ANTIOXIDANT DEFENCE AND AUTOXIDATIVE DAMAGE IN NEOPLASTIC DISEASE

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by

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and

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and fresh knowledge, the possibility of a titanic breakthrough with the work he had been doing still was possible, still could come at any time. Within the parameters of organic chemistry an answer existed — an answer to his questions posed through countless experiments over ten long years of grinding research.

The quenching of free radicals.

Along with the answer Vincent Lord sought would come enormous therapeutic benefits, plus unlimited commercial possibilities which Sam Hawthorne and others in the company, in their scientific ignorance, had so far failed to grasp.

What would the quenching of free radicals achieve?

The answer: something essentially simple but magnificent.

Like all scientists in his field, Vincent Lord knew that many drugs, when in action in the human body and as part of their metabolism, generated ‘free radicals’. These were elements harmful to healthy tissue, and the cause of adverse side effects and sometimes death.

Elimination, or ‘quenching’, of free radicals would mean that beneficial drugs, other drugs, which previously could not be used on humans because of dangerous side effects, could be taken by anyone with impunity. And restricted drugs, hitherto used only at great risk, could be absorbed as casually as aspirin.

No longer need physicians, when prescribing for their patients, worry about toxicity of drugs. No longer need cancer patients suffer agonies from the near-deadly drugs which sometimes kept them alive, but equally often tortured then killed them from some other cause than cancer. The beneficial effects of those and all other drugs would remain, but the killing effects would be nullified by the quenching of free radicals.

What Vincent Lord hoped to produce was a drug to add to other drugs, to make them totally safe.

And it was all possible. The answer existed. It was there. Hidden, elusive, but waiting to be found.

And Vincent Lord, after ten years’ searching, believed he was close to that elusive answer. He could smell it, sense it, almost taste the nectar of success.

But how much longer? Oh, how much longer would he have to wait?

ABSTRACT

The principal factors governing antioxidant defence and lipid peroxidation in vitro have been determined for neoplastic and normal tissues, from mice, bearing Lewis lung carcinomas. These parameters were measured at varying intervals after the intramuscular transplantation of Lewis lung carcinoma cells in C57BL6 mice.

The activities of glutathione peroxidase (EC 1.11.1.9), superoxide dismutase (EC 1.15.1.1), gamma glutamylcysteine synthetase (EC 6.3.2.2), and catalase (EC 1.11.1.6) were similar in tumour tissue to those of lung, the tissue of origin. Glutathione reductase (EC 1.6.4.2) and glutathione-S-transferase (EC 2.5.1.18) activities were considerably greater than those of lung. Gamma-glutamyltranspeptidase (EC 2.3.2.2) activity was about 25% of the corresponding value in murine lung.

Lewis lung carcinoma tissue contained considerable quantities of lipid peroxide (as determined by the thiobarbituric acid test). The in vitro lipid peroxidation of tumour microsomes was less than that of pulmonary tissue.

Tissue from the tumour "core" had significantly greater reduced glutathione concentration, lower mitochondrial superoxide dismutase, glutathione peroxidase and gamma-glutamyltranspeptidase activities, and lower lipoperoxide concentration than the periphery. Glutathione peroxidase, gamma glutamylcysteine synthetase and superoxide dismutase activities from Lewis lung carcinoma cells in monolayer culture (18% O2 concentration) were greater than those of solid tumour.

Histological sections of lung and liver from Lewis lung carcinoma-bearing mice were found to have many pathological changes. The livers of tumour-bearing mice had raised glutathione and DNA concentrations, and increased glutathione reductase, glutathione-S-transferase and gamma-glutamyltranspeptidase activities; hepatic catalase and mitochondrial glutathione peroxidase activities were decreased. Hepatic microsomes from carcinoma-bearing mice had increased thiobarbituric acid-reactivity and were more susceptible to iron/ascorbate lipid peroxidation. Storage, as a suspension in a glycerol-containing phosphate buffer at -80 °C, conferred resistance to in vitro lipid peroxidation to liver microsomes from healthy animals, but not to those from tumour-bearing mice.

Disturbances in Lewis lung carcinoma-bearing mice could generally only be determined at 9 or more days after tumour cell implantation, when the tumour was just palpable. The livers of genetically obese C57BL6 mice had some similar biochemical characteristics to those of tumour-bearing hosts. Kidney and erythrocytes of Lewis lung carcinoma-bearing mice, and tissues of mice, bearing B16 melanomas, also displayed perturbations of antioxidant defence and lipid peroxidation.

The significance of the above observations is discussed. Further experiments are suggested to improve the understanding of the role of autoxidation and antioxidant defence in neoplastic disease.
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1. Analysis of Rat & Mouse No. 1 animal diet
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CHAPTER ONE

INTRODUCTION
Cancer and Degenerative Diseases - Twentieth Century Epidemics

Degenerative diseases are maladies in which there is progressive chemical deterioration of tissues to functionally less active forms. Although all are potentially degenerative, the term "degenerative disease" is commonly applied to diseases that do not generally have an aetiology of microbial infection. Cancer, or malignant neoplastic disease, can be defined as a degenerative disorder in which aberrant internal changes of cells cause them to divide and grow hyperplastically and anarchically in the "autonomy" of the organism from which they originated (Harris, 1976; Prehn, 1977). This uncoordinated cell division usually produces a mass of tissue or tumour, although in leukaemias, that is cancerous myeloid or lymphoid cells, the neoplastic cells are dispersed throughout the bone marrow or lymphoid tissues, and into the blood (Halliwell and Gutteridge, 1985). Benign tumours are contained at the site of origin, often by a fibrous outer capsule; malignant tumours are able to invade local tissues and circulate in the vasculature to form secondary tumours (metastatic foci) at new sites. Human cancer is a group of at least 100 different diseases (Carter, 1979).

Before the turn of the century, infectious diseases were the major cause of death even in developed countries. The discoveries of vaccines and antibiotics, and major advances in standards of public health and hygiene have decreased the mortalities due to infection to approximately 5% of all deaths. The consequence of the diminished incidence of death from microbial infections has been a dramatic rise in human lifespan (Cutler, 1984). In the United States, for example, life expectancy has risen from an average of 47.2 years in 1900 to 73.3 years by 1980 (Harman, 1981; fig. 1.1a). The incidence of death due to cardiovascular/renal diseases, diabetes mellitus, and cancer, the three
  b) Incidence of Cancer (per 100,000 persons) Against Age in Years in the United States during 1979.

(from Prescott and Flexer, 1982)
major forms of degenerative disease, have however increased this century by 29, 38 and 179% respectively (Prescott and Flexer, 1982). Cancer and cardiovascular disease, together account for almost 70% of the present mortalities in the Western World (Prescott and Flexer, 1982).

The estimate of deaths caused by cancer in the United States in the year 1850 is only 1 per 190 mortalities (Silverberg, 1985). Despite significant advances in cancer therapy, neoplastic diseases presently kill one fifth of the citizens of the Western World, and one quarter will succumb to the disease at some time during their lives (American Cancer Society, 1982; Prescott and Flexer, 1982; see fig. 1.1a). In the period 1968-1977 the age-adjusted mortality rate for cancer in the United States increased by approximately 2%, suggesting that the incidence of death in the developed nations due to malignant neoplastic diseases is still rising (Prescott and Flexer, 1982). The scientific and medical understanding of cancer remains relatively inadequate and it thus has a poor, though improving, prognosis - an observed 5 year survival rate of 38% (American Cancer society, 1982). Western society is victim of an epidemic of cancer and other degenerative diseases. Whereas in previous centuries, contagious diseases such as typhoid and bubonic plague were greatly feared, today, these dreads have been extensively transferred to cancer and other disorders associated with old age (Currie and Currie, 1982). Arguably only acquired immune deficiency syndrome (AIDS) can arouse more public interest than cancer, but even this disease is frequently expressed by an otherwise rare neoplasm, Karposi's sarcoma.

In man the incidence of cancer increases to approximately the fourth power with age (Peto et al., 1975; Dix et al., 1980; Ames, 1983). Major degenerative diseases, such as hypertension, atherosclerosis, diabetes, arthritis, immune disorders and senile dementia, are all associated with old age (Harman, 1982). This phenomenon presides in part because exposure
to the factors mediating these diseases is greater with advancing age (Peto et al., 1975; Symington, 1980). Degenerative diseases such as atherosclerosis and cancer have latent periods before their onset: the fatty streaks, postulated to be the progenitors of atheroma in middle-aged and elderly adults, are commonly found in the arterial intima of children (Boyd, 1970); the time elapsing between exposure to environmental carcinogen and the appearance of the resulting cancer can range from 5 to 50 years (Muir, 1983). Age could also be an important factor in the genesis of degenerative diseases due to age-related changes in endocrine function (Dilman, 1971). Older animals may be more susceptible to the factors that cause degenerative disease because their immunological surveillance, enzymic defence and DNA-repair mechanisms are less efficient than those of young animals (Albright and Albright, 1978; Kent, 1977; Pitot, 1977; Bennett, 1979; Hocman, 1981).

It has been proposed that aging and age-related disorders are at least partially due to perturbative actions of free radical reactions (Harman, 1956). Molecular oxygen \( \text{O}_2 \), can be extremely toxic: clinically this toxicity has been observed through the pathological changes in the lungs of patients ventilated with \( \text{O}_2 \) for several days (Balentine, 1982). Oxygen toxicity is probably mediated by free radical reactions (Pryor, 1976; Fridovich, 1983; Frank and Massaro, 1980). The "free radical theory of aging" postulates that organisms have evolved defence mechanisms to limit the deleterious actions of free radicals to levels compatible with normal function (Harman, 1982); as an organism becomes older, the ability of its tissues to detoxicate oxygen-derived free radicals and their reaction products decreases, and the resultant accumulation of cellular oxidative damage provokes dysfunction or death (Harman, 1981). Fluorescent age pigments (lipofuscins; see later), which amass in post mitotic cells of an organism as a function of age, are proposed to be formed by the oxidative polymerization of lipids and proteins due to the
formation of Schiff bases between amide groups and the breakdown products of radical chain reactions (Donato, 1981; Harman, 1981). Tissues from aged animals are more susceptible to oxidative damage in vitro (Stege et al., 1982). The inverse relationship between basal metabolic rate and longevity in aerobes, and the observation that dietary antioxidants (substances that quench oxygen-derived radicals) can sometimes prolong lifespan indicate that oxygen and its metabolites are involved in the aging process (Pryor, 1977; Hocman, 1981; Harman, 1982; Sohal, 1984).

As oxidative cellular injury and the aging process are associated, oxygen-derived radicals may also have an important role in the aetiology of at least some degenerative disorders. Activated oxygen intermediates have been suggested to be the primary causative factors of numerous pathological conditions (McCay, 1981; Autor, 1982; Petkau, 1982a & b). Atherosclerosis (Parke and Ioannides, 1981b), Down's syndrome (Elroy-Stein et al., 1986), cerebral ischaemia and spinal cord injury (Demopoulos et al., 1982), cancer (Totter, 1980), haemolytic anaemia (Hebbel et al., 1982), ischaemic heart disease (Hess et al., 1982), pancreatic disease (Braganza et al., 1983), emphysema and other forms of pulmonary injury (Dooley and Pryor, 1982; Tanswell, 1983), rheumatoid arthritis (Blake et al., 1981), Parkinson's disease (Cohen, 1982), immune disorders and senile dementia (Harman, 1982) are among the many diseases and injury states in which active oxygen species have been implicated. The outbreak of poisoning in Spain in the early 1980's, due to the illicit trade in cooking oil, produced by the distillation of aniline-containing industrial rape-seed oil, caused at least 350 deaths and affected over 20,000 others (Halliwell and Gutteridge, 1985). The symptoms of the "Spanish cooking-oil syndrome" were very similar to those of free radical induced toxicity, and it has thus been proposed that aniline-derived toxins, such as oleanaldehyde, in
the adulterated oil exerted their effects by a free radical mechanism (Pestana and Munoz, 1982).

Oxidative Cellular Injury

Free radicals: A free radical is an atom or molecule that possesses one or more odd (unpaired) electrons (Freeman and Crapo, 1982; Pryor, 1984). Free radicals are formed by the homolytic (symmetrical) cleavage of molecular bonds, by the abstraction of protons, or by one-electron transfers. Free radicals are energy-rich species and are thus almost always extremely reactive because of the tendency to become stabilized by forming new bonds. The three major types of reaction are electron transfer, radical addition, and abstraction of univalent atoms such as hydrogen atoms or halides (Freeman and Crapo, 1982).

Chemistry and biology of oxygen: Oxygen is an unusual gas because it displays a paramagnetism due to having two unpaired electrons residing in the outermost molecular orbitals (Fridovich, 1977; Malmström, 1982). These \( \pi_p^* \) orbitals are of equal energy, that is degenerate (Paine, 1978; Malmström, 1982). \( \text{O}_2 \) in its triplet ground state is relatively inert kinetically because by accepting a further electron pair to fill its outer molecular orbitals, one of its \( \pi_p^* \) electron spins must be inverted. The spin restriction of \( \text{O}_2 \) can be overcome when sufficiently electronically excited, however, and two possible non-paramagnetic states of molecular oxygen are formed; these excited species are termed singlet oxygen (\( \text{O}_2^1 \)).

During aerobic respiration, \( \text{O}_2 \) is reduced bivalently to \( \text{H}_2\text{O} \) by transition metal ions, which are also paramagnetic, donating unpaired electrons to molecular oxygen (Fridovich, 1977; Malmström, 1982). The other means to circumvent the spin restriction of \( \text{O}_2 \) is univalent reduction; that is electrons are accepted one at a time. The addition of one electron to \( \text{O}_2 \) produces the superoxide radical (superoxy anion;
Further reduction produces \( \text{H}_2\text{O}_2 \); and the penultimate reduction step results in the formation of the hydroxyl radical ('\( \cdot \)OH):

\[
\begin{align*}
\text{O}_2 + e^- & \rightarrow \cdot \text{O}_2^- \\
\cdot \text{O}_2^- + e^- + 2\text{H}^+ & \rightarrow \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + e^- + \text{H}^+ & \rightarrow \text{H}_2\text{O} + \text{OH}' \\
\cdot \text{OH} + e^- + \text{H}^+ & \rightarrow \text{H}_2\text{O}
\end{align*}
\]

There are three main classifications of enzyme that can use \( \text{O}_2 \) as substrate. The reduction of \( \text{O}_2 \) to \( \cdot \text{O}_2^- \), \( \text{H}_2\text{O}_2 \) or \( \text{H}_2\text{O} \) is catalysed by a group of enzymes, termed oxidases. Dioxygenases catalyse the bonding of \( \text{O}_2 \) to an organic molecule. The third category, the mixed-function oxidases (mono-oxygenases), are arguably a cross between the two former classifications, as they catalyse the bonding of one atom of \( \text{O}_2 \) to organic substrate and the reduction of the other to \( \text{H}_2\text{O} \). Monoxygenases and dioxygenases are collectively referred to as oxygenases (Malmstrom, 1982). All enzymes, using \( \text{O}_2 \) as substrate, are conjugated proteins, containing transition metal-centred prosthetic groups, such as haems and flavins (Malmstrom, 1982). Reactive oxygen species are produced as side- or main-products of many of these enzymes.

Sources of free radicals encountered by biological systems: Free radicals are intermediates and by-products of many biological processes. Oxygen-centred radicals, the principal radical species produced by biochemical reactions in aerobic organisms, are involved in the catalytic mechanisms of enzymes such as xanthine oxidase, tyrosinase, and tryptophan 2,3 dioxygenase (Paine, 1978; Malmstrom, 1982). Peroxisomes contain \( \text{H}_2\text{O}_2 \)-generating enzymes, such as urate oxidase and glycollate...
oxidase, which are involved in fatty acid β-oxidation (Sies, 1977; Reddy et al., 1982; Halliwell and Gutteridge, 1985). Semiquinone radicals are intermediates in the mitochondrial electron transport chain (Freeman and Crapo, 1982). Hypochlorite radicals and active oxygen species are produced by activated phagocytic leukocytes (Del Maestro et al., 1980; Weiss and LoBuglio, 1982). Melanins, the pigments, present for example in skin, hair, the eye and substantia nigra, contain relatively stable free radicals (Sealy et al., 1980; Lohman and Neubacker, 1984).

Up to 5% of oxygen consumed during mitochondrial respiration is postulated to be converted to $\cdot O_2^-$ by one electron reduction (Fridovich, 1978). The reduction of $O_2$ to $H_2O$ by cytochrome c oxidase, at the terminal end of the mitochondrial electron transport chain is not, however, the source of $O_2^-$. This enzyme is thought to bind all reactive oxygen intermediates tightly, and so prevent their escape (Freeman and Crapo, 1982; Halliwell and Gutteridge, 1985); cytochrome oxidase might even be a $O_2^-$ scavenger in itself (Naqui et al., 1986). The leakage of electrons by other components of the chain, particularly ubiquinone (coenzyme Q) and NADH coenzyme Q reductase, causes a univalent reduction of $O_2$ to $O_2^-$ (Halliwell and Gutteridge, 1985; fig. 1.2). The $O_2^-$ is converted to $H_2O_2$, a less harmful oxygen intermediate, by an enzyme called superoxide dismutase (Dionisi et al., 1975; Boveris, 1977; Fridovich, 1978).

The source of free radicals to which an organism can be exposed can also be environmental or xenobiotic (Pryor, 1976). Electromagnetic radiations (x-rays, γ-rays, UV radiation) and particulate radiation (electrons, protons, neutrons, deuterons, and β-particles) are able to transfer their energy to biological molecules to generate radical species (Freeman and Crapo, 1982). Some xenobiotics, such as NO and $'NO_2$ of cigarette smoke, are already free radicals (Pryor et al., 1983); other
FIG. 1.2 - Scheme of Respiratory Chain, Showing Sites of Substrate Entry, Inhibitor Action and Potential Sites of Superoxide Formation.

(from Turrens et al, 1982a)
xenobiotics, such as ozone, are so reactive they will interact with cell components to form radical species.

The endoplasmic reticulum of liver and other mammalian tissues have a mono-oxygenase system which metabolises, mainly by hydroxylation, a great variety of lipophilic xenobiotics and endogenous substrates to polar derivatives. The main component of this system is cytochrome P₄₅₀, a mixed-function oxygenase of multiple forms, which requires NADPH and O₂ for activity (Parke and Ioannides, 1981b). The overall reaction, catalysed by cytochrome P₄₅₀, in which AH is the substrate can be represented:

\[
\text{AH} + \text{O}_2 + \text{RH}_2 \rightarrow \text{AOH} + \text{R} + \text{H}_2\text{O}
\]

NADPH-cytochrome P₄₅₀ reductase provides electrons for the cytochrome P₄₅₀ system by transfer from NADPH. Cytochrome P₄₅₀ usually converts its substrates to less harmful derivatives, but, paradoxically, some xenobiotics, such as paracetamol and benzo(a)pyrene are hydroxylated to the free radical intermediates, responsible for the toxicity of these substances (Parke and Ioannides, 1981a). Carbon tetrachloride, as a further example, is converted to the trichloromethyl radical ('CCl₃) by cytochrome P₄₅₀ (Parke and Ioannides, 1981b; Trush et al, 1982).

Some substrates for cytochrome P₄₅₀, for example phenobarbitone, have been shown to induce the synthesis of some forms of this enzyme. The endoplasmic reticulum also contains a related enzyme, cytochrome P₄₄₈, which can be induced by polycyclic aromatic hydrocarbons, such as 3-methylcholanthrene (Parke and Ioannides, 1984a). Cytochrome P₄₄₈, particularly in adult liver, is less abundant than the main P₄₅₀ cytochromes and is thought to be mainly concerned with the metabolism of endogenous steroids (Parke and Ioannides, 1984b). Cytochrome P₄₄₈ has a less restricted active site than the cytochrome P₄₅₀ and can catalyse the
oxygenation of chemicals in "bay region" positions, that is at molecules that are conformationally inaccessible to cytochrome P450 (Parke and Ioannides, 1981a). Many of the metabolites of cytochrome P448 oxygenation are highly reactive electrophiles, which are not readily detoxicated by enzymic conjugases, such as epoxide hydrolase (Parke et al., 1985). These reactive intermediates can be free-radicals, for example semiquinones, nitro-aromatic cations and azo anions, or may interact with cell components to form tertiary free radicals. The radical intermediates can react with molecular oxygen to form \( \cdot O_2^- \) and regenerate the oxidized parent compound, which then becomes a substrate again for the mixed-function oxidases; a redox cycle is thereby established (Trush et al., 1982).

The microsomal mixed function oxidase system has been postulated to be leaky, such that reactive oxygen species can escape into the rest of the cell (Paine, 1978; Cohen and Cederbaum, 1979). The microsomal and mitochondrial electron transport chains are probably the most important sources of \( \cdot O_2^- \) in vivo (Halliwell and Gutteridge, 1985).

**Radical intermediates of pharmacological agents:** Anticancer agents are prominent among drugs, thought to be metabolized to free-radical intermediates (Trush et al., 1982): these drugs include the anthracycline antibiotics, adriamycin and daunorubicin; the quinones, mitomycin D, mytomycin C and streptonigrin; and the protein antitumour drug, neocarzinostatin (Kennedy et al., 1981; Lown et al., 1982; Oberley et al., 1982). Bleomycin is a glycopeptide anticancer drug which binds to DNA, and will degrade it in a reaction dependent on iron and \( O_2 \). The incubation of bleomycin with an Fe(II) salt in aqueous solution has been reported to cause the formation of \( \cdot O_2^- \) and \( \cdot OH \) radicals (Halliwell and Gutteridge, 1985). Nitrogen mustards, such as cyclophosphamide and melphalan, the nitrosoureas (see Chapter 6), sesquiterpene lactones, and
an acridine derivative, AMSA may promote free radical reactions due to their ability to deplete glutathione (GSH), an important antioxidant (Gurtoo et al., 1981; Arrick and Nathan, 1984; see later). Although the main mode of action of many of the above drugs might not involve free radicals, their adverse effects on the cancer patient may well be mediated by such species.

**Effects of free radical-induced injury:** Autoxidation is the spontaneous autocatalytic oxidation of organic substrate under mild conditions at ambient or subambient temperatures in the presence of molecular oxygen (Burton and Ingold, 1983). These reactions characteristically have a single free radical initiation step, and if the production of active oxygen intermediates is not adequately controlled then autoxidation can occur. Activated oxygen species, produced generally by side reactions of aerobic metabolism or through exposure to some toxic chemicals, can react detrimentally with potentially all the constituents of biological cells (Freeman and Crapo, 1982; Singh, 1982). Superoxide radical-generating systems have been used to demonstrate that $\cdot O_2^-$ can damage subcellular components, including membranes and DNA; inactivate enzymes such as catalase, glutathione peroxidase and rat brain membrane Na$^+$, K$^+$ ATPase; and kill bacterial cells in culture (Bus and Gibson, 1982; Fridovich, 1986).

The damaging actions of the reactive intermediates of oxygen metabolism, upon polyunsaturated fatty acids (PUFA) are expressed by a radical chain reaction, termed lipid peroxidation (Slater, 1984; see later). The abstraction of a hydrogen atom from a methylene group in a polyunsaturated fatty acid leaves behind a carbon-centred radical which, after rearrangement to a conjugated diene, reacts with $O_2$ to form a hydroperoxy radical, $R-\cdot OO^-$ (Halliwell and Gutteridge, 1985; fig 1.3). The peroxy radical abstracts a hydrogen from another lipid molecule to give a
The Stages of Lipid Peroxidation: Sites of Generation of Chemiluminescent Species.

(from Boveris et al, 1981)
lipid hydroperoxide (R-OOH) and a new hydroperoxy radical: this process is repeated - a chain reaction has been established. Lipid hydroperoxides (lipoperoxides) are relatively stable, under mild conditions, but transition-metal complexes catalyze their decomposition to cyclic peroxides and cyclic endoperoxides (Halliwell and Gutteridge, 1985). Transition metal complexes, that have this ability, include salts of Fe(III) and Fe(II), haemoglobin, haem, cytochromes and horseradish peroxidase (Halliwell and Gutteridge, 1985). The cyclic peroxides and endoperoxides are degraded by a series of complex reactions to a variety of carbonyl compounds, such as alkenals, alkanals, hydroxyalkenals, ketones, alkanes, hydroxy-acids and keto-acids (Esterbauer, 1982; Slater, 1984; fig 1.3).

The oxidative deterioration of biomembranes is primarily caused by the uncontrolled peroxidation of phospholipid (Mead, 1976; Bus and Gibson, 1979; Porter, 1984). As a result of lipid peroxidation peptide scission can occur and proteins become denatured: there can be strand scission and base modification of DNA; the activity of thiol-containing enzymes is inhibited; and loss of membrane PUFA can cause changes in membrane permeability and impair membrane function (Freeman and Crapo, 1982). The oxidation of carbohydrates can cause changes in cell surface receptors. Many important cofactors, such as ascorbate, flavins and porphyrins become unavailable when oxidized. Lipid peroxidation can thus cause major disruption of cell functions, and cell death (Tappel, 1973; Plaa and Witschi, 1976; Freeman and Crapo, 1982).

Autoxidation of membrane PUFA has been implicated as a primary factor in the toxicity of numerous xenobiotics, such as paraquat, and in the pathogenesis of many diseases (Shu et al, 1979; Reddrop et al, 1983; Smith et al, 1983; see earlier). Lipoperoxides inhibit prostacyclin synthetase (Ham et al, 1979). The aldehydic products of lipoperoxide
composition are also toxic, and have been shown to inhibit DNA and protein synthesis and to block lipoprotein secretion in isolated rat hepatocytes (Dianzani, 1982). One aldehyde product, malondialdehyde (fig 1.6) has been reported to have lethal and mutagenic effects (Yonei and Furei, 1981). Malondialdehyde can cause cross-linkages between proteins by reacting with their side chain amino groups to form highly fluorescent, conjugated Schiff bases (Donato, 1981). Age pigments (lipofuscin) contain cross-linked proteins, various lipids and transition metal ions (particularly iron), and display fluorescence similar to that of Schiff bases; they are thus thought to be end products of lipid peroxidation (Gutteridge, 1984). These pigments have been observed to accumulate much faster in the muscles of house flies of high flight activity than in insects of low activity; the rate of lipofuscin accumulation correlated inversely with the life spans of flies (Sohal, 1984). Age pigments are a prominent feature of certain recessively inherited disorders, such as neuronal ceroid lipofuscinosis (Batten's Disease and Chediak-Higashi syndrome (Armstrong, 1984). The cerebral spinal fluid of patients with Batten's Disease, has been shown to contain increased amounts of non-protein bound iron and to be extra-sensitive to the damaging effects of $O_2^-$; further indicating that free radicals are involved in the aetiology of this syndrome (Gutteridge, 1982b).

Amyloid is an extracellular pathological pigment, comprising largely of protein, which is associated with advancing age and numerous diseases (Halliwell and Gutteridge, 1985). Amyloid is one of the acute-phase proteins, synthesised by the liver in response to tissue damage, and might, like lipofuscin, be a product of autoxidation.

**Biologically useful free radical reactions:** Not all free radical-mediated reactions are deleterious. The controlled production of reactive oxygen species is involved in normal oxidation and hydroxylation
reactions, including those indicated earlier. \( \cdot O_2^- \) might be an intermediate in vitamin K-dependent carboxylation reactions, such as those of the blood clotting system (Halliwell, 1981). Free radical intermediates are thought to associated with the formation of cyclic GMP by guanylate cyclase, and with the biosynthesis of prostanoids and leukotrienes (Hammar and Lands, 1980; Parantainen, 1982).

Leukocytes capable of phagocytosis include neutrophils (a form of polymorphonuclear leukocyte), macrophages and their precursor cells, the monocytes. When phagocytic cells recognise a foreign particle, such as a bacterial cell, there is a pronounced increase in oxygen consumption and in pentose monophosphate shunt activity, and reactive oxygen species are produced: this process is known as the "respiratory burst" (Fantone and Ward, 1982). It is probable that reactive oxygen species are used to kill invading microbes (Fantone and Ward; 1982; Weiss and LoBuglio, 1982). Basophils, eosinophils, mast cells and the Kupffer cells of the liver, have also been observed to produce \( \cdot O_2^- \) during a respiratory burst (Halliwell and Gutteridge, 1985).

**Defence Against Autoxidation - The Antioxidant Defence System**

Animals, and plant cells contain multiple defences against the damaging actions of reactive oxygen species (Fridovich, 1978; Sies, 1984). These defence mechanisms are collectively known as the antioxidant defence system. There are two main groups of antioxidants: the preventative (primary) antioxidants, which decrease the rate of chain initiation; and the chain-breaking (secondary) antioxidants, which intercept the free radicals, responsible for autoxidation (Burton and Ingold, 1983). Secondary antioxidants (ArOH) generally prevent lipid peroxidation by donating hydrogen atoms to hydroperoxy radicals; the resultant radical (ArO') is not sufficiently reactive to continue the
reaction chain: 

\[ \text{ROO}^* + \text{ArOH} \rightarrow \text{ROOH} + \text{ArO}^* \]

Superoxide dismutase: In 1938, Mann and Keilin discovered a copper-containing protein in bovine blood, and called it haemocuprein. During the years following, similar proteins were also isolated from other tissues and in other species. It was not until the late 1960's that McCord and Fridovich showed that erythrocuprein contained zinc in the same proportion as copper, and that the function of the enzyme was to accelerate the dismutation (disproportionation) of \( \cdot O_2^- \) to \( H_2O_2 \) (Fridovich, 1976):

\[ 2 \cdot O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \]

This enzyme exists in virtually all eukaryotic cells: is generally absent in obligate anaerobes; and was the first of the superoxide dismutases (SOD) to be discovered (Fridovich, 1978). Cuprozinc superoxide dismutases (Cu/Zn-SOD) are composed of two subunits, each containing one copper and one zinc ion at the active site, and have molecular weights of about 32,000 (Fridovich, 1982a).

In addition to Cu/Zn-SODs there are at least three other forms of the enzyme. A high molecular weight (135,000) copper-containing SOD has been discovered to be present in extracellular fluids and, in small amounts, in other tissues, especially lung (Marklund, 1982). Many bacteria, algae and a few species of plant possess an iron-containing SOD, (Fe-SOD; Halliwell and Gutteridge, 1985). These enzymes are generally dimeric and have amino acid sequences dissimilar to those of Cu/Zn-SODs. A related enzyme, manganese SOD (Mn-SOD), has been found in bacteria, plants and animals. In higher species, such as the rat, this enzyme has been reported to have a tetrameric structure and to have a
molecular weight of 89,000 (Salin et al., 1978).

Cu/Zn-containing superoxide dismutases are mainly confined to the cytosol of mammalian cells, although some is possibly located between the inner and outer mitochondrial membranes (Weisinger and Fridovich, 1973; Peskin et al., 1977). Cu/Zn-SOD is inhibited by cyanide, and, like the Fe-SODs, can be inactivated by azide and prolonged exposure to H$_2$O$_2$ (Hassan et al., 1980). Mn-SOD is mainly located in the mitochondrial matrix, although in human liver, extramitochondrial Mn-SOD has been determined (Marklund et al., 1982). The manganous enzyme is not inhibited by cyanide, and is less sensitive to inactivation by reactive oxygen species than other dismutases (Weisinger and Fridovich, 1973).

Catalase: The catalases are haemoproteins, primarily located in the peroxisomes of aerobic cells (Chance et al., 1979). Catalases remove H$_2$O$_2$, generated by peroxisomal oxidases by catalysing the following reaction:

$$2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2$$

The catalase reaction mechanism has two stages: H$_2$O$_2$ interacts with the enzyme to form a complex, called compound I, which reacts with further H$_2$O$_2$ to produce free catalase, O$_2$ and H$_2$O (Cohen et al., 1970). Compound I will catalyze the oxidation of the alcohols, methanol and ethanol to their corresponding aldehydes, formaldehyde and acetaldehyde, respectively (Halliwell and Gutteridge, 1985).

Catalases generally comprise four haem-containing sub-units, and are inhibited by cyanide, azide and aminotriazole.

Glutathione and glutathione-centred enzymes: Glutathione in its reduced form (GSH) is a tripeptide, consisting of glutamate, glycine and cysteine, and represents over 90% of the non-protein thiol of the liver (Meister, 1982; Levine, 1983). GSH has a central role in many aspects of
metabolism, as it is a co-factor for several enzymes, such as glyoxylase and prostaglandin endoperoxide isomerase; GSH is also concerned with the metabolism of insulin (Kosower and Kosower, 1976b; Halliwell and Gutteridge, 1985). GSH is not vital for all aerobic organisms, as there are many strains of bacteria that use other low molecular weight thiols as an alternative (Fahey et al., 1984). The depletion of GSH in rodent livers has been reported to cause hepatic lipid peroxidation (Younes and Siegers, 1980). The protection from peroxidation might be partly due to the ability of GSH to scavenge \(^{•}\text{OH}\) and \(\text{O}_2^{•}\) (Meister and Anderson, 1983). GSH and other thiol compounds at appropriate concentrations will, however, catalyse the formation of \(^{•}\text{OH}\) from \(\text{O}_2^{•}\) (Rowley and Halliwell, 1982). GSH might also help to regenerate sulphhydryl groups of enzymes that have been subjected to autoxidation (Isaacs and Binkley, 1977). GSH is conjugated to some electrophilic xenobiotics by glutathione-S-transferases to make them water-soluble so that they can be more readily excreted from the body (Chasseaud, 1979). GSH might also help to prevent liver damage by acting as a cofactor in the pathways that oxidize formaldehyde, formed during demethylation reactions (Levine, 1983).

The main way in which GSH acts as an antioxidant is probably via its role as co-substrate for the enzyme glutathione peroxidase (GSH peroxidase). This enzyme catalyzes the reduction of \(\text{H}_2\text{O}_2\) and lipid hydroperoxides, while converting GSH to its oxidized form, GSSG, in which two molecules of GSH are linked by a disulphide bridge between the cysteine residues (Ganter et al., 1976; Sunde and Hoekstra, 1980):

\[
\text{H}_2\text{O}_2 + 2 \text{GSH} \rightarrow \text{GSSG} + \text{H}_2\text{O}
\]

\[
\text{ROOH} + 2 \text{GSH} \rightarrow \text{ROH} + \text{GSSG} + \text{H}_2\text{O}
\]

Hydrogen peroxide, produced by the peroxisomes is metabolized by
catalase, while that of other cellular compartments is reduced by GSH peroxidases (Jones et al., 1981). The best characterized form of GSH peroxidase was discovered by Mills in 1957: has a molecular weight of about 85,000; and consists of four identical subunits, each containing a selenium atom at the active site (Ganther et al., 1976; Kraus et al., 1980). GSH peroxidase has very high substrate specificity for GSH but will reduce a wide range of peroxides (Flohe, 1982). The enzyme has been shown to be relatively durable in vitro to adverse conditions of storage and so is probably particularly suitable as a defence against autoxidation (Condell and Tappel, 1983). GSH peroxidase can be inhibited in vitro by iodoacetic acid, iodoacetamide, NADPH, and by metal ions, particularly cadmium (II) and zinc (II) (Zakowski and Tappel, 1978; Splittgerber and Tappel, 1979). In animals, GSH peroxidase is present in the mitochondrial matrix and cytosol of cells and in the plasma (Ganther et al., 1976). A related membrane-bound selenoenzyme, called phospholipid hydroperoxide GSH peroxidase has been found in various mammalian tissues, such as pig heart (Ursini et al., 1985).

The activity of GSH peroxidase is dependent upon the dietary intake of selenium (Valentine et al., 1980). However the tissues of rodents, fed a selenium-deficient diet, still exhibit GSH peroxidase activity (Lawrence and Burk, 1976; Cikryt et al., 1982). This non-selenium GSH peroxidase activity is probably due to some of the glutathione-S transferases; these enzymes will reduce organic peroxides but not $\text{H}_2\text{O}_2$ (Cikryt et al., 1982). Non-selenium GSH peroxidases are mainly located in the cytosol and mitochondria, although activity has been reported to occur in rat liver microsomes (Lawrence and Burk, 1978; Reddy et al., 1981).

The GSSG, produced by GSH peroxidases, is reduced back to GSH by glutathione reductases, which catalyze the reaction:--
The NADPH, required for this reaction, is provided by the pentose phosphate pathway. GSSG can inactivate a number of enzymes, such as adenylate cyclase, phosphofructokinase, and phosphorylase phosphatase, by forming mixed disulphides, and inhibits protein synthesis. Glutathione reductases thus help to maintain the high GSH/GSSG ratios required for normal cell function (Kosower and Kosower, 1978; Halliwell and Gutteridge, 1985).

Other antioxidant enzymes: The cytochrome $P_450$ system is probably involved in the metabolism of lipoperoxides (White and Coon, 1980; Wheeler, 1983). The primary role of cytochrome $P_450$ in single cell organisms has been suggested to be oxygen detoxification (Wickramsinghe and Villee, 1975). The enzyme thioredoxin reductase has been reported to quench free radicals at the surface of the epidermis, indicating that this enzyme may help to prevent damage to the skin by radicals, generated from UV light (Schallreuter and Wood, 1985). Caeruloplasmin, the copper-carrying glycoprotein of the extracellular fluids has ferroxidase activity, that is it catalyses the oxidation of Fe(II) to Fe(III). Caeruloplasmin might thus inhibit lipid peroxidation by iron salts, as Fe(III) is a less potent catalyst of autoxidation reactions than Fe(II) (Gutteridge et al., 1985). Caeruloplasmin has been shown to scavenge 'O$_2$' but much less effectively than SOD (Goldstein et al., 1979). Transferrin and lactoferrin are extracellular proteins that prevent lipid peroxidation by binding iron salts (Halliwell and Gutteridge, 1985).

Non-enzymic antioxidant defence: The constituents of lipid membranes can influence the susceptibility to autoxidation: the presence of cholesterol, for example, can confer resistance against peroxidation (Burlakova, 1975; Halliwell and Gutteridge, 1985). Vitamin E is a lipid-
soluble vitamin, that concentrates in the interior of membranes, and is a mixture of four phenols (tocopherols) of which the $\alpha$-form is most active (Burton and Ingold, 1983b). Vitamin E quenches and reacts with $^{1}\text{O}_2$ and scavenges $'\text{O}_2^-$. It is also a chain-breaking antioxidant as it reacts with lipid peroxide radicals to form tocopheryl radicals, which cannot abstract protons from membrane PUFA (Halliwell and Gutteridge, 1985). $\alpha$-Tocopherol has been shown to protect against lipid peroxidation in vitro and in vivo (Burton et al, 1983b).

**Ascorbate:** Ascorbic acid (vitamin C) is a reducing agent which may help to detoxicate radical species by reacting with them to produce a comparatively unreactive species, the semidihydroascorbate radical:

\[
'\text{OH} + \text{ascorbate} \rightarrow \text{semidihydroascorbate} + \text{OH}^-
\]

\[
2 \text{semidihydroascorbate} \rightarrow \text{ascorbate} + \text{dehydroascorbate}
\]

Ascorbic acid is possibly an important extracellular antioxidant in lung, because it has been shown to accumulate in the fluid lining of pulmonary air spaces (Halliwell and Gutteridge, 1985). Ascorbate does not react with peroxide radicals as readily as vitamin E; it has been hypothesised a major antioxidant function of ascorbate is to regenerate vitamin E from tocopheryl radicals (Niki et al, 1984).

Dehydroascorbate has been shown to cause diabetes in animals. Ascorbate, in the presence of $\text{H}_2\text{O}_2$ and at concentrations too low to scavenge 'OH, can reduce Fe(III) to Fe(II) to stimulate 'OH formation (see Chapter 4).

**Selenium:** The essential trace element, selenium (Se) resembles sulphur in many of its properties, but in vivo selenium compounds tend to undergo reduction reactions, whereas sulphur compounds usually undergo oxidations.
The reduction of GSH by selenium-dependent GSH peroxidase is an example of this generalisation. Although GSH peroxidase is the best characterized, other selenoproteins, such as selenoflagellin of muscle and selenium-transport protein are also present in animals (Reddy and Massara, 1983). The main antioxidant properties of selenium are due to its presence in GSH peroxidase (Vernie, 1984). Selenium does, however, have a synergistic effect on vitamin E: as part of GSH peroxidase it lessens the amount of vitamin required to protect membranes from peroxidation; it assists normal dietary absorption of vitamin E by preserving the integrity of the pancreas; and aids the retention of vitamin E in the plasma by an unknown mechanism (Scott, 1986). Selenium has been reported to enhance both humoral and cellular immunity (Reddy and Massara, 1983).

Other antioxidants: Uric acid, at physiological concentrations in human plasma, has been reported to be a scavenger of $'OH$ (Ames et al., 1981). Glucose is a weak scavenger of $'OH$, but is present in substantial concentrations in biological systems, and so might be a significant antioxidant (Sagone et al., 1983). Zinc possibly has antioxidative properties (Willson, 1977): spermine has been reported to be a $O_2^-$ scavenger (Vanella et al., 1982); ethanol can scavenge $'OH$ radicals (Halliwell and Gutteridge, 1985). $\beta$-carotene, an orange-yellow pigment and a pro-vitamin for vitamin A, is obtained in the diet from vegetables, and has been shown to be a powerful $^1O_2$ quencher (Willson, 1983). Dietary riboflavin has been observed to act synergistically with $\alpha$-tocopherol in inhibiting $CCl_4$-induced hepatic lipid peroxidation in rats (Miyazawa et al., 1984). Synthetic antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate, promethazine and ethoxyquin, can be found in foodstuffs and in certain over-the-counter medicines (Halliwell and Gutteridge, 1985). There are a number of synthetic and natural antioxidants developed by the Pharmaceutical
oxidized by hydroperoxides:

$$\text{ROOH} + 2\text{H}^+ + 2\text{I}^- \longrightarrow \text{ROH} + \text{H}_2\text{O} + \text{I}_2$$

The concentration of liberated iodine can be measured by titration with thiosulphate, or tri-iodide anions:

$$\text{I}^- + \text{I}_2 \rightarrow 2\text{I}_3^-$$

The iodometric assay has to be applied under anaerobic conditions to prevent interference in the reaction system by molecular oxygen (Pryor and Castle, 1984)

Hydroperoxide concentrations can be determined using peroxidases (Pryor and Castle, 1984). Peroxidases catalyse the reduction of hydroperoxides to the corresponding alcohols, using hydrogen, abstracted from a suitable donor ($\text{DH}_2$):

$$\text{ROOH} + \text{DH}_2 \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{D}$$

The concentrations of the oxidized product, $\text{D}$, corresponds to that of hydroperoxide, and can be estimated by various suitable means. Lipoperoxides may be determined, for example, using GSH peroxidase and excess GSH. The lipoperoxide concentrations would then be quantified, by determining the concentration of GSSG (Pryor and Castle, 1984):

$$\text{ROOH} + 2\text{GSH} \rightarrow \text{ROH} + \text{GSSG} + \text{H}_2\text{O}$$

The peroxidase (haematin)-catalyzed oxidation of dichlorofluorescin to dichlorofluorescein, has been applied as an assay to determine hydroperoxide concentrations (Cathcart et al., 1983). As the hydroperoxide substrate and dichlorofluorescein product were reported to have 1:1 stoichiometry, hydroperoxide concentration can be ascertained from the fluorimetric determination of dichlorofluorescein.
The Detection of Lipid Peroxidation

Lipid peroxidation is a complex process (fig. 1.3). The diversity of the products of lipid peroxidation has enabled numerous methods of monitoring lipid peroxidation to be devised (Slater, 1982; summarized in fig. 1.4). Most methods, however, are only suitable for in vitro lipid peroxidation systems, such as microsomal fractions and lipid membranes (Slater, 1984).

Diene conjugation: Lipids, that contain diene or polyene groups, have been observed to show a shift in the position of their double bonds, when peroxidized (Logani and Davies, 1980). The resultant conjugated dienes and trienes have intense optical absorptions at 233 nm and 288 nm respectively, which can be detected, using UV spectrophotometry (Cawood et al., 1984). The detection of lipid peroxidation in biological samples, by this method has the disadvantage of being relatively non-specific (Recknagel and Ghoshal, 1966).

Fluorescence: Secondary carbonyls, produced by lipid peroxidation, react with amino acids to form conjugated Schiff bases, which have characteristic optical absorptions and fluorescence (Gutteridge et al., 1982). Lipid peroxidation in biological systems can thus be quantified using fluorescent analysis (Tappel, 1975; Wickens et al., 1981). Lipid peroxidation in vivo can be assessed by studying the autofluorescence of ceroid and lipofuscin pigments in histological sections of tissue, using fluorescence microscopy (Gedigt, 1969; Tappel, 1980; Chapters 2 and 3).

Direct detection of hydroperoxides: Hydroperoxides can be determined directly, by an iodometric assay (Pryor and Castle, 1984). Iodide is
Oxygen-derived free radical

**Polyunsaturated fatty acid**

- Molecular rearrangement
- OXYGEN UPTAKE
- Peroxides
  - IODOMETRY, HPLC, TLC, DICHLORFLORESCEIN ASSAY

**Chemiluminescence**

- Excited carbonyls
  - Alddehydes (e.g., malondialdehyde)
  - Polymers
    - Hydrocarbons (Ethane, pentane etc)

**Dichlorofluorescein Assay**

**Indirect Detection**

(Superoxide dismutase, hydroxylation)

**GLC**

**UV Absorption**

**Thiobarbituric Acid Reactivity**

**Fluorimetry**

**FIG. 1.4 - Summary of the Direct and Indirect Methods Available for the Detection and Measurement of Lipid Peroxidation.**

(from Gutteridge and Stocks, 1981)
The various procedures to measure lipid peroxidation by determination of lipoperoxides, are generally sensitive and comparatively specific. The peroxidase methods have the advantage of determining only hydroperoxides and \( \text{H}_2\text{O}_2 \): endoperoxides, such as prostaglandin \( \text{H}_2 \) are not substrates for peroxidases (Cathcart et al., 1983; Pryor and Castle, 1984). Assays that estimate hydroperoxide concentrations in biological samples must, however, include rigorous controls and precautions to preclude various sources of interference, such as hydroperoxide degradation by transition metals, thiols or endogenous peroxidases (Slater, 1984).

**Chromatographic techniques:** Lipid peroxidation in *in vitro* systems can be monitored by determining the loss of polyunsaturated fatty acids (PUFA), using gas liquid chromatography (GLC). The loss in the amount of PUFA in the peroxidized lipid is determined by comparison with the PUFA concentration before oxidation (May and McCay, 1968).

Short-chain, saturated hydrocarbon gases are produced during lipid peroxidation in biological systems (Cohen, 1979). Pentane and ethane, for example, probably originate from the decomposition of \( \omega-6 \)- and \( \omega-3 \)-hydroperoxides respectively (Bus and Gibson, 1979; figure 1.5). Measurement of exhaled hydrocarbons by GLC, has enabled noninvasive monitoring of *in vivo* lipid peroxidation (Lawrence and Cohen, 1984; see earlier). Lipid peroxidation *in vitro* can also be monitored by assay of hydrocarbon gases (Cohen, 1979; Muller and Sies, 1984).

Aldehydes, and other carbonyl compounds, produced during the peroxidative degradation of lipid biomembranes, can be isolated to quantify lipid peroxidation (fig. 1.4). Hydroxy-aldehydes, for example, can be derivatized with 2,4-dinitrophenylhydrazine into their corresponding hydrazone derivatives to enable their separation from complex carbonyl mixtures, resulting from peroxidation (Esterbauer, 1982). The aldehydes
FIG. 1.5 - Production of Pentane by Iron-catalysed Decomposition of ω-6-hydroperoxides.
are separated into their individual carbonyl classes, using a combination of thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The hydrazone derivatives have characteristic UV absorption spectra, and thus may be applied to assay the individual aldehydes (Esterbauer, 1982).

Hydroxy fatty acids are believed to be formed in biological tissues, by enzymic conversion of lipoperoxides during the initial stages of the lipid peroxidation cascade (Slater, 1984). These hydroxy fatty acids have been separated by HPLC, and used to estimate lipid peroxidation (Capdevila et al., 1982). Hydroxylated derivatives of unsaturated fatty acids of peroxidized murine liver phosphatidylcholines have been detected, using HPLC and GLC mass spectrometry (Hughes et al., 1983).

Chromatographic methods, using the available technology, to determine various carbonyls resulting from lipid peroxidation, are, however, time-consuming and complex to perform (Slater, 1984). These methodologies thus have not yet become routine means of estimating lipid peroxidation in biological samples.

Measurement of malondialdehyde: Cyclic endoperoxides, formed by the autoxidation of PUFA containing 3 or more methylene-interrupted double bonds (notably arachidonic acid), are cleaved to produce malondialdehyde (malonaldehyde, malonyldialdehyde; Pryor et al., 1976b; Esterbauer, 1982; fig. 1.6). The determination of malondialdehyde has been used extensively to detect oxidative rancidity of foods that have high lipid contents, and to measure oxidative changes in biological materials (Bird and Draper, 1984; Halliwell and Gutteridge, 1985). Malondialdehyde has been reported also to be produced by the eicosanoid cascade, and by free-radical damage to amino acids, carbohydrates and nucleic acids (Gutteridge, 1982a).

Malondialdehyde exists in biological cells as a free form, or as
FIG. 1.6 - Cleavage of Cyclic Endoperoxides Group to Form Malondialdehyde.
complexes, such as lipofuscin, with various tissue components (Suege and Aust, 1977; Bird and Draper, 1984). Malondialdehyde has been reported to be metabolized mostly by mitochondrial aldehyde dehydrogenases to a semialdehyde, followed by decarboxylation to acetate and CO$_2$ (Siu and Draper, 1982). Free malondialdehyde can be quantified by its UV absorption, at acid pH, by polarography, and by HPLC separation (Bird and Draper, 1984; Esterbauer et al., 1984).

The most widely adopted method to estimate lipid peroxidation is based upon the reaction between malondialdehyde and 2-thiobarbituric acid (TBA), under acidic conditions, to form a chromogenic adduct (Gutteridge and Stocks, 1976; Ohkawa et al., 1979; Bird and Draper, 1984; fig. 1.7). This reaction product is a pink-red crystalline substance, which has an optical absorption maximum of 532-535 nm, and a fluorescence spectrum with an excitation maximum at 532 nm and emission maximum of 553 nm (Yagi, 1976; Bird and Draper, 1984). The concentration of the TBA-reactive product is determined by comparison with the reaction of malondialdehyde standards, produced by heating 1,1,3,3-tetraethoxypropane or 1,1,3,3-tetramethoxypropane (malonaldehyde bisdimethyl acetal), at acid pH. The complete hydrolysis of one molecule of tetramethoxypropane produces one molecule of malondialdehyde and four molecules of methanol (Gutteridge, 1982a).

The procedure to monitor lipid peroxidation in biological materials, by determining reactivity with TBA (often referred to as the TBA method or TBA test), has been employed in several different ways and with a variety of different acidifying reagents (Uchiyama and Mihara, 1978; Gutteridge, 1982a; Slater, 1984). The most usual procedure has been to mix tissue samples with an acid precipitant, such as trichloroacetic acid (TCA): the mixture is then centrifuged; and the supernatant is used for the reaction with TBA (Slater, 1984). This method is used to ascertain...
FIG. 1.7 - Reaction of Malondialdehyde with Thiobarbituric Acid.
the concentration of free malondialdehyde in a biological sample. An alternative method determines the concentration of TBA-reactants in the precipitate (Satoh, 1976), whereas other procedures use acidified homogenates for the TBA reaction (Uchiyama and Mihara, 1978; Ohkawa et al, 1979). With assays that employ either the precipitate or non-acid precipitant-treated samples, most of the malondialdehyde, detected by the TBA reaction, is produced by endoperoxides which break down during the acid-heating conditions of the reaction (Gutteridge, 1982a). TBA tests, using homogenates or acid-precipitates, thus determine the concentrations of comparatively stable peroxidic precursors. These tests are possibly more accurate indicators of lipid peroxidation than assay procedures using the acid-soluble components of tissues, as free malondialdehyde in biological systems is rapidly metabolized (Barber and Bernheim, 1967; Siu and Draper, 1982). Studies of microsomal peroxidation have shown that the production of ethane, but not pentane, correlates with that of TBA-reactive material. Ethane can be detected to pmol level and TBA-reactive substances only to nmol concentrations, but the stoichiometry of the peroxidation reaction is 2200 molecules of malondialdehyde for every ethane, so the measurement of either substance affords a similar sensitivity (Wendel and Reiter, 1984).

Chemiluminescence: The emission of light from chemical reactions, at non-extreme temperatures, is termed chemiluminescence (Thorpe et al, 1982). Chemiluminescent reactions yield an electronically excited product or intermediate, which can either release energy in the form of light, or will transfer its energy to another molecule, which then radiates light (fig. 1.8). The energy released, during chemiluminescence, must be sufficient to produce light quanta, that is at least 40 kcal/mol (Lloyd, 1982). Chemiluminescent reactions are therefore comparatively uncommon: chemical energy is more usually dissipated in the form of heat (Thorpe et
Molecules in high energy excited state

Molecules in low energy ground state

ENERGY INPUT (OXIDATION)

ENERGY RELEASED AS CHEMILUMINESCENCE

Molecules in low energy ground state

FIG. 1.8 - Chemiluminescence.
al, 1982). Most chemiluminescence reactions are thus oxidations, in which large amounts of energy are generally transferred (Rauhut, 1979).

The decomposition of hydroperoxides yields carbonyl products in an excited triplet state, and singlet oxygen (Krinsky, 1979; Rauhut, 1979; fig. 1.3). The emission of light from these excited species (low-level chemiluminescence) can be quantitated to monitor lipid peroxidation (Cadenas and Sies, 1984; Boveris et al., 1981):

\[ 2 \text{R}_2\text{CHOO}^- \rightarrow \text{R}_2\text{C}=\text{O} + \text{R}_2\text{COH} + ^1\text{O}_2 + \text{H}^+ \]

secondary peroxy radical
(lipid peroxy radical)

Singlet oxygen, when decaying to the triplet ground state, emits light at 634 nm and 703 nm (dimol emission):

\[ ^1\text{O}_2 + \text{O}_2 \rightarrow 2 ^3\text{O}_2 + \text{LIGHT} \text{ (634, 703 nm)}. \]

Singlet oxygen can also decay to produce monomol light emission:

\[ ^1\text{O}_2 \rightarrow ^3\text{O}_2 + \text{LIGHT} \text{ (1268, 1406 nm)} \]

The decay of secondary peroxy radicals generates excited carbonyl species, which emit light at between 380 and 460 nm (Rauhut, 1979; Boveris et al., 1981; Cadenas and Sies, 1984; fig. 1.9). Excited carbonyl groups have also been reported to be produced from the reaction between \(^1\text{O}_2\) and substituted unsaturated lipids (Boveris et al., 1981):

\[ \text{R}_2\text{C}=\text{CR}_2 + ^1\text{O}_2 \rightarrow ^0\text{O} \longrightarrow \text{R}_2\text{C}^* \text{CR}_2 \rightarrow \text{R}_2\text{CO} + \text{R}_2\text{CO}^* \]

dioxetane
intermediate
excited
 carbonyl

Excited carbonyls have been postulated to be quenched by molecular
FIG. 1.9 - Chemiluminescence Termination Step for Secondary Peroxy Radicals.
oxygen to produce singlet oxygen (Cadenas et al, 1981):

\[ R_2CO^* + O_2 \rightarrow R_2O + ^1O_2 \]

The detection of light, emitted during the degradation of lipoperoxides, can be used as a method to monitor lipid peroxidation (Boveris et al, 1981; Kricka and Thorpe, 1983). The chemiluminescence, emitted from peroxidizing liver microsomes has been reported to correlate with malondialdehyde formation, detected using the TBA test (Wright et al, 1979). In studies to evaluate various methods to characterize lipid peroxidation in isolated hepatocytes, measurements of expired alkanes, malondialdehyde production, fluorescence and chemiluminescence were all found to correlate (Smith et al, 1982). Chemiluminescence detection has also been reported to be one of the most sensitive means to measure lipid peroxidation (Smith et al, 1982).

The light emission from biological oxidations is termed low level or ultraweak chemiluminescence (Boveris et al, 1981). Chemiluminescence is detected using photomultipliers of suitable sensitivity, and with sufficient spectral range for the wavelengths of light emitted. Chemiluminescence is generally expressed as counts/sec. The reproducibility of the method has been reported to be appreciably good (Cadenas and Sies, 1984).

Luminol \((\text{5-amino-2,3-dihydro-1,4-phthalazinedione})\) is oxidized by active oxygen intermediates to the aminophthalate anion, which emits light at around 450 nm, upon relaxation to the triplet ground state (Cadenas and Sies, 1984). Luminol can thus be used to amplify chemiluminescence due to \(^1O_2\), and as a non-specific method to detect the generation of other reactive oxygen metabolites (Chapter 4).

**Determination of in vivo lipid peroxidation:** Lipid peroxidation can be
studied in vivo, by assaying alkanes in the exhaled breath (Cohen, 1979). Precautions have to be taken, however, to account for the possible influence of microbial flora in the digestive tract (Slater, 1984). The site of lipid peroxidation cannot be located by this technique (Lawrence and Cohen, 1984).

The chemiluminescence, associated with lipid peroxidation, can be measured in exposed organs of anaesthetized animals (Boveris et al., 1980). The chemiluminescence of human breath samples has been postulated to represent pulmonary peroxide excretion due to hyperoxic stress (Williams and Chance, 1983).

A limited assessment of lipid peroxidation in vivo can be achieved by determining the concentrations of the more stable products of lipid peroxidation in suitable tissues, taken from experimental animals, after they have been killed. Interpretation of the results of these types of study are complicated due to metabolism of lipid peroxidation products in vivo, and their possible post mortem degradation or production.

Autoxidative damage and cancer

Deleterious oxidation reactions (for example, lipid peroxidation) may be significant in the initiation and possibly the progression of many types of cancers (Apffel, 1976; Demopoulos et al., 1980; Ames, 1983; Cerutti, 1985).

Carcinogenesis: The comparison of sera from human subjects in whom cancer developed in the following few years with that of cancer-free subjects has shown that there is a greater incidence of malignant disease in those who have below average levels of antioxidants (Willett et al., 1984). Other epidemiological studies have indicated that there is a negative correlation between serum selenium concentration and the incidence of cancer (Robinson et al., 1979; Clark, 1985). The dietary
supplementation of antioxidants, such as vitamin E and selenium, have been shown to decrease the incidence of tumours in animals, treated with carcinogens (Demopoulos et al., 1980; Vernie, 1984; Ip, 1985). It has been reported that vitamin E, though ineffective on its own, potentiates the inhibitory effect of selenium upon the development of chemically-induced mammary tumours in rats (Horvath and Ip, 1983). The cancer inhibitory effects of the antioxidant vitamins and selenium may however be due to mechanisms, such as the alteration of enzymes involved in the metabolism of carcinogens, rather than their ability to detoxicate free radicals (Milner, 1986).

It has been reported that paraquat can cause mutations in Salmonella typhimurium and that the mutagenicity of paraquat was inversely related to the superoxide dismutase activity of these bacteria (Moody and Hassan, 1982). The dietary intake of PUFA can enhance the concentration of TBA-reactive material, and subsequent incidence of tumours in mice exposed to ultraviolet radiation (Black et al., 1985). It has been observed that malondialdehyde increases the incidence of skin tumours in mice (Shamberger et al., 1974). The topical application of the carcinogen, 7,12-dimethylbenz(a)anthracene to the skin of mice, has been demonstrated to cause an increase in epidermal TBA-reactive material concentrations (Shamberger, 1972). The free radical generator, benzoyl peroxide, has been reported to promote the progression of benign tumours on mouse skin into malignant tumors (O'Connell et al., 1986). Tumour promoters have been shown to enhance lipid peroxidation in murine skin (Logani et al., 1982).

Many carcinogens form free radical metabolites (Demopoulos et al., 1980; Mason et al., 1982). It has been hypothesised that carcinogenic aromatic amines are converted by the liver into nitroxides which enter into a free radical-mediated redox cycle (Stier et al., 1980). Benzo(a)-pyrene is probably activated in the liver by cytochrome P448 to the
carcinogen, 7,8-epoxide, a generator of reactive oxygen species (Parke and Ioannides, 1984a; see earlier).

Free radical production in vitro: A few investigators have reported that neoplastic tissues can produce reactive oxygen species in vitro, but usually to a lesser extent than normal tissues. Mitochondrial membrane fragments from Ehrlich ascites tumours, H6-hepatomas and Morris hepatomas have been shown to produce appreciable superoxide (Dionisi et al, 1975; Oberley et al, 1982). The slow-growing Morris hepatoma has been observed to have greatly decreased Mn-SOD activity but a capacity to generate $'O_2^-$ at a rate comparable to that of normal rat liver (Oberley et al, 1981). The NADPH-stimulated production of $'O_2^-$ by human renal adenocarcinoma tissue has, however, been reported to be considerably less than that by normal human kidney (Wickramasinghe et al, 1976). Nuclear membranes from rodent tumours have also been observed to be able to produce $'O_2^-$ in vitro (Bartoli et al, 1977; Peskin et al, 1980).

