute Weight (g)</th>
<th>Relative Weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>2.82 ± 0.07</td>
<td>1.22 ± 0.03</td>
</tr>
<tr>
<td>Zinc only</td>
<td>2.76 ± 0.07</td>
<td>1.18 ± 0.06</td>
</tr>
<tr>
<td>Cadmium only</td>
<td>3.01 ± 0.10</td>
<td>1.36 ± 0.07</td>
</tr>
<tr>
<td>Zinc and Cadmium</td>
<td>2.67 ± 0.08</td>
<td>1.18 ± 0.05</td>
</tr>
</tbody>
</table>

All values are means ± SEM

No abnormalities were detected histologically in the testes from saline, zinc only or zinc plus cadmium-treated animals. Cadmium only-treated animals however, showed varying degrees of damage. Three of the five had interstitial capillary dilatation and haemorrhage with tubular oedema and necrosis of the outer spermatogonia. A fourth animal showed minimal changes as above and in a fifth, no abnormalities were detected.

Haematology

3.3.2.6 Haematocrit

Treatment had no significant effect on haematocrit (results not presented).

3.3.2.7 Total White Cell Count

Figure 3.7c shows an apparently reduced white cell count following zinc treatment. However, the individual values all fell within the wide range of 5.16 to 8.60 x 10⁷ cells/l displayed by the saline control group and so this result should be interpreted with caution.
3.3.2.8 Differential White Cell Count

Cadmium treatment alone caused an upward shift in the relative numbers of neutrophils as compared to the saline (p < 0.01), zinc only (p < 0.01) and zinc plus cadmium (p < 0.05) and a reduced percentage of lymphocytes compared to saline (p < 0.01) and zinc (p < 0.05) groups as shown below:

Table 3.6 Percentage of Neutrophils and Lymphocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>% Neutrophils</th>
<th>% Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>23.5 ± 1.0</td>
<td>70.8 ± 1.0</td>
</tr>
<tr>
<td>Zinc only</td>
<td>24.9 ± 2.3</td>
<td>67.6 ± 2.4</td>
</tr>
<tr>
<td>Cadmium only</td>
<td>42.4 ± 4.1</td>
<td>52.8 ± 4.2</td>
</tr>
<tr>
<td>Zinc and Cadmium</td>
<td>28.8 ± 3.6</td>
<td>62.5 ± 3.9</td>
</tr>
</tbody>
</table>

All values are means ± SEM

Figure 3.7d shows that absolute numbers of neutrophils were significantly higher in the cadmium only group as compared to the saline (p < 0.05) and zinc (p < 0.05) groups, whilst lymphocytes were slightly reduced in numbers in all groups as compared to the saline group (p < 0.05). No significant differences were seen in the numbers of monocytes or eosinophils.

3.3.2.9 Plasma Caeruloplasmin

No statistically significant differences in plasma caeruloplasmin concentration were apparent between treatment groups although there was a trend towards higher levels in all three groups given zinc and/or cadmium (Figure 3.7e).

3.3.2.10 Plasma Iron

Both cadmium alone and zinc plus cadmium treatment caused a significant fall in plasma iron concentration (p < 0.01) whilst zinc alone produced only a slight reduction (Figure 3.7f).
Discussion

The purpose of the present study was two-fold: (i) to compare the effects of s.c. administration of cadmium with those of other metals as well as with two well recognised models of inflammation, and (ii) to investigate whether pretreatment with zinc could ameliorate the cadmium-induced inflammatory response. Despite the small group sizes of the comparative study, which were chosen because the effect of administration of some of the solutions was unpredictable, a number of conclusions could be reached.

The prominent microscopical features distinguishing the local inflammatory reaction induced by cadmium from that produced by the other metals were the extent of the oedema and necrosis. The cadmium-induced local reaction was of interest since it was characterised not only by extensive and marked oedema and necrosis but also by apparently rather limited numbers of inflammatory cells, of which most were necrosed, and a general absence of repair processes. This contrasted with the type of lesion induced by a similar mode of administration of turpentine where, although oedema was significant, subcutaneous abscesses formed with large numbers of inflammatory cells seemingly active in containing the lesion and aiding repair. Although zinc and copper produced significant inflammatory responses at injection site (copper moreso than zinc) these were generally restricted to the s.c. tissue and contained numerous apparently intact polymorphonuclear and mononuclear leucocytes. There were signs of fibrosis and general containment of the lesions.

Even the two injections of zinc sulphate given in the zinc pretreatment study, at six times the dose level used in the comparative study, failed to produce either the same devastating lesion or the same systemic response as cadmium. The gross appearance of injection sites following s.c. zinc administration has been reported previously. Immediately following administration of a high dose of zinc acetate (3mmol/kg) to rats in divided doses there was an 'intense local inflammatory
response with ulceration of overlying skin after 2-3 weeks followed by healing and scarring' (Gunn et al., 1964). In the present study the local response provoked by zinc at 5mg Zn\(^{2+}\)/kg was similar to that which followed 0.87mg Zn\(^{2+}\)/kg but more extensive. The microscopic appearance of the injection sites at the two dose levels differed most markedly in one respect. The high dose group (with or without subsequent cadmium treatment) exhibited numerous large deposits of a mineralised appearance which stained positively by the Von Kossa silver substitution technique for the demonstration of calcium. This method depends on the presence of phosphates and carbonates and not on the presence of calcium. Since in animal tissues insoluble phosphates and carbonates are nearly always those of calcium, this test is usually regarded as specific for this element (Pearse, 1972). The large amount of zinc administered in this study however, may well have precipitated as phosphates or carbonates and thus become Von Kossa positive. The significance of this finding is therefore unclear since the deposits may be either zinc or of calcium, the deposition of which could have been caused by zinc.

It is possible that the nature of the local reaction to the s.c. administration of metal salt solutions is dependent on their solubility at injection sites, which will determine the availability of metals for interaction with target cells. Tumours have arisen at injection sites following administration of both soluble cadmium salts such as cadmium chloride (Lucis et al., 1972) and water insoluble derivatives, cadmium sulphide and oxide (Kazantzis and Hanbury, 1966). This suggests either (a) that poorly soluble salts may become sufficiently solubilised to permit a confined interaction of Cd\(^{2+}\) with target sites which by some mechanism proceeds to tumour development, or (ii) that soluble salts are precipitated on contact with body fluids and it is the interaction of the cells with the surface of the particles, and probably the persistence of the metal at the site, that is the stimulus to the formation of tumours. This would then mimic a non-specific foreign body reaction to an implanted solid which has been reported for a large number of substances and which
in almost all cases in rodents will lead to formation of fibrosarcoma (Furst, 1981; Grasso and Golberg, 1966).

Acute damage caused by the s.c. administration of solutions of metal salts is not always succeeded by the development of tumours. Despite local inflammation produced by a high concentration of s.c. zinc, its administration alone has not produced sarcoma (Gunn et al., 1964). This indicates that factors other than those related to carcinogenesis as a sequel to general acute local damage and subsequent uncontrolled fibroblast proliferation may determine what leads to tumour development. In the case of some metals, such as cadmium, it could involve the precise mechanism of locally induced injury. Differential retention of metals at injection sites may account for the observed differences in the local reactions elicited by the compounds investigated. The physico-chemical properties of the substances probably also plays a significant part in determining the type of reaction; for example, surface activity, lipid solubility, enzyme inhibitory ability and protein binding ability, especially if the latter is associated with alterations in protein structure (Furst, 1981; Grasso and Golberg, 1966). Tumorigenesis in the testis however, was thought to be linked to the specific acute action of cadmium, perhaps via the injury to the vessels and the subsequent process of cell regeneration (Lucis et al., 1972).

The results of this study confirm the findings of previous workers (see Introduction) that zinc pretreatment provides complete protection against the acute effects of cadmium on rat testes. Furthermore it was demonstrated that an additional consequence of cadmium intoxication to those already described (see Introduction) could be alleviated by zinc. The acute local inflammatory reaction provoked at s.c. sites of cadmium administration was moderated by the pre- and simultaneous treatment with zinc at the same injection site. Even though the zinc itself was found to produce a significant local reaction it appeared to offer some protection against the inflammatory response normally provoked by cadmium, as
evidenced by reduced oedema formation and necrosis. There was some evidence also that certain systemic responses might also be prevented, such as the neutrophilic leucocytosis.

After the administration of irritant substances, a second experimental inflammation is inhibited, especially as regards oedema, with maximal inhibition at a time interval of 24 hours. Natural inhibitory factors such as the acute phase protein, foetal $\alpha_2$ globulin have been found in inflammatory exudate and appear to function to inhibit the further development of inflammatory oedema (Van Gool et al., 1974). This may account for the reduced oedema following zinc pretreatment in cadmium-treated animals but the reduced necrosis is more difficult to explain. It seems plausible that zinc may protect by competing for available target binding sites normally having an affinity for cadmium, thus preventing the attachment of cadmium and subsequent toxic action. Biochemically, zinc as well as cadmium has an affinity for sulphydryl groups.

The enhanced uptake of cadmium from injection sites might be predicted in zinc-pretreated animals if cadmium was not able to bind to tissue. However, a recent study in this department indicated that significantly more of the administered cadmium was retained at the injection site in the zinc pretreatment group ($72 \pm 9\%$) as compared to the cadmium group alone ($38 \pm 8\%$). This resulted in slightly less cadmium in the liver and testes of zinc plus cadmium-treated animals (Parsons et al., 1987). These are preliminary results which are not presented as they warrant further verification since some technical difficulties were encountered. The trauma of injection and subsequent tissue reaction to zinc may have led to retention of more cadmium possibly as protein-metal complexes since the cadmium was injected into a reactive lesion where serum proteins were presumably abundant. Cationic ions are known to bind to serum components and thereby be effectively unavailable for uptake by cells (Morgan, 1981). This could be considered as a protective mechanism involving priming of the inflammatory processes for the subsequent onslaught of cadmium.
Alternatively, the areas of dense mineralisation caused by high levels of zinc may have caused increased retention of cadmium as zinc/calcium-cadmium complexes, thus diverting cadmium away from the usual target binding sites. This diminished availability of cadmium for interaction with the tissue and infiltrating inflammatory cells might therefore explain the reduced necrosis following zinc pretreatment. To test these hypotheses, high levels of zinc could be administered at a site distant from the cadmium injection to investigate whether inflammation could similarly be alleviated. This would indicate whether zinc was acting merely by a local physico-chemical mechanism or by preventing cadmium interactions with zinc-dependent biological processes to be discussed later.

The effect of administration of other metals on cadmium uptake from injection sites has been studied. Poirier et al., (1983) demonstrated that the development of tumours at the site of a s.c. cadmium chloride injection could be prevented by simultaneous injections of magnesium acetate at the same place whilst injections of calcium acetate did not produce such protective effects. Early cadmium retention at the injection site was found to be reduced by magnesium but enhanced by calcium (Kasprzak and Poirier, 1985). It was thought that the preventative effects of magnesium might result from decreased binding of cadmium by target tissue. A number of theories for the different effects were discussed and it was proposed that calcium by its contracting action on the blood vessel smooth muscle would, like cadmium (Bondia et al., 1980, cited Kasprzak and Poirier, 1985), hinder blood flow thus slowing mobilisation of cadmium from the extravascular space. Magnesium, which is antagonistic to calcium in the vascular contraction mechanism, would produce the opposite effect. The interaction of cadmium with these essential metals at the site of injection may play a role in the cadmium-induced local inflammatory effect.

The pH of the dosing solution did not seem to be a factor in the severity of the reaction since the more acid solutions of aluminium salts produced only minimal
responses resulting in small fibrosing encapsulated lesions. The intensity of the acute local reaction exhibited by cadmium, zinc and copper fitted in with the hypothesis of (Williams et al., 1982) that the most toxic ions, as determined from LD$_{50}$ studies on mice given metal salts intraperitoneally, are those with a greater tendency towards softness as an acid, i.e., Cd > Cu > Zn.

Though not showing statistical significance, whole body retention of intestinally-absorbed cadmium in rats was found to be lowest amongst those fed cadmium sulphate, somewhat higher with cadmium chloride and highest with cadmium acetate (Moore et al., 1973). Small group sizes limit any firm conclusions from the present study but results similarly suggest a higher uptake into the liver and kidneys of the cadmium given as acetate by s.c. injections. This was despite there being no observable differences in the nature of the local inflammatory reaction nor in the systemic response and the explanation is not apparent.

The intense neutrophilic leucocytosis characteristic of the response to s.c. administration of cadmium was not seen after administration of the other metals. Even administration of an equivalent weight of copper which produced a significant local reaction failed to alter the numbers of circulating leucocytes. In the zinc pretreatment study, zinc injected twice at six times the molarity of cadmium did not raise numbers of circulating neutrophils whilst a significant neutrophilia was apparent only 24 hours after a single injection of the cadmium alone. The neutrophilic leucocytosis and monocytosis are probably therefore a response to the high turnover of neutrophils and monocytes which was evident in the necrotic lesions of the injection sites and testes in cadmium-treated animals. This is supported by the demonstration that zinc pretreatment by preventing the testicular damage and local subcutaneous destruction of inflammatory cells also prevented the significant compensatory rise in circulating neutrophils.

The only other metal to produce a rise in circulating leucocytes was zinc sulphate at 13.35μmol Zn$^{2+}$/kg and this was due to slightly elevated numbers of
lymphocytes whilst zinc at 76.45 μmol Zn\(^{2+}\)/kg appeared to reduce numbers of lymphocytes. Turpentine surprisingly caused no leucocytosis after 24 hours and indeed, a slight drop in lymphocytes was observed whilst 24 hours after FCA challenge there was a marked neutrophilic leucocytosis. The response to FCA, although still significant, had subsided by three days when it was accompanied by a monocytosis. The systemic response to FCA challenge would be expected to be more rapid than in the turpentine model since the former is a cell-mediated response. This probably also accounts for the larger spleens seen only in this group and which seemed to be attributable primarily to increased myelopoietic activity.

The plasma concentrations of the acute phase proteins haptoglobin and caeruloplasmin were raised after s.c. administration of all metals but most markedly, with cadmium and copper which produced the most significant local reactions. Aluminium surprisingly produced a more significant effect when given as the chloride, than as the sulphate; the reason for this is unclear. The finding of raised plasma caeruloplasmin following copper administration is in contrast to the unchanged caeruloplasmin activity after an identical dosing schedule at a dose level of 3.0mg Cu\(^{2+}\)/kg carried out on mice by Sugawara and Sugawara (1987). No mention was made of any inflammatory response provoked by copper in their animals; the discrepancy in results is difficult to explain.

Although caeruloplasmin activity showed a similar increase in response to cadmium in the present study as that following administration of 1.5mg Cd\(^{2+}\)/kg to mice, Sugawara and Sugawara (1987) did not observe the reduced biliary copper as seen by Ashby et al., (1980). They stated that although hepatic copper may contribute to stimulation of caeruloplasmin activity and/or biosynthesis (as proposed by Ashby et al., 1980) it did not explain why caeruloplasmin activity was not also stimulated by excess copper in copper-injected mice. The simultaneous s.c. administration of 5mg Zn\(^{2+}\)/kg in conjunction with 1.5mg Cd\(^{2+}\)/kg for two days did not eliminate the inhibitory effect of cadmium on the biliary excretion of copper (Ashby et al., 1980).
This was seen to provide further evidence for the involvement of metallothionein in the hepatic copper levels since this protein is induced by zinc. However, the injection with zinc alone (5mg/kg) which should also therefore have stimulated metallothionein production had no effect on the biliary excretion of copper (Ashby et al., 1980). If as proposed in Chapter 2, biliary copper is reduced in order to provide a pool of copper for caeruloplasmin synthesis, then this result is also surprising in view of the observation in the present study that zinc causes inflammation. Plasma caeruloplasmin was increased following three daily injections of only 0.87mg Zn^{2+}/kg consistent with its induction as an acute phase protein in response to the inflammation at injection sites. However, local inflammation was not as intense and caeruloplasmin levels not as high as following cadmium administration so possibly hepatic copper levels were adequate to sustain caeruloplasmin synthesis without reducing biliary copper excretion. Caeruloplasmin was not measured by Ashby et al., (1980) following zinc injection but plasma copper measurements indicated a marginal rise which could have been attributable to caeruloplasmin. The subcutaneous administration of lead was also found to stimulate caeruloplasmin, but not to the same degree as with cadmium, whilst hepatic copper remained unchanged (Sugawara and Sugawara, 1987). It was not mentioned but perhaps lead also causes moderate s.c. inflammation.

Caeruloplasmin showed a small increase, although not statistically significant, following administration of zinc at 5mg/kg for two days, with or without cadmium, in the zinc pretreatment study. Rats given cadmium alone attained a plasma caeruloplasmin concentration after 24 hours which was in the same range as the zinc-treated animals and was much lower than seen after three days cadmium treatment. There was no difference in caeruloplasmin levels between the zinc-pretreated cadmium group and the cadmium only group. A protective effect of zinc on this systemic response might not be expected to become apparent until at least 48 hours after administration of cadmium since acute phase proteins do not reach their maximum plasma levels until this time.
The very high concentrations of caeruloplasmin seen one day after turpentine administration and three days after FCA challenge, in comparison to the levels induced by the metals, was interesting particularly since haptoglobin appeared to be elevated to similar levels after most treatments. This suggests that various components of the systemic inflammatory response respond differentially and very specifically depending on the type of local response. Alternatively, the biosynthesis of caeruloplasmin may in fact be limited following metal administration, possibly by hepatic metallothionein synthesis and copper retention. Bremner (1987) stated that the finding by Ashby et al., (1980) that cadmium treatment of rats reduces biliary excretion of copper is contrary to that which might be predicted on the basis of evidence that he presented suggesting that the binding of hepatic copper to metallothionein favours its secretion into bile. Further studies are required to elucidate the effect of inflammation- or metal-induced metallothionein synthesis on the biliary excretion of copper, hepatic copper levels and caeruloplasmin synthesis.