The concentrations of the metabolites of lipid peroxidation in tumour tissues, and the susceptibility of neoplastic tissues to undergo lipid peroxidation, have generally been reported to be considerably less than that, determined for comparable normal tissues (Thiele and Huff, 1960; Lash, 1966; Player et al, 1979; Bartoli and Galeotti, 1979; Ahmed and Slater, 1981; Rossi and Cecchini, 1983). Oxidized methyl linolenate has been observed to inhibit cell division (Wilbur et al, 1954). Many of the oxidation products of PUFA, have been reported to inhibit the metabolism and growth of tumour cells (Shuster, 1955; Dianzani, 1982; Schauenstein, 1982). It has been suggested that an increase in the ability to detoxicate free radicals may promote cell multiplication (Burlakova and Pal'mina, 1967). It has also been hypothesised that neoplasia is a direct consequence of excessively high cellular concentration of antioxidants or an extremely low propensity to produce radical
There have, however, been reports of tumours which have greater lipoperoxide concentrations than comparative normal tissues: human gastric cancers and colon cancers have, for example been reported to have increased concentrations of TBA-reactive material and diene conjugates (Baur and Wendel, 1980; Vainshtein and Zvershkhanovskii, 1984).

**Radical production in vivo:** Although there is evidence that at least some types of neoplastic transformations can be initiated by free radical mechanisms, the role of free radicals in the cancer phenotype is uncertain (Swartz, 1984). The direct means to determine whether there are free radicals in tissues is to measure paramagnetism, using a technique called electron spin resonance (ESR; Swartz, 1979). Almost all ESR studies of experimental tumours have indicated that their concentrations of free radicals are lower than those of comparable normal tissues (Swartz, 1982a). Exceptions, such as melanin-containing tumours, dimethylbenzanthracene-induced mammary carcinomas of rats have, however, been observed to have substantial free-radical concentrations (Swartz, 1982b; Lohman and Neubacher, 1984). It has been reported that the concentrations of free radicals in sarcoma-37 tumours change as the neoplasm increases in size (Saprin et al, 1967). Ehrlich ascites tumour cells have been observed to have significant chemiluminescence (Cheng et al, 1983), and this was concluded to be due to unusually high concentrations of free radicals. The ascorbyl radical has been identified in a number of rat tumour tissues, in the murine B16 melanoma and at raised concentrations in the erythrocytes of patients with acute lymphatic leukaemia (Lohman and Neubacher, 1984). The occurrence of an ESR spectrum, characteristic of the ascorbyl radical is not, however, proof for the existence of free radicals, because the preparation of tissues, usually by freeze drying for these studies, can create spurious ESR
signals due to the presence of blood (Mueller and Tannert, 1986). Oxygen radicals may be involved in melanin reactions because melanin can consume \( \text{O}_2 \) and produce \( ^\cdot\text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) (Sealy et al., 1980). It has been reported that there is an increase in free radical concentration during the early stages of cancer, followed by a decrease as neoplastic tissue enlarges (Emanuel, 1982).

The activities of the major antioxidant enzymes, superoxide dismutase, catalase and GSH peroxidase, have been reported to comparatively diminished in many tumour cells (Bozzi et al., 1976; Peskin et al., 1977; Nathan et al., 1980; Oberley, 1982; Tisdale and Mahmoud, 1983; Corrocher et al., 1986). Tumour cells might therefore be sensitive to reactive oxygen species, even though most evidence suggests that they have low susceptibility to lipid peroxidation.

Antioxidants, such as vitamin C (Cameron et al., 1979), vitamin A (Oberley and Beuttner, 1979) and selenium (Milner, 1984), however, have been reported to slow the growth of animal tumours and human cancers. The OH\(^-\) scavenger, dimethyl thiourea, has been observed to induce cell differentiation and inhibit tumour growth of B16 melanomas (Nordenberg et al., 1985). It has been shown that the administration of vitamin E causes neuroblastoma and B16 melanoma cells, in culture, to differentiate (Prasad et al., 1979; Rama and Prasad, 1983).

**Experimental Rationale**

It has been concluded that the treatment of common adult malignancies by cytotoxic chemotherapy has generally been disappointing (Kearsley, 1986). Clinical oncologists very often have to decide whether there is sufficient therapeutic benefit from anticancer drugs to warrant the suffering of distressing side effects. Safer cancer treatments are urgently required.
Activated oxygen species may be implicated in the antineoplastic actions of ionizing radiations, many chemotherapeutic drugs, and some products of cellular metabolism. Cells of the immune system can attack neoplastic cells and destroy them, using the reactive oxygen species of the respiratory burst. The ability of cancer cells to detoxify reactive oxygen intermediates and the susceptibility of neoplasms to autoxidative injury may thus be relevant to neoplastic growth and to anticancer therapy. The purpose of the following studies was to characterise a transplantable animal tumour in terms of these parameters, as a model for improved therapy of clinical cancer.

The Lewis lung carcinoma: The Lewis lung carcinoma is a malignant, essentially anaplastic tumour which, when transplanted either subcutaneously or intramuscularly into syngeneic mice, regularly produces metastatic foci in the lungs (Trope, 1974; Isakov et al., 1981). The carcinoma arose spontaneously in 1951 as a pulmonary tumour in a C57BL mouse, and was discovered by Dr Margaret Lewis of the Wistar Institute (Sugiura and Stock, 1955; Gundersen et al., 1981).

Previous experiments: Shortly before the studies described in the following chapters, preliminary studies to determine antioxidant defence and lipid peroxidation in liver, lung and tumours of mice, bearing Lewis lung carcinomas, were reported (Capel and Thornley, 1982; Capel and Thornley, 1983). In this earlier work, SOD activities and TBA-reactive material concentrations in Lewis lung tumour homogenates, removed from animals at various intervals after tumour implantation, were found to be comparatively pronounced. GSH concentrations and GSH peroxidase activities in Lewis lung carcinomas were, however, relatively limited. In other preliminary studies, the vitamin E and selenium concentrations of Lewis lung carcinomas were observed to increase appreciably between 13 and 18 days after transplantation (Capel, 1984).
Systemic effects of cancer: It has been known for many years that malignant tumours induce changes in tissues of the host that have not been infiltrated by neoplastic cells (Greenstein, 1954; Begg, 1958; Busch, 1962; Shapot et al, 1972; Grigor et al, 1979). Perturbations of normal tissues, remote from a tumour or its metastatic foci, have been referred to as "systemic" changes (Begg, 1958). The systemic effects of neoplastic disease have been the subject of considerable study because they may contribute to the death of tumour-bearing animals or cancer patients (Hardy, 1962; Costa, 1977).

Catalase was one of the first enzymes to be discovered and isolated and thus was one of the first enzymes to be investigated in cancerous tissues (Busch, 1962; Aebl, 1974). Earlier this century, the catalase activities of livers, removed post mortem from cancer patients, were reported to be appreciably less than those of livers from people who had died from other causes (Brahn, 1916). This was probably the first description of cancers affecting the enzyme systems of non-involved host tissues. Decreased hepatic catalase activity has since been one of the most conspicuous observations of the considerable literature, concerned with the systemic effects of tumours (Greenstein, 1954; Begg, 1958).

Superoxide dismutase, another enzyme associated with the detoxification of reactive oxygen species, might also be affected by neoplasia. Tumour-bearing animals have been reported to have decreased hepatic Cu/Zn-SOD activities (Tarakhovsky et al, 1980; Takada et al, 1982. The livers of mice, bearing Ehrlich ascites carcinomas, have been observed to have less SOD activity (particularly Mn-containing isoenzyme) than the livers of tumour-free mice. (Oberley et al, 1981). In preliminary studies of mice bearing Lewis lung carcinomas, however, hepatic Cu/Zn-SOD and GSH peroxidase activities were found not to differ from those of tumour-free animals (Capel and Thornley, 1982; Capel and
The hepatic GSH/GSSG ratio of Lewis lung carcinoma-bearing mice has been observed to rise as the tumour increased in size (Capel and Thornley, 1983). Mice, bearing Lewis lung carcinomas, were however observed to have considerably greater hepatic TBA-reactive material concentrations than tumour-free controls, indicating that the tumour was adversely affecting the livers of these animals.

Objectives of the studies described by this thesis: The Lewis lung carcinoma is among the most widely used animal tumours in the screening of compounds for antineoplastic activity (Berndal et al., 1983), but is comparatively resistant to most conventional cancer drugs. Apart from the preliminary studies described above, however, relatively little is known of the antioxidant defence capability of Lewis lung tumour cells: SOD activity is significantly less in Lewis lung than in the tissue of origin, murine lung (Peskin et al., 1977; Van Balgooy and Roberts, 1978).

The biochemical parameters, determined in the preliminary studies were expressed in terms of the wet weight of the tumour. The Lewis lung carcinoma, in common with other solid tumours has a heterogenous composition (Fidler, 1978; Takenaga, 1984; Zupi et al., 1984): variation of biochemical parameters in homogenates of whole tumour with time after implantation (tumour size?) could thus be explained by a change in the ratio of different cell types. Change in the concentration of neoplastic cells due to alterations in extracellular factors such as microbial infection, cell-mediated immunity or dilution by extracellular fluids (oedema) could also affect the biochemistry of neoplastic tumours. The inverse relationship between SOD activity and tumour weight (Capel and Thornley, 1982) for example, could have resulted from a greater contribution to tumour mass by water as tumour volume increased. Although neoplastic cells can produce factors that stimulate the growth of new blood vessels (angiogenesis factors; Folkman and Tyler, 1977), regions of
growing tumours may become so detached from vascular capillaries that they become hypoxic and ischaemic, and cell death ensues. Necrosis could therefore contribute to biochemical changes, determined at various days after tumour transplantation.

The studies of this thesis were therefore instigated to expand and improve the earlier investigations upon antioxidant defence and lipid peroxidation in tumour-bearing mice. Many anticancer drugs influence tissue concentrations and the redox status of glutathione (Beck, 1980; Arrick and Nathan, 1984). The activities of the enzymes, responsible for the metabolism of glutathione were therefore determined. As the observation of pronounced TBA-reactive material concentrations in the Lewis lung carcinoma conflicted with reports for most other animal tumours and human cancers, particular attention was afforded to the determination of lipoperoxides. Degraded tissues, removed post mortem undergo autoxidation much more readily than when in the living animal (Halliwell and Gutteridge, 1986). The observations of the preliminary studies must therefore be interpreted cautiously, and measures taken to determine in vivo lipid peroxidation.

It has been hypothesised that serial passaging of rodent tumours allows longer exposure of the neoplastic cells to the internal milieu of the organism (Leibovici, 1984). The Lewis lung carcinoma, due to passaging over many years, might thus be better a reflection of the disease in long-life span animals, such as human beings, than say chemically induced tumours, or cancer cells maintained in tissue culture. The Lewis lung carcinoma was considered still to be a relevant model with which to continue the studies. Another advantage of the Lewis lung carcinoma is that there are many reports in the biomedical literature of its ability to form metastatic foci, and of its prostanoid metabolism: antioxidant defence and autoxidative damage might be of relevance to
To determine whether the systemic effects of bearing Lewis lung carcinomas were common to other tumour models, studies were also undertaken using mice, implanted with B16 melanomas. This tumour has similar growth kinetics to the Lewis lung and is also passaged in mice of the same strain. Like the Lewis lung, there are many reports of the biology and biochemistry of the B16 melanoma with which to compare the findings of the following studies. Animals, bearing B16 melanomas, have been reported elsewhere to have increased concentrations of TBA-reactive material in their livers (Pierson and Meadows, 1985). One parallel with the Lewis lung carcinoma has thus already been established.
CHAPTER TWO

TUMOUR GROWTH AND ANTIOXIDANT DEFENCE
INTRODUCTION

The studies described in the following chapter were directed to improve and expand the earlier work to determine antioxidant defence in Lewis lung tumours (Capel and Thornley, 1982; Capel and Thornley, 1983; Chapter 1). A number of modifications to make the biochemical assessment of the intramuscularly-implanted Lewis lung carcinoma less ambiguous, were attempted. All determinations were related to protein concentrations of the applicable tumour-derived samples, rather than to wet weights. Estimations of biochemical parameters in whole-tissue homogenates could conceal differences between normal and neoplastic cells due to subcellular compartmentation (Sies, 1982; Kohen et al, 1983). Tumour homogenates, when practicable, were therefore differentially centrifuged into appropriate subcellular fractions. During the latter procedure, homogenates would undergo an initial low-speed centrifugation (at 660g) to precipitate and remove cell debris and the necrotic components of the tumour (Oberley and Spitz, 1984). An additional approach was to physically subdivide Lewis lung tumours into arbitrary cortical "hypoxic" and peripheral "oxygenated" zones (Kennedy et al, 1980).

Determinations were also made using Lewis lung cells maintained by tissue culture. Tumour cells grown in an in vitro system for a number of passages are unlikely to be contaminated by host-derived cells, such as fibroblasts and activated macrophages, that extensively infiltrate transplantable tumours borne by experimental animals. (Stephens et al, 1978; Gundersen et al, 1981; Isakov et al, 1981b). The 95:5 air/CO₂ atmosphere under which cells in culture were maintained has a much greater partial oxygen pressure than that of tumours in situ (Bump et al, 1982). The syntheses of the major antioxidant defence enzymes, that is
GSH peroxidase, GSSG reductase and SOD in rat pulmonary tissue are inducible by molecular oxygen (Kimball et al., 1976). The evaluation of enzymic antioxidant systems in the well-oxygenated cultured Lewis lung cells should thus make a useful comparison to that of relatively ischaemic Lewis lung tumours borne by mice.

Tumour growth was assessed by determining tumour weight in situ (the difference in weight between the tumour-free and the tumour-bearing limb); the maximum diameter of the tumour; and DNA concentration (an estimation of cell number).

The cellular concentration of reduced glutathione (GSH) has recently been postulated to be a major determinant of the efficacy of the great majority of anticancer regimens (Arrick and Nathan, 1984). In previous studies, the ratio of GSH to its oxidized form, GSSG was shown to correlate significantly with tumour weight (Capel and Thornley, 1983). This observation may have been a result of a shift in the composition of Lewis lung tumours in favour of cells containing relatively greater GSH and smaller GSSG concentrations, as the mass of the neoplasm increased. Change in GSH/GSSG ratio could alternatively be caused by an alteration in GSH or GSSG generation and utilization (Kosower and Kosower, 1978). Activities of the principal enzymes of GSH synthesis, \( \gamma \)-glutamylcysteine synthetase and GSSG reductase, and of the major enzymes of metabolism, GSH peroxidase, GSH-transferase and \( \gamma \)-glutamyl transpeptidase were therefore determined. NADPH, the cofactor for GSSG reductase is primarily produced from the reduction of \( \text{NADP}^+ \) by the pentose phosphate pathway or shunt (Elsayed et al., 1982; fig 2.1). Glucose-6-phosphate dehydrogenase (EC. 1.1.1.49) is a rate limiting enzyme of the pentose phosphate shunt (Hosoda and Nakamura, 1970), and therefore its activity was determined.
FIG. 2.1 - The Relationship Between the Enzymic Antioxidant Defence System and the Pentose Phosphate Shunt.
EXPERIMENTAL

Chemicals

[8-\textsuperscript{14}C]Styrene oxide (specific activity 607 MBq/m mole) and L-[1-\textsuperscript{14}C]-glutamic acid (specific activity 2 GBq/m mole) were purchased from the Radiochemical Centre, Amersham, Bucks.

Riboflavin and cumene hydroperoxide were supplied by Koch Light, Colnbrook, Bucks.; metaphosphoric acid, orthophosphoric acid and diaminoethane-tetra-acetic acid (EDTA) by British Drug Houses Ltd., Poole, Dorset; and 2-vinyl pyridine by Aldrich Chemical Co. Ltd., Gillingham, Dorset. Bactotrypsin was purchased from Difco Laboratories, Detroit, Mich., U.S.A. The tissue culture medium, Dulbecco's modification of Eagles minimal essential medium, was supplied by the Institute of Cancer Research, Sutton, Surrey (a detailed description is given in the Appendices). All other reagents were of the purest grade commercially available and were obtained from either Sigma London Chemical Co. Ltd., Poole, Dorset or Fisons, Loughborough, Leics.

Animals and Treatment

All studies were undertaken with male C57BL6 mice (Olac) which were maintained on R&M experimental SQC diet (BP Nutritional; see Appendix I for details), and housed on sterilized sawdust bedding in high density polypropylene cages at 21 + 1°C. Food and water were available ad libitum.

The Lewis lung tumour cell line was obtained in 1977 from Dr. G.G. Steel of the Institute of Cancer Research (ICR), Sutton, Surrey, and has since been maintained by regular intramuscular passage in syngeneic...
hosts. The tumour cell line at the ICR was itself supplied by Professor K. Hellman of the Imperial Cancer Research Fund, London (Steele and Adams, 1975).

The C57BL10 mouse, of the initial studies of Capel and Thoruley (1982, 1983) was replaced by C57BL6 as host for Lewis lung carcinoma because the C57BL6 is the substrain of preference in the great majority of publications in the literature concerned with the Lewis lung. Since the exact substrain in which the Lewis lung carcinoma originated is uncertain, and the BL10 and BL6 mice only differ by two relatively minor histocompatibility loci, both substrains are effectively syngeneic to the Lewis lung carcinoma (Gundersen et al, 1981; Isakov et al, 1981a). Co-workers at the Marie Curie Memorial Foundation Research Institute (Whur et al, 1980) found no significant differences between the C57BL6 and the C57BL10 substrains, as tumour host, in the growth kinetics of the primary Lewis lung or its pulmonary metastases.

Lactate dehydrogenase virus is associated with a 5 to 10 fold increase in the plasma lactate dehydrogenase activity of infected mice (Bailey et al, 1963; Riley, 1968). Host macrophages, that infiltrate transplantable tumours, are potential carriers of this murine tumour-associated virus (Johnson and Shin, 1983). To minimize the carry-over of host cells, Lewis lung cells were cultured in vitro for 5 consecutive passages (Isakov et al, 1981b). Cells were allowed to grow to subconfluency and released from the culture dishes by means of trypsin (5ml of 5% w/v Difco Bactotrypsin) at 37°C. The Lewis lung cells were resuspended in growth medium at a concentration of $2.5 \times 10^6$ cells/ml. Dimethylsulphoxide (DMSO) was added to a concentration of 10% v/v, and aliquots (1ml) of cell suspension were transferred to sterile ampoules. The ampoules were sealed and cooled gradually at a rate of approximately
1°C/min to a final temperature of -70°C. Lewis lung tumour cells from this fifth passage (P5) were stored in a liquid nitrogen container, as a primary stock for all future experiments using Lewis lung tumour cells.

Lewis lung tumours were maintained by one in vivo passage of the P5 stock into one flank of a C57BL6 mouse (see later). The tumour was allowed to develop for 14 days. The mouse was killed by cervical dislocation, and the tumour dissected out of the limb, aseptically. The tumour was finely chopped with crossed scalpel blades and washed with phosphate buffered saline (20ml at 37°C). To disaggregate the tumour cells the fragmented tissue was incubated in phosphate-buffered saline containing Bacto-trypsin (2mg/ml) and DNase (0.1 mg/ml) at 37°C (Stephens et al., 1984). The tumour fragments were allowed to settle (10 min) and the supernatant discarded. The precipitate was suspended in phosphate-buffered saline containing fresh enzymes, and incubated for 20 min with continuous gentle agitation.

The suspension was vigorously shaken to dislodge loosely attached cells from the smaller tumour fragments remaining. Further DNase (0.1 mg/ml final concentration) was added and the suspension filtered through monofil polyester mesh (35mm aperture, Henry Simon Ltd, Stockport, Cheshire). The filtrate was centrifuged (1000 rpm for 5 min) and the resultant pellet washed twice in phosphate-buffered saline (20ml). DNase was added to prevent re-aggregation of tumour cells, to ensure that all the final cell suspension was composed of single cells and not small clumps of cells (Shipley et al., 1975). After discarding the second wash, the cells were resuspended in buffered saline and the concentration of viable, trypan blue excluding, cells (normally >90%) estimated using an improved Neubauer haemocytometer. Mice designated by random selection to bear tumours, received single hindlimb i/m injections (0.2ml) of $5 \times 10^5$
Lewis lung cells suspended in phosphate-buffered saline (PBS). Animals (age-matched) selected to be donors of the reference tissue, lung, received i/m injections of phosphate-buffered saline only. Mice had attained body weights of 21 + 3 g (8-10 weeks of age) by the time of tumour implantation.

Six male C57BL6 mice were inoculated as described previously, with Lewis lung carcinoma cells and six age-matched controls were inoculated with vehicle only. The tumours were allowed to develop for 14 days before the animals were killed by ether asphyxiation and blood removed aseptically by cardiopuncture. Blood from the six individuals of each group was pooled and the serum analyzed for 12 common murine viruses (Abtek Biologicals Ltd, Liverpool), including Murine Hepatitis, Sendai, Reo 3 and Lactate Dehydrogenase virus. Tumour tissue from the mice implanted with Lewis lung carcinoma cells was also screened for Lactate Dehydrogenase virus. There was no evidence of any contamination of either the tumour-bearing (Tum) or the tumour-free controls (Con) by any of the viruses (see Appendix II for full virus profile).

Tissue Culture

Cells from the P5 stock in liquid nitrogen were brought to room temperature and washed twice in phosphate-buffered saline by gentle centrifugation (1000 rpm for 5 min). The cells were resuspended in a small volume of phosphate-buffered saline (approximately 10⁶ cells/ml). The concentration of viable (trypan blue excluding) cells was determined using an improved Neubauer haemocytometer. Cells (5 x 10⁵) were plated onto tissue culture dishes (14cm diameter) containing 1x Dulbecco's modification of Eagles medium (40ml; Appendix III), supplemented with 10% foetal calf serum and 0.2M L-glutamine, penicillin (0.25mg/ml), neomycin (0.1mg/ml) and streptomycin (0.05mg/ml). Cultures were maintained in a
Humidified atmosphere of 95% air and 5% CO₂.

**Histology**

Mice bearing Lewis lung tumours were killed, at intervals from 10-16 days after implantation of tumour cells, by CO₂ narcosis and exsanguination via cardiopuncture. The weight of the tumour was determined by subtracting the weight of the disarticulated tumour-free limb from that of the tumour-bearing limb (Steele and Adams, 1975), and the maximum tumour diameter ascertained by means of callipers. Skin and fur over the tumour were carefully removed. The tumour-bearing and the tumour-free hindlimbs were detached from the carcass at the hip joint, and immersed in phosphate-buffered 10% v/v formol saline for 24 hours. Using a scalpel blade, an incision of approximately 5mm depth was made in the tumour 3-6mm above and below the plane in which sections were to be cut. This procedure facilitated the perfusion of fixing fluid into the body of the tumour. The limbs were immersed for a further 3 days in formol saline before being decalcified in Gooding and Stewart's solution (5% v/v formic acid in 5% v/v formalin) for 4 days. A section of 2-3mm thickness was cut from each limb in the plane perpendicular to the femur and tibia, at the level across the femur just proximal to the knee joint. These pieces of tissue were then processed overnight in a "Histokinette" type E7326 (British American Optical Co. Ltd, Slough, Bucks.). This involved immersion in baths containing increasing strengths of ethanol (70, 85, 95, 100% v/v) before being cleared in 100% toluene and impregnated with wax (Culling, 1974). The tissues were then embedded in paraffin-wax blocks, sectioned using a rotary microtome (to 6 microns thickness) and mounted onto glass microscope slides.

Sections were stained with haematoxylin and eosin (H&E) for routine
examination using the light microscope (Appendix IV.a). Other sections were stained using van Gieson's preparation (1889) which stains for connective tissues (principally collagen) or with Perls' Prussian blue (1867) for ferric iron salts (Appendices IV.b and IV.c). A number of unstained sections were mounted in 3:1 glycerol/phosphate-buffered saline, and examined using a fluorescence microscope for lipofuscin-like autofluorescence (Porta and Hartroft, 1969; Reddy et al., 1982; Katz et al., 1984). The histological sections of tumour, and host tissues (Chapter 3) were inspected by an independent examiner, and his appraisal of their pathology is given in the results section.

Separation of Tumour Tissue into "Cortical" and "Peripheral" Regions

Tumour-bearing mice were killed by ether asphyxiation on days 13, 15 and 17 after tumour implant. Blood was removed from the thoracic cavity by cardiopuncture. Livers and lungs (the reference tissues) were rapidly excised: rinsed in phosphate-buffered saline; and then immersed in isopentane which had been cooled in liquid nitrogen, in which they were stored until the day of assay. Isopentane (freezing point: $-160^\circ$ C) was used as refrigerant because it has been reported to significantly decrease the freezing time of tissues (Faupel et al., 1972). Skin and fur were stripped from the tumour-bearing limb and the entire leg detached at the hip joint. The in situ mass of each tumour was determined as described in the previous section. The tumours were then immersed in isopentane/liquid nitrogen until required for analysis.

Frozen tumours were removed from liquid nitrogen and oriented such that the skeletal components of the limb were all in the same horizontal plane, and displaced to one side of the muscle- and fascia-encapsulated tumour (see fig. 2.2). The tumour was secured between the polypropylene-
FIG. 2.2 - Schematic Diagram of Procedure to Subdivide Intramuscularly-implanted Lewis Lung Tumours into Arbitrary Cortical and Peripheral Regions.
lined jaws of a vice and placed under an overhead-drive bench drill. Using a 10mm length polythene tube of 7mm internal diameter (adapted from a 10ml capacity disposable blowout pipette), revolving at 500 rpm, a bore of frozen tumour tissue was drilled. The cylindrical section of tissue was pushed out of the tube with a loose-fitting rod and 3-4mm of tissue from each end was discarded. The residual tissue was thought to correspond to the hypoxic cortex of the tumour. The tumour tissue remaining in the tumour-bearing limb, considered to be broadly representative of the tumour periphery, was also dissected out.

Preparation of Tissues

Frozen tissue was thawed to 4°C. Tumour tissues were homogenized at 10 or 20% wet wt/vol in 1.15% w/v KCl using 3-5 passes of a teflon pestle in a Potter-Elvehjem homogenizer at 4°C. When a mitochondrial fraction of tumour tissue was required, 20% w/v homogenates were diluted 1:1 with 0.5 M sucrose (pH 7.4) containing 1 mM TRIS and 0.1 mM ethyleneglycol-bis-[B-amino-ethyl ether]N,N'-tetra-acetic acid (EGTA). To separate tissue homogenates into subcellular fractions the standard differential centrifugation procedure (Hogeboom, 1955) was used. Portions of the homogenates (<10 ml) were centrifuged in an MSE 65 ultra-centrifuge at 660 £ for 10 min. The pellets, presumed to contain cell nuclei, intact cells, connective tissue, necrotic material, erythrocytes and a relatively small proportion of mitochondria (Hogeboom, 1955), were discarded. The supernatants were centrifuged at 9770 £ for 20 min and the resultant pellets (the mitochondrial fractions) retained. The supernatants from the 9770 £ centrifugation were centrifuged at 105 000 £ for 60 min to provide the microsomal (the pellet) and cytosolic or soluble fractions (the supernatant). Microsomal and mitochondrial pellets were washed twice by resuspension and centrifugation. The microsomal and mitochondrial
fractions were stored as refrigerated (5°C) or frozen pellets (-20°C), as required, and resuspended in appropriate buffers immediately prior to analysis.

Determination of GSH peroxidase, SOD, GSH-transferase and γ-glutamylcysteine synthetase activities, and GSH/GSSG concentrations were determined within 24 hr of homogenization. All other determinations were performed within 14 days of refrigeration at -20°C.

**Instrumentation**

$^{14}$C-Radioactivities were quantified using a Packard Tricarb$^R$ 2650 liquid scintillation spectrometer. Optical absorbance was determined with either a Pye Unicam SP1800 twin-beam ultraviolet/visible spectrophotometer or a Pye Unicam SP6-550 single beam ultraviolet/visible spectrophotometer. Fluorescence was quantified with a Baird Atomic SFR100 ratio-recording spectrofluorimeter.

Membranous subcellular fractions were sonicated, when required, using a Soniprobe$^R$ type 1130A (Dawe Instruments Ltd., London). Photomicroscopy was achieved using a Nikon Optiphot$^R$ HFM-T visible light microscope with a M-35FA 35 mm film photomicrography attachment. A Leitz Orthoplan/Orthomat$^R$ ultraviolet fluorescence microscope was used to examine unstained histological sections for autofluorescence.

**Biochemical Assays**

**DNA:** DNA concentration was determined by a procedure in which the nucleic acid component of tissue samples was first isolated by means of preferential hydrolysis in hot perchloric acid (PCA). The DNA could then be
quantified by the reaction between deoxyribose sugars and diphenylamine to give a dark-blue product (Burton, 1956).

1.7% w/v PCA (3.5ml) was added to duplicate 0.5ml samples of tissue homogenate, mixed by Vortex and centrifuged at 2500 rpm for 15 min to precipitate nucleic acid and proteins. The supernatants were carefully decanted and the acid-insoluble pellets resuspended in 5% w/v PCA (4ml). After further centrifugation, the pellets were suspended in defatting solvent (4ml of 2:2:1 by vol mixture of ether, ethanol and chloroform) and centrifuged as before. The pellets were washed again in defatting solvent: resuspended in 5% PCA, containing 0.1 mM diethylene-triaminopenta-acetic acid (DETPAC); and incubated for 15 min at 70° C. The chelating agent, DETPAC was included to remove free iron which could render DNA non-hydrolyzable and thereby prevent its extraction (Hall and Axelrod, 1977).

The hydrolysates were cooled to room temperature and centrifuged at 2500 rpm for 15 min: 87 mM diphenylamine solution (4ml in 0.01: 1.45: 98.54% by vol acetaldehyde, sulphuric acid and glacial acetic acid) was then added to 2ml aliquots of the supernatant. The diphenylamine reagent (4ml) was also added to duplicate 2ml volumes (in 5% w/v PCA) of a range (0-400 μg) of calf-thymus DNA standards. The assay tubes were sealed with Parafilm® and left for 16-20 hr in a dark cupboard at room temperature, before their spectrophotometric absorbances at 595nm were determined. DNA concentration in the samples was estimated by comparison with the standards. The recoveries of 50 μg and 200 μg calf-thymus standards, when added to 0.5 ml of 10% w/v liver homogenate, as evaluated by the above procedure, were 93 ± 2% and 94 ± 3% respectively.

Glutathione: Total glutathione (reduced + oxidized) was assayed by a standard cyclo-reduction procedure in which the GSH in the sample reduces 5,5'-dithiobis[2-nitrobenzoic acid] (Ellman's reagent, DTNB) to form a
yellow ion product of characteristic absorbance at 412nm, and a mixed disulphide (Tietze, 1969). The disulphide reacts with further GSHG to liberate another ion and GSSG. GSSG was then enzymically reduced to GSH by GSSG reductase, included in the assay mixture, and this GSH then re-enters the main reaction cycle. The rate of formation of the yellow ion product is proportional to the concentration of glutathione in the sample (Owens and Belcher, 1965).

Oxidized glutathione (GSSG) and mixed disulphides were determined by masking GSH by derivitization with 2-vinyl pyridine prior to the assay as described above (Griffith, 1980).

Tissues in which glutathione concentrations were to be determined were homogenized at 10% w/v in a protein precipitant of 1.67% w/v metaphosphoric acid in saturated saline solution containing 0.2% w/v EDTA. The homogenate was centrifuged at 2500 rpm for 15 min and the supernatant used for assay. All samples were refrigerated at 4° C and assayed within 24 hours of removal from donor mice.

To ascertain glutathione concentrations, duplicate reaction mixtures comprising 0.21 mM NADPH, 0.6 mM DTNB, 40 mM Na₂HPO₄, 0.1 M Na phosphate buffer (PH 7.5) and 0.1 ml of sample (suitably diluted in metaphosphoric acid reagent) in total volumes of 1.0 ml were preincubated at 30° C. The Na₂HPO₄ was included in the reaction mixture to neutralize the metaphosphoric acid. The reaction was started by addition of 0.5 units (Sigma) of GSSG reductase (10μl) and the change in absorbance at 412 nm (at 30° C in a heated cuvette) monitored using a spectrophotometer, and recorded on a chart recorder. The gradient of the linear portion of the chart-recorder tracings corresponded to the glutathione concentration of the samples, which was evaluated by comparison with the gradients produced by known GSSG standards (0.5-5.0 nmoles). In determinations of GSSG concentration, the derivitization of GSH was achieved by incubating
the samples with 0.2% v/v 2-vinyl pyridine for 20-30 min at 25°C, pH 6-7. The content of GSH was estimated by subtracting the GSSG content (GSH equivalents) from the total glutathione (GSH equivalents) of the sample. Recovery of known additions of GSH from samples of liver homogenate, as determined by the above procedure was >90%.

**Protein:**

a) Method using Coomassie blue (Spector, 1978)

In the initial studies outlined in this thesis, protein concentration was determined by a recently-developed procedure using Coomassie blue reagent (Spector, 1978). This relatively quick and convenient method involves the addition of 1 ml dye reagent (comprising 0.01% w/v Coomassie Brilliant blue G-250, 4.7% w/v ethanol and 8.5% w/v orthophosphoric acid) to 0.1 ml of sample appropriately diluted in 0.1 M phosphate buffer (pH 7.0). The reaction mixtures were mixed by Vortex and the optical absorption at 595nm determined spectrophotometrically within 5-20 min. Protein concentrations were then estimated by comparison with a range (0-20 μg) of protein (bovine serum albumin; BSA) standards.

b) Method using Folin-Ciocalteu reagent (Lowry et al., 1951)

Samples were diluted appropriately in distilled water to within the range of the assay. Alkaline copper reagent, comprising 100ml of 2% w/v Na₂CO₃ in 0.05 M NaOH, 1ml of 1% w/v CuSO₄, and 1ml of 2% w/v Na tartrate was freshly prepared. Aliquots (0.4ml) of diluted samples and a range (5-25 μg) of BSA standards was dispensed, in duplicate, into test-tubes. Alkaline copper regent (2ml) was added to each tube and the contents mixed by Vortex. After 10 min, Folin-Ciocalteu's phenol reagent (0.2ml), freshly diluted 2:3 in water, was pipetted into each assay tube and then vortexed. The reaction mixtures were left for 30 min at room temperature. The optical absorbances at 500nm were then read against a blank in which
water had been substituted for protein solution. The protein concentrations of the samples were then determined by comparing their optical absorbances with those of the standards.

The protein assay using Coomassie brilliant blue dye has the advantage over the procedure using Folin-Ciocalteu reagent (Lowry et al., 1951) because it is a relatively more convenient method to operate, and is four times more sensitive (Bradford, 1976). There were however discrepancies between the protein concentrations estimated by the two different methods (see Results). The procedure of Lowry and co-workers is considerably more established than the method using Coomassie blue reagent. Comparison of results with those published in the biomedical literature can thus be achieved more readily with values indexed to protein concentrations determined by the standard Lowry method than using Coomassie blue reagent. The Lowry procedure was thus employed preferentially from a relatively early stage of the present studies. The tables of results that follow the present section indicate the method of protein assay, where applicable.

**Lipoperoxide concentration:** The concentration of lipoperoxides (thiobarbituric acid-reactive material) was estimated by a procedure in which samples were heated under mildly acidic conditions to release intermediates of peroxide origin which form malondialdehyde (MDA). One mole MDA reacts with two moles of thiobarbituric acid (TBA) to form a chromogenic adduct which has characteristic fluorescence (Gutteridge, 1982a).

All glassware, used in the assay procedure, was acid-washed to remove any residual detergent from usual washing, that could cause fluorescent interference. Samples (0.1ml), in duplicate, were added to 2.4ml of 0.1 M potassium hydrogen phthalate buffer pH 3.5 in screw-capped test tubes: 2.5ml of 1% w/v TBA in 0.05 M NaOH was pipetted into the
reaction tubes which were then sealed with teflon-lined screw caps. The tubes were incubated for 15 min at 100° C. After cooling to room temperature, the fluorescent reaction products were extracted into 4ml of butan-l-ol. A range (0.125-1.00 nmol) of malonaldehyde bis-[dimethyl acetal] standards (1,1,3,3 tetramethoxypropane) in 2.5ml phthalate buffer was similarly incubated with 2.5ml of TBA and the TBA/MDA fluorophore extracted into 4ml of butan-l-ol. The concentrations of TBA-reactive substances in the samples were evaluated by comparison of their spectrofluorescence (535 nm excitation and 553 nm emission) with those of the standards.

**Catalase:** Catalase activity \( \text{H}_2\text{O}_2 : \text{H}_2\text{O}_2 \text{ oxoreductase; } \text{EC}1.11.1.6 \) was assayed in tissue homogenates by a procedure in which the concentration of unreacted substrate, \( \text{H}_2\text{O}_2 \) was measured by reaction with excess potassium permanganate \( \text{KMnO}_4 \). The concentration the residual \( \text{KMnO}_4 \) was then determined spectrophotometrically (Cohen et al., 1970).

Lung and tumour homogenates were diluted with ice-cold triton\(^R\) X-100 solution to give a final tissue concentration of 2.5-5.0 % wet wt/vol and triton\(^R\) concentration of 1.0% w/v. The samples were then sonicated at maximum output for 3 bursts of 15 sec. Sonication and the reaction procedure were carried out over an ice water bath (0-2° C).

Duplicate aliquots (0.25ml) of the diluted samples were pipetted into test tubes. The enzymic reaction was started sequentially at timed intervals (10 sec) by adding 0.25ml of 6 mM \( \text{H}_2\text{O}_2 \) in 0.01 M potassium phosphate buffer, pH 7.0: after exactly 3 min, the reactions were stopped by rapid addition of 0.5ml of 3 M \( \text{H}_2\text{SO}_4 \) and mixing by Vortex.

A photometric standard was prepared by pipetting 3.5ml of 1 mM \( \text{KMnO}_4 \) into 10 mM 2.5ml of phosphate buffer, 0.5ml of 3 M \( \text{H}_2\text{SO}_4 \) and diluted tissue 0.25ml of homogenate. An aliquot of the standard was placed into a
flow-through cuvette and the digital spectrophotometric reading, at an absorbance of 480 nm, for convenience adjusted to be 1.0. Each reaction tube was then taken one at a time and 3.5 ml of 1 mM KMnO₄ added. The absorbance at 480 nm was determined within 30-60 sec of mixing by Vortex. Zero-time standards were prepared by adding H₂SO₄ to reaction mixtures before the addition of buffered H₂O₂.

Under the conditions of the assay, the decomposition of H₂O₂ by catalase follows first order kinetics. Enzyme activities in tumour and lung homogenates, was thus expressed as a function of the first order reaction rate constant (k) at 2 ± 1°C, pH 7.4:

$$k = \log_e \left( \frac{S_0}{S_3} \right) / t$$

where:-

- t is the reaction time (3 min)
- S₀ is the substrate concentration in absorbance units at zero time
- S₃ is the substrate concentration in absorbance units at 3 min.

When standard bovine catalase (Sigma) was assayed in the presence of liver or Lewis lung carcinoma homogenates, of predetermined catalase activities, there was no significant loss of catalase activity.

Glucose-6-phosphate dehydrogenase: Glucose-6-phosphate dehydrogenase activity was assayed by a standard spectrophotometric method in which the rate of formation of NADPH was monitored by determining the increase in absorbance at 340 nm (Lohr and Waller, 1974).

Cytosolic fractions, from tumour and lung homogenate, were dialyzed
overnight at 4°C against 1.15% w/v KCl in 50 mM triethanolamine buffer, pH 7.5 containing 0.6 mM EDTA. To duplicate 0.1ml aliquots of sample, 1.64 mM NADP (0.9ml) in the dialysis buffer, containing 13.2 mM maleimide, was added. Maleimide was included to inhibit 6-phosphogluconate dehydrogenase activity (EC.1.1.1.44), which might also produce NADPH. The contents of the reaction tubes were mixed (Vortex) and left to stand at 37°C for 5-10 min. The reaction was initiated by the addition of 2ml of 1.05 mM glucose-6-phosphate, in triethanolamine buffer, to each tube, at timed intervals. Each reaction mixture, after rapid vortexing, was placed into a heated cuvette (37°C) of a spectrophotometer and the absorbance at 340 nm determined against a water blank. The reaction mixtures were incubated at 37°C and their absorbances at 340 nm redetermined at 5 and 10 min after the initiation of the reaction. The mean change in absorbance over the 10 min reaction period was calculated for each sample. NADPH concentration was evaluated from the molar extinction coefficient for NADPH at 37°C (9.42 x 10^3 /cm). Specific glucose-6-phosphate dehydrogenase activities were expressed as nmol NADPH formed/min per mg cytosolic protein at 37°C, pH 7.5.

GSH peroxidase: Glutathione peroxidase activity (GSH peroxidase; EC 1.11.1.9) was assayed by a procedure in which the rate of enzymic oxidation of the co-substrate, GSH was ascertained (Hafeman et al., 1974). The GSH concentration of the reaction mixtures, at timed intervals after the initiation of the reaction, were determined using the reaction of GSH with DTNB to form a yellow product of characteristic absorbance at 412 nm (see earlier under glutathione).

Mitochondrial pellets were resuspended in 1.15% w/v KCl in 0.1 M sodium phosphate buffer, pH 7.4 containing 0.5% w/v triton X-100 to a concentration of approximately 1 mg protein/ml, and sonicated as described previously. Cytosolic fractions from tumour and lung tissue,
were diluted in 1.15% w/v KCl to approximately 5% original tissue wt/vol.

Reaction mixtures (4ml) comprising 0.5ml of sample, 2ml of 1 mM GSH, 1ml of 0.4 M sodium phosphate buffer, pH 7.0 and 0.5ml of 10 mM NaN₃ were mixed by Vortex and incubated at 37° C for 5 min. NaN₃ was included to inhibit any catalase activity present in the sample (Hafeman et al., 1974). Reactions were initiated at timed intervals by the addition of 1ml of 1.25 mM H₂O₂ (prewarmed to 37° C). At intervals of 3, 6, 9 and 12 min after peroxide addition, aliquots (1ml) were pipetted out of the reaction mixtures into test tubes containing 4ml of 1.67% w/v metaphosphoric acid, in a saturated solution of NaCl containing 0.4 mM EDTA. The metaphosphoric acid-containing tubes were centrifuged at 2500 rpm for 15 min to precipitate the macromolecule contents of the mixtures. A 2ml aliquot of the clear supernatant from each tube was then added to tubes, containing 2ml of 0.4 M Na₂HPO₄, to neutralize the acid: 1 mM DTNB (1ml), in 1% w/v trisodium citrate, was pipetted sequentially into the tubes and, after mixing by Vortex, the spectrophotometric absorbance at 412 nm of the mixtures was determined. Blanks, with boiled tissue sample substituted for enzyme source, were carried through the assay procedure, simultaneously with the samples, to evaluate non-enzymic oxidation of GSH by H₂O₂. Zero time standards were prepared by substituting H₂O for H₂O₂ in the reaction mixtures. A range (25-500 μM) of GSH standards in metaphosphoric acid was mixed 2:2:1 by volume with 0.4 M Na₂HPO₄ and 1 mM DTNB respectively. The GSH concentrations of the reaction mixtures, at the various times after initiation of the reaction, were then evaluated by comparison with the absorbances of the above standards. A unit of GSH peroxidase activity (EU) was defined as the decrease in log₁₀ millimolar GSH concentration per min at 37° C, pH 7.0 (after correction for non-enzymic GSH oxidation), multiplied by 1000 (Hafeman et al., 1974).

**GSSG reductase:** Glutathione reductase activity (NADPH: GSSG oxido-
NADPH oxidation was measured by determining the absorbance change at 340 nm.

Tissue homogenates (50–100 μl) were diluted in 0.1 M sodium phosphate buffer, pH 7.4 to a volume of 9 ml. The samples were centrifuged at 2500 rpm for 15 min. Duplicate aliquots (3 ml) were removed from the supernatants and placed in test tubes. To each aliquot was added 0.1 ml 0.3 mM FAD and 0.2 ml of 2 mM NADPH. The mixtures were pre-incubated at 37° C for 5 min, and then the reactions were initiated by addition of 0.1 ml of 7.6 mM GSSG. The change in optical absorbance at 340 nm of the reaction mixtures between 1 and 9–16 min after initiation, were determined against blanks containing 3.3 ml of buffer plus sample mixture only. The dilution of the samples was sufficient to produce a change in absorbance of 0.02–0.05 units/min. Glutathione reductase (GSSG reductase) activity was calculated from the absorbance changes of the reaction mixtures, utilizing the standard molar extinction coefficient for NADPH, at 37° C, of 9.42 x 10^3 /cm. Activities were expressed as the disappearance (oxidation) of NADPH/min, per mg protein, at 37° C, pH 7.4.

**Gamma-glutamylcysteine synthetase:** γ-Glutamylcysteine synthetase (L-glutamate: L-cysteine γ-ligase [ADP]; EC 6.3.2.2) is the rate-limiting enzyme in the biosynthesis of glutathione. γ-Glutamylcysteine, produced by γ-glutamylcysteine synthetase, is linked to a glycine residue by glutathione synthetase (γ-L-glutamyl-L-cysteine: glycine ligase [ADP]; EC 6.3.2.3) to form glutathione (Meister and Anderson, 1983).

γ-Glutamylcysteine synthetase activity was ascertained by determining the radioactivity due to enzymic addition of ^14C-glutamate to cysteine (Paniker and Beutler, 1972). Samples (cytosol) were dialyzed...
overnight, at 4°C, against 1.15% w/v KCl in 0.1 M potassium phosphate buffer, pH 7.4. Reaction mixtures, in duplicate, contained 0.1 M imidazole/ HCl buffer, pH 8.25, 20 mM MgCl$_2$, 4 mM ATP, 10 mM cysteine, 5 mM dithiothreitol, 1.15% w/v KCl, 10 mM $^\text{14}$C-glutamate (containing 0.278 kBq/mmol) and 0.2ml of cytosol to a total volume of 1.0ml. Cysteine was titrated with hydroxide to neutrality before adding to the reaction mixtures. Dithiothreitol (Cleland's reagent) was included in the assay system to help to maintain sulphydryls in a reduced state. The reactions were initiated by addition of 0.1ml of $^\text{14}$C-glutamate. The reactions were terminated, after incubation at 37°C for 60 min, by adding 1ml of 10% w/v trichloroacetic acid (TCA) to the assay mixtures. The mixtures were then centrifuged at 1500 rpm for 15 min. To aliquots of the supernatant (1.5ml) were added 0.1ml of 0.2 M GSH, 0.1ml of 0.75 M CdSO$_4$, 50μl of 0.04% w/v bromocresol green indicator and 50μl of 0.04% bromocresol purple indicator were added: the mixtures were then titrated with 0.5 M NaOH, while constantly mixing by Vortex, until the colour became blue-green (aquamarine, pH 5.25 ± 0.05). If a blue colour was reached, the reaction mixture was back titrated with 7% w/v TCA (Minnich et al., 1971). The mixtures were centrifuged at 2500 rpm for 15 min, and the precipitates, containing the calcium mercaptide salt of γ-glutamylcysteine, were washed three times by resuspension in 2ml of 0.9% w/v NaCl and centrifugation. The washed precipitates were dissolved in 0.25ml of 7% w/v TCA and and transferred to scintillation vials. The inside of the tubes was washed out with further TCA (0.25ml) and two 5ml portions of Tritosclint$^\text{R}$ scintillation fluid. The $^\text{14}$C-radioactivity associated with the γ-glutamylcysteine, was determined by a liquid scintillation counter. The counts were corrected by subtracting blanks in which the cysteine of the reaction mixtures had been replaced by water. The concentrations of γ-glutamylcysteine were calculated by comparison with the radioactivity of a range of $^\text{14}$C-glutamate standards.
γ-Glutamylcysteine synthetase activity was expressed as nmol γ-glutamylcysteine formed /min at 37°C, pH 8.25.

**Gamma-glutamyl transpeptidase:** γ-Glutamyl transpeptidase ([γ-glutamyl]-peptide: amino acid 5-glutamyltransferase; EC 2.3.2.2.) catalyzes the transfer of a γ-glutamyl residue from a γ-glutamyl-containing donor molecule to a number of possible amino acids or peptides (Tate and Meister, 1974). The method to determine γ-glutamyl transpeptidase activity utilized L-γ-glutamyl-p-nitroanilide as the γ-glutamyl donor and glycylglycine as the acceptor (Tate and Meister, 1974). The concentration of p-nitroaniline may then be determined via its characteristic absorbance at 410 nm (Fujiwara et al., 1982). In tissues with relatively low γ-glutamyl transpeptidase activity, however, where a relatively large sample might be required, there may be appreciable interference at this wavelength (Naftalin et al., 1969). The p-nitroaniline was therefore diazotized according to a modified Bratton-Marshall reaction:-

1) p-Nitroaniline + NaNO₂ + acid → Diazo compound
2) Diazo compound + N-(1-naphthyl)-ethylenediamine → Pink azo dye

Employing the Bratton-Marshall reaction achieved a gain in sensitivity of the procedure (Naftalin et al., 1969). The optical absorbance of the azo dye at 530-550 nm corresponded to the concentration of p-nitroaniline.

To determine γ-glutamyl transpeptidase activity, 0.2ml aliquots of sample, in triplicate, were incubated at 37°C for 5 min. Samples of homogenate, mitochondrial or microsomal fraction had 0.5% w/v (final concentration) triton X-100 added to them; and were then sonicated to obtain maximal activity of γ-glutamyl transpeptidase. Mixtures (0.8ml, prewarmed to 37°C), containing 0.1 M glycylglycine and 4.6 mM γ-glutamyl-p-nitroanilide and 10 mM MgCl₂ in 80 mM Tris/ HCl buffer, pH
7.8 were added, at timed intervals, to each tube. The contents of the reaction tubes were mixed thoroughly by Vortex. The reaction of one tube from each triplicate was then terminated after exactly one minute of incubation at 37° C, by the addition of 1ml 25% w/v TCA. The reactions in the remaining mixtures were similarly terminated after a further 20 min (samples of cytosol, obtained from 10-20% w/v homogenates, were incubated for 61 min). After centrifugation, an 8ml portion of the supernatant from the reaction mixtures was diazotised by addition of 0.8ml of 58 mM sodium nitrite. Excess nitrite was removed after 3 min by addition of 0.8ml of 0.175 M ammonium sulphamate to each tube. The diazo compound was then coupled to produce the coloured dye by adding 8ml of 5.8 mM ethanolic N-(-l-naphthyl) ethylenediamine solution 2 min later. After thorough mixing by Vortex, the optical absorbances at 550 nm for each mixture, given a longer incubation, were measured against the corresponding sample incubated for 1 min. Corrections were made for the non-enzymic cleavage of the γ-glutamyl moiety from γ-glutamyl-p-nitroaniline, by using reaction mixtures containing boiled tissue sample: these were all relatively minimal. The concentrations of p-nitroaniline were determined by comparison with p-nitroaniline standards. γ-Glutamyl transpeptidase activities were expressed as nmol p-nitroaniline formed per min, per unit wt of protein, at 37° C, pH 7.8.

Glutathione S-epoxide-transferase: Glutathione S-transferases (GSH-transferases; EC 2.5.1.18) are a group of enzymes with overlapping substrate specificities, which catalyze the conjugation of GSH to the electrophilic sites of potential alkylating agents, and thus increase their water solubility (Habig et al, 1974). GSH-transferase activity was determined by using the ability of this enzyme(s) to transfer GSH to a 14C-labelled epoxide and thereby render it water soluble (James et al, 1976).
Cytosolic fractions of the samples were diluted with 1.15% w/v KCl to a concentration of approximately 2.5 mg protein/ml. Duplicate incubation mixtures, containing 767μl of 0.1 M N-[2-hydroxyethyl]-l-piperazine-ethanesulphonic acid (Hepes) buffer, pH 7.2, 100μl of 50 mM GSH and 100μl of cytosol were pre-incubated for 5 min at 37° C. Reactions were initiated, at timed intervals, by adding 33μl of 10.41 mM [8-14C] styrene oxide (0.185 MBq/ml) to each mixture. The reactions were terminated after 10 min by addition of ethyl acetate (4ml). After thorough mixing, the reaction mixtures (in screw-capped vials) were placed in the deep freeze at -20° C. An hour later, the unfrozen organic layer was carefully decanted off and fresh ethyl acetate (4ml) added. The vials were returned to ambient temperature, and their contents mixed and returned to the deep freeze. This procedure was repeated and then 10ml of Triton X scintillation fluid was added to the frozen aqueous layer of each vial. The residual 14C- radioactivity associated with the GSH-conjugated styrene oxide was determined by liquid scintillation counting. Corrections were made for non-enzymic transfer of GSH, using reaction mixtures in which tissue sample was replaced by 1.15% w/v KCl. The concentration of the conjugated styrene oxide was determined by comparison with the radioactivity of known styrene oxide standards. GSH-transferase activity was expressed as nmol styrene oxide-GSH conjugate formed per min, per mg cytosolic protein, at 37° C, pH 7.2.

Superoxide dismutase: Superoxide dismutase activity (SOD; 'O2-' oxido-reductase; EC 1.15.1.1) was ascertained using a photochemical augmentation assay (Misra and Fridovich, 1977). This procedure can be used to determine activities of Cu/Zn, Fe, and Mn-SOD isoenzymes (Misra and Fridovich, 1977; Benovic et al, 1983). The principle of the reaction procedure is shown below:
The dye, riboflavin, when illuminated by visible light, absorbs protons to become electronically excited (reaction 1). The excited riboflavin (Rb*) then oxidizes dianisidine to yield a flavin semi-quinone and a dianisidine radical (DH*; reaction 2). Univalently-oxidized dianisidine can dismute to a brown-coloured product (D; reaction 3), with characteristic optical absorbance at 460 nm. Flavin semiquinones (RbH) however, react with O₂ to form *O₂⁻ which, in turn, reduces the dianisidine radical back to dianisidine (Fridovich, 1983; reactions 4 & 5). Under the above circumstances, the riboflavin-sensitized photo-oxidation of dianisidine is self-limiting and the reaction proceeds very slowly. SOD scavenges the *O₂⁻ and thereby accelerates the formation of the dye product of dianisidine oxidation (Misra and Fridovich, 1977).

Cytosolic fractions from 10-20% wet wt/vol homogenates, were cleared of haemoglobin using the Tsuchihashi procedure (Misra and Fridovich, 1977; Loven et al, 1980). This involved adding 1ml of ethanol and 0.6ml of chloroform to 1.0ml of cytosol. The mixture was then vigorously shaken for one min and centrifuged at 2500 rpm for 15 min. The clear aqueous layer was used for the assay. The Tsuchihashi procedure also would have deactivated any activity in the cytosol due to Mn-SOD (Loven et al, 1980; Fridovich, 1982b). Mitochondrial pellets were resuspended to a concentration of approximately 1 mg protein/ml in 0.1 M
sodium phosphate buffer, pH 7.4, containing 1.15% w/v KCl and 0.5% w/v triton X-100. The mitochondrial suspensions were sonicated at 0-4°C, as described previously.

Samples for assay (0.1ml, in duplicate) were added to a solution, containing 0.1ml of 0.39 mM riboflavin and 10 mM sodium, 2.74ml of potassium phosphate buffer, pH 7.5. Reactions were initiated, at timed intervals, by addition of 0.06ml of 10 mM o-dianisidine dihydrochloride and, after rapid vortexing, the optical absorbances of the reaction mixtures at 460 nm were determined against a blank containing buffer and riboflavin only. The reaction mixtures were then illuminated midway between two fluorescent tubes in an aluminium foil-lined open-ended cabinet. The temperature of the airspace between the fluorescent tubes was 25 ± 2°C. The absorbances at 460 nm of the mixtures was redetermined after exactly 8 min of illumination. SOD activity, which was directly proportional to the formation of oxidized dianisidine dye, was calibrated by comparison with the reactions of a 1-10 "unit" range of bovine blood-derived standards (Sigma). Corrections were made for non-enzymic formation of oxidized dianisidine, using reaction mixtures in which boiled sample (5 min) was substituted for sample.

Inclusion of 2 mM KCN into reaction mixtures containing Tsuchihashi procedure-pretreated cytosol inhibited SOD activity of hepatic cytosol by 98 ± 1% (n=20) and cytosol of Lewis lung carcinoma by 82 ± 14% (n=20). SOD activity in mitochondrial samples was not significantly inhibited by 2 mM KCN. Increasing the concentration of cyanide beyond 2 mM, that is to 6 mM, a concentration required to inhibit Cu/Zn-SOD by 100%, interfered with the reaction, possibly due to bleaching of riboflavin/oxidized dianisidine. SOD activity of mitochondrial fractions was completely lost after the Tsuchihashi procedure. To verify further that SOD activity of mitochondrial fractions was due to Mn-SOD, cytosol and
mitochondrial fractions, from 5 murine livers and Lewis lung carcinomas, were incubated for 60 min at 37°C with 2% w/v sodium dodecyl sulphate (SDS). This procedure has been reported to deactivate Mn-SOD but not Cu/Zn-SOD (Geller and Winge, 1983). After this treatment, there was no significant SOD activity remaining in the mitochondrial fractions, but SOD activity of the cytosols was only 8 ± 3% less than in untreated cytosols.

Detergents have been reported to inhibit riboflavin-sensitized photo-oxidation of o-dianisidine (Ljutakova, 1984). Triton® X-100 was therefore included, at a concentration comparable to that of samples (0.02% w/v), when calibrating mitochondrial SOD activities, using bovine SOD (Sigma) standards.

Statistical analyses

Data from experimental groups containing 3 or more observations were statistically evaluated by analysis of variance (ANOVA). Means of estimations from tumour tissue were compared with those of the reference tissue, lung, by use of the Student-type range statistic in the Newman Keuls procedure (Winer, 1971). Results were considered to be significant when P<0.05. Data from experiments where there were only two groups (containing three or more observations) were evaluated by Student's t-test.

Tumour weight and time, after tumour implantation (days) were compared by linear regression analysis (Winer, 1971).
RESULTS

Histopathology

Animals bearing Lewis lung carcinomas were healthy in appearance and there was no indication of any pronounced stress. Although articulation by the tumour-bearing limb was almost entirely restricted, the mice were remarkably mobile. Mice were able to tolerate a tumour burden of greater than 20% of their body weight before becoming moribund. Fatalities were presumably caused by asphyxiation due to the accumulation of metastatic lesions in the lungs. All studies described in this thesis used mice bearing tumours of maximum size of 18% of body weight.

Removal of fur and skin overlying the tumour-bearing limb revealed the muscles of the leg to be grossly swollen around the carcinoma. There were no external exudates or lesions. The surface of the fascia and muscle bed was smooth, regular and of normal colouration, although a number of focal haemorrhages (tumour cells?) were usually present. Invasion of the tumour into the peritoneum was infrequent and usually only occurred when tumour volume was relatively great.

Sections of tumour-bearing limbs, stained with H&E, show the Lewis lung carcinomas, transplanted in the present studies, to be invasive, undifferentiated and anaplastic (table 2.1). The tumour cells were polyhedral to spindle shaped and possessed irregular nuclei. Large tumour cells with multiple nuclei were sometimes observed. Tumour cells were in close proximity to blood vessels which were poorly formed, especially at the periphery of the carcinoma. Blood vessels were absent in the central necrotic areas. There were extensive areas of haemorrhage and necrosis (plates I and II). In some sections focal aggregates of golden-brown pigment were present (plate IIb). In sections stained with van Gieson's preparation, no connective tissue stroma was observed in the body of the
<table>
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<th>Time after transplantation tumour (days)</th>
<th>Maximum diameter of tumour (cm)</th>
<th>Weight in situ (g)</th>
<th>Appraisal of histopathology</th>
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<tr>
<td>10</td>
<td>1.00</td>
<td>1.3</td>
<td>Well developed anaplastic carcinoma. A portion of the tumour is necrotic with brisk acute inflammatory infiltrate, associated with tracts of haemorrhage</td>
</tr>
<tr>
<td>11</td>
<td>1.20</td>
<td>1.6</td>
<td>Anaplastic epitheloid carcinoma with areas of haemorrhage and necrosis. The overlying fascia is blood filled.</td>
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<td>13</td>
<td>1.46</td>
<td>2.1</td>
<td>Massive invasive anaplastic carcinoma with necrotic and haemorrhagic foci.</td>
</tr>
<tr>
<td>14</td>
<td>1.70</td>
<td>2.5</td>
<td>Massive invasive anaplastic carcinoma with extensive necrosis and tracts of haemorrhage containing agglutinated erythrocytes and focal aggregates of (?) haemosiderin.</td>
</tr>
<tr>
<td>15</td>
<td>1.84</td>
<td>2.9</td>
<td>Massive invasive undifferentiated carcinoma containing extensive tracts of haemorrhage and areas of necrosis.</td>
</tr>
<tr>
<td>16</td>
<td>2.00</td>
<td>3.4</td>
<td>Massive invasive undifferentiated anaplastic carcinoma containing extensive haemorrhage and necrosis</td>
</tr>
</tbody>
</table>
tumour (plate III). Sections stained with Perls' Prussian blue had appreciable blue haemosiderin-like pigmentation, and also substantial areas of yellow-gold pigment, probably formalin-derived artefact (plate IV).

Inspection of unstained sections of tumour by u.v. fluorescence microscopy did not reveal any lipofuscin-like autofluorescence.

Growth Kinetics

Linear regression analysis of tumour weight against time (days) after implantation, showed a correlation coefficient of 0.95 (Fig. 3.1; Chapter 3). Mean doubling time of the intramuscularly-implanted Lewis lung carcinoma was approximately 50 hr when 0.5g, and approximately 100 hr when 1.0g in weight. The growth rate of the Lewis lung tumour was essentially similar in all experiments described in this thesis.

Protein and DNA

Protein concentration and DNA concentration did not vary significantly as tumour mass increased (table 2.2). Values for protein concentration in tissue homogenates, obtained by the assay using Coomassie blue reagent (Spector, 1978) were approximately half those obtained by the alternative procedure using Folin-Ciocalteu reagent (Lowry et al., 1951). This observation was confirmed by repeated determinations with batches of Coomassie blue and Folin-Ciocalteu reagents, and by similar findings by other operators in the laboratories of the Marie Curie Memorial Foundation. Both assays showed Lewis lung tissue to have approximately double the protein concentration of the reference tissue, lung. Pulmonary and tumour DNA concentrations, expressed per mg protein, were not significantly different (table 2.2).
<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Relative weight of tumour to body wt (%)</th>
<th>Protein concentration (mg/ g wet wt)</th>
<th>DNA concentration (µg DNA equiv/ mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(wet wt [g] of tumours shown in brackets)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1.6 ± 0.5 (0.3 ± 0.1)</td>
<td>51 ± 3</td>
<td>84 ± 3</td>
</tr>
<tr>
<td>11</td>
<td>3.3 ± 0.5a (0.8 ± 0.1)</td>
<td>49 ± 1</td>
<td>93 ± 2</td>
</tr>
<tr>
<td>13</td>
<td>6.8 ± 0.9b (1.4 ± 0.1)</td>
<td>53 ± 1</td>
<td>95 ± 1</td>
</tr>
<tr>
<td>15</td>
<td>8.0 ± 1.2b (1.7 ± 0.1)</td>
<td>48 ± 1</td>
<td>94 ± 4</td>
</tr>
<tr>
<td>17</td>
<td>10.7 ± 0.8c (2.5 ± 0.2)</td>
<td>51 ± 2</td>
<td>91 ± 2</td>
</tr>
<tr>
<td>19</td>
<td>15.2 ± 1.9d (3.4 ± 0.3)</td>
<td>50 ± 2</td>
<td>89 ± 2</td>
</tr>
</tbody>
</table>

a–d Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
Activities of Enzymes that May Influence Glutathione Status

Cytosolic GSH peroxidase activity in Lewis lung tumour was significantly less than that of lung (table 2.3). Activity was significantly greater at 17 days after implantation than at 13 and 15 days (table 3.3). Mitochondrial GSH peroxidase activity in Lewis lung tumour tissue was essentially similar to that of control lung, although it decreased significantly at 17 days post implantation (table 2.3). GSH peroxidase activities of lung and tumour homogenates, from mice killed 15 day after implantation of Lewis lung carcinomas, when assayed with 3 mM cumene hydroperoxide as substrate, were $13.2 \pm 0.9 \text{ (n=5)}$ enzyme units/ mg protein and $3.7 \pm 0.4 \text{ (n=5)}$ enzyme units/ mg protein respectively. The same tissues, when assayed with 1.25 mM $\text{H}_2\text{O}_2$ as substrate, had activities of $12.5 \pm 0.8$ enzyme units/ mg protein (lung) and $3.4 \pm 0.2$ enzyme units/ mg protein (tumour): these activities were not significantly different from activities using cumene.

GSSG reductase and GSH-transferase activities in Lewis lung tumour tissue were approximately 50% greater than those of normal pulmonary tissue (table 2.4), and did not vary significantly with tumour size. The activities of glucose-6-phosphate dehydrogenase and $\gamma$-Glutamylcysteine synthetase were significantly less in tumour than in lung (table 2.5). Glucose-6-phosphate dehydrogenase activity did not vary with time after tumour implantation (tumour size). $\gamma$-Glutamylcysteine synthetase activity increased from approximately 55% of the pulmonary value at 13 days to a value similar to that of control lung by 17 days post implantation. $\gamma$-Glutamyl transpeptidase activity, estimated in whole tissue and in subcellular fractions was considerably less in Lewis lung tumour than in pulmonary tissue (table 2.6). The difference was most pronounced in the cytosolic fraction, where the activity for tumour was only 0.3% of that determined for murine lung (table 2.6).
<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Cytosolic Fraction (Enzyme units / mg protein)</th>
<th>Mitochondrial Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>$17 \pm 3^a$</td>
<td>$14 \pm 2$</td>
</tr>
<tr>
<td>15</td>
<td>$17 \pm 1^a$</td>
<td>$14 \pm 2$</td>
</tr>
<tr>
<td>17</td>
<td>$21 \pm 3^b$</td>
<td>$10 \pm 1^c$</td>
</tr>
</tbody>
</table>

Control lung day 13-17:

<table>
<thead>
<tr>
<th></th>
<th><strong>Enzyme units / mg protein</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$29 \pm 3$</td>
</tr>
</tbody>
</table>

* Enzyme units are defined as $1000 \times \log_{10}$ (decrease in GSH/min) at $37^\circ C$, pH 7.0.

# See tables 3.7 and 3.8 for lung values in full.

$^a-c$ Significantly different ($P<0.05$) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter. Tumour values compared with those of control lung.


<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>GSSG Reductase</th>
<th>GSH S-transferase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol NADPH oxidized/min/mg protein at 37°C, pH 7.4)</td>
<td>(nmol styrene oxide conjugated/min/mg cytosolic protein at 37°C, pH 7.2)</td>
</tr>
<tr>
<td>9</td>
<td>172 + 4&lt;sup&gt;a&lt;/sup&gt; (160)</td>
<td>51 + 2&lt;sup&gt;bc&lt;/sup&gt; (170)</td>
</tr>
<tr>
<td></td>
<td>167 + 5&lt;sup&gt;a&lt;/sup&gt; (150)</td>
<td>49 + 1&lt;sup&gt;bc&lt;/sup&gt; (160)</td>
</tr>
<tr>
<td>13</td>
<td>164 + 1&lt;sup&gt;b&lt;/sup&gt; (150)</td>
<td>58 + 3&lt;sup&gt;c&lt;/sup&gt; (170)</td>
</tr>
<tr>
<td>15</td>
<td>170 + 6&lt;sup&gt;a&lt;/sup&gt; (160)</td>
<td>45 + 3&lt;sup&gt;b&lt;/sup&gt; (130)</td>
</tr>
<tr>
<td>Control lung day 9-15&lt;sup&gt;#&lt;/sup&gt;</td>
<td>108 + 4</td>
<td>30 + 3</td>
</tr>
</tbody>
</table>

# See tables 3.14 and 3.15 for lung values in full.

<sup>a-c</sup> Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter. Tumour values compared with those of control lung.
TABLE 2.5 - Glucose-6-phosphate Dehydrogenase and γ-Glutamylcysteine Synthetase Activities of Tumour Tissue from Lewis Lung Carcinoma-bearing C57BL6 Mice

Results represent mean ± SEM for 6 mice at each time interval. Values expressed as % of that of corresponding control lung shown in brackets. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Glucose-6-phosphate dehydrogenase (nmol NADPH/min/mg protein at 37° C, pH 7.5)</th>
<th>γ-Glutamylcysteine synthetase (nmol/min/mg cytosolic protein at 37° C, pH 8.25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>14 ± 1&lt;sup&gt;a&lt;/sup&gt; (27)</td>
<td>0.8 ± 0.2&lt;sup&gt;b&lt;/sup&gt; (55)</td>
</tr>
<tr>
<td>15</td>
<td>14 ± 1&lt;sup&gt;b&lt;/sup&gt; (150)</td>
<td>1.0 ± 0.1&lt;sup&gt;b&lt;/sup&gt; (60)</td>
</tr>
<tr>
<td>17</td>
<td>14 ± 1&lt;sup&gt;a&lt;/sup&gt; (27)</td>
<td>1.7 ± 0.2 (115)</td>
</tr>
<tr>
<td>Control lung day 13-17#</td>
<td>52 ± 2</td>
<td>1.5 ± 0.1</td>
</tr>
</tbody>
</table>

# See table 3.16 for lung values in full.

<sup>a-b</sup> Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter. Tumour values compared with those of control lung.
**TABLE 2.6 - Whole Tissue and Subcellular γ-Glutamyl Transpeptidase Activities of Tumour Tissue from Lewis Lung Carcinoma-bearing C57BL6 Mice**

Results represent mean ± SEM for 6 mice, bearing tumours of weight 2.4 ± 0.6g, killed 14 days after inoculation with Lewis lung carcinoma cells. Values expressed as % of that of corresponding control lung shown in brackets. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Cellular fraction</th>
<th>Lewis lung carcinoma</th>
<th>Control lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol p-nitroaniline/ min/ mg protein at 37° C, pH 7.8)</td>
<td></td>
</tr>
<tr>
<td>Whole tissue</td>
<td>1.0 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(29)</td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>2.4 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>(21)</td>
<td></td>
</tr>
<tr>
<td>Microsome</td>
<td>1.3 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>(13)</td>
<td></td>
</tr>
<tr>
<td>Cytosol</td>
<td>0.04 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.29 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>(14)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly different (P<0.05) by unpaired Student's t-test from corresponding control pulmonary value.
Catalase and SOD activities

Catalase activity in Lewis lung carcinoma tissue was significantly less than that of murine lung (table 2.7). Mean catalase activity increased from approximately 52% of the pulmonary value, 13 days after implantation, to approximately 70% of the reference by 17 days (table 2.7). Cytosolic SOD activity in Lewis lung approximated to 30% of that of pulmonary tissue (table 2.8). Mitochondrial SOD activity of Lewis lung carcinoma was 440 to 620% greater than that of normal lung. The activity of either SOD isoenzyme did not vary significantly with time after tumour implantation (table 2.8).

Activities of Antioxidant Defence Enzymes in Cells Maintained by Monolayer Tissue Culture.

Subcellular GSH peroxidase activity and cytosolic γ-glutamylcysteine synthetase activity in cultured Lewis lung cells were similar to those determined in tumours borne by mice (table 2.9). Cytosolic SOD activity in cultured cells was only 13% of that of the solid Lewis lung tumour while mitochondrial SOD activity was approximately double that of tissue from the i/m-implanted tumour (table 2.9).

Antioxidant Defence and Autoxidative Damage in Subdivided Lewis Lung Tumours

Cytosolic GSH-peroxidase and SOD activities of tissue derived from the cortex of Lewis lung carcinomas were not significantly different from that of the residual neoplastic tissue (tables 2.10 & 2.11). Mitochondrial GSH-peroxidase and SOD activities in tissue from tumour cortex were significantly less than those of the remaining tissue (tables 2.10 & 2.11).

Cortical γ-glutamylcysteine synthetase activity from Lewis lung
TABLE 2.7 - Catalase Activity and Protein Concentration of Tumour Tissue from Lewis Lung Carcinoma-bearing C57BL6 Mice

Results represent mean ± SEM for 6 mice at each time interval. Values expressed as % of that of corresponding control lung shown in brackets. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Catalase (k / min / mg protein)</th>
<th>Protein (mg / g wet wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>0.12 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>113 ± 10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(52)</td>
<td>(190)</td>
</tr>
<tr>
<td>15</td>
<td>0.14 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>102 ± 7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(61)</td>
<td>(170)</td>
</tr>
<tr>
<td>17</td>
<td>0.16 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>106 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(70)</td>
<td>(180)</td>
</tr>
<tr>
<td>Control lung day 13-17&lt;sup&gt;#&lt;/sup&gt;</td>
<td>0.23 ± 0.01</td>
<td>60 ± 4</td>
</tr>
</tbody>
</table>

<sup>#</sup> See tables 3.6 and 3.11 for lung values in full.

* k is the first-order reaction rate constant at 2 ± 1°C, pH 7.4.

<sup>a–b</sup> Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter. Tumour values compared with those of control lung.
TABLE 2.8 - Subcellular Superoxide Dismutase Activities of Tumour Tissue from Lewis Lung Carcinoma-bearing C57BL6 Mice

Results represent mean ± SEM for 5 mice at each time interval. Values expressed as % of that of corresponding control lung shown in brackets. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Cytosolic fraction</th>
<th>Mitochondrial fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(units (\text{Sigma}^R)/ mg protein at 25 ± 2°C, pH 7.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(units [\text{Sigma}^R]/ mg protein at 25 ± 2°C, pH 7.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(30)</td>
<td>(720)</td>
</tr>
<tr>
<td>13</td>
<td>2.9 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.0 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(720)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>3.2 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.8 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(610)</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>2.7 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(540)</td>
<td></td>
</tr>
<tr>
<td>Control lung day 13-17&lt;sup&gt;#&lt;/sup&gt;</td>
<td>10.0 ± 0.8</td>
<td>0.3 ± 0.03</td>
</tr>
</tbody>
</table>

<sup>#</sup> See tables 3.9 and 3.10 for lung values in full.

<sup>a-b</sup> Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter. Tumour values compared with those of control lung.
TABLE 2.9 - GSH Peroxidase, Superoxide Dismutase and $\gamma$-Glutamylcysteine Synthetase Activities of Lewis Lung Carcinoma Cells that Have been Maintained by Monolayer Tissue Culture

Results represent mean $\pm$ SEM of duplicate determinations from 4 dishes, containing approximately $2 \times 10^7$ confluent cells, incubated at $37^\circ$C in a humidified atmosphere comprising 95% air and 5% CO$_2$. Values expressed as an approximate % of the corresponding value determined for Lewis lung carcinomas passaged in vivo, shown in brackets. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th></th>
<th>Cytosolic Mitochondrial</th>
<th>Cytosolic Mitochondrial</th>
<th>Cytosolic</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH peroxidase</td>
<td>Enzyme units* / mg protein</td>
<td>Superoxide dismutase units [Sigma R] / mg protein at 25 $\pm$ 2°C, pH 7.5</td>
<td>$\gamma$-Glutamylcysteine (nmol/ min/ mg protein at 37$^\circ$C, pH 8.25)</td>
</tr>
<tr>
<td></td>
<td>$21 \pm 2$ 16 $\pm 1$</td>
<td>$0.4 \pm 0.1$ $3.8 \pm 0.4$</td>
<td>$1.7 \pm 0.2$</td>
</tr>
<tr>
<td></td>
<td>(110) (130)</td>
<td>(13) (220)</td>
<td>(120)</td>
</tr>
</tbody>
</table>

* Enzyme units are defined as $1000 \times \log_{10}$ (decrease in GSH/ min) at $37^\circ$C, pH 7.0.
TABLE 2.10 - GSH Peroxidase Activities of Tissue from the "Hypoxic" Cortex, and from the Residual Tissue of Lewis Lung Carcinomas Borne by C57BL6 Mice

Results represent mean ± SEM for 6 mice at each time interval. Values expressed as % of that of corresponding control lung, shown in brackets. Protein determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after transplantation of tumour (days)</th>
<th>Cytosolic Cortex</th>
<th>Cytosolic Periphery</th>
<th>Mitochondrial Cortex</th>
<th>Mitochondrial Periphery</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>16 ± 1&lt;sup&gt;a&lt;/sup&gt; (73)</td>
<td>14 ± 1&lt;sup&gt;a&lt;/sup&gt; (67)</td>
<td>7.1 ± 1.5&lt;sup&gt;bd&lt;/sup&gt; (59)</td>
<td>8.2 ± 0.9&lt;sup&gt;d&lt;/sup&gt; (68)</td>
</tr>
<tr>
<td>15</td>
<td>14 ± 1&lt;sup&gt;a&lt;/sup&gt; (65)</td>
<td>14 ± 1&lt;sup&gt;a&lt;/sup&gt; (63)</td>
<td>5.8 ± 0.8&lt;sup&gt;bc&lt;/sup&gt; (48)</td>
<td>8.5 ± 0.6&lt;sup&gt;d&lt;/sup&gt; (70)</td>
</tr>
<tr>
<td>17</td>
<td>14 ± 2&lt;sup&gt;a&lt;/sup&gt; (64)</td>
<td>13 ± 1&lt;sup&gt;a&lt;/sup&gt; (60)</td>
<td>4.9 ± 0.9&lt;sup&gt;c&lt;/sup&gt; (41)</td>
<td>7.7 ± 0.9&lt;sup&gt;d&lt;/sup&gt; (64)</td>
</tr>
<tr>
<td>Control lung day 13-17&lt;sup&gt;#&lt;/sup&gt;</td>
<td>22 ± 2</td>
<td></td>
<td>12.1 ± 1.7</td>
<td></td>
</tr>
</tbody>
</table>

<sup>#</sup> See tables 3.7 and 3.8

* Enzyme units are defined as 1000 x log<sub>10</sub> (decrease in GSH/min) at 37°C, pH 7.0.

<sup>a-d</sup> Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter. Tumour values compared with those of control lung.
TABLE 2.11 - Superoxide Dismutase Activities of Tissue from the "Hypoxic" Cortex, and from the Residual Tissue of Lewis Lung Carcinomas Borne by C57BL6 Mice

Results represent mean ± SEM for 6 mice at each time interval. Values expressed as % of that of corresponding control lung, shown in brackets. Protein determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after transplantation of tumour (days)</th>
<th>Cytosolic Cortex</th>
<th>Cytosolic Periphery</th>
<th>Mitochondrial Cortex</th>
<th>Mitochondrial Periphery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(units [Sigma^R]/ mg protein at 25 ± 2° C, pH 7.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>2.2 ± 0.2^a</td>
<td>3.2 ± 0.3^a</td>
<td>1.2 ± 0.3^b</td>
<td>2.1 ± 0.4^c</td>
</tr>
<tr>
<td></td>
<td>(33)</td>
<td>(34)</td>
<td>(480)</td>
<td>(850)</td>
</tr>
<tr>
<td>14</td>
<td>2.8 ± 0.4^a</td>
<td>3.3 ± 0.3^a</td>
<td>1.1 ± 0.1^b</td>
<td>1.8 ± 0.4^c</td>
</tr>
<tr>
<td></td>
<td>(24)</td>
<td>(28)</td>
<td>(370)</td>
<td>(630)</td>
</tr>
<tr>
<td>17</td>
<td>2.9 ± 0.4^a</td>
<td>3.5 ± 0.3^a</td>
<td>0.6 ± 0.1^b</td>
<td>2.4 ± 0.2^c</td>
</tr>
<tr>
<td></td>
<td>(31)</td>
<td>(38)</td>
<td>(210)</td>
<td>(860)</td>
</tr>
<tr>
<td>Control lung day 12-17#</td>
<td>10 ± 1</td>
<td>0.3 ± 0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

# See tables 3.9 and 3.10

^a-c Significantly different (p<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter. Tumour values compared with those of control lung.
TABLE 2.12 - Cytosolic \( \gamma \)-Glutamylcysteine Synthetase Activity and Mitochondrial \( \gamma \)-Glutamyl Transpeptidase Activity of Tissue from the "Hypoxic" Cortex, and from the Residual Tissue of Lewis Lung Carcinomas Borne by C57BL6 Mice

Results represent mean ± SEM for 6 mice at each time interval. Values expressed as % of that of corresponding control lung, shown in brackets. Protein determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after transplantation of tumour (days)</th>
<th>( \gamma )-Glutamylcysteine synthetase (nmol/min/ protein at 37° C, pH 8.25)</th>
<th>( \gamma )-Glutamyl transpeptidase (nmol p-nitroanilide/min/protein at 37° C, pH 7.8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortex</td>
<td>Periphery</td>
</tr>
<tr>
<td>13</td>
<td>0.7 ± 0.1(^a) (48)</td>
<td>0.7 ± 0.1(^a) (46)</td>
</tr>
<tr>
<td>15</td>
<td>1.0 ± 0.1(^a) (62)</td>
<td>0.7 ± 0.1(^a) (42)</td>
</tr>
<tr>
<td>17</td>
<td>1.4 ± 0.2 (94)</td>
<td>0.7 ± 0.1(^a) (49)</td>
</tr>
</tbody>
</table>

Control lung day 13-17#

1.5 ± 0.2

11 ± 1

# See tables 3.12 and 3.16

\(^a-c\) Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter. Tumour values compared with those of control lung.
TABLE 2.13 - Mitochondrial and Cytosolic Protein Yields from Lewis Lung Carcinomas Divided into Cortex and Periphery

Results represent mean ± SEM for 6 mice at each time interval. Values expressed as % of that of corresponding control lung shown in brackets. Protein determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after transplantation of tumour (days)</th>
<th>Cytosolic Cortex (mg protein/g wet wt)</th>
<th>Mitochondrial Cortex</th>
<th>Periphery</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>42 ± 4 (130)</td>
<td>15 ± 1 (80)</td>
<td>9.6 ± 0.5 (50)</td>
</tr>
<tr>
<td>14</td>
<td>41 ± 2 (120)</td>
<td>21 ± 1 (94)</td>
<td>9.6 ± 0.3 (43)</td>
</tr>
<tr>
<td>17</td>
<td>54 ± 5 (170)</td>
<td>19 ± 1 (90)</td>
<td>11 ± 1 (50)</td>
</tr>
</tbody>
</table>

Control lung day 12-17#

<table>
<thead>
<tr>
<th></th>
<th>Cortex</th>
<th>Periphery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control lung day 12-17# (n=18)</td>
<td>33 ± 3</td>
<td>21 ± 2</td>
</tr>
</tbody>
</table>

\(a-c\) Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter. Tumour values compared with those of control lung.
TABLE 2.14 - Glutathione Status of the "Hypoxic" Cortical Portion and the Residual Peripheral Portion of Lewis Lung Carcinomas Borne by C57BL/6 Mice

Results represent mean ± SEM for 6 mice at each time interval. Protein determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Total Glutathione (nmol GSH/mg protein)</th>
<th>GSH (nmol/mg protein)</th>
<th>GSSG (nmol/mg protein)</th>
<th>GSH/GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortical Tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>18 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.1 ± 0.1</td>
<td>12 ± 2&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>18 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.5 ± 0.3</td>
<td>10 ± 2&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>18</td>
<td>18 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.2 ± 0.2</td>
<td>8.3 ± 2.6&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peripheral Tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>10 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6 ± 1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.4 ± 0.2</td>
<td>6 ± 2&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>11 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8 ± 1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.7 ± 0.3</td>
<td>6 ± 1&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>18</td>
<td>11 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6 ± 1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.9 ± 0.3</td>
<td>4 ± 1&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control lung day 18</td>
<td>20 ± 2</td>
<td>18 ± 2</td>
<td>1.1 ± 0.2</td>
<td>16 ± 3</td>
</tr>
</tbody>
</table>

<sup>a-c</sup> Significantly different (P<0.05) by Newman Keuls test (after analysis of variance) from all values except those bearing the same superscript letter. Corresponding values from "cortical" and "peripheral" tumour tissue compared.
TABLE 2.15 - Thiobarbituric Acid-reactive Material Concentration of Tissue from the "Hypoxic" Cortex, and from the Residual Tissue of Lewis Lung Carcinomas Borne by C57BL6 Mice

Results represent mean ± SEM for 6 mice at each time interval. Thiobarbituric acid-reactive chromophores obtained by incubation for 15 min at 100°C in 0.1M phthalate buffer (pH 3.5). No antioxidant additives were used. Protein determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Cortex</th>
<th>Periphery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol malondialdehyde equiv./ mg protein)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>0.9 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.2 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>0.7 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.7 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18</td>
<td>0.7 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.8 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control lung day 18</td>
<td>0.5 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a-b</sup> Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter. Tumour values compared with those of control lung.
TABLE 2.16 - Weight in situ and Percentage of Tumour Mass Arbitrarily Taken to Represent the "Hypoxic" Cortex of Lewis Lung Carcinomas Borne by C57BL6 Mice

Results represent mean ± SEM for 6 mice at each time interval. Weight of intact tumour (g) shown in brackets.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Tumour Wt (as a % of body wt)</th>
<th>Cortex Wt* (as a % of periphery wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>6.6 ± 0.5\textsuperscript{a}</td>
<td>29 ± 5\textsuperscript{d}</td>
</tr>
<tr>
<td></td>
<td>(1.6 ± 0.1)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>10.8 ± 0.8\textsuperscript{b}</td>
<td>27 ± 5\textsuperscript{d}</td>
</tr>
<tr>
<td></td>
<td>(2.5 ± 0.2)</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>15.2 ± 1.9\textsuperscript{c}</td>
<td>21 ± 2\textsuperscript{d}</td>
</tr>
<tr>
<td></td>
<td>(3.4 ± 0.3)</td>
<td></td>
</tr>
</tbody>
</table>

* Periphery refers to the residual tumour tissue after the cortex has been removed

\textsuperscript{a-d} Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter. Tumour values compared with those of control lung.
Mitochondrial γ-glutamyltranspeptidase activity of the cortical region was significantly less than that of the residual tumour tissue, on the 15th and 17th days after transplantation (table 2.12).

Protein yield, expressed as mg/g wet weight, was significantly greater for the mitochondrial fractions from the "cortex" than from the tumour periphery (table 2.13). Cytosolic protein yield was significantly greater for the tumour tissue from the periphery than that from the cortical region at 12 and 14 days after implantation (table 2.13).

GSH concentration and GSH/GSSG ratio were significantly greater in tissue removed from tumour "cortex" than periphery (table 2.14). The concentration of TBA-reactive material was approximately 100% greater in the peripheral neoplastic tissue than in the tissue of the "cortex" (table 2.15).

The ratio of the weight of tissue removed as "cortex" to the weight of residual tissue was kept essentially constant with increasing tumour size (table 2.16).

Comparison with control lung: The major differences between intramuscularly-implanted Lewis lung carcinoma and control murine lung, as determined in the present studies, are summarized in table 2.17.
### TABLE 2.17 - Summary of Major Differences Between Intramuscularly-implanted Lewis Lung Carcinoma and Control Pulmonary Tissue (as determined in the studies of Chapter 2)
All values are specific enzyme activities.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Subcellular fraction</th>
<th>Mean day 13 tumour value as a % of corresponding control lung value</th>
<th>Trend with increasing time after implantation (post day 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH peroxidase</td>
<td>Cytosolic</td>
<td>60</td>
<td>No significant change.</td>
</tr>
<tr>
<td></td>
<td>Mitochondrial</td>
<td>110</td>
<td>Decreases to 80% by 17 days after implant.</td>
</tr>
<tr>
<td>GSSG reductase</td>
<td>Whole tissue</td>
<td>160</td>
<td>No significant change.</td>
</tr>
<tr>
<td>GSH-transferase</td>
<td>Cytosolic</td>
<td>175</td>
<td>No significant change.</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>Cytosolic</td>
<td>30</td>
<td>No significant change.</td>
</tr>
<tr>
<td></td>
<td>Mitochondrial</td>
<td>720</td>
<td>Decreases to 540% by 17 days after implant.</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>Cytosolic</td>
<td>27</td>
<td>No significant change.</td>
</tr>
<tr>
<td>γ-Glutamyl-cysteine synthetase</td>
<td>Cytosolic</td>
<td>55</td>
<td>Increases to 120% by 17 days after implant.</td>
</tr>
<tr>
<td>Catalase</td>
<td>Whole tissue</td>
<td>52</td>
<td>Increases to 70% by 17 days after implant.</td>
</tr>
<tr>
<td>γ-Glutamyl transpeptidase</td>
<td>Cytosolic</td>
<td>14</td>
<td>Not determined.</td>
</tr>
<tr>
<td></td>
<td>Mitochondrial</td>
<td>21</td>
<td>Not determined.</td>
</tr>
<tr>
<td></td>
<td>Microsomal</td>
<td>13</td>
<td>Not determined.</td>
</tr>
</tbody>
</table>
The Lewis lung carcinoma has been described as an epidermoid carcinoma (squamous cell carcinoma; Sugiura and Stock, 1955). In a more recent study however, where sections of subcutaneously-implanted Lewis lung carcinoma were observed with an electron microscope, many of the characteristic features of epidermoid carcinoma cells were absent (Sato et al, 1982). The authors concluded that, due to successive transplantations, the Lewis lung tumour had become more undifferentiated and thus would be more appropriately categorized as a large-cell carcinoma (Sato et al, 1982).

Inspection of sectioned Lewis lung carcinomas, revealed the "healthy" red-coloured (hyperaemic) peripheral region and amorphous mushy "necrotic" tissue of the central regions of the tumour, that have been reported elsewhere (Salsbury et al, 1974; Dobrossy et al, 1980). Sections, stained with H&E (plates I & II), when examined by the light microscope, were observed to have similar appearance to photomicrographs of Lewis lung tumours in other publications (Sugiura and Stock, 1955; Salsbury et al, 1974; Sato et al, 1982). The histology of the Lewis lung carcinoma, in respect to the relation of tumour cells to blood vessels, has been described as being more similar to that of a sarcoma than that of a carcinoma (Salsbury et al, 1974). The "transformation" of epithelial tumours of mice and rats to sarcomas has been reported (Greenstein, 1954). The lack of reaction between tumour cells and van Gieson's stain (plate III) may be verification that the tumour, used in the present studies, was not derived from connective tissue (that is sarcomatous).

Histological Evidence of Autoxidation

The golden brown pigment observed in sections stained with H&E (plate IIb), was possibly haemosiderin, a non-functional iron-containing
Plate I - Section (H&E) through hindlimb:

a) tumour-free mouse (x 45)

b) Lewis lung carcinoma-bearing mouse (x 45).

Note: 13 days post implantation of tumour.
Plate II - Section (H&E) through Lewis lung carcinoma:

a) Haemorrhagic region containing inflammatory cells (x 275)

b) Region containing golden-brown pigment (x 730).

Note: 13 days post implantation of tumour.
Plate III - Section (Van Gieson's) through hindlimb:

a) tumour-free mouse (x 110)

b) Lewis lung carcinoma-bearing mouse (x 730).

Note: 13 days post implantation of tumour.
pigment associated with autooxidative injury (Culling, 1974). The apoprotein, siderin is a glycoprotein that binds, with great affinity, to inorganic iron (Gedigk, 1969). Haemosiderin occurs when the concentration of free iron in the cytosol exceeds the capacity for chelation by apoferritin to form ferritin (Gedigt, 1969). Haemosiderin is also considered to be the product of lysosomal digestion of ferritin (Halliwell and Gutteridge, 1984a), and can be shown histochemically using Perls' Prussian blue reaction for ferric salts (Culling, 1974). The yellowish pigmentation could alternatively have been haematoidin, which is a pigment derived from bile pigment during the breakdown of old haemorrhages (Sturgeon and Shoden, 1969; Culling, 1974). Haematoidin, owing to its relatively low iron content, does not give a positive stain with Perls' Prussian blue reaction, and therefore can be distinguished histochemically from haemosiderin (Sturgeon and Shoden, 1969). Sections of Lewis lung carcinoma had extensive aggregates of Perls' Prussian blue-positive foci which indicates that the yellow-brown pigment was not haematoidin. The extracellular distribution, the localization mainly to haemorrhagic areas, and the birefringence of the pigmentation (plate IVa), however, suggest that it was largely artefactual acid formaldehyde-haematin pigment (formalin pigment). The occurrence of formalin pigment is due to the reaction between formaldehyde at low pH with haemoglobin or its breakdown products (Culling, 1974; Drury and Wallington, 1980); the Gooding and Stewart's fluid, used to decalcify the tumour-bearing limbs, contained 5% v/v formic acid. Most of the ferric iron-containing pigment in sections of Lewis lung carcinoma was thus more probably the result of post-mortem changes in the presence of formaldehyde than a consequence of a pathological excess of inorganic iron in vivo. There were, however, occurrences of blue-staining (Perls') pigment, possibly haemosiderin, in regions of tumour, remote from haemorrhagic areas (plate IVb).

Lipofuscin (haemofuscin, age pigment) is class of yellow-brown
Plate IV - Section (Perls') through Lewis lung carcinoma:

a) Haemorrhagic region containing formalin pigment (x 110)

b) Region containing blue haemosiderin-like pigment (x 275).

Note: 13 days post implantation of tumour.
of antioxidant nutrients (Porta and Hartroff, 1969; Katz et al., 1984). Lipofuscin is formed by the autoxidative polymerization of intracellular lipids, nucleic acids and proteins with the Schiff base product of malondialdehyde (Tappel, 1975; Donato, 1981). A u.v. fluorescence technique (Reddy et al., 1982) was used in preference to the more commonly applied Schmorl's ferric-ferricyanide staining procedure to histologically demonstrate lipofuscin pigment. The ferric-ferricyanide method, however, incorporates the Perls' Prussian blue reaction at acidic pH (Culling, 1974), and thus the presence of formalin pigment artefacts could have made the interpretation of the haemorrhagic Lewis lung carcinoma ambiguous. The presence of reducing sites, such as sulphhydryl groups, ascorbate, uric acid, phenols and indols, might also have made distinction between lipofuscin and haem-derived artefacts more difficult (Lillie, 1962): a series of controls would be required to make the distinction possible. Examination of unstained sections of Lewis lung with a u.v. microscope did not however reveal any yellow-green to orange fluorescence, characteristic of lipofuscin (Reddy et al., 1982).

The sections of tumour could have been treated with saturated alcoholic solution of picric acid to dissolve formalin pigment (Culling, 1974; Drury and Wallington, 1980), prior to staining for pathological pigments. The histochemical search for evidence of autoxidative damage in Lewis lung carcinomas could possibly be furthered by use of staining techniques for lipid peroxides: such as the haem-catalyzed leuco-dichloroindophenol reaction, or the application of the Winkler-Schulze reaction for lipoperoxides (Lillie, 1962). The studies, described in the present chapter, do not however provide significant evidence of autoxidative lesions in i/m-embedded Lewis lung carcinomas.
Contribution by infiltrating host cells

Sections of Lewis lung carcinoma, when examined using a light microscope, were revealed to have an estimated 5-10% contribution to the total cell population by host inflammatory cells (plate IIa). It has been reported elsewhere that inflammatory cells were seldom observed in histological sections of Lewis lung carcinoma (Sato et al, 1982). The proportion of host-derived cells (primarily macrophages) in the nucleated cell population of Lewis lung carcinoma has been estimated as 10-15% (Stephens et al, 1978), and at less than 10% (Gundersen et al, 1981). As much as 44% by volume of i/m-implanted Lewis lung carcinoma has been contended to comprise of host cells, blood vessels, connective tissue and necrotic material (Stephens et al, 1984). Most of the contamination of Lewis lung tumours by necrotic and endothelial tissues was probably removed by the preparative procedures described earlier. The residual 5-15% contribution by other host cells (mainly macrophages; Stephens et al, 1978) could possibly have attenuated some of the biochemical values determined in the present studies for Lewis lung tumour. The proportion of inflammatory cells was however, probably insufficient to affect appreciably the biological character inferred for the neoplastic component of the tumour.

Origin of Lewis lung carcinoma

A monoclonal antibody has recently been developed that binds to a surface antigen on Lewis lung carcinoma cells but not to other murine malignant cell lines (Hadas et al, 1984). When this monoclonal antibody was screened immunohistochemically against various normal tissues of C57BL6 mice, only alveolar epithelial cells were stained. As Lewis lung cells express an antigen found exclusively in the tissue of origin, lung, at least some of the original pulmonary phenotype is evidently still preserved in Lewis lung tumour cells even though the tumour originated
pigment associated with autooxidative injury (Culling, 1974). The apoprotein, siderin is a glycoprotein that binds, with great affinity, to inorganic iron (Gedigk, 1969). Haemosiderin occurs when the concentration of free iron in the cytosol exceeds the capacity for chelation by apoferritin to form ferritin (Gedigt, 1969). Haemosiderin is also considered to be the product of lysosomal digestion of ferritin (Halliwell and Gutteridge, 1984a), and can be shown histochemically using Perls' Prussian blue reaction for ferric salts (Culling, 1974). The yellowish pigmentation could alternatively have been haematoidin, which is a pigment derived from bile pigment during the breakdown of old haemorrhages (Sturgeon and Shoden, 1969; Culling, 1974). Haematoidin, owing to its relatively low iron content, does not give a positive stain with Perls' Prussian blue reaction, and therefore can be distinguished histochemically from haemosiderin (Sturgeon and Shoden, 1969). Sections of Lewis lung carcinoma had extensive aggregates of Perls' Prussian blue-positive foci which indicates that the yellow-brown pigment was not haematoidin. The extracellular distribution, the localization mainly to haemorrhagic areas, and the birefringence of the pigmentation (plate IVa), however, suggest that it was largely artefactual acid formaldehyde-haematin pigment (formalin pigment). The occurrence of formalin pigment is due to the reaction between formaldehyde at low pH with haemoglobin or its breakdown products (Culling, 1974; Drury and Wallington, 1980); the Gooding and Stewart's fluid, used to decalcify the tumour-bearing limbs, contained 5% v/v formic acid. Most of the ferric iron-containing pigment in sections of Lewis lung carcinoma was thus more probably the result of post-mortem changes in the presence of formaldehyde than a consequence of a pathological excess of inorganic iron in vivo. There were, however, occurances of blue-staining (Perls') pigment, possibly haemosiderin, in regions of tumour, remote from haemorrhagic areas (plate IVb).

Lipofuscin (haemofuscin, age pigment) is class of yellow-brown
Tumour growth

The growth kinetics of Lewis lung carcinomas, borne by C57BL6 mice in the present studies, were similar to those of other reports (Mayo, 1972; DeWys, 1972; Dobrossy *et al.*, 1980). The tumour normally became palpable at 8-10 days after implantation. During the period in which analyses were performed, 9-19 days after inoculation of mice with tumour cells, the rate of increase of tumour mass was essentially linear (fig. 3.1; Chapter 3). This suggests that Lewis lung tumours had progressed beyond the exponential phase characterized in early growth (DeWys, 1972) and that growth rate was slowing as the carcinoma increased in size. As DNA and protein concentrations did not vary significantly with increasing tumour mass, tumour cell division after an average of 9 days post implantation, might have equilibrated with cell death and quiescence. Studies elsewhere, in which the cell cycle of Lewis lung carcinomas was monitored using flow cytometry, have however shown that the proportion of cells in proliferative, S-phase was 52-55% between 8 and 15 days after implantation but only 35% at 23 days (Starace *et al.*, 1982). During the 9-19 day post implantation period, the balance of the mitotic cell cycle in Lewis lung carcinoma probably was not sufficiently disturbed, to contribute significantly to biochemical changes associated with increase in tumour size. Alterations in enzymic activities or in concentrations of cellular constituents, reported in this chapter, were more likely to have been due to specific changes in the synthesis, degradation and transport processes of Lewis lung cells than non-systematic factors such as oedema and necrosis.

Protein assay

Protein concentrations of murine tissue homogenates when determined
by the more established technique of Lowry and co-workers (1951) were
approximately twice those determined using Coomassie blue dye (Bradford,
1977; Spector, 1978). These observations differ from those of a study in
which the two methods were compared for the estimation of protein
concentration in human tissue homogenates (Westman and Marklund, 1981):
no significant quantitative differences were found. The sensitivity of
Coomassie blue reagent to differing types of protein varies substantially
(Pierce and Seulter, 1977; Van Kley and Hale, 1977). The Lowry procedure
has also been reported to show variability in its quantitation of
differing proteins (Bradford, 1977; Sedmark and Grossberg, 1977). In the
present studies, however, the qualitative estimations of protein
concentration of differing murine tissue homogenates were similar for
both assays. For reasons given in the Methods section, the procedure
using Folin-Ciocalteu reagent was preferred to that employing Coomassie
blue reagent, in all except the preliminary experiments of this thesis.

Antioxidant defence enzymes

Superoxide dismutase: The SOD activities of cytosolic and mitochondrial
fractions from Lewis lung carcinomas and other murine tissues were heat
labile, and were appropriately resistant or sensitive to treatment with
various regimens reported to differentiate Cu/Zn- and Mn-SOD (Fridovich,
1982b; Geller and Winge, 1983). The activity determined for cytosolic
fractions from murine tissues probably corresponded to cuprozinc SOD
whereas that of the mitochondrial fractions was analogous to the
manganous isoenzyme. Lewis lung carcinoma cells, in common with other
tumours, have mitochondria with aberrant ultrastructural features (Sato
et al, 1982). Mitochondrial SOD is not membrane-associated but localized
in the mitochondrial interspace (Stein et al, 1982). Mitochondrial
fragility might therefore be expressed by leakage of Mn-SOD, during
tissue processing into the soluble fraction. The absence of significant
Diminution of SOD activity in tumours, relative to their tissue of origin, has been a comparatively common observation (Oberley and Beuttner, 1979; Tisdale and Mahmoud, 1983; Hoffman et al., 1985). The relatively low Cu/Zn-SOD activity of Lewis lung carcinoma is consistent with observations elsewhere for Lewis lung (Peskin et al., 1977; Van Balgooy and Roberts, 1978). Cytosolic SOD of other rodent neoplasms have also been reported to be lowered in comparison to normal tissues (Bozzi et al., 1976; Peskin et al., 1977; Van Balgooy and Roberts, 1978; Dinescu-Romalo and Mihai, 1979; Bartoli et al., 1980; Bize et al., 1980; Takada et al., 1982). Cu/Zn-SOD activities of human tumours have been generally reported to be less than those of the tissues of origin, although there is large overlap between the ranges of activities determined for normal and transformed tissues (Sykes et al., 1978; Galeotti et al., 1980; Westman and Marklund, 1981; Hoffman et al., 1985). Human leukaemic cells (Yamanka et al., 1979) and colon carcinomas (Baur and Wendel, 1980) have, however, been observed to have greater Cu/Zn-SOD activities than their non-neoplastic counterparts.

Comparison of a variety of rat and mouse neoplasms have shown that the faster the rate of growth of the tumour, the lower the cytosolic SOD activity (Bartoli et al., 1980). Total SOD activity (assumed to be primarily the Cu/Zn isoenzyme; Oberley and Beuttner, 1979) of Ehrlich ascites carcinomas however has been observed to be greatest during the period of maximum tumour growth, shortly after transplantation, but decreased considerably during advanced tumour growth (Lankin and Gurevich, 1976). The observations of Lankin and Gurevich compare with those of studies in which Cu/Zn SOD activities of Lewis lung carcinoma.
were observed to decrease from a value similar to that of lung, at 9 days post implantation, to activities significantly less by the 15th day (Capel and Thornley, 1982). The present studies, however, did not show any variation in the activity of the cytosolic fraction from Lewis lung tumours of 13 to 17 days after transplantation. As tumour volume enlarged, tissue oxygenation will have decreased and the relative proportion of hypoxic cells and necrotic material will have subsequently increased. The comparatively greater Cu/Zn-SOD activity reported for day 9 Lewis lung carcinoma thus might have been associated with the better oxygenation of tumours of smaller volume (see later). Lewis lung carcinomas of 9 days post implantation were only just palpable. There thus could have been a relatively greater contribution to Cu/Zn-SOD activity by host tissues in Lewis lung tumours of early growth.

The greater specific Mn-SOD activity of Lewis lung carcinoma than parent tissue, lung, determined in the present studies, is possibly the first report of its kind concerning an animal tumour model. This observation conflicts with those of other reports (Dinescu-Romalo and Mihai, 1979; Oberley and Beuttner, 1979; Takada et al., 1982), in which little or no Mn-SOD was determined in tumours. In human tumours and neoplastic cell lines depression of Mn-SOD is not so apparent: Mn-SOD in some human tumours has been reported to be greater than comparative normal tissues (Westman and Marklund, 1981; Marklund et al., 1982). The discrepancy in the pattern of Mn-SOD activities between human and animal neoplasms might have been due to the presence of Mn-SOD in the cytosol of human tissues but not in that of animal tissues, which contain Mn-SOD only in the mitochondrial matrix (Marklund et al., 1982). The many observations of diminished mitochondrial SOD in animal tumours have served as the basis of hypotheses that diminution of Mn-SOD is the cause of neoplastic transformation (Oberley et al., 1981). The conclusions of Oberley and co-workers are however based on a limited number of cancer
models, primarily hepatomas (Marklund et al., 1982). Studies in which SOD isoenzymes of a range of rat and mouse tumours, including Lewis lung carcinoma (two samples), were detected using polyacrylamide gel electrophoresis showed that Mn-SOD was barely detectable in tumours (Van Balgooy and Roberts, 1978). Photographs of gels, used for the electrophoretic separation of murine lung (the reference tissue to Lewis lung carcinoma) from the studies of Van Balgooy and Roberts did not however show bands corresponding to Mn-SOD activity. The methods used in these studies to separate and detect SOD were possibly too insensitive to compare Lewis lung carcinomas with pulmonary tissue.

Catalase: Ethanol is included in many assay procedures to determine catalase activity because it apparently decomposes the inactive catalase complex with $H_2O_2$ (complex II) and thereby maximizes the catalase activity that can be detected (Cohen et al., 1970). When ethanol (1% w/v) was incorporated with the samples and bovine liver standards (Sigma), used in the present studies, no catalase activity could be detected. Catalase is inactivated by a wide variety of substances (Kampschmidt, 1965), which might have included a contaminant in the ethanol (absolute alcohol) used in the present investigations.

Catalase activity was determined to be significantly less in Lewis lung carcinoma than pulmonary tissue. Necrosis and post-mortem autolysis might have liberated substances that inhibited catalase activity of Lewis lung carcinoma in vitro (Hargreaves et al., 1959). Catalase activity has been reported to be comparatively low or absent in other animal tumours (Ono, 1966; Bozzi et al., 1976; Docampo et al., 1979; Pinto et al., 1980; Sun and Cederbaum, 1980; Tisdale and Mahmoud, 1983; Williams-Smith et al., 1984). Studies of human cancers have also reported lower catalase activities than in comparative normal tissues (Wickramsinghe et al., 1976; Baur and Wendel, 1980; Hoffman et al., 1985). Many of the above tumour models
were however compared with tissues such as liver or kidney, which have relatively greater catalase activities than other non-neoplastic tissues. No general differences in catalase activity can be observed between normal tissues and transformed tissues (Greenstein, 1956; Marklund et al, 1982). Investigations of 6 differing murine tumour models have demonstrated an approximately 60-fold variation in catalase activity (Nathan et al, 1980). The relatively low catalase activity of Lewis lung carcinoma was not necessarily characteristic of neoplastic tissues.

Glutathione peroxidase: The observation that GSH peroxidase activities of Lewis lung carcinoma and murine lung, when assayed with cumene as substrate, did not significantly differ from determinations using H$_2$O$_2$, infers that all GSH peroxidase activity in lung and Lewis lung carcinoma was attributable to the selenium-dependent enzyme. Selenium-free GSH peroxidase has been reported to represent only 10% of the GSH peroxidase activity of rat lung (Jenkinson et al, 1983), and to be absent in human foetal lung (Polidoro et al, 1982). GSH peroxidase occurs predominantly in the cytosol of mammalian cells (Cikryt et al, 1982). The observation that cytosolic GSH peroxidase activity was significantly less in Lewis lung carcinoma therefore affirms preliminary studies in which GSH peroxidase activity in homogenates of Lewis lung carcinoma was reported to be relatively low (Capel and Thornley, 1982). Mitochondrial GSH peroxidase activity of Lewis lung carcinoma was however similar to that of murine lung. Mitochondrial GSH peroxidase activity in Ehrlich ascites tumours has also been demonstrated to be relatively pronounced (Hosoda and Nakamura, 1970). H$_2$O$_2$, possibly generated by the comparatively elevated Mn-SOD activity of the Lewis lung tumour mitochondria, could presumably have been adequately detoxified by mitochondrial GSH peroxidase. Cytosolic GSH peroxidase, which metabolizes extra-mitochondrial peroxides and peroxides produced by the outer mitochondrial membrane (Ganther et al, 1976), might however have been limiting in Lewis
GSH peroxidase activities of rodent and human hepatomas have been reported to be significantly less than that of comparative normal livers (Bozzi et al., 1976; Peskin et al., 1977; Corrocher et al., 1980; Rossi et al., 1983; Casaril et al., 1985). Hepatic tissue however has considerably greater GSH peroxidase activity than other tissues (Ganther et al., 1976). Investigations of murine and human tumours, derived from tissues other than liver, have not generally shown any differences between the GSH peroxidase activities of neoplastic tissues and normal tissues (Hosoda and Nakamura, 1970; Marklund et al., 1982; Lane and Medina, 1983; Tisdale et al., 1983; Hoffman et al., 1985). GSH peroxidase activities of human colon carcinoma excisions, have been reported to be more than twice that of non-neoplastic colon tissue (Baur and Wendel, 1980). The relatively low cytosolic GSH peroxidase activity of Lewis lung carcinoma was thus not evidence of any general perturbation of the expression of cytosolic GSH peroxidase in neoplastic cells (Oberley and Spitz, 1984).

Other enzymes mediating GSH turnover

GSH production: Intracellular GSH can be oxidized by free radical reactions (Kosower and Kosower, 1976b), or non-enzymically by GSH peroxidase and transhydrogenases (Meister and Anderson, 1983). GSSG is reduced back to GSH by GSSG reductase. In the procedure of the present studies to determine GSSG reductase activity, reactions were initiated by adding GSSG to the assay mixtures, which contained the co-substrate, NADPH. It has recently been reported, however, that preincubation of GSSG reductase with NADPH, in the absence of GSSG, causes substantial time-dependent loss of GSSG reductase activity (Podrazky and Steven, 1984). The consistent values obtained for GSSG reductase activities (table 2.4) in the present studies, suggested that inhibition of the enzyme by NADPH was not excessive. The presence of endogenous thiols or the inclusion of
co-enzyme, FAD were possibly sufficient to counteract inhibition of GSSG reductase by NADPH.

The relatively pronounced GSSG reductase activity evaluated for Lewis lung carcinoma has been observed elsewhere (Manso et al., 1958). Elevated GSSG reductase activities have been reported for murine mammary adenocarcinomas and Ehrlich ascites tumours, rat R320AC tumours and human hepatomas (Hosoda and Nakamura, 1970; Hilf et al., 1978; Suojanen et al., 1980; Corrocher et al., 1980). A relatively high GSSG reductase activity has been postulated to be prerequisite of neoplastic growth (Hilf et al., 1978). Exceptions, such as the relatively low activity observed for Ridgeway osteogenic sarcoma (Manso et al., 1958), rat hepatomas (Rossi et al., 1983) and cancer cells of lymphocyte origin (Tisdale and Mahmoud, 1983), however negate any generalizations concerning GSSG reductase activities of neoplastic cells.

The reduction of GSSG to GSH uses NADPH furnished by the pentose phosphate shunt (Elsayed et al., 1982). Hydroperoxide metabolism, via the GSH peroxidase/GSSG reductase couplet is thus directly linked to glucose metabolism (figure 2.1; Hosoda and Nakamura, 1970). Relatively elevated GSSG reductase activity in Lewis lung and other tumours, could provide NADP⁺ for the pentose phosphate pathway and other catabolic processes. The pentose phosphate pathway generates pentose sugars necessary for the biosynthetic pathways of dividing tissues, such as tumours (Weber, 1977).

Glucose-6-phosphate dehydrogenase activities, determined for Lewis lung carcinoma, were approximately one quarter of that of pulmonary tissue. As glucose-6-phosphate dehydrogenase is the rate-limiting enzyme of the pentose phosphate pathway (Weber, 1977; Elsayed et al., 1982), the activity of the pathway in Lewis lung carcinoma could have been significantly low. NADPH, which also acts as a reducing agent in many biosynthetic processes (Weber, 1977; Ross et al., 1982), might have been
been reported to be relatively diminished in murine tumours (Greenstein, 1956; Smith and King, 1970), and in rat hepatomas (Nishizuka and Hayaishi, 1966; Ross et al, 1982). GSSG reductase activity, as determined in the present studies, may merely have represented the concentration of the enzyme in the carcinoma (and other murine tissues) because its activity in vivo was limited by the availability of NADPH, and possibly FAD.

In common with Lewis lung carcinoma, murine mammary carcinomas have been observed to have comparatively low glucose-6-phosphate dehydrogenase activities (Kopelovich et al, 1966; Smith and King, 1970). Normal murine lactating mammary gland utilizes a significant proportion of its glucose via the pentose phosphate shunt whereas Barrett mammary adenocarcinomas of mice have been reported to metabolize glucose by glycolysis only (Abraham and Chaikoff, 1964). Other studies of preneoplastic and neoplastic murine adenocarcinomas, have shown that inactive monomeric and dimeric forms of glucose-6-phosphate dehydrogenase predominate over the normal active tetrameric enzyme (Hilf et al, 1978). Pentose monophosphate pathway activities and glucose-6-phosphate dehydrogenase activities of rodent hepatomas have been generally observed to be greater than that of normal liver (Abraham and Chaikoff, 1964; Ono, 1966; Shonk et al. 1965; Weber, 1967). Hepatic tissue of rats and mice, however, have relatively low pentose phosphate pathway activities (Abraham and Chaikoff, 1964; Knox, 1967). Glucose-6-phosphate dehydrogenase activities (and pentose phosphate pathway activities) of neoplastic tissues has therefore been concluded to be generally intermediate in value between the lower and upper extremes determined for normal tissues (Knox, 1967).

Gamma-glutamylcysteine synthetase activity in Lewis lung carcinomas, 14 days after i/m implantation, was significantly less than that of
normal murine lung. Rat Novikoff hepatomas have been reported to have considerably less $\gamma$-glutamylcysteine synthetase activities than non-neoplastic adult rat liver (Wirth and Thorgiersson, 1978). The increase in $\gamma$-glutamylcysteine synthetase activity, observed for Lewis lung carcinoma removed at 15 and 17 days post implantation might have been indicative of a requirement for greater GSH concentrations as tumour volume increased. An inverse relation has been shown between cellular GSH concentration and susceptibility of murine tumour cells to externally-generated H$_2$O$_2$ (Nathan et al, 1980). The increase in GSH-synthesizing ability, detected in Lewis lung carcinomas, might have represented an adaptation by the cells of the tumour to counteract H$_2$O$_2$, produced by invading macrophages. Elevation of $\gamma$-glutamylcysteine synthetase activity possibly correlates with GSH concentration and GSH/GSSG ratio which were reported to increase with increasing weight of Lewis lung carcinoma (Capel and Thornley, 1983). The change in $\gamma$-glutamylcysteine synthetase activity and the appreciable GSSG reductase activity of Lewis lung tumours may well have been causative of the disturbance in glutathione concentrations, observed in preliminary studies (Capel and Thornley, 1983).

**GSH degradation:** Subcellular GSH concentration must also reflect the rate of GSH breakdown and utilization (Tateishi et al, 1974). GSH may be lost from the cell due to oxidation, conjugation to electrophiles via GSH S-transferases, or transport out of the cell across the plasma membrane (Meister and Anderson, 1983). The degradation of GSH (and also of GSSG and S-substituted GSH) is catalyzed by $\gamma$-glutamyl transpeptidase (glutathionase) which breaks down GSH by hydrolysis or transpeptidation (Meister, 1982). A major proportion of $\gamma$-glutamyl transpeptidase is located on the external surface of the plasma membrane and has been postulated to be involved in the transport of amino acids ($\gamma$-glutamyl acceptors) into cells (Griffith and Meister, 1979). The
translocation of amino acids into the cell by γ-glutamyl transpeptidase and the regeneration of GSH via a 5 enzyme pathway (which includes γ-glutamylcysteine synthetase) is termed the γ-glutamyl cycle (Fiala et al, 1976; Meister and Anderson, 1983).

Lewis lung carcinomas were observed to have relatively little γ-glutamyl transpeptidase activity compared to murine lung (although substantially greater activity than liver), indicating that γ-glutamyl transpeptidase probably did not account for any significant utilization of GSH in the carcinoma. The comparatively diminished γ-glutamyl transpeptidase activity might have helped to conserve GSH. Lewis lung carcinoma cells possibly had alternative mechanisms of transporting amino acids and small peptides than the γ-glutamyl cycle. The comparatively low γ-glutamyl transpeptidase activity implies that Lewis lung carcinoma cells had limited capacity to form mercapturic acids from GSH conjugates, a pathway involved in the biosynthesis of leukotriene D (Meister and Anderson, 1983). The impaired ability to synthesize certain leukotrienes could have affected homeostatic regulatory processes in Lewis lung carcinoma.

In contrast to Lewis lung carcinoma, many neoplasms, particularly hepatomas, have been observed to have greater γ-glutamyl transpeptidase activities than their tissues of origin (Wirth and Thorgeirsson, 1978; Richards et al, 1982). The presence of significant γ-glutamyl transpeptidase has been used as a marker for preneoplastic foci in rat livers (Dragosics et al, 1975; Cameron et al, 1978) and for neoplasia itself (Rosalki, 1975; Richards et al, 1982). γ-Glutamyl transpeptidase activity is not however elevated in all hepatomas (Fiala et al, 1972; Goldfarb and Pugh, 1981). γ-glutamyl transpeptidase activity in rat hepatomas has been reported to be within the same range of activity as normal tissues such as small intestine, kidney and lactating mammary gland (Fiala et al,
1976), indicating that there are probably no specific generalizations concerning \(\gamma\)-glutamyl transpeptidase activities of neoplastic tissues.

GSH can also be utilized by conjugation reactions, catalyzed by GSH S-transferases (GSH-transferases; Chassaud, 1979). As the assay procedure of the present studies, used styrene oxide as substrate, the GSH-transferase activity determined for murine lung and Lewis lung carcinoma primarily corresponded to GSH S-epoxide transferase activity (James et al., 1976). Cytosolic GSH transferase activity was significantly greater in Lewis lung carcinoma than pulmonary tissue. Yoshida AH-130 and Morris 3924A hepatomas have been observed to have considerably less soluble GSH-transferase activity than normal rat liver (Rossi et al., 1983). Neoplastic tissue from metastatic foci on human livers has been found to have less GSH S-aryl transferase activity than normal liver (Siegers and Younes, 1983). The GSH-transferase activity of rat hepatomas was however determined with 1-chloro-2, 4-dinitrobenzene (CDNB), a different substrate to that of the present studies, which has high specificity for the basic GSH-transferases (Habig and Jakoby, 1981). In the rat the basic transferases (those isoenzymes with comparatively alkaline isoelectric points) include A, AA, B, C and D (Hayes and Chalmers, 1983). Other GSH transferases, such as GSH transferases E and X of rat liver, are reported to have comparatively little reactivity with CDNB (Habig and Jakoby, 1981; Friedberg et al., 1983). The diminished general GSH-transferase activities, reported for rat hepatomas, might have concealed an unchanged or increased activity of one or more GSH-transferase isoenzymes. The observed GSH-transferase activities of Lewis lung carcinomas could have reflected a relatively conspicuous activity of just one enzyme. Other GSH-transferases, present in normal lung but with differing substrate specificities, were possibly absent or diminished in Lewis lung carcinoma. Future determinations of GSH-transferase activity in Lewis lung carcinoma should include procedures to differentiate the
various species of GSH-transferases (Habig and Jakoby, 1981) to overcome the ambiguities of the present investigations.

Lewis Lung Carcinoma Cells Maintained by Tissue Culture: A Single Cell Population

Lewis lung carcinoma cells from in vitro tissue culture system differed from those of solid tumour primarily because they were a population of one cell type, and had essentially unlimited access to essential nutrients. The oxygen tension of approximately 20% under which cells in culture were maintained, would also have been considerably greater than that of the Lewis lung tumour, which probably contained areas of hypoxia. Cultured Lewis lung carcinoma cells would have been proportionately more in metaphase than the cells of the solid Lewis lung tumour. Observations elsewhere have confirmed that i/m implanted Lewis lung carcinomas, of equivalent volume to those of the present studies, have a significantly lower proportion of cells in S phase of the cell cycle than cells maintained in tissue culture (Starace et al., 1982).

The relatively low cytosolic SOD activity of Lewis lung cells from the in vitro system infers that much of the activity determined for tissue from solid carcinoma, was possibly contributed by host cells, such as erythrocytes. Cytosolic GSH peroxidase and γ-glutamylcysteine synthetase activities were however similar between the in vivo and the in vitro systems. The contribution to tumour enzyme activities by erythrocyte enzymes was thus probably not significant.

Mitochondrial SOD of neonatal rat lung has been reported to be inducible by oxygen (Stevens and Autor, 1977). Catalase and GSH peroxidase activities of parenchymal cells from neonatal rat lung have also been reported to have greater activities at elevated oxygen tensions than at normal oxygen tension (Autor et al., 1979). As Lewis lung carcinoma
originated in lung, the activities of its antioxidant defence enzymes might be inducible by $O_2$. The comparatively greater mitochondrial SOD and GSH peroxidase activities of cultured Lewis lung cells could possibly reflect the greater oxygen tension of the in vitro growth system compared to that of the solid tumour.