Zinc administration alone did not significantly decrease plasma iron although at both dose levels a slight drop was seen. Despite amelioration of the local response to cadmium by zinc pretreatment, the hypoferraemic response still took place in these animals. Bonner (1980) found that simultaneous administration of zinc (5mg/kg) i.p. and cadmium (1.5mg/kg) s.c. caused a greater decrease of plasma iron than either cadmium or zinc given individually. This was not the case in the present study probably because zinc and cadmium were given at the same site whereas Bonner may have initiated two separate local inflammatory reactions intraperitoneally and subcutaneously which might therefore be expected to give rise to an enhanced systemic response.

Copper chloride (1.5mg Cu/kg) and copper sulphate were the only metal salts besides cadmium to produce a significant drop in plasma iron although the decrease with copper was not as great as with cadmium. Plasma iron does not therefore appear to be as sensitive a marker of inflammation as caeruloplasmin and
haptoglobin. A marked hypoferraemic response however accompanied turpentine-induced inflammation and FCA challenge after one day. Interestingly, the return to normal plasma iron levels three days after FCA challenge coincided with the greatly elevated plasma caeruloplasmin. This would seem to support the proposal of Piercy (1979) that the protein may be synthesised in order to stimulate the fresh release of iron for restoration of plasma iron levels at the end of an inflammatory response.

A comparison of the effects of the various inflammatory stimuli investigated shows that the different irritants induced both local and systemic responses which varied considerably in the magnitude of individual components. The nature of each systemic response is probably appropriate to the level and type of local damage. These differential responses support a hypothesis of differences in the mechanism of inflammation suggesting the involvement of different mediators to varying degrees.

The mechanism of zinc-induced tolerance to cadmium toxicity in vivo has been attributed to induction of metallothionein synthesis with resulting altered subcellular and organ distribution of cadmium such that less of the metal binds to critical organelles and macromolecules in target organs and instead binds preferentially to metallothionein in the cytosol where it is less toxic (Goering and Klaassen, 1983). Webb (1972) suggested that pretreatment with zinc prevented testicular damage by inducing liver metallothionein which immobilised cadmium more rapidly. However, it is not generally thought to be the primary mechanism for testicular tolerance and cannot account for the reduced tissue damage at injection sites since cadmium levels here appeared to be higher in the zinc-pretreated cadmium group.

Although several reports have indicated the presence of metallothionein like protein in rodent testes, Waalkes et al., (1984) reported that the low molecular weight cadmium-binding protein in rat testis is not metallothionein. They proposed that this could account for the sensitivity of the organ to the toxic effects of cadmium when levels of the metal are known to be low in comparison to the liver
and kidney (Gunn et al., 1968c). Induction of metallothionein synthesis at s.c. cadmium injection sites does not seem to have been investigated. Murine peritoneal macrophages and human blood lymphocytes and monocytes readily accumulate cadmium in vitro, associated with the synthesis of metallothionein (Patierno et al., 1983; Hildebrand and Cram, 1979; Peavy and Fairchild, 1987) as do the phagocytic Kupffer cells of the liver following s.c. injection of 1.5mg Cd^{2+}/kg to rats (Caperna and Failla, 1984). Protection against subcutaneous cadmium-induced damage by zinc pretreatment could therefore involve the induction of metallothionein synthesis in infiltrating inflammatory cells and possibly other resident cell types, with subsequent uptake of cadmium into the protein where it is less toxic. This would then account for the survival of cells at injection sites following zinc pretreatment.

Cadmium may exert a direct toxic action on inflammatory cells resulting in cell lysis with release of lysosomal enzymes and free radical oxygen species (see Introduction, Chapter 4) giving rise to indirect cytotoxicity of the s.c. tissue. Lundborg et al., (1987) proposed that the increased lysozyme activity displayed by macrophages and in lung lavage fluid following inhalation of low concentrations of cadmium chloride by rabbits was probably a secondary effect subsequent to inflammation. Nevertheless, cadmium has been shown to be toxic to rabbit pulmonary alveolar macrophages in vitro (Coin and Stevens, 1986; Lundborg et al., 1987) and to impair the phagocytic activity of mouse mononuclear phagocytes in vivo (Levy et al., 1984) mouse peritoneal and alveolar macrophages and polymorphonuclear leucocytes in vitro (Loose et al., 1978) rat pulmonary macrophages both in vivo and in vitro (Greenspan and Morrow, 1984) and human polymorphonuclear leucocytes in vitro (Baginski, 1985). Conversely, Koller and Roan (1977) found that cadmium given orally to mice had no effect on cell viability and in fact stimulated phagocytosis and increased acid phosphatase levels in peritoneal macrophages. Chvapil et al., (1977) meanwhile, showed that cadmium was not inhibitory to granulocyte phagocytosis, bacterial killing or migration in
vitro. It thus remains unclear whether local tissue damage at s.c. sites of cadmium injection is attributable to effects of a direct interaction with cadmium and/or as a secondary consequence of inflammation.

Zinc is an essential component of many enzyme systems and functions in a wide range of cellular activities including many aspects of the inflammatory response (Nriagu, 1980). Cadmium, as an antagonist of zinc, may cause an excessive local s.c. reaction by replacing functional zinc or by altering the availability of this essential trace element in one or more zinc-dependent processes, the effect being overcome by the exogenously administered zinc. The zinc-dependent processes with which cadmium could interfere fall broadly into two categories. Those involved in the development of the acute inflammatory response and those connected with resolution and repair, ie, wound healing. The same holds true for copper-dependent processes which could also suffer from a copper deficiency through the effects of cadmium.

Throughout the inflammatory response cadmium may affect the function of phagocytic cells by interfering with zinc-dependent processes. These include the mobilisation of macrophages/monocytes and polymorphonuclear leucocytes into tissues, cellular activation, phagocytosis and the stabilisation of cell and lysosomal membranes, all of which are known to be inhibited by zinc deficiency (Norris, 1985). However, it should be noted that excessive zinc can also be inhibitory to certain functions of these cells (Chvapil et al., 1979a). Brewer (1981) has shown that zinc acts in a reciprocal or antagonistic manner to calcium and postulated that the mechanism of this action is through calcium activation and zinc inhibition of the function of a small ubiquitous calcium binding protein called calmodulin. There is evidence that calcium activation of a variety of cells occurs through calmodulin. Calcium-dependent cellular responses include platelet aggregation, neutrophil chemotaxis and phagocytosis and mast cell histamine secretion. These functions are inhibited by zinc. The homeostatic control of inflammatory cells is clearly complex.
and involving a critical balance of metals which it is predicted could be easily upset by cadmium.

Another important component of the initial inflammatory response is the increased local blood flow and vascular permeability leading to extravasation of proteins and fluid which contribute to and largely determine the extent of tissue oedema. The release of mediators which are vasoactive in response to tissue injury is thought to explain these acute vascular responses. Oedema probably serves to dilute the offending substance with its magnitude in some way being determined by the toxicity of the substance. Although the process by which cadmium causes such pronounced oedema and necrosis in comparison to the other metals administered in this study is not clear, further investigations into the partial protection offered against these events by zinc pretreatment may provide important clues.

A common mechanism of acute toxicity may be related to the initial interaction of cadmium with the vascular endothelium of the tissue (Nolan and Shaikh, 1986a). Electron microscopic studies by Gabbiani et al., (1974) on rat testis, epididymis and gasserian ganglion and by Schlaepfer (1971) on rat spinal sensory ganglia showed that selective injury to vascular endothelial cells preceded all other changes. Degenerative changes included increased vacuolation and widening of intercellular junctions of arteriolar, capillary and venular epithelium and the oedema and haemorrhage in the testes which follow are an indication of the interference with the permeability of the vasculature. It appears that cadmium alters this by direct insult rather than through a secondary effect on permeability factors.

Some tissues, such as testis, seem to be predisposed towards susceptibility to vascular injury, for which a number of speculations have been made to explain (Gabbiani et al., 1974; Gunn et al., 1963a; Schlaepfer, 1971). In addition, cadmium-induced lesions are not uniformly distributed among the endothelial cells of small vessels. Some endothelial cells of sensory ganglia appeared less susceptible to
cadmium-induced injury and showed features of endothelial regeneration and repair. The selective survival and perpetuation of these resistant cells may explain the known development of tolerance of tissues to a second exposure to cadmium (Schlaepfer, 1971). If cadmium also causes endothelial cell damage to blood vessels at s.c. sites of injection, resulting in increased vascular permeability, this could have some bearing on the very oedematous condition of the lesions formed. As discussed in relation to testis by Schlaepfer (1971) a marked oedema could be expected to lead to increased intra-organ pressure and diminution of blood flow which would result in ischaemic necrosis. Such a process might contribute to the necrotic nature of the s.c. lesion. Gunn et al. (1968b) suggested that the protective agents, selenium and zinc, exerted their protective effects on the primary site of cadmium injury, i.e., at the vascular level. They suggested the formation of a Se-Cd complex which thus inactivates cadmium. Zinc pretreatment might prevent increased vascular permeability and hence oedema and necrosis either by complexing with cadmium and preventing it reacting with the vascular endothelial cells or by providing competitive inhibition at the level of common binding sites.

Immunocytochemical staining has indicated that endothelial lining of blood vessels in mouse liver contain metallothionein (Nolan and Shaikh, 1986b) suggesting this to be a target cell type. Susceptibility of vascular endothelial cells to cadmium toxicity might therefore be determined by their metallothionein content with zinc pretreatment providing protection by inducing metallothionein.

In inflammation, protease inhibitors are important in limiting proteolytic damage to surrounding tissue, evoked by lytic enzymes released both by infiltrating phagocytes and locally activated cells such as fibroblasts. Chowdhury et al., (1983) found a great decrease in the level of serum $\alpha_1$ antitrypsin or reduced serum trypsin inhibitor capacity after cadmium exposure of rats. They suggested that the imbalance of the protease-antiprotease system was responsible for disturbances in
the integrity of the lung connective tissue and for the development of pulmonary lesions typical of cadmium intoxication. Similar imbalances of protease-antiprotease systems at s.c. cadmium injection sites might also lead to enhanced tissue destruction.

α₂ Macroglobulin of humans is a zinc metalloprotein which is a protease inhibitor synthesised not only by the liver but also by fibroblasts, monocytes and lymphocytes (Powanda, 1981). The affinity of cadmium for α₂ macroglobulin has been shown (Watkins et al., 1977; Carson, 1984). If cadmium modulates the suppressing effects of such proteins in the inflammatory response then tissue damage could be potentiated.

Zinc accumulates at sites of tissue injury and has a role in various aspects of wound healing by having an effect on collagen synthesis through a generalised effect on protein synthesis and nucleic acid metabolism. In the process of tissue repair after injury the presence of zinc facilitates the formation of granulation tissue and healing (Nriagu, 1980). If cadmium displaces zinc from active sites this might explain the persistent necrosis and apparently impaired healing following s.c. cadmium administration.

Cadmium could also interfere with copper-dependent enzymes such as cytochrome oxidase which is a component of the aerobic energy production system and which increases during wound healing. A number of studies have indicated that fibrous proteins of connective tissue are affected by cadmium intoxication. A decrease in collagen and elastin content with impaired extracellular maturation of the collagen fibres was found in skin amongst a number of other tissues following oral administration of cadmium to rats (Kucharz, 1988). Studies into the acute inflammatory effects in rat lung tissue following a single intratracheal instillation of cadmium chloride demonstrated a clear-cut interference with the metabolism of lung fibrous proteins (Kobrle et al., 1986). The disturbed polymerisation of collagenous proteins in the acute phase indicated insufficient activity of lysyl
oxidase, a copper-dependent enzyme which catalyses the covalent cross-linking of collagen, probably connected with the reduced supply of copper in lung tissue due to cadmium-induced formation of metallothionein. Iguchi and Sano (1985) demonstrated reduced lysyl oxidase activity and higher amounts of soluble collagen in bone related to the inhibition of the enzyme. Lysyl oxidase is also decreased in zinc deficiency probably because zinc is required for protein synthesis (Van Rij and Pories, 1980). Persistence of cadmium in subcutaneous tissue or a systemic effect of cadmium on zinc and copper homeostasis might therefore be predicted to affect the metabolism of fibrous proteins during the repair process.

In conclusion, cadmium may interfere with the integrated host defence/repair system and the ability of zinc to alleviate cadmium-induced local damage could be mediated in a number of ways. It is not clear whether the protective effects of zinc on acute local damage is the mechanism by which zinc in some way prevents tumour formation. Testicular damage became evident after a prolonged period of time had elapsed after cadmium treatment in zinc-pretreated animals (Gunn et al., 1961) and this was attributed to a delayed effect dependent on the turnover of zinc and re-availability of binding sites for cadmium. The effect on subcutaneous tissue may also be only temporary and would clearly depend on the dose of zinc used in pretreatment.

It is apparent that a clearer understanding of the interactions between trace metals and cadmium during inflammation could be fruitful not only to our understanding of the mechanisms of acute cadmium toxicity and chronic carcinogenicity, but also to the role of trace metal metabolism during inflammation.
CHAPTER 4

EFFECT OF CADMIUM AND OTHER SELECTED TREATMENTS ON FREE RADICAL PRODUCTION FROM SUBCUTANEOUS TISSUE AS MEASURED BY CHEMILUMINESCENCE: IN VIVO AND IN VITRO STUDIES
CHAPTER 4

4.1 Introduction

4.1.1 General Introduction

The results of Chapters 2 and 3 have highlighted the oedematous and necrotic nature of the local reaction to the subcutaneous administration of cadmium. It was decided to investigate the response further to see if it was primarily a consequence of the destructive effects of oxidative products released from infiltrating inflammatory cells or whether cadmium might exert a more direct effect on the subcutaneous tissue, possibly involving free radical mechanisms and the process of lipid peroxidation.

During the inflammatory process polymorphonuclear leucocytes (PMNL) and macrophages accumulate at the site of injury. An increase in the oxidative metabolism of these cells, commonly known as the respiratory burst, results in the release of several reactive metabolites of oxygen including superoxide anion (${O_2^-}$), hydroxyl radical (${\cdot OH}$) and hydrogen peroxide (${H_2O_2}$). Several reactions responsible for production of these oxygen radicals and hydroperoxides have been identified. Reference to this work is given in a number of excellent reviews which have been published in recent years dealing with phagocyte oxygen metabolism, the molecular basis and role of transition metals in free radical formation as well as the process and detection of lipid peroxidation (Babior, 1982; Clark, 1986; Flohe et al., 1985; Halliwell and Gutteridge, 1985, 1986; Hamers and Roos, 1985; Slater, 1984; Southorn and Powis, 1988; Torielli and Dianzani, 1984; Tribble et al., 1987). The subject will therefore only be dealt with briefly in this introduction.

4.1.2 Free Radicals and the Production of Reactive Oxygen Species

The outermost orbitals of most non-radical molecules are occupied by a pair of electrons that spin in opposite directions. This is the most stable energetic configuration. A free radical, in contrast, is defined as any species that has one or
more unpaired electrons. A single electron confers instability and high reactivity on the molecule. This broad definition therefore embraces the atom of hydrogen (one unpaired electron), most transition metals and the ground state diatomic oxygen molecule \((O_2)\) is itself a radical with two unpaired electrons in parallel spin.

The production of superoxide radical anion \((O_2^-)\) depicted as \(O_2^-\) in this thesis) appears to be caused by activation of an NADPH oxidase in the cell membrane which possibly coupled with a unique b-type cytochrome causes a one-electron reduction of \(O_2\) to \(O_2^-\)

\[
2O_2 + \text{NADPH} + H^+ \xrightarrow{\text{Oxidase}} 2O_2^- + \text{NADP}^+ + 2H^+
\]

The superoxide anion itself shows poor reactivity but it can serve as an intermediate in radical redox reactions which produce further oxygenating agents. Dismutation of \(O_2^-\) can occur spontaneously or can be catalysed by superoxide dismutase (SOD), which acts as a natural intracellular scavenger of \(O_2^-\) to produce hydrogen peroxide \((H_2O_2)\) by a reaction which can be written overall as:

\[
2O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2
\]

\(H_2O_2\) has no unpaired electrons, is not a radical and has limited reactivity, but it can be converted into more reactive radicals. In the presence of metal catalysts \(O_2^-\) and \(H_2O_2\) can give rise to the highly reactive hydroxyl radical \(({}^\cdot OH)\) by reactions which can be summarized as:

\[
O_2^- + H_2O_2 \xrightarrow{\text{Fe-complex}} O_2 + {}^\cdot OH + OH^- \text{Haber Weiss Reaction}
\]

This reaction can potentially be supported by iron-containing proteins such as haemoglobin, ferritin, lactoferrin, transferrin and simple iron chelates such as ATP, ADP and citrate although the role of some as major physiological catalysts of \({}^\cdot OH\) production is still questionable (Halliwell and Gutteridge, 1985, 1986).
H$_2$O$_2$ serves as a substrate for myeloperoxidase which in the presence of a halide ion, probably Cl\textsuperscript{-}, catalyses the formation of hypochlorite anion (OCl\textsuperscript{-}): \[
H_2O_2 + Cl^- \rightarrow OCl^- + H_2O
\]

This constitutes an exceedingly potent microbicidal system.

Additionally, alkyl hydroperoxides (ROOH), which react rapidly with O$_2^\cdot$ to generate alkyl radicals (RO\textsuperscript{•}): \[
O_2^\cdot + ROOH \rightarrow RO\textsuperscript{•} + O_2 + OH^- \]

are produced by the oxidation of polyunsaturated fatty acids (PUFAs) in neutrophils undergoing the respiratory burst.

4.1.3 Lipid Peroxidation

Free radicals are important in many biological processes and oxygen-centred radicals play a crucial role during inflammation in the intracellular destruction of endocytosed micro-organisms. However, they may also be liberated into the extracellular space and cause undesirable damage to surrounding tissue by peroxidation of membrane PUFAs and depolymerisation of structural macromolecules such as hyaluronic acid and collagen. Lipid peroxidation, the process by which oxidative degradation of PUFAs occurs, thus leads to disruption of cell membranes and cell death.

The initiation of a lipid peroxidative sequence in a membrane or PUFA is due to the attack by any species that has sufficient reactivity to abstract a hydrogen atom (H\textsuperscript{•}) from a methylene (-CH$_2$-) group (Figure 4.1). Since a hydrogen atom has only one electron, this leaves behind an unpaired electron on the carbon(-CH-). The carbon-centred radical undergoes a molecular rearrangement to form a conjugated diene that then combines with oxygen to form a peroxy radical, itself able to
abstract a hydrogen atom from another fatty acid and so start up a chain reaction. Species which can initiate lipid peroxidation include hydroxyl radical (·OH), alkoxy radicals (RO·), peroxy radicals (ROO·) and possibly HO2· but not H2O2 or O2− (Halliwell and Gutteridge, 1985).