The transfer of Lewis lung carcinoma cells from the conditions of intramuscular implantation to those of tissue culture might have caused selection of a new cell population. Those cells able to survive at comparatively greater oxygen tension, that is those cells with greater antioxidant defence enzyme activities, would be expected to persist.

Differences in the activities of antioxidant defence enzymes between cultured and i/m embedded Lewis lung carcinoma cells might also have been caused by the better nourishment of cells in culture (Tannock, 1982). $H_2O_2$ production by lung mitochondria and microsomes has been reported to be increased by hyperoxia (Turrens et al, 1982b). The increase in mitochondrial SOD activity of cultured cells in comparison to cells from solid Lewis lung carcinomas, was proportionately greater than the increase in mitochondrial GSH peroxidase. At the relatively hyperoxic conditions of tissue culture there was possibly excessive production of $H_2O_2$ by Mn-SOD of Lewis lung tumour cells. The generation of $H_2O_2$ may have exceeded the capacity for reduction by GSH peroxidase. Cu/Zn SOD of the cytosol might thus have been deactivated by the flux of $H_2O_2$ in the cultured Lewis lung tumour cells (Bray et al, 1974; Hodgson and Fridovich, 1975a). The concentration of $H_2O_2$ would have to have been generally sublethal to the tumour cells. 20% oxygen has been reported to be toxic to Lewis lung tumour cells in culture. Plating efficiency, that is the number of colonies arising per 100 cells plated onto tissue culture dishes, of Lewis lung carcinoma cells has been observed to be significantly improved by decreasing oxygen concentration from 20% to 5%
Other tumour cell lines have been observed to grow more readily at oxygen concentrations of 0.1% to 10%, as compared to 20% (Gupta and Eberle, 1984). Oxygen toxicity appears to have been mediated through the $O_2^{-}$ anion because the colony size and plating efficiency of Ehrlich ascites cells grown at 20% $O_2$ was enhanced (Gupta and Eberle, 1984).

The Effects of Hypoxia in Lewis Lung Carcinoma

The model of a solid tumour which is aerated at the periphery and hypoxic at the centre (Kennedy et al., 1980; figure 2.3) is probably an oversimplification because the distribution of blood vessels in solid tumours is probably not uniform and cell proliferation is irregular. The relation between aerated and relatively anoxic zones of Lewis lung carcinoma was therefore unlikely to have been symmetrical: there may well have been numerous foci of oxygenated or hypoxic cells within the body of the tumour. The technique, described in the present studies, may have, at best, isolated cortical regions of Lewis lung carcinoma which, on average, were proportionately more hypoxic than the residual tumour tissue.

The proportion of the cell population of subcutaneously-implanted Lewis lung carcinomas (of similar size to the i/m implanted tumours used in the present studies) occupied by hypoxic cells, has been estimated to be 36% (Shipley et al., 1975). The use, in the present studies, of a bore of tissue, representing 21-29% of the total mass of the tumour, was thus a reasonable estimate of the proportion of Lewis lung carcinoma that would have been hypoxic.

The observation of lower mitochondrial SOD activity at the centre of the tumour was similar to that elsewhere of the differential distribution of SOD activity in rat mammary carcinomas (Petkau et al., 1977). In ESR
FIG. 2.3 - Schematic Diagram of Oxygen Gradients in Solid Tumours.
This neoplasm is well vascularized on the surface by blood vessels extending from nearby normal tissues. Cells on the edge of necrotic area, located at the centre of the tumour, are remote from blood vessels, and are thought to be severely hypoxic, but viable. Hypoxic cells may be quiescent (Q) or may have prolonged cell cycle times. (from Kennedy et al, 1980)
studies, the spin concentrations of pulmonary tumours were observed to be highest at the tumour rim, decreasing towards the centre (Lohmann and Neubacher, 1984). This finding indicates that the lower TBA-reactive material concentration at the "core" of Lewis lung carcinomas reflected lower oxygen-derived free radical generation (and therefore autoxidation) in the hypoxic tumour centre.

As the tumour increases in size, its vascular network and thereby tissue oxygenation, decrease proportionately (Millar, 1982). In the present studies, mitochondrial SOD and GSH peroxidase activities decreased and \( \gamma \)-glutamylcysteine synthetase activities increased with time after implantation (increasing tumour volume). A positive correlation between weight of Lewis lung tumours and GSH/GSSG ratio was demonstrated in previous studies (Capel and Thornley, 1983). All these observations might be explained by the increasing contribution to the biochemical character of the enlarging Lewis lung carcinoma by its hypoxic cells.
CHAPTER THREE

EFFECT OF TUMOURS UPON ANTIOXIDANT DEFENCE
SYSTEMS OF MURINE HOST TISSUES
INTRODUCTION

The purpose of the studies, described in the present chapter, was to continue and improve the preliminary work ascertaining the influence of intramuscularly-implanted Lewis lung carcinoma upon the antioxidant defence systems of various murine tissues (Capel and Thornley, 1982; Capel and Thornley, 1983). The investigations were mainly concerned with lung, liver and blood of tumour-bearing mice. The lungs of mice, bearing Lewis lung carcinomas are likely to be infiltrated by metastatic deposits whereas the livers of these animals should be essentially free of tumour cells (Chapter 2). Hepatic tissue, from tumour-bearing mice, as an uninvolved tissue and pulmonary tissue, with its direct contribution from neoplastic cells, should therefore be of useful comparison.

GSH peroxidase and SOD activities, and glutathione concentrations, determined in the earlier investigations for tissues from Lewis lung carcinoma-bearing mice, were expressed in terms of wet weight of tissue (Capel and Thornley, 1982; Capel and Thornley, 1983). The tissues of cancer patients have been reported to contain a greater proportion of water than those of their healthy peers (Theologides, 1972). Therefore, to avoid ambiguity caused by possible changes in water concentration of the tissues of tumour-bearing mice, due to effects such as oedema, all determinations of the present studies were indexed to protein concentrations. Hepatic and pulmonary tissues were subdivided into mitochondrial and cytosolic fractions, and the appropriate SOD and GSH peroxidase activities determined. The activity of the major antioxidant defence enzyme, catalase was also determined.

Changes in biochemical parameters of host tissues, due to the
presence of a tumour, could possibly be caused by a shift in the cell population of a given tissue. DNA concentrations of pulmonary and hepatic tissues of mice, implanted with Lewis lung carcinomas, were ascertained accordingly as an estimation of cell numbers.

Hepatic GSH/GSSG ratio of Lewis lung carcinoma-bearing mice has been observed to increase as the tumour increased in size (Capel and Thornley, 1983). The alteration of glutathione status might have been caused by a change in the rate of generation or degradation of GSH or GSSG (Kosower and Kosower, 1978). Activities of the major enzymes, concerned with GSH synthesis, that is γ-glutamylcysteine synthetase, GSSG reductase and glucose-6-phosphate dehydrogenase (see Chapter 2), were therefore determined for hepatic and pulmonary tissues from mice bearing Lewis lung carcinomas. The activities of the major enzymes of GSH utilization (other than GSH peroxidase), namely GSH S-transferase and γ-glutamyl transpeptidase were also determined (Chapter 2).

Kidneys and blood, from Lewis lung carcinoma-bearing mice, were also investigated to ascertain whether murine tissues, other than liver and lung, were affected by tumour implantation. Renal GSH peroxidase and GSSG reductase activities, DNA concentration and TBA-reactive material concentration were determined. GSH and GSSG concentrations, and GSH peroxidase, GSSG reductase, γ-glutamylcysteine synthetase and catalase activities were determined for erythrocytes from mice bearing Lewis lung carcinomas.

The pattern of biochemical changes in non-neoplastic murine tissues, induced by the presence of a tumour, may be influenced by factors including the type and size of tumour, the species of the host and the site(s) of neoplastic growth (Greenstein, 1954). There have been comparatively few reports, in the biomedical literature, of the systemic effects of tumours upon host antioxidant defence systems, other than
catalase. Hepatic catalase activities of tumour-bearing animals and cancer patients have been observed to be almost invariably decreased (Greenstein, 1954; Begg, 1958; Kampschmidt, 1965). To ascertain whether the perturbations of enzymic antioxidant defence systems, exercised by Lewis lung carcinoma, were common to other murine tumour models, a limited investigation of the B16 melanoma was undertaken. Activities of GSH peroxidase, Cu/Zn-SOD, catalase, GSSG reductase and \( \gamma \)-glutamyl transpeptidase, and concentrations of DNA, GSH, GSSG and TBA-reactive substances were determined for liver and tumours of mice bearing intramuscularly (i/m) implanted B16 melanomas. Plasma caeruloplasmin oxidase activities, erythrocyte glutathione concentrations, and erythrocyte SOD, GSH peroxidase and GSSG reductase activities of B16 melanoma-bearing mice were compared with equivalent parameters from the tissues of tumour-free mice.

**EXPERIMENTAL**

**Chemicals**

\[ 8^{14} \text{C} \] Styrene oxide (specific activity 607 MBq/ mmol; purity 90.0 - 92.7%) and \( L-[1^{14} \text{C}] \) glutamic acid (specific activity 2 GBq/ mmol; purity 97.1 - 99.0%) were purchased from the Radiochemical Centre, Amersham, Bucks.

The ferricyanide/ cyanide solution (Drabkin's reagent) and the cyanomethaemoglobin standards, for the determination of haemoglobin concentration were supplied by Sigma London Chemical Co., Ltd., Poole, Dorset. All other chemicals were of the purest grades commercially available and were obtained from the suppliers, listed in Chapter 2.

**Animals and Treatment**
Animals and Treatment

Studies of animals implanted with Lewis lung carcinomas were undertaken with male C57BL6 mice (Olac). The mice and the Lewis lung carcinoma were maintained as described previously (Chapter 2). Mice had attained body weights of 21±3 g (8-10 weeks of age) by the time of inoculation with Lewis lung carcinoma cells.

B16 melanoma cells were implanted i/m into one hindlimb of male C57BL10 ScSn mice (Bantin and Kingman, Hull, Yorks), which had attained body weights of 28±3 g (11-13 weeks). Suspensions of cells from i/m implanted B16 melanomas were prepared essentially as detailed for Lewis lung carcinomas (Chapter 2), except that the duration of the second incubation with DNase and trypsin solutions, to disaggregate melanoma cells, was however extended to 45 min (Stephens et al, 1977).

B16 melanoma arose spontaneously in a C57BL mouse about thirty years ago, and has since been maintained in many laboratories world wide (Stephens and Peacock, 1978; Giavazzi et al, 1980). The B16 melanoma, like the Lewis lung carcinoma, has been in common use to screen compounds for anticancer activity (Berndal et al, 1983). The B16 melanoma cell line was obtained from Dr G.G. Steel of the Institute of Cancer Research (ICR), Sutton, Surrey, and has since been maintained by regular intramuscular passage in syngeneic C57BL10 mice. The tumour cell line was supplied to the ICR in 1970 by the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, USA (Stephens et al, 1977).

Mice, designated by random selection to bear tumours, received single hindlimb i/m inoculations (0.2ml) of 5 x 10^5 Lewis lung or B16 melanoma cells, as appropriate. Animals (age-matched), selected to be tumour-free controls, were injected with phosphate-buffered saline (0.2ml) only. Biochemical analyses of non-neoplastic tissues were
Histology

Mice bearing Lewis lung carcinomas, and tumour-free control animals were killed, at intervals from 10 to 16 days after implantation of tumour cells, by CO\textsubscript{2} narcosis and exsanguination via cardiopuncture. Skin and fur were stripped of the hindlimbs and thorax. The hindlimb, that had not been inoculated was disarticulated at the hipjoint. After 24 hrs immersion in phosphate-buffered 10% v/v formol saline, the hindlimbs were decalcified in Gooding and Stewart's fluid, as described previously (Chapter 2). Livers and lung were also excised from the mice, and immersed in 10% v/v phosphate-buffered formol saline for 8 days. Tissues were processed, embedded in paraffin-wax blocks, sectioned and mounted onto glass microscope slides, as described previously (Chapter 2).

Sections were stained with haematoxylin and eosin (H&E) for routine examination, using the microscope (Appendix IV.a). Other sections were stained with van Gieson's preparation, or with Perls' Prussian blue (Appendices IV.b and IV.c). A number of unstained sections of liver and lung were mounted in 3:1 glycerol/phosphate-buffered saline, and examined using a fluorescence microscope for lipofuscin-like autofluorescence (Porta and Hartroft, 1969; Reddy et al, 1982; Katz et al, 1984). The histological sections of liver, lung and hindleg were inspected by an independent examiner, and his appraisal of their pathology is given in the results section.

Preparation of Tissues and Instrumentation

Tissues were homogenised at 10% (lung) or 20% (liver, kidney, B16 melanoma) wet wt/ vol in 1.15% KCl, using a Potter-Elvehjem homogenizer. When mitochondrial fractions of tissues were required, the tissues were
homogenized in 0.25 M sucrose buffered by 1 mM TRIS, pH 7.4, containing 0.1 mM EGTA. Tissue homogenates were separated into subcellular fractions using a standard differential centrifugation procedure, described in Chapter 2 (Hogeboom, 1955). The storage conditions of samples for the various biochemical assays were as detailed previously (chapter 2).

Blood was removed from ether-anaesthetized animals via cardiopuncture with heparinized syringes and was transferred to heparinized screw-capped tubes. To obtain sufficient blood for assay, 2-3 samples of blood from each experimental group of mice were combined. Aliquots of blood were removed to estimate packed cell volume (PCV), haemoglobin concentration and glutathione concentrations (see later). The residual blood samples were centrifuged at 2500 rpm for 15 min. The supernatants (plasma) were transferred to fresh screw-capped tubes and, after storage at 4°C, were used for the determination of caeruloplasmin (B16 melanoma-bearing mice only). The red blood cells (RBC) were resuspended in 5 volumes of cold 0.9% w/v NaCl and centrifuged at 2500 rpm for 10 min to remove residual plasma and the majority of leucocytes and platelets (Beutler, 1971). After discarding the supernatant and buffy coat, the red cells were washed twice more. This procedure has been reported to result in a 10-fold decrease in the contamination of the RBC fraction by leucocytes (Beutler, 1971).

To prevent GSH oxidation by oxygen released during haemolysis of blood samples, carbon monoxide was gently bubbled through blood to displace oxygen from oxyhaemoglobin (Owens and Belcher, 1965). As the contribution to blood by plasma and other formed elements is negligible, whole blood was used for the determination of erythrocyte glutathione concentration (Beutler, 1971). Aliquots of deoxygenated blood (50μl) were added to metaphosphoric acid reagent (3.95ml) at 4°C, for immediate analysis of glutathione concentration. The metaphosphoric acid precipit-
ating solution, comprising of 1.67% w/v glacial metaphosphoric acid, 30% w/v NaCl and 0.2% EDTA, has been reported to be particularly suitable for the qualitative estimation of glutathione concentration because contaminating metal ions are chelated (Beutler, 1971).

All other determinations with erythrocytes, used haemolysates of washed red blood cells. For the assay of GSSG reductase activity, an aliquot of erythrocytes (50μl) was added to 0.1 M ice-cold sodium phosphate buffer (8.95ml), pH 7.4, containing 0.1% w/v EDTA. The red cells were lysed by adding 1% w/v saponin (0.2ml) to the haemolysates. For the remaining assay procedures, 10% v/v haemolysates in ice-cold H₂O were prepared. These haemolysates were diluted a further 5-fold and 20-fold for the GSH peroxidase and catalase determinations respectively.

In the assays to determine erythrocyte SOD activity, haemolysates were cleared of haemoglobin using the Tsuchihashi procedure (Misra and Fridovich, 1971). Ethanol (1ml) and chloroform (0.6ml) were added to 4ml of ice-cold 10% v/v haemolysate, and centrifuged at 2500 rpm for 15 min. The clear aqueous layer was used for assay of SOD activity.

The instrumentation used in the present studies was that described previously (Chapter 2).

Biochemical Assays

All biochemical assays, except those whose descriptions are to follow, have been detailed previously (Chapter 2). The concentrations of renal and hepatic samples, used in the assay procedures, were generally the same as those for pulmonary tissues (Chapter 2). Hepatic cytosol was however diluted 25-fold (from that obtained from 20% wet wt homogenate) for the determination of GSH peroxidase activity. Liver homogenates were diluted 1:1 by volume with ice-cold 25 w/v triton X-100 solution, sonicated and then diluted a further 100 fold before assay of catalase.
activity. B16 melanoma tissue was analysed at dilutions similar to those used for Lewis lung carcinoma (Chapter 2), except for the assay of γ-glutamyl transpeptidase activity, which required further dilution of homogenate, due to a relatively pronounced activity in B16 melanoma. The 20% wet wt/vol B16 melanoma homogenate was diluted 1:1 by volume with ice-cold 1% w/v triton, sonicated and then diluted 5 fold before assay of γ-glutamyl transpeptidase activity.

Haemoglobin concentration: Blood haemoglobin concentration (Hb) was determined by a standard procedure in which haemoglobin was converted to its cyano-derivative under alkaline conditions, and the concentration of the derivative estimated from its optical absorbance (Eilers, 1967). Duplicate aliquots (20ul) of blood were added to Drabkin's reagent (5ml), a solution of 0.02% w/v K₃Fe(CN)₆, 0.005% w/v KCN and 0.1% w/v NaHCO₃. The Drabkins's reagent was reconstituted from a commercially-supplied powdered preparation (Sigma). The reaction mixtures were vortexed and left for 15 min at room temperature. The optical absorbances, at 540 nm, of the mixtures were measured spectrophotometrically against a blank of Drabkin's solution only. The haemoglobin concentrations of the blood samples were quantified by comparison with the optical absorbances obtained from the reaction of a range (5-45 g/100ml) ofmethaemoglobin standards (Sigma).

Packed cell volume of blood samples was estimated by the standard method of centrifugation (2500 rpm for 15 min) of whole blood in graduated capillary tubes of uniform bore.

Caeruloplasmin: Plasma caeruloplasmin was determined from its oxidase activity with o-dianisidine (Schosinsky et al, 1974). Quadruple reaction mixtures, comprising 0.75ml of 0.1 M acetate buffer, pH 5.0, and 50ul of plasma were preincubated for 5 min at 30°C. Reactions were started by the addition of 0.2ml of 7.9 mM o-dianisidine dihydrochloride solution
(prewarmed to 30°C), at timed intervals. After 5 min, reactions in one pair from each set of tubes, were terminated by adding 9 M H₂SO₄ (2m1). The tubes were mixed by Vortex and cooled to ambient temperature. This procedure was repeated for the remaining reaction mixtures after a further 10 min of incubation. The absorbances, at 540 nm, of the mixtures were measured spectrophotometrically against distilled water. Caeruloplasmin oxidase activity of the plasma samples was proportional to the rate of formation of oxidized dianisidine. A unit of caeruloplasmin oxidase activity was defined as 6.25 x the increase in optical absorbance units, at 540 nm, /min at 30°C, pH 5.0 (Schosinsky et al, 1974). Caeruloplasmin oxidase activities were expressed as units per ml plasma.

Protein: For reasons given in the preceding chapter, two different methods to estimate protein concentration were employed in the present studies. DNA, glutathione and TBA-reactive material concentrations, GSSG reductase activity and GSH S-transferase activities of samples were related to protein concentrations, determined using Coomassie blue reagent (Spector, 1978). Whole tissue GSH peroxidase and Cu/Zn-SOD activities from mice bearing B16 melanomas were also indexed to protein concentrations, quantified by the Coomassie blue method. All other determinations were linked to protein concentrations, estimated by the standard procedure, using Folin-Ciocalteu reagent (Lowry et al, 1951). The tables of results that follow the present section, indicate the method of protein assay, where applicable.

Lipoperoxide (Thiobarbituric acid reactive material): Lipoperoxide concentrations were estimated by a different application of the method to determine the concentrations of TBA-reactive substances (Satoh, 1978) to that used in the studies described previously (Chapter 2). In this procedure, only the trichloroacetic acid (TCA) insoluble components of the tissue samples were assayed. Dilute H₂SO₄ was used instead of
phthalate buffer, for the acid-heating stage of the procedure. The concen-
tration of TBA-reactive material (primarily TBA-malondialdehyde adduct; Gutteridge, 1977), liberated by from the samples was again quantified
fluorimetrically. Fluorimetric measurement of TBA-reactive substances was
preferred to spectrophotometric determination (via optical absorbance at
540 nm) because it has been reported to be less affected by optical
interference due to endogenous substances such as bilirubin (Ohkawa et
al., 1979). The fluorimetric method has been observed to be a more
sensitive technique, with greater specificity for the TBA-malondialdehyde
adduct, than the spectrophotometric method (Gutteridge and Tickner,
1978).

All glassware, used in the assay procedure, was acid-washed (in
nitric acid) to remove any residual detergent from usual washing, a
possible source of fluorescent interference. Duplicate tissue samples
(0.1ml) were added to 20% w/v TCA, in screw-capped tubes: mixed by
Vortex; and left to stand for 10 min at room temperature. The mixtures
were centrifuged at 2500 rpm for 20 min. The supernatants were decanted
and the precipitates washed twice in 5ml volumes of 0.05 M \( \text{H}_2\text{SO}_4 \). The
precipitates were resuspended in 2.5ml of 0.05 M \( \text{H}_2\text{SO}_4 \), and 3ml of 0.2%
TBA in \( 2\text{M} \ \text{Na}_2\text{SO}_4 \) was added. The mixtures were vortexed: the screw caps
were placed tightly on the tubes; and the mixtures were incubated at 100°
C for 30 min. After cooling the mixtures to room temperature, the fluo-
rescent reaction products were extracted into butan-1-ol (4ml). The \( \text{Na}_2\text{SO}_4 \)
was used, in the reaction mixtures, to prevent emulsification during the
extraction into organic solvent (Satoh, 1978). A range (0.125-1.000 mmol)
of 1,1,3,3- tetramethoxypropane standards in 2.5ml of 0.05 M \( \text{H}_2\text{SO}_4 \) was
similarly incubated with TBA (3ml), and the TBA-malondialdehyde fluoro-
phore extracted into butan-1-ol (4ml). The concentrations of TBA-reactive
substances in the samples were evaluated by comparison of their spectro-
fluorescence (535 nm excitation and 553 nm emission) with those of the
Statistical Analyses

Results were expressed as mean ± SEM. Data from experimental groups containing 3 or more observations were statistically evaluated by analysis of variance. Means of the estimations for tissues from tumour-bearing animals were compared with those of tumour-free controls through use of the Student-type range statistic in the Newman-Keuls procedure (Winer, 1971). Results were considered to be significant when P<0.05.

RESULTS

Lewis Lung Carcinoma-Bearing Mice

Histology of liver and lung: The external appearance of mice, bearing i/m implanted Lewis lung carcinoma, has been described previously (Chapter 2).

Liver sections, stained with H&E and removed from mice implanted with Lewis lung carcinomas, had considerably greater incidence of inflammatory aggregates and mitotic cells than liver sections from tumour-free animals (Table 3.1; plate V). Livers from mice, with Lewis lung carcinomas, also displayed foci of atypical cells (table 3.1). The histological differences between the livers of tumour-bearing and tumour-free mice became more apparent at 13 days and beyond, after inoculation with Lewis lung carcinoma cells (table 3.1).

Sections from the liver of a mouse, killed 13 days after implantation with Lewis lung carcinoma, showed pronounced histological abnormalities, which included fatty vacuolation, hypertrophy of hepatocytes and the presence of golden brown pigment and hepatocytes with granular cytoplasm (table 3.1). Hepatic sections, from this animal, when stained
TABLE 3.1 - Histopathology of the Livers from Lewis Lung Carcinoma-bearing C57BL6 Mice. Livers from tumour-free litter mates were used as controls.

<table>
<thead>
<tr>
<th>Time after transplantation of tumour (days)</th>
<th>Appraisal of histopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>10</td>
<td>Occasional inflammatory focus</td>
</tr>
<tr>
<td>11</td>
<td>Normal</td>
</tr>
<tr>
<td>13</td>
<td>Plant cells. Occasional inflammatory cells.</td>
</tr>
<tr>
<td>14</td>
<td>Extensive plant cell formation. Occasional focus of inflammatory cells.</td>
</tr>
<tr>
<td>15</td>
<td>Extensive plant cell formation.</td>
</tr>
<tr>
<td>16</td>
<td>Extensive plant cell formation.</td>
</tr>
</tbody>
</table>

* "Plant cell phenomenon" - Cell wall, plasma membrane and cytoplasm resemble those of plant cells. Observed when there is considerable glycogen in the cells. Not a vacuole because the cell walls are not smooth.
Plate V - Section through liver (H&E):

a) tumour-free mouse (x 450)

b) Lewis lung carcinoma-bearing mouse (x 450).

Note: inflammatory infiltrates and loss of structural organisation. 13 days post implantation of tumour.
with Perls' Prussian blue, contained considerably more haemosiderin-like pigments than comparative sections from tumour-free mice (plate VI).

Lipofuscin-like autofluorescence of unstained sections of murine liver was minimal for control animals. Liver sections, from tumour-bearing mice, particularly one killed 13 days after implantation of a Lewis lung carcinoma, did, however, show significant yellow-orange autofluorescence when illuminated with u.v. light.

Inspection of pulmonary sections from mice, bearing Lewis lung carcinomas for 11 or more days, revealed the presence of tumour nodules (table 3.2; plate VII). Some sections of pulmonary tissue, from tumour-bearing mice, contained occasional inflammatory cells. There were appreciable blue haemosiderin-like pigments, associated with metastatic cells, in sections of lung, stained with Perls'. Histological sections of pulmonary tissue, from Lewis lung carcinoma-bearing mice, did not otherwise differ from pulmonary sections from tumour-free animals (table 3.2). Sections of the hindlimb of tumour-bearing mice, which was not inoculated with Lewis lung carcinoma cells, were of normal appearance (plates Ia and IIIa).

Organ weights: The wet weights of livers and lungs from mice, bearing Lewis lung carcinomas, did not differ from those of tumour-free controls (table 3.3). The weight of the kidneys from mice, killed 15 days after tumour-implantation, was significantly less than that of control mice (table 3.17). Hepatic and pulmonary weights, expressed as a percentage of body weight (minus tumour weight) were significantly greater in mice, killed 15 days after inoculation with Lewis lung carcinoma cells, than in tumour-free controls (table 3.3). The percentage of body weight, accounted by kidneys, in tumour-bearing mice did not differ from that of control mice (table 3.17). The spleens of mice (n=18) that had borne Lewis lung carcinomas for 9-15 days weighed 160 ± 20% more than the
Plate VI - Section (Perls') through liver of Lewis lung carcinoma-bearing mouse (x 450).
Note: 15 days post implantation of tumour.
<table>
<thead>
<tr>
<th>Time after transplantation of tumour (days)</th>
<th>Control</th>
<th>Histopathology</th>
<th>Tumour-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Agonal haemorrhage in some bronchi.</td>
<td>Agonal haemorrhage in some bronchi.</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Agonal haemorrhage in some bronchi.</td>
<td>Tumour nodule present.</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Normal</td>
<td>Occasional interstitial focus of inflammatory cells in one lobe. A second lobe contains a zone of apical haemorrhage associated with an anaplastic epitheloid carcinoma and a number of islets of (?)tumour cells. A second nodule is also present. A low grade mucoid exudate is present in some bronchi.</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Agonal haemorrhage in some bronchi.</td>
<td>Tumour nodule present.</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Normal</td>
<td>Inflammatory cells in alveolar wall. One sub-pleural tumour nodule and one in the body of the section. Tumour cells scattered elsewhere in the parenchyma.</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Peribronchiolar lymphoid hyperplasia and perivascular &quot;cuffing&quot;.</td>
<td>Sub-pleural tumour nodule associated with outer zone of haemorrhage. A larger more diffuse area of haemorrhage with a sheet of tumour cells also present.</td>
<td></td>
</tr>
</tbody>
</table>
Plate VII - Sections (H&E) through lung of Lewis lung tumour-bearing mouse, showing metastatic tumour deposits (x 275).
Note: 12 days post implantation of tumour.
Plate VIII- Section (Perls') through lung of Lewis lung carcinoma-bearing mouse:

a) Region of non-neoplastic lung containing small metastatic deposit (x 450)

b) Region of neoplastic cells, containing extensive blue haemosiderin-like pigment (x 450).

Note: 15 days post implantation of tumour.
TABLE 3.3 - Liver and Lung Weights of Lewis Lung Carcinoma-bearing C57BL6 Mice and of Tumour-free Controls.
Results represent mean ± SEM for 6 mice at each time interval

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Liver Control</th>
<th>Liver Tumour-bearing</th>
<th>Lung Control</th>
<th>Lung Tumour-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight as a % of (body wt minus tumour wt)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(wet wt [g] of tissues shown in brackets)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5.4 ± 0.5</td>
<td>4.9 ± 0.1</td>
<td>0.85 ± 0.05</td>
<td>0.85 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>(1.4 ± 0.1)</td>
<td>(1.4 ± 0.05)</td>
<td>(0.19 ± 0.01)</td>
<td>(0.19 ± 0.01)</td>
</tr>
<tr>
<td>7</td>
<td>5.4 ± 0.5</td>
<td>5.4 ± 0.4</td>
<td>0.85 ± 0.01</td>
<td>0.85 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>(1.4 ± 0.1)</td>
<td>(1.5 ± 0.2)</td>
<td>(0.20 ± 0.01)</td>
<td>(0.20 ± 0.01)</td>
</tr>
<tr>
<td>9</td>
<td>5.4 ± 0.5</td>
<td>5.1 ± 0.2</td>
<td>0.90 ± 0.05</td>
<td>0.85 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>(1.4 ± 0.2)</td>
<td>(1.3 ± 0.1)</td>
<td>(0.22 ± 0.01)</td>
<td>(0.21 ± 0.01)</td>
</tr>
<tr>
<td>11</td>
<td>5.6 ± 0.2</td>
<td>5.4 ± 0.4</td>
<td>0.90 ± 0.05</td>
<td>0.87 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>(1.4 ± 0.1)</td>
<td>(1.4 ± 0.1)</td>
<td>(0.19 ± 0.01)</td>
<td>(0.19 ± 0.01)</td>
</tr>
<tr>
<td>13</td>
<td>5.6 ± 0.4</td>
<td>5.4 ± 0.5</td>
<td>0.85 ± 0.04</td>
<td>0.85 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>(1.4 ± 0.1)</td>
<td>(1.4 ± 0.1)</td>
<td>(0.21 ± 0.01)</td>
<td>(0.20 ± 0.01)</td>
</tr>
<tr>
<td>15</td>
<td>5.5 ± 0.3</td>
<td>7.4 ± 0.4</td>
<td>0.85 ± 0.05</td>
<td>1.02 ± 0.01b</td>
</tr>
<tr>
<td></td>
<td>(1.4 ± 0.1)</td>
<td>(1.4 ± 0.2)</td>
<td>(0.20 ± 0.01)</td>
<td>(0.19 ± 0.01)</td>
</tr>
</tbody>
</table>

a-b Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
Protein and DNA: Hepatic, pulmonary and renal protein concentrations of mice, bearing Lewis lung carcinomas, did not differ significantly from those of tumour-free controls (tables 3.4, 3.6 and 3.17). As determined in the studies of chapter 2, values for protein concentrations of tissue homogenates, when estimated using Coomassie blue reagent (Spector, 1978) were approximately half those of estimations by the procedure, using Folin-Ciocalteu reagent (Lowry et al, 1951).

Hepatic DNA concentrations of mice, killed 9, 13 and 15 days after inoculation with Lewis lung carcinoma cells, were significantly greater than those of tumour-free mice (table 3.5). Pulmonary DNA concentration was significantly increased, in mice that had borne Lewis lung carcinomas for 13 days (table 3.5). Renal DNA concentrations of Lewis lung carcinoma-bearing mice did not differ significantly from those of control mice (table 3.17).

Antioxidant defence enzymes: Cytosolic GSH peroxidase activities of the livers and lungs of mice with Lewis lung carcinomas, were not significantly different from those of controls (table 3.7). Renal GSH peroxidase activities, determined for whole tissue homogenate, of tumour-bearing mice did not differ from those of control animals (table 3.17). Mitochondrial GSH peroxidase activities of livers from mice, killed 15 and 17 days after implantation of Lewis lung carcinomas, were significantly less than those of tumour-free mice (table 3.8).

Neither cytosolic (Cu/Zn-) nor mitochondrial (Mn-) SOD activities in liver and lung tissue of Lewis lung carcinoma-bearing mice were significantly different from the activities of tumour-free controls (tables 3.9 and 3.10). Hepatic and pulmonary catalase activities of animals, killed 13-17 days after implantation of Lewis lung carcinomas, were signifi-
**TABLE 3.4 - Liver and Lung Protein Concentrations of Lewis Lung Carcinoma-bearing C57BL6 Mice and of Tumour-free Controls.**
Results represent mean ± SEM for 6 mice at each time interval. Protein was determined with Coomassie blue reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Liver Control</th>
<th>Liver Tumour-bearing</th>
<th>Lung Control</th>
<th>Lung Tumour-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg/ g wet wt of tissue)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>69 ± 3</td>
<td>72 ± 4</td>
<td>26 ± 2</td>
<td>27 ± 1</td>
</tr>
<tr>
<td>7</td>
<td>72 ± 2</td>
<td>74 ± 2</td>
<td>30 ± 2</td>
<td>29 ± 2</td>
</tr>
<tr>
<td>9</td>
<td>69 ± 3</td>
<td>72 ± 3</td>
<td>26 ± 2</td>
<td>28 ± 1</td>
</tr>
<tr>
<td>11</td>
<td>71 ± 3</td>
<td>76 ± 4</td>
<td>31 ± 1</td>
<td>27 ± 1</td>
</tr>
<tr>
<td>13</td>
<td>72 ± 3</td>
<td>73 ± 4</td>
<td>27 ± 1</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>15</td>
<td>74 ± 2</td>
<td>69 ± 2</td>
<td>30 ± 2</td>
<td>28 ± 1</td>
</tr>
</tbody>
</table>

No significant differences (P>0.05) by Newman-Keuls test (after analysis of variance).
<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Liver</th>
<th>Lung</th>
<th>Liver</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Tumour-bearing</td>
<td>Control</td>
<td>Tumour-bearing</td>
</tr>
<tr>
<td></td>
<td>(µg foetal calf DNA equiv./mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>13.7 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.1 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>113 ± 10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>108 ± 4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>12.8 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.2 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>106 ± 10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>110 ± 6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>12.2 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.2 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>103 ± 10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>112 ± 9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>11</td>
<td>12.3 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.6 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>98 ± 5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>136 ± 8&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>13</td>
<td>12.6 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.0 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>105 ± 3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>138 ± 8&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>12.5 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.8 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>113 ± 6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>124 ± 11&lt;sup&gt;ce&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-d</sup> Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 3.6 - Liver and Lung Protein Concentrations of Lewis Lung Carcinoma-bearing C57BL6 Mice and of Tumour-free Controls. Results represent mean ± SEM for 6 mice at each time interval. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Liver Control</th>
<th>Liver Tumour-bearing</th>
<th>Lung Control</th>
<th>Lung Tumour-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>160 ± 8</td>
<td>153 ± 5</td>
<td>58.3 ± 4.4</td>
<td>68.3 ± 4.7</td>
</tr>
<tr>
<td>15</td>
<td>153 ± 8</td>
<td>149 ± 5</td>
<td>60.0 ± 4.7</td>
<td>63.7 ± 3.6</td>
</tr>
<tr>
<td>17</td>
<td>155 ± 7</td>
<td>151 ± 5</td>
<td>60.6 ± 2.9</td>
<td>67.0 ± 3.3</td>
</tr>
</tbody>
</table>

No significant differences (P>0.05) by Newman-Keuls test (after analysis of variance).
TABLE 3.7 - Cytosolic GSH Peroxidase Activities in Hepatic and Pulmonary Tissues of Lewis Lung Carcinoma-bearing C57BL6 Mice, and of Tumour-free Controls. Results represent mean ± SEM for 5 mice at each time interval. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Liver</th>
<th>Lung</th>
<th>Liver</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Tumour-bearing</td>
<td>Control</td>
<td>Tumour-bearing</td>
</tr>
<tr>
<td></td>
<td>(Enzyme units * / mg protein)</td>
<td>(Enzyme units * / mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>118 ± 8</td>
<td>104 ± 6</td>
<td>29.4 ± 2.8</td>
<td>29.0 ± 1.4</td>
</tr>
<tr>
<td>15</td>
<td>120 ± 3</td>
<td>119 ± 2</td>
<td>29.6 ± 3.4</td>
<td>31.9 ± 3.5</td>
</tr>
<tr>
<td>17</td>
<td>123 ± 8</td>
<td>120 ± 7</td>
<td>28.7 ± 3.7</td>
<td>29.7 ± 2.1</td>
</tr>
</tbody>
</table>

* Enzyme units are defined as 1000 x log₁₀ (decrease in GSH/min) at 37°C, pH 7.0.

No significant differences (P>0.05) by Newman-Keuls test (after analysis of variance).
TABLE 3.8 - Mitochondrial GSH Peroxidase Activities in Hepatic and Pulmonary Tissues of Lewis Lung Carcinoma-bearing C57BL6 Mice, and of Tumour-free Controls.
Results represent mean ± SEM for 5 mice at each time interval. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Liver</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Tumour-bearing</td>
</tr>
<tr>
<td></td>
<td>Enzyme units*/mg protein</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>17 ± 2</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>15</td>
<td>17 ± 1</td>
<td>12 ± 1a</td>
</tr>
<tr>
<td>17</td>
<td>17 ± 2</td>
<td>11 ± 1a</td>
</tr>
</tbody>
</table>

* Enzyme units are defined as 1000 x log 10 (decrease in GSH/min) at 37°C, pH 7.0.

a significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 3.9 - Cytosolic Superoxide Dismutase Activities in Hepatic and Pulmonary Tissues of Lewis Lung Carcinoma-bearing C57BL6 Mice, and of Tumour-free Controls.

Results represent mean ± SEM for 3 control or 6 tumour-bearing mice at each time interval. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Liver Control</th>
<th>Liver Tumour-bearing</th>
<th>Lung Control</th>
<th>Lung Tumour-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(units [Sigma^/- mg protein at 25 ± 2° C, pH 7.5)</td>
<td></td>
<td>(units [Sigma^/- mg protein at 25 ± 2° C, pH 7.5)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>40 ± 2</td>
<td>42 ± 2</td>
<td>9.5 ± 0.8</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>14</td>
<td>41 ± 2</td>
<td>42 ± 2</td>
<td>12 ± 1</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>17</td>
<td>37 ± 2</td>
<td>36 ± 2</td>
<td>9.2 ± 1.6</td>
<td>13 ± 1</td>
</tr>
</tbody>
</table>

No significant differences (P>0.05) by Newman-Keuls test (after analysis of variance).
TABLE 3.10 - Mitochondrial Superoxide Dismutase Activities in Hepatic and Pulmonary Tissues of Lewis Lung Carcinoma-bearing C57BL6 Mice, and of Tumour-free Controls.
Results represent mean ± SEM for 3 control or 6 tumour-bearing mice at each time interval. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Liver Control</th>
<th>Liver Tumour-bearing</th>
<th>Lung Control</th>
<th>Lung Tumour-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.8 ± 0.3</td>
<td>4.2 ± 0.9</td>
<td>0.25 ± 0.8</td>
<td>0.30 ± 0.01</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>4.3 ± 0.8</td>
<td>3.0 ± 0.7</td>
<td>0.29 ± 0.02</td>
<td>0.30 ± 0.07</td>
</tr>
<tr>
<td>17</td>
<td>3.1 ± 0.2</td>
<td>3.7 ± 0.9</td>
<td>0.28 ± 0.07</td>
<td>0.25 ± 0.06</td>
</tr>
</tbody>
</table>

No significant differences (P>0.05) by Newman-Keuls test (after analysis of variance).
Activities of other enzyme systems that may influence glutathione status:

Hepatic γ-glutamylcysteine synthetase activities were significantly less than control values, 15 days after inoculation with Lewis lung carcinoma cells, but significantly greater than the activities of control animals, 17 days post implantation (table 3.12). Pulmonary γ-glutamylcysteine synthetase activities were significantly less than those of control mice, 13 and 15 days after tumour-implantation, but significantly raised at 17 days (table 3.12).

Mitochondrial γ-glutamyl transpeptidase activities, determined for liver and lungs of mice, killed 14 days after inoculation with Lewis lung tumour cells, were significantly greater than the activities of control mice (table 3.13). Whole tissue, microsomal and cytosolic γ-glutamyl transpeptidase activities of tumour-bearing mice did not differ from those of tumour-free mice (table 3.13).

Hepatic GSSG reductase activities of Lewis lung carcinoma-bearing mice, at 13 and 15 days post implantation, were significantly greater than those of controls (table 3.14). Pulmonary GSSG reductase activities of mice, bearing Lewis lung carcinomas, were significantly increased (above control levels) at 11, 13 and 15 days after inoculation with tumour cells (table 3.14). Renal GSSG reductase activities of carcinoma-bearing mice did not differ from those of control animals (table 3.17).

Cytosolic GSH S-transferase activities, determined with styrene oxide as substrate, were significantly greater in the livers of Lewis lung carcinoma-bearing mice, at 9 to 15 days post implantation, than in those of tumour-free controls (table 3.15). Pulmonary GSH S-transferase activities of carcinoma-bearing mice did not differ generally from control values, except at 7 days post implantation, when GSH
TABLE 3.11 - Catalase Activities in Hepatic and Pulmonary Tissues of Lewis Lung Carcinoma-bearing C57BL6 Mice, and of Tumour-free Controls. Results represent mean ± SEM for 6 mice at each time interval. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Liver</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>Tumour-bearing</td>
<td>Tumour-bearing</td>
</tr>
<tr>
<td></td>
<td>(k* / min / mg protein)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>2.8 ± 0.2</td>
<td>1.8 ± 0.2^a</td>
</tr>
<tr>
<td>15</td>
<td>2.7 ± 0.3</td>
<td>1.8 ± 0.2^a</td>
</tr>
<tr>
<td>17</td>
<td>2.5 ± 0.2</td>
<td>1.4 ± 0.1^a</td>
</tr>
</tbody>
</table>

* k is the first-order reaction rate constant at 2 ± 1°C, pH 7.4.

^a^-^b Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 3.12 - γ-Glutamylcysteine Synthetase Activities in Hepatic and Pulmonary Tissues of Lewis Lung Carcinoma-bearing C57BL6 Mice, and of Tumour-free Controls.
Results represent mean ± SEM for 6 mice at each time interval. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Liver</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Tumour-bearing</td>
</tr>
<tr>
<td></td>
<td>(nmol/min/mg cytosolic protein at 37°C, pH 8.25)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>3.5 ± 0.2</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>15</td>
<td>3.2 ± 0.2</td>
<td>1.8 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>17</td>
<td>3.3 ± 0.1</td>
<td>4.5 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-f</sup> Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 3.13 — Whole Tissue and Subcellular \( \gamma \)-Glutamyl Transpeptidase Activities in Hepatic and Pulmonary Tissues of Lewis Lung Carcinoma-bearing C57BL6 Mice, and of Tumour-free Controls.

Results represent mean ± SEM for 6 mice, killed 14 days after inoculation of tumour cells (or with PBS for the controls. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Tumour-bearing</td>
</tr>
<tr>
<td>Whole tissue</td>
<td>0.06 ± 0.01</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.25 ± 0.02</td>
<td>0.35 ± 0.01\textsuperscript{a}</td>
</tr>
<tr>
<td>Microsomes</td>
<td>0.34 ± 0.04</td>
<td>0.33 ± 0.05</td>
</tr>
<tr>
<td>Cytosol</td>
<td>0.19 ± 0.02</td>
<td>0.16 ± 0.03</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Significantly different (P<0.05) by unpaired Student's \( t \)-test from the corresponding control value.
TABLE 3.14 - Liver and Lung GSSG Reductase Activities of Lewis Lung Carcinoma-bearing C57BL6 Mice and of Tumour-free Controls.

Results represent mean ± SEM for 6 mice at each time interval. Protein was determined with Coomassie blue reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Liver Control</th>
<th>Tumour-bearing</th>
<th>Lung Control</th>
<th>Tumour-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol NADPH oxidized/ min/ mg protein at 37°C, pH 7.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>99 ± 3</td>
<td>95 ± 5</td>
<td>104 ± 5</td>
<td>91 ± 6</td>
</tr>
<tr>
<td>7</td>
<td>93 ± 3</td>
<td>90 ± 1</td>
<td>101 ± 5</td>
<td>106 ± 5</td>
</tr>
<tr>
<td>9</td>
<td>99 ± 4</td>
<td>92 ± 3</td>
<td>105 ± 4</td>
<td>106 ± 3</td>
</tr>
<tr>
<td>11</td>
<td>97 ± 7</td>
<td>93 ± 5</td>
<td>114 ± 3</td>
<td>148 ± 10b</td>
</tr>
<tr>
<td>13</td>
<td>102 ± 3</td>
<td>142 ± 5a</td>
<td>107 ± 2</td>
<td>152 ± 3b</td>
</tr>
<tr>
<td>15</td>
<td>97 ± 3</td>
<td>127 ± 4a</td>
<td>105 ± 6</td>
<td>149 ± 4b</td>
</tr>
</tbody>
</table>

a-b Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
S-transferase activity was significantly decreased (table 3.15).

Cytosolic glucose-6-phosphate dehydrogenase activities of the livers of mice with Lewis lung carcinomas were significantly less than control activities, at 15 and 17 days after tumour implantation (table 3.16). Pulmonary glucose-6-phosphate dehydrogenase activities of Lewis lung carcinoma-bearing animals were significantly greater than those of control mice (table 3.16).

Renal TBA-reactive material: concentrations of Lewis lung carcinoma-bearing mice were not significantly different from those of tumour-free control animals (table 3.17).

Antioxidant defence systems of blood: Mice became severely anaemic by 11-13 days after implantation with Lewis lung carcinomas (table 3.18). Erythrocyte GSH concentration of mice, killed 15 days after inoculation with Lewis lung carcinoma cells, was greater than that of control animals (table 3.19). Red blood cells, of mice that had borne Lewis lung carcinomas for 9 to 15 days, had greater GSSG reductase activities and lower GSH peroxidase activities, than those of tumour-free mice (table 3.20). Erythrocyte γ-glutamylcysteine synthetase activities of carcinoma-bearing mice were raised above the levels of control animals (table 3.21). Catalase activities of erythrocytes from mice, bearing Lewis lung carcinomas, did not differ from those of controls (table 3.21).

The major effects of bearing a Lewis lung carcinoma, upon non-neoplastic murine tissues, distal to the site of tumour implantation, as determined in the present studies, are summarized in table 3.36.

B16 Melanoma-Bearing Mice

Growth and appearance of B16 melanomas: Intramuscularly-embedded B16
### TABLE 3.15 - Liver and Lung GSH S-Transferase Activities of Lewis Lung Carcinoma-bearing C57BL6 Mice and of Tumour-free Controls.

Results represent mean ± SEM for 6 mice at each time interval. Protein was determined with Coomassie blue reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Liver Control</th>
<th>Liver Tumour-bearing</th>
<th>Lung Control</th>
<th>Lung Tumour-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol styrene oxide conjugated/ min/ mg cytosolic protein at 37°C, pH 7.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>38 ± 5</td>
<td>51 ± 4</td>
<td>32 ± 3&lt;sup&gt;cd&lt;/sup&gt;e</td>
<td>33 ± 3&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>38 ± 1</td>
<td>49 ± 2</td>
<td>32 ± 2&lt;sup&gt;cd&lt;/sup&gt;e</td>
<td>24 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>39 ± 2</td>
<td>52 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29 ± 1&lt;sup&gt;ce&lt;/sup&gt;</td>
<td>26 ± 1&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>11</td>
<td>37 ± 1</td>
<td>48 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31 ± 2&lt;sup&gt;de&lt;/sup&gt;</td>
<td>36 ± 1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>13</td>
<td>40 ± 3</td>
<td>51 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34 ± 1&lt;sup&gt;de&lt;/sup&gt;</td>
<td>41 ± 2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>42 ± 3</td>
<td>53 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34 ± 1&lt;sup&gt;de&lt;/sup&gt;</td>
<td>36 ± 1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-e</sup> Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 3.16 - Glucose-6-Phosphate Dehydrogenase Activities in Hepatic and Pulmonary Tissues of Lewis Lung Carcinoma-bearing C57BL6 Mice, and of Tumour-free Controls. Results represent mean ± SEM for 6 mice at each time interval. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after Liver implantation of tumour (days)</th>
<th>Liver Control</th>
<th>Tumour-bearing</th>
<th>Lung Control</th>
<th>Tumour-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol NADPH produced/ min/ mg cytosolic protein at 37° C, pH 7.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>17 ± 2</td>
<td>14 ± 2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>51 ± 2</td>
<td>86 ± 5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>16 ± 2</td>
<td>11 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>53 ± 1</td>
<td>92 ± 8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>17</td>
<td>16 ± 2</td>
<td>10 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>52 ± 3</td>
<td>77 ± 5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a–c</sup> Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
**TABLE 3.17 - Weight, Protein Concentration, DNA Concentration, GSSG Reductase Activity, GSH Peroxidase Activity and Thiobarbituric Acid-reactive Material Concentration of Kidneys from Lewis Lung Carcinoma-bearing C57BL6 Mice, and from Tumour-free Controls.**

Results represent mean ± SEM for 6 mice at each time interval. Protein was determined with Coomassie blue reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th></th>
<th>13 days after tumour implantation</th>
<th>15 days after tumour implantation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Tumour-bearing</td>
</tr>
<tr>
<td>% of body wt</td>
<td>0.59 ± 0.02</td>
<td>0.59 ± 0.06</td>
</tr>
<tr>
<td>Kidney weight (g wet wt)</td>
<td>117 ± 2</td>
<td>117 ± 6</td>
</tr>
<tr>
<td>protein conc(^n) (mg/ g wet wt)</td>
<td>70 ± 5</td>
<td>69 ± 2</td>
</tr>
<tr>
<td>DNA conc(^n) (μg/ mg protein)</td>
<td>38 ± 2</td>
<td>43 ± 3</td>
</tr>
<tr>
<td>GSSG reductase (nmol/min/ mg protein)</td>
<td>175 ± 9</td>
<td>174 ± 6</td>
</tr>
<tr>
<td>GSH peroxidase(_S) (Enzyme units /mg protein)</td>
<td>142 ± 9</td>
<td>149 ± 9</td>
</tr>
<tr>
<td>TBA reactivity(#) (nmol MDA equiv. /mg protein)</td>
<td>0.8 ± 0.1(^b)</td>
<td>1.1 ± 0.2</td>
</tr>
</tbody>
</table>

* Enzyme units are defined as 1000 x log\(_{10}\) (decrease in GSH/min) at 37°C, pH 7.0.

\# Chromophores obtained by incubation for 30 min at 100°C in 0.05 M H\(_2\)SO\(_4\), in the absence of any antioxidant additive.

\(^a\)-\(^c\) Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 3.18 - Haematocrits, and Haemoglobin Concentrations of Blood from Lewis Lung Carcinoma-bearing C57BL6 Mice, and from Tumour-free Controls. Results represent mean of two observations (each from the blood composite of 3 mice) at each time interval.

<table>
<thead>
<tr>
<th>Time after transplantation of tumour (days)</th>
<th>Haematocrit (% v/v)</th>
<th>Haemoglobin (mg/ 0.1 ml blood)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Tumour-bearing</td>
</tr>
<tr>
<td>5</td>
<td>36</td>
<td>35</td>
</tr>
<tr>
<td>7</td>
<td>41</td>
<td>40</td>
</tr>
<tr>
<td>9</td>
<td>41</td>
<td>38</td>
</tr>
<tr>
<td>11</td>
<td>39</td>
<td>38</td>
</tr>
<tr>
<td>13</td>
<td>40</td>
<td>17</td>
</tr>
<tr>
<td>15</td>
<td>41</td>
<td>20</td>
</tr>
</tbody>
</table>
TABLE 3.19 - Erythrocyte Glutathione Status of Blood from Lewis Lung Carcinoma-bearing C57BL6 Mice, and from Tumour-free Controls.

Results represent mean of two observations (each from the blood composite of 3 mice) at each time interval.

<table>
<thead>
<tr>
<th>Time after transplantation of tumour (days)</th>
<th>GSH (nmol/ mg Hb)</th>
<th>GSSG (nmol/ mg Hb)</th>
<th>GSH / GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Tumour-bearing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4.1</td>
<td>4.2</td>
<td>0.8</td>
</tr>
<tr>
<td>7</td>
<td>4.5</td>
<td>4.6</td>
<td>0.9</td>
</tr>
<tr>
<td>9</td>
<td>4.4</td>
<td>4.8</td>
<td>0.9</td>
</tr>
<tr>
<td>11</td>
<td>4.8</td>
<td>5.1</td>
<td>0.8</td>
</tr>
<tr>
<td>13</td>
<td>4.7</td>
<td>5.2</td>
<td>0.9</td>
</tr>
<tr>
<td>15</td>
<td>4.6</td>
<td>8.2</td>
<td>0.8</td>
</tr>
</tbody>
</table>
TABLE 3.20 - Erythrocyte GSSG Reductase and GSH Peroxidase Activities of Blood from Lewis Lung Carcinoma-bearing C57BL6 Mice, and from Tumour-free Controls.
Results represent mean of two observations (each from the blood composite of 3 mice) at each time interval.

<table>
<thead>
<tr>
<th>Time after transplantation of tumour (days)</th>
<th>GSSG Reductase (nmol NADPH/min/mg Hb)</th>
<th>GSH Peroxidase (Enzyme units / mg Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Tumour-bearing</td>
</tr>
<tr>
<td>5</td>
<td>71</td>
<td>67</td>
</tr>
<tr>
<td>7</td>
<td>64</td>
<td>60</td>
</tr>
<tr>
<td>9</td>
<td>66</td>
<td>94</td>
</tr>
<tr>
<td>11</td>
<td>71</td>
<td>85</td>
</tr>
<tr>
<td>13</td>
<td>72</td>
<td>127</td>
</tr>
<tr>
<td>15</td>
<td>70</td>
<td>91</td>
</tr>
</tbody>
</table>

* Enzyme units are defined as $1000 \times \log_{10}$ (decrease in GSH/min) at $37^\circ$ C, pH 7.0.
TABLE 3.21 - Erythrocyte Catalase and $\gamma$-Glutamylcysteine Synthetase of Blood from Lewis Lung Carcinoma-bearing C57BL6 Mice, and from Tumour-free Controls. Results represent mean of two observations (each from the blood composite of 3 mice) at each time interval.

<table>
<thead>
<tr>
<th>Time after transplantation of tumour (days)</th>
<th>Catalase (k [1st-order reaction rate constant]/min/mg Hb at $2 \pm 1^\circ C$, pH 7.4)</th>
<th>$\gamma$-glutamylcysteine synthetase (nmol/min mg Hb at $37^\circ C$, pH 8.25)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Tumour-bearing</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>1.36</td>
<td>1.37</td>
</tr>
<tr>
<td>15</td>
<td>1.28</td>
<td>1.33</td>
</tr>
<tr>
<td>17</td>
<td>1.25</td>
<td>1.23</td>
</tr>
</tbody>
</table>
melanomas usually became palpable at 10 to 12 days after implantation. Thereafter, the rate of weight gain by B16 melanomas, was similar to that of Lewis lung carcinomas (figure 3.1). B16 melanomas had a mean doubling time of 43.5 hr when 0.5 g, and 87 hr when 1.0 g in weight. Linear regression analysis of tumour weight against time (days) after implantation, revealed a correlation coefficient of 0.96 for B16 melanoma.

DNA concentrations of B16 melanomas were pronounced. Protein concentrations and DNA concentrations of B16 melanoma did not vary significantly, as tumour mass increased (tables 3.23).

Inspection of sectioned B16 melanomas showed the tumour to have a mushy constitution of intense black colouration. The melanoma was uniform in appearance and there were no visibly apparent regions of hyperaemia or necrosis.

Activities of antioxidant defence and related enzymes in B16 melanomas:

As the B16 melanomas cannot be compared with the tissue in which it originated, the relative levels of the biochemical estimations for the melanoma had to be indexed to those of other normal murine tissues. In the present studies, these tissues were limited to liver, lung, kidney and blood.

GSH peroxidase and GSSG reductase activities of B16 melanomas were within the range of activities, expressed by normal murine tissues, and did not vary significantly with time after tumour implantation (tables 3.24 and 2.25). Cu/Zn-SOD activities and catalase activities of B16 melanoma were comparatively low, being less than that determined for Lewis lung carcinomas (tables 3.26 and 3.27). The level of melanoma catalase activity did not vary between 13 and 21 days after implantation (table 3.27). Cu/Zn- SOD activities of B16 melanomas in animals, killed 17 and 21 days after inoculation with tumour cells, were significantly
FIG. 3.1 - Graph of Tumour Weight Against Time After Intramuscular Transplantation of Lewis Lung Carcinomas or B16 Melanomas into C57BL Mice

Tumour weight expressed as a % of carcass weight.
Results represent mean ± SEM at each time interval.
TABLE 3.22 - Liver and Tumour Weights of B16 Melanoma-bearing C57BL10 Mice
Tumour-free litter mates served as the controls.
Results represent mean ± SEM for 6 mice at each time interval.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Tumour-bearing</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>(Weight as % body wt minus tumour wt)</td>
<td>(wet wt of tissues shown in brackets)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>3.9 ± 0.1 (1.03 ± 0.07)</td>
<td>5.3 ± 0.2 (1.57 ± 0.08)</td>
</tr>
<tr>
<td>17</td>
<td>6.4 ± 0.8a (1.60 ± 0.17)</td>
<td>5.3 ± 0.1 (1.55 ± 0.02)</td>
</tr>
<tr>
<td>21</td>
<td>14.2 ± 1.8b (3.27 ± 0.26)</td>
<td>5.5 ± 0.2 (1.60 ± 0.15)</td>
</tr>
</tbody>
</table>

a-b Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 3.23 - Liver and Tumour DNA and Protein Concentrations of Bl6 Melanoma-bearing C57BL10 Mice
Tumour-free litter mates served as the controls.
Results represent mean ± SEM for 6 mice at each time interval. Protein was determined with Coomassie blue reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Tumour Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Tumour-bearing</td>
</tr>
<tr>
<td></td>
<td>(DNA conc<em>n as μg foetal calf DNA equiv./ mg protein) (protein conc</em>n [mg/g] shown in brackets)</td>
</tr>
<tr>
<td></td>
<td>(protein conc*n [mg/g] shown in brackets)</td>
</tr>
<tr>
<td></td>
<td>(DNA conc*n as μg foetal calf DNA equiv./ mg protein)</td>
</tr>
<tr>
<td></td>
<td>(protein conc*n [mg/g] shown in brackets)</td>
</tr>
<tr>
<td>13</td>
<td>67 ± 5 (68 ± 3)</td>
</tr>
<tr>
<td>17</td>
<td>67 ± 6 (66 ± 3)</td>
</tr>
<tr>
<td>21</td>
<td>66 ± 2 (68 ± 1)</td>
</tr>
</tbody>
</table>

*a-b* Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 3.24 - Liver and Tumour GSH Peroxidase Activities of Bl6 Melanoma-bearing C57BL10 Mice Tumour-free litter mates served as the controls. Results represent mean ± SEM for 6 mice at each time interval. Protein was determined with Coomassie blue reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Tumour</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Tumour-bearing</td>
</tr>
<tr>
<td></td>
<td>(Enzyme units*/mg protein)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>14 + 1</td>
<td>124 + 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>121 ± 4</td>
</tr>
<tr>
<td>17</td>
<td>13 + 1</td>
<td>128 + 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>111 ± 3(^a)</td>
</tr>
<tr>
<td>21</td>
<td>13 + 1</td>
<td>120 + 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>118 ± 9</td>
</tr>
</tbody>
</table>

* Enzyme units are defined as 1000 x log\(_{10}\) (decrease in GSH/min) at 37\(^\circ\) C, pH 7.0.

\(^a\) Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 3.25 - Liver and Tumour GSSG Reductase Activities of B16 Melanoma-bearing C57BL10 Mice Tumour-free litter mates served as the controls. Results represent mean ± SEM for 6 mice at each time interval. Protein was determined with Coomassie blue reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Tumour Control</th>
<th>Liver Tumour-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol NADPH oxidized/ min/ mg protein at 37° C, pH 7.4)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>58 ± 1</td>
<td>90 ± 4</td>
</tr>
<tr>
<td>17</td>
<td>53 ± 2</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>21</td>
<td>41 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>91 ± 5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 3.26 - Liver and Tumour Cu/Zn Superoxide Dismutase Activities of B16 Melanoma-bearing C57BL10 Mice Tumour-free litter mates served as the controls. Results represent mean ± SEM for 6 mice at each time interval. Protein was determined with Coomassie blue reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Tumour Control (units [Sigma^R]/ mg protein at 25 ± 20 C, pH 7.5)</th>
<th>Liver Tumour-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>13 7.8 ± 0.8</td>
<td>115 ± 3 112 ± 7</td>
</tr>
<tr>
<td></td>
<td>17 3.5 ± 0.7^a</td>
<td>117 ± 5 111 ± 5</td>
</tr>
<tr>
<td></td>
<td>21 3.8 ± 0.8^a</td>
<td>116 ± 3 118 ± 6</td>
</tr>
</tbody>
</table>

^a Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
less than the activity of melanoma tissue, determined 13 days post implantation (Table 3.26).