The Chain Reaction of Lipid Peroxidation

The end-products of the chain reaction are a variety of lipid peroxides and cyclic peroxides. Transition metals and metal complexes catalyse the decomposition of these to alkoxy (lipid -O·) radicals:

\[
\text{Lipid -O}_2\text{H} + Fe^{2+} - \text{complex} \rightarrow Fe^{3+} - \text{complex} + OH^- + \text{Lipid -O·}
\]
and peroxy (lipid $O_2^\cdot$) radicals:

$$\text{Lipid } -O_2H + Fe^{2+} \text{ complex } \rightarrow Fe^{3+} \text{ complex } + H^+ + \text{ lipid } -O_2^\cdot$$

which in turn stimulate the chain reaction of lipid peroxidation by abstracting further hydrogen atoms. Other decomposition products include hydrocarbon gases and cytotoxic aldehydes.

4.1.4 Free Radicals and Oxygen-Derived Species in Tissue Injury

The inappropriate release of short-lived oxygen metabolites from phagocytes is attracting much attention as playing a possible key role in tissue injury that takes place in regions of inflammation. Oxygen radicals and oxygen-derived species together with the chain reaction of lipid peroxidation have thus been implicated in the pathogenesis of many diseases particularly where there is massive or persistent phagocytic infiltration (Clark, 1986; Halliwell and Gutteridge, 1985; Slater, 1984; Southorn and Powis, 1988; Tribble et al., 1987).

A possible function for the hypoferraemic response of inflammation (discussed in Chapter 2) in addition to its bacteriocidal effect, might be to reduce the risk of iron being available at the local sites of inflammation as well as elsewhere in the circulation where it could enhance the destructive effects of reactive oxygen intermediates released during phagocytosis. This might also explain the high concentration of certain acute phase proteins, such as caeruloplasmin, which have antioxidant activity.

The generation of harmful radical species and resulting lipid peroxidation has been suggested to be responsible also for the toxic and carcinogenic action of a wide range of compounds, some taken for therapeutic effect and others unwittingly (Parke, 1982).
4.1.5 Cadmium and Lipid Peroxidation

Lipid peroxidation has been demonstrated, usually using the thiobarbituric acid reaction for measuring malondialdehyde, in a number of tissues after exposure to cadmium both in vitro and in vivo, the latter most frequently via a parenteral route. The s.c. administration of cadmium stimulated lipid peroxidation in testes of rats (Evans, 1983; Gabor et al., 1978; Omaye and Tappel, 1975; Sajiki et al., 1981) and mice (Sugawara and Sugawara, 1984b), rat liver (Klimczak et al., 1984; Sato et al., 1983), and kidney (Gabor et al., 1978). Cadmium given by an i.p. route also induced lipid peroxidation in rat testes and kidney (Hussain et al., 1987) and brain (Shukla et al., 1987) and the intratracheal instillation of cadmium provoked lipid peroxidation in lungs of rats (Cross et al., 1979) and hamsters (Hoidal et al., 1985). Cultures of isolated hepatocytes incubated with cadmium have also shown increased lipid peroxidation (Stacey et al., 1980; Muller, 1986) as have homogenates of liver and kidney (Hussain et al., 1987) and liver microsomes (Evans, 1983) exposed to cadmium in vitro.

These data have led to speculation that lipid peroxidation may be involved in the mechanism of cadmium-induced toxicity in these tissues. Conversely, however, ethane exhalation in vivo (another indicator of lipid peroxidation) was not increased after cadmium administration via an i.v. route to mice (Siegers et al., 1986) or i.p. to rats (Harvey and Klaassen, 1983) and no lipid peroxidation was detected in liver and kidney following s.c. administration of cadmium to rats (Evans, 1983). Rat liver perfused with cadmium in vitro similarly showed no enhanced lipid peroxidation (Lupo et al., 1986). Several studies have found evidence to refute lipid peroxidation as the cause of cadmium-induced hepatotoxicity (Lupo et al., 1986; Muller, 1986, Stacey et al., 1980) although the exact mechanisms involved have not been clearly elucidated.

4.1.6 Chemiluminescence

In 1972, Allen and co-workers observed that human neutrophils displayed chemiluminescence (CL) during phagocytosis. CL is defined as the emission of light
accompanying chemical reactions in which molecules in an electronically excited state are generated, some of which will lose the excess energy as light, to regain their original stability (known as ground state) as depicted below:

The initial change from ground state to an excited state could be accompanied by an oxidation of the initial molecule in the ground state (Van Dyke, 1985). CL thus reflects and is quantitatively related to the occurrence of excited states generated during the course of oxidative chain reactions such as occur during phagocytosis (Allen et al., 1972) and also as a consequence of tissue damage (Cadenas and Sies, 1984).

4.1.7 Sources of Chemiluminescence in Biological Systems

One major source of chemiluminescence in biological systems appears to be due to the chemical generation of singlet oxygen as a secondary product of a variety of reactions (Allen et al., 1972). Singlet molecular oxygen (\(^{1}O_2\)) abbreviated to \(^{1}O_2\) is an excited state of molecular oxygen. The ground state oxygen molecule (\(^{3}Z\) \(O_2\)) has its lowest energy configuration with two unpaired electrons each located in a different outer orbital and with these electrons spinning in the same direction (Figure 4.2).

Oxygen molecules in a higher energy state have paired electrons with opposite spin, either in a common or in two different orbitals. Singlet molecular oxygen is formed when absorption of energy shifts a valence electron to an orbital of higher energy with an inversion of spin. Two singlet
**Figure 4.2**

Arrangement of Electrons in Outmost Orbitals of Molecular Oxygen, Delta and Sigma Singlet Oxygen and Superoxide Anion

Arrows indicate direction of electron spin

<table>
<thead>
<tr>
<th>State</th>
<th>Orbital</th>
<th>Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>( ^3 \Delta gO_2 )</td>
<td>( \uparrow \uparrow )</td>
<td>Low</td>
</tr>
<tr>
<td>( ^1 \Delta gO_2 )</td>
<td>( \downarrow \uparrow )</td>
<td>High</td>
</tr>
<tr>
<td>( ^1 \Delta g^+ )</td>
<td>( \uparrow \downarrow )</td>
<td>V.High</td>
</tr>
<tr>
<td>( O_2^- )</td>
<td>( \uparrow \downarrow )</td>
<td></td>
</tr>
</tbody>
</table>

states of oxygen exist, the most important in biological systems (\( ^1 \Delta gO_2 \)) has no unpaired electrons and is not a radical. These molecules are highly unstable and release photons upon relaxation. The generation of visible light from singlet \( ^1O_2 \) is derived from the combined relaxation of 2 \( ^1O_2 \) molecules, a so-called 'dimol' emission at specified wavelengths of 634 and 703nm.

\[
^1O_2 + ^1O_2 \rightarrow 2^3O_2 + h\nu \ (634 + 703\text{nm})
\]

Alternatively, the energy released on decay of \( ^1O_2 \) may react with other molecules forming excited products that subsequently emit light.

The photoemission observed during phagocytosis is ascribed to the generation of \( ^1O_2 \) arising in a secondary fashion from reactions involving oxygen species such as \( O_2^- \), \( H_2O_2 \) and \( ^1OH \). Several reactions are hypothesised to evolve \( ^1O_2 \) (reviewed by Cadenas and Sies, 1984) although whether some of these operate to any significant extent in vivo is in dispute (Foote et al., 1980; Halliwell and Gutteridge, 1985; Hamers and Roos, 1985).

The spontaneous reactivity of \( O_2^- \) (or electron transfer reaction of the \( O_2^- \)) was regarded as a source of \( ^1O_2 \).
\[
2H^+ + O_2^- + O_2^- \rightarrow H_2O_2 + 'O_2
\]

as was the interaction of \( O_2^- \) and \( H_2O_2 \) through a Haber-Weiss reaction:

\[
O_2^- + H_2O_2 \rightarrow 'OH + OH^- + 'O_2
\]

and the reaction of \('OH\) and \( O_2^- \)

\[
O_2^- + 'OH + H^+ \rightarrow H_2O + 'O_2
\]

The spontaneous reactivity of \( H_2O_2 \) was also indicated as generating \('O_2\)

\[
H_2O_2 + H_2O_2 \rightarrow 2H_2O + 'O_2
\]

and the reaction of \( H_2O_2 \) and hypochlorite anion

\[
H_2O_2 + OCl^- \rightarrow 'O_2 + H_2O + Cl^- \]

Another source of visible range photons has been attributed to the spontaneous transition of excited carbonyl groups denoted as \( RO^* \):

\[
RO^* \rightarrow RO + \nu \text{ (between 420 - 450nm)}
\]

These compounds in addition to \('O_2\) are now thought to arise principally from the degradation of lipid peroxyl radicals (\( ROO^* \)) during the free radical-mediated process of lipid peroxidation.

\[
H^+ + ROO^* + ROO^* \rightarrow ROH + O_2 + RO^*
\]

\[
H^+ + ROO^* + ROO^* \rightarrow ROH + RO + 'O_2
\]
The mechanism invoked to interpret the formation of \('O_2\) has however been subject to criticism. A decomposition of hydroperoxides by metals could yield alcohols, ketones and alkyl radicals which are capable of generating CL and CL arising from transition metal ion- or haem compound-catalysed decomposition of hydroperoxides has been observed (references in Cadenas and Sies, 1984).

CL has been observed in association with lipid peroxidation in exposed organs in situ, isolated perfused organs, isolated intact tissues, homogenates, cells, subcellular organelles and enzyme reaction models (Boveris et al., 1981; Cadenas and Sies, 1984; Dowling et al., 1987; Iwaoka et al., 1987; Nakano et al., 1975; Noll et al., 1987) and reflects the generation of short-lived free radicals or excited states derived from the termination reactions of the process.

A third source of \('O_2\) has been proposed to involve arachidonic acid metabolism which leads to the production of free radicals during prostaglandin formation via the cyclooxygenase pathway.

4.1.8 The Technique of Chemiluminescence Detection to Monitor Oxidative Processes

\('O_2\) arises in very low yield from these reactions but nevertheless measurement of this weak CL provides a convenient method of monitoring oxidative processes in cells and tissues. One system for detecting this low level light is by the use of a single photon counting apparatus containing a red-sensitive photomultiplier tube with filters to select out the wavelengths of light attributable to \('O_2\) dimol emission (Cadenas and Sies, 1984). More commonly, the highly sensitive photomultiplier tubes available in liquid scintillation counters are employed. The spectral sensitivity of this instrument however is limited by the sensitivity of the photomultiplier tube that is used. Most scintillation counters are equipped with photomultipliers sensitive only up to approximately 600nm so that \('O_2\) dimol emission which occurs at 634 and 703nm may remain undetected.
One way of overcoming this and the problem of detecting the very low level of light associated with native CL, is to incorporate a substance known as a chemiluminogenic probe which is able to intensify the CL signal. The phthalazine diones, or cyclic phthalhydrazides as they are often known, of which luminol is the most well known example, act as an exogenous substrate whose oxygenation is associated with a high yield of electronically excited products. This enhancement also enables a response to be detected from a smaller number of cells than would be possible with native CL.

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) is converted to an electronically excited aminophthalate ion in the presence of several oxidising species ($O_2^-$, $H_2O_2$, 'OH, 'O$_2$) which upon relaxation to the ground state emits photons. This reaction, illustrated in Figure 4.3, emits blue light at 425nm making the photomultiplier tube of the liquid scintillation counter applicable for luminol-amplified chemiluminescence (LAC) measurements.

![Chemiluminescence Properties of Luminol: Schematic](image)

Figure 4.3

Chemiluminescence Properties of Luminol: Schematic

Representation of the Oxidation of Luminol Which Results in the Generation of Photons

(Trush et al., 1978)
Although the LAC method generally lacks specificity for identifying the precise chemical origin of the CL, the application of the technique to biological systems is useful for estimating the cellular efflux of oxygen species as an assay for detecting oxidative stress and cellular damage. Non-invasive, whole organ to subcellular investigations can be made and there are certain techniques available to make LAC more specific (see Discussion).

4.1.9 Applications of Chemiluminescence Detection

An intense LAC was measured directly from inflamed tissue during the development of the foot pad oedema model of inflammation by Dowling et al., (1986). By using modifications of the technique it was demonstrated that at least three types of reactive oxygen species (\(H_2O_2\), 'OH and 'O_2) contributed to this burst of oxidative activity. This together with the finding of an increase in products derived from lipid peroxidation suggested a role for free radical generation and lipid peroxidation in this model of inflammation (Dowling, 1985; Dowling et al., 1986, 1987). It has been proposed by many investigators that oxygen-derived species are most likely responsible for much of the injury that takes place in regions of inflammation.

CL in the presence of enhancers such as luminol has been extensively employed as a very convenient and highly sensitive method for monitoring the oxygen radical production associated with the respiratory burst of phagocytic cells (reviewed by Allen et al., 1985; Campbell et al., 1985; Trush et al., 1978). It has made possible the identification of a wide range of physiological, pathological and experimental stimuli which provoke oxygen metabolite production from cells as well as from acellular reactions. LAC provides a means of screening phagocytes for defects in oxidative metabolism and for investigating their interaction with pharmacological and toxicological agents.
4.1.10 The Effect of Cadmium on Phagocyte Function

Both depressed phagocytosis (Baginski, 1985; Greenspan and Morrow, 1984; Levy et al., 1984; Loose et al., 1978) enhanced phagocytosis (Greenspan and Morrow, 1984; Koller and Roan, 1977) and inhibited oxidative metabolism (Amoruso et al., 1982; Castranova et al., 1980; Hilbertz et al., 1986; Loose et al., 1977) as well as enhanced oxidative metabolism (Amoruso et al., 1982; Elsasser et al., 1986; Hilbertz et al., 1986) have been reported for a variety of types of activated phagocytes, assessed by a variety of techniques including CL (Castranova et al., 1980; Elsasser et al., 1986; Hilbertz et al., 1986) following both in vivo and in vitro exposure to cadmium. The outcome seems to depend on the type of stimulus and hence the mechanism to activate the cells, the concentration of cadmium as well as the timing of measurement. The oxidative metabolism of mouse peritoneal macrophages in vitro, measured by CL in the presence of the chemiluminogenic probe lucigenin, was enhanced within the first hour of exposure to cadmium when phorbol myristate acetate was used as the stimulus, whereas zymosan-induced oxidative metabolism was reduced (Hilbertz et al., 1986). Phagocytosis by rat pulmonary macrophages was reduced by in vivo and in vitro exposure to cadmium but a brief stimulatory effect was seen by low levels of cadmium in vivo (Greenspan and Morrow, 1984). A similar concentration effect was seen by Amoruso et al., (1982) who showed that cadmium at high levels inhibited the production of $O_2^-$ in digitonin-stimulated rat alveolar macrophages and human granulocytes in vitro. However, at low concentrations likely to be achieved in vivo, enhanced production of $O_2^-$ in vitro was observed and it was suggested that such a mechanism might explain the oxidising effects of the metal in vivo.

4.1.11 Consideration of the Parenteral Route of Cadmium Administration

Whilst the doses of cadmium used in the present study are very high and the subcutaneous route of administration does not simulate any known exposure to cadmium in man, a basic knowledge of the acute effects of cadmium in tissues may
help to elucidate the mode of action of this metal. An understanding of the development of the non-malignant s.c. lesion may be important in understanding the way in which cadmium results in tumour development (Chapter 1). The extent of oedema and necrosis and the apparent inadequacy of the repair processes to contain the s.c. lesion is in contrast to the rapid onset of regeneration observed in the kidney and testes and the minimal damage observed in the liver despite a high cadmium load (Chapter 2). This may relate to the amount of cadmium in the different tissues and the presence of cadmium-binding proteins which prevent its availability for interaction with tissue binding sites. However, of note is that cadmium-induced recruitment of inflammatory cells has been observed at s.c. sites of injection (Chapter 2), in the peritoneal cavity following i.p. administration (Giri et al., 1979) and in the lung following intratracheal instillation (Cross et al., 1979; Hoidal et al., 1985). In the lung, this was accompanied by evidence of lipid peroxidation.

4.1.12 Aims of the Study

The mechanism of increased oxidative metabolism of phagocytes induced by cadmium is not clear. However, it is speculated that enhanced production of reactive oxygen species by PMNLs and macrophages recruited into the s.c. cadmium injection sites and testes and subsequent free radical chain reactions, possibly potentiated by an inhibitory effect of cadmium on antioxidant systems (as discussed in Chapter 3) could be the basis for the observed severe tissue damage seen at these sites. The present study was undertaken to investigate this hypothesis.

4.1.13 Outline of the Investigation

Evaluation of the role of reactive oxygen species released from inflammatory cells in cadmium-induced damage was undertaken by using the detection of LAC directly from inflamed tissue as a measure of free radical production during the development of acute inflammation at s.c. sites of cadmium administration. The
The proposed direction of work involved assessing the early sequence of events in the formation of the s.c. lesion by measuring LAC at various time points up to 32 hours after cadmium administration and correlating the results with a histological assessment of the extent and nature of the local inflammatory reaction.

The investigation also aimed to compare the LAC response 24 hours after cadmium administration with that following administration of other metals and also to determine the effect of zinc pretreatment on free radical activity in the cadmium-induced lesion.

The results of this study prompted the development of an 'in vitro skin explant' system offering a controlled environment from which LAC could be monitored during various treatments. Such a system would thus be free from the effects of infiltrating inflammatory cells and make it possible to explore whether they played an essential part in the production of LAC in inflamed tissue or whether the response resulted from a more fundamental primary action of cadmium on the tissue.

A number of variables have been shown to modify CL production by phagocytosing leucocytes (Campbell et al., 1985; Dahlgren and Briheim, 1985; Easmon et al., 1980; Halstensen et al., 1986; Hastings et al., 1982; Westman, 1986). This can be by altering the production and release of metabolites responsible for the CL or by interfering with their measurement. An attempt was therefore made to standardise and optimise the experimental conditions for the detection of CL from tissue samples in vitro. The term 'in vitro' is used to make the distinction from the in vivo treatment study but is employed loosely since skin explants were not maintained metabolically viable by the use of culture medium. Despite this, the system allowed for the capacity of various pretreatments to inhibit LAC to be tested in the hope of shedding some light on the origins of the LAC and on the mechanism of the toxic interaction of cadmium with tissue.
Experimental Section

The Detection of Luminol-Amplified Chemiluminescence Directly From Tissue Taken From Subcutaneous Injection Sites Following Various In Vivo Treatments

4.2.1 Materials and Methods

Substances were administered by subcutaneous (s.c.) injection to male Wistar albino rats (180-200g) as previously described in the relevant sections in Chapter 2 and 3. The presence of free radicals in inflamed tissue arising at s.c. injection sites was determined by using a modification of the luminol-amplified chemiluminescence (LAC) technique described by Dowling et al., (1986).

A stock solution of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical Company, Poole) was prepared by dissolving 10mg/ml \(5.64 \times 10^{-2}\) M in dimethylsulphoxide (DMSO; May and Baker Limited, Dagenham). It was stored in a dark container and diluted 1:250 in Dulbecco's Formula phosphate buffer (modified), without calcium or magnesium, pH 7.2 (Flow Laboratories, Irvine) at 4°C. immediately before use. The final concentration of luminol in the working solution was \(2.26 \times 10^{-4}\) M. The use of luminol in chemiluminescence detection has obviated the need to work under dark-adapted conditions (Dechatelet and Shirley, 1981) but nevertheless materials were stored in the dark prior to use.

Problems were encountered in equating quantitation and standardisation of samples. Speed in the preparation of tissue samples is of the utmost importance in order to avoid missing the CL response, but if accuracy of sampling is to be precisely achieved delay is inevitable. In the subsequent experiments where LAC was detected directly from inflamed tissue, the most suitable method of tissue sampling, incorporating a compromise to the problem, was found to be as follows:

Skin injection sites were shaved post mortem and the skin and s.c. tissue comprising the inflamed area was dissected from the underlying abdominal muscle. The problems of defining the boundary of the lesions was overcome by determining
the approximate surface area of the inflamed region from a 24-hour cadmium-treated animal and taking samples of a similar size from injection sites following all treatments. Each inflamed region was divided into five strips of approximately equal surface area using a scalpel. Five pieces of a similar size from the opposite flank constituted the control group.

Each sample was placed in a 6ml capacity, 55 x 15mm polypropylene scintillation vial (LKB Products Limited, Croydon). 4ml of the working luminol solution at 4°C was added to each vial which was then capped and mixed by inverting 2-3 times. Immediately after preparation of the samples CL was measured for 30 seconds on an LKB-Wallac 1216 scintillation counter (LKB Instruments Limited, Croydon) operating at ambient temperature and set in the out of coincidence mode. This mode is used due to the low energy of the light emitting species. It maximises the counting efficiency as both photomultiplier tubes count the low energy light emission separately and the counts for each tube are then summed automatically.

Vials containing luminol solution only (blanks) were included in each run to measure background CL. The operation mode of the instrument was set to continuous repetition and the number of vials in each rack was such that the cycle was repeated automatically at intervals of 10 minutes to enable the peak response and decay of CL to be observed. After measurement the vials and their contents were weighed to determine the weight of tissue samples which were then transferred into 10%v/v neutral buffered formalin for subsequent histological examination (see Chapter 2 for method).

4.2.2 Presentation of Results

Results were corrected for background CL and expressed as arbitrary units of CL rate, counts per minute (cpm) per sample. It was considered a valid approach to compare samples of a similar surface area, even though inflamed oedematous tissue weighed more, on the basis of the results of a preliminary experiment into the
The effect of tissue weight on LAC (CL was measured on a range of weights of skin from an untreated rat. Levels of light production were fairly low (0.22 to 0.63 \times 10^4 \text{ cpm}) and there did not appear to be any correlation between weights of tissue samples and the counts recorded).

The measurements revealed a differential response across the inflamed site with the highest counts being recorded at the centre of the lesion. For this reason the results at the time of maximum CL response are individually presented. Repeated readings after the addition of luminol to tissue showed that the response usually peaked at 15 to 20 minutes and then decayed rapidly with time. The weights of the individual samples are also presented on the figures.

4.2.3 Experimental Details and Results

1. Comparison of the Effect of Various In Vivo Treatments on the LAC Detected at s.c. Injection Sites

LAC was measured directly from freshly-dissected injection sites 24 hours after a single s.c. administration of saline, solutions in saline of the chloride salts of aluminium, cadmium, copper or iron (13.35 \mu \text{mol metal/kg}) or turpentine (0.5ml/rat) to male W/A rats. There were three animals per group except for turpentine, where \( n \) was 5 and for iron (\( n = 2 \)).

Despite the small group size, Figure 4.4 shows that pronounced differences were observed between treatment groups. There was a dramatic increase in the LAC arising from inflamed tissue following cadmium treatment. The response from non-inflamed tissue (as assessed microscopically) was consistently low and ranged from 0.4 to 1.1 \times 10^4 \text{ cpm}. Individual basal counts are presented to give an indication of the low variation. The values for inflamed tissue from cadmium-treated animals ranged from 1.1 to 16.9 \times 10^4 \text{ cpm} with samples from central regions of the inflamed site giving much higher responses than those from the margins. 16.9 \times 10^4 \text{ cpm} represented a 25-fold increase in light emission over the mean control value.
Figure 4.4
Luminol-Amplified Chemiluminescence (LAC) at Subcutaneous Injection Sites at 24 Hours After Various Treatments

Each injection site was divided into five pieces of approximately equal surface area and results, expressed as cpm $\times 10^4$ per sample, are individually presented with those of control samples taken from the opposite flank. Sample weights (○, ●) are also shown.
Saline, aluminium and iron produced little or no response. LAC was enhanced in copper-induced inflammation, ranging from 0.4 to $2.6 \times 10^4$ cpm but this was minimal in comparison to the effect seen with cadmium. Turpentine produced variable results but counts were on the whole also much lower than those following cadmium treatment despite a marked oedema making the weights of the samples generally much greater than in the cadmium-induced lesion.

Histological Examination of control skin showed that autolytic changes resulting from the incubation of samples in luminol solution during measurement prior to fixation were minimal. The microscopic appearance of the cadmium- and turpentine-induced inflammation was as described fully in Chapters 2 and 3. Cadmium caused an intense acute inflammatory response with infiltrating PMNLs, marked oedema and extensive necrosis. Large s.c. abscesses resulted from turpentine administration with broad rims of intense suppuration around residual pockets of turpentine. There was also an intense oedema and large numbers of mononuclear and polymorphonuclear leucocytes.

Copper attracted much less oedema, few polymorphs and there was little necrosis. Cells were predominantly mononuclear and confined to the subcutis and panniculus carnosus. Aluminium produced a minimal reaction which was highly localised (Chapter 3). Although inflammation was not apparent macroscopically following iron administration, an orange colouration of the s.c. tissue indicated the presence of the metal. Histologically, oedema was minimal and the inflammatory lesion was confined mainly to the subpannicular connective tissue with a few inflammatory cells infiltrating the panniculus carnosus (Figure 4.5a). Macrophages predominated and were shown by Perls Prussian Blue reaction to contain phagocytosed iron. There was little evidence of necrosis.

2. The Effect of Zinc Pretreatment on LAC Detected at s.c. Sites of Cadmium Administration

Four rats were given zinc pretreatment (76.45 μmol/kg; 5mg/kg) 24 hours prior to simultaneous zinc (76.45 μmol/kg; 5mg/kg) and cadmium (13.35 μmol/kg;
Figure 4.5(a)
Subcutaneous Tissue at Injection Site 24 Hours After Administration of Ferric Chloride (13.35 μmol Fe³⁺/kg)
Note: predominantly mononuclear inflammatory cells, I, confined mainly to the subpannicular connective tissue.
Stain: haematoxylin and eosin. Magnification: x40

Figure 4.5(b)
Subcutaneous Tissue at Injection Site 4 Hours After Administration of Cadmium (1.5 mg Cd²⁺/kg)
Note: large numbers of polymorphs, PMNL, capillary dilatation, CD, haemorrhage, H, and oedema of panniculus carnosus, PC, and subpannicular connective tissue, SCT.
Stain: haematoxylin and eosin. Magnification: x100
1.5mg/kg) treatment as described in Chapter 3. The LAC emitted from injection sites 24 hours after the final injection was compared with that from five animals given cadmium only and three animals given the zinc treatment only. A further group of three rats were examined 24 hours after a single injection of zinc at a dose of 13.35μmol/kg.

A much higher LAC was observed in this study (Figure 4.6) which was probably attributable to the use of a freshly opened jar of luminol (see section on evaluation of the method). A minimal response was seen following the administration of zinc at 13.35μmol/kg whilst a more significant LAC was evident after two injections of 76.45μmol Zn^{2+}/kg. Very high levels of free radical activity were produced by three out of the five inflamed lesions caused by cadmium alone with moderate activity exhibited by the other two. Zinc pretreatment resulted in variable responses. In one animal zinc almost completely inhibited the cadmium response whilst at the other end of the scale, another animal showed a typical cadmium effect with high levels of activity.

Histological findings were as reported in Chapter 3. Zinc at 13.35μmol/kg produced a diminished inflammatory response in comparison to 76.45μmol/kg.

3. The Time Course of LAC During Development of Acute Inflammation at s.c. Sites of Cadmium Administration

Figure 4.7 shows the LAC response in inflamed tissue at specific time points, between 4 and 32 hours following a single s.c. injection of 13.35μmol Cd^{2+}/kg to rats (n = 3). LAC was slightly (up to 4-fold) and fairly consistently raised as compared to control skin after 4, 6 and 8 hours. Very high levels of free radical activity were detected after longer intervals (up to 25-fold at 24 hours) but there was individual variation within the groups in the magnitude of the response.

Four hours after cadmium administration histological examination revealed numerous PMNLs, especially within blood vessels where they displayed margination, but also in the s.c. tissue and some were also present in the dermis (Figure 4.5b).
Figure 4.6  Zn (13.35\,\mu mol/kg)  
Saline  
Cd (13.35\,\mu mol/kg)  
Zn (78.45\,\mu mol/kg)  
Zn (78.45\,\mu mol/kg)  
\textit{+} Cd (13.35\,\mu mol/kg)  
\textit{+} 2nd Injection

The Effect of Zinc Pretreatment on Luminol-Amplified Chemiluminescence (LAC) at Subcutaneous Sites of Cadmium Administration

Each injection site was divided into five pieces of approximately equal surface area and results, expressed as cpm x 10^{5} per sample, are individually presented \textbullet with those of control samples taken from the opposite flank \circled{1}. Sample weights (\textcircled{1}, \textcircled{2}) are also shown.
Figure 4.7
Time Course of Luminol-Amplified Chemiluminescence (LAC) During Development of Acute Inflammation at Subcutaneous Sites of Cadmium Administration
Each injection site was divided into five pieces of approximately equal surface area and results, expressed as cpm x 10^4 per sample, are individually presented with those of control samples taken from the opposite flank. Sample weights (○, ●) are also presented.
Oedema was quite marked but the tissue remained fairly intact although some
degeneration of leucocytes and degranulation of mast cells was apparent. From 6 to
8 hours there was increasing evidence of necrosis and although polymorphs still
predominated there were increasing numbers of mononuclear cells. By 12 to 16
hours oedema was more intense and the lesion occupied a larger area. Numerous
inflammatory cells were present in all layers of skin and there was extensive
necrosis. The lesion had further developed by 24 to 32 hours and showed a similar
picture as described previously in Chapter 2.

4.3 Evaluation of the Method for Detection of LAC
Directly From Tissue and its Modification for In Vitro Studies

The addition of cadmium to vials containing untreated skin samples (0.5g) in
luminol solution was unexpectedly found to produce intense chemiluminescence.
Figure 4.8 shows a typical response demonstrating the time course of
chemiluminescence production and decay. There was a dramatic 23-fold
enhancement of light emitted at the peak as compared to the response in the
absence of cadmium. In order to arrive at the optimal experimental conditions to
undertake in vitro examination of the effect of cadmium on tissue free radical
activity, a series of experiments were carried out in which untreated skin was
incubated in the presence or absence of cadmium under a range of different
conditions. A number of factors likely to influence the chemiluminescence response
were taken into account including concentration of luminol and DMSO, type and pH
of the buffer, temperature of the scintillation counter and luminol solution and
tissue weight and integrity. Cadmium addition was achieved by the substitution of
100µl of solution in each vial with 100µl of 8.125 x 10^{-1}M or 8.125 x 10^{-2} M Cd^{2+}
to give a final cadmium concentration of 1 x 10^{-2}M or 1 x 10^{-3}M.

4.3.1 Effect of Type of Buffer and pH of Luminol Solution

The addition of cadmium solution to luminol made up in phosphate buffer, as
described in Section 4.2.1, resulted in the formation of a precipitate, presumably
Saline or $2.26 \times 10^{-4}$M luminol solution $\pm 1 \times 10^{-2}$M Cd$^{2+}$ was added to 0.5g skin samples at zero time. Chemiluminescence is expressed as cpm $\times 10^4$ per 0.5g sample. Each value represents mean $\pm$ SEM of five samples.
cadmium phosphate. This probably reflects what happens in vivo when cadmium solution, administered by s.c. injection, comes into contact with tissue fluids. Nevertheless all in vitro studies were performed using 0.9% w/v saline as diluent to minimise the possible interference that the presence of precipitate might have on light detection and to maximise the amount of cadmium available to interact with the tissue.

Phosphate buffer and saline, were compared for background chemiluminescence with and without the addition of luminol; all four solutions gave similar values. No differences were apparent in the LAC response of skin with phosphate buffer or saline as diluent.

Each of the substances to which skin was exposed during in vitro studies was tested in blanks also to ensure that the chemiluminescence being detected was not due to secondary light-producing reactions caused by a chemical interaction of luminol with the substances in question. No such reactions altering light emission were evident.

Following chemiluminescence measurements in all studies, the pH of the solution was determined since this has been shown to affect the CL of phthalazine dione derivatives (Dahlgren and Briheim, 1985; Hastings et al., 1982; Westman, 1986). The pH at which luminol reacts best and with greatest light yield has proven to be 10.5 in cell free systems (Bostick and Hercules, 1975) although luminescence can be observed at lower pH values. Usually a compromise has to be reached between physiological pH values (7.2 - 7.4) at which cells function most efficiently and the much higher optimal value for luminol oxidation. Saline had a pH of about 6.5 which was reduced to about 5.9 by the addition of luminol. Addition of skin however had a buffering effect resulting in a solution of pH between 6.4 and 7.0. 1 x 10^{-2} M cadmium caused a slight shift in pH of 0.2 towards acidity. The presence of cadmium in luminol solution alone did not alter background counts despite slight differences in the pH of solutions.
Hastings et al. (1982) showed a 5-fold increase in LAC of zymosan-stimulated neutrophils with shift a in pH from 7.4 to 8.5, however only a slight rise in chemiluminescence with alkalinity occurred between pH 6.5 and 7.4. Since the pH values of all solutions in the present study were below 7.4 and cadmium tended to cause slight shift towards acidity, it is concluded that the LAC response of skin in the presence of cadmium is not attributable to a pH effect on luminol activity.

4.3.2 **Effect of the Temperature of Scintillation Counter and Luminol Solution**

Comparison of the LAC response using a refrigerated scintillation counter (15°C) and one at room temperature showed, as expected (Easmon et al., 1980; Halstensen et al., 1986; Westman, 1986) that background counts were reduced and the interval to maximum response was longer at the lower temperature. However, the magnitude of the response appeared to be similar (results not presented). Measurements were subsequently carried out at room temperature as recommended by Trush et al., (1978).

Maximum light was emitted rapidly from samples of inflamed tissue following in vivo treatment (section 4.2.2) so adding the luminol solution at 4°C served to delay the response until the vials had been loaded into the instrument. This was not found to be necessary for in vitro studies since the maximum response was not usually reached until approximately one hour after the addition of cadmium; hence luminol solution was added at room temperature. Unfortunately the ambient temperature of the scintillation counter room was not controlled and varied between 22-27°C. This resulted in inter-experiment variability in background counts, which ranged between 23,000-42,000cpm, and time to maximum response (30-100min). The inclusion of a group of untreated control skin samples in each run however showed that there was little variation in the magnitude of the response.

4.3.3 **Comparison of Native and Luminol-Amplified Chemiluminescence**

A weak but detectable CL signal above background was observed when 0.5g tissue samples were immersed in saline in the absence of luminol and cadmium
The light emission was amplified 3-4 fold in the presence of $2.26 \times 10^4 \text{M}$ luminol but declined gradually towards native CL values during the three hour measurement period. This basal activity probably reflects the degree of tissue damage resulting from dissection procedures and decreased with time.

Native CL was increased by the addition of $1 \times 10^{-2} \text{M}$ cadmium. A fairly low but significant difference was apparent between control and cadmium-treated tissue from 45 minutes ($p < 0.05$) which increased during the following 135 minutes and light emission was still significantly higher ($p < 0.001$) 24 hours after the addition of cadmium. Addition of cadmium to skin in luminol solution resulted in an intense CL response exhibiting maximum levels of $3.287 \pm 1.375 \times 10^5 \text{cpm}$ at 60 minutes and equal to a 23-fold increase in light production over basal values of $0.142 \pm 0.013 \times 10^5 \text{cpm}$ ($p < 0.05$). Luminol amplified the light emission at peak response 42-fold over the native CL response to cadmium. LAC declined rapidly to 7% of the maximal levels seen at 60 minutes over the ensuing two hours but remained 8-fold higher ($p < 0.001$) even 24 hours after cadmium was added.

### 4.3.4 Effect of Luminol Concentration

Stock solutions ranging in concentration from 0 to $5.64 \times 10^{-1} \text{M}$ luminol in DMSO were prepared and used to make working solutions containing 0, $2.26 \times 10^{-3}$, $10^{-4}$, $10^{-5}$ and $10^{-6} \text{M}$ luminol in saline. Each luminol solution was added to four skin samples each weighing 0.5g in separate vials, to which cadmium was added to give a $1 \times 10^{-3} \text{M}$ solution. The light emitted from vials was measured at 15 minute intervals and the temporal response is shown in Figure 4.9a.