γ-Glutamyl transpeptidase activities of B16 melanoma tissue were relatively pronounced and did not vary with time after implantation (Table 3.28). GSH concentrations of B16 melanoma tissue were appreciable (approximately half that of liver) and did not change between 13 and 21 days after tumour implantation (Table 3.29). The GSSG concentration of B16 melanomas, 21 days after transplantation, was significantly less than at 13 or 17 days (Table 3.29). The ratio of GSH to GSSG in B16 melanomas, increased significantly between 13 to 21 days post implantation (Table 3.29).

B16 melanoma tissue had a considerable concentration of TBA-reactive material, approximately half that of hepatic tissue (Table 3.31).

**Effect of B16 melanomas upon the livers of tumour-bearing mice:** The weights and protein concentrations of the livers of mice bearing B16 melanomas did not differ from those of tumour-free mice (Tables 3.22 and 3.23). Hepatic DNA concentrations of B16 melanoma-bearing mice were significantly greater than those of control animals (Table 3.23).

GSH peroxidase, GSSG reductase and Cu/Zn SOD activities of the livers of mice, with B16 melanomas, did not differ from the appropriate values in tumour-free mice (Tables 3.24, 3.25 and 3.26). Hepatic catalase activities in mice, bearing B16 melanomas, were about half those of tumour-free controls (Table 3.29).

Hepatic γ-glutamyl transpeptidase activities of B16 melanoma-bearing mice were significantly greater than those of the controls (Table 3.28). GSH concentrations in the livers of mice, killed 13 days after inoculation with B16 melanoma cells, were significantly greater than those of tumour-free animals (Table 3.30). Hepatic GSH concentrations of
TABLE 3.27 - Liver and Tumour Catalase Activities of B16 Melanoma-bearing C57BL10 Mice Tumour-free litter mates served as the controls.
Results represent mean ± SEM for 6 mice at each time interval. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Tumour Control</th>
<th>Liver Tumour-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K x 10^-2/min/mg protein)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>6.8 ± 0.8</td>
<td>280 ± 20</td>
</tr>
<tr>
<td>17</td>
<td>6.5 ± 1.1</td>
<td>290 ± 20</td>
</tr>
<tr>
<td>21</td>
<td>6.5 ± 1.0</td>
<td>280 ± 30</td>
</tr>
</tbody>
</table>

^k is the first-order reaction rate constant at 2 ± 1°C, pH 7.4.

^a Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 3.28 - Liver and Tumour \( \gamma \)-Glutamyl Transpeptidase Activities of B16 Melanoma-bearing C57BL10 Mice Tumour-free litter mates served as the controls. Results represent mean ± SEM for 6 mice at each time interval. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Liver</th>
<th>Control</th>
<th>Tumour-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol p-nitroanilide/ min/ mg protein at 37°C, pH 7.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>15 ± 1</td>
<td>0.072 ± 0.002</td>
<td>0.085 ± 0.002 (^a)</td>
</tr>
<tr>
<td>17</td>
<td>16 ± 1</td>
<td>0.070 ± 0.001</td>
<td>0.090 ± 0.003 (^a)</td>
</tr>
<tr>
<td>21</td>
<td>15 ± 1</td>
<td>0.068 ± 0.002</td>
<td>0.093 ± 0.003 (^a)</td>
</tr>
</tbody>
</table>

\(^a\) Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 3.29 - Weight in situ and Glutathione Status of B16 Melanoma Tumours Borne by C57BL10 Mice. Results represent mean ± SEM for 6 mice at each time interval. Protein was determined with Coomassie blue reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Weight (% of body wt)</th>
<th>GSH (nmol/mg)</th>
<th>GSSG (nmol/mg)</th>
<th>GSH/GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>1.7 ± 0.3</td>
<td>26 ± 4</td>
<td>1.7 ± 0.3</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>17</td>
<td>3.5 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26 ± 2</td>
<td>1.5 ± 0.1</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>21</td>
<td>6.9 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26 ± 2</td>
<td>1.1 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>26 ± 4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-d</sup> Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 3.30 - Glutathione Status of Livers from B16 Melanoma-bearing C57BL10 Mice, and from Tumour-free Controls

Results represent mean ± SEM for 6 mice at each time interval. Protein determined with Coomassie blue reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>GSH</th>
<th>GSGG</th>
<th>GSH/GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Tumour-bearing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumour-bearing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>49 ± 4</td>
<td>68 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>17</td>
<td>49 ± 3</td>
<td>49 ± 2</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>21</td>
<td>52 ± 2</td>
<td>19 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.3 ± 0.1</td>
</tr>
</tbody>
</table>

<sup>a-d</sup> Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
animals, that had borne Bl6 melanomas for 21 days, were significantly less than control values (table 3.30). Hepatic TBA-reactive material concentrations of mice, killed 17 and 21 days after transplantation of Bl6 melanomas, were significantly greater than those of tumour-free mice (table 3.31).

**Effect of Bl6 melanoma upon the blood of tumour-bearing mice:** During the period of investigation, 13 to 21 days after tumour implantation, Bl6 melanoma-bearing mice had pronounced decreases in haematocrit and haemoglobin concentration (table 3.34). The anaemia was not as severe as that ascertained for mice, bearing Lewis lung carcinomas.

Mice with Bl6 melanomas had moderately increased erythrocyte GSH concentrations (table 3.33). Red blood cell GSSG concentrations of mice, that had borne Bl6 melanomas for 21 days, was half that of control animals (table 3.33). Erythrocyte GSSG reductase and GSH peroxidase activities of Bl6 melanoma-bearing mice were considerably greater than those of tumour-free mice (table 3.34). SOD activity of erythrocytes from mice, that had borne Bl6 melanomas, did not differ from that of control mice (table 3.35). Plasma caeruloplasmin oxidase activities of mice with Bl6 melanomas were appreciably greater than control plasma activities (table 3.35).

The major effects of i/m embedded Bl6 melanomas upon murine liver and blood, as determined in the present studies, are summarized in table 3.37.
TABLE 3.31 - Liver and Tumour Thiobarbituric Acid Material
Concentration of B16 Melanoma-bearing C57BL10 Mice
Tumour-free litter mates served as the controls.
Results represent mean ± SEM for 6 mice at each time
interval. Protein was determined with Coomassie blue
reagent, using BSA as the standard.
Chromophores obtained by incubation for 30 min at 100°C
in 0.05 M H₂SO₄. No antioxidant additives were used.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Tumour</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Tumour-bearing</td>
</tr>
<tr>
<td></td>
<td>(nmol malondialdehyde equiv. /mg protein)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>0.75 ± 0.05</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>17</td>
<td>0.68 ± 0.09</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>21</td>
<td>0.70 ± 0.04</td>
<td>1.3 ± 0.2</td>
</tr>
</tbody>
</table>

a Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 3.32 - Haematocrits and Haemoglobin Concentrations of Blood from B16 Melanoma-bearing C57BL10 Mice, and from Tumour-free Controls
Results represent the mean of two observations (each from the blood composite of 3 mice) at each time interval.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Haematocrit (%) v/v</th>
<th>Haemoglobin (mg/ 0.1ml blood)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Tumour-bearing</td>
</tr>
<tr>
<td>13</td>
<td>39</td>
<td>31</td>
</tr>
<tr>
<td>17</td>
<td>38</td>
<td>30</td>
</tr>
<tr>
<td>21</td>
<td>41</td>
<td>28</td>
</tr>
<tr>
<td>Time after implantation of tumour (days)</td>
<td>GSH (nmol/ mg Hb)</td>
<td>GSSG (nmol/ mg Hb)</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Tumour-bearing</td>
</tr>
<tr>
<td>13</td>
<td>4.4</td>
<td>5.1</td>
</tr>
<tr>
<td>17</td>
<td>4.0</td>
<td>5.4</td>
</tr>
<tr>
<td>21</td>
<td>4.6</td>
<td>6.0</td>
</tr>
</tbody>
</table>
TABLE 3.34 - Erythrocyte GSSG Reductase and GSH Peroxidase Activities of Blood from B16 Melanoma-bearing C57BL/10 Mice, and from Tumour-free Controls

Results represent the mean of two observations (each from the blood composite of 3 mice) at each time interval.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>GSSG Reductase (nmol NADPH/min/mg Hb at 37°C, pH 7.4)</th>
<th>GSH Peroxidase* (Enzyme units / mg Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Tumour-bearing</td>
</tr>
<tr>
<td>13</td>
<td>69</td>
<td>101</td>
</tr>
<tr>
<td>16</td>
<td>69</td>
<td>100</td>
</tr>
<tr>
<td>21</td>
<td>75</td>
<td>123</td>
</tr>
</tbody>
</table>

*Enzyme units are defined as 1000 x log_{10} (decrease in GSH/min) at 37°C, pH 7.0.
**TABLE 3.35 - Erythrocyte Cu/Zn Superoxide Dismutase Activities and Plasma Caeruloplasmin Oxidase Activities of Blood from B16 Melanoma-bearing C57BL10 Mice, and from Tumour-free Controls**

Results represent the mean of two observations (each from the blood composite of 3 mice) at each time interval.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Erythrocyte SOD (units [Sigma]/ mg Hb at 24 ± 2°C, pH 7.5)</th>
<th>Plasma caeruloplasmin (Oxidase units/ mg Hb at 30°C, pH 5.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Tumour-bearing</td>
</tr>
<tr>
<td>13</td>
<td>2.7</td>
<td>2.6</td>
</tr>
<tr>
<td>17</td>
<td>2.7</td>
<td>2.5</td>
</tr>
<tr>
<td>21</td>
<td>2.5</td>
<td>2.9</td>
</tr>
</tbody>
</table>
TABLE 3.36 - Summary of Major Effects of the Lewis Lung Carcinoma upon Tissues of Host C57BL6 Mice, Distal to the Site of Implantation (as determined in the studies of Chapter 3)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tissue</th>
<th>Day after implantation when first significant</th>
<th>Value as a % of corresponding tumour-control</th>
<th>Trend with further time after implantation.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>liver</td>
<td>9</td>
<td>150</td>
<td>Stays at this level</td>
</tr>
<tr>
<td></td>
<td>lung</td>
<td>11</td>
<td>140</td>
<td>Stays at this level</td>
</tr>
<tr>
<td>GSH peroxidase</td>
<td>liver mitochondria</td>
<td>15</td>
<td>70</td>
<td>Decreases to 60% by day 17</td>
</tr>
<tr>
<td></td>
<td>erythrocyte</td>
<td>9</td>
<td>90</td>
<td>Stays at this level</td>
</tr>
<tr>
<td>Catalase</td>
<td>liver</td>
<td>13*</td>
<td>70</td>
<td>Decreases to 50% by day 17</td>
</tr>
<tr>
<td></td>
<td>lung</td>
<td>13*</td>
<td>80</td>
<td>Decreases to 70% by day 17</td>
</tr>
<tr>
<td>GSSG reductase</td>
<td>liver</td>
<td>13</td>
<td>140</td>
<td>Stays at this level</td>
</tr>
<tr>
<td></td>
<td>lung</td>
<td>11</td>
<td>130</td>
<td>Stays at this level</td>
</tr>
<tr>
<td></td>
<td>erythrocyte</td>
<td>9</td>
<td>140</td>
<td>Stays at this level</td>
</tr>
<tr>
<td>Cytosolic γ-glutamylcysteine synthetase</td>
<td>liver</td>
<td>15</td>
<td>55</td>
<td>Increases to 130% by day 17</td>
</tr>
<tr>
<td></td>
<td>lung</td>
<td>13*</td>
<td>70</td>
<td>Increases to 140% by day 17</td>
</tr>
<tr>
<td></td>
<td>erythrocyte</td>
<td>13*</td>
<td>140</td>
<td>Increases to 160% by day 17</td>
</tr>
<tr>
<td>Mitochondrial γ-glutamyl transpeptidase</td>
<td>liver</td>
<td>14*</td>
<td>140</td>
<td>No further determinations</td>
</tr>
<tr>
<td></td>
<td>lung</td>
<td>14*</td>
<td>160</td>
<td>No further determinations</td>
</tr>
<tr>
<td>Cytosolic GSH S-transferase</td>
<td>liver</td>
<td>9</td>
<td>130</td>
<td>Stays at this level</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>liver</td>
<td>15</td>
<td>70</td>
<td>Decreases to 60% by day 17</td>
</tr>
<tr>
<td>Organ weight</td>
<td>kidney</td>
<td>15</td>
<td>80</td>
<td>No further determinations</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>blood</td>
<td>13</td>
<td>40</td>
<td>Stays at this level</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>blood</td>
<td>11</td>
<td>80</td>
<td>Decreases to 40% by day 15</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td>erythrocyte</td>
<td>15</td>
<td>180</td>
<td>No further determinations</td>
</tr>
</tbody>
</table>

* No determinations prior to this day after implantation.
All values (except *) are specific activities or concentrations.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tissue</th>
<th>Day after implantation when first significant</th>
<th>Value as a % of corresponding tumour-control</th>
<th>Trend with further time after implantation</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>liver</td>
<td>13*</td>
<td>120</td>
<td>Increases to 170% by day 21.</td>
</tr>
<tr>
<td>GSH peroxidase</td>
<td>liver</td>
<td>17</td>
<td>90</td>
<td>Only one occasion of sig. difference.</td>
</tr>
<tr>
<td>Catalase</td>
<td>liver</td>
<td>13*</td>
<td>54</td>
<td>Decreases to 48% by day 21</td>
</tr>
<tr>
<td>( \gamma )-Glutamyl</td>
<td>liver</td>
<td>13*</td>
<td>120</td>
<td>Increases to 140% by day 21.</td>
</tr>
<tr>
<td>transpeptidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione (GSH)</td>
<td>liver</td>
<td>13*</td>
<td>140</td>
<td>Decreases to 37% by day 21. increases to 130% by day 21.</td>
</tr>
<tr>
<td>erythrocyte</td>
<td></td>
<td>13*</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>Haematocrit</td>
<td>blood</td>
<td>13*</td>
<td>80</td>
<td>Decreases to 70% by day 21.</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>blood</td>
<td>13*</td>
<td>80</td>
<td>Decreases to 55% by day 21.</td>
</tr>
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</table>

* No determinations prior to this day after implantation.

All values are specific activities or concentrations.
DISCUSSION

Histological Evidence of Biochemical Lesions in Normal Tissues of Mice Bearing Lewis Lung Carcinomas

The profound histological changes observed for livers of mice, bearing Lewis lung carcinomas, indicated that the presence of a tumour had evoked significant biochemical disturbances in these animals. These morphological perturbations were similar to those of sublethal injury, resulting from toxic insult or viral infection (Trump and Arstilla, 1971; Reynolds and Treinen Moslen, 1980). A viral screen of mice bearing Lewis lung carcinomas did not, however, show any evidence of infection by common murine viruses (see Appendix III). Hepatic function of cancer patients, assessed by several biochemical tests, has been reported to be considerably impaired (Greenstein, 1954).

The liver cells of mice, bearing Ehrlich ascites tumours, have been reported to have morphological changes in the endoplasmic reticulum and other cytoplasmic components, and display lipid vacuolization (Baum and Nishimura, 1964). Histological changes in the adrenal glands of tumour-bearing rats have also been reported (Dalton, 1944). Tumour-bearing mice have been reported to focal necrosis and amyloid infiltration in their livers (Parsons et al, 1947). Morphological changes to the livers of tumour-bearing animals have not, however, been a universal finding (Begg, 1958; Stein et al, 1976). Tumour-bearing rats, for example, have been reported to have numerous biochemical abnormalities but to be histologically normal (Greengard, 1979).

The increase in the number of mitotic cells in the livers of mice, bearing Lewis lung carcinomas agreed with similar findings, reported previously for tumour-bearing animals (Annae et al, 1951; Malmgren, 1956; Baserga and Kisieleski, 1961; Morgan and Cameron, 1973). The dividing
cells in the livers of mice, bearing Lewis lung carcinomas had the appearance of hepatocytes. No infiltrating neoplastic cells were seen in liver sections from Lewis lung carcinoma-bearing mice. Although numerous metastatic cells were observed in sections of lung from mice, bearing Lewis lung carcinomas, the residual pulmonary tissue had similar histological appearance to that of tumour-free animals. This suggested that the abnormalities of the liver were not caused by metastasis.

Liver sections from Lewis lung carcinoma-bearing mice contained haemosiderin-like pigments, which are associated with autoxidative injury (Lippman, 1983; Chapter 2). This pigment was unlikely to have been artifactual formalin pigment because livers were fixed in formol saline, buffered at neutral pH. The observation of lipofuscin-like autofluorescence was further evidence of hepatic injury due to autoxidation, in these animals (Chapter 2). These observations might correlate with the biochemical finding of increased hepatic TBA-reactive material concentrations in mice, bearing Lewis lung carcinomas (Capel and Thornley, 1982). The study was, however, limited to only 6 tumour-bearing and 6 tumour-free mice and further investigations, using a greater number of animals and other histological procedures to detect free-radical metabolites (Chapter 2), would therefore be justified.

**Effect of Tumours Upon Body and Organ weights**

There have been numerous reports of increased hepatic mass of tumour-bearing animals (Medigreceanu, 1910; Yeakel, 1948; Annae et al, 1951; Stewart and Begg, 1953; Theologides, 1971). Much of the increase in liver weight may generally be attributable to the greater water content observed for livers of tumour-bearing animals, in comparison to tumour-free controls (Rechner et al, 1961; Theologides, 1971; Lundholm et al, 1979). The liver weights of mice bearing either Lewis lung carcinomas or B16 melanomas did not differ from those of control animals. The ratio of
Liver weight to carcass weight (body weight minus tumour weight) was, however significantly greater in animals, bearing comparatively advanced tumours (>8% body weight) than that of the controls. Animals, bearing Lewis lung carcinomas or B16 melanomas, were thus able to maintain a constant liver weight despite the decrease in carcass weight. The ratio of dry liver weight to total animal weight of tumour-bearing animals has been reported elsewhere to be greater than that of tumour-free animals (Theologides, 1971). Pulmonary weights of Lewis lung carcinoma-bearing mice were also unchanged, although loss of pulmonary mass might have been masked by the weight of metastatic foci.

Loss of body weight in man or animals with cancer, has frequently been reported (Costa, 1977; deWys, 1982). Much of this loss of biomass is probably caused by the catabolism of host tissues, primarily of skeletal muscle, to meet the nutrient requirements of tumours (Costa, 1977; Bennegard et al, 1982; Pain et al, 1984). The livers of mice, bearing Lewis lung carcinomas or B16 melanomas, possibly had resistance to this catabolism.

The increase in spleen weights of animals, implanted with Lewis lung carcinomas or B16 melanomas, was in agreement with similar previous observations for tumour-bearing animals (Greenstein, 1954), and indicates that the tumour had evoked an immune response in the host animal (see Chapter 5).

Effect of Tumours Upon Protein and DNA Concentrations

Hepatic protein synthesis and protein turnover, have been reported to be greater than control levels, in tumour-bearing mice and rats, and in cancer patients (Norberg and Greenberg, 1951; Stein et al, 1976; Lundholm et al, 1978; Lundholm et al, 1979; Norton et al, 1981; Pain et al, 1984). In the present studies, hepatic protein concentrations of
Lewis lung carcinoma-bearing and B16 melanoma-bearing mice were essentially unchanged. The levels and turnovers of some hepatic proteins might, however, have been increased, possibly at the expense of a lowered synthesis or increased catabolism of other proteins (Stein et al., 1976).

The protein concentrations of Lewis lung carcinoma tissue was almost twice that of the reference tissue, lung from tumour-free mice (Chapter 2). As the protein concentrations of the lungs of mice, bearing Lewis lung carcinomas were essentially the same as those of control animals, metastatic cells _per se_ probably did not make many significant direct contributions to the biochemical character of the lungs of mice, bearing Lewis lung carcinomas.

The present investigations confirm previous reports of increased DNA concentrations of the livers of tumour-bearing animals (Greenstein, 1954; Begg, 1958). This finding has not, however, been universal, as the DNA concentrations of, for example, sarcoma-bearing mice have been reported not to differ from that of healthy animals (Karlberg et al., 1981). The increase in the hepatic DNA concentrations of tumour-bearing mice may have correlated with with the increased incidence of mitotic hepatocytes, observed elsewhere and in the present studies for mice bearing Lewis lung carcinomas. The lungs and kidneys of tumour-bearing mice have also been reported to have greater DNA concentrations than those of tumour-free mice (Begg, 1958). The greater DNA concentration of pulmonary tissue from Lewis lung carcinoma-bearing mice than tumour-free controls, was probably, at least in part, due to the contribution from metastatic cells.

The presence of a tumour has been reported to cause increases in the RNA concentrations of various normal tissues in laboratory animals and man (Begg, 1958; Karlberg et al., 1981; Lundholm et al., 1978; Lundholm et al., 1979). Liver cells, isolated from sarcoma-bearing mice, have been
observed to have greater rates of nucleic acid transcription and protein translation in vitro than cells from healthy mice (Ternell et al., 1983). The greater nucleic acid concentrations of non-neoplastic tissues in tumour-bearing hosts (Begg, 1958), supports the earlier suggestion that synthesis of at least some hepatic enzymes in mice, bearing Lewis lung carcinomas or B16 melanomas was increased. Many of the biochemical changes in the normal tissues of tumour-bearing mice, observed in the current investigations, may thus have originated at the level of DNA replication and protein synthesis (Begg, 1958).

**Effect of Tumours Upon Antioxidant Defence Enzymes of Host Tissues**

**Catalase activity:** The observations of the present studies that hepatic catalase activities of mice, bearing Lewis lung carcinomas or B16 melanomas, were decreased, agreed with the many similar reports elsewhere for tumour-bearing rodents (Greenstein, 1954; Begg, 1958; Busch, 1962; Nishimura et al., 1962; Higashi et al., 1968). Tumours borne by other species, such as frogs or chick embryos, have also been observed to cause a depression in the hepatic catalase activities of the host animal (Busch, 1962). Patients, in the advanced stages of cancer, have been reported to have lower hepatic catalase activities than non-cancer patients (Maldia and Holland, 1962). Reports of decreased hepatic catalase activities for cancer patients have, however, been much less common than those for tumour-bearing animals, possibly because the tumour-burden tolerable to human beings is considerably less than that generally inflicted upon laboratory animals (Busch, 1962).

Animal tissues were first observed to contain substances that can inhibit catalase activity in vitro in 1905 (Battelli and Stern). Catalase activity of rat liver homogenates has been observed to be inhibited by a wide variety of substances including nucleic acid catabolites, and substances released by post mortem autolysis of normal tissues and
necrosis of neoplastic tissues (Hargreaves et al., 1959). A considerable number of substances, such as talcum, colloidal carbon, amino acids, purines, certain bacteria and viruses, and several anticancer drugs and carcinogens, have been reported to cause decreased hepatic catalase activity when injected into normal animals (Kampschmidt, 1965). It has been reported that patients, with gastric carcinomas, have a hepatic catalase-inhibiting factor in their gastric juices (Takamura, 1962). The decreased catalase activity of the livers of mice, bearing Lewis lung carcinomas or Bl6 melanomas, might thus have been caused by enzyme inhibition by substances secreted directly by, or as a consequence of, the tumour.

The decrease in catalase activity in the livers of tumour-bearing animals has also been considered to be caused by repression of the synthesis of the enzyme (Nishimura et al., 1962; Higashi et al., 1968). The biosynthesis of hepatic catalase in rats, bearing ascites hepatomas, has been shown, using immunochemical techniques, to be less than that of tumour-free rats (Kashiwagi et al., 1972).

The depression of hepatic catalase synthesis in mice, following tumour implantation or injury with talcum, has been associated with ultrastructural changes in the livers of these animals (Baum and Nishimura, 1964). The gross histological changes of Lewis lung carcinoma-bearing mice, observed in the present studies, might thus have been related to the decreased hepatic catalase activities.

**GSH peroxidase and SOD activities:** The decrease in the mitochondrial GSH peroxidase activities, observed for mice bearing Lewis lung carcinomas, was probably the first report of a tumour having a systemic effect upon this enzyme. In previous studies the mean GSH peroxidase activities of hepatic homogenate from mice, with Lewis lung carcinomas, were invariably less than those of tumour-free controls, but a statistical significance
could not be achieved (Capel and Thornley, 1982). The unchanged cytosolic GSH peroxidase activities of the livers of animals, bearing Lewis lung carcinomas, might have made the lowered activity of the mitochondrial compartment less discernable. Mitochondrial GSH peroxidase may have been inhibited or repressed by similar factors to those discussed previously, concerning catalase. Liver cells, isolated from sarcoma-bearing mice, have been reported to have lower oxygen metabolism than comparative cells from tumour-free mice (Ternell et al 1983). The depressed hepatic mitochondrial GSH peroxidase activities of Lewis lung carcinoma-bearing mice might have been a result of a lowered production of reactive oxygen species by hepatic mitochondria. The normal level of Mn-SOD in the livers of these animals, however, indicates that the hepatic oxygen tension of these animals was not depressed. The present studies did not agree with those of other investigations, in which Cu/Zn-SOD and Mn-SOD activities of the livers of tumour-bearing animals were reported to be decreased (see earlier).

Enzymes of GSH Synthesis

GSSG reductase: Studies of transplantable murine tumour have shown that 18 out of 23 of the tumour models caused increased hepatic GSSG reductase activities in the animals bearing them: one of the 18 tumours was the Lewis lung carcinoma; tumour-bearing rats were also observed to have greater GSSG reductase than control animals (Manso et al, 1958). Partial hepatectomy and pregnancy were observed to have no effect upon the hepatic GSSG reductase activities of mice, indicating that an increased activity of this enzyme was probably specific to the bearing of a tumour (Manso et al, 1958). In the present studies, the hepatic GSSG reductase activity of mice, bearing Lewis lung carcinomas, was significantly increased, whereas that of B16 melanoma-bearing mice did not differ from control values. The effect upon liver GSSG reductase activity was thus
common to many types of tumour, although there are a few exceptions.

GSSG reductase activities, of the livers of Lewis lung carcinoma-bearing mice, may have increased due to decreases in the ratios of GSH to GSSG or NADP⁺ to NADPH (Elsayed et al., 1982; see fig. 2.3). These ratios would decrease in response to a diminution in pentose phosphate shunt activity or to oxidative stress (Hosoda and Nakamura, 1970; Elsayed et al., 1982). The increase in GSSG reductase activity may have correlated with the elevated hepatic GSH/GSSG ratio, reported for Lewis lung carcinoma-bearing mice (Capel and Thornley, 1983).

The GSSG reductase activities, determined for non-neoplastic tissues of tumour-bearing mice, possibly only represented the concentrations of the enzyme and not in vivo activity. The capacity to reduce GSSG in vivo, might have been limited by the availability of the co-factors, FAD and NADPH. NADPH is synthesized via the phosphorylation of NAD⁺. Tumour-bearing mice have been reported to have relatively low hepatic NAD⁺ and NADH concentrations (Smith and King, 1970). The livers of rats, bearing hepatomas, have been observed, however, to have unchanged or elevated NAD⁺ concentrations (Nishizuka and Hayaishi, 1966).

Glucose 6-phosphate dehydrogenase: Cytosolic glucose 6-phosphate dehydrogenase activities of the livers of tumour-bearing rats and mice, have been reported to be greater than those of tumour-free animals (Wu and Homberger, 1969; Herzfeld and Greengard, 1972; Lundholm et al., 1983). Cancer patients have been shown to have marginally increased hepatic cytosolic glucose 6-phosphate dehydrogenase activities (Herzfeld et al., 1980). These observations conflict with those of the present studies, in which mice, bearing Lewis lung carcinomas, had lower activities than control mice. As soluble glucose 6-phosphate dehydrogenase is the rate-limiting enzyme of the pentose phosphate shunt, the activity of this pathway, in the livers of Lewis lung carcinoma-bearing mice, was possibly
impaired. The increased GSSG reductase activities of the livers of these animals might have been a biochemical response to stimulate the pentose phosphate pathway by providing further NADPH.

**Gamma-glutamylcysteine synthetase**: The γ-glutamylcysteine synthetase activities of the livers of Lewis lung carcinoma-bearing mice varied according to the duration in which the tumour had been borne. In preliminary studies (Capel and Thornley, 1983), the hepatic GSH concentrations of mice, bearing Lewis lung carcinomas, was observed to be generally less than that of control animals at 5 to 11 days, and to be significantly increased at 15 days post implantation. The present studies indicated that γ-glutamyl cysteine synthetase activity might have been the major determinant of the variable hepatic GSH in mice, bearing Lewis lung carcinomas. It would be appropriate to undertake a study in which the hepatic GSH concentrations were determined concomitantly with γ-glutamylcysteine synthetase and GSSG reductase activities to ascertain the relation between GSH synthesis and GSH concentration in tumour-bearing mice.

**Enzymes of GSH Degradation**

**Gamma-glutamyl transpeptidase**: Hepatic γ-glutamyl transpeptidase activities of cancer patients (Herzfeld et al, 1980) and tumour-bearing rats (Koss and Greengard, 1982) have been reported to be greater than those of tumour-free controls. The γ-glutamyl transpeptidase activities of several non-neoplastic tissues, such as spleen, liver, lung and bone marrow, from tumour-bearing rats have been observed to be substantially greater than those of comparative tissues in tumour-free rats (Koss and Greengard, 1982). This indicated that γ-glutamyl transpeptidase of many types of tissue in the tumour-bearing host, may respond to the presence of neoplastic cells. The above investigations supported the observations of the present studies, in which hepatic γ-glutamyl transpeptidase...
activities of B16 melanoma-bearing mice, and pulmonary and hepatic
activities of Lewis lung carcinoma-bearing mice were all greater than
control values. An increased hepatic $\gamma$-glutamyl transpeptidase activity,
in tumour-bearing animals, has not been a universal finding, as the
activity of rats, bearing Morris or Novikoff hepatomas, has been reported
not to be unperturbed, and that of rats, bearing Walker 256 carcinomas,
was decreased below control values (Wu and Bauer, 1960; Wu et al., 1965).
As Lewis lung carcinoma tissue was observed to have relatively low
$\gamma$-glutamyl transpeptidase activity (Chapter 2), the increased pulmonary
activity of mice, bearing Lewis lung carcinomas, was probably not due to
metastatic cells per se.

The alteration in $\gamma$-glutamyl transpeptidase activity of mice,
bearing Lewis lung carcinomas, was observed to preponderate to the mito-
chondrial fractions of liver and lung. There thus might have been an
increase in the translocation of amino acids or glutamate across the
mitochondrial, or possibly the peroxisomal or lysosomal, membranes of the
liver and lungs of Lewis lung carcinoma-bearing mice. An increased mito-
chondrial $\gamma$-glutamyl transpeptidase activity might have caused a decrease
in mitochondrial glutathione concentrations.

The glutamine synthetase activities of the livers of tumour-bearing
rats, have been reported to be less than that of control animals (Wu et
al., 1965). $\gamma$-Glutamyl transpeptidase activity, in normal tissues of
tumour-bearing mice, was possibly increased to supply glutamate, which
could then be used to synthesize glutamine, via a transamination
reaction.

**GSH S-epoxide transferase**: The increased GSH S-epoxide transferase
activity of the livers of mice, bearing Lewis lung carcinomas, was
possibly the first observation of a neoplasm having a systemic effect
upon this species of GSH-transferase. The hepatic GSH-S transferase
activity of rats, bearing benzo[a]pyrene-induced lung tumours, has been observed to be decreased (Dogra et al, 1985). The GSH S-aryl transferase activity of non-neoplastic hepatic tissue, removed post mortem from patients whose cancers had disseminated to the liver, has been reported to be less than that of non-cancer patients (Siegers and Younes, 1983). The activities of GSH S-transferase isoenzymes, might thus have been affected differently by the transplantation of a Lewis lung carcinoma. Future studies of GSH-transferase activities of the livers of tumour-bearing mice should therefore include procedures to differentiate the various species of the enzyme (Chapter 2).

The present studies indicated that disturbed hepatic metabolism in Lewis-lung carcinoma-bearing mice, might have caused the excessive production of epoxide-like electrophiles. Lipid epoxides are a major product of lipid peroxidation (Sevanian et al, 1981). The increased hepatic GSH S-epoxide transferase activity of tumour-bearing mice possibly reflected an elevated degree of autoxidation in the livers of these animals (Chapter 4). The raised GSH S-epoxide transferase activity might have contributed to the decreased hepatic GSH concentrations of Lewis lung carcinoma-bearing mice, as both these parameters were observed to be altered, comparatively soon after tumour implantation.

**Perturbations of the Blood of Tumour-bearing Mice**

The present studies agreed with a report that blood GSH concentrations of tumour-bearing animals were greater than those of healthy controls (Mills et al, 1981). The increased GSSG reductase and γ-glutamylcysteine synthetase activities, and decreased GSH peroxidase activity of the erythrocytes of Lewis lung carcinoma-bearing mice, might have caused the increased GSH concentration and GSH to GSSG ratio. In studies of cancer patients, erythrocyte catalase and GSH peroxidase activities were found to be normal; erythrocyte SOD in patients with
leukaemia or lymphoproliferative syndromes, but not visceral cancer were, however, observed to be raised (Gonzales et al, 1984); changes in red blood cell antioxidant defence is not therefore general to all cancers.

The anaemia of B16 melanoma-bearing and Lewis lung carcinoma-bearing mice, may have been caused by excessive destruction of red cells (Price and Greenfield, 1958; Costa, 1977). Erythrocyte GSSG reductase activity and GSH concentration have been reported to decrease with increasing red cell age (Abraham et al, 1978; Powers et al, 1981; Vanella et al, 1982). The raised GSH concentrations and GSSG reductase activity thus might have resulted from a proportionately younger erythrocyte population than that of tumour-free animals.

Erythrocyte SOD activity, which was increased in Lewis lung carcinoma-bearing mice (Capel and Thornley, 1982) and in B16 melanoma-bearing mice (these studies), does not, however, vary with red cell age (Michelson et al, 1977; Vanella et al, 1982). Glutathione is considered to be a major determinant of the susceptibility of erythrocytes to oxidative damage (Fuji et al, 1984). The erythrocytes of tumour-bearing mice, might thus have been survivors of oxidative stress, having been selected out or adapted to the stress by means of increased antioxidant defence capability. The blood concentrations of reactive oxygen species may have been increased, for example due to oxyhaemoglobin breakdown (Misra and Fridovich, 1972), or by direct generation from activated inflammatory cells (Nathan et al, 1980; Weiss and Lobuglio, 1982).

Mice, bearing Lewis lung carcinomas were more anaemic than mice, bearing B16 melanomas, of equivalent mass. The hypothetical oxidative destruction of erythrocytes might thus have been greater in Lewis lung carcinoma-bearing mice than those bearing B16 melanomas. The lungs of carcinoma-bearing mice had conspicuous metastatic foci, whereas the incidence of metastatic deposits in animals, bearing i/m-implanted B16
melanomas, has been reported to be relatively low (Weiss et al., 1982). Blood-borne tumour cells, disseminated from the primary tumour, might thus have been the trigger for the destruction of erythrocytes.

**Plasma caeruloplasmin oxidase:** activities of C57BL mice were only about a tenth of the activities, reported for rat or human plasma (Schosinsky et al., 1974), implying that murine caeruloplasmin has no significant role as an oxygen radical scavenger. The caeruloplasmin of C57BL mice has, however, been reported to be less than that of most other strains of mice (Meter and MacPike, 1968). The caeruloplasmin of mice probably mainly functions as a carrier of plasma copper, although it might help to prevent autoxidation by aiding the binding of iron to its carrier proteins (Halliwell and Gutteridge, 1985; Chapter 1). The plasma iron concentrations of cancer patients is generally decreased, although there are exceptions, such as leukaemia, breast cancer and Hodgkin's disease, in which plasma iron concentrations are increased (Whiting et al., 1981). The plasma copper concentrations of cancer patients and tumour-bearing animals have been observed to be increased (Fisher and Shifrine, 1978; Pizzolo et al., 1978; Roguljic et al., 1980). The raised plasma caeruloplasmin oxidase activities of B16 melanoma-bearing mice, probably reflected an elevation in serum copper, or possibly iron, in these animals.

**Comparison of the Systemic Effects of B16 Melanomas With Those of Lewis Lung Carcinomas.**

Most of the biochemical perturbations, observed for normal tissues from B16 melanoma-bearing mice, were common to mice, bearing Lewis lung carcinomas. The only differences between the systemic effects of the two murine tumour models, as determined in the present studies, were the increased hepatic GSSG reductase activity of Lewis lung carcinoma-bearing mice (unaltered in B16 melanoma-bearing mice) and the increased erythro-
cyte GSH peroxidase activity of B16 melanoma-bearing mice (decreased in Lewis lung carcinoma-bearing mice). These differences might have been attributable to the considerably greater ability of the Lewis lung carcinoma to metastasize, as discussed previously. Most of the factors, causing the perturbations of normal tissues of tumour-bearing mice, were probably general to mice bearing either type of tumour. The biochemical changes in the non-neoplastic tissues of tumour-bearing mice were possibly caused by one or more facets of neoplastic disease, which may have included stress (Sklar, 1981), the immune reaction of the host, changes in nutritional status of the host, and the production of chemical modulators by the tumour (Greengard and Cayanis, 1983). These possibilities will be given more detailed consideration in Chapters 5 and 6.

Anticancer Therapy

Lewis lung carcinomas, implanted into mice, have been reported to be comparatively insensitive to most anticancer drugs (Mayo, 1972). Active oxygen species have been implicated to be involved in the cytotoxic actions of many clinically-useful anticancer drugs (Doroshow and Hochstein, 1982; Lown et al., 1982; McGuiness et al., 1982; Arrick and Nathan, 1984). The toxicity of chemotherapeutic agents to non-neoplastic tissues often limits the use of these drugs in doses sufficiently large to be curative (Perry and Yarbro, 1984). Systemic therapy with anticancer drugs to mice bearing Lewis lung carcinomas might be further limited due to exacerbation of autoxidative injuries, already present in host tissues. The effects of Lewis lung carcinoma upon non-neoplastic tissues could possibly decrease the efficacy of chemotherapeutic regimens that generate reactive oxygen metabolites and might contribute to the comparative insensitivity of the tumour to systemic therapy by many anticancer drugs.
CHAPTER FOUR

LIPID PEROXIDATION IN TISSUES OF TUMOUR-BEARING MICE
CHAPTER FOUR: LIPID PEROXIDATION IN TISSUES OF TUMOUR-BEARING MICE

INTRODUCTION

Previous studies (Capel and Thornley 1982; Capel and Thornley, 1983) and investigations, described earlier in this thesis (Chapter 2) have shown that homogenates and microsomal fractions of Lewis lung carcinoma, contain substantial concentrations of substances that react with TBA. The livers of mice, bearing Lewis lung carcinomas, were also observed to have significantly increased concentrations of TBA-reactive material (Capel and Thornley, 1982; Capel and Thornley, 1983). A similar observation was also determined, in the present studies for the livers of mice, bearing B16 melanomas (Chapter 3). These TBA-reactive substances had fluorescence spectra, characteristic of the TBA-malondialdehyde adduct.

Experimental Rationale

The pronounced reactivity of Lewis lung carcinoma tissue with TBA, and the increased hepatic TBA-reactive material concentrations of tumour-bearing mice, may signify an appreciable degree of lipid peroxidation in vivo. The TBA-reactive substances, determined for tissues of tumour-bearing mice, might, however, have been produced during storage or processing of these tissues, prior to assay for lipoperoxides (Mead, 1976). The components necessary for lipid peroxidation, that is PUFA, iron (particularly in the membranous fraction), and soluble pro-oxidants, such as GSH or ascorbate, were probably present in the reaction mixtures, used in the TBA tests of the above studies (Barber and Bernheim, 1967). The aerobic incubation of homogenates, during the TBA tests might thus have promoted further lipid peroxidation. The concentrations of malondialdehyde-like substances, determined for the tissues of tumour-bearing
mice may have been over-estimated.

The purpose of the investigations, described in the following chapter was to characterize the TBA-reactants, determined for hepatic and tumour tissue of Lewis lung carcinoma-bearing mice. The TBA test has a number of limitations (Slater, 1984). It was thus first necessary to scrutinize the procedures, used in the present studies, to determine whether the results, obtained with these procedures, had any biological meaning.

The TBA-using method has been routinely applied, in investigations of autoxidation in animal tissues, by colleagues at the Marie Curie Memorial Foundation Research Institute, and in the Department of Biochemistry at the University of Surrey. The method, originally adopted, was adapted from an assay designed to quantify lipid peroxidation in plasma, using dilute sulphuric acid as the acid medium (Satoh, 1978). This procedure used only the pellet from serum or plasma, precipitated with TCA, because the acid-soluble components in the supernatant include sialic acid residues, a source of interference in the TBA method (Satoh, 1978). As neoplastic tissues have been reported to have aberrant metabolism, they could possibly contain a considerable number of acid-soluble substances, such as sialic acid, that might interfere with the TBA reaction. This procedure was therefore employed in preliminary studies of lipid peroxidation in mice bearing Lewis lung carcinomas (Capel and Thornley, 1982; Capel and Thornley, 1983).

The TBA-test, using $\text{H}_2\text{SO}_4$, had reliably shown qualitative differences between tissues from different experimental groups of animals. The TBA-reactive material concentrations, determined by this procedure were, however, quantitatively inconsistent and had comparatively large standard errors. Much of the error, observed with this TBA-test, may have been caused by the loss of substrate during the precipitation and washing
of the tissue pellet, after addition of protein precipitant; it has been reported that the removal of the acid-soluble components of tissues, prior to the TBA-reaction, is not necessary (Uchiyama and Mihara, 1978). In the studies, described in the present chapter, therefore, tissues were not treated with protein precipitant (TCA).

**Collaborative studies:** The pH of the reaction mixture in the TBA-test has been reported to be a critical factor governing the breakdown of the tissue precursors of malondialdehyde (Ohkawa et al., 1978; Bird and Draper, 1984). An application of the TBA method, which employs a buffer system, might be more satisfactory than systems using inorganic acid because the pH of the reaction could be held at its optimum level. The optimum pH of the reaction to determine TBA-reactivity of animal tissues has been variably reported to be 2.0 (Uchiyama and Mihara, 1978), 2 to 3 (Bird and Draper, 1984), and 3.5 (Ohkawa et al., 1979).

A collaborative study with a co-worker at the Marie Curie Memorial Foundation (Smallwood, 1983), was initiated to compare the above application of the TBA test with a recently reported method that used 0.1 M phthalate buffer, pH 3.5 as the acidifying reagent (Gutteridge, 1982a). These studies showed that the incubation times, used in the TBA-reaction procedures (see Experimental) were appropriate, and that the pH of 3.5 produced the optimum TBA-reactive material concentration from murine liver (Smallwood, 1983). The TBA-reactivity of murine liver was observed to be affected by the manner of homogenisation and storage of the tissue homogenate (Smallwood, 1983). The inclusion of whole blood (50 or 100 µl) or ascorbate (10^{-4} to 10^{-6} M) in the reaction mixture was observed to inhibit the concentration of TBA-reactant detected, by approximately 50% (Smallwood, 1983). Ascorbate, at higher concentrations (10^{-4} to 10^{-3} M), however, inhibited the reaction between malondialdehyde standard (1 nmole) and TBA by about 40% (Smallwood, 1983).
In the collaborative studies, the TBA-reactive material concentrations of liver and tumour tissue, from mice bearing Lewis lung carcinomas (15-20 days) and from tumour-free controls, were estimated both using the \( \text{H}_2\text{SO}_4 \)-method and the phthalate buffer-centred method. Free malondialdehyde concentrations in the same tissues was also determined, using an HPLC separative technique (Esterbauer et al, 1984), for comparison with the levels of malondialdehyde-like substances, ascertained by the TBA-tests. As the results of these studies were of relevance to the present investigations, they have been included in the present chapter (tables 4.1 - 4.3).

**Further experiments:** The reported effect of blood or ascorbate upon the TBA reaction could have been caused by the possible inhibition of further lipid peroxidation, promoted by the conditions of the reaction itself. The present studies thus investigated the effect of including an antioxidant or iron-chelator in the reaction mixtures, to inhibit any lipid peroxidation. Previous studies have shown that the differences in the TBA-reactive material concentrations between tissues of mice, bearing Lewis lung carcinomas, and tumour-free control animals, were more pronounced in microsomes than in other subcellular fractions (Capel and Thornley, 1982). The following investigations were therefore conducted, using the microsomal fractions of murine tissues.

The TBA-method can produce ambiguous evaluations of tissue lipid peroxidation. It has been suggested that it would be prudent to verify the results of such determinations, by employing as many other methods for the detection of lipid peroxidation as practicable (Slater, 1984). Chemiluminescence-measurement is a comparatively sensitive and easy-to-perform technique (Cadenas and Sies, 1984; Chapter 1). In the following studies, microsomes from hepatic, pulmonary and tumour tissues of Lewis lung carcinoma-bearing mice, and equivalent tissues from tumour-free
controls, were examined for their chemiluminescence during in vitro lipid peroxidation.

The light-emitting species of chemiluminescence reactions are transient, unlike the comparatively stable malondialdehyde precursors of lipoperoxides. An in vitro lipid peroxidation system was thus required. A pilot study was first performed to determine the most suitable type of autooxidative challenge. The microsomal lipid peroxidation reaction, initiated by the challenge, would have to be of convenient duration, and of sufficient intensity to be detected, with precision, by the available instrumentation (liquid scintillation counter).

The chemiluminescence, arising from the microsomal samples, was equated with the amount of TBA-reactive material, generated by a similar in vitro system. Experiments were also undertaken, using various suitable antioxidants and free-radical scavenging systems, to characterize the free-radical species, concerned with the peroxidation reactions that gave rise to chemiluminescence and malondialdehyde-like substances.

EXPERIMENTAL

Chemicals

All chemicals were of the purest quality commercially available, and were supplied by either Fisons Scientific Apparatus, Loughborough, Leics., or by Sigma London Chemical Co., Ltd., Poole, Dorset.

Animals and Treatment

Male C57BL6 mice (Olac) were used and were maintained as described previously (Chapter 2). Mice (20 ± 2g body wt, 8-10 weeks), designated to bear Lewis lung carcinomas, were inoculated with tumour cells, as described earlier (Chapter 2). Equal numbers of age-matched male mice,
injected with phosphate-buffered saline only, served as the tumour-free controls.

**Preparation of Tissues**

Animals were killed by CO₂ narcosis: blood was removed by cardiopuncture with a 1ml heparinized syringe. The vasculature was perfused by injecting saline (1ml) into the hepatic portal vein. Tissues were immediately excised and rinsed in ice-cold 1.15% w/v KCl.

Tissues were homogenized in 1.15% w/v KCl, as described previously (Chapter 2): livers and tumours at 25% wet wt/vol; lungs at 20% wet wt/vol. Microsomal fractions were obtained from tissue homogenates, using the differential centrifugation technique, described earlier (Chapter 2). Microsomal pellets were obtained from the 20 000 g supernatants, by centrifugation at 105 000 g for 60 min.

In the studies to determine microsomal concentrations of TBA-reactive substances, and in the pilot chemiluminescence studies, the microsomal pellets were washed twice by resuspension in 1.15% w/v KCl followed by centrifugation. The washed pellets were resuspended at 25% original wet wt/vol in KCl, and stored in the refrigerator. These samples were assayed within 36 hr of excision of tissues from the experimental animals.

In the remaining experiments, microsomal pellets were washed once in 1.15% w/v KCl, and stored as 100% original wet wt/vol suspensions in 50 mM phosphate buffer, pH 7.4, containing 20% w/v glycerol and 1 mM EDTA, at -80°C. Prior to their use in the following investigations, these microsomes were washed twice in 1.15% w/v KCl: resuspended at 25% original wet wt/vol in isotonic KCl; and diluted appropriately with 0.1 M phosphate buffer, pH 7.4, to a final concentration of approximately 1mg
Biochemical Investigations

TBA-reactive material concentrations:

a) Sulphuric acid method Reaction mixtures, containing 0.1ml of tissue homogenate or microsomal suspension, 2.4ml of 0.05 M \( \text{H}_2\text{SO}_4 \), and 2.5ml of 0.2% w/v TBA in 2 M \( \text{Na}_2\text{SO}_4 \) were vortexed and incubated at 100° C for 30 min (Satoh, 1978; Chapter 3). The TBA-reactive adduct was extracted into 4ml of butan-1-ol, and its fluorescence (535 nm excitation, 553 nm emission) measured. The concentration of TBA-reactive substances in the samples was determined by comparison with the fluorescence obtained from the reaction between TBA and 1,1,3,3-tetramethoxypropane standards (0.25 and 0.75 nmols).

b) Phthalate buffer method Reaction mixtures, containing 0.1 ml of tissue homogenate or microsomes, 2.4ml of 0.1 M potassium hydrogen phthalate buffer, and 2.5ml of 1% w/v TBA in 0.05 M NaOH were vortexed and incubated at 100° C for 15 min (Gutteridge, 1982a). The concentrations of TBA-reactive material in the samples were determined fluorimetrically, as described above.

Addition of BHT or DETAPAC: The TBA-reactivity in microsomal fractions, determined by the two procedures, outlined above, were compared with that obtained after adding an antioxidant or a metal chelator into the reaction mixtures. The antioxidant, butylated hydroxytoluene (BHT) was dissolved in ethanol (0.1ml) and added to a final concentration of 0.01% w/v (Svingen et al., 1979; Kornbrust and Mavis, 1980). The chelator, diethylene-triaminepenta-acetic acid (DETAPAC) was dissolved in 0.1 M phthalate buffer or 0.05 M \( \text{H}_2\text{SO}_4 \), as appropriate, to a final reaction mixture concentration of 0.2 mM.

Determination of free malondialdehyde concentration: Ice-cold 20% w/v
TCA (2ml) was added to 1ml of freshly-prepared tissue homogenate. The mixture was centrifuged for 30 min at 2500 rpm: 1ml of supernatant was made up to 10ml with 9:1 by vol solution of 0.03 M Tris/HCl buffer, pH 7.4, and acetonitrile (Esterbauer et al, 1984). Aliquots (20μl) were injected, via a Rheodyne injection valve, into the Spherisorb R column of the HPLC. The eluent, used for equilibration of the instrument and separation of malondialdehyde was 9:1 v/v Tris/HCl buffer/CH₃CN. The flow rate of eluent was 1ml/ min. Malondialdehyde was detected spectro-photometrically by its absorption at 270nm (Lang et al, 1984).

A 10 mM stock solution of 1,1,3,3-tetramethoxy propane, in 0.05 M H₂SO₄, was freshly prepared and then diluted with Tris/HCl buffer to a concentration of 20 μM. 1ml volumes of the standard solution, containing 20 nmoles of malondialdehyde, were used to calibrate the HPLC and thereby determine the concentration of malondialdehyde in the tissue samples.

Chemiluminescence

Detection system: Chemiluminescence, from peroxidizing microsomes, was detected using a liquid scintillation counter (Seitz, 1978; Smith et al, 1982; Repine et al, 1984). Liquid peroxidation was initiated at timed intervals by the addition of oxidant (0.1ml of FeCl₃) to 4ml volumes of 1 mg protein/ml microsomal suspensions in 0.1 M phosphate buffer, pH 7.4, prewarmed to 37°C. The reaction mixtures were shaken: the caps were screwed onto the vials (two at each occasion); and then the mixtures were placed into the liquid scintillation counter for automated counting (30 sec). The counter mechanism was set at 100% gain, with discriminator fully open from 1 to 256. The coincidence mode of the counter was disabled so that both photomultiplier tubes could be used to discriminate and summate the comparatively low light emission, resulting from lipid
To minimize the phosphorescence, that can arise from plastics, the caps and vials, used for the chemiluminescence procedures were dark-adapted (Seitz, 1981). The phosphate buffer was stored in a light proof container, and the procedure was performed in a room with minimal feasible lighting (Trush et al, 1978). The background counts for the scintillation vials, containing buffer alone, were approximately 5000 cpm.

After counting, the vials were uncapped and returned to a 37°C water bath. Chemiluminescence was monitored at regular intervals (every 4-5 min) until the reaction had peaked and returned to basal levels. The vials were regularly shaken to ensure oxygenation of the reaction mixtures. Chemiluminescence of the samples was expressed as CPM/ mg microsomal protein (after correction for background luminescence).

**Autoxidative challenge:** Three different systems to promote microsomal lipid peroxidation were evaluated (Bartoli and Galeotti, 1979). The following autoxidative challenges were applied to fresh hepatic microsomal suspensions (final concentrations):

1) 4 mM ADP + 0.05 mM FeCl₃ + 0.4 mM NADPH

2) 4 mM ADP + 0.05 mM FeCl₃ + 0.33 mM xanthine + 5 x 10⁻² units (Sigma®) xanthine oxidase.

3) 4 mM ADP + 0.05 mM FeCl₃ + 0.25 mM sodium ascorbate.

Reactions were initiated by addition of 0.1ml of FeCl₃.

**Luminol:** A stock solution of luminol was prepared by dissolving 0.5 mg of Luminol® in 0.05ml of dimethyl sulfoxide (DMSO) and then diluting to
a concentration of 10 mg/100ml in phosphate-buffered saline (Trush et al., 1978). The stock solution was refrigerated for 2 to 3 days to stabilize, before use (Seitz, 1978).

The stock solution of Luminol was diluted 1 in 100, 1 in 200 and 1 in 400 by volume in 0.1 M phosphate buffer, pH 7.4, and 0.1ml of one of the dilutions was added to the reaction mixture, immediately before initiating peroxidation.

Monitoring of In Vitro Lipid Peroxidation by Detection of TBA-reactive Material

Reaction mixtures (prewarmed to 37°C) were assembled at the same concentrations as those used for the chemiluminescence procedure, except that all the volumes were doubled to a final volume of 8ml. Reactions were started, at timed intervals, by addition of 0.2ml of 0.05 mM (final concentration) FeCl₃. The mixtures were gently agitated in a shaking water bath at 37°C, throughout the procedure. At timed intervals (every 5 min) a 0.1ml aliquot of each reaction mixture was added to tubes, containing 0.02% w/v BHT and 0.2 mM DETAPAC in 0.1 M phthalate buffer, pH 3.5, in an ice-water bath. At the end of the reaction procedure, 2.5ml of 1% w/v TBA in 0.05 M NaOH was added to each tube: the TBA-adduct fluorophore was extracted into butan-l-ol and the concentration of malondialdehyde-like material, in the microsomal samples, determined as described previously for the TBA-test.

Characterization of Free-radical Species, Involved in Microsomal Lipid Peroxidation

The antioxidants and free-radical quenchers used in the present studies to characterize the free radical species, evolved during the microsomal lipid peroxidation reaction are named in the tables of results (tables 4.10 - 4.12). The rationale for using these various substances
will become apparent in the Discussion of the present chapter.

**Instrumentation**

The HPLC separative technique used a Du Pont 850 High Performance Liquid Chromatograph, incorporating a UV-visible detector and Spherisorb\textsuperscript{R} ODS HPLC column (internal measurements of 25 ml/5mm). Chemiluminescence was measured using a LKB Wallac 1217 Rackbeta liquid scintillation counter with refrigeration unit.

**Statistical Analyses**

Results were expressed as mean ± SEM. Data was statistically evaluated by analysis of variance; means of experimental group data were compared using the Newman-Keuls procedure.

**RESULTS**

**Collaborative Studies**

**TBA reactivity, using sulphuric acid as the acid reagent (table 4.1):**
The concentration of malondialdehyde-like substances in Lewis lung carcinoma tissue, determined by the TBA test using sulphuric acid as the acidifying medium, was considerable (more than twice that of liver) and increased marginally, but not significantly, with time after tumour implantation (15-20 days). The TBA-reactive material concentrations of liver homogenate from tumour-bearing mice were greater than those of tumour-free animals, almost doubling between 15 days and 20 days after tumour implantation.

**TBA reactivity, using phthalate buffer as the acid reagent (table 4.2):**
The concentration of TBA-reactive material in Lewis lung carcinoma homogenate, when determined using phthalate buffer, was also substantial but of similar magnitude to that of liver. TBA-reactive material concen-
TABLE 4.1 - Thiobarbituric Acid-reactive Material Concentrations in Hepatic and Tumour Tissue of Lewis Lung Carcinoma-bearing C57BL6 mice, and in Hepatic Tissue of Tumour-free Controls - AS DETERMINED BY INCUBATION IN 0.05M H₂SO₄

Results represent mean ± SEM for 6 mice at each time interval. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Liver Control</th>
<th>Tumour-bearing Tumour</th>
<th>Tumour-bearing Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Tumour</td>
<td>Tumour-bearing</td>
</tr>
<tr>
<td></td>
<td>(nmol MDA equiv /mg protein)</td>
<td>(nmol MDA equiv /mg protein)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(nmol MDA equiv /mg protein)</td>
<td>(nmol MDA equiv /mg protein)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.57 ± 0.08</td>
<td>0.82 ± 0.08</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>18</td>
<td>0.57 ± 0.09</td>
<td>1.0 ± 0.16¹</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>20</td>
<td>0.57 ± 0.11</td>
<td>1.5 ± 0.25¹</td>
<td>1.7 ± 0.2</td>
</tr>
</tbody>
</table>

¹ Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 4.2 - Thiobarbituric Acid-reactive Material Concentrations in Hepatic and Tumour Tissue of Lewis Lung Carcinoma-bearing C57BL6 Mice, and in Hepatic Tissue of Tumour-free Controls - AS DETERMINED BY INCUBATION IN 0.1M PHTHALATE BUFFER (pH 3.5)

Results represent mean ± SEM for 6 mice at each time interval. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Liver Control</th>
<th>Liver Tumour-bearing</th>
<th>Tumour-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol MDA equiv /mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.16 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>18</td>
<td>0.15 ± 0.01</td>
<td>0.19 ± 0.01</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>20</td>
<td>0.16 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.25 ± 0.01</td>
</tr>
</tbody>
</table>

a-b Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
Concentrations of "free" malondialdehyde (table 4.3): The concentration of malondialdehyde in the acid-soluble components of murine tissues, determined by HPLC, was at least an order of magnitude less than the concentration of TBA reactants of the intact tissues (tables 4.1 to 4.3). The malondialdehyde concentrations of Lewis lung carcinoma tissue was marginally greater than that of control liver tissue. The malondialdehyde concentration of livers, from tumour-bearing mice did not differ significantly from those of tumour-free animals.

Microsomal TBA-reactive Material Concentrations (tables 4.4 and 4.5)

a) No additives to inhibit lipid peroxidation in the reaction mixtures: The concentration of TBA-reactive substances in hepatic microsomes, when determined, using H₂SO₄ as the acid medium, was approximately 8 times greater than the concentration, determined, using phthalate buffer. The procedure, using H₂SO₄, revealed a significantly greater TBA-reactive material concentration for hepatic microsomes from tumour-bearing mice (table 4.4): the method, using phthalate buffer did not show any significant differences (table 4.5).

The TBA-reactive material concentration of microsomes, from Lewis lung carcinoma, was about 50% greater when estimated by the procedure, using phthalate buffer, than that using H₂O₄.

b) Effect of an anti-oxidant (BHT): The inclusion of 0.01% w/v ethanolic BHT in the reaction mixture, decreased the concentration of TBA-reactive
TABLE 4.3 - Malondialdehyde Concentrations in Hepatic and Tumour Tissue of Lewis Lung Carcinoma-bearing C57BL6 Mice, and in Hepatic Tissue of Tumour-free Controls - AS DETERMINED BY HPLC SEPARATIVE TECHNIQUE.

Results represent mean ± SEM for 6 mice at each time interval. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Liver Control</th>
<th>Tumour-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(pmol MDA equiv /mg protein)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>11 ± 1</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>18</td>
<td>15 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>20</td>
<td>17 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 4.4 - Microsomal Thiobarbituric Acid-reactive Material Concentrations in Hepatic and Tumour Tissue of Lewis Lung Carcinoma-bearing C57BL6 Mice, and in Hepatic Tissue of Tumour-free Controls - AS DETERMINED BY INCUBATION IN 0.05M H$_2$SO$_4$

Effect of incorporating an antioxidant (BHT) or an iron-chelator (DETAPAC) into the reaction mixture

Results represent mean ± SEM for 6 mice, killed 14 days after inoculation with tumour cells (or with PBS for the controls). Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th></th>
<th>Liver Control (nmol MDA equiv /mg protein)</th>
<th>Tumour-bearing (nmol MDA equiv /mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additives</td>
<td>12 ± 1</td>
<td>17 ± 2$^b$</td>
</tr>
<tr>
<td>+0.01% BHT</td>
<td>4.1 ± 1.0$^a$</td>
<td>4.6 ± 0.8$^a$</td>
</tr>
<tr>
<td>+0.1mM DETAPAC</td>
<td>12 ± 0.5</td>
<td>11 ± 0.6</td>
</tr>
</tbody>
</table>

$^a-c$ Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 4.5 - Microsomal Thiobarbituric Acid-reactive Material Concentrations in Hepatic and Tumour Tissue of Lewis Lung Carcinoma-bearing C57BL6 Mice, and in Hepatic Tissue of Tumour-free Controls

- AS DETERMINED BY INCUBATION IN 0.1M PHTHALATE BUFFER (pH 3.5)

Effect of incorporating an antioxidant (BHT) or an iron-chelator (DETAPAC) into the reaction mixture

Results represent mean ± SEM for 6 mice, killed 14 days after inoculation with tumour cells (or with PBS for the controls). Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th></th>
<th>Liver Control</th>
<th>Tumour-bearing</th>
<th>(nmol MDA equiv /mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additives</td>
<td>1.6 ± 0.1</td>
<td>1.8 ± 0.2</td>
<td>4.5 ± 1.1</td>
</tr>
<tr>
<td>+0.01% BHT</td>
<td>0.84 ± 0.07</td>
<td>0.88 ± 0.09</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>+0/1mM DETAPAC</td>
<td>1.7 ± 0.1</td>
<td>1.5 ± 0.2</td>
<td>3.8 ± 0.4</td>
</tr>
</tbody>
</table>

a Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
material in hepatic microsomes by 65%, when assayed by the procedure using H$_2$SO$_4$, and by 48% when assayed by the procedure using phthalate buffer. Tests that included BHT in their reaction mixtures, did not show any significant differences between the TBA-reactivity of microsomes from tumour-bearing mice and their tumour-free controls.

BHT caused the TBA-reactive material concentrations of tumour microsomes to decrease by 47%, when assayed by the procedure using H$_2$SO$_4$, and by 29% when determined by the procedure using phthalate buffer.

c) **Effect of metal-chelator (DETPAC):** DETPAC, incorporated at 0.1 mM in the reaction mixture, did not significantly affect the TBA-reactive material concentrations of control hepatic, or tumour microsomes, determined by either application of the TBA method. The increased hepatic microsomal TBA-reactivity of tumour-bearing mice, observed using the H$_2$SO$_4$ assay system, did not occur in the presence of DETPAC (table 4.4).

d) **Effect of BHT, DETPAC or type of acid reagent upon reactivity of malondialdehyde standards**

BHT and DETPAC, at the concentrations used above, had no appreciable effect upon the fluorescence, resulting from the reaction of malonaldehyde bisdimethyl acetal standards (0.25 and 1.0 nmole) with TBA. The fluorescence of the TBA-adduct of standard malondialdehyde was essentially the same whether using phthalate buffer or H$_2$SO$_4$ as the acidifying reagents.

**Studies of In Vitro Lipid Peroxidation**

**Selection of autoxidative challenge:** The iron/ascorbate autoxidative challenge evoked considerably greater chemiluminescence from fresh hepatic microsomes than did iron/xanthine-xanthine oxidase or iron/NADPH (fig. 4.1). The omission of ADP from the reaction mixture caused an approximately 80% decrease in chemiluminescence and production of TBA-
FIG. 4.1 - Chemiluminescence of Hepatic Microsomes after Peroxidative Insult by Various Toxic Challenges.

- NADPH \( \leftrightarrow \) Xanthine + xanthine oxidase
- Ascorbate \( \leftrightarrow \) Ascorbate + ADP

[Reactions initiated with FeCl\(_3\) - see main text for details]
FIG. 4.2 - Effect of Luminol on FeCl\textsubscript{3}/ascorbate-induced Chemiluminescence of Fresh Hepatic Microsomes.

Note: Luminol was a x 400 dilution of stock solution (Results = mean ± SEM - see main text for details).

(→) + Luminol  (↔) - Luminol
TABLE 4.6 - Ascorbate/FeCl$_3$-induced Peak Chemiluminescence of Tumour and Pulmonary Microsomes from Lewis Lung Carcinoma-bearing C57BL6 Mice, and of Microsomes of Tumour-free Controls

Results represent mean ± SEM for 6 mice at each time interval. Microsomes were stored as suspensions in 50mM phosphate buffer (pH 7.4), containing 20% w/v glycerol and 1mM EDTA, at -80°C prior to analysis. Time (min) to maximal change in chemiluminescence shown in brackets. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Lung Tumour-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Tumour-bearing</td>
</tr>
<tr>
<td></td>
<td>(cpm x 10$^{-3}$/mg protein, at 37°C, pH 7.4)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>130 ± 26</td>
</tr>
<tr>
<td></td>
<td>(19 ± 4)</td>
</tr>
<tr>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>117 ± 30</td>
</tr>
<tr>
<td></td>
<td>(15 ± 2)</td>
</tr>
<tr>
<td>17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>139 ± 28</td>
</tr>
<tr>
<td></td>
<td>(19 ± 4)</td>
</tr>
</tbody>
</table>

$^a$ Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 4.7 - Ascorbate/FeCl\textsubscript{2}-induced Peak Chemiluminescence of Hepatic Microsomes from Lewis Lung Carcinoma-bearing C57BL6 mice-and of Microsomes of Tumour-free Controls

Results represent mean ± SEM for 6 mice at each time interval. Microsomes were stored as suspensions in 50mM phosphate buffer (pH 7.4), containing 20% w/v glycerol and 1mM EDTA, at -80°C prior to analysis. Time (min) to maximal change in chemiluminescence shown in brackets. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Control</th>
<th>Tumour-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(cpm x 10\textsuperscript{-3}/mg protein, at 37°C, pH 7.4)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>22 ± 9</td>
<td>667 ± 63\textsuperscript{a} (44 ± 3)</td>
</tr>
<tr>
<td>15</td>
<td>36 ± 17</td>
<td>724 ± 87\textsuperscript{a} (47 ± 3)</td>
</tr>
<tr>
<td>17</td>
<td>30 ± 11</td>
<td>653 ± 53\textsuperscript{a} (52 ± 11)</td>
</tr>
</tbody>
</table>

Chemiluminescence was monitored for 90 min.

\textsuperscript{a} Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 4.8 - Total Chemiluminescence of Ascorbate/FeCl$_3$-induced Hepatic Tumour and Pulmonary Microsomes from Lewis Lung Carcinomabearing C57BL6 Mice.

Results represent mean surface area of microsomal chemiluminescence plotted vs time, for 6 mice. Values are expressed as % of corresponding control. Tumour microsomes were compared with control pulmonary microsomes. Microsomes were stored as suspensions in 50mM phosphate buffer (pH 7.4), containing 20% w/v glycerol and 1mM EDTA, at 80°C, prior to analysis.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Liver</th>
<th>Lung</th>
<th>Tumour</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>2390</td>
<td>84</td>
<td>22</td>
</tr>
<tr>
<td>15</td>
<td>1850</td>
<td>87</td>
<td>30</td>
</tr>
<tr>
<td>17</td>
<td>2230</td>
<td>97</td>
<td>36</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
reactive material. 4 mM ADP, 0.05 mM FeCl₃ and 0.25 mM ascorbate were therefore used to initiate lipid peroxidation in all subsequent studies.

Iron/ascorbate-initiated chemiluminescence had a linear relationship with microsomal concentration, in the range of 0.2 to 5 mg protein/ml.

Luminol: The addition of 1ml of luminol in the dilution range given earlier (see Experimental) did not greatly increase the chemiluminescence of iron/ascorbate-challenged hepatic microsomes (table 4.9; fig. 4.2). Luminol thus was not used in the studies of the present chapter.

Background chemiluminescence: Microsomal suspensions in buffer (1mg protein/ml), at 37° C, emitted light of about 2000 cpm greater than that of buffer alone. The unchallenged chemiluminescence of hepatic microsomes from tumour-bearing mice did not differ from that of control animals. None of the free radical scavengers, antioxidant defence enzymes, singlet oxygen quenchers or ascorbate affected the chemiluminescence of the microsomal suspensions prior to the addition of FeCl₃.

Chemiluminescence of tumour and pulmonary microsomes (tables 4.6 and 4.8): Storage of pulmonary and tumour microsomes as suspensions in 20% w/v glycerol in phosphate buffer at -80° C, did not cause any appreciable differences in their chemiluminescence during lipid peroxidation. The peak of the chemiluminescence, emitted during lipid peroxidation of microsomes from Lewis lung carcinomas was approximately 65% less than that of the reference tissue, control lung (table 4.6). The amount of light emitted, during lipid peroxidation was estimated to have been about 70% less in tumour microsomes than pulmonary microsomes (table 4.7; fig. 4.4). The chemiluminescence of tumour microsomes generally peaked at 7-15 min after the initiation of the reaction: a shorter duration than that required by control pulmonary microsomes (15-20 min).
The chemiluminescence of microsomes from the lungs of mice, bearing Lewis lung carcinomas peaked earlier than that of control mice, but the peak chemiluminescence level and total chemiluminescence did not differ from that of the controls (fig. 4.4).

Chemiluminescence of hepatic microsomes (tables 4.8 and 4.9): Hepatic microsomes, that had been stored as suspensions in glycerol/phosphate buffer emitted comparatively little chemiluminescence when exposed to FeCl₃/ascorbate (fig. 4.5). Doubling the concentration of FeCl₃ or ascorbate did not result in any appreciable increase in chemiluminescence. Chemiluminescence was however monitored for only 90 min after the initiation of peroxidation. The chemiluminescence of hepatic microsomes, from tumour-bearing mice, that had been stored under the same conditions was, however, only marginally less than that observed for fresh microsomes: took 20 to 30 min to peak; and was approximately 20-fold greater than that of control hepatic microsomes (table 4.7; fig. 4.5).

In a limited study, the chemiluminescence of glycerol/phosphate buffer-stored microsomes was monitored for more than 90 min after the initiation of the reaction. A rise in chemiluminescence, equivalent to that observed for fresh hepatic microsomes, occurred at about 120 min after adding FeCl₃ to the mixture (figs. 4.7 and 4.8). The inclusion of SOD or vitamin E acetate prevented this delayed chemiluminescence emission (figs. 4.7 and 4.8). A second parallel chemiluminescence reaction did not arise from the microsomes of tumour-bearing animals.

TBA-reactive material production during iron/ascorbate-induced lipid peroxidation (table 4.9)

Lipid peroxidation, monitored by determination of TBA-reactive substances, of hepatic microsomes that had been stored as suspensions in
TABLE 4.9 - Ascorbate/FeCl$_3$-induced THIOBARBITURIC ACID-REACTIVE MATERIAL PRODUCTION by microsomes of hepatic and tumour tissue from Lewis lung carcinoma-bearing C57BL6 mice, and by hepatic microsomes of tumour-free controls

Results represent mean ± SEM for 6 mice at each time interval. Microsomes were stored as suspensions in 50mM phosphate buffer (pH 7.4), containing 20% w/v glycerol and 1mM EDTA, at -80°C prior to analysis. Time (min) to maximal change in TBA reaction shown in brackets. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after implantation of tumour (days)</th>
<th>Liver</th>
<th>Tumour-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control-bearing</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>51 ± 4</td>
<td>84 ± 9$^a$</td>
</tr>
<tr>
<td></td>
<td>(46 ± 2)</td>
<td>(42 ± 4)</td>
</tr>
<tr>
<td>15</td>
<td>49 ± 5</td>
<td>79 ± 10$^a$</td>
</tr>
<tr>
<td></td>
<td>(42 ± 3)</td>
<td>(42 ± 4)</td>
</tr>
<tr>
<td>17</td>
<td>53 ± 4</td>
<td>102 ± 6$^a$</td>
</tr>
<tr>
<td></td>
<td>(46 ± 2)</td>
<td>(45 ± 5)</td>
</tr>
</tbody>
</table>

$^a$ Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
FIG. 4.3 - Effect of Vitamin E or BHT upon FeCl$_2$/ascorbate Induced Chemiluminescence of Fresh Hepatic Microsomes.

(•--•) Control (×--×) + Vitamin E
(○--○) + BHT

[Results = mean ± SEM - see main text for details]
glycerol/ phosphate buffer, peaked at about 40 to 45 min after addition of FeCl₃. The production of malondialdehyde-like material by microsomes, from the livers of tumour-bearing mice, was significantly greater than that of control mice. The generation of TBA-reactive material from iron/ ascorbate-challenged tumour microsomes was approximately 20% of that produced by control liver microsomes, and took 20 to 25 min to reach a maximum.

**Effect of free-radical scavengers and active oxygen quenchers**

Catalase caused a pronounced inhibition of iron/ascorbate-mediated chemiluminescence of liver, lung and tumour microsomes (table 4.10; fig. 4.6). Adding catalase at the peak of the chemiluminescence response, or use of denatured catalase, caused negligible inhibition of chemiluminescence. Catalase inhibited TBA-reactive material production by about 60% (table 4.12).

SOD did not significantly affect the degree of hepatic microsomal chemiluminescence or TBA-reactive material production (tables 4.10 and 4.12), but did delay the peak of TBA-reactivity by about 30 min (table 4.12). The chemiluminescence of tumour microsomes was almost completely inhibited by SOD (table 4.10). SOD also inhibited the slow onset chemiluminescence of hepatic phosphate-buffer/ glycerol-stored microsomes from tumour-free animals (see earlier; fig. 4.8)).

Vitamin E (α-tocopherol acetate) inhibited the chemiluminescence of peroxidizing hepatic microsomes by about 9% (table 4.10; fig. 4.3) and the TBA-reactive material production by 25% (table 4.12). The peak of the hepatic microsomal TBA-reactive material production was delayed by about 30 min, in the presence of α-tocopherol. Ethanol, at the same final concentration in the reaction mixture as when used to solubilize α-tocopherol, probably accounted for about half the inhibition of lipid
FIG. 4.4 - FeCl$_3$/ascorbate-induced Chemiluminescence of Phosphate buffer/glycerol-stored microsomes from Lungs and Tumour of Mice that had Borne Lewis Lung Carcinomas for 15 days.

- Lung of tumour-free mice [n=6]
- Lung of tumour-bearing mice [n=6]
- Lewis lung carcinoma [n=6]

[Results = mean ± SEM - see main text for details]
FIG. 4.5 - FeCl$_3$/ascorbate-induced Chemiluminescence of Phosphate buffer/glycerol-stored microsomes from Livers of Mice that had Borne Lewis Lung Carcinomas for 15 days.

(•••) Tumour-free Controls [n=6]
(••••) Tumour-bearing [n=6]
[Results = mean ± SEM - see main text for details]
TABLE 4.10 - Effect of Catalase, SOD or Vitamin E Acetate Upon Ascorbate/FeCl$_3$-induced Microsomal Chemiluminescence

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Final concentration</th>
<th>Number of samples</th>
<th>Tissue</th>
<th>Maximal chemiluminescence (% of expected luminescence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>1300 Sigma$^R$ units/ml</td>
<td>3</td>
<td>liver*</td>
<td>0</td>
</tr>
<tr>
<td>Catalase</td>
<td>1300 Sigma$^R$ units/ml</td>
<td>3</td>
<td>liver#</td>
<td>85</td>
</tr>
<tr>
<td>Catalase</td>
<td>1300 Sigma$^R$ units/ml</td>
<td>2</td>
<td>lung*</td>
<td>0</td>
</tr>
<tr>
<td>Catalase</td>
<td>1300 Sigma$^R$ units/ml</td>
<td>2</td>
<td>Lewis lung* carcinoma</td>
<td>6</td>
</tr>
<tr>
<td>Catalase (boiled)</td>
<td>1300 Sigma$^R$ units/ml</td>
<td>2</td>
<td>liver*</td>
<td>88</td>
</tr>
<tr>
<td>SOD</td>
<td>1400 Sigma$^R$ units/ml</td>
<td>3</td>
<td>liver*</td>
<td>103</td>
</tr>
<tr>
<td>SOD</td>
<td>1400 Sigma$^R$ units/ml</td>
<td>2</td>
<td>liver#</td>
<td>97</td>
</tr>
<tr>
<td>SOD</td>
<td>1400 Sigma$^R$ units/ml</td>
<td>2</td>
<td>Lewis lung* carcinoma</td>
<td>5</td>
</tr>
<tr>
<td>SOD</td>
<td>1400 Sigma$^R$ units/ml</td>
<td>2</td>
<td>PO$_4$/glycerol buffer-stored liver</td>
<td>15</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.7mM</td>
<td>2</td>
<td>liver*</td>
<td>91</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.7mM</td>
<td>2</td>
<td>liver#</td>
<td>116</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.7mM</td>
<td>2</td>
<td>Lewis lung* carcinoma</td>
<td>122</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2.3% w/v</td>
<td>2</td>
<td>liver*</td>
<td>94</td>
</tr>
</tbody>
</table>

* Added before peroxidation reaction was initiated with FeCl$_3$
# Added when chemiluminescence was maximal.
FIG. 4.6 - Effect of Vitamin E or Catalase upon FeCl$_2$/ascorbate-induced Chemiluminescence of Phosphate buffer/glycerol-stored Tumour Microsomes from Mice that had Borne Lewis Lung Carcinomas for 17 Days.

(○--○) Control  (•••••) + Vitamin E
(•---•) + Catalase

[Results = mean ± SEM – see main text for details]
FIG. 4.7 - Effect of Vitamin E upon FeCl₃/ascorbate-induced Chemiluminescence of Phosphate buffer/glycerol-stored Microsomes from the Livers of Tumour-free Mice.

(—•—•) Control (○—○) + Vitamin E

[Results = mean ± SEM - see main text for details]
FIG. 4.8 - Effect of Superoxide Dismutase upon FeCl$_3$/ascorbate-induced Chemiluminescence of Phosphate buffer/glycerol-stored Microsomes from the Livers of Tumour-free Mice.

(●—●) Control (○—○) + SOD

[Results = mean ± SEM - see main text for details]
### TABLE 4.11 - Effects of Various Free Radical Scavengers Upon Ascorbate/FeCl\textsubscript{3}-induced Microsomal Chemiluminescence

<table>
<thead>
<tr>
<th>Scavenger</th>
<th>Final concentration</th>
<th>Number of samples</th>
<th>Tissue</th>
<th>Maximal chemiluminescence (% of expected luminescence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Mannitol</td>
<td>10 mM</td>
<td>3</td>
<td>liver*</td>
<td>74</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>10 mM</td>
<td>2</td>
<td>liver#</td>
<td>61</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>10 mM</td>
<td>2</td>
<td>Lewis lung carcinoma*</td>
<td>61</td>
</tr>
<tr>
<td>Thiourea</td>
<td>1 mM</td>
<td>3</td>
<td>liver*</td>
<td>43</td>
</tr>
<tr>
<td>Thiourea</td>
<td>1 mM</td>
<td>2</td>
<td>Lewis lung carcinoma*</td>
<td>38</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>10 mM</td>
<td>2</td>
<td>liver*</td>
<td>92</td>
</tr>
<tr>
<td>DMSO</td>
<td>30 mM</td>
<td>2</td>
<td>liver*</td>
<td>92</td>
</tr>
<tr>
<td>BHT</td>
<td>0.5 mM</td>
<td>3</td>
<td>liver*</td>
<td>19</td>
</tr>
<tr>
<td>BHT</td>
<td>0.5 mM</td>
<td>2</td>
<td>liver*</td>
<td>58</td>
</tr>
<tr>
<td>DABCO</td>
<td>100 mM</td>
<td>3</td>
<td>liver*</td>
<td>6</td>
</tr>
<tr>
<td>DABCO</td>
<td>100 mM</td>
<td>2</td>
<td>liver#</td>
<td>41</td>
</tr>
<tr>
<td>DABCO</td>
<td>100 mM</td>
<td>2</td>
<td>Lewis lung carcinoma*</td>
<td>2</td>
</tr>
<tr>
<td>DABCO</td>
<td>100 mM</td>
<td>2</td>
<td>Lewis lung# carcinoma</td>
<td>61</td>
</tr>
</tbody>
</table>

* Added before peroxidation reaction was initiated with FeCl\textsubscript{3}.

# Added when chemiluminescence was maximal.
<table>
<thead>
<tr>
<th>Reaction System</th>
<th>% of expected TBA-reactive material production</th>
<th>Time to max TCA-reactive material production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control: 20 mM ascorbate + 4 mM FeCl₃ + 4 mM ADP</td>
<td>100</td>
<td>40</td>
</tr>
<tr>
<td>Blank: control system with no ADP</td>
<td>29</td>
<td>50</td>
</tr>
<tr>
<td>Blank: Control system with no FeCl₃</td>
<td>6</td>
<td>60</td>
</tr>
<tr>
<td>0.1 mM H₂O₂ + 4 mM FeCl₃</td>
<td>17</td>
<td>50</td>
</tr>
<tr>
<td>Control + SOD (1400 Sigma units/ml)</td>
<td>102</td>
<td>70</td>
</tr>
<tr>
<td>Control + Catalase (1300 Sigma units/ml)</td>
<td>42</td>
<td>40</td>
</tr>
<tr>
<td>Control + α-tocopherol (0.7 mM)</td>
<td>75</td>
<td>70</td>
</tr>
<tr>
<td>Control + Ethanol (2.3% v/v)</td>
<td>86</td>
<td>40</td>
</tr>
<tr>
<td>Control + D-Mannitol (25 mM)</td>
<td>82</td>
<td>70</td>
</tr>
<tr>
<td>Control + BHT (0.5 mM)</td>
<td>18</td>
<td>50</td>
</tr>
<tr>
<td>Control + Thiourea (1 mM)</td>
<td>68</td>
<td>60</td>
</tr>
<tr>
<td>Control + Sodium Azide (10 mM)</td>
<td>85</td>
<td>40</td>
</tr>
<tr>
<td>Control + DABCO (50 mM)</td>
<td>12</td>
<td>50</td>
</tr>
</tbody>
</table>
Antioxidant added

FIG. 4.9 - Effect of DABCO or Mannitol upon FeCl$_3$/ascorbate-induced Chemiluminescence of phosphate buffer/glycerol-stored Tumour Microsomes from Mice that had Borne Lewis lung Carcinomas for 17 Days.

(•—•) Control (o—o) + DABCO
(*—*) + Mannitol

[Results = mean ± SEM - see main text for details]
FIG. 4.10 - Effect of DABCO upon FeCl₂/ascorbate-induced Chemiluminescence of Phosphate buffer/glycerol-stored Microsomes from the Livers of Mice that had Borne Lewis Lung Carcinomas for 15 days.

(•—•) Control - no DABCO
(○—○) DABCO added at peak of reaction
(♦—♦) DABCO added before reaction initiated

[Results = mean ± SEM - see main text for details]
peroxidation by $\alpha$-tocopherol (tables 4.10 and 4.12). Addition of $\alpha$-tocopherol to the reaction mixture at the peak of chemiluminescence response, caused a 16% increase in light emission (table 4.10). $\alpha$-Tocopherol increased the chemiluminescence of tumour microsomes by 22% (table 4.10, fig 4.6).

The 'OH radical scavengers, DMSO, mannitol and thiourea caused appreciable inhibition of the chemiluminescence of peroxidizing hepatic and tumour microsomes (table 4.11; fig. 4.9). TBA-reactive material production was partially inhibited by 'OH radical scavengers (table 4.12). Thiourea was the most potent of the scavengers used.

Sodium azide inhibited the chemiluminescence of hepatic microsomes by 8% (table 4.11), and the TBA-reactive material production of these microsomes by 15% (table 4.12). DABCO completely inhibited the chemiluminescence of hepatic and tumour microsomes and considerably inhibited the TBA-reactive material production by hepatic microsomes (tables 4.11 and 4.12; fig 4.10). Addition of DABCO to peroxidizing tumour or hepatic microsomes, when chemiluminescence was maximal, substantially blocked the ensuing light emission (table 4.11; figs 4.9 and 4.10).

BHT inhibited the chemiluminescence and TBA-reactive material production of peroxidizing hepatic microsomes, by about 80% (tables 4.11 and 4.12; fig. 4.3).

DISCUSSION

Concentrations of Malondialdehyde-like Material

The two different TBA-procedures, used in the present studies to determine malondialdehyde-like substances, agreed qualitatively with previous studies (Capel and Thornley, 1983). Whole tissue homogenates
were used instead of the TCA-precipitates of the earlier work; the increased TBA-reactive substances in the livers of tumour-bearing mice were thus not due to tumour-induced changes in hepatic concentrations of acid-soluble antioxidants or pro-oxidants, such as ascorbate or GSH. As free-radical mediated damage to virtually all types of cell constituent can be expressed by TBA-reactivity (Gutteridge, 1982a), that of hepatic and tumour tissues, from Lewis lung carcinoma-bearing mice, was probably a tissue component, such as membrane lipid, protein or DNA, precipitated by TCA.

Free malondialdehyde concentrations of murine tissues were considerably less than TBA-reactive material; indicating that TBA-reactive material was probably generated from the breakdown of lipoperoxides during the test itself (Gutteridge, 1982a). The similarity in the free malondialdehyde concentrations between the livers of tumour-free and tumour-bearing mice suggests that, while the animals were alive, lipid peroxidation in tumour-bearing mice did not differ from that of the controls. However the levels of malondialdehyde were very variable, probably because the concentrations of malondialdehyde were at the limits of detection. As malondialdehyde is rapidly metabolized in vivo (Siu and Draper, 1982), it may have arisen from autoxidation during preparation and storage of tissues.

Lewis lung carcinomas, used in the present studies, had substantial necrotic/hypoxic and haemorrhagic components (Chapter 2). Homogenisation of Lewis lung carcinoma tissue would have exposed these components to the air, and might have initiated autoxidation. The disruption of tissue by homogenisation might have enabled oxidants, such as haemoglobin or iron (Misra and Fridovich, 1972), to interact with cell components that were protected in vivo by compartmentalization and the comparatively low oxygen tensions in the tumour. Hepatic tissue might have similarly been
exposed to oxidative stress, after removal from the animal, indicating that a substantial component of malondialdehyde and TBA-reactive substances, detected in animal tissues, may have been experimental artefacts. This artefactual component might have been more indicative of the susceptibility of tissues to lipid peroxidation, during autoxidative insult than of in vivo peroxidation. The present studies may have been less ambiguous if an antioxidant had been included in the homogenizing medium and efforts had been made to exclude oxygen during the preparation of tissue samples.

The TBA-Test

Studies, comparing the effectiveness of various acid reagents in promoting the decomposition of malondialdehyde precursors from autoxidized pure polyunsaturated fatty acids, have shown that phthalate buffer, pH 3.5, was the most effective promoter of TBA-reactivity (Gutteridge, 1982a). Inorganic acid (25% w/v HCl), however, promoted greater TBA-reactive material production from membrane phospholipid, a complex lipid mixture, than did phthalate (Gutteridge, 1982a). It thus might be concluded that different oxidizable substrates require different acid conditions to obtain optimal TBA-reactivity. The results of the present studies indicate that H₂SO₄ is a more suitable medium for determining the TBA-reactive material concentrations of hepatic tissue, while phthalate buffer, which allowed a greater production of TBA-reactive material from Lewis lung than did H₂O₂, might be the better acid medium for tumour tissue.

The antioxidant, BHT, did not interfere with the reaction between malondialdehyde standards and TBA, but blocked iron/ascorbate-promoted lipid peroxidation, inhibiting both applications of the TBA-test by about 50-60%. This inferred that 40-50% of the products of the test were formed by lipid peroxidation during the assay itself. Studies elsewhere have
shown that BHT significantly inhibits the TBA-reactivity of rat liver, decreasing the TBA-reactive material concentrations of aging or chemically-intoxicated livers to that of healthy young tissue (Mihara and Uchiyama, 1983). The pattern of TBA-reactivity of hepatic tissue from tumour-bearing mice possibly resembled that of pathologically-damaged liver.

The present studies, in which it was observed that DETAPAC did not affect the TBA-reactivity of normal liver or that of tumour tissue, agreed with other studies in which the addition of iron salt or EDTA was observed not to affect the TBA-reactive material concentrations of hepatic tissue (Kornbrust and Mavis, 1980a; Mihara and Uchiyama, 1983). DETAPAC did, however, decrease the TBA-reactive material concentration of hepatic tissue from Lewis lung tumour-bearing mice, to a level below that of control liver. The increased hepatic TBA-reactivity of tumour-bearing mice might thus have been caused by raised concentrations of free iron in the livers of these animals. Cancer has been reported to cause changes in the distribution of body iron: decreasing in the blood; and accumulating in the liver, spleen and bone marrow (Weinberg, 1981).

The TBA-test, using $\text{H}_2\text{SO}_4$ as the acid medium was considerably more sensitive than that using phthalate buffer. The disparity might have been caused by the difference between the length of the incubation periods, employed by the two procedures. As the phthalate-buffer method required only a 10 minute incubation but generally yielded much lower concentrations of TBA-reactive material, this procedure might have been more susceptible to interference from side-reactions, that possibly occur in impure substrates such as biological samples. In the present studies, oxidized cellular constituents, other than PUFA, may have contributed to the TBA-reactive material, determined for microsomes or tissue homogenates. As the $\text{H}_2\text{SO}_4$-using application of the TBA-test had a much
longer incubation phase than the phthalate buffer method, a relatively greater proportion of the TBA-reactive substances, detected by this method, might have been of non-lipid origin.

The application of the TBA method, employing phthalate buffer had a smaller standard error and was more reproducible than the alternative procedure. Further experiments are required to determine which procedure gives the most accurate quantification of lipoperoxides in animal tissues. One approach might be to treat tissue samples at 100° C, in various acid reagents and then determine the time course of malondialdehyde production, using the HPLC separative technique (described earlier). The results of the present studies would advocate the use of antioxidants, to prevent spurious autoxidation during the acid/heating conditions of the TBA-test.

Correlation between chemiluminescence and TBA-reactive material production: Iron/ascorbate-induced chemiluminescence of rat hepatic microsomes has been shown to be closely related to TBA-reactive material production (Wright et al., 1979). The FeCl$_3$/ascorbate-induced system was additionally suitable for the present investigations because it was not dependent upon any endogenous enzymes (the NADPH-system requires cytochrome P450 reductase). This type of reaction system has been referred to as non-enzymic (Wright et al., 1979). Any peroxidation occurring during the 100° C temperatures of the TBA-test must also be non-enzymic; thus a non-enzymic in vitro peroxidation system was more relevant to the assays of TBA-reactive material concentration than an enzymic one.

The maximal levels of chemiluminescence and TBA-reactive material production were observed in these studies to occur at 90 to 120 min, after the start of the reaction. In the present investigations, the chemiluminescence and TBA-reactive material production of fresh hepatic microsomes maximized at about 40 min, indicating a possible species
difference in susceptibility to non-enzymic lipid peroxidation.

Antioxidants and free-radical scavengers had similar effects upon both the generation of chemiluminescence and TBA-reactive material. The two detection techniques were therefore probably monitoring the same type of chemical reaction. The onset of lipid peroxidation in hepatic microsomes, that had been stored as suspensions in phosphate buffer/glycerol, was considerably delayed whereas the generation of TBA-reactive material by these microsomes had a similar time-course to that of fresh microsomes. The maximal production of TBA-reactive material by similarly-stored tumour microsomes took approximately double the time that chemiluminescence took to maximise. As microsomal lipid peroxidation was measured by the TBA-method, several hours after that by chemiluminescence, the disparity between the two methods was possibly caused by changes arising in the microsomal suspensions during prolonged refrigeration. This indicates that the effect of storage in phosphate buffer/glycerol was reversible.

It has been reported that malondialdehyde is the only substance, generated by ADP/Fe-induced lipid peroxidation, capable of reaction in the TBA-test (Esterbauer, 1982). Malondialdehyde and light-emitting species are formed by different pathways and at different times of the lipid peroxidation cascade (Boveris et al, 1981; Chapter 1). This might have been reflected by the poor kinetic correlation between the two methods, used in the present studies to monitor lipid peroxidation.

Characterisation of Reactive Species in Iron/ascorbate-induced Peroxidation

Light-emitting species: The photomultipliers of most liquid scintillation counters are sensitive to light of wavelengths of up to 600 nm (Cadenas and Sies, 1984). The dimol emission of $^{1}O_2$, arising at 634 and 703 nm,
with a weak band at 580 nm is thus likely to be largely undetected by most liquid scintillation counters. Singlet oxygen can be produced chemically by the peroxide-hypochlorite reaction (Kanofsky, 1983):

\[ \text{OCl}^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{H}_2\text{O} + \text{Cl}^- \]

A reaction system, comprising 0.1% w/v NaOCl and 0.01% v/v H₂O₂ in 0.01 M NH₄OH, has been observed, using the chemiluminescence detection system of the present studies, to produce a prolonged light emission of about 2 x 10⁵ cpm, lasting in excess of 50 min (Phillipson et al., 1983). The addition of 2µM Luminol (400x dilution; see methods) was observed to increase this light emission to greater than 1.8 x 10⁶ cpm, that is beyond the detection limits of the counter.

A proportion of the non-luminol mediated luminescence, observed in the present studies, might have been weak emission at 580 nm. As Luminol had little effect, the ascorbate-induced microsomal chemiluminescence, detected in the present studies, was probably mainly the emission of excited carbonyl species.

Characterisation of Species Mediating Microsomal Lipid Peroxidation

Singlet oxygen: DABCO and azide are believed to quench \( \text{O}_2^+ \) by electron transfer (Foote, 1979):

\[ \text{O}_2^+ + Q \rightarrow \cdot\text{O}_2^- + \cdot\text{Q}^- \rightarrow \text{O}_3^- + Q \]  
(where Q=quencher)

Azide has been reported to be considerably more reactive than DABCO (Foote, 1979). In the present studies, however, DABCO was a conspicuously greater inhibitor of lipid peroxidation than azide. Azide, at the concentrations used in these investigations, may have inhibited endogenous microsomal catalase (Feierman and Cederbaum, 1983; Morehouse et al.,
The inhibitory actions of azide upon lipid peroxidation may thus have been counteracted by the inhibition of H₂O₂ metabolism (Kakinuma et al., 1979).

DABCO, at 50-100 mM concentration, has been shown to enhance the dimol chemiluminescence of chemically-produced O₂ (Denke and Krinsky, 1977). DABCO and another tertiary amine, dimethylpiperaazine, however, have been reported to inhibit the chemiluminescence of myristic acid-treated polymorphonuclear leukocytes (Kakinuma et al., 1979). As azide did not have a significant inhibitory effect upon this chemiluminescence, it was concluded that O₂ did not directly participate in the chemiluminescence reaction. Most O₂ quenchers have low oxidation potentials and would probably react with strong oxidants in an oxidation system (Foote, 1979); O₂ quenchers have been reported to react with organic peroxy radicals (Packer et al., 1981). DABCO might thus have inhibited microsomal lipid peroxidation by a mechanism other than O₂ quenching; for example by reaction with radical species such as 'OH (Halliwell, 1982).

H₂O₂: Catalase, in the present investigations, had a pronounced inhibitory effect upon murine microsomal lipid peroxidation, in accord with the findings of other studies using rat liver microsomes (Koster and Slee, 1980; Feierman and Cederbaum, 1983; Girotti and Thomas, 1984). Studies elsewhere, however, have shown that catalase did not inhibit microsomal lipid peroxidation (Kornbrust and Mavis, 1980a; Morehouse et al., 1983). Antioxidants, such as thymol, present in many commercial catalase preparations, may have been the inhibitory agent rather than enzyme (Pederson and Aust, 1970). Commercial catalase preparations (including one from Sigma), which had previously inhibited lipid peroxidation, were observed to be ineffective after chromatography to remove low molecular weight contaminants (Morehouse et al., 1983). In the present
investigations, a thymol-free grade of catalase was used; when this catalase was denatured by boiling, it had minimal inhibitory effect upon the chemiluminescence of peroxidizing microsomes. As only active catalase was effective, and inhibition was much more pronounced than that of the antioxidants, BHT and vitamin E, the inhibitory action of catalase was more probably due to catabolism of H$_2$O$_2$ than to antioxidant activities. The commercially-obtained catalase might, however, have been contaminated by another antioxidant enzyme, such as SOD.

When microsomes were incubated with 0.1mM H$_2$O$_2$, the resultant lipid peroxidation, detected by TBA-test activity, was only about 17% of that obtained with the ADP-FeCl$_3$/ascorbate system (table 4.13). This indicated that H$_2$O$_2$ may well have been an initiating species in the reaction, but was not sufficient alone to elicit the full lipid peroxidation cascade. H$_2$O$_2$, in 0.1 M phosphate buffer, pH 7.4, had a prolonged chemiluminescence: the chemiluminescence was not linear with respect to H$_2$O$_2$ concentration (1 $\mu$M to 1 mM). Phosphate buffers are sometimes contaminated by metal ions, sufficient even to initiate microsomal lipid peroxidation (Cadenas et al., 1983). Iron is only sparingly soluble in phosphate buffer (Feierman and Cederbaum, 1983), but trace amounts of iron, however, or more probably copper salts, might have reacted with H$_2$O$_2$ to produce 'OH (Halliwell and Gutteridge, 1985):

$$Fe^{2+} + H_2O_2 \rightarrow 'OH + Fe^{3+} + OH^-$$ \text{ Fenton reaction}

$$Cu^{2+} + H_2O_2 \rightarrow 'OH + Cu^{3+} + OH^-$$

Chemiluminescence emission occurs from many organic materials, such as plastics, paper, rubber, cloth and resins (Thorpe et al., 1982). The chemiluminescence, arising from the peroxide/hypochlorite reaction, and that of H$_2$O$_2$ in phosphate buffer might have been caused by the reaction
of \( \cdot \text{OH} \) and \( ^1\text{O}_2 \) with the plastic material of the vials, used to contain the reaction mixtures. Experiments are thus required to determine whether plastic is a significant substrate for iron/ascorbate-mediated autoxidation. The chemiluminescence of \( \text{H}_2\text{O}_2 \) or chemically-generated \( ^1\text{O}_2 \) might be studied, using glass reaction vessels.

**Hydroxyl radical:** Scavengers of \( \cdot \text{OH} \) radicals, particularly thiourea, caused appreciable inhibition of lipid peroxidation, monitored by chemiluminescence and TBA-reactive material production. \( \cdot \text{OH} \) may well have been an important component of the microsomal lipid peroxidation reaction.

**Superoxide anion:** As SOD had little effect upon hepatic microsomal chemiluminescence or TBA-reactive material production, \( ^-\text{O}_2 \) was possibly not involved in the peroxidation reaction. SOD did, however, inhibit the chemiluminescence of iron/ascorbate-challenged tumour microsomes and the delayed chemiluminescence of phosphate buffer/glycerol-stored hepatic microsomes. The SOD, used in the present studies, originated from bovine liver and was probably mainly the Cu/Zn isoenzyme. The ineffectiveness of SOD upon hepatic chemiluminescence might have been caused by inactivation of Cu/Zn SOD by \( \text{H}_2\text{O}_2 \), or other reactive oxygen species (Hodgson and Fridovich, 1973b). The deactivation of SOD by the tumour microsomal system may have been insignificant, as the lipid peroxidation reaction was relatively unpronounced. The generation of reactive oxygen metabolites, during the prolonged initiation phase of phosphate buffer/glycerol-stored hepatic microsomes, was possibly so low that SOD could adequately dismutate \( ^-\text{O}_2 \) and prevent the autoxidation of microsomal lipid. It might be better in future experiments to use Mn-SOD, because this enzyme is less sensitive to \( \text{H}_2\text{O}_2 \) (Misra and Squatrito, 1982).

**Lack of effect of vitamin E:** Vitamin E is a hydrophobic, peroxyl-radical trapping, \( ^1\text{O}_2 \) quenching, \( \cdot \text{OH} \)-scavenging, chain-breaking antioxidant (Chapter 1), which has been reported to prevent lipid peroxidation in
vivo (Tappel, 1979; Mihara et al, 1980) and in vitro (Pryor et al, 1976a; McCay et al, 1978; Nakamura and Hishinuma, 1978). Studies elsewhere, however, have agreed with the findings of the present investigation: preincubation of rat liver microsomes with vitamin E, dissolved in ethanol, was observed to have little effect upon iron/cysteine-induced peroxidation (Willson, 1983); the addition of vitamin E to membrane preparations has been observed to inhibit lipid peroxidation by only 12% (Gutteridge, 1978). The comparative inability of vitamin E to prevent iron/ascorbate-initiated microsomal peroxidation might have been caused by the inaccessibility of the vitamin to the peroxidizing membranes (Willson, 1983). The form of vitamin E, used in the present studies, was α-tocopherol acetate (Sigma); reported not to be as potent as natural vitamin E, although some free vitamin E is generally detectable in α-tocopherol acetate preparations (Gutteridge, 1978). Natural α-tocopherol has been reported to bind strongly to the molecules of the lipid membrane, whereas the acetate could only form a loose hydrophobic bond and, unlike the natural vitamin, was unable to decompose H₂O₂ (Srivastava et al, 1983). The vitamin E, used in the present investigations, may thus have been unsuitable.

Rat liver microsomes have to be pre-incubated with α-tocopherol for a few minutes before any protection from lipid peroxidation could be observed (Mak et al, 1983). Vitamin E prevented the delayed chemiluminescence of phosphate buffer/glycerol-stored hepatic microsomes (fig 4.7) possibly because there was sufficient time, due to delay in the onset of the reaction, for the vitamin to interact with the membrane lipid.

Vitamin E caused a moderate increase in the chemiluminescence of tumour microsomes (fig. 4.6). It might have promoted lipid peroxidation under these circumstances, by acting as a pro-oxidant in catalyzing the
reduction of ferric iron to ferrous iron.

Conclusions Concerning the Chemical Nature of Iron/ascorbate-induced Microsomal Lipid Peroxidation

The iron/ascorbate-challenged microsomal lipid peroxidation reaction, of this study, probably had 'OH and H₂O₂ as intermediates, and possibly involved 'O₂⁻ but not O₂. However, the identification of active oxygen intermediates, by use of radical scavengers or reactive oxygen quenchers, can be equivocal because these agents can cause excited species to transfer their energy to other molecules, which then become new excited species (Clifford and Repine, 1984).

\(^1\text{O}_2\) might have been generated during the lipoperoxide breakdown phase of the reaction (Chapter 1); this could be verified using a photomultiplier, sensitive to \(^1\text{O}_2\) emissions in the infra-red end of the electromagnetic spectrum. Optical filters could possibly be used to analyse the wavelengths of light emitted (Cadenas and Sies, 1984). As the lifetime of \(^1\text{O}_2\) has been reported to be 10-fold longer in deuterium than water, prolongation of chemiluminescence, in a system using deuterium as solvent, would indicate the presence of \(^1\text{O}_2\) (Cadenas and Sies, 1984).

NADPH-dependent lipid peroxidation in azide pretreated hepatic microsomes (to inhibit endogenous catalase) has been observed to be inhibited by H₂O₂, indicating that free H₂O₂ is not associated with microsomal lipid peroxidation (Morehouse et al., 1983). The ascorbate/FeCl₃-induced peroxidation reaction could possibly have been independent of active oxygen species. Metal complexes, such as ferrous iron with phosphate esters or EDTA, haem, haemoproteins and cytochromes, are reported to be able to catalyze the decomposition of pre-formed lipoperoxides (Halliwell and Gutteridge, 1984a). The chain-reaction of lipid peroxidation (Chapter 1) may be stimulated accordingly:
Alkoxyl (RO') and peroxy (ROO') radicals are believed to abstract hydrogen atoms from PUFA to initiate the chain-reaction stage of the lipid peroxidation reaction. This phase of lipid peroxidation has been reported to account for greater than 90% of the products of the total reaction (Halliwell and Gutteridge, 1984a).

The results of the present studies, however, implicate reactive oxygen intermediates in the initiation of iron/ascorbate-mediated microsomal peroxidation, and experiments to detect the electron paramagnetic resonance (EPR) of radicals, using suitable spin traps, have shown that \( \cdot O_2^- \), \( \cdot OH \) and the lipid peroxy radical are the principal radical species of hepatic microsomal lipid peroxidation (Rosen and Rauckman, 1981). It has been reported, from EPR studies, that complexes of di- and triphosphate nucleotides with ferrous iron, catalyse the formation of \( \cdot OH \) from \( H_2O_2 \) (Floyd, 1983). A possible mechanism, for the lipid peroxidation reactions of the present studies, involving \( H_2O_2 \), \( \cdot O_2^- \) and \( \cdot OH \) may have been an iron-catalyzed Haber-Weiss reaction (Svingen et al, 1979):

\[
ADP-Fe^{3+} + \cdot O_2^- \rightarrow ADP-Fe^{2+} + O_2
\]

\[
2\cdot O_2^- + 2H^+ \rightarrow H_2O_2 + O_2
\]

\[
ADP-Fe^{2+} + H_2O_2 \rightarrow ADP-Fe^{3+} + \cdot OH + OH^- \quad \text{(Fenton reaction)}
\]

Ascorbate probably would have replenished \( \cdot O_2^- \) in reducing Fe(III) to Fe(II), leaving \( \cdot O_2^- \) free to dismute into further \( H_2O_2 \) (Halliwell and
Gutteridge, 1985). It has been reported that the reaction between ferrous ions and molecular oxygen forms the perferryl iron, which has been postulated to be an initiator of lipid peroxidation (Aust and Svingen, 1982):

\[
\begin{align*}
Fe^{2+} + O_2 & \leftrightarrow [Fe^{2+} - O_2] \leftrightarrow Fe^{3+} \cdot O_2^- \\
2 \cdot O_2^- + 2H^+ & \rightarrow H_2O_2 + O_2 \text{ (dismutation)} \\
H_2O_2 + Fe^{3+} & \rightarrow Fe^{3+} + \cdot OH + OH^- \text{ (Fenton reaction)}
\end{align*}
\]

The perferryl ion, however, has been reported to have comparatively poor reactivity, and thus the ferryl ion is believed to be a more likely initiator of lipid peroxidation (Halliwell and Gutteridge, 1984a):

\[
Fe^{2+} + H_2O_2 \rightarrow [FeOH^{3+} \leftrightarrow FeO^{2+}] + OH^- \text{ ferryl ion complex}
\]

Microsomal Lipid Peroxidation in Tissues of Tumour-bearing Mice

Lewis lung carcinoma: The production of TBA-reactive material, during iron/ascorbate-mediated peroxidation was considerably less in tumour microsomes than in hepatic microsomes. Pulmonary tissue has been shown to be one of the least susceptible rodent tissues to microsomal lipid peroxidation (Kornbrust and Mavis, 1980b). As peroxidizing Lewis lung carcinoma microsomes emitted even less chemiluminescence than pulmonary microsomes, it can be concluded that Lewis lung carcinoma has very low susceptibility to in vitro lipid peroxidation. This property of the Lewis lung carcinoma was in accord with the observations of lipid peroxidation in other tumour systems (Bartoli and Galeotti, 1979; Player et al, 1979;
Ahmed and Slater, 1980).

The chemiluminescence of peroxidizing tissues or membranes has been reported to be proportional to the degree of unsaturation of the lipid substrate (Cadenas et al., 1980). Lewis lung carcinomas, like other tumours such as rat hepatomas (Galeotti et al., 1984), might have a relatively low unsaturated lipid concentration. Tumours have been observed to have comparatively pronounced concentrations of antioxidants (Chapter 6); the resistance of microsomes from the Lewis lung carcinoma to in vitro lipid peroxidation may have been due to having a possibly greater membrane antioxidant concentration than normal murine tissues.

The substantial concentration of malondialdehyde-like substances in Lewis lung carcinoma might indicate that the tumour membranes had such poor antioxidant defence that they readily oxidize during preparation and storage of the tissue. Tumour homogenate, and microsomal fractions would thus have contained considerable concentrations of lipoperoxides, which would decompose to form malondialdehyde during the TBA-test. Further significant peroxidation in vitro was possibly prevented by a comparative lack of remaining unoxidized PUFA.

Lipid peroxidation of Lewis lung carcinoma microsomes was monitored for 90 min. The present studies have shown that the major chemiluminescence emission can be delayed until beyond this time. It is possible, therefore, that lipid peroxidation in tumour microsomes was not monitored for sufficient time to detect the principal peroxidation reaction.

The TBA-reactive material and chemiluminescence, measured in the present studies, may have been largely attributable to host cells infiltrating the Lewis lung carcinoma, or may have been derived from the necrotic components of the tumour. Haemoglobin has been reported to be an
effective catalyst of lipid peroxidation (Misra and Fridovich, 1972). It has been observed that BHT inhibits the TBA-reactivity of animal tissues, catalyzed by haem iron (Mihara and Uchiyama, 1983). The Lewis lung carcinoma is a haemorrhagic tumour: BHT interfered with the TBA reaction of tumour microsomes; haemoglobin may thus have promoted the synthesis of TBA-reactive substances, during the TBA-test.

Alternatively, the TBA-reactive material of Lewis lung carcinomas might have reflected a pronounced eicosanoid cascade activity (Chapter 6). The malondialdehyde-like substances would thus have originated from the degradation of endoperoxide precursors, synthesised by the enzymic oxidation of arachidonic acid, and not be generalized oxidation of PUFA.

Lipid peroxidation in livers of tumour-bearing mice: When using the more sensitive $\text{H}_2\text{SO}_4$-based application of the TBA-test, the present studies confirmed that the livers of mice, bearing lewis lung carcinomas, had greater concentrations of malondialdehyde-like material than tumour-free controls. This additional TBA-reactive material was possibly not produced in vivo but by metal-catalyzed autoxidation during the acid/heating conditions of the TBA-test.

Studies elsewhere have also shown that tumour-bearing rodents have elevated concentrations of TBA-reactive material in their livers (Neyfakh and Kagan, 1969; Gorkin et al, 1973; Khuzhamberdyev et al, 1973; Pierson and Meadows, 1985). Tumour-bearing mice have been observed to have greater hepatic chemiluminescence than tumour-free animals (Boveris et al, 1985). Iron/NADPH, NADPH/CCl$_4$, or iron/ascorbate initiated peroxidation of microsomes, from apparently normal liver taken from rats bearing ethionine-induced hepatomas, however, has been observed not to differ from that of tumour-free rats (Ahmed and Slater, 1981); investigations of the electron spin resonance of tissues of tumour-bearing mice have revealed that the free radical concentration in host livers
decreased during tumour development (Emanuel, 1982). Mice, bearing B16 melanomas were, however, observed to have increased concentrations in their livers, spleens and lungs, which increased further upon metastasis to these organs (Emanuel, 1982). Mice, bearing B16 melanomas, have been observed in the present investigations, and in studies elsewhere, to have elevated hepatic TBA-reactive material concentrations (Pierson and Meadows, 1985; Chapter 3). The increased TBA-reactivity of the livers of Lewis lung carcinoma-bearing mice, might thus have been due to raised free radical concentrations.

The present studies have shown that hepatic tissue from mice, bearing Lewis lung carcinomas, was probably more susceptible to lipid peroxidation than normal liver. Storage as suspensions in phosphate buffer/glycerol had profoundly different effects upon iron/ascorbate-mediated chemiluminescence of microsomes from tumour-free and tumour-bearing mice. This mode of storing hepatic microsomes may have exaggerated the biochemical differences between the two experimental groups and thereby may provide clues which might help to determine the exact nature of the tumour-induced perturbations of the liver.

Future studies are required to ascertain how storage of normal murine liver microsomes as suspensions in phosphate buffer/glycerol at -80°C, confers protection against lipid peroxidation. The effects of, for example, freezing, changing the type or pH of the buffer, omitting glycerol or EDTA, or using other types of metal chelator, should all be investigated.

The differences between the livers of tumour-free and tumour-bearing mice may have been caused by accumulation of transition metals, such as iron, in the livers of the tumour-bearers (as discussed previously). Phosphate buffer has been reported to inhibit iron-induced lipid peroxidation, such that comparatively large concentrations of iron salt are
required to initiate peroxidation (see earlier). Treatment with phosphate buffer, containing EDTA, might have removed most of the free iron from normal hepatic microsomes, but left a significant residue in the microsomes from the livers of tumour-bearing mice. If the endogenous microsomal iron were depleted as a result of the mode of storage, a greater concentration of FeCl₃ would have been needed to initiate a reaction equivalent to that of fresh hepatic microsomes. As microsomes from tumour-bearing mice were possibly not depleted of iron, due to a pronounced endogenous level prior to storage, the iron/ascorbate-induced chemiluminescence was not significantly affected.

Mice, bearing Lewis lung carcinomas, had significantly lower catalase and mitochondrial GSH peroxidase activities than their healthy counterparts (Chapter 3). The greater susceptibility of hepatic microsomes, from carcinoma-bearing mice, to peroxidation possibly resulted from a deficiency in an antioxidant enzyme. Rat liver microsomes have been reported to be protected from iron/ascorbate-induced lipid peroxidation by a GSH-dependent, heat-labile factor, which is believed to inhibit peroxidation by preventing the formation of the initiating species, rather than by peroxidase activity or radical scavenging (Haenen and Bast, 1983). In the present studies, microsomes were washed several times and thus probably did not contain sufficient GSH for a factor, such as that above, to mediate any effect. In future studies, however, microsomes could be preheated, say for 90 sec in boiling water, to deactivate any enzymes that might influence lipid peroxidation; caution must be used to ensure that even this short time of heating will not initiate any significant autoxidation.

Tumours have been observed to concentrate antioxidants at the expense of the normal tissues of their hosts (Chapter 6). The storage of hepatic microsomes in phosphate buffer/glycerol possibly promoted access
lipid. The protection afforded by vitamin E against the peroxidation of methyl linoleate has been reported to be in the form of delaying the peroxidation reaction (Pryor et al, 1976a). The length of the induction period, before peroxidation became apparent, was observed to be proportional to the amount of the vitamin added to the reaction mixture. Once the autoxidation started, it proceeded at a rate, independent of the concentration of vitamin E (Pryor et al, 1976a). This effect of vitamin E was remarkably like that of storage in phosphate buffer/glycerol at -80°C upon hepatic microsomes. The intramuscularly-embedded tumour might have evoked a decrease in the ability of the host liver to detoxify reactive oxygen intermediates.

Conclusions

The results of the present studies indicate that the increased concentrations of malondialdehyde-like material in the livers of tumour-bearing mice were substantially mediated by the increased sensitivity of this tissue to in vitro lipid peroxidation. The TBA-test, commonly used to determine the lipoperoxide concentrations of animal tissues, probably also reflects the susceptibility of tissues to lipid peroxidation in vitro. Unless precautions are taken to prevent autoxidation of tissues during processing, storage and the test itself, the results of determinations, using the TBA-method, must be viewed with caution.

Most or all of the TBA-reactive material, determined for Lewis lung carcinomas and at increased levels in the livers of tumour-bearing mice, was possibly artefactual. This does not, however, invalidate these consistent empirical observations. Further studies are necessary to determine the precursors of the TBA-reactive material. It may be useful to evaluate the physiological significance of the results of the present investigations and determine whether they are applicable to other forms of neoplastic disease, particularly human cancers.
CHAPTER FIVE

FURTHER STUDIES OF SYSTEMIC EFFECTS OF LEWIS LUNG CARCINOMAS
CHAPTER FIVE: FURTHER STUDIES OF THE SYSTEMIC EFFECTS OF LEWIS LUNG CARCINOMAS

INTRODUCTION

Post-mortem studies of cancer patients have shown that the tumour mass rarely exceeds 1% of body weight (Costa, 1977). Cachexia (Chapter 3), the most apparent expression of the systemic disturbances, exerted by cancers, can occur when a clinical tumour is only the size of a pinhead (Morrison et al, 1984). Cachexia is preceded by biochemical alterations in the tissues of the host, particularly the liver (Greengard, 1979).

Most investigations of the systemic effects of cancer, have used animal models in which the tumour occupies about 5% of the body weight (Morrison et al, 1984). The validity of these types of study is therefore questionable.

In the present investigations, the Lewis lung (and B16 melanoma) murine tumour models have weighed up to 15% of the body weight of the animals into which they were implanted (Chapters 2 and 3). The earliest day at which normal tissues of a tumour-bearing animal were investigated was 5 days after implantation, when the Lewis lung carcinoma was not yet palpable and probably occupied considerably less than 1% of body weight. An increased hepatic GSH S-epoxide transferase activity, have been observed for animals that had borne Lewis lung carcinomas for 5 days (Chapter 3). Hepatic DNA concentrations and GSSG reductase activities at 5 days post implantation, however, did not differ from those of tumour-free mice. Catalase, GSH peroxidase and SOD activities have not been determined for mice that have borne Lewis lung carcinomas for less than 12 days; perturbations of these enzymes in tumour-bearing mice might have been the primary cause of the deleterious systemic changes in non-neoplastic tissues.
The purpose of the present investigations was to examine the above hypothesis by determining whether there were any changes in antioxidant defence or susceptibility to lipid peroxidation in normal tissues of mice, within 3 days of implantation with Lewis lung carcinomas. As the intramuscularly-implanted Lewis lung carcinoma would probably weigh less than 10 mg, at this juncture, any observed biochemical variations would be unlikely to be caused by the physical burden of the tumour or by deprivation of essential nutrients. Short-term systemic effects would probably only result from the immune reaction of the host mouse, or from reactive substances elaborated by the tumour into the circulation of the host. Thus, in addition to the usual Lewis lung carcinoma-implanted mice and their saline-injected controls, an experimental group was used, in which mice were implanted with Lewis lung carcinoma cells that had been pretreated with mitomycin C. The mitomycin C-treated cells would have been non-viable but probably could produce the same active substances and have the same antigenicity as viable Lewis lung carcinoma cells.

The second part of the present chapter is concerned with the possibility that many of the biochemical alterations, reported for the livers of tumour-bearing animals and cancer patients, are caused by changes in their lipid composition (Chapter 3). Tumour-bearing rats have been observed to have impaired feedback regulation of hepatic cholesterol biosynthesis (Shapot et al., 1972); profound changes in the lipid composition of the livers of tumour-bearing animals has been reported (Baum and Nishimura, 1964; Carruthers and Kim, 1968; Polyakov et al., 1977). Tumour-bearing animals and cancer patients generally have depressed activities of hepatic catalase (Chapter 3). It has been postulated that peroxisomes have a significant regulatory role in lipid metabolism (Crane and Masters, 1984). As catalase is an integral component of peroxisomes, change in hepatic catalase activity might alter the lipid composition of the livers of animals or man with neoplastic
The livers of genetically-obese C57BL6 mice have been reported to have decreased GSH peroxidase activity and TBA-reactive material concentrations, and to undergo a more pronounced NADPH-mediated microsomal lipid peroxidation reaction than lean mice (Capel and Dorell, 1984). These perturbations were similar to those of the livers of mice, bearing Lewis lung carcinomas. The following investigations have thus used the liver of the obese mouse as a model for the liver of the Lewis lung carcinoma-bearing mouse. DNA concentration, TBA-reactive material concentration, and SOD, catalase, GSH peroxidase and γ-glutamyl transpeptidase activities of the livers of obese mice and lean controls were determined. The non-enzymic (iron/ascorbate-mediated) lipid peroxidation reaction of hepatic microsomes, from lean and obese mice, was also studied. The variations of these parameters from the levels observed for lean mice, were then compared with that observed for mice, bearing Lewis lung carcinomas. These studies showed that obese mice, like tumour-bearing animals, exhibited evidence of increased hepatic autooxidative injury.

EXPERIMENTAL

Chemicals

All chemicals were of the highest purity, commercially available, and were supplied by either Fisons Scientific Apparatus, Loughborough, Leics., or by Sigma London Chemical Co. Ltd., Poole, Dorset.

Animals and Treatment

Animals were maintained as described previously (Chapter 2). The
studies to determine the short-term effects of implanting Lewis lung carcinomas, upon normal murine tissues, used male C57BL6 mice of body weight 24.2 ± 0.8g (8 wks of age). The studies of genetically-obese mice were undertaken with male C57BL6 ob/ob mice of body weight 34.7 ± 0.9g (aged 8 wks), and with male age-matched C57BL6 lean controls of body weight 26.0 ± 0.7g. All animals were supplied by Olac, Bicester, Oxon. Mice were killed by CO₂ narcosis and cardiopuncture.

Lewis lung carcinoma cells were taken from the primary frozen stock and grown in culture, as described previously (Chapter 2), until they were semi-confluent. The cells were then dispersed with trypsin, resuspended in phosphate-buffered saline and counted, using a haemocytometer. The cell suspensions were then divided into two approximately equal parts and reintroduced to dishes, containing culture medium. To one half of the cells, mitomycin C was added, at a concentration of 5 μg/10⁶ cells. All cells were maintained in culture overnight. The following day, the cells were disaggregated into single cell suspensions, as described previously (Chapter 2). Mice were then divided, by random selection, into 3 experimental groups of appropriate size. Animals from one group were inoculated in the hindlimb with a 0.1ml injection of 5 x 10⁵ untreated tumour cells. A second group were inoculated with the same number of mitomycin C-pretreated cells, and the third group received 0.1ml of phosphate-buffered saline only.