Background counts of the various concentrations of luminol did not differ, Luminol at all concentrations produced a statistically significant enhancement of CL over native CL of skin in the presence of cadmium. A concentration-response relationship between CL and increase in concentration of luminol was seen up to a level of $2.26 \times 10^{-4} \text{M}$. A further increase in concentration to $2.26 \times 10^{-3} \text{M}$ resulted in a slightly lower LAC than at $2.26 \times 10^{-5} \text{M}$ which may have been due to quenching.
Figure 4.9
Effect of (a) Luminol Concentration (b) DMSO Concentration (c) Tissue Weight and (d) Intact Versus Finely Divided Tissue on the Luminol-Amplified Chemiluminescence (LAC) Response of Skin to Cadmium In Vitro

All samples of tissue, except those in the two saline groups in Figure (d) were exposed to cadmium from zero time at concentrations of $1 \times 10^{-3}$M (a and b) and $1 \times 10^{-2}$M (c and d). Luminol concentration was $2.26 \times 10^{-4}$M in (b), (c) and (d). Each value represents mean ± SEM where $n = 4$ (a and b), or $n = 5$ (c and d). Statistically significant differences between intact and divided tissue in (d) are shown as ** $p < 0.01$. 
of light since a precipitate was formed on addition of the stock solution of the highest concentration of luminol to saline. Alternatively a high concentration of luminol may inhibit the response. The peak response occurred 60 minutes after addition of the reagents in all groups except $2.26 \times 10^{-4}$M which showed a main peak 30 minutes earlier. It was decided to employ luminol at a concentration of $2.26 \times 10^{-4}$M since this gave the highest response. Since luminol concentration may become limiting during the reaction, it was felt that this would ensure the presence of sufficient luminol.

During the course of these studies carried out over a period of two years it was found that luminol appeared to deteriorate once a jar had been opened. Direct comparison showed that 'old' luminol produced a markedly lower magnitude of response than that from a newly-opened jar (results not presented). Although the magnitude of the response was decreased the same features of the curve were retained. This variation in magnitude inevitably gave rise to some difficulty in comparison of inter-experiment results.

4.3.5 Effect of DMSO Concentration

Small amounts of DMSO used as a solvent for the preparation of stock luminol solutions have been found to have no effect on the CL generated (De Chatelet et al., 1982). However, DMSO has \textsuperscript{•}OH radical scavenging activity (Kahl et al., 1987) and increasing concentrations of DMSO have been reported to have a detrimental effect on CL from zymosan-stimulated granulocytes (Hastings et al., 1982). Therefore a range of concentrations of DMSO in the working solution containing a fixed concentration of luminol ($2.26 \times 10^{-4}$M) were tested using 0.5g samples of skin and $1 \times 10^{-3}$M Cd$^{2+}$. The final concentrations of DMSO were 0.04, 0.4, 0.8, 2.0 and 4.0% v/v.

Similar marked CL responses were produced in the presence of 0.04, 0.4 and 0.8% DMSO (Figure 4.9b). There was a slight precipitate in the working solution containing 0.04% DMSO since the stock solution contained a high concentration of
luminol which precipitated on addition to saline. Hence, the reduction in use of DMSO achieved by adding a more concentrated stock luminol solution appears to be limited. 2% and 4% DMSO produced a concentration-related inhibitory action on the CL response which was exhibited at the lower concentrations of DMSO ($p < 0.05$ at peak response). It was concluded that 0.4% DMSO was acceptable since it had no inhibitory effect on the response.

4.3.6 Effect of Tissue Weight

Comparison of the response of 0.25, 0.5 or 1.0g samples of tissue to $1 \times 10^{-2}$ M cadmium is shown in Figure 4.9c. A clear weight response was evident in contrast to previous observations with non-treated tissue (Section 4.2.2). However differences only showed statistical significance between 0.5 and 1.0g groups and between 0.25 and 1.0g groups at zero time ($p < 0.001$) and between 0.25 and 1.0g at 10 and 20 minutes ($p < 0.05$). Doubling the tissue weight almost doubled the CL at 0 and 10 minutes. The finding of such an immediate difference suggested that the area of cut surface may be important.

4.3.7 The Effect of Intact Versus Finely Divided Tissue

To test the effect of exposing a greater surface area of tissue, 20 samples of 0.5g weight were prepared. Ten of these were placed intact in vials whilst the rest were divided into small pieces, approximately $1 \text{mm}^3$, using a scalpel blade. Cadmium was added to half of each group to give a $1 \times 10^{-2}$ M solution whilst saline was added to the others.

The results are shown in Figure 4.9d. Initially there was a significantly elevated response ($p < 0.01$) immediately cadmium was added to finely divided tissue as compared to intact tissue. Similarly with saline-treated skin there was a transient higher response in the finely divided samples but not showing statistical significance until 16 minutes ($p < 0.01$). Following this immediate response, the significance of which is considered in the discussion, there was little difference.
between sample preparation groups. Hence, exposure of a greater surface area of tissue does not appear to be a significant factor in the time scale nor the magnitude of the peak response. In order to measure the CL of samples as quickly as possible after removal from the animal it was decided to use intact samples.

4.4 The Detection of Luminol-Amplified Chemiluminescence Directly From Skin During Various In Vitro Treatments

4.4.1 Method

Based on the results of the evaluation of CL measurement, in vitro experiments were carried out as follows: 0.5g samples of skin and s.c. tissue from male W/A rats (180-200g) were prepared as quickly as possible. Working luminol solution comprised of $2.26 \times 10^{-4}$ M luminol and 0.4%v/v DMSO in normal saline and was added at room temperature. Addition of test substances in solution was made by substitution of 100µl of the 4ml volume of working luminol solution. All vials, including blanks, were filled, capped, mixed by inversion and CL measured within 30 seconds of the addition of solutions. A sample from each treatment group was measured in turn to avoid differences due to any incidental effect that a delay in counting might have on prepared skin samples. CL was repeatedly measured as described earlier, usually at 15 minute intervals over a period of at least two hours, until the peak response had passed.

4.4.2 Presentation of Results

With the experimental conditions standardised as far as possible good reproducibility and sensitivity of the system was obtained. Temporal responses are illustrated in Figures 4.10 and 4.11; the zero mark on the time axis was the time of the first CL measurement which was not usually more than five minutes after removal of the tissue from the animal. All results are expressed in cpm as means ± SEM where the number of samples per group is 5 and groups within the experiments were compared using the Students t-test. The statistical significance of differences
between groups at maximum response is presented as * p < 0.05; ** p < 0.01; *** p < 0.001 in the tables and the value of peak response is also expressed as a multiple of the value for the saline group. It should be pointed out however that variability within the groups tended to be lower before and after the maximum response so in some of these cases differences reached statistical significance or t values were higher.

4.4.3 Dose Response to Cadmium

Stock solutions of cadmium chloride were prepared such that addition of 100μl to the contents of vials gave 1 x 10^{-2}, 1 x 10^{-3} or 1 x 10^{-4}M Cd^{2+}. A further group received saline only. In the 1 x 10^{-2}M solution, the skin was exposed to a comparable concentration of cadmium to that which s.c. tissue experienced during in vivo administration of the metal (1.335 x 10^{-2}M).

Figure 4.10a shows that a significant enhancement of light emission occurred immediately after the addition of cadmium. The response increased in a dose-dependent manner and reached maximum intensity after 75 minutes. At this time the increase in CL over saline-treated skin and statistical significance of differences between groups was as shown in Table 4.1:

Table 4.1

<table>
<thead>
<tr>
<th>Cd^{2+} Conc.(M)</th>
<th>Peak CL (75min) Compared to Saline Group</th>
<th>Statistical Significance of Difference Between Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>x 2.3</td>
<td>*</td>
</tr>
<tr>
<td>1 x 10^{-4}</td>
<td>x 2.3</td>
<td>*</td>
</tr>
<tr>
<td>1 x 10^{-3}</td>
<td>x 12.1</td>
<td>**</td>
</tr>
<tr>
<td>1 x 10^{-2}</td>
<td>x 22.4</td>
<td>**</td>
</tr>
</tbody>
</table>

* p < 0.05, ** p < 0.01
Figure 4.10
Effect of (a) Cadmium Concentration (b) Compound of Cadmium (c) Heat Pretreatment of Tissue and (d) Zinc Pretreatment on the Luminol-Amplified Chemiluminescence (LAC) Response of Skin to Cadmium In Vitro

Cadmium was added to give $1 \times 10^{-2}$M solution except in the dose response study (a). In (a) (c) and (d) the chloride salt of cadmium was used. Cadmium was added with luminol solution at zero time except in (d) when it was added to and A following 30 minutes pretreatment of A and A with zinc sulphate ($1 \times 10^{-2}$M Zn$^{2+}$). LAC is expressed as cpm $x 10^4$ per 0.5g sample. Each value represents mean $\pm$ SEM where n = 5.
Statistically significant, dose-dependent differences between groups still persisted 3.5 hours after the addition of cadmium.

4.4.4 **Comparison of Various Compounds of Cadmium**

The following five compounds of cadmium, dissolved in distilled water were added individually to vials to give \(1 \times 10^{-2} \text{M Cd}^{2+}\):

**Table 4.2**

<table>
<thead>
<tr>
<th>Cadmium Compound</th>
<th>Formula</th>
<th>Solubility at Room Temp. (g/ml)</th>
<th>pH of Solution After CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloride</td>
<td>(\text{CdCl}_2 \cdot 2\frac{1}{2}\text{H}_2\text{O})</td>
<td>1.8</td>
<td>6.13</td>
</tr>
<tr>
<td>Acetate</td>
<td>(\text{Cd(C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O})</td>
<td>1.9</td>
<td>6.40</td>
</tr>
<tr>
<td>Iodide</td>
<td>(\text{CdI}_2)</td>
<td>0.7</td>
<td>6.22</td>
</tr>
<tr>
<td>Nitrate</td>
<td>(\text{Cd(NO}_3)_2 \cdot 4\text{H}_2\text{O})</td>
<td>2.3</td>
<td>6.20</td>
</tr>
<tr>
<td>Sulphate</td>
<td>(3\text{CdSO}_4 \cdot 3\text{H}_2\text{O})</td>
<td>1.0</td>
<td>5.89</td>
</tr>
</tbody>
</table>

(Saline) 6.40

Figure 4.10b and the table 4.3 shows that there was a marked variation in the CL response of skin to the different compounds of cadmium:

**Table 4.3**

<table>
<thead>
<tr>
<th>Cadmium Compound</th>
<th>Peak CL (45min) Compared to Saline</th>
<th>Statistical Significance of Difference Between Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodide</td>
<td>x 1.5</td>
<td>-</td>
</tr>
<tr>
<td>Acetate</td>
<td>x 1.5</td>
<td>*</td>
</tr>
<tr>
<td>Sulphate</td>
<td>x 13.5</td>
<td>-</td>
</tr>
<tr>
<td>Chloride</td>
<td>x 17.8</td>
<td>***</td>
</tr>
<tr>
<td>Nitrate</td>
<td>x 29.7</td>
<td>***</td>
</tr>
</tbody>
</table>

* Saline

* p < 0.05, *** p < 0.001
Both iodide and acetate produced only a 1.5-fold increase in the light emitted above basal values. The iodide group was not significantly different from the saline group at any point measured whilst acetate showed increasing significance with time to $p < 0.001$ at 120 minutes.

In contrast cadmium chloride, sulphate and particularly nitrate produced a notable CL response. Although skin exposed to cadmium nitrate reached a maximum intensity of light production which was 30-fold that of saline, a significant difference ($p < 0.05$) was only apparent at 0, 15 and 90 minutes.

4.4.5 The Effect of Heat Treatment of Skin on LAC Response to Cadmium

Half of the tissue samples was heat-treated by placing in boiling saline for three minutes whilst the other half was put in saline at room temperature. All samples were then laid on filter paper to drain and cool for 15-20 seconds and then placed in vials with luminol solution and $1 \times 10^{-2} \text{M} \text{Cd}^{2+}$.

Fresh tissue responded to cadmium as in the previous experiments with a very intense CL whilst the response was almost completely inhibited by heat pretreatment (Figure 4.10c). The CL from fresh tissue peaked at 32 minutes and was 24.2-fold higher than light emitted following heat treatment ($p < 0.001$).

It was evident that the peak response of the fresh tissue in this experiment was about four times higher than in the cadmium dose response experiment. This may in part be due to a higher ambient temperature which could also be responsible for the shortened time to maximum response. However, skin samples for the heat treatment study were obtained from a slightly older animal and hence were composed of more adipose tissue which may have some bearing on the magnitude of the response.

Results of a preliminary experiment (results not presented) comparing the effects of cadmium on LAC of equivalent weights of various tissues seemed to indicate that obviously fattier tissues such as epididymal fat body and skin gave much higher responses than tissues such as liver and kidney. This suggests that the
The presence of lipid may have a role to play in the cadmium-induced LAC observed in this system and the possible significance of this will be dealt with in the Discussion.

4.4.6 Effect of Zinc Pretreatment of Skin on LAC Response to Cadmium

Zinc sulphate solution was added to half the skin samples immediately prior to CL measurement to give a $1 \times 10^{-2}$M solution of zinc. The other samples received an equal volume of saline. CL was measured twice during 30 minutes zinc pretreatment after which cadmium was added to half of the saline and half of the zinc-treated samples to give 10mM solution whilst saline was added to the others. All vials were recapped and mixed and then counted immediately and repeatedly every 15 minutes for a further 90 minutes. The results are shown in Figure 4.10d and Table 4.4:

Table 4.4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peak CL (45 min)</th>
<th>Statistical Significance of Difference Between Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td></td>
<td>Saline</td>
</tr>
<tr>
<td>Zn only</td>
<td>x 1.9</td>
<td>** Zn only</td>
</tr>
<tr>
<td>Zn + Cd</td>
<td>x 2.5</td>
<td>* * Zn + Cd</td>
</tr>
<tr>
<td>Cd only</td>
<td>x 26.0</td>
<td>* * Zn + Cd</td>
</tr>
</tbody>
</table>

* p < 0.05, ** p < 0.01

The cadmium only group gave a marked CL response which peaked at 15 minutes and again at 60 minutes after the addition of cadmium. Zinc treatment enhanced CL slightly by 15 minutes ($p < 0.05$) and the zinc only group remained slightly though significantly higher than saline until 75 minutes. The zinc-pretreated cadmium group remained significantly higher until 45 minutes. The response to cadmium was completely prevented by zinc pretreatment.
4.4.7 Effect of Pretreatment with Metal Chelators on the LAC Response of Skin to Cadmium

To test whether the cadmium-induced CL response shown by skin in vitro was possibly mediated through the release of iron, pretreatment with each of four chelators, two of which are purported to be specific for iron was carried out.

Skin samples were incubated in the presence of each chelator at a concentration of $1 \times 10^{-3} \text{M}$ in working luminol solution for 30 minutes, during which time CL was monitored twice before the addition of cadmium to give a $1 \times 10^{-3} \text{M}$ solution. Groups given cadmium, chelator or saline only were included and each sample and blank underwent the same procedures of addition of cadmium, chelator or saline, mixing and recapping. As in the zinc pretreatment study, a transient slight peak was seen after the addition of the second solution probably as a result of agitation, causing increased contact between the tissue and reagents.

(a) Ethylenediaminetetraacetic Acid (EDTA)

The results of pretreatment with ethylenediaminetetraacetic acid, disodium salt (Sigma Chemical Co, Poole) a general heavy metal chelator are shown in Figure 4.11a and the table below:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peak CL (60 min) Compared to Saline</th>
<th>Statistical Significance of Differences Between Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA only</td>
<td>$x 0.6$</td>
<td>Saline</td>
</tr>
<tr>
<td>EDTA + Cd</td>
<td>$x 0.4$</td>
<td>* EDTA only</td>
</tr>
<tr>
<td>Cd only</td>
<td>$x 8.0$</td>
<td>** - EDTA + Cd</td>
</tr>
</tbody>
</table>

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$
Figure 4.11

Effect of Pretreatment with (a) EDTA, (b) DTPA, (c) DFX and (d) BDS on the Luminol-Amplified Chemiluminescence (LAC) Response of Skin to Cadmium In Vitro

Each chelator was added with luminol solution at zero time to ▲ and ▲ to give 1 x 10^{-3}M solution whilst saline was added to ○ and ●. Cadmium chloride solution was added to ▲ and ○ to give 1 x 10^{-3}M Cd^{2+} solution and saline to ▲ and ● immediately prior to the third measurement. LAC is expressed as cpm x 10^4 per 0.5g sample. Each value represents mean ± SEM where n = 5.
EDTA pretreatment completely abolished the cadmium-induced CL response. There was no statistically significant difference between the EDTA alone and EDTA-Cd treated groups at any time point. EDTA only treated samples exhibited a lower CL than the saline group, statistically significant at 15, 60 and 90 minutes (p < 0.05). Light emitted from the EDTA + Cd group was similarly reduced showing statistical significance between 30 and 105 minutes.

Addition of EDTA to luminol lowered the pH from 5.72 to 4.72 and a further drop to 3.2 was caused by the addition of cadmium. However the buffering effect of the skin on the solution meant the pH only fell from 6.4 to 5.3 in the presence of EDTA and cadmium and is unlikely to have been responsible for the reduced CL.

(b) Diethylenetriaminepentaacetic Acid (DTPA)

Diethylenetriaminepentaacetic acid (Sigma Chemical Company, Poole) is a general heavy metal chelating agent which has frequently been used for cadmium. DTPA is insoluble in distilled water so a stock solution was prepared by adding in order 3:1:1 mole ratios of NaOH:DTPA:Ca(OH)₂ to distilled water as described by Cantilena and Klaassen (1982).