Animals were killed at 1, 3 or 14 days after the inoculation procedure. Blood was removed by cardiopuncture with a heparinized 1ml syringe. The mice, killed at 14 days post implantation, were used to ensure that the non-viable cells did not produce a tumour, and to check that the growth rate and systemic actions of the viable Lewis lung carcinomas were similar to that observed in previous studies. Obese mice and their lean controls were not inoculated with tumour cells.
Biochemical Assays

Tissues were homogenised and divided into subcellular fractions as described previously (Chapter 2). Protein concentrations were determined by the procedure, using Folin-Ciocalteu reagent (Lowry et al., 1951). DNA concentrations, and activities of SOD, GSH peroxidase, catalase and γ-glutamyl transpeptidase were determined by previously described methods (Chapter 2). Lipoperoxide concentrations were estimated by the two applications of the TBA test, evaluated in Chapter 4. Iron/ascorbate-induced microsomal lipid peroxidation was monitored by determining chemiluminescence and TBA-reactive material production, as described previously (Chapter 4).

DNA concentrations and catalase activities were ascertained, using whole tissue homogenates that had been stored at -20°C for not more than 2 weeks. All other studies were undertaken with haemolysates or subcellular fractions that had been refrigerated at 4°C for not more than 48 hrs after killing the experimental animals.

Blood packed cell volumes and haemoglobin concentrations were estimated as described previously (Chapter 3). The instrumentation employed in the present investigations, has been described in previous chapters.

Leukocyte-induced Chemiluminescence at the Inoculation Site

A study was undertaken to determine whether the introduction of tumour cells had evoked a phagocyte-mediated inflammatory reaction at the intramuscular site of inoculation (Dowling et al., 1985). Activated phagocytes generate reactive oxygen species which may oxidize Luminol in a chemiluminescent reaction (Chapters 1 and 4). The appropriate limb was excised from mice, killed at 1 or 3 days after the inoculation procedure. The skin and fur of the leg were removed and the portion of the leg, proximal to the knee joint, retained. The muscle was excised from the
femur, and finely chopped, using a scalpel blade. The pieces of muscle were then placed into plastic scintillation vials (dark adapted), containing phosphate-buffered saline, prewarmed to 37° C. The phosphate-buffered saline contained luminol at a 100-fold dilution of the Luminol\(^R\) stock (Chapter 4). The luminol–mediated chemiluminescence of hindlimb muscle was measured within 15 min of mice being killed.

Statistical Analyses

Results were expressed as mean ± SEM. Experimental group means, from the study of the short-term systemic effects of Lewis lung carcinomas, were compared by analysis of variance and the Newman-Keuls test. The experimental means from the studies of obese mice were compared with those of lean mice, using the Student's t-test.

RESULTS

Short-term Systemic Effects of Lewis Lung Carcinomas

Organ weights: There were no differences between the liver and lung weights of mice from the three experimental groups, at 1, 3 or 14 days after the inoculation procedure. The spleen weights of mice, implanted with tumour cells did not differ from those of the saline-injected controls, at 1 and 3 days post-inoculation. The spleens of mice that had borne Lewis lung carcinomas for 14 days or more weighed 120% more than those of mice that had been injected with saline or non-viable cells.

Antioxidant defence enzymes of liver: Mice, that had borne Lewis lung carcinomas for 14 days, had significantly decreased hepatic catalase and mitochondrial GSH peroxidase activities (tables 5.5 and 5.2), and increased hepatic DNA concentrations (table 5.6). Cytosolic GSH peroxidase and SOD activities of the livers of these animals did not differ from those of the tumour-free controls (tables 5.1 and 5.3).
**TABLE 5.1 - Hepatic Cytosolic GSH Peroxidase Activities of C57BL6 Mice Implanted with Lewis Lung Carcinomas**

Control mice were injected with phosphate-buffered saline and the positive controls were inoculated with non-viable cells. Results represent mean ± SEM at each time interval. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after inoculation (days)</th>
<th>CONTROL (saline)</th>
<th>+VE CONTROL (non-viable cells)</th>
<th>TUMOUR-BEARING (viable cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 3</td>
<td>n = 4</td>
<td>n = 5</td>
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(Enzyme units*/mg protein)

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<thead>
<tr>
<th></th>
<th>Enzyme units</th>
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<tr>
<td>1</td>
<td>128 ± 9</td>
<td>115 ± 10</td>
<td>122 ± 11</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>118 ± 4</td>
<td>128 ± 13</td>
<td>112 ± 6</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>119 ± 9</td>
<td>121 ± 8</td>
<td>115 ± 5</td>
<td></td>
</tr>
</tbody>
</table>

* Enzyme units are defined as 1000 x log₁₀ (decrease in GSH/min) at 37°C, pH 7.0.

No significant differences (p>0.05) by Newman-Keuls test (after analysis of variance).
TABLE 5.2 - Hepatic Mitochondrial GSH Peroxidase Activities of C57BL6 Mice Implanted with Lewis Lung Carcinomas

Control mice were injected with phosphate-buffered saline and the positive controls were inoculated with non-viable cells. Results represent mean ± SEM at each time interval. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after inoculation (days)</th>
<th>CONTROL (saline) n = 3</th>
<th>+VE CONTROL (non-viable cells) n = 4</th>
<th>TUMOUR-BEARING (viable cells) n = 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17 ± 0.5</td>
<td>19 ± 0.7</td>
<td>17 ± 0.5</td>
</tr>
<tr>
<td>3</td>
<td>18 ± 2.4</td>
<td>20 ± 1.5</td>
<td>16 ± 1.7</td>
</tr>
<tr>
<td>14</td>
<td>17 ± 1.0</td>
<td>18 ± 0.7</td>
<td>11 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Enzyme units are defined as 1000 x log<sub>10</sub> (decrease in GSH/min) at 37°C, pH 7.0.

<sup>a</sup> Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 5.3 - Hepatic Cytosolic Superoxide Dismutase Activities of C57BL6 Mice Implanted with Lewis Lung Carcinomas

Control mice were injected with phosphate-buffered saline and the positive controls were inoculated with non-viable cells. Results represent mean ± SEM at each time interval. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after inoculation (days)</th>
<th>CONTROL (saline) n = 3</th>
<th>+VE CONTROL (non-viable cells) n = 4</th>
<th>TUMOUR-BEARING (viable cells) n = 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23 ± 0.5</td>
<td>26 ± 0.6</td>
<td>23 ± 1.8</td>
</tr>
<tr>
<td>3</td>
<td>21 ± 2.1</td>
<td>28 ± 1.7</td>
<td>24 ± 0.5</td>
</tr>
<tr>
<td>14</td>
<td>24 ± 1.9</td>
<td>24 ± 0.9</td>
<td>25 ± 0.7</td>
</tr>
</tbody>
</table>

No significant differences (P>0.05) by Newman-Keuls test (after analysis of variance).
TABLE 5.4 - Hepatic Mitochondrial Superoxide Dismutase Activities of C57BL6 Mice Implanted with Lewis Lung Carcinomas
Control mice were injected with phosphate-buffered saline and the positive controls were inoculated with non-viable cells. Results represent mean ± SEM at each time interval. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after inoculation (days)</th>
<th>CONTROL (saline) (units [Sigma]²/mg protein at 25 ± 2°C, pH 7.5)</th>
<th>+VE CONTROL (non-viable cells)</th>
<th>TUMOUR-BEARING (viable cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 3</td>
<td>6.0 ± 0.1</td>
<td>4.9 ± 0.1</td>
<td>5.7 ± 0.4</td>
</tr>
<tr>
<td>3</td>
<td>4.8 ± 0.3</td>
<td>5.6 ± 0.3</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td>14</td>
<td>5.3 ± 0.4</td>
<td>5.3 ± 0.4</td>
<td>5.0 ± 0.1</td>
</tr>
</tbody>
</table>

No significant differences (p>0.05) by Newman-Keuls test (after analysis of variance).
Hepatic GSH peroxidase, SOD and catalase activities, at 1 and 3 days after the inoculation procedure, did not vary significantly between mice injected with viable tumour cells or non-viable cells and saline-injected controls (tables 5.1 to 5.5). The hepatic DNA concentrations of mice, killed 1 or 3 days after inoculation with viable or non-viable Lewis lung carcinoma cells, did not differ from those of animals injected with saline only (table 5.6).

Antioxidant defence enzymes of blood (table 5.7): The erythrocyte GSH peroxidase and catalase activities of mice, injected with viable Lewis lung carcinoma cells, did not vary from those of control animals, at 1 or 3 days post inoculation. The erythrocyte GSH peroxidase activities of tumour-bearing mice, killed 14 days after implantation, was about 17% less than that of the control groups. Erythrocyte SOD activities of mice, inoculated with non-viable cells, were considerably greater than those of animals, inoculated with saline only. This effect was observed at 1 and 3 days but not 14 days after the inoculation procedure. The erythrocyte SOD activities of mice, implanted with viable tumour cells were greater than the levels observed for saline-injected controls, by 87% at 3 days and 210% at 14 days, post inoculation.

Lipoperoxide concentrations: Pulmonary microsomal lipoperoxide (TBA-reactive material) concentrations of mice, injected with non-viable cells, did not differ from those of saline injected controls (table 5.8). At 3 and 14 days post implantation, the TBA-reactive material concentrations of pulmonary microsomes from Lewis lung carcinoma-bearing mice, were about 180% and 70% respectively greater than those of control animals (table 5.8).

The hepatic microsomal TBA-reactive material concentrations of animals, injected with viable or non-viable tumour cells, were marginally less than that of untreated mice, at 1 day post inoculation (table 5.9).
TABLE 5.5 - Hepatic Catalase Activities of C57BL6 Mice Implanted with Lewis Lung Carcinomas

Control mice were injected with phosphate-buffered saline and the positive controls were inoculated with non-viable cells. Results represent mean ± SEM at each time interval. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after inoculation (days)</th>
<th>CONTROL (saline) n = 3</th>
<th>+VE CONTROL (non-viable cells) n = 4</th>
<th>TUMOUR-BEARING (viable cells) n = 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k/min/mg protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.7 ± 0.3</td>
<td>2.4 ± 0.1</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>2.8 ± 0.4</td>
<td>2.5 ± 0.3</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>14</td>
<td>2.8 ± 0.3</td>
<td>2.6 ± 0.2</td>
<td>1.8 ± 0.1a</td>
</tr>
</tbody>
</table>

* k is the first order reaction rate constant at 2 ± 1°C, pH 7.4.

a Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 5.6 - Hepatic DNA Concentrations of C57BL6 Mice Implanted with Lewis Lung Carcinomas

Control mice were injected with phosphate-buffered saline and the positive controls were inoculated with non-viable cells. Results represent mean ± SEM at each time interval. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after inoculation (days)</th>
<th>CONTROL (saline) n = 3</th>
<th>+VE CONTROL (non-viable cells) n = 4</th>
<th>TUMOUR-BEARING (viable cells) n = 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µg foetal calf DNA equivalents/mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.2 ± 0.3</td>
<td>6.2 ± 0.1</td>
<td>6.1 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>6.3 ± 0.6</td>
<td>6.1 ± 0.1</td>
<td>6.3 ± 0.1</td>
</tr>
<tr>
<td>14</td>
<td>6.3 ± 0.2</td>
<td>6.3 ± 0.2</td>
<td>10.0 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
**TABLE 5.7** - Erythrocyte GSH Peroxidase, Superoxide Dismutase and Catalase Activities of C57BL6 Mice Implanted with Lewis Lung Carcinomas

Control mice were injected with phosphate-buffered saline and the positive controls were inoculated with non-viable cells. Results represent mean of duplicate determinations from the blood composites of 2-3 mice, at each time interval.

<table>
<thead>
<tr>
<th>Time after inoculation (days)</th>
<th>GSH Peroxidase (Enzyme units/mg Hb)</th>
<th>Superoxide Dismutase (units [Sigma]/mg Hb at 25 ± 2°C, pH 7.5)</th>
<th>Catalase (** k/min/mg Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Positive</td>
<td>Tumour-bearing</td>
</tr>
<tr>
<td></td>
<td>n=3</td>
<td>n=4</td>
<td>n=5</td>
</tr>
<tr>
<td>1</td>
<td>14.5</td>
<td>15.0</td>
<td>14.9</td>
</tr>
<tr>
<td>3</td>
<td>14.5</td>
<td>14.8</td>
<td>14.7</td>
</tr>
<tr>
<td>14</td>
<td>15.1</td>
<td>15.0</td>
<td>12.4</td>
</tr>
</tbody>
</table>

* Enzyme units are defined as 1000 x log_{10} (decrease in GSH/min) at 37°C, pH 7.0.

** k is the first-order reaction rate constant at 2±1°C, pH 7.4.
TABLE 5.8 - Pulmonary and Tumour Microsomal Thiobarbituric Acid-Reactive Material Concentrations of C57B16 Mice Implanted with Lewis Lung Carcinomas

Control mice were injected with phosphate-buffered saline and the positive controls were inoculated with non-viable cells. Results represent mean ± SEM at each time interval. Microsomes were refrigerated overnight at 4°C prior to analysis. Thiobarbituric acid-reactivity was determined using 0.1 M phthalate buffer (pH 3.5) as the acid medium. Reaction mixtures contained 0.01% w/v BHT and 0.1 mM DETAPAC. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after inoculation (days)</th>
<th>CONTROL (saline)</th>
<th>+VE CONTROL (non-viable cells)</th>
<th>TUMOUR-BEARING (viable cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>Lung</td>
<td>Lung</td>
<td>Tumour</td>
</tr>
<tr>
<td>n = 3</td>
<td>n = 4</td>
<td>n = 5</td>
<td>n = 5</td>
</tr>
</tbody>
</table>

(nmol malondialdehyde equivalents/mg protein)

1 1.1 ± 0.3 1.2 ± 0.2 1.0 ± 0.1 -

3 1.0 ± 0.2 1.3 ± 0.3 3.2 ± 0.3a -

14 1.0 ± 0.2 1.0 ± 0.1 1.8 ± 0.2 3.0 ± 0.4a

- Insufficient tissue for assay.

a-b Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter. Tumour values compared with those of control lung.
### TABLE 5.9 - Hepatic Microsomal Thiobarbituric Acid-Reactive Material Concentrations of C57BL6 Mice Implanted with Lewis Lung Carcinomas

Control mice were injected with phosphate-buffered saline and the positive controls were inoculated with non-viable cells. Results represent mean ± SEM at each time interval. Microsomes were refrigerated overnight at 4°C prior to analysis. Thiobarbituric acid-reactivity was determined using 0.1 M phthalate buffer (pH 3.5) as the acid medium. Reaction mixtures contained 0.01% w/v BHT and 0.1 mM DETAPAC. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after inoculation (days)</th>
<th>CONTROL</th>
<th>+VE CONTROL</th>
<th>TUMOUR-BEARING</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 3</td>
<td>n = 4</td>
<td>n = 5</td>
<td></td>
</tr>
</tbody>
</table>

(nmol malondialdehyde equivalents/mg protein)

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>+VE CONTROL</th>
<th>TUMOUR-BEARING</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.2 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>1.4 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>14</td>
<td>1.4 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
</tbody>
</table>

No significant differences (p>0.05) by Newman-Keuls test (after analysis of variance).
At 3 and 14 days post inoculation, there were no differences in the hepatic TBA-reactivities between any of the experimental groups (table 5.9). The TBA-reactive material concentrations, described thus far, were determined using the procedure, employing phthalate buffer. When the method using $H_2SO_4$ was used, the hepatic microsomal TBA-reactive material concentrations of mice, implanted with viable or non-viable cells, did not differ from that of saline-injected controls (table 5.10).

Iron/ascorbate-mediated lipid peroxidation: The maximal iron/ascorbate chemiluminescence of mice, that had been injected with tumour cells did not differ from that of saline-injected controls (table 5.11). The total chemiluminescence (integral of chemiluminescence against time of reaction plots) was appreciably greater in peroxidizing pulmonary microsomes from tumour-implanted mice than tumour-free controls; and about 30% greater than control values in mice, 3 days after implantation with non-viable cells (table 5.12; fig. 5.1). The maximal chemiluminescence of iron/ascorbate-challenged tumour microsomes was approximately half that of control pulmonary microsomes (table 5.11), and the total chemiluminescence was 66% of that of lung (table 5.12; fig. 5.1).

The peak chemiluminescence of peroxidizing hepatic microsomes from mice, implanted with viable cells, was marginally less than the control levels, at 1 and 3 days post-inoculation, but significantly greater than the control levels at 14 days (table 5.13; fig. 5.2). The total hepatic microsomal chemiluminescence of tumour-bearing mice, was 88% of control values at 1 day and 151% at 14 days post inoculation (table 5.13). Mice injected with non-viable tumour cells, had lower peak and total hepatic microsomal chemiluminescences, at 1 day post-inoculation, than the saline-injected controls (tables 5.13 and 5.14).

The basal chemiluminescence, in the presence of Luminol$^R$, of muscle tissue into which Lewis lung carcinoma cells had been inoculated, did not
TABLE 5.10 - Hepatic Microsomal Thiobarbituric Acid-Reactive Material Concentrations of C57BL/6 Mice Implanted with Lewis Lung Carcinomas

Control mice were injected with phosphate-buffered saline and the positive controls were inoculated with non-viable cells. Results represent mean ± SEM at each time interval. Microsomes were refrigerated overnight at 4° C prior to analysis. Thiobarbituric acid-reactivity was determined using 0.05 M H₂SO₄ as the acid medium. Reaction mixtures contained 0.01% w/v BHT and 0.1 mM DETAPAC. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Time after inoculation (days)</th>
<th>CONTROL</th>
<th>+VE CONTROL</th>
<th>TUMOUR-BEARING</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 3</td>
<td>n = 4</td>
<td>n = 5</td>
</tr>
<tr>
<td></td>
<td>(nmol malondialdehyde equivalents/mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.5 ± 0.3</td>
<td>4.4 ± 0.2</td>
<td>4.8 ± 0.3</td>
</tr>
<tr>
<td>3</td>
<td>3.6 ± 0.3</td>
<td>4.9 ± 0.3</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>14</td>
<td>5.1 ± 0.5</td>
<td>5.1 ± 0.5</td>
<td>9.2 ± 0.9⁴</td>
</tr>
</tbody>
</table>

⁴ Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 5.11 - Ascorbate/FeCl$_2$-induced Peak Chemiluminescence of Pulmonary and Tumour Microsomes from C57BL6 Mice, Bearing Lewis Lung Carcinomas

Control mice were injected with phosphate-buffered saline and the positive controls were inoculated with non-viable cells. Results represent mean ± SEM at each time interval. Microsomes were refrigerated overnight at 4° C prior to analysis. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard. Time (min) to maximum chemiluminescence shown in brackets.

<table>
<thead>
<tr>
<th>Time after inoculation (days)</th>
<th>CONTROL</th>
<th>+VE CONTROL</th>
<th>TUMOUR-BEARING</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(cpm x 10$^{-3}$/mg protein at 37° C, pH 7.4)

1

- 130 + 9
  (8)

- 131 + 2
  (8)

- 140 + 3
  (8)

- Insufficient tissue for assay.

3

- 142 + 27
  (8)

- 175 + 9
  (8)

- 137 + 8
  (8)

14

- 205 ± 13
  (6)

- 197 + 25
  (8)

- 170 ± 13
  (8)

- 82 + 6$^a$
  (7)

$^a$ Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter. Tumour values compared with those of control lung.
TABLE 5.12  Ascorbate/FeCl₃-induced Total Chemiluminescence of Pulmonary and Tumour Microsomes from C57BL6 Mice, Bearing Lewis Lung Carcinomas

Control mice were injected with phosphate-buffered saline and the positive controls were inoculated with non-viable cells. Results represent mean surface area enclosed by graphs of chemiluminescence plotted against time. Microsomes were refrigerated overnight at 4°C prior to analysis.

<table>
<thead>
<tr>
<th>Time after inoculation (days)</th>
<th>CONTROL Lung n = 3</th>
<th>+VE CONTROL Lung n = 4</th>
<th>TUMOUR-BEARING Lung n = 5</th>
<th>TUMOUR-BEARING Tumour n = 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(%) of corresponding control pulmonary value</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>110</td>
<td>154</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>130</td>
<td>150</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>100</td>
<td>110</td>
<td>130</td>
<td>66</td>
</tr>
</tbody>
</table>

- Insufficient tissue for assay.

*Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter. Tumour values compared with those of control lung.*
TABLE 5.13 - Ascorbate/FeCl₃-induced Peak Chemiluminescence of Hepatic Microsomes from C57BL/6 Mice, Implanted with Lewis Lung Carcinomas

Control mice were injected with phosphate-buffered saline and the positive controls were inoculated with non-viable cells. Results represent mean ± SEM at each time interval. Microsomes were refrigerated overnight at 4°C prior to analysis. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard. Time (min) to maximum chemiluminescence shown in brackets.

<table>
<thead>
<tr>
<th>Time after Inoculation (days)</th>
<th>CONTROL</th>
<th>+VE CONTROL</th>
<th>TUMOUR-BEARING</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 3</td>
<td>n = 4</td>
<td>n = 5</td>
</tr>
<tr>
<td></td>
<td>(cpm x 10⁻³/mg protein at 37°C, pH 7.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>523 ± 19</td>
<td>410 ± 29</td>
<td>438 ± 8</td>
</tr>
<tr>
<td></td>
<td>(19 ± 1)</td>
<td>(36 ± 2)</td>
<td>(35 ± 4)</td>
</tr>
<tr>
<td>3</td>
<td>500 ± 14</td>
<td>539 ± 34</td>
<td>466 ± 40</td>
</tr>
<tr>
<td></td>
<td>(24 ± 2)</td>
<td>(22 ± 3)</td>
<td>(24 ± 3)</td>
</tr>
<tr>
<td>14</td>
<td>489 ± 2</td>
<td>482 ± 9</td>
<td>624 ± 44a</td>
</tr>
<tr>
<td></td>
<td>(22 ± 4)</td>
<td>(21 ± 4)</td>
<td>(20 ± 4)</td>
</tr>
</tbody>
</table>

*Significantly different (P<0.05) by Newman-Keuls test (after analysis of variance) from all values except those bearing the same superscript letter.
TABLE 5.14 - Ascorbate/FeCl$_2$-induced Total Chemiluminescence of Hepatic Microsomes from C57BL6 Mice, Implanted with Lewis Lung Carcinomas

Control mice were injected with phosphate-buffered saline and the positive controls were inoculated with non-viable cells. Results represent mean surface area enclosed by graphs of chemiluminescence plotted against time. Microsomes were refrigerated overnight at 4°C prior to analysis.

<table>
<thead>
<tr>
<th>Time after inoculation (days)</th>
<th>CONTROL n = 3</th>
<th>+VE CONTROL n = 4</th>
<th>TUMOUR-BEARING n = 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>78</td>
<td>88</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>109</td>
<td>103</td>
</tr>
<tr>
<td>14</td>
<td>100</td>
<td>97</td>
<td>151</td>
</tr>
</tbody>
</table>
FIG. 5.1 - FeCl$_2$/ascorbate-induced Chemiluminescence of Pulmonary and Tumour Microsomes at:
(a) 1 day, (b) 3 days, (c) 14 days, After Inoculation of Mice with Lewis Lung Carcinoma Cells.

(---) Saline-injected controls. (---) Controls inoculated with non-viable cells. (---) Inoculated with viable cells.
FIG. 5.2 - FeCl$_2$/ascorbate-induced Chemiluminescence of Hepatic Microsomes From Mice that Had Borne Lewis Lung Carcinomas for 14 Days

(---) Saline-injected controls. (*--*) Controls inoculated with non-viable cells. (A----A) Inoculated with viable cells.
differ from that of muscle tissue of saline-injected controls. This chemiluminescence was approximately 5000 cpm greater than that of buffer and Luminol\textsuperscript{R} alone.

Studies of the Livers of Genetically Obese Mice

Liver weights and yields of subcellular fractions: The liver wet weights of obese and lean mice were 3.25 ± 0.34 g and 1.44 ± 0.12 g respectively. The yields of protein in the mitochondrial and soluble fractions of liver did not differ between lean and obese mice. The yield of hepatic microsomes from obese mice (5.4 ± 0.7 mg protein/g wet wt) was about a third of that of lean mice (15.7 ± 4.6 mg/g wet wt).

Protein and DNA concentrations: Hepatic protein concentrations of obese mice, determined either by the procedure using Folin-Ciocalteu reagent or that using Coomassie blue reagent, did not differ from those of lean mice (table 5.15). The hepatic DNA concentrations of obese mice were 34% of those of lean mice.

Antioxidant defence enzymes: Cytosolic GSH peroxidase and SOD activities, and mitochondrial \(\gamma\) glutamyl transpeptidase activities of obese mice did not differ significantly from those of lean mice (tables 5.16 and 5.17). Mitochondrial GSH peroxidase and SOD activities (table 5.16), and catalase activities (table 5.17) of obese mice were less than those of lean controls.

Lipid Peroxidation: The concentration of TBA-reactive material in hepatic microsomes from obese mice did not differ from that of lean mice (table 5.18). BHT and DETAPAC considerably decreased the TBA-reactivity of hepatic microsomes, and there were no differences between lean and obese mice (table 5.18).

The chemiluminescence of iron/ascorbate-challenged microsomes, from
TABLE 5.15 - Protein and DNA Concentrations of Livers of C57BL6 ob/ob Mice

Lean C57BL6 mice served as the controls. Results represent mean ± SEM for 5 mice in each group. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard. Protein determinations with Coomassie blue reagent are shown in brackets.

<table>
<thead>
<tr>
<th>Protein (mg/ g wet wt)</th>
<th>DNA (μg DNA equiv/ mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td>Obese</td>
</tr>
<tr>
<td>155 ± 7</td>
<td>160 ± 9</td>
</tr>
<tr>
<td>6.2 ± 0.6</td>
<td>2.2 ± 0.2^a</td>
</tr>
</tbody>
</table>

^a Significantly different (P<0.05) by unpaired Student's t-test from the corresponding value in the lean mouse.
TABLE 5.16 - GSH Peroxidase and Superoxide Dismutase Activities of Livers of C57BL6 ob/ob Mice

Lean C57BL6 mice served as the controls. Results represent mean ± SEM for 5 mice in each group. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th></th>
<th>GSH Peroxidase (Enzyme units/mg protein)</th>
<th>Superoxide Dismutase (units [μg]/mg protein at 25 ± 2°C, pH 7.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytosolic</td>
<td>Mitochondrial</td>
</tr>
<tr>
<td>LEAN</td>
<td>118 ± 11</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>OBESE</td>
<td>129 ± 3</td>
<td>9.7 ± 0.4a</td>
</tr>
</tbody>
</table>

*Significantly different (P<0.05) by unpaired Student's t-test from the corresponding value in the lean mouse.
TABLE 5.17 - Catalase and Mitochondrial $\gamma$-Glutamyl Transpeptidase Activities in the Livers of C57BL6 ob/ob Mice

Lean C57BL6 mice served as the controls. Results represent mean ± SEM for 5 mice in each group. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th></th>
<th>Catalase (k*/min/ mg protein)</th>
<th>Mitochondrial $\gamma$-Glutamyl transpeptidase (nmol p-nitroaniline/ min/ mg protein at 37° C, pH 7.8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td>2.4 ± 0.2</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td>Obese</td>
<td>1.0 ± 0.1*</td>
<td>0.25 ± 0.02</td>
</tr>
</tbody>
</table>

* k is the first order reaction rate constant at 2 + 1° C, pH 7.4.

a Significantly different (P<0.05) by unpaired Student's t-test from the corresponding value in the lean mouse.
obese mice, had a greater maximum and took a shorter time to peak than microsomes from lean mice (table 5.19). Total chemiluminescence, and the lipid peroxidation reaction of hepatic microsomes, monitored by TBA-reactive material production, were similar between the two experimental groups (tables 5.19 and 5.20).

Comparison of Liver of Obese Mice with that of Tumour-bearing Mice

The effects of congenital obesity upon antioxidant defence enzymes and lipid peroxidation in murine liver, have been compared with those of bearing a Lewis lung carcinoma (table 5.21). In common with tumour-bearing mice, protein concentration was unchanged, catalase and mitochondrial GSH peroxidase activities were decreased, and TBA-reactive material concentration and maximal chemiluminescence were increased. The increased DNA concentration, mitochondrial γ-glutamyl transpeptidase activity, increased total chemiluminescence and TBA-reactive material production of peroxidizing microsomes, were observed for the livers of tumour-bearing mice but not obese mice. The livers of obese mice, but not those of tumour-bearing mice, displayed decreased mitochondrial SOD activity, and a shortened induction time for iron/ascorbate-mediated chemiluminescence.

DISCUSSION

The Immune Status of Lewis Lung carcinoma

The defence of the host against neoplasia is mediated by T-lymphocytes, macrophages, and natural killer cells (Young and Hoover, 1986). Some types of lymphoid cell, produced by tumour-bearing animals, are toxic both to normal and neoplastic tissues (Del Maestro et al, 1980; Olsson et al, 1981). The unchanged spleen weights and the absence of a leukocyte-dependent inflammatory reaction, as evidenced by lack of
TABLE 5.18 - Microsomal Thiobarbituric Acid-reactive Material
Concentrations of the Livers of C57BL6 ob/ob Mice
Livers from lean C57BL6 mice served as the controls.
Results represent mean ± SEM for 5 mice in each group.
Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th>Conditions of incubation to produce TBA-reactive material chromaphore</th>
<th>LEAN (nmol malondialdehyde equiv/mg protein)</th>
<th>OBESE (nmol malondialdehyde equiv/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min in 0.1 M phthalate buffer (pH 3.5) at 100°C.</td>
<td>3.8 ± 0.5</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>15 min in 0.1 M phthalate buffer (pH 3.5) at 100°C + 0.01% w/v BHT + 0.1 mM DETAPAC</td>
<td>1.8 ± 0.2</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>30 min in 0.05 M H₂SO₄ at 100°C.</td>
<td>6.4 ± 0.7</td>
<td>7.4 ± 0.4</td>
</tr>
<tr>
<td>30 min in 0.05 M H₂SO₄ at 100°C + 0.01% w/v BHT + 0.1 mM DETAPAC.</td>
<td>2.1 ± 0.1</td>
<td>2.0 ± 0.1</td>
</tr>
</tbody>
</table>

No significant differences (P>0.05) by unpaired Student's t-test between corresponding values of lean and obese mice.
TABLE 5.19 - Ascorbate/FeCl₃-induced Chemiluminescence by Hepatic Microsomes from C57BL6 ob/ob Mice.

Hepatic microsomes from C57BL6 lean mice were used as the controls. Total chemiluminescence is represented as the mean surface area enclosed by graphs of chemiluminescence plotted against time. Results represent mean (+ SEM where appropriate) for 5 mice in each group. Microsomes were refrigerated overnight at 4°C prior to analysis. Protein was determined with Folin-Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th></th>
<th>Total Chemilumin-</th>
<th>Peak Chemilumin-</th>
<th>Time to Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>escence</td>
<td>escence</td>
<td>Chemiluminescence</td>
</tr>
<tr>
<td>(%) of corresponding hepatic value in the lean mouse</td>
<td>(cpm x 10⁻³/ mg protein at 37°C pH 7.4)</td>
<td>(min)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Total Chemilumin-</th>
<th>Peak Chemilumin-</th>
<th>Time to Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>escence</td>
<td>escence</td>
<td>Chemiluminescence</td>
</tr>
<tr>
<td>LEAN</td>
<td>100</td>
<td>531 ± 39</td>
<td>20 ± 7</td>
</tr>
<tr>
<td>OBSESE</td>
<td>95</td>
<td>729 ± 46ᵃ</td>
<td>12 ± 2ᵃ</td>
</tr>
</tbody>
</table>

ᵃ Significantly different (P<0.05) by unpaired Student's t-test from the corresponding value in the lean mouse.
FIG. 5.3 - FeCl\textsubscript{3}/ascorbate-induced Chemiluminescence of Hepatic Microsomes from Genetically Obese Mice, and from Lean Controls.

(– – –) C57BL6 lean controls. (▲ ▲ ▲) C57BL6 ob/ob mice.
TABLE 5.20

Ascorbate/FeCl$_2$-induced Thiobarbituric Acid-Reactive
Material Production by Hepatic Microsomes from C57BL6
ob/ob Mice.

Hepatic microsomes from C57BL6 lean mice were used as the
controls. Results represent mean ± SEM for 5 mice in each
group. Microsomes were refrigerated overnight at 4°C
prior to analysis. Protein was determined with Folin-
Ciocalteu reagent, using BSA as the standard.

<table>
<thead>
<tr>
<th></th>
<th>Peak TBA-reactive Material Production (nmol malondialdehyde equiv/mg protein at 37°C, pH 7.4)</th>
<th>Time to Peak TBA-reactive Material Production (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEAN</td>
<td>45 ± 4</td>
<td>39 ± 6</td>
</tr>
<tr>
<td>OBESE</td>
<td>47 ± 3</td>
<td>36 ± 6</td>
</tr>
</tbody>
</table>

No significant differences (P>0.05) by unpaired Student's t-test between the corresponding values of lean and obese mice.
TABLE 5.21 - A Comparison of the Effect of Congenital Obesity With the Effect of Bearing a Lewis Lung Carcinoma, upon the Livers of C57BL6 Mice (as mainly determined by the studies of chapters 3-5)  
All values are specific activities or concentrations.

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>SUBCELLULAR FRACTION</th>
<th>OBESE MOUSE</th>
<th>TUMOUR-BEARING MOUSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>whole tissue</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>DNA</td>
<td>whole tissue</td>
<td>34% of lean control</td>
<td>150% of tumour-free control</td>
</tr>
<tr>
<td>GSH peroxidase</td>
<td>cytosol</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>mitochondria</td>
<td>33% of lean control</td>
<td>66% of tumour-free control</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>cytosol</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>mitochondria</td>
<td>33% of lean control</td>
<td>NS</td>
</tr>
<tr>
<td>Catalase</td>
<td>whole tissue</td>
<td>43% of lean control</td>
<td>60% of tumour-free control</td>
</tr>
<tr>
<td>γ-Glutamyl transpeptidase</td>
<td>mitochondria</td>
<td>NS</td>
<td>140% of tumour-free control</td>
</tr>
<tr>
<td>TBA-reactive material</td>
<td>whole tissue</td>
<td>Significantly increased</td>
<td>Significantly increased</td>
</tr>
<tr>
<td></td>
<td>microsomes</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Ascorbate/FeCl₃- induced production of TBA-reactive material</td>
<td>microsomes</td>
<td>NS</td>
<td>180% of tumour-free control</td>
</tr>
<tr>
<td>Ascorbate/FeCl₃- induced chemiluminescence (CL)</td>
<td>microsomes</td>
<td>Max CL increased by 37%. Time to max CL decreased by 42%. No significant difference in total CL. increased by 50%</td>
<td>Max CL increased by 50%. Time to max CL not significantly different. Total CL. increased by 50%</td>
</tr>
</tbody>
</table>


NS - No significant differences from lean control or tumour-free mouse.
Luminol-mediated chemiluminescence at the site of inoculation with tumour cells, indicated that Lewis lung carcinomas elicited little, if any, cell-mediated immune response in the host mice, within 3 days of tumour implantation. Studies elsewhere also support this conclusion (Steel and Adams, 1975; Gundersen et al, 1981; Olsson et al, 1981). The cytotoxicity of host lymphocytes towards tumour cells has, however, been reported to be enhanced in mice following the first few days after implantation of Lewis lung carcinomas (Young and Hoover, 1986).

Natural killer cells are cells, other than polymorphonuclear leukocytes and macrophages, produced in response to neoplasia or viral infections; they are cytotoxic without prior sensitization (production of antibodies) to antigens (Woodruff, 1986). Natural killer cell activity is induced by lymphokines, such as interferon and interleukin 2 (Woodruff, 1986). It has been reported that natural killer cell activity in spleen cells increases after intra-footpad inoculation of Lewis lung carcinoma cells into mice, reaching a plateau level at 3 days post-implantation (Shoham and Klein, 1984). The limited host response, shortly after transplantation of Lewis lung carcinomas, may well be primarily mediated by natural killer cells.

Lewis lung carcinomas can be transplanted into allogenic mice, although metastatic deposits are not formed in these animals (Isakov et al, 1983). The carcinoma might thus have the ability to suppress the immune systems of the host. It has been reported that Lewis lung carcinomas produce a carboxylic acid-like substance, possibly prostaglandin E2, that inhibits macrophage-mediated resistance to the tumour (Young et al, 1982). The suppression of the host-immune response is possibly governed by T-lymphocytes, found in the spleens of mice, bearing Lewis lung carcinomas, as thymectomy inhibits the tumour's growth (Malave et al, 1979; Olsson et al, 1981; Young et al, 1986). The carcinoma may
well promote its own growth by elaborating factors into the vasculature or lymphatics of the host to modulate cell-mediated immunity. These hypothetical substances were possibly not specific to lymphoid cells; affecting other tissues of tumour-bearing mice, such as blood and liver (Chapter 6).

As the Lewis lung carcinoma can establish itself across allogenic barriers (Isakov et al, 1983), its means to overcome the immune system of the host must be appreciable, particularly shortly after transplantation when the number of neoplastic cells would be comparatively low. A major systemic effect, observed for mice within 3 days of inoculation with tumour cells, common to mice that had borne tumours for 14 days, was increased erythrocyte SOD activity. Erythrocyte SOD may have been more sensitive to tumour-associated substances than other tissue enzymes, such as hepatic catalase, which only changed when the tumour had attained a relatively large size. The abnormalities, that were not observed until the Lewis lung carcinomas were of comparatively advanced size, might alternatively have been secondary to other biochemical changes, not determined in the present studies.

**Increased Erythrocyte SOD Activity and Interferon**

Increased erythrocyte SOD (erythrocuprein) activity has been associated with several human degenerative diseases, such as Duchenne muscular distrophy (Burri et al, 1980), thalassaemia (Gerli et al, 1980), diabetes (Michelson et al, 1977), and some types of human cancers (Gonzales et al, 1984). SOD activity does not vary with the age of erythrocytes (Michelson et al, 1977; Vanella et al, 1982), indicating that the raised activity in tumour-bearing mice was not caused by a change in the distribution of the red cell population. Disturbances of red cell SOD probably indicate pathological change, possibly due to excessive oxygen radical production, and thus this enzyme might be a
sensitive marker for some diseases. As the erythrocyte SOD activities of mice, bearing B16 melanomas, did not differ from those of the controls (Chapter 3), increased erythrocuprein activity probably is not a general indicator of murine neoplastic disease.

Comparative studies of erythrocyte antioxidant defence enzymes have shown that SOD activity varies relatively little among different species (Maral et al., 1977). SOD might therefore have a more primary role in protecting erythrocytes from uncontrolled autoxidation than for example, catalase and GSH peroxidase. The raised erythrocyte SOD activities of Lewis lung carcinoma-bearing mice indicated that 'O$_2^-$ was generated, in pathological excess, in the blood streams of these animals. The survival times of tumour-bearing animals have been observed to be prolonged by the intravenous injection of SOD, suggesting that there may have been excess extracellular 'O$_2^-$ (Michelson et al., 1977; Oberley et al., 1982). The 'O$_2^-$ radicals were unlikely to have been produced by activated granulocytes or phagocytes as there was no evidence of any local inflammatory reaction. The gene, coding for sensitivity to interferon, a mediator of antiviral defence, and that for synthesis of Cu/Zn SOD, have been reported to be assigned to the same chromosomal segment (Cox et al., 1980; Lin et al.; Pottathil et al., 1981). In the present studies, even the injection of non-viable cells caused increased red cell SOD activity, indicating that this was the only systemic effect, determined for Lewis lung carcinoma-bearing mice, that may have a significant component from the immune response of the host. Lewis lung tumour cells could possibly have caused increased production of erythrocyte SOD, in association with activating the synthesis of interferon. Macrophages, activated by interferon (sometimes referred to as macrophage-stimulating factor) produce H$_2$O$_2$ by an enzymic reaction, reported to be almost entirely mediated by Mn-SOD (Freund and Pick 1986). Sensitivity to interferon may well be coupled to the biosynthesis of SOD in cells other than erythro-
Murine serum has been reported to contain a factor, possibly interferon, which inhibits the growth of Lewis lung carcinoma (Gundersen et al., 1981). Interferon, synthesised in response to inoculation with Lewis lung carcinoma cells might have had a limited effect in preventing the growth of the tumour, but possibly contributed to the perturbations of non-neoplastic tissues (see Chapter 6).

Pulmonary Lipid Peroxidation

The increased iron/ascorbate-mediated chemiluminescence and TBA-reactive material concentration of pulmonary microsomes from mice, killed 1-3 days after inoculation with viable tumour cells, indicated that at least one type of lung cell was sensitive to the presence of Lewis lung cells. During the intramuscular injection of Lewis lung carcinoma cells into mice, tumour cells may have entered blood vessels, damaged by the syringe needle. The blood-borne tumour cells might have been arrested in the capillary bed of the lungs, where they elicited the changes in pulmonary lipid peroxidation.

The early alterations in lung peroxidation were probably not due to tumour cells per se, because the chemiluminescence of peroxidizing tumour microsomes was less than that of control lung, and changes in pulmonary peroxidation were less pronounced at 14 days post implantation, when metastatic foci would have been established, than at 1-3 days. Metastatic tumour cells might, however, have contributed to the less aberrant TBA-reactive material and chemiluminescence values, observed for mice that had borne Lewis lung carcinomas for 14 days.

Histological examinations of the lungs of mice, have shown substantial endarteritis obliterans by 1 day, and infiltration by lymphoid and plasma cells at 3 days onward, after transplantation of
Lewis lung carcinomas (Salsbury et al., 1974). The increased TBA-reactivity and iron/ascorbate-induced chemiluminescence of pulmonary microsomes may thus have resulted from an immune reaction in response to the presence of neoplastic cells in the lungs. The lungs of mice, bearing Lewis lung carcinomas, possibly had a more apparent immune reaction than that evidenced by the spleen, or chemiluminescence in the muscle into which cells were injected.

Systemic Changes in Mice That had Borne Lewis Lung Carcinomas for 14 Days

The changes in hepatic DNA concentration and in enzyme activity of blood and liver, seen in mice that had borne Lewis lung carcinomas for 14 days, reaffirm previous observations (Chapter 3), and demonstrate that these earlier results were reproducible. The present investigations supported the studies of chapter 4, which indicated that hepatic microsomes, from mice bearing carcinomas of appreciable size, were more susceptible to iron/ascorbate-mediated peroxidation. It is also now apparent that Lewis lung carcinomas, derived directly from cells maintained in tissue culture, caused the same systemic abnormalities as tumours, transplanted by in vivo passage.

Lewis lung carcinomas, of pronounced size, might have accrued essential nutrients or trace elements at the expense of the non-neoplastic tissues, thereby causing harmful effects to tumour-bearing mice. In experiments elsewhere, rats were implanted with an inert artificial tumour, inflated with saline to simulate the growth of a neoplasm (Morrison et al., 1984). The artificial tumour mimicked much of the effect that a neoplastic tumour exerts upon the energy expenditure of the host animal. The stress of tumour burden might therefore contribute substantially to the systemic effects of animal tumour models, and of human cancer (Chapters 3 and 6). It might thus be appropriate to implant
a similar artificial tumour into C57BL6 mice, and determine whether any of the abnormalities of normal tissues in Lewis lung carcinoma-bearing mice were attributable to the encumbrance of the tumour.

The immune response of mice may well have been largely repressed during the first few days after tumour implantation. The enlarged spleens of mice, that had borne Lewis lung carcinomas for 14 days, however, suggests that a significant host immune response might have been elicited in these animals. As the major perturbations of host tissue antioxidant defence and lipid peroxidation seemed to be present only when tumour growth was relatively advanced, the immune response of the host mice was possibly a significant causative factor.

**Studies of Obese Mice**

The present studies showed that the only abnormalities of hepatic metabolism, common to obese mice and to mice, bearing Lewis lung carcinomas, were decreased catalase and mitochondrial GSH peroxidase activities, and increased TBA-reactive material concentrations (table 5.21). The livers of obese mice weighed more than twice those of lean animals, and the DNA concentration of their livers was only a third of that of lean animals; this indicated that the total number of hepatocytes in livers from obese mice did not exceed that of normal liver. The biochemical disturbances of the livers of the obese mice, unlike that of tumour-bearing mice, probably did not involve an increase in cell division.

The protein concentrations and mitochondrial yields of the livers of obese mice were the same as those of lean animals. The general level of enzymically-mediated metabolism, particularly that of mitochondria, was thus maintained, possibly in proportion to the size of the liver. Obese
mice have been reported to have decreased concentrations of hepatic endoplasmic reticulum, supporting the present observation of a relatively low microsomal yield (Rouer and Leroux, 1980). The concentration of membranous enzymes in the livers of obese mice, has been reported to be 2- to 3-fold less than that of lean mice; the amount of these enzymes per liver did not differ between lean and obese mice, as the obese mouse has a much larger liver (Murphy et al., 1979). This indicates that the quantity of microsomal components per liver of obese mice was probably similar to that of normal mice.

Uncontrolled autoxidation, due to deficiency of catalase and mitochondrial GSH peroxidase, might have been a common mechanism for causing the metabolic perturbations of livers from obese or Lewis lung carcinoma-bearing mice. The abnormalities of the genetically-obese mouse have been attributed to a lack of thyroid hormone-inducible (Na\(^+\), K\(^+\) dependent)-ATPase (York et al., 1978). The Na\(^+\), K\(^+\) pump activity of the plasma membrane has been reported to be inactivated by lipid peroxidation (Trush et al., 1982). The decreased Na\(^+\), K\(^+\) pump activity and other manifestations of obesity, such as impaired drug metabolism and cirrhosis, have been postulated to be caused by excessive hepatic lipid peroxidation (Capel and Dorrell, 1984).

The present studies indicate that the concentration of malondialdehyde-like material and the susceptibility to non-enzymic lipid peroxidation of hepatic microsomes from obese mice were probably not sufficiently different from that of lean mice to be considered biologically significant. The chemiluminescence response (figure 5.3) inferred that the lipid composition of liver microsomes from obese mice did not differ substantially from that of normal mice. It has been reported that hepatic TBA-reactivity of obese mice is greater than that of lean mice (Capel and Dorrell, 1984). In these studies, however, the
assay procedure used did not include any antioxidants. Liver homogenates, from obese mice, were observed in the present studies to contain conspicuous amounts of lipid. The increased TBA-reactive material concentration of hepatic tissue was probably an experimental artefact, reflecting a comparatively large lipid peroxidation reaction during the TBA-test itself, due to a pronounced PUFA concentration.

The microsomal concentration of cytochrome P450 of obese mice has been reported to be slightly greater than that of lean mice (Rouer and Leroux, 1980). The relatively increased NADPH-dependent microsomal lipid peroxidation reaction, reported for the livers of obese mice (Capel and Dorrell, 1984), might have reflected increased NADPH-dependent flavoprotein cytochrome P450 reductase.

The hepatic catalase activities of genetically obese mice have been reported elsewhere not to differ from those of lean mice (Murphy et al, 1979). In the present studies, liver tissue was frozen at -20°C prior to the determination of catalase activity. The catalase of liver homogenate from obese mice might have been less stable under these conditions than that from lean mice.

The decreased SOD and GSH peroxidase activities of hepatic mitochondria from obese mice might have been a dilution effect, caused by increased synthesis of other mitochondrial enzymes. It has been shown, however, that the specific activity of the mitochondrial marker enzyme, glutamate dehydrogenase, was the same whether from the lean or obese mouse (Murphy et al, 1979). This indicates that there was a specific repression or inhibition of mitochondrial SOD and GSH peroxidase in the livers of obese mice. Obese mice have lowered O2 consumption than their lean counterparts (Murphy et al, 1979). As the liver of the obese mouse is considerably enlarged, it might have lower oxygenation than control liver. Mitochondrial SOD and GSH peroxidase are possibly inducible by O2.
Conclusions

Relatively few of the biochemical alterations, ascertained for the livers of obese mice, were the same as those of Lewis lung carcinoma-bearing mice. The liver of the obese mouse was therefore a comparatively limited model of the liver of Lewis lung carcinoma-bearing mouse. However, the observation that hepatic catalase activity is decreased both in tumour-bearing and in obese mice, and the postulated role of catalase in lipid metabolism (Crane and Masters, 1984) may well be related. The results of the present studies do not, therefore, preclude the possibility that altered fat metabolism is an important antecedent of many of the hepatic abnormalities of tumour-bearing animals (Chapter 6).

The studies of the short-term systemic effects of Lewis lung carcinomas have indicated that the tumour has to be at least externally palpable before most of the biochemical abnormalities of non-neoplastic tissues were detectable. As the systemic effects of human cancers can occur when tumours are still relatively minute in size, changes in antioxidant defence and lipid peroxidation follow other biochemical perturbations, not determined by the present investigations, rather than directly cause them. The present studies do not militate against cell-mediated immunity having a significant role in the disturbances of murine tissues; lymphokines, such as interferon, may well be involved. The possible causes of the systemic abnormalities of mice, bearing Lewis lung carcinomas, will be discussed further in the following chapter.
CHAPTER SIX

GENERAL DISCUSSION
INTRODUCTION

One of the primary objectives of this thesis was to ascertain whether any major facets of antioxidant defence in a model tumour system, were significantly different from those of normal tissues. It might then be possible to devise methods to exploit any biochemical differences so that neoplastic cells could either be destroyed or compelled to differentiate. Such stratagems must, however, have an irreversible effect upon malignant tissues but cause little or no toxicity to normal tissues (Greenstein, 1956). The antioxidant defence systems of non-neoplastic tissues, from the tumour-bearing animals, have also been monitored because changes in the ability of the host animal to detoxify reactive oxygen intermediates, could possibly promote or, conversely, attenuate anticancer regimens, dependent upon these species.

In this concluding chapter of the thesis, some of the earlier observations, concerning antioxidant defence and lipid peroxidation in the Lewis lung carcinoma, are discussed further, and their implications with regard to tumour metabolism and anti-tumour therapy are speculated. The causes of the effects of the Lewis lung carcinoma upon the antioxidant defence systems and lipid peroxidation of normal tissues in the tumour-bearing mouse are also postulated, consideration being given to the ways in which these perturbations might interfere with systemic antitumour therapy.

Further Considerations of Antioxidant Defence in the Lewis Lung Carcinoma

Superoxide dismutase: The observation of a substantially greater mitochondrial SOD in Lewis lung carcinoma than murine lung (Chapter 2) was
not necessarily a contradiction of the belief that decreased Mn-SOD is characteristic of cancer cells (Oberley et al., 1981). SOD has been reported to have a non-homogenous distribution throughout the lung (Minchin and Boyd, 1983): lung comprises of at least 40 cell types (Oberley and Spitz, 1984); and so the type of cell from which Lewis lung carcinoma arose (possibly an alveolar epithelial cell; Chapter 2) might have had even greater Mn-SOD than Lewis lung cells (Westman and Marklund, 1981).

The relatively elevated Mn-SOD activity determined for Lewis lung tumours indicates that in vivo, the tumour cells may have been subjected to considerable oxygen radical flux of mitochondrial origin. The aberrant morphology of Lewis lung cell mitochondria (Sato et al., 1982) might reflect a perturbation of the mitochondrial electron transport chain in these cells. Blockage of respiration enhances \( \cdot O_2^- \) production by mitochondria (Boveris, 1977; Turrens et al., 1982a; Oberley and Spitz, 1984). The \( \cdot O_2^- \), postulated to have been generated by the mitochondria of Lewis lung carcinoma cells, would be intercepted by SOD and dismuted to \( H_2O_2 \). Some \( H_2O_2 \) might escape reduction by GSH peroxidase, as the activity of this enzyme in the tumour cytosol was comparatively low. \( H_2O_2 \) could possibly have deactivated the Cu/Zn-SOD of the cytosol, which was observed to have relatively low activity in Lewis lung carcinoma. As a large proportion of anticancer regimens involve \( \cdot O_2^- \) in their mode of action (Oberley and Beuttner, 1979). The relative resistance of Lewis lung carcinoma to conventional chemotherapy might be explained by the comparatively high Mn-SOD activity.

**Procedures to determine SOD activity in tumours:** The determination of SOD activity in biological samples is subject to endogenous sources of interference. Any compound, present in the assay mixture (such as ascorbate, GSH, transition metals, PUFA, nucleic acids) able to act as a
source of 'O$_2^-$, other than the prime 'O$_2^-$ generator, or a sink of 'O$_2^-$ other than SOD, or the indicating scavenger of 'O$_2^-$, could have caused erroneous estimations of SOD activity (Hassan et al., 1980; Eldred and Hoffert, 1981). Thiols, such as GSH, at physiological concentrations inhibit the production of photo-oxidized dianisidine, and thereby might have caused an underestimation of SOD activity, as determined by the present studies (Roth and Gilbert, 1984).

H$_2$O$_2$, produced by SOD, could have, in the presence of free iron, reacted in a Fenton-type manner with 'O$_2^-$ (generated by electronically-excited riboflavin) to form hydroxyl radicals (OH') and singlet oxygen (O$_2^1$) (Oberley and Beuttner, 1979; Halliwell, 1981). These reactive oxygen species are capable of initiating lipid peroxidation (Bus and Gibson, 1979; Cheeseman, 1982; Singh, 1982). Reaction mixtures, used in the determination of SOD activity in biological samples, may contain all the components required to initiate an autooxidative cascade (namely: O$_2$, a transition metal catalyst, 'O$_2^-$, H$_2$O$_2$, and PUFA). Lipoperoxides are reported to be unstable in the presence of iron salts or iron-containing proteins (Halliwell and Gutteridge, 1984a), and will break down to form further reactive oxygen species. The resultant amplification of the generation of reactive oxygen intermediates, particularly H$_2$O$_2$, could cause the inactivation of Cu/Zn-SOD (Bray et al., 1974; Hodgson and Fridovich, 1975a; Fuchs and Borders, 1983). Cytosolic SOD, deactivated by H$_2$O$_2$ can even act as a promoter of lipid peroxidation (Hodgson and Fridovich, 1975b). SOD activities, determined by the procedure of the present studies, could thus have been, at least in part, a reflection of susceptibility for autoxidation and the antioxidative capacity of the samples. Many animal tumours have been observed to have greater resistance to lipid peroxidation than comparative normal tissues (Bartoli and Galeotti, 1979; Player et al., 1979; Rossi and Gecchini, 1983). SOD activities of Lewis lung carcinoma, may thus have been over-estimated in
10 mM phosphate, which was used to buffer the reaction mixture in the determinations of SOD activity, has been observed to inhibit bovine Cu/Zn SOD activity by about 50% (de Freitas and Valentine, 1984). This inhibition could be partially caused by contamination of phosphate buffer by heavy metals (Fridovich, 1982b). Cu/Zn-SOD activity in biological samples, and that of bovine SOD standards (Sigma), will probably have been equally affected by phosphate anions and other sources of interference, such as detergents (Ljutakova, 1984). Cu/Zn-SOD activities, determined by the procedure described in Chapter 2, should thus have been quantitatively correct. Mn-SOD activity, which is also reported to be inhibited by anions such as phosphate (Benovic et al., 1983) however was standardized, using bovine Cu/Zn SOD which might have been inappropriate due to differences in susceptibility to interference.

The present series of experiments could be improved by re-evaluating SOD activity, using an assay system containing a suitable metal chelator, such as DETAPAC (Oberley and Spitz, 1984) or desferrioxamine (Halliwell and Gutteridge, 1984a), which do not form significant radical-reaction initiating complexes with iron. Desferrioxamine would have the advantage of being a potent scavenger of OH• radicals (Halliwell, 1985). 10 mM phosphate should be substituted by another buffer system, such as 50 mM Hepes (de Freitas and Valentine, 1984). Tris buffer must not be used because it has SOD-like activity (Oberley and Spitz, 1984). Samples could also be dialyzed to remove low molecular weight sources of interference (Eldred and Hoffert, 1981; Fridovich, 1982b). Catalase might be added to the reaction mixtures for the determination of SOD activity, to prevent lipid peroxidation, and end-product inhibition of Cu/Zn SOD by H2O2 (Oberley and Spitz, 1984). Commercial preparations of catalase must be inspected for SOD activity before use (Fridovich, 1982b).
SOD activity of Lewis lung carcinoma and murine reference tissues, might be usefully determined, employing other procedures. A recent review of methods to evaluate SOD activity, contended that only two procedures could be reliably used to determine the SOD activities of neoplastic tissues (Oberley and Spitz, 1984). One of these methods is the modified xanthine oxidase/ nitroblue tetrazolium (NBT) assay (Loven et al., 1980), a procedure which is, however, affected by back-reactions (Fridovich, 1982b). The second method assesses SOD activity by the ability of SOD to catalyze the disproportionation of $\cdot O_2^-$ at alkaline pH (Marklund et al., 1982), but this assay is relatively sensitive to interference by impurities (Oberley and Spitz, 1984). Both methods therefore require a number of controls and have the disadvantage of being comparatively difficult to operate.

One of the authors of the theory that the neoplastic phenotype is associated with diminished Mn-SOD activity (Oberley et al., 1982), has recently discovered the inclusion of NaCN in the xanthine oxidase/ NBT procedure, applied by his laboratory, causes underestimation of Mn-SOD activity (Oberley and Spitz, 1984). Using a modified assay, Mn-SOD could be demonstrated in samples where activity was not previously detected (Oberley and Spitz, 1984). The determination of SOD activities in biological tissues by the available methods, is hampered by interferences that might have resulted in spurious assessments of SOD activities in neoplastic tissues. It would thus be desirable, in future studies of Lewis lung carcinoma, to verify the biochemical estimations of SOD activity, using techniques such as radioimmunoassay or polyacrylamide-gel electrophoresis, to determine absolute quantities of SOD isoenzymes.

Glutathione metabolism in Lewis lung carcinomas: GSH peroxidase activities, determined in biological samples, may reflect the concentration of the enzyme rather than the ability to detoxify peroxides in
GSH peroxidase can react with a great variety of hydroperoxide substrates (hydrogen acceptors), but has pronounced specificity for the hydrogen-donating substrate, GSH (Flohe, 1982). GSH peroxidase activity in Lewis lung carcinoma could thus have been limited by the availability in vivo of GSH, which is generated by \( \gamma \)-glutamylcysteine synthetase and GSSG reductase (see following section). GSH of Lewis lung carcinoma cells might have been relatively inaccessible to GSH peroxidase of cytosol, mitochondria (or other subcellular compartments; Cikryt et al, 1982), due to appropriation by other subcellular compartments or sulphhydril pools (Kosower and Kosower, 1976b; Romero et al, 1984). Approximately 35% of the GSH of Ehrlich ascites tumour cells, for example, has been reported to be bound to protein as mixed disulphides (Modig, 1968). GSH is required for biosynthetic pathways of cells (Harington, 1967; Kosower and Kosower, 1978). Tumours, being relatively rapidly-dividing tissues, probably require substantial GSH, and possibly can segregate GSH to the biosynthetic components of their cells. GSH for competing reactions, such as hydroperoxide metabolism, might thus be limiting in some tumours.

GSH peroxidase activity of lung, the tissue from which Lewis lung carcinoma originated, has been postulated to be regulated by the availability of selenium, and by hyperoxic stress (Forman and Fisher, 1981). The comparatively low cytosolic GSH peroxidase activity of Lewis lung carcinoma was probably not the result of limited supply of selenium because the tumour mitochondria were observed to have pronounced GSH peroxidase activity. Many tumours have been shown to accumulate selenium (Milner, 1984). The diminished cytosolic GSH peroxidase activity of the carcinoma might therefore have reflected a low potential for free radical reactions in the soluble compartment of Lewis lung tumour cells.

Rat lung has been observed to have a relatively high proportion of
GSH-transferases with neutral-to-acidic isoelectric points (Nicholls and Ahokas, 1984). GSH-transferase AA and GSH transferase B (ligandin) are basic transferases postulated to correspond to selenium-independent GSH peroxidase (Tan et al, 1984). Non-selenium dependent GSH peroxidase has been reported to represent about 10% of the total GSH peroxidase activity of rat lung (Jenkinson et al, 1983). In the present studies, no significant selenium-independent GSH peroxidase activity was detected in murine lung or Lewis lung carcinoma. The relatively pronounced cytosolic GSH-transferase activities of carcinoma and pulmonary tissues were thus unlikely to have contained a significant component from either a ligandin-like or a GSH-transferase AA-like enzyme.

The comparatively raised GSH S-epoxide transferase activity determined for Lewis lung carcinomas was possibly a reflection of disturbed metabolism in neoplastic cells which might have resulted in the production of excessive quantities of hydrophobic electrophiles. Lipid epoxides are reported to be a major product of lipid peroxidation reactions in biological membranes (Sevanian et al, 1981). Examination of a range of rat tissues has revealed lung to have the greatest concentration of lipid epoxide (Sevanian et al, 1981). It has been postulated that membrane phospholipases can cleave epoxide moieties from the membrane phospholipid to be hydrated by epoxide hydrolases to fatty acid diols (Sevanian et al, 1981). These diols and epoxides are probably removed from the cell cytosol by conjugation to GSH. The substantial GSH S-epoxide transferase of Lewis lung carcinoma and pulmonary tissue might therefore have functioned, in part, to transport lipid epoxide and lipid diol residues. A substantial GSH-transferase activity might also assist in the removal of electrophiles, such as many anticancer drugs, rendering the Lewis lung carcinoma more resistant to their actions.