Figure 4.11b and the Table 4.6 show that DTPA completely prevented the response to cadmium:

Table 4.6

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peak CL (45min) Compared to Saline</th>
<th>Statistical Significance of Differences Between Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>x 1.2</td>
<td>Saline</td>
</tr>
<tr>
<td>DTPA</td>
<td>x 1.2</td>
<td>-</td>
</tr>
<tr>
<td>DTPA + Cd</td>
<td>x 1.2</td>
<td>-</td>
</tr>
<tr>
<td>Cd only</td>
<td>x 5.8</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>*</td>
</tr>
</tbody>
</table>

* p < 0.05
There was no significant difference between saline and DTPA groups either in the presence or absence of cadmium suggesting that DTPA has different chelation properties to EDTA. Very little difference was found in the pH of solutions following CL measurements.

(c) Desferrioxamine Mesylate (DFX)

Results of pretreatment with desferrioxamine mesylate BP, Desferal \(^R\) (Ciba Geigy Limited, Horsham) a highly specific chelator for iron, are shown in Figure 4.11c and the Table 4.7:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peak CL (60min)</th>
<th>Statistical Significance of Differences Between Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFX Only</td>
<td>x 0.6</td>
<td>Saline</td>
</tr>
<tr>
<td>DFX + Cd</td>
<td>x 5.0</td>
<td>*** ** DFX + Cd</td>
</tr>
<tr>
<td>Cd only</td>
<td>x 7.3</td>
<td>** ** DFX + Cd</td>
</tr>
</tbody>
</table>

** p < 0.01, *** p < 0.001

DFX treatment alone reduced CL slightly as compared to the saline group but this only showed significance at 30 minutes (p < 0.05). DFX pretreatment appeared to produce a slight reduction in the response to cadmium, significant between 90 and 135 minutes (p < 0.05).

(d) Bathophenanthroline Disulphonate (BDS)

Figure 4.11d shows that cadmium-induced CL was not influenced by pretreatment with the iron chelator, bathophenanthroline disulphonate (Sigma Chemical Company, Poole). There were no significant differences between the BDS
pretreated cadmium group and the cadmium group alone at any time point. Unfortunately a saline group was not included for comparison with the effect of BDS alone.

4.5 Discussion

Chemiluminescence in biological systems is thought principally to reflect the occurrence of excited states generated during the course of oxidative chain reactions such as occur during the production of reactive oxygen species by phagocytes and during the process of lipid peroxidation. Luminal-amplified chemiluminescence, used as a measure of tissue levels of free radical generation in the foot pad oedema model of inflammation (Dowling et al., 1987) showed significant correlation with tissue and plasma levels of products (ie, malondialdehyde) derived from lipid peroxidation.

In this investigation the detection of LAC directly from intact tissue has similarly been used as a measure of the generation of free radicals following both in vitro and in vivo exposure of s.c. tissue to cadmium. The study would obviously have benefitted from the use of larger groups and more time points to validate the findings but the results were nevertheless of interest. High levels of free radical activity were demonstrated during development of cadmium-induced local inflammation and a significant involvement of reactive species is implicated in the pathogenesis of the intense local tissue injury accompanying the inflammation. In addition LAC was pronounced on exposure of skin and s.c. tissue to cadmium in vitro and the significance of this finding will be discussed later.

The magnitude of the LAC following various in vivo treatments reflected the histologically-verified variation in the intensity of inflammation across each injection site as well as to a certain extent the duration and type of treatment. The quantitation of results posed problems because of the difficulty in defining the area of lesions and achieving rapid sampling. Despite subsequently discovering, during evaluation of the method, that in the presence of cadmium in vitro the CL response
of tissue is related to tissue weight, it is believed that the dramatic response by inflamed samples following in vivo cadmium treatment could not be accounted for solely on the basis of the higher weight of the tissue. A number of factors support this view. Firstly, there did not seem to be any consistency in the relationship between tissue weight and CL response. Samples from the centre of the cadmium lesion which generally displayed the highest CL value did not always differ markedly in weight from those on either side giving perhaps only half the CL response. The most intense light emission by a sample from a cadmium lesion was 25 times that emitted by the non-infamed tissue whilst the weight of the inflamed tissue was only three times higher. Secondly, the high weights of the turpentine-treated tissue samples did not produce proportionally higher results. It is felt that the recognised limitations of this apparently crude sampling technique in no way detracts from the usefulness of this method for indicating free radical activity in intact tissue.

The detection of free radical activity by LAC directly from inflamed tissue has been seldom employed. Belotsky et al., (1987) found that CL of the primary inflammatory focus of guinea pigs infected i.m. with S.aureus at a low dose, which produced inflammatory infiltration, peaked at Day 1 and was mainly due to a neutrophil reaction. With higher doses, which gave rise to abscess formation, a later peak between 3 and 20 days was observed and attributed to a macrophage reaction. Later time points were unfortunately not investigated in the present study but of interest was the observation that the high LAC did not occur until 12 hours after cadmium administration whereas Dowling et al., (1986) investigating the foot pad oedema model of inflammation, found a peak of activity in inflamed tissue earlier at eight hours. This then declined to 10% of maximum levels by 24 hours at which time in the cadmium model very high levels were still evident. This is perhaps consistent with the view that the CL response represents a notable production of active oxygen species by inflammatory cells (Dowling, 1985) since a more rapid response would be expected in the cell-mediated hypersensitivity response of the foot pad oedema model than in the acute inflammation induced by cadmium.
The marked variation in CL exhibited by individual rats from 12 hours after cadmium administration may be explained by inter-animal variation in the time to peak acute inflammatory response although no histological differences between local reactions to cadmium were apparent to account for the variability. The general of free radicals and/or active states of oxygen at injection sites by cadmium in vivo seems likely to be mediated in part by infiltrating phagocytic cells. The extent to which a substance produces free radical formation might then be determined by the numbers of cells recruited into the area. However, this was not supported by the findings in the present study. The intensity of the inflammatory infiltrate assessed histologically did not seem to bear a clear relationship with the magnitude of the CL response. Even at four and six hours after cadmium administration, when the CL was low, there were numerous PMNLs present. Copper and zinc produced negligible CL as compared to cadmium in spite of the presence of quite substantial numbers of inflammatory cells.

It was surprising that turpentine in spite of giving rise to broad rims of densely packed inflammatory cells did not produce as much free radical activity as cadmium at 24 hours. The more intense oedema exhibited during turpentine-induced inflammation suggests that oedema itself is not a factor in the magnitude of the CL response either. If it is the number of inflammatory cells that is important then pretreatment with zinc in vivo prior to cadmium treatment might have been expected to enhance the free radical activity since the tissue was being pre-irritated with a resulting infiltration of phagocytes and their potential for production of reactive oxygen species. This was not the case; in fact the results were very variable. In one animal the CL response was completely inhibited whilst in the others it remained to varying degrees. The explanation for this is unclear but may relate to difficulty encountered in placing the second injection precisely at the same location as the first.

It is possible that the type of cell present determines the CL response and the comparative study into the effect of various in vivo treatments indicated that a
predominance of PMNLs as seen in response to cadmium might be needed although again turpentine attracted large numbers of these cells without an intense CL being observed. The significance of the marked rise in the LAC response 12-24 hours after cadmium administration is difficult to explain but may be associated with the increasing numbers of necrotic phagocytes of all types that were evident as the inflammation progressed and which would presumably release active oxygen species. However, the CL response was still low at eight hours when necrosing PMNLs were evident in substantial numbers as well as increasing numbers of macrophages.

The intense CL response may not therefore be directly dependent on the presence and activity of substantial numbers of phagocytes of a particular type. It may ultimately reflect free radical-mediated tissue damage arising from an interaction of the tissue with reactive oxygen species. The involvement of self-propagating chain reactions of lipid peroxidation would require only a small number of reactive products released from phagocytes for initiation of the process. Alternatively, similar processes might result from a direct interaction of cadmium with the tissue by mechanisms to be postulated later in discussion. The balance between adequate protection against, and the potential destructive action of reactive oxygen species and free radicals is critical in determining the overall severity of the inflammatory reaction. Cadmium has been shown to enhance the production of active oxygen species from phagocytes (Amoruso et al., 1982; Elsasser et al., 1986; Hilbertz et al., 1986). An excess of oxygen species might overwhelm the radical scavenging capacity of the phagocytes and tissue cells which might anyway be compromised directly by cadmium, causing free radical damage to surrounding tissue and resulting in lipid peroxidation and depolymerisation of macromolecules.

The stimulatory effect of cadmium on O$_2^-$ production by phagocytes was not produced by an equivalent concentration of zinc (Amoruso et al., 1982) and in contrast to the significant increase in CL response of fish phagocytes to cadmium a significant reduction in CL was seen with copper and aluminium (Elsasser et al.,
These findings are interesting in view of the marked CL response seen at injection sites following the administration of cadmium in this study compared with the minimal response provoked by an equivalent molarity of aluminium, copper or zinc.

The observation by Sedgwick et al., (1985) that the depletion of circulating leucocytes reduces both inflammatory cell accumulation and oedema formation supported the concept that PMNLs are important in oedema formation. It has been proposed that some of the increased permeability seen during inflammation may be related to a flux of oxygen radicals produced by activated leucocytes at the site (Bjork et al., 1980). In particular the participation of 'OH and possibly 'O₂ have been implicated. The marked oedema seen at injection sites and in the testes may therefore be related to an enhanced production of these species in response to cadmium.

Oxygen metabolites may also mediate endothelial cell damage (Sacks et al., 1978) and cause tissue injury not only at the site of injury where polymorphs localise along vessels but also at sites distant to the area (Hoover et al., 1987). The generation of free radicals may initiate chain reactions within membranes with the release of hydroperoxides and the presence of these and possibly others such as endoperoxide products of arachidonic acid may result in both reversible and irreversible endothelial cell alterations. This, together with radical-induced degradation of vessel wall components such as hyaluronic acid may result in disruption of the endothelial cell barrier with subsequent haemorrhage (Bjork et al., 1980). Close physical inter-digitation between PMNLs and the endothelium is probably necessary and Gannon et al., (1987) have recently suggested that iron, which plays an important role in oxygen-mediated killing of endothelial cells by neutrophils, is derived from the target (endothelial) cells themselves. The results of Thomas et al., (1985) indicated that O₂⁻ can mediate the reductive release of iron from ferritin and it was suggested that ferritin may function as a source of iron for promotion of O₂⁻ dependent lipid peroxidation. As discussed in Chapter 3 selective
injury to vascular endothelial cells precedes other changes following exposure to cadmium in some tissues and results in haemorrhage. The above mechanism may be involved and additionally cadmium itself or cadmium-enhanced $O_2^-$ production may promote the release of iron from ferritin within the endothelial cells increasing its availability to catalyse free radical reactions.

The in vivo treatment study was complemented by in vitro experiments allowing for the study of a particular captive population of cells free from the effects of infiltrating inflammatory cells, etc, which occurs in vivo. An attempt was made to differentiate the contribution of resident skin and s.c. cells from infiltrating phagocytes with respect to the production of free radical activity in response to cadmium. It was demonstrated that CL can be used to assess free radical activity from intact tissue samples during various in vitro treatments. Discussion of a number of the technical details is included in the experimental section on evaluation of the method.

Untreated skin was found to produce consistently low levels of light. A dramatic dose related increase in LAC accompanied cadmium exposure and up to 30-fold higher levels of light were recorded. The response could also be inhibited by certain pretreatments. The amplification of CL by luminol increases the sensitivity of the system but also introduces an additional variable. Interference with the luminol reaction or the excited state may alter the observed light emission in the absence of effects on radical production itself. That the LAC response was a function of the interaction between tissue and cadmium rather than some incidental chemical effect of an interaction between cadmium and luminol or other cadmium-induced artefact of the luminol enhancement system was demonstrated by the following findings: (i) A negligible response to cadmium was seen after heat treatment of skin. The procedure clearly altered the tissue in some way indicating that the reaction is not just a chemical effect but a biological one dependent on the integrity and activity of the tissue. Heat treatment may alter the binding sites involved in the cadmium interaction responsible for the response, (ii) the CL of
blanks was not enhanced on addition of cadmium to the luminol solution in the absence of tissue, (iii) A significant CL was detectable on exposure of skin to cadmium in the absence of luminol although it was much lower than that detected with the enhancer. It is probable that excited carbonyls (RO*) primarily contribute to the native CL signal since \( ^1O_2 \) is likely to escape detection by the liquid scintillation counter (Cadenas and Sies, 1984).

As in the present study, the peak LAC response by fish phagocytes in vitro was observed 30-60 minutes after the addition of cadmium (Elsasser et al., 1986). The luminol-dependent CL curve for PMNLs consists of two peaks the significance of which has been investigated in some detail by Bender and Van Epps, (1983); Briheim et al., (1984); Dahlgren et al., (1985) and Westman, (1986) with the following conclusions. The first peak is probably extracellular in origin, possibly at the membrane surface. Luminol is thought to readily diffuse into cells so the second peak may represent intracellular events such as the reaction of luminol with the oxidising species produced during phagocytosis. Thus both intracellular and extracellular events contribute to the measured CL and CL of intracellular origin appears to be limited not by the generation of oxidative metabolites but by the diffusion of luminol into the cell.

Only one peak was apparent in the present study. The earlier peak may have been missed and the maximum response may therefore represent primarily an intracellular event with the delay being associated with the time required for luminol to become intracellular. During the study in which tissue preparation technique was evaluated, there was an initial higher response with finely-divided tissue which may represent an extracellular event attributable to the presence of more cellular contents 'spilled out' from cells disrupted by the slicing procedure. The shortened time to peak response seen following the addition of cadmium subsequent to measurements made during pretreatment may be because intracellular localisation of luminol had already commenced. The time to maximum response was longer during in vitro cadmium treatment than following in vivo exposure,
suggesting perhaps that the CL response may additionally be limited by the diffusion of cadmium into tissues. The inhibition of the response to cadmium by heat treatment of skin might be attributable to alterations to the tissue preventing not only cadmium interacting but equally luminol becoming intracellular. This might then account for the slight progressive rise in CL, similar to native CL, seen on exposure of heat treated skin to cadmium. The possibility arises that cadmium may alter cell membranes in some way thus allowing more luminol to enter the cell. It is not thought that this would produce the clear dose response to cadmium which was observed but could be tested by examining the native CL response to different doses of cadmium in the absence of luminol. It is more likely that cadmium directly affects the free radical production responsible for the CL by some mechanism.

The oxidation of luminol by reactive species produced intracellularly seems to be less sensitive to environmental conditions such as pH than the oxidation of luminol taking place extracellularly (Westman, 1986). Therefore, if the in vitro response of tissue to cadmium reflects primarily intracellular events, variation in the pH of the extracellular solution may not be important in altering the activity of luminol as discussed in the method evaluation section. Though the pH of solutions would also be expected to influence the reactivity of the tissue, pH measurements indicated that tissue seemed to have a buffering effect on the solutions so this was probably not a significant factor.

The rapid decline in CL after the peak may reflect an exhaustion of the luminol. If this is the case, the observation that a similar rate of decay occurred at the same time independent of luminol concentration or the magnitude of the response is difficult to explain. The decay may reflect a decline in cell activity either due to reduced viability with time or due to the endogenous anti-oxidant detoxification system of cells coming into action. This seems unlikely, however, especially since the native CL response to cadmium continued to increase with time and surprisingly both native CL and LAC were still significantly higher 24 hours after the addition of cadmium. This suggests that cadmium may be promoting the
spontaneous endogenous peroxidation of the tissue. The viability of the skin explants was not maintained by the use of elaborate culture medium but all measurements were compared to those of untreated skin to take account of CL due to autolytic degradation.

There are a number of limitations in the use of CL as a research or clinical technique. A major disadvantage is the transient nature of the signal; each excited molecule emits light only once and thus the CL substrate is usually used up during the reaction. CL production is expressed as a peak value although by measuring at intervals the peak value might be missed. One of the greatest limitations to be overcome is variability since several factors such as temperature, luminol activity and characteristics of the photomultiplier tube in a given instrument, often beyond the control of the operator, have been shown to influence the magnitude of the response. A further drawback is the lack of specificity. The identification of the pertinent activated oxygen species responsible for the LAC response was not established from the results of this study.

The characterisation of the photo-emission with regard to identification of the excited species could be extended with the use of alternative enhancers although the mechanism of action of these is complex and at present appears to be poorly understood whilst their specificity remains in question. Luminol probably reacts with $O_2^-$, $^\cdot$OH and $^\cdot$O$_2$ $^\cdot$ (Campbell et al., 1985) whereas the chemiluminescent probe lucigenin may be more specific for $O_2^-$ (Aasen et al., 1987; Aniansson et al., 1984; Edwards, 1987; Meretey et al., 1987). DABCO, 1-4-diazobicyclo-2,2,2-octane, an enhancer of singlet oxygen dimol emission, has been used to enhance the ultra-weak CL of $O_2^-$ (Dowling et al., 1986).

Specific scavengers and inhibitors, or quenchers, can be employed to determine the relative contribution of different reactive species to the response and the use of this technique has been discussed by Campbell et al., 1985; Kahl et al., (1987) and Rao et al., (1988). For example, a role for $O_2^-$ is indicated if addition of superoxide dismutase reduces LAC, whilst catalase demonstrates a role for $H_2O_2$. 
If both of these enzymes are necessary, a contribution by 'OH is implied which can be confirmed by the use of the 'OH scavengers DMSO, benzoate or mannitol. A role for 'O₂ is indicated if L-methionine, an 'OH and 'O₂ scavenger, further inhibits CL.

As might be expected, high concentrations (2 and 4%) of DMSO in the luminol solution were found to significantly reduce the LAC response of skin to cadmium in vitro suggesting a possible involvement of 'OH in the response. However, caution should be adopted in inferring the formation of particular species from the protective effects of scavengers since the specificity of some of these agents is poor (Halliwell and Grootveld, 1987; Halliwell and Gutteridge, 1985). The use of enhancers and scavengers in chemiluminescence is regarded by some workers (Kahl et al., 1987; Wilhelm and Vilim, 1986) as an unsuitable method for discrimination among individual oxygen species and their quantitative determination in biological systems. However continued research in this field will probably improve upon this situation.

The detection of light emission from biological samples remains a useful method for studying oxidative reactions in intact systems. The generation of electronically excited states during oxidative conditions can result from free radical interactions that may or may not be associated with the peroxidation of membrane fatty acids. CL may therefore reflect peroxidative chain reactions. The demonstration in skin of high levels of free radical activity by CL following the in vivo and in vitro exposure to cadmium concurs with the findings of previous investigations (see Introduction) that have demonstrated that cadmium produces lipid peroxidation both in vivo and in vitro in other tissues. However, other methods of detecting the process of lipid peroxidation are required to determine the significance of the CL results obtained in this study.

Because free radicals are highly reactive, their simple detection in biological materials is not at present possible. Indirect evidence has therefore to be sought by the application of methods to detect their degradation products, the transient nature of which nevertheless makes quantitative detection difficult. Spin trapping
and electron spin resonance (ESR) spectroscopy (Reviewed by McCay, 1987) is based on the property of spin trapping agents (nitrones) to react readily with reactive free radicals to produce stable radical adducts at the site of their origin in target organs. These radical adducts can then be detected by ESR spectroscopy to determine the intensity of radical products and in most cases, identify the nature of the radical produced. A common approach also is to measure the end products of lipid peroxidation in body fluids and tissue extracts and a number of methods in addition to CL are available and have been reviewed by Halliwell and Gutteridge, (1985); Slater, (1984) and Southorn and Powis, (1988). The two most popular are (1) the measurement of diene conjugation by the detection of characteristic light absorption, and (2) the thiobarbituric acid (TBA) reaction which is usually described as measuring malondialdehyde (MDA). Also used are: (3) the measurement of fluorescent products formed by the interaction of products of lipid peroxidation and other tissue components, (4) measurement of oxygen uptake (in vitro), (5) loss of PUFAs in membrane phospholipids, (6) detection of lipid hydroperoxides, (7) detection of specific aldehydes such as alkenals, and finally, (8) the estimation of alkane formation by measuring ethane and pentane exhalation by gas-liquid chromatography. The latter is the only genuinely non-invasive technique for measuring lipid peroxidation in vivo. A number of limitations and drawbacks are associated with current methodology which will not be discussed here since they have been excellently reviewed by Halliwell and Grootveld (1987) and Slater (1984). Since each technique is based upon the measurement of products originating from the process at different stages no one method can be said to be an accurate measurement of lipid peroxidation (Halliwell and Gutteridge, 1985). It is safest to use two or three different methods the results of which should correlate.

A criticism of the work presented in this Chapter is the absence of other methods to detect the process of lipid peroxidation. Other methods generally require tissue homogenisation and a major problem with skin is the presence of structural proteins that are resistant to conventional homogenising techniques that
work well for other tissue. In consequence the greater severity of the procedures that have to be used to obtain a homogeneous mixture may itself result in the production of lipid peroxides. Homogenisation of skin was attempted with a view to measuring MDA but without success since the composition of the tissue made it impossible to obtain sufficient tissue disruption. An alternative technique which could be attempted is that adopted by Dowling et al., (1987) who used intact pieces of tissue of approximately 50mg weight in the thiobarbituric acid reaction.

The limits to conclusions that can be drawn from the results in this Chapter concerning the possible source of CL and macromolecular targets of cadmium are recognised. It is interesting, nevertheless, to speculate on the mechanism by which cadmium produces oxidising effects, both in vivo and in vitro, which can be detected by CL. It is difficult to explain how sufficient reactive oxygen species might be produced to account for the CL observed in vitro in the absence of the substantial numbers of infiltrating inflammatory cells which were present at s.c. injection sites. The in vitro interaction of cadmium with a small number of endogenous phagocytes in skin might generate enough free radicals to initiate the chain of events which occur in lipid peroxidation. Other cell types may also be promoted to generate free radical species; for instance, epidermal cells, as shown by means of CL by Fischer and Adams (1984). In support of lipid peroxidative processes taking place is the observation from the present study that cadmium promoted much higher CL responses with equivalent weights of tissues of an apparently high lipid content which would presumably be more susceptible to peroxidative damage.

So, whilst toxic products from inflammatory cells may well provide a predominant contribution to the CL in inflamed tissue, the pronounced effect of cadmium on the CL response of skin in vitro supports the contention that an alternative process such as lipid peroxidation may play an important role in cadmium-induced s.c. damage and possibly injury at other sites. This would be in agreement with the finding that acute lung injury and lipid peroxidation following the intratracheal instillation of cadmium was the same in hamsters which had been
depleted of PMNLs as those which had not (Hoidal et al., 1985). This suggested that the products of PMNLs were not essential for the development of lung injury and that other mechanisms must operate.

Transition metals can affect many reactions leading to CL (Introduction). Iron stimulates the decomposition of lipid peroxides into alkoxy (RO*) and peroxo (ROO*) radicals and also the formation of hydroxyl radical (‘OH) in the Haber Weiss reaction. All three of these radicals can initiate peroxidation. There is no evidence to suggest that cadmium participates directly in the redox reactions leading to radical formation and it is difficult to imagine where and how cadmium might promote iron release. Aluminium can accelerate the peroxidation induced by Fe II salts in an in vitro system (Gutteridge et al., 1985) and it was suggested that Al III ions produce a subtle rearrangement in membrane structure that facilitates the peroxidative action of iron salts. A similar mechanism could be envisaged for cadmium with perhaps a cadmium-facilitated release of iron from proteins, such as ferritin, by mechanisms described by Halliwell and Gutteridge (1986) and Thomas et al., (1985) thus making it available to promote lipid peroxidation.

This possibility was tested by the use of chelators which should have trapped any iron and thus prevented the response. The dramatic inhibition of the cadmium-induced CL response following DTPA and EDTA pretreatment is in sharp contrast to the lack of inhibitory effect with BDS and DFX and probably reflects the metal specificity of the different chelators. DTPA and EDTA are general heavy metal chelators which probably acted by binding cadmium thus preventing its interaction with the tissue. A protective ability by DFX and BDS, both fairly specific chelators of iron, was not demonstrated suggesting that the cadmium effect is not mediated through iron release. It could be speculated that the inhibition of CL exhibited by EDTA in the absence of cadmium could be the result of EDTA chelating small amounts of iron present which might have promoted autoxidation of the tissue. A similar effect was surprisingly not produced by the other chelators.
Chelation of iron ions does not always prevent their reactions (Wilhelm and Vilim, 1986). Gutteridge et al., (1979) showed that the ratio of Fe^{2+} to chelator appears to be a critical factor in determining pro-oxidant or antioxidant activity. Thus iron sequestered by DTPA is no longer active (Buettner and Oberley, 1978) whereas iron in EDTA can be reactive depending on the ratio (Dunford, 1987; Gutteridge et al., 1979). Since the effectiveness of the various chelators tested may depend on the metal-chelator ratio the testing of a range of concentrations of chelators in the present system is required before firm conclusions about the involvement of iron can be made.

Chelators themselves are not inert towards reactive oxygen species. Desferal was shown to react slowly with O_2^-; this chelator and its complex with iron is also a powerful scavenger of *OH (Halliwell, 1985). This scavenging action might explain the slight inhibition shown by Desferal on the CL provoked by cadmium in vitro. Clearly complex interactions are involved which need further investigation but results would tend to discount cadmium facilitation of iron-promoted lipid peroxidation as being the primary mechanism at work. Investigations into the in vivo induction of lipid peroxidation by cadmium using iron-deficient or iron-loaded animals may illuminate the role of iron. Evaluation might be hampered however by the known interactions of cadmium with iron metabolism, such as the enhanced intestinal uptake of cadmium in iron deficiency, which could modify toxicity.

Chelating agents, such as EDTA and DTPA, are used as antidotes for in vivo metal intoxication. The present study confirmed the effectiveness of these two chelators in reducing the availability of cadmium for interaction with target cells. Chelating agents are being continually sought which will remove cadmium or other toxic metals already incorporated into sites whilst not interfering with endogenous metals. The in vitro CL technique described here could be used to assess the direct effect of chelators on metal-tissue interactions and to test the effect of various chelators in reversing the cadmium response. Results could be correlated with other in vitro screening methods.
The differential effectiveness of the various salts of cadmium to produce the CL response in vitro, in spite of inflammatory responses of similar magnitude being provoked by their in vivo administration (Chapter 3), is difficult to explain. It cannot be related to a difference in pH of the resulting solutions, neither does it seem to correlate with the solubility of the compounds since nitrate and acetate salts of cadmium, both showing high solubility, gave a very different magnitude of peak CL response. The results might suggest that it is the anion itself which causes the response in some way and this could be tested by the addition of equivalent amounts of anion of another salt. However, a more likely explanation is that acetate and iodide anions somehow exert a modulating effect either on the cadmium interaction with the tissue or on the intracellular localisation of luminol. Pretreatment of samples with, for instance, sodium acetate before cadmium chloride solution might indicate whether this is the case. Interestingly, Loose et al., (1978) found that the phagocytic capacity of mouse PMNLs was impaired more when cells were incubated with the chloride salt of cadmium than it was with the acetate salt although no explanation for this effect was given.

The effect of zinc pretreatment in vivo was variable and did not show conclusively that zinc reduces the CL response from injection sites. However, the finding of prevention of the cadmium response in vitro by zinc pretreatment was interesting and several mechanisms can be suggested to explain this antagonism. It is possible that zinc prevents cadmium reacting with the tissue either by forming complexes with cadmium in the luminol solution or more likely, by non-specific attachment to the tissue itself thus blocking any potential sites for cadmium interaction and perhaps preventing the passage of cadmium and/or luminol into the tissue. A protective action of zinc by competition with cadmium for more specific cellular sites such as sulphhydryl groups could also be envisaged thus diverting cadmium away from usual targets to a less sensitive site.

It is possible also that a more subtle biochemical relationship between zinc and cadmium exists that depends on the interference by cadmium in the biochemical...
role of zinc. Protective mechanisms are required by tissues in order to withstand the toxic effects of substances released by phagocytes. Cadmium may cause depletion of extracellular or intracellular antioxidant defence reserve. The superoxide dismutase (SOD)-catalase system provides intracellular defence against oxygen toxicity. Since $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ if not scavenged efficiently are known to give rise to potentially toxic intermediates (\'OH and \'O$_2$), depletion or inactivity of these enzymes will result in tissue injury. A likely target of cadmium is zinc-dependent SOD and cadmium has been shown to replace zinc in this enzyme (Bauer et al., 1980; Beem et al., 1974). Reduced activity of SOD accompanying lipid peroxidation has been observed in rat liver and kidney (Hussain et al., 1987) and brain (Shukla et al., 1987) following i.p. administration of cadmium. Assessment of SOD activity at s.c. sites of inflammation would be desirable. Zinc pretreatment may protect by preserving the activity of this enzyme. Cadmium-induced lipid peroxidation has been inhibited by zinc treatment in vivo in testis and kidney (Gabor et al., 1978) and in vitro in liver (Stacey and Klaassen, 1981).

Endothelial cells are important in affecting the apparent reduction in toxic oxygen products derived from polymorphonuclear leucocytes attached to their surface. The presence of detoxifying enzymes such as SOD in endothelial cells of blood vessels is thought to explain protection of these cells against the potential dangers of active oxygen species released from circulating polymorphs (Hoover et al., 1987). The toxic effect of cadmium on endothelial cells (Chapter 3, Discussion) may be mediated through inactivation of SOD which could be prevented by zinc pretreatment. Hence, testicular and possibly subcutaneous injury may involve neutrophil-dependent oxygen radical-mediated injury of vascular endothelial cells due to interference of cadmium with the antioxidant system of endothelial cells. The differential susceptibility of endothelial cells to cadmium toxicity might then be explained by differing superoxide dismutase levels.

Increasing evidence indicates the importance of zinc as a stabiliser of macromolecules and biological membranes by a mechanism restricted to the surface
of the membrane, of which zinc appears to be an integral part (Chvapil, 1976). Zinc in a concentration-related manner, as well as a variety of chelating agents, have been shown to stabilise hepatic lysosomal membranes in vitro whilst cupric and mercuric ions labilise the membrane (Chvapil et al., 1972a, 1972b). The mechanism by which such agents affect membrane stability is discussed by Chvapil, 1973, 1976; Chvapil et al., 1972b) in terms of their reactivity with components of the membrane which control structure and function, resulting in a change in the conformation of macromolecules involved and/or an effect on some enzymes controlling the integrity of the membrane. Evidence also indicates that one of the various possible mechanisms by which zinc stabilises a variety of biomembranes, both in vivo and in vitro is related to the inhibition of peroxidation of membrane lipids (Chvapil, 1973; Chvapil et al., 1972c) specifically to the NADPH oxidation-related formation of a free radical which can initiate the peroxidation reaction (Chvapil et al., 1976). Chvapil et al., (1972c) found that zinc prevents or significantly reduces the CCl₄-induced formation of lipid peroxides in liver, both in vivo and in vitro. A number of proposed mechanisms for the metal ion effect on biological membranes have been discussed by Ludwig and Chvapil (1981) and Diplock et al., (1986).

The well-recognised antagonistic effect that cadmium displays with respect to various functions of zinc might be postulated to include the membrane stabilising properties of zinc thus resulting in cadmium having a labilising effect on membranes and facilitating lipid peroxidative processes. The protective properties exhibited by zinc against the oxidising effects of cadmium in present work and by Gabor et al., (1978) and Stacey and Klaassen, (1981) may be associated with the stabilising action of zinc. Thus pretreatment with zinc or certain chelators may have enhanced the stabilisation of the membrane and protected it against a subsequent labilising action of cadmium. Contrary to this hypothesis however, is the finding that stabilising properties on hepatic lysosomal membranes were exhibited not only by zinc but also by cadmium and lead (Chvapil et al., 1972b), all of which are metals having only one oxidation state, suggesting that they may act by interfering with the oxidation of
membrane components. This throws doubt on a direct antagonism by cadmium on
the role of zinc in these membranes although other membrane systems and tissues
may respond differently. Cadmium may affect the integrity of membranes by other
mechanisms, possibly through reaction with sulphydryl groups of proteins, most
notably enzymes such as Na⁺/K⁺ ATPase or through a direct effect on phospholipid
metabolism (Diplock et al., 1986).

It is appropriate here to draw attention briefly to reports that high levels of
dietary zinc render erythrocytes more resistant to haemolysis (Settlemire and
Matrone, 1967; Chvapil et al., 1974) whilst zinc deficiency and low plasma zinc
reduce the level of zinc in the cell membrane and increase osmotic fragility
(Bettger et al., 1978; O'Dell et al., 1987). Zinc is a component of the erythrocyte
membrane linked both to protein as well as lipid and contributing to the integrity of
the cell (Chvapil et al., 1979b). This is of possible relevance to the cadmium-
induced alterations to erythrocytes, reported in Chapter 2. The increased fragility
shown by some cells may be related to the low plasma zinc levels known to occur in
inflammation (Sobocinski et al., 1978) and as a consequence of the acute s.c.
administration of cadmium (Bonner, 1980; Ashby et al., 1980). However, if the
stabilising effect that cadmium exerts on hepatic lysosomal membranes (Chvapil et
al., 1972b) also occurs in the erythrocyte membrane then cadmium would be
expected to protect the cell from osmotic stress. This could provide another possible
explanation for the reduced fragility of red blood cells seen after s.c.
administration of cadmium (Chapter 2).

Increasing amounts of zinc, supplied both in vitro and in vivo, exert an
inhibitory effect on various functions of granulocytes and macrophages, such as
chemotaxis, oxygen consumption and phagocytosis (Chvapil et al., 1977). It is
believed that zinc may be one of the many homeostatic factors regulating the
reactivity of these cells rather than the effect being a toxic one. The molecular
mechanism underlying the effect of zinc at the cell or tissue level is still uncertain
but a scheme implicating the interaction of zinc with membrane components of the
cell and with enzymes controlling the structure and function of the membrane seems likely. Zinc has been shown to inhibit the activity of membrane bound Na⁺/K⁺ stimulated, Mg²⁺-dependent ATPase system of activated alveolar macrophages, which is essential for phagocytosis, pinocytosis and active ionic transport processes of these cells (Chvapil et al., 1976). It was also found that zinc completely inhibits NADPH oxidase, the activity of which is essential for the formation of reactive oxygen species needed for the bacteriocidal activity of phagocytes (see Introduction).

It may be worthwhile investigating whether an interaction of cadmium with membranes of phagocytes can reverse this inhibitory effect of zinc on NADPH oxidase since this might provide an explanation for the enhanced O₂⁻ production shown by cadmium-exposed phagocytes (Amoruso et al., 1982). Such an effect was not observed with other divalent cations such as Pb²⁺, Zn²⁺, Hg²⁺ and Sr²⁺ (Amoruso et al., 1982).

In conclusion, even without the identification of the electronically excited states responsible for the CL detected, the results presented in this Chapter proved to be of considerable interest. Their significance could be further elucidated in future work by application of some of the techniques which have already been discussed for the identification of particular oxygen species and detection of lipid peroxidation. A concept of the possible sources of LAC at s.c. sites of cadmium administration which have been proposed is depicted in Figure 4.12. It was demonstrated that free radical activity and perhaps lipid peroxidative processes are closely associated with the local injury provoked by the s.c. administration of cadmium. The question arises as to whether lipid peroxidation is a major cause of the tissue damage or merely a consequence of it. Disrupted tissues undergo lipid peroxidation more readily than healthy ones. Even if the peroxidation is not the primary cause of the damage, its occurrence as a consequence of damage is still biologically important in view of the cytotoxicity of the end products of the process.
Figure 4.12
A Tentative Scheme to Explain the Source of Luminol-Amplified Chemiluminescence at Subcutaneous Sites of Cadmium Administration

The sites of action of cadmium which might lead to the production of reactive oxygen species and lipid peroxidative processes detectable by luminol-amplified chemiluminescence are depicted. It should be stressed that the involvement of these pathways, though proposed in discussion, has not been verified in the present study and thus remain highly speculative. It is conceivable however, that such processes could be involved in the development of the tissue injury at subcutaneous injection sites.

The following hypothetical mechanisms are presented:

1. Toxic effects on phagocytes causing the release of active oxygen species.
2. Enhancement of $O_2^-$ production by phagocytes.
3. Inhibition of intracellular protective antioxidant systems, eg, Zn dependent SOD in phagocytes and resident cells.
4. Lipid peroxidation as a consequence of toxic injury caused by cadmium.
5. Inhibition of extracellular antioxidant (eg, Cp) or antiprotease (eg, Zn-dependent $\alpha_2$ MG) systems.
6. Interaction with enzymes controlling the integrity of the membrane or interference with zinc-dependent macromolecular components of the membrane causing reduced membrane stability.
7. Enhancement of metal-catalysed lipid peroxidation via the release of transition metals, eg, Fe.
Subcutaneous tissue

Release of Fe/Cu

Release of metals which promote Lpx

Release of oxygen species

Enhanced O$_2^-$ production

(? via antagonism of Zn inhibition of NADPH oxidase)

Impaired intracellular antioxidant defence

Impaired extracellular antioxidant defence

Interference with enzymic or non-enzymic stabilising effect of Zn on biomembranes

Impaired antiprotease activity

O$_2^-$ = superoxide anion

$\mathcal{P}$ represents luminol-amplified chemiluminescence

Small arrows $\uparrow$ and $\downarrow$ refer to the increase and decrease respectively of the parameters indicated.

The phagocyte is depicted as a polymorphonuclear leucocyte but could also be a macrophage.

Abbreviations:
SOD = superoxide dismutase
Cp = caeruloplasmin
$\alpha_2$MG = $\alpha_2$ macroglobulin
Lpx = lipid peroxidation
Cd = cadmium
Zn = zinc
O$_2^-$ = superoxide anion

Subcutaneous tissue
Scepticism about the role of lipid peroxidation in cadmium-induced toxicity is derived mainly from in vitro studies in rat hepatocyte cultures exposed for short periods to high concentrations of cadmium (eg, Muller, 1986; Stacey et al., 1980). It is questionable whether the hypothesis that toxicity induced by cadmium can be dissociated from concurrently observed lipid peroxidation holds for the in vivo effect of cadmium in other tissues. Zinc appears to play a significant role in the control of the production of oxygen species and the process of lipid peroxidation by various inhibitory and stabilising actions. A wealth of potential sites at which cadmium could antagonise this function of zinc in a range of processes thus exist. Further studies may elucidate whether cadmium does indeed interfere and cause the initial structural damage to the membrane, allowing access for released iron to susceptible membrane sites where rapid peroxidation may ensue.

The parenteral route of administration appears to have almost exclusively been used for in vivo studies on the effect of cadmium on lipid peroxidation (see Introduction). The possibility arises that the findings of free radical production and lipid peroxidative processes associated with in vivo cadmium-induced injury are dependent on a parenteral mode of administration, thus implicating a role for inflammatory processes in their development. This might explain some of the conflicting reports of the occurrence of lipid peroxidation. Interestingly, acute inflammation induced by the s.c. administration of turpentine to rats has been shown to cause elevated hepatic lipid peroxidation even though turpentine does not reach the liver by the s.c. route (Nadkarni and D'Souza, 1986). Furthermore, in contrast to cadmium-induced hepatic lipid peroxidation (Hussain et al., 1987) it was accompanied by an increase in SOD activity.

Additional studies are needed to define the pathogenic role of phagocytic cells and their products in the oxidising effects of cadmium in vivo. An enhanced or prolonged production of reactive oxygen species may be due to altered regulation of the cells' metabolism by cadmium and/or an impairment of the self-limitation system which detoxifies oxygen derivatives. A possible line of research could be the
evaluation of the direct effect of cadmium exposure in vivo and in vitro on the oxidative metabolism of different isolated populations of cells by LAC in combination with other techniques. The protective action of zinc, a property which may be exhibited by other metals, may provide a useful tool for further investigation of the possible role of lipid peroxidation in cadmium-induced cellular injury.
CHAPTER 5

FINAL DISCUSSION
CHAPTER 5

FINAL DISCUSSION

5.1 A Consideration of the Implications of Inflammation
Provoked by the Parenteral Administration of Cadmium

Many of the effects observed after repeated s.c. administration of cadmium to rats at two dose levels (Chapter 2) mimicked the well-established response manifest after administration of a wide variety of substances that cause acute inflammation. A perusal of the literature on experimental cadmium toxicology shows that parenteral administration routes have almost exclusively been employed in acute experiments. These have been recognised as not being relevant to human exposure on the grounds that acute mortality is due to severe hepatic necrosis and the critical organ in male animals is the testis. The results of this study highlight an additional important effect, namely, acute inflammation, which substantiates this view. A major role of inflammation has been implicated in many of the changes observed after the s.c. administration of cadmium (Chapter 2) and must raise serious doubts about the interpretation of much published work, in particular relating to protein synthesis, metal homeostasis and the development of anaemia. Parenteral modes of administration have also been used for sub-acute and chronic studies with lower doses of cadmium where the primary effects are not on the liver and testes. Nevertheless it seems likely from scant observations in the literature that an acute inflammatory response accompanies s.c., i.m. and i.p. modes of administration of cadmium to a number of species and at a wide range of doses.

In many of the papers cited it seems that emphasis was laid on the primary organ or system under investigation and local reactions appear to have been overlooked or little account taken of their effects. Complex physiological alterations can be caused by the interaction of cadmium-induced toxicity and the inflammatory response. In cases where local reactions were identified, seldom were
systemic responses considered nor the possible consequences of these responses on the parameters under investigation. It is questionable whether many of the effects reported after administration of cadmium by parenteral routes are directly attributable to toxic effects of the metal on an organ or system under investigation or arise as a consequence of the irritant properties of cadmium which provoke an intense acute inflammatory response. In particular, it was shown that caution should be adopted in attributing metal changes directly to cadmium (or any administered substance that might cause inflammation) when using a parenteral route which induces lesions which of their own volition, can alter metal homeostasis. The differential effects that s.c. and oral cadmium exposure can have on iron and copper homeostasis were discussed and it is suggested that inflammation may play an important part in the development of anaemia following the s.c. administration of cadmium. Many of the effects of inflammation on metal homeostasis may have been under-estimated and results obtained following parenteral administration of cadmium may require reassessment in view of the findings of this thesis.

It was shown that other metals also provoke local reactions of variable intensity at s.c. sites of administration (Chapter 3). These were not as destructive in nature as seen with cadmium but nevertheless some systemic inflammatory changes were also evident. Hence, no discussion of the complex interrelationships that occur between substances under investigation and various endogenous processes should be complete without detailed consideration of the possible involvement of inflammatory responses provoked by the mode of administration.

Lipid peroxidation has been demonstrated in various tissues following the parenteral administration of cadmium (Introduction, Chapter 4). Inflammation has been implicated in the alterations in metal homeostasis and development of anaemia associated with the subcutaneous administration of cadmium. The question of whether lipid peroxidation may also arise as a consequence of cadmium-induced inflammation remains to be investigated.
Our understanding of copper, zinc and iron metabolism during inflammation and the involvement of these metals in free radical reactions and lipid peroxidation in vivo is still inadequate. It merits further improvement particularly in view of possible pharmacological manipulation of these metals as a new approach to the therapy of inflammatory disorders. Several postulated mechanisms to explain the acute toxic action of cadmium have been discussed in Chapters 2, 3 and 4 of this thesis, some of which involve antagonism by cadmium of one or more zinc-dependent processes. Investigations into the interaction of parenterally administered cadmium with endogenous metals, such as zinc, during cadmium-induced inflammation may expand our knowledge not only of the mechanisms of acute cadmium toxicity but also the role of trace metal metabolism during inflammation. The experimental use of other heavy metals as tools to improve our understanding should also be considered.

5.2 The Extrapolation of Rat Data to Cadmium Exposure in Man

With regard to iron metabolism, extrapolation of rat data to human should be done with extreme caution. The rat is more likely to rapidly display the consequences of a restricted flow of iron into the plasma than man because of its rapid growth rate and associated expansion of red cell mass and demand for iron. The importance of the nutritional status of the animal cannot be over-emphasised since the susceptibility of the rat to the toxic effects of cadmium can be enhanced by deficiency of essential metals such as iron and copper.

The need to use a parenteral mode of cadmium administration arises because of the low absorption rate of cadmium and the difficulties of undertaking laboratory investigations in short-lived rodents. Critics of the model state that it is not relevant for investigating the mechanisms by which cadmium expresses its toxicity after low oral exposure in man.
Despite drawbacks, most important of which would seem to be the induction of an inflammatory response, use of the parenteral dosing model over a number of years has nevertheless added substantially and will probably continue to do so, to our knowledge of cadmium toxicity. Many of the current concepts on the mechanisms involved have been extrapolated from parenteral dosing studies. Such methods should remain suitable for investigating the early sequence of events in acute cadmium-tissue interactions with a view to further understanding the mechanisms of both toxic and carcinogenic actions of cadmium. The model should however be used with caution and alternative methods of administration should be sought.

5.3 Alternative Methods of Cadmium Administration

Local tissue toxicity might be minimised by consideration of such factors as the choice of vehicle and buffering of parenterally-administered solutions. However, local reactions are most probably engendered by a mass action effect. An additional consequence of parenteral administration is the generation of transient high blood levels of cadmium. An alternative method to be considered is the use of a subcutaneously-implanted osmotic pump that would deliver constant amounts of cadmium over relatively long periods. The use of such a technique for the administration of cadmium has been reported by Duval and Grubb (1986). Of interest was their finding that the administration of 1.5mg Cd/day to rabbits for 28 days by continuous infusion caused no depression of body weight gain or lowered haematocrit as has been reported for rabbits as well as for other species following repeated parenteral injections (Introduction, Chapter 2). This supports the view that these effects may be related to inflammation or alternatively they may be a consequence of transient acute toxic levels of cadmium. Nevertheless, the possibility that the s.c. implant itself could provoke a local and systemic inflammatory reaction should not be overlooked.
Further studies are required to compare the effects of different routes of cadmium administration on a multitude of factors. Also, results in this study and those of other workers (Chapters 3 and 4) have indicated that the potency of various compounds of cadmium may vary and this remains a subject for future investigation.

5.4 **Proposals for Future Studies on Haematological Effects of Cadmium**

In addition to the subjects for future investigation which have already been proposed within the experimental chapters, research could be conducted along the following lines.

The spleen and bone marrow act as functional parts of the haemopoietic system as well as the reticuloendothelial and immune systems. A closer look at the mechanism of cadmium accumulation, its effects and its relationship with metallothionein in these organs will therefore be important in view of the possible correlation of cadmium with the manifestations of haematologic or immunologic anomalies. This could be achieved by employing techniques such as metal histochemistry or immunocytochemistry to identify the intracellular localisation of cadmium as well as of endogenous metals and metallothionein. The application of these techniques to tissues such as spleen and marrow, which displays a very heterogeneous population of cells, has the advantage that differences within individual cells can be detected which are often not apparent by biochemical assay. In addition, the inclusion of such techniques in future investigations involving s.c. injection sites would eliminate the need for the homogenisation of the tissue which is required for biochemical methods and which has proved difficult to apply to skin. Of potential use also is a recently described method for the histochemical detection of lipid peroxidation (Pompella et al., 1987).
Evaluation of the Technique of Luminol-Amplified Chemiluminescence Detection Applied to Intact Tissue

The development of in vitro systems such as the luminol-amplified chemiluminescence technique described in Chapter 4 is essential to circumvent the use of animals in research.

The potential of chemiluminescence as an analytical tool for the investigation of pathogenic mechanisms as well as for diagnostic clinical applications is considerable because of a number of particular features. The technique has the advantages of being relatively quick, simple and inexpensive to perform and can be carried out and give highly reproducible results on standard laboratory liquid scintillation counters. It is highly sensitive whilst being non-radioactive and non-hazardous and can be made more sensitive by the use of chemiluminogenic probes. Measurement is non-invasive and tissue disruption due to preparative homogenisation procedures can be eliminated. In spite of the limitations discussed in Chapter 4, these features make chemiluminescence a very convenient method of allowing the direct continuous quantitative monitoring of free radical activity in structurally intact, small numbers of cells, tissue samples or whole organs in response to various stimulation.

It was demonstrated that intact tissue samples can be successfully used to assess free radical activity as demonstrated by detection of chemiluminescence both following in vivo administration of substances and during in vitro treatment. The use of intact tissue samples may have some advantage over isolated cells or tissue homogenates. They can be prepared more rapidly and cell to cell contact and tissue organisation is preserved. Studies on intact tissue may reflect more accurately the integrated biochemical and physiological processes that occur in vivo. Maintenance of tissue integrity also avoids the need for cell separation and tissue homogenisation procedures, which need to be particularly harsh when applied to skin, and which may have a promoting effect on free radical processes.
The Involvement of Free Radical Mechanisms in the Toxicity of Cadmium

Experimental evidence was presented which indicated intense free radical formation at s.c. sites of cadmium injection as well as from skin and s.c. tissue exposed to cadmium in vitro. This endorses the concept of the involvement of reactive oxygen species and free radicals in the development of the intense local reaction seen at s.c. sites of cadmium administration. Whether produced by invading inflammatory cells or resident tissue cells, cadmium appears to cause the production of reactive species by an as yet unidentified mechanism. They may play a direct role in the toxic action of cadmium on s.c. tissue or be a consequence of it. Conclusions drawn from the results in this thesis did not allow much more than pure speculation on the possible mechanisms involved. It was shown that the in vitro chemiluminescence response of tissue to cadmium was inhibited by zinc pretreatment and further work is necessary to ascertain the possible common target sites of action involved.

Substantial evidence has accumulated which implicates the involvement of free radicals and reactive oxygen species in the process of tumour promotion and this has been the subject of many recent reviews (eg, Kensler and Taffe, 1986; Troll and Wiesner, 1985). Tumours may arise as a consequence of damage by reactive oxygen species derived from phagocytic cells. The intense free radical activity which was detected at s.c. sites of cadmium injection may well have some bearing on the subsequent development of tumours which has been reported by other workers (Chapter 3). Chronic free radical production may cause depletion of protective antioxidants and such a depletion, possibly intensified by a more direct effect of cadmium on scavengers as discussed in Chapter 4, may well be associated with a heightened cancer risk. Thus, cadmium may disrupt the homeostasis that normally exists between the rate of radical formation and the rate of radical dissipation with the resulting imbalance leading to the defence mechanism being overwhelmed.
Potential Use of Luminol-Amplified Chemiluminescence
to Study Free Radical Formation

Further studies using the chemiluminescence system described in Chapter 4 could offer an opportunity to determine the possible importance of reactive oxygen species in tissue injury and gain a unique insight into the molecular mechanisms underlying cadmium toxicity and carcinogenicity. The system could be suitable for inclusion in a battery of in vitro tests on acute metal cytotoxicity, metal-metal and metal-chelator interactions and for the in vitro preliminary screening of possible chelating agents for the treatment of metal poisoning in vivo.

The characterisation of free radical formation in vivo will require the development of methods with increased sensitivity, specificity and improved limits of detection. Further research and validation are needed to develop the potential of chemiluminescence detection as a technique for monitoring free radical activity. At present the advantages of the system must be weighed against the problems of standardising the variables and quantifying the results.

An increasing awareness of the variables to which chemiluminescence measurements are prone and the use of tightly controlled conditions together with additional knowledge of the source of chemiluminescence could improve the potential for its application as an index of free radical activity. Further investigations into the concept of selective scavenging may provide the basis for the specific identification of radical species.

Much attention in toxicology is being focussed on new methods which eliminate or reduce the need for animal experimentation. With refinement, the chemiluminescence technique applied to tissue explants or cell separations may provide a useful tool for testing the potency of cytotoxic agents which act by free radical mechanisms and the rapid screening of potentially therapeutic agents under controlled in vitro conditions. A convenient development could be the use of currently available luminometers which have added features of strict temperature
control and agitation of samples. Additions can be made whilst the photomultiplier is in operation thus avoiding the interruption of measurement and the missing of rapid responses. Possibly of value also could be incorporation of a system to allow for the regulation of oxygen consumption which may be an important factor in limiting the response (Campbell et al., 1985).

A further approach could involve the provision of in vitro culture medium to retain optimal cell/tissue viability in order to minimise the component of free radical activity due to the spontaneous auto-oxidative degradation that occurs during cell degeneration and death. This would permit longer-term studies on cell/organ cultures with the chemiluminescence technique able to provide continuous monitoring and the quantitation of chemical events under differing controlled conditions whilst the cells remain viable. Since detection of chemiluminescence is non-invasive and would not necessitate the sampling of tissue or culture medium in order to detect the product, as is required by other in vitro methods, this would allow for subsequent treatments to be carried out.

Another area worthy of future development is single cell analysis of oxygen radical production, allowing not only cellular heterogeneity but also the threshold responses in cells to be defined (Campbell et al., 1985). It has recently become possible to visualise and quantify oxyradical burst from individual cells by using ultrasensitive video intensifier microscopy in the presence of a chemiluminogenic probe (Suematsu et al., 1987), thus showing both spatial as well as temporal alterations. The results suggested functional heterogeneity of the ability to release oxyradicals among human neutrophils. The development of this technique raises exciting possibilities of obtaining direct evidence of oxidative stress on various target cells during tissue injury in acute inflammation and toxic injury.

Single photon-counting devices for the measurement of low level chemiluminescence are available, which can be applied to the study of in vivo exposed organs or in vitro perfused organs as well as cells, subcellular fractions or
model systems (Boveris et al., 1981; Cadenas and Sies, 1984). Halliwell and Grootveld, 1987 have attempted to evaluate some of the many promising new techniques that are continually becoming available and that are required to further develop our understanding of the role of free radicals in human disease. Light emission has potential as a non-invasive technique for measuring oxidative stress in intact tissues and shows considerable promise as an in vivo assay of lipid peroxidation and other free radical reactions. It might prove difficult to apply to humans although as pointed out by Halliwell and Grootveld (1987) the pace of advance in fibre optic technology is so great that one cannot rule it out.

5.8 Conclusions

The results of this thesis have indicated that, in view of the acute inflammation which follows the subcutaneous administration of cadmium to rats, this model of cadmium intoxication is unsuitable for the study of many aspects of the toxicity of this metal.

The development of in vitro techniques such as the chemiluminescence method described in this study might advance our understanding of the mechanism of cadmium toxicity, in particular whether or how cadmium might promote the process of lipid peroxidation. Such knowledge could help to clarify the questionable involvement of cadmium in the pathogenesis of human conditions such as emphysema, cancer and hypertension and could also lead to the discovery of improved methods of antidotal treatment of cadmium intoxication.
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