It has been suggested that cytosolic GSH-transferases are concerned
with the transport of non-polar molecules between the plasma membrane and the intracellular membranes of cells (Boyer et al., 1983). Lewis lung carcinomas could thus have had enhanced requirement to transport hydrophobic substances such as fat-soluble vitamins, steroids and prostanoids. Rat and mouse tumours have been reported to accumulate vitamin E (Burobina and Nefakh, 1970; Burton et al., 1983a). Soluble GSH-transferases however have been reported to be unable to bind with membrane-bound substrates (Boyer et al., 1983). γ-Glutamyl transpeptidase is a membrane-bound enzyme, able to react with S-substituted GSH as part of the pathway to form mercapturic acids from GSH-conjugated electrophiles (Meister and Anderson, 1983). GSH-transferases and γ-glutamyl transpeptidase possibly combine as an intracellular transport system of non-polar compounds. As Lewis lung carcinoma had comparatively low γ-glutamyl transpeptidase activity, this hypothetical transport system may be less significant in Lewis lung carcinoma than in the tissue of origin, lung. Lewis lung tumours possibly have alternative means, such as α-tocopherol transport protein and phosphatidylcholine exchange protein, to translocate non-polar intracellular constituents (Boyer et al., 1983).

Comparisons between tumours and their tissues of origin: The present studies have been largely concerned with ascertaining activities of enzymes involved with metabolism of either free radicals, xenobiotics or carbohydrate. The liver is the major organ of carbohydrate metabolism (Waterhouse and Kemperman, 1971) and generally has greater activities of reactive oxygen- and xenobiotic-detoxicating enzymes than non-hepatic tissues (Tappel, 1980; Orrenius and Sies, 1982). Hepatocellular carcinomas (hepatomas), neoplasms of hepatic origin, have consequently served as the major animal model for investigations of cancer biochemistry (Knox, 1978). The biomedical literature, concerning the activities of antioxidant defence enzymes and enzymes of GSH metabolism,
has been dominated by reports of these activities in hepatomas. Liver
tumours are generally reported to have biochemical features contrary to
those of Lewis lung carcinoma, that is greater γ-glutamyl transpeptidase
and glucose-6-phosphate dehydrogenase activities, and lower catalase,
SOD, GSH peroxidase, GSH-transferase, GSSG reductase and γ-glutamyl-
cysteine synthetase activities than the tissue of origin. Glucose-6-
phosphate and γ-glutamyl transpeptidase are almost undetectable in normal
adult rodent liver whereas the activities of the six other enzymes are
greater in liver than in any other tissue. The activities, determined for
hepatomas, were thus less extreme in comparison to most normal tissues
than the highly-specialized tissue of liver. The varied patterns of
enzyme activity of normal tissues have been proposed to converge towards
a common pattern after malignant transformation (Greenstein, 1956; Knox,
1967). It has been concluded that tumours of different origins resemble
one another more closely than do the tissues from which they arose
(Greenstein, 1956). The concept of enzymic convergence in neoplastic
cells is possibly the biochemical counterpart of anaplasia, which is the
tendency of malignant cells to adopt morphological uniformity (Prehn,
1977). There might therefore be more similarities in the antioxidant
defence enzyme activities between Lewis lung carcinomas and hepatomas,
than between normal liver and lung. It is notable that the enzyme
activities, determined in the present studies for the B16 melanoma, were
not appreciably different from those of the Lewis lung carcinoma. The one
major exception was the pronounced γ-glutamyl transpeptidase activity of
the B16 melanoma, but even this activity was considerably less than that
of kidney, a non-neoplastic tissue.

Liver cancers account for less than 1% of the total incidence of
human cancer in the Western World (Silverberg, 1985). The extensive use
of hepatomas as biochemical models of neoplastic disease might thus be
inappropriate. Many generalizations or theories of neoplasia have been
largely based upon observations of biochemical perturbations of hepatocellular carcinomas. Some of these interpretations may be spurious because liver, the tissue of reference, is so biochemically specialized. The Lewis lung carcinoma, in some respects, is a better cancer model because lung cancer is the principal cause of death by neoplastic disease in the Developed World (Prescott and Flexer, 1982). Epidermoid and large cell carcinomas, of which Lewis lung may be representative, are two of the four major categories of pulmonary cancer (Becker and Gazdar, 1985). As lung comprises of many cell types, the reflection of biochemical specialization of one type of cell is less probable than liver (which is relatively homogenous). Pulmonary tissue is possibly a more representative non-neoplastic reference tissue than liver.

The requisite control: Alteration of single enzyme pathways, even to levels comparable to that of other normal cells, could possibly evoke biochemical imbalances in specialized cells that may cause cell damage leading to death or transformation. In cells that have substantial oxygen metabolism, for example, a given decrease in SOD or GSH peroxidase activity would be considerably more deleterious than for cells whose metabolism is more anaerobic. The principle of the rational approach to the improvement of chemotherapy, is to exploit differences in biochemical pathways between normal and tumour cells (Greenstein, 1956). This approach is not necessarily concerned with absolute concentrations of subcellular constituents or enzyme activities in tumours (Oberley and Spitz, 1984). The only tenable normal tissue control, with which a tumour can be compared, has thus been concluded to be a single cell population of the differentiated cell type from which the tumour originated (Oberley and Spitz, 1984). Pulmonary tissue might therefore have been a poor control for Lewis lung carcinoma because it is so heterogenous.

The identity of the cell from which Lewis lung arose is uncertain.
The yield of "control" cells from lung tissue would probably be so small that excessive numbers of animals would be required to provide sufficient samples to make statistical comparison with Lewis lung tumour cells. Similar rigorous isolation of a single cell population of Lewis lung carcinoma cells would also be justified. Disaggregated neoplastic cells of tumours can be separated from infiltrating host cells, by velocity sedimentation techniques for example (Gillespie, 1982), or by establishing tumour cell spheroids in tissue culture (Twentyman, 1983); both procedures, if applied to the present studies would probably be limited by relatively poor cell yield. The processing of intact lung and tumour cells might also cause in vitro induction of antioxidant defence enzymes by molecular oxygen (Kimball et al., 1976; Autor et al., 1979; see later). The application of the premise, that tumour cells may only be compared with a single cell population of their differentiated normal cell counterparts, in studies with Lewis lung carcinoma, would clearly be subject to many practical difficulties.

Infiltration of tumour by host cells: The argument of using single cell populations might also be applied to tumours, such as Lewis lung carcinoma, which are significantly infiltrated by host cells. Erythrocytes have been observed to have pronounced activities of catalase, and cytosolic GSH peroxidase, SOD, γ-glutamylcysteine synthetase, glucose-6-phosphate dehydrogenase (Marklund et al., 1982); alveolar macrophages have been reported to have pronounced Mn-SOD activities (see later). The activities of these enzymes were comparatively low in Lewis lung carcinomas (Chapter 2) and therefore a significant proportion of all these activities might have been contributed by host-derived cells.

Influence of hypoxia: Histological examinations of Lewis lung carcinomas have supported the concept that regions of the tumour, particularly at
the centre, are comparatively hypoxic (Salsbury et al., 1974; Dobrossy et al., 1980). Oxygenation of tissues is dependent upon supply by blood vessels (Millar, 1982); hypoxic cells arise because the growth of tumour cells exceeds that of the supportive vascular tissue (Stone, 1979). Unbalanced tumour cell proliferation causes excessive pressures on blood capillaries, causing them to collapse (Teicher and Rose, 1984). The supposition, of the present studies, was that a bore of tissue removed from the centre of i/m embedded Lewis lung carcinoma, would approximate to the bulk of the hypoxic cells and necrotic components of the tumour. Tumours were separated into hypothetical oxygenated and hypoxic zones, using anatomical considerations because direct determination of regional oxygen tension in solid tumours was not technically possible (Kennedy et al., 1980). The distribution of antioxidant defence enzyme activity and TBA-reactivity with solid Lewis lung carcinomas (Chapter 2), however, indicated that the simplified model, used in the present studies, that of a solid tumour which is aerated at the periphery and hypoxic at the centre, may well have had some relevance to in vivo tumour oxygenation.

The haemoglobin distribution within Lewis lung carcinomas, could possibly be determined by injecting radiolabelled iron into a tumour-free syngeneic mouse. The red blood cells of this mouse could then be injected i/v into a mouse, bearing a Lewis lung carcinoma. The autoradiograph of the tumour, from this animal, might then indicate the locations of haemoglobin and, by inference, those of hypoxia within the carcinoma. A similar technique to estimate tissue oxygenation has been reported: tritiated radiosensitizer, misoxidazole is injected into the circulation of a cancer patient or tumour-bearing animal; histological sections of neoplasm show intense radioactive labelling in regions that were hypoxic in vivo (Urtasun et al., 1986).

Mice were observed to be grossly anaemic after 13 days of bearing
Lewis lung carcinomas (Chapter 3). The lack of erythrocytes, that is oxygen-carrying capacity, of the blood of tumour-bearing mice might have exacerbated the hypoxic state of Lewis lung carcinomas (Teicher and Rose, 1984).

The relatively greater γ-glutamyl cysteine synthetase activity, determined for the innermost zone of Lewis lung carcinoma may have been a cause of the greater GSH concentration of the cortical region. The environment of hypoxic tumour cells has been postulated to be more conducive to reductive reactions than that of better aerated cells. A reducing environment would probably help to maintain the GSH/GSSG balance in favour of GSH. It has been proposed (Chapter 2) that there was possibly less lipid peroxidation at the cortex of Lewis lung carcinoma than the periphery. At the lower oxygen tensions of the cortex, production of H₂O₂ and lipid peroxides would be comparatively low and there would be correspondingly less oxidation of GSH by GSH peroxidase.

Alveolar macrophages have been reported to have specific Mn-SOD activities approximately 12 fold greater than that of rat lung, from which they were isolated (Forman and Fisher, 1981). Lewis lung tumour tissue, from the periphery of the carcinoma, was observed to have 6-8 fold greater Mn-SOD activity than murine lung and about 2-4 fold greater activity than that of the cortical region (table 2.11). The concentration of invading macrophages was probably greater at the periphery of Lewis lung carcinomas than at the cortex because the vasculature at the periphery was theoretically more intact. The difference in the activity of Mn-SOD, and possibly other antioxidant defence enzymes, between the two regions of Lewis lung carcinoma might have been caused by the differential distribution of macrophages in the tumour. The greater concentration of TBA-reactive material at the outermost regions of Lewis lung carcinoma might have been the result of active oxygen species,
produced by invasive macrophages (Del Maestro et al., 1980; Weiss and LoBuglio, 1982).

The studies described in this thesis were intended to be a preliminary investigation of the possible effects of diminishing tissue oxygenation upon antioxidant defence enzymes. By considering subcellular activities of enzymes and the effects of tumour growth, the present investigations have arguably improved upon the only similar experiments to have been reported elsewhere (Petkau et al., 1977).

The ambiguities of the present studies might be partially resolved by determining the relative contributions of all subcellular organelles in cortical and peripheral regions of Lewis lung carcinomas. The greater yield of protein in the mitochondrial fractions of the cortical regions, for example, could have been the result of lysosomal proliferation in these ischaemic tissues. Observations elsewhere of the "florid" and necrotic areas of Lewis lung carcinomas, however, have shown that the florid, presumably aerated regions of tumour, have significantly greater lysosomal enzyme activities than tissue of the necrotic (hypoxic) region (Dobrossy et al., 1980). As the extent of hypoxia in Lewis lung carcinomas was unknown, it may be appropriate to remove bores of differing diameter, for a given volume of tumour. This procedure might optimize the differences in antioxidant defence enzyme activities that can be obtained between outer and inner zones of Lewis lung carcinoma. The inevitable overlap in hypoxic and aerobic "character" between tumour cortex and the residual tumour might have attenuated the apparent differences in antioxidant defence enzymes and TBA-reactive material. Future studies could possibly use smaller samples, removed by biopsy from the centre and margin of the tumour, rather than separating the tumour into two arbitrary divisions.

The effect of manipulating the oxygenation of Lewis lung carcinoma
might also be studied. Tumour antioxidant defence enzymes may be
determined after hyperoxic exposure of Lewis lung carcinoma-bearing mice
(Petkau et al, 1977). An alternative means to increase the oxygenation of
Lewis lung carcinoma is to perfuse tumour-bearing mice intravenously with
oxygen-carrying perfluorochemical emulsion, which has superior oxygen-
transporting capacity than blood (Teicher and Rose, 1984). Perfluro-
chemical emulsion particles are considerably smaller than haemoglobin
molecules and may thus allow delivery of oxygen to areas of the tumour,
inaccessible to haemoglobin. Intravenous administration of perfluro-
chemical emulsion, with concomitant hyperoxic exposure, has been
successfully applied to increase effectiveness of radiation therapy
against i/m-implanted Lewis lung carcinomas (Teicher and Rose, 1984).
Another experimental approach to the investigation of the effects of
oxygenation upon Lewis lung carcinoma might be to vary the oxygen
concentrations under which cultured Lewis lung carcinoma cells are
incubated. Multicellular spheroids are alternative in vitro models of
oxygen gradients in solid tumours (Jones et al, 1982) which might be
usefully employed for future studies of Lewis lung carcinoma.

Other antioxidants: Many other cellular constituents, such as
ascorbate, uric acid, α-tocopherol, riboflavin, retinoids, zinc and
selenium, are reported to function as antioxidants (Willson, 1977; Ames,
1983; Bollag, 1983; Burton and Ingold, 1983; Willson, 1983; Miyazawa et
al, 1984). Neoplastic tissues have been shown to concentrate selenium
more than normal tissues (Baumgartner et al, 1978; Milner, 1984). Vitamin
E concentrations of murine and rat tumours have been reported to be
comparatively greater than those of non-neoplastic tissues (Burobina and
have demonstrated that their neoplastic tissues selectively concentrate
ascorbate at the expense of normal tissues (Cameron et al, 1979). Amines,
such as spermine, have been postulated to be cell antioxidants (Vanella
et al., 1980). Lewis lung carcinoma has been reported to have comparatively raised concentrations of histamine and polyamines (Bartholeyns and Bouclier, 1984), which possibly contribute to the antioxidant capacity of the carcinoma. An increase in serum uric acid has been associated with malignant tumours, especially those with extensive necrosis (Hepler, 1957). The relatively lower antioxidant defence enzyme activities of many tumours might result from a shift in biochemical character in which enzymic systems to detoxify active oxygen species are displaced by non-enzymic mechanisms (Peskin et al., 1977). The sequestration of non-enzymic antioxidants by tumours might explain their general resistance to lipid peroxidation.

It would be appropriate to determine concentrations of antioxidant systems, other than SOD, catalase and GSH-centred systems, in future studies of Lewis lung carcinoma. Cytochrome P₄₅₀, or a related cytochrome, may have had an important role in the enzymic metabolism of oxygen radicals in Lewis lung carcinomas. As vitamin E is the most effective endogenous chain-breaking antioxidant known (Burton and Ingold, 1983), it would be particularly important to ascertain its concentrations in the carcinoma to complete the antioxidant profile of the Lewis lung.

Lipid Peroxidation and the Lewis Lung Carcinoma

The substantial TBA-reactive material concentrations of Lewis lung carcinomas did not necessarily conflict with observations of other animal tumours (Barber and Bernheim, 1967), as the carcinoma showed limited ability to undergo lipid peroxidation in vitro. Potential sources of TBA-reactivity in the Lewis lung carcinoma have been discussed previously (Chapter 4). Lewis lung carcinoma cells may feasibly have produced free radicals to aid the growth of the tumour (see later). The carcinoma cells would thus, by necessity, have been relatively resistant to lipid peroxidation, as indicated by the studies in which tumour microsomes were
insulted with FeCl$_3$ and ascorbate. The ability of Lewis lung carcinoma cells to proliferate in the 95% air atmosphere of cell culture, also infers a comparative resistance to oxygen-mediated toxicity. The low susceptibility of tumour cells to damage from reactive oxygen species has been suggested to be caused by the inability to convert 'O$_2^\cdot$ to more toxic species, such as 'OH, or because intrinsic cell perturbations may have made tumour cells less sensitive to oxygen toxicity (Oberley et al, 1980). The "immortality" of neoplastic cells has been postulated to be due to this resistance to autoxidation, although biochemical abnormalities, characteristic of cancer cells, may well be caused by reactive oxygen intermediates (Oberley et al, 1980).

The present studies might thus be furthered by determining the ability of Lewis lung carcinomas to generate 'O$_2^\cdot$ relative to normal tissues. Adrenochrome formation could, for example, be used to indicate the presence of 'O$_2^\cdot$ (Oberley et al, 1982). Nuclear membranes from rodent tumours have also been reported to be able to produce 'O$_2^\cdot$ in vitro (Bartoli et al, 1977; Peskin et al, 1980). Studies of 'O$_2^\cdot$ generation by the Lewis lung carcinoma might therefore use mitochondrial, microsomal and nuclear subcellular fractions. The determination of oxygen radical formation by Lewis lung carcinoma cells in culture might also be warranted, as the oxygen tension of this type of system can be varied.

Although some types of tumour cell might be able to produce reactive oxygen species in vitro, there is very little evidence that tumour cells produce these species in vivo. Melanin is a stable free radical, reported to produce 'O$_2^\cdot$ and H$_2$O$_2$ (Swartz, 1982a). The significant TBA-reactive material concentrations of B16 melanomas (Chapter 3) may well have reflected the occurrence of free radicals in this tumour.

Rat neonatal lung tissue has been reported to have a greater lipo- peroxide concentration than adult tissue (Kehrer and Autor, 1977). This
indicates that neoplastic lung tissue might, feasibly, be susceptible to lipid peroxidation. The Lewis lung carcinoma is possibly another apparently unusual tumour which can produce reactive oxygen species in vivo.

Relevance of GSH Concentration and Antioxidant Defence Enzyme Activities to Tumour Progression and Therapy

Tumour progression: The present studies and previous observations (Capel and Thornley, 1982) have shown significant concentrations of TBA-reactive material in Lewis lung carcinoma, indicating that there may have been in vivo lipid peroxidation. To have been biologically significant, the postulated generation of oxygen-centred radicals must have outweighed the antioxidant defence systems of Lewis lung carcinoma to a sublethal extent: otherwise the tumour could not have continued to increase in size. Activated oxygen species in Lewis lung carcinoma might have been generated by one or more of the oxidase systems of the cell, such as the mitochondrial electron transport chain, peroxisomal oxidases, or xanthine oxidase of the cytosol (Freeman and Crapo, 1982). Lung, the tissue in which Lewis lung carcinoma originated, has been demonstrated to have a functional cytochrome \( P_{450} \) mono-oxygenase system (Minchin and Boyd, 1983). NADPH-dependent cytochrome \( P_{450} \) reductase of the microsomal mixed function oxidase system can catalyze the reduction of \( {\cdot}O_2^- \) to \( O_2^- \) (Paine, 1978; Ernester et al, 1982). It would be useful in future experiments to determine the activity of this enzyme in the Lewis lung carcinoma.

Tumour cells have been reported to accumulate haematoporphyrin more than most normal tissues (Beuttner and Oberley, 1980). Haemoglobin and its derivatives have been observed to be able to catalyze the production of active oxygen intermediates and lipoperoxides (Rotilio et al, 1977; Szefinski et al, 1984; Thornally et al, 1984). As Lewis lung carcinoma, used in the present studies, was haemorrhagic, haemoglobin might be
implicated in the hypothetically-elevated production of reactive oxygen species. The targeting of oxygen metabolites by invasive inflammatory cells, invading the Lewis lung carcinoma, was probably a significant source of autoxidative injury in the tumour.

Excessive production of reactive species in biological tissues has many consequences (Chapter 1), such as the inactivation of sulphhydryl-dependent proteins, the loss of nicotinamide and flavin co-factors, disturbance of membrane permeability, and damage to cell proteins and DNA (Freeman and Crapo, 1982; Singh, 1982). Adenylate cyclase and phosphodiesterase are sulphhydryl-dependent enzymes concerned with the regulation of cyclic AMP. Both enzymes have been reported to have abnormally low activities in tumours (Hocman, 1981). The concentration of pyridine nucleotides has observed to be less in murine tumours than in comparative normal tissues (Greenstein, 1956). Sublethal autoxidative injury might therefore explain many of the morphological and biochemical abnormalities, documented for Lewis lung carcinoma (and possibly other neoplasms). In models for cancer, which might be applicable to Lewis lung carcinoma, it has been proposed that a comparative over-production of oxygen-derived radicals prevents differentiation and thereby causes neoplasia (Oberley et al, 1981). The growth of the B16 melanoma has been reported to be inhibited by antioxidants, suggesting that free radicals might be implicated in the neoplastic character of this tumour (Nordenberg et al, 1985).

H$_2$O$_2$ can freely permeate cell membranes. $^{\cdot}$O$_2^-$ can also cross cell membranes, where it may be protonated to the perhydroxy radical (HO$_2^-$) which is a stronger oxidant than $^{\cdot}$O$_2^-$ (Freeman and Crapo, 1982). Oxygen metabolites, that have escaped detoxification, possibly could have leaked through the plasma membranes of Lewis lung carcinomas into the extracellular fluid, which has comparatively low concentrations of SOD and
catalase (Del Maestro et al., 1982). These reactive oxygen species might have been able to initiate further free radical reactions to degrade endothelial cell and basement membranes. This hypothetical process may assist the invasion of Lewis lung carcinoma into the surrounding host tissues and promote the intravasation of tumour cells into the circulation of the tumour-bearing mouse. Macrophage-mediated tumour cell destruction has been reported to be inhibited by oxidized plasma lipoproteins (Justement et al., 1984). Leakage of active oxygen species by Lewis lung carcinomas might cause oxidation of plasma lipoproteins which would impair macrophage activity and thereby aid tumour invasion.

Oxygen intermediates can cause strand scission and base modification of DNA which may result in cell death or mutations (Freeman and Crapo, 1982; Moody and Hassan, 1982). An excessive production of reactive oxygen species would increase the frequency of mutations in rapidly-dividing tissue such as Lewis lung carcinomas, and might be a cause of the heterogenous cell composition of the Lewis lung (Starace et al., 1982; Takenaga, 1984; Zupi et al., 1984).

Active oxygen species might disturb the metabolism of Lewis lung carcinoma due to their influence upon the synthesis of eicosanoids (prostanoids). OH⁻ and O₂¹⁻ have been postulated to be able to cause the release of arachidonic acid from the cell membrane, either through stimulation of phospholipase A₂ or autoxidative disruption of the membrane (Del Maestro et al., 1982). Lipid hydroperoxides, particularly those of the lipoxygenase pathway, stimulate cyclo-oxygenase activity (Hemler and Lands, 1980). Prostaglandins G₂ and H₂, the initial products of the cyclo-oxygenase pathway, and 5-hydroperoxy-eicosatetraenoic acid, the first product of the lipoxygenase pathway, are all hydroperoxide forms of arachidonic acid (Parantainen, 1982). Lipid peroxidation reactions in Lewis lung carcinoma could thus have resulted in the
undescribed synthesis of prostanoids, which might influence tumour growth and progression (Droller, 1981). Mice, bearing Lewis lung carcinomas, have been observed to have elevated concentrations of prostaglandin E in their sera, and Lewis lung cells in culture were found to produce considerable amounts of prostaglandin E (Young and Knies, 1984). The seral prostaglandin E of mice bearing Lewis lung carcinomas was probably of tumour origin (Young and Knies, 1984).

The pattern of antioxidant defence and free radical generation could also influence the distribution of arachidonic acid metabolites in Lewis lung carcinomas. The isomerases that catalyze the synthesis of prostaglandins $E_2$ and $D_2$ are possibly GSH S-transferases (Parantainen, 1982). As Lewis lung carcinoma was derived from pulmonary tissue, a significant lipoxygenase pathway might have been present (Parantainen, 1982). Leukotriene C, which is reported to be produced by GSH conjugation, can be further metabolized to leukotriene D, one of the slow-reacting substances associated with bronchial anaphylaxis, in a reaction catalyzed by $\gamma$-glutamyl transpeptidase. As the observed activity of GSH-transferase of Lewis lung carcinoma was comparatively pronounced whereas that of $\gamma$-glutamyl transpeptidase was comparatively low, the reactions of the eicosanoid cascade could possibly have favoured the syntheses of prostaglandins $E_2$ and $D_2$ and leukotriene C, but not leukotriene D. GSH peroxidase may be involved in the production of prostacyclin (Parantainen, 1982), and with the conversion of hydroperoxy eicosatetraenoic acids (HPETE) to hydroxy eicosatetraenoic acids (HETE; Bryant and Bailey, 1980). The relatively low GSH peroxidase activity of cytosol from Lewis lung carcinoma may well have influenced prostacyclin and HETE production by the tumour. The reductions of 15-HPETE and 12-HPETE, and of prostaglandin $G_2$ to prostaglandin $H_2$ generate active oxygen species that selectively inhibit the synthesis of prostacyclin (Ham et al., 1979). Thromboxane formation has been reported to be unaffected by free radical
reactions of the eicosanoid cascade (Parantainen, 1982). Autoxidation of the cellular membranes of Lewis lung carcinoma would thus promote the synthesis of thromboxane, and possibly other radical insensitive pathways of the eicosanoid cascade, but suppress the production of prostacyclin.

The reactions of blood coagulation may share some common mechanisms with factors promoting tumour growth and dissemination (Zacharski, 1983). The Lewis lung carcinoma is probably the most studied model of the relationship between tumour cell metastasis and the clotting mechanism (Zacharski, 1983). Lewis lung carcinoma cells from subcutaneously-implanted tumours have been observed to induce platelet aggregation in vitro (Menter et al, 1984), and in vivo (Donati et al, 1981). It has been proposed that the vascular endothelium produces prostacyclin to impede metastasis by disseminated tumour cells (Honn et al, 1983a). The formation of artificial pulmonary metastatic foci in mice injected intravenously with Lewis lung carcinoma cells has been reported to be impaired by prostacyclin, prostacyclin enhancers or by thromboxane inhibitors (Honn et al, 1981; Donati et al, 1982). Other workers have been unable to show any effect of prostacyclin upon the development of metastatic deposits, after i/v injection of Lewis lung tumour cells (Karpatick et al, 1984). The prostacyclin potentiating agents, nafazatrom and ticlopidine have been reported to decrease pulmonary metastasis in mice bearing Lewis lung carcinomas (Kohga et al, 1981; Honn et al, 1983b). Other studies of mice bearing Lewis lung carcinomas, however have demonstrated that prostaglandin synthetase inhibitors have no significant effect upon pulmonary metastasis (Hilgard et al, 1976; Donati et al, 1982). These discrepancies might have resulted from the use of carcinoma cells, taken from an implanted tumour, by the investigators who have demonstrated an inhibitory action of prostacyclin and prostacyclin potentiators (Karpatick et al, 1984); those experiments giving apparently contradictory results, employed Lewis lung carcinoma cells that had been
maintained by tissue culture. The present studies have demonstrated differences in the antioxidant defence activities between Lewis lung carcinoma cells from in vivo and in vitro systems, which might have caused changes in the pattern of prostanoid generation. If the production of eicosanoids by Lewis lung carcinomas were substantially more due to autoxidation than enzyme-catalyzed oxidation of arachidonic acid, prostaglandin synthetase (cyclo-oxygenase) inhibitors might not have any significant effect upon prostanoid-linked processes, such as metastasis.

As the eicosanoid cascade involves lipid hydroperoxide intermediates, the malondialdehyde determined in Lewis lung carcinoma tissue, using the TBA test, might have been the breakdown product of enzymatically-mediated prostanoid synthesis, and not the product of generalized lipid peroxidation. Malondialdehyde has been regarded to be a by-product primarily of thromboxane generation (Parantainen, 1982). Prostaglandin concentrations of rat mammary adenocarcinomas have been observed to be greater than those of normal tissues (Karmali et al, 1983). Concentrations of thromboxanes and other prostanoids were, however, greater than could be accounted by prostaglandin synthetase activity (Karmali et al, 1983). The rat mammary carcinoma might thus have been a tumour model in which a significant proportion of eicosanoid production was caused by autoxidation.

Prostaglandins of the E series, and prostacyclin have cytoprotective activities and have been observed to reverse cellular degeneration (Parantainen, 1982). Prostaglandin \( E_2 \) has been reported to be a significant immune suppressant, in Lewis lung carcinoma-bearing mice (Young and Hoover, 1986; Chapter 5). Prostaglandins of the F series have been reported to have considerable growth factor activity (Armato and Andreis, 1983). Prostacyclin and prostaglandins of D and E series have been reported to stimulate cyclic AMP production (Hammarstrom, 1982). The
It has been reported that prostacyclin concentration is less in Lewis lung carcinoma than in normal pulmonary tissue (Donati et al., 1981). Other workers have not been able to detect prostacyclin in Lewis lung carcinoma (Honn et al., 1983b). Thromboxane B₂ concentrations in Lewis lung carcinoma have been reported to be greater than that of murine lung (Chiabrando et al., 1985). The balance of eicosanoid synthesis possibly favouring prostaglandin E and thromboxane, might have had substantial influence upon the metabolism, and thereby growth and dissemination of Lewis lung carcinomas.

Much of the prostaglandin extracted from tumours may reflect the activity of host stromal and inflammatory cells, particularly macrophages (Bennett et al., 1980). Future studies of prostanoid metabolism in Lewis lung carcinomas should therefore use only single cell populations of neoplastic cells, separated from the carcinoma. Research is warranted to elucidate the relationships between antioxidant defence, free radical generation and eicosanoid production upon the growth and dissemination of Lewis lung carcinomas, and of other tumour models.

Antitumour therapy: The present studies have been greatly concerned with the metabolism of GSH in Lewis lung carcinomas, because of the importance of GSH in the detoxification of reactive oxygen species and peroxides. GSH, as the most important non-protein sulphhydryl constituent, has a major role in the metabolic processes of most mammalian cells (Meister and Anderson, 1983). The manipulation of the GSH concentration of Lewis lung carcinomas might therefore have profound consequences upon the progress of the tumour and its resistance to anticancer therapy.

Decreasing the GSH concentration of Lewis lung carcinoma cells would probably increase their sensitivity to lysis by reactive oxygen species, produced by invasive macrophages (Arrick et al., 1982). Depleting cellular
GSH has been reported to cause a compensatory increase in prostacyclin production (Rouzer et al., 1982). A change in GSH concentration might therefore alter the pattern of eicosanoid synthesis in Lewis lung carcinomas and thereby affect tumour metabolism. The increased autoxidation caused by a lower GSH concentration, may however promote the synthesis of thromboxane which could increase metastasis of Lewis lung carcinoma cells (Honn et al., 1983a).

Increasing the GSH concentration of Lewis lung carcinoma is thought to favour the production of the stable, "classical" prostaglandins, PGD$_2$, PGE$_2$ and PGF$_2\alpha$ (Karmali et al., 1983), which tend to have cytoprotective effects upon cells (Parantainen, 1982). The neoplastic character of Lewis lung carcinoma possibly resulted directly from poorly regulated free-radical production (see earlier). The supplementation of GSH, and other antioxidants, might therefore reverse the biochemical abnormalities of Lewis lung carcinoma and thereby promote differentiation. Administration of GSH has been reported to cause regression of aflatoxin B$_1$-induced rat hepatomas (Novi, 1983). Other workers, however, have not succeeded in demonstrating any significant effect of GSH upon aflatoxin B$_1$-induced or 3'-methyl-4-dimethyl—aminoazobenzene-induced neoplastic lesions of rat liver (Neal and Legg, 1983; Cook et al., 1984). Other antioxidants, such as vitamin C (Cameron et al., 1979), vitamin A (Oberley and Beuttner, 1979) and selenium (Milner, 1984) have been reported to slow the growth of animal tumours and human cancers. The OH$^+$ scavenger, dimethyl thiourea, has been observed to induce cell differentiation and inhibit tumour growth of B16 melanomas (Nordenberg et al., 1985). Administration of vitamin E has been observed to cause neuroblastoma cells, in culture, to differentiate (Prasad et al., 1979).

GSH is involved in the detoxification and repair of cellular injury caused by radiation, hyperthermia and many anticancer drugs (Arrick and
Nathan, 1984; Russo et al., 1984). A possibly more subtle stratagem for the antineoplastic therapy of Lewis lung carcinoma might be to deplete GSH as an adjuvant to other suitable cytotoxic regimens. Inhibition of GSH synthesis of murine tumour cells, has been reported to considerably enhance their sensitivity to enzymically-generated \( \text{H}_2\text{O}_2 \) (Nathan et al., 1980) and to sulphhydryl-reactive anticancer drugs (Arrick et al., 1983).

In the present studies, it was observed that tissue from the central, apparently hypoxic region, of Lewis lung carcinoma, had greater GSH concentration than the peripheral region of the tumour. The survival of cell populations in Lewis lung carcinomas after irradiation (Stephens et al., 1984) and after drug therapy (Stephens et al., 1978) might have been partly due to the relatively greater GSH concentration of some Lewis lung tumour cells. It has been reported that nitroimidazole radiosensitizers, such as misonidazole, can cause significant loss of GSH from hypoxic cells (Hall et al., 1982). Depletion of GSH has been shown to sensitize hypoxic tumour cells to the cytotoxic effects of misonidazole (Bump et al., 1982; Clark et al., 1984). Decreasing the GSH concentration of Lewis lung carcinomas might therefore increase the efficacy of many cancer regimens against both aerated and hypoxic cells.

The GSH concentration of Lewis lung carcinomas may be decreased using the \( \gamma \)-glutamylcysteine synthetase inhibitor, buthionine sulfoximine (BSO; Meister, 1983). The use of BSO would probably be most effective, if administered before 17 days after tumour implantation, when \( \gamma \)-glutamyl synthetase activity of Lewis lung carcinoma was determined to be comparatively low. As Lewis lung carcinomas were observed to have appreciable GSSG reductase activity, GSH concentration might be depleted by administration of nitrosoureas, a group of compounds which inhibit this enzyme (Babson and Reed, 1978). BCNU (1,3-bis [2-chloroethyl]-1-nitrosourea; carmustine), a potent inhibitor of GSSG reductase, has been reported to limit the growth of Lewis lung
carcinomas, though not as effectively as a related compound, methyl-CCNU (1-[2-chloroethyl]-3[4-methylcyclohexyl]-1-nitrosourea; semustine; Mayo, 1972). BCNU has been demonstrated to cause severe generalized inhibition of GSSG reductase activity in cancer patients (Frischer and Ahmad, 1977). The effectiveness of BCNU might therefore have been limited by its toxicity to normal tissues. Methyl-CCNU could possibly have been selectively metabolized by Lewis lung carcinoma, but not significantly by normal tissues, to its active cyclohexyl isocyanide form (Babson and Reed, 1978). Cyclophosphamide, a nitrogen mustard anticancer drug, has been demonstrated to cause a significant depletion of hepatic non-protein sulphhydryls, primarily GSH, in mice (Gurtoo et al, 1981). Methyl-CCNU and cyclophosphamide have been reported to act synergistically in causing regression of Lewis lung carcinomas (Mayo et al, 1972). This effect was possibly mediated by a decrease in GSH, and may well be preliminary evidence that the stratagem of decreasing the GSH concentration of the Lewis lung carcinoma could increase the efficacy of some anticancer regimens.

The administration of suitable substrates, such as phorone, for conjugation to GSH, might be a means to decrease the GSH concentration of Lewis lung carcinoma, as it was observed to have a pronounced GSH S-epoxide transferase activity. GSH, conjugated to an epoxide would probably be irreversibly lost because the tumour was observed to have comparatively low γ-glutamyl transpeptidase activity.

It might be possible to increase the GSH concentration of Lewis lung carcinomas by oral administration of cysteine or methionine (Meister, 1983). Cysteine is however rapidly metabolized and may be toxic (Meister, 1983). It has been reported that the injection of L-2-oxothiazolidine-4-carboxylate (OTZ) to raise murine tissue concentrations of GSH (Williamson et al, 1982). OTZ is converted to L-cysteine in a reaction
catalysed by 5-oxo-L-prolinase, an enzyme found in almost all tissues (Williamson et al., 1982). It might thus be useful to determine 5-oxo-L-prolinase activities of Lewis lung carcinoma, and other tumour models. If the activity of this enzyme were comparatively low in neoplastic tissues, the administration of OTZ may afford protection of non-neoplastic tissues, but not neoplastic tissues, during sulphhydryl-depleting anticancer therapy. OTZ has been reported to protect normal human lung fibroblasts, but not lung adenoma cells from the cytotoxic effects of the anticancer drug, neocarzinostatin (Russo et al., 1986).

It has been shown that treatment of mice, bearing Lewis lung carcinomas with phenylthioethylamine, an inhibitor of SOD activity increases the survival time of these animals (Distephano et al., 1983). The sensitivity of Lewis lung carcinoma to reactive oxygen species might thus be increased after administration of SOD inhibitors, such as phenylthioethylamine.

The depletion of GSH concentration of Lewis lung carcinomas may warrant anticancer therapy that involves production of reactive oxygen species. The effectiveness of $H_2O_2$ as an anticancer agent has been reported to be comparatively limited (Holman, 1957; Green and Westrop, 1958; Sugiura, 1958; Mealey, 1965), probably due to its rapid metabolism and its toxicity to normal tissues. Reactive oxygen species are an important agency by which activated inflammatory cells of the immune system destroy tumour cells (Del Maestro et al., 1980; Nathan and Cohn, 1981; Weiss and LoBuglio, 1982). It might therefore be appropriate to use various means, such as irradiation, to target active oxygen to GSH-depleted Lewis lung carcinoma. Peroxidase enzymes or free-radical producing drugs might be linked to a monoclonal antibody specific for Lewis lung carcinoma (Collier and Kaplan, 1984; Hadas et al., 1984). Albumin microspheres, containing the anticancer drug doxorubicin
(adriamycin), have been magnetically directed onto Yoshida sarcomas, borne by rats: this procedure has been observed to cause total remissions of the tumours (Widder et al, 1983). Magnetically-targeted microspheres might be used to deliver an oxygen-radical producing toxicant to Lewis lung carcinomas. The toxicity of active oxygen metabolites of the drug could possibly be amplified by catalysis, caused by the iron oxide incorporated into the albumin microspheres.

H$_2$O$_2$, generated enzymically in situ, is probably less toxic to the tumour-bearing host than the systemic injection of preformed peroxide (Nathan and Cohn, 1981). Mice, bearing Lewis lung carcinomas, could possibly be given an infusion of glucose, and then injected with glucose oxidase, attached to polystyrene microspheres (or the albumin microspheres described above), which would then generate H$_2$O$_2$. This peroxide-delivery system has been reported to have selective toxicity towards neoplastic cells (Nathan and Cohn, 1981). Anticancer regimens that generate active oxygen species, might also be significantly improved by increasing the oxygenation of the tumour, using perflurochemical emulsion and hyperbaric oxygen (Teicher and Rose, 1984; see earlier).

The determination of the activities of antioxidant defence enzymes and principal antioxidant constituents of tumours may thus be useful in choosing the most suitable stratagems to treat cancer. The present studies have indicated that the presence of a tumour can perturb the antioxidant defence systems of normal tissues in the host, and these impairments must also be considered.

The Systemic Actions of the Lewis Lung Carcinoma

Cancer patients and tumour-bearing animals suffer from pronounced degenerative changes in their non-neoplastic tissues which may result in cachexia (Chapter 3). These disturbances cannot not be adequately
interpreted as being caused by stress, by an alteration in nutritional state, or by activation of the host immune system (Costa, 1977; Greengard, 1979). The physical deterioration of cancer patients and tumour-bearing animals has been attributed to biochemical perturbations mediated by as yet unknown substances, released to the circulation by tumours (Costa, 1977).

**Experiments using parabiotic animals:** Parabiotic "twins" are artificial "siamese twins", in which the blood circulation of two animals have been joined so that they are continuous. The small amount of blood crossing the parabiotic union (capillary anastomoses) is diluted with the much larger volume of blood in the main circulation of each animal. In experiments where Walker hepatoma-bearing rats were parabiotically linked to non tumour-bearing controls, the hepatic activities of three glycolytic enzymes (hexokinase, pyruvate kinase and phosphofructo kinase) were found to increase in intact animals in parallel with those of their tumour-bearing partners (Suda et al., 1966). The decreased hepatic catalase activities of tumour-bearing rats (Lucké et al., 1953), and changes in splenic and hepatic enzyme activities of rats, bearing lymphomas or fibrosarcomas (Herzfeld et al., 1978) were also observed to occur in tumour-free animals, parabiotically linked to tumour-bearing hosts. The changes in tumour-free parabiotic animals tend to be less pronounced than those of the tumour-bearing partner. The changes in tumour-bearing animals, linked to a healthy partner, were usually less appreciable than those of unlinked animals, bearing neoplasms. These observations have been interpreted to indicate that the blood of the tumour-bearing rats contained a chemical modulator, originating from the tumour. The modulator possibly acted like a hormone, targeting to and modifying the metabolism of sensitive tissues, distant to the tumour (Lucké et al., 1953). It is also remotely possible that the blood of the healthy animal was supplementing a critical factor, absent, or present at
much decreased concentration, in the blood of the parabiotically-linked, tumour-bearing partner (Herzfeld et al., 1978).

The "chemical modulator" could have been, for example, a hormone, prostanoid, kinin or growth factor. The Lewis lung carcinoma has been reported to secrete a number of different substances such as plasminogen activator (Whur et al., 1980; Eisenbach et al., 1985), prostaglandin E₂ (Young and Knies, 1984), and a macrophage-modulating factor of low molecular weight (Cheung et al., 1979). These substances, or some other tumour-elaborated factor, such as "toxohormone" (see later), possibly caused many of the systemic changes, observed for mice, bearing Lewis lung carcinomas.

"Toxohormone": An acid-resistant, water-soluble peptide, isolated from extracts of human gastric or rectal carcinomas, has been observed to cause a pronounced decrease in hepatic catalase activity, when injected into mice (Nakahara and Fukuoka, 1949). This peptide, subsequently named "toxohormone", was found to be present in normal tissues, but at considerably lower concentrations than in neoplastic tissues (Busch, 1963; Kampschmidt, 1965). "Toxohormone" has also been reported to have other physiological effects, such as thymus involution, increase in liver weight, decrease in hepatic NAD⁺, anaemia, and increased plasma Cu: effects similar to changes observed for tumour-bearing animals and cancer patients (Busch, 1963; Kampschmidt, 1965).

The identity of "toxohormone" is unknown. As hepatic catalase activity has been reported to be inhibited by several different substances (Hargreaves et al., 1959), many substances, elaborated by tumours such as the Lewis lung carcinoma, probably have "toxohormone"-like activity. Depressed catalase activity has been associated with alterations in the morphology of liver cells (Baum and Nishimura, 1964), and with alterations in hepatic lipid metabolism (Crane and Masters,
Hormones: Rats subjected to cold-restraint stress, have been observed to have decreased hepatic GSH peroxidase activities and increased hepatic TBA-reactive material concentrations: an effect that was probably mediated by glucocorticoids (Capel et al., 1983). Hepatic GSSG reductase, GSH peroxidase (Pinto and Bartley, 1969b; Capel and Smallwood, 1983), and glucose-6-phosphate dehydrogenase activities (Demus-Oole and Swierczewski, 1969) have been reported to be induced by oestrogen and progesterone, and repressed by testosterone. The livers of female rats also have been observed to have greater susceptibility to in vitro lipid peroxidation (Pinto and Bartley, 1979a) and greater TBA-reactive material concentrations (Capel and Smallwood, 1983) than their male counterparts. Adrenalectomy, castration of male mice, injections of cortisone, thyroxin or stilboesterol have all been observed to produce decreased hepatic catalase activities (Busch, 1962; Kampschmidt, 1965); tissue GSH concentrations have been observed to have circadian variation (Farooqui and Ahmed, 1984). Antioxidant defence and lipid peroxidation are clearly influenced by the endocrine system.

Many types of tumour have been observed to produce hormones, which are secreted into the blood stream of the host (Rees, 1975). Inappropriate adrenocorticotrophic hormone (ACTH) production has been associated with many bronchial tumours, for example (Neville and Symington, 1975). As the Lewis lung carcinoma, originated from the lung, it could possibly have secreted ACTH. Probably no single hormone can mirror the effects, exerted by Lewis lung carcinomas upon hepatic antioxidant defence and lipid peroxidation of the host mice. The carcinoma
might, however, have synthesised substances, which by means of hormone-like activity or ability to influence the endocrine system of the host mouse, contributed significantly to the perturbations of non-neoplastic tissues.

Growth factors: It has been observed that hypophysectomy prevents the decrease in catalase activity of tumour-bearing rodents, an effect counteracted by the administration of growth hormone (Utsugi, 1960). The growth-promoting activity of growth hormone is mediated by somatomedins, substances thought to be growth factors, that is they stimulate cell mitosis (Gospodarowicz, 1983). Neoplastic cells are known to produce conspicuous amounts of mitogens, referred to as transforming growth factors. Tumour-derived growth factors have been reported to stimulate normal cells to divide in vitro and to cause normal cells to adopt some of the morphology and phenotype of transformed cells (DeLarco and Todaro, 1978). It has been postulated that neoplastic cells maintain their malignant character by synthesising, in concert, growth factors and cell membrane receptors for growth factors (Alexander and Currie, 1984). The B16 melanoma has been proposed to incite its own growth by secreting a substance into the blood, which stimulates the release of growth hormone by the pituitary gland (Bajzer et al., 1984). The Lewis lung carcinoma might also produce pronounced quantities of mitogens.

The physiological effects of growth factors include hepatic hyperplasia and hypertrophy, increased fatty acid concentration, increased skin disulphide concentration, and generalized increases in glycolysis, and in nucleic acid and protein biosynthesis (Carpenter and Cohen, 1979): effects similar to some of the systemic actions of tumours. Transforming growth factors, in common with "toxohormone" have been reported to be peptides, which are comparatively resistant to heat and acid (Nickell et al., 1983). The systemic effects of tumours have been suggested to be
caused by growth factors, elaborated by neoplastic cells (Morgan and Cameron, 1973; Greengard et al, 1984). "Toxohormone" and growth factors may well be identical groups of substances.

Hepatic proliferation during liver regeneration is thought to be mediated by hepatocyte growth factors, hepatopoietins, present in the blood (Thaler and Michalopoulos, 1985). As liver has the capacity to regenerate after partial hepatectomy, it may well be comparatively sensitive to any tumour-elaborated mitogen. Prostaglandins of the F-series, for example, are reported to be very potent growth factors for undifferentiated rat hepatocytes (Armato and Andreis, 1983). Prostaglandins $\text{PGF}_{2\alpha}$ and $\text{6-keto-F}_{1\alpha}$ have been reported to be major eicosanoid metabolites of Lewis lung carcinomas (Chiabrando et al, 1985). A growth factor, secreted into the circulation of the tumour-bearing mice, might have stimulated mitosis in liver, and possibly other tissues, such as kidney.

The injection of mice with Corynebacterium parvum vaccine stimulates cells in tissues, such as liver and spleen, to grow exponentially (Karlberg et al, 1981). Twenty-one of 29 different systemic abnormalities, observed for sarcoma-bearing mice, were also present in Corynebacterium-vaccinated animals (Karlberg et al, 1981). This indicates that many, if not most, of the systemic effects of tumours are related to cell proliferation per se, and not necessarily to malignancy. As growth factors are associated with cell division, the studies, using Corynebacterium-vaccinated mice, infer that growth factors are major mediators of the perturbations of normal tissues in tumour-bearing animals and cancer patients.

Comparison with immature liver tissues: It has been proposed that the hepatic enzymes of tumour-bearing rodents tend to change such that their activities become similar to those of the tumour (Greenstein, 1954). An
alternative generalization that the enzyme pattern of the livers of tumour-bearing animals and cancer patients shifts towards that of immature or re-generating liver, has also been postulated (Suda et al., 1972; Herzfeld and Greengard, 1972).

Histological studies and observations of liver DNA concentration (Chapter 3), have suggested that there was a substantial increase in the proportion of mitotic cells in the livers of mice, bearing Lewis lung carcinomas. Foetal rat livers have been reported to have comparatively low $\gamma$-glutamylcysteine synthetase, GSH peroxidase and GSSG reductase activities, and increased $\gamma$-glutamyl transpeptidase activities (Pinto and Bartley, 1969; Wirth and Thorgeirsson, 1978). Rat liver tissue, regenerating after partial hepatectomy, has been observed to have decreased catalase and GSH peroxidase activities, unchanged SOD activity, and an increased TBA-reactive material concentration, compared to intact liver (Ueda et al., 1983). Foetal liver has been reported to have relatively low GSH peroxidase, GSSG reductase and $\gamma$-glutamylcysteine synthetase activities (Demus-Oole and Swierczewski, 1969; Pinto and Bartley, 1969; Wirth and Thorgeirsson, 1978). Comparison of the hepatic alterations of Lewis lung carcinoma-bearing mice with appropriate observations of foetal, regenerating and neoplastic liver (table 6.1) indicates that the changes in hepatic antioxidant defence and lipid peroxidation were largely consistent with the two generalizations, given in the previous paragraph. The substantial increase in the population of immature cells in the livers of mice, bearing Lewis lung carcinomas, might thus have contributed significantly to the perturbations in hepatic antioxidant defence and lipid peroxidation.

The pattern of antioxidant defence and lipid peroxidation, reported for undifferentiated hepatocytes, does not, however correspond exactly to the changes, observed for the livers of Lewis lung carcinoma-bearing mice.
### TABLE 6.1 - Pattern of Antioxidant Defence and Lipid Peroxidation Reported for Undifferentiated Rodent Liver Tissue and for Livers from Mice, Bearing Lewis Lung Carcinomas.

<table>
<thead>
<tr>
<th></th>
<th>FOETAL</th>
<th>REGENERATING</th>
<th>NEOPLASTIC</th>
<th>EFFECT OF BEARING LEWIS LUNG CARCINOMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH peroxidase</td>
<td>▼2,3</td>
<td>▼4</td>
<td>▼8</td>
<td>▼</td>
</tr>
<tr>
<td>γ-Glutamyl transpeptidase</td>
<td>▼1</td>
<td>▼1,7</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td></td>
<td>No significant differences</td>
<td>▼10</td>
<td>No significant differences</td>
</tr>
<tr>
<td>GSSG reductase</td>
<td>▼2</td>
<td>▼4</td>
<td>▼8</td>
<td>▼</td>
</tr>
<tr>
<td>Catalase</td>
<td>▼1</td>
<td>▼11</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>γ-Glutamyl-cysteine synth.</td>
<td>▼6</td>
<td>▼7</td>
<td>▼</td>
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<tr>
<td>Glucose-6-phosph dehydrogenase</td>
<td>▼6</td>
<td>▼7</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>TBA-reactive material conc&lt;sup&gt;n&lt;/sup&gt;</td>
<td>▼6</td>
<td>▼7</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>Fe/ascorbate microsomal perox&lt;sup&gt;n&lt;/sup&gt;</td>
<td>▼5</td>
<td>▼9</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>Fe/NADPH microsomal perox&lt;sup&gt;n&lt;/sup&gt;</td>
<td>▼5</td>
<td>▼5</td>
<td>▼</td>
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<tr>
<td>GSH conc&lt;sup&gt;n&lt;/sup&gt;</td>
<td>▼1</td>
<td>▼1</td>
<td>▼</td>
<td>variable</td>
</tr>
</tbody>
</table>

1. Wirth & Thorgeirsson (1978)
2. Pinto & Bartley (1969a)
5. Player (1982)
10. Oberley & Buettner (1979)
11. Greenstein (1956)
(table 6.1); GSSG reductase activities of the tumour-bearing mice were increased for example, whereas the activity reported for foetal and neoplastic liver is less than that of adult animals. The observations in the Biomedical literature, have mainly been undertaken with undifferentiated rat liver tissue: the discrepancies might thus represent species differences between mice and rats. Increased cell division in non-neoplastic tissues of mice, bearing Lewis lung carcinomas, may thus represent only a partial explanation of the biochemical changes, observed for these animals.

**Tumour-host Interaction**

The abnormalities in host metabolism, thought to be initiated by unidentified tumour-elaborated substances (Costa, 1977) might not include significant changes in antioxidant defence enzyme activity or susceptibility to lipid peroxidation. The perturbations, observed for mice, bearing Lewis lung carcinomas, were possibly caused by other mechanisms, such as the "parasytic" activities of the tumour, or were not caused by the tumour per se, but by the response of the host animal to the presence of neoplastic cells.

**Unappreciated metastasis:** Mice, injected intrasplenically with Lewis lung carcinoma cells, have been observed to have metastatic foci in their livers, eight days later (Kopper et al., 1982). It has been reported that mice, bearing subcutaneously-implanted B16 melanomas, have extensive metastatic deposits in their lungs, and occasional foci in organs, such as kidney, adrenal and brain; liver secondary involvement in these animals was evident exclusively as visibly-undetectable micrometastatic growths, which could only be determined by bioassay (Alterman et al., 1985). Such micrometastatic cells were possibly also be present in the livers of mice, bearing i/m implanted Lewis lung carcinomas. The biochemical abnormalities, observed for tumour-bearing mice in the
bearing mice, was therefore probably not caused by stress.

Host immune response: Studies of Corynebacterium parvum-inoculated mice have indicated that even non-malignant cell proliferation can stimulate systemic effects in rodents, similar to those of tumour-bearing animals (see earlier). It has been reported, however, that pregnant mice, or mice, bearing embryonic implants do not have depressed hepatic catalase activities (Greenstein and Andervont, 1943). These animals were probably immunosuppressed, whereas vaccination of mice with Corynebacterium would have elicited an immune reaction. This suggests that mitogenic agents, associated with immune defence, that is the lymphokines, might be implicated in the systemic effects of cancer.

It has been reported that hepatic cytochrome \( P_{450} \) and other drug metabolizing enzymes of tumour-bearing animals are less than those of tumour-free controls (Strittmatter, 1979; Dogra et al., 1985). Interferon and interferon inducers have been observed to depress hepatic cytochrome \( P_{450} \) and to induce xanthine oxidase activity, indicating that the impairment of hepatic drug metabolism, known to be caused by interferon, is mediated by reactive oxygen species (Ghezzi et al., 1985; Crowe et al., 1986). Other lymphokines, tumour-necrosis factor and interleukin 1, have also been shown to cause a decrease in murine cytochrome \( P_{450} \)-dependent microsomal drug metabolism (Ghezzi et al., 1986).

Tumour-necrosis factor (TNF) causes haemorrhagic necrosis of neoplasia, and is homologous with other macrophage/monocyte products, such as tumour differentiation-inducing factor (Takeda et al., 1986) and cachectin (Old, 1985; Dinarello et al., 1986; Nawroth et al., 1986). Cachectin is a pyrogen which has many diverse biological properties, including the promotion of muscle catabolism, activation of immune effector cells, activation of hepatic acute phase protein synthesis, and inhibition of adipocyte lipoprotein lipase. Insulin treatment has been
observed to largely reverse cachexia in tumour-bearing rats (Moley et al, 1985). The anabolic effects of insulin may well have counteracted the catabolic disposition of the host metabolism. It has been reported that insulin may inhibit TNF production from macrophages, and that TNF can cause hypoglycaemia (Satomi et al, 1985). Cachectin has thus been implicated as the cause of cachexia, associated with cancer and the acute-phase response of severe infections (Old, 1985; Bachwich et al, 1986). TNF/cachectin has been reported to stimulate the release of interleukin 1 by endothelial cells, and the production of interleukin 1 and prostaglandin E\textsubscript{2} by macrophages (Nawroth et al, 1986; Bachwich et al, 1986).

Interleukin 1 refers to a family of peptides, which like TNF, affect several different tissue targets, such as the leukocytes, liver, bone, muscle, synovial fibroblasts, brain and pancreas (Dinarello and Mier, 1986). Interleukin 1 is also a mediator of the acute phase response, and has multiple effects including the suppression of appetite, resorption of cartilage and bone, and muscle catabolism; many of these effects may be due to the stimulation of prostaglandin E\textsubscript{2} production (Dinarello and Mier, 1986). The haemorrhagic nature of the Lewis lung carcinoma indicated that substantial TNF, at least, was being synthesised in the host mice. Lymphokines, produced by the immune-defence cells of the host mice, may well have been the major mediators of the systemic abnormalities, observed for mice, bearing Lewis lung carcinomas. The unidentified "toxohormone" of the parabiosis studies, described earlier, is possibly synonymous with the lymphokine family.

Interleukin 1 is primarily synthesized by the monocytes of blood and by the phagocytic lining cells of organs, such as liver (Dinarello and Mier, 1986). Sinusoidal rat liver cells have been discovered to kill tumour cells \textit{in vitro} without prior immunisation and with greater potency
positioned in the circulatory system to be an effective filter, it has thus been hypothesised that the liver might function as a tumour cell killing organ (Malter et al., 1986). The biochemical changes, observed in the present studies for the livers of tumour-bearing mice, were possibly part of an adaptive response to the presence of disseminated cancer cells in the vasculature.

**Lewis lung carcinoma acting as a parasite:** Tumour-bearing rodents have been reported to have depleted liver glycogen stores (Shapot and Blinov, 1974). The liver glycogen of B16 melanoma-bearing mice has been shown to be about two-thirds of that of tumour-free animals (Rofe et al., 1985). Lewis lung carcinoma-bearing mice, however, were observed in the present studies to have plant cells in their livers, indicating that there were significant glycogen stores (Chapter 3); these animals were therefore unlikely to have been anorexic.

The nutritional demands of a comparatively large tumour mass, such as the Lewis lung, may well have limited the supply of vital nutrients to the non-neoplastic tissues of the host. The possible concentration of substances, necessary for the normal metabolism of the host mouse may have been a major cause of the abnormalities in non-neoplastic tissues. The amassing of antioxidants, for example vitamin E, in the Lewis lung carcinoma might have caused the increased susceptibility of hepatic tissue to in vitro lipid peroxidation.

Selenium has been found to inhibit cell proliferation in partially-hepatectomised rat livers, an effect possibly associated with an accompanying increase in hepatic GSH/GSSG ratio (LeBoeuf et al., 1985). The increased mitosis in the livers of tumour-bearing mice might thus have been initiated by a decrease in selenium concentration. Although tumours possibly concentrate selenium (Milner, 1985), they may protect themselves
from selenium's antiproliferative effects by sequestering it to a less active form. A deficiency of selenium might also have caused the decreased mitochondrial GSH peroxidase activities of the livers of Lewis lung carcinoma-bearing mice.

Mitochondria from kidneys and livers of rats, bearing Walker 256 carcinomas, have been reported to be about 20% deficient in magnesium and to have impaired respiratory function due to uncoupling of oxidative phosphorylation in mitochondria (Cummings et al., 1984). The uncoupling of mitochondrial respiration could cause excessive generation of reactive oxygen species (Chapter 1), leading to autoxidation and cell damage. The hepatic perturbations of Lewis lung carcinoma-bearing mice possibly originated by this mechanism.

Altered metabolism is a prominent feature of the systemic effects of malignant tumours upon animals and human beings (Waterhouse and Kemperman, 1971; Theogides, 1972; Costa, 1977; DeWys, 1983; Greengard and Cayanis, 1983). The antioxidant defence system, via glutathione and possibly catalase, is closely associated with fat, protein and carbohydrate metabolism (Magnani et al., 1980; Meister and Anderson, 1983; Crane and Masters, 1984). It is thus almost impossible to determine whether a change in antioxidant defence precedes an abnormality in general metabolism or vice versa.

**Decreased hepatic mitochondrial GSH peroxidase:** The feeding of high concentrations of cholesterol to rats and guinea pigs has been reported to cause an increase in the rate of hepatic lipid peroxidation in vitro, and to decrease liver GSH peroxidase activity (Tsai et al., 1977). The livers of tumour-bearing rats have been observed to have an impaired feedback mechanism for controlling the synthesis of cholesterol (Shapot, 1980). Alterations in the cholesterol/phospholipid ratios of hepatic membranes can cause changes in their physico-chemical properties:
phospholipids, for example, are allosteric regulators of many enzymes, such as adenylate cyclase (Burlakova, 1975). The decreased hepatic mitochondrial GSH peroxidase activities of mice, bearing Lewis lung carcinomas, may thus have resulted from an increase in the cholesterol concentration of liver membranes.

It is considered that GSH peroxidase can not directly react with lipoperoxides, that are incorporated in lipid membranes (McCay et al, 1976). Fatty acyl hydroperoxides have to be cleaved from the peroxidized membrane in a reaction, catalyzed by phospholipase A$_2$, before GSH peroxidase can reduce them (Tan et al, 1984; Sevanian and Kim, 1985). The hydrolysis of fatty acid/lysophospholipid bilayers by phospholipase A$_2$ has been observed to be inhibited by a variety of structurally dissimilar organic solutes; this indicated that many phospholipase A$_2$ inhibitors act by altering the substrate interface, as opposed to directly interacting with the enzyme (Jain et al, 1984). It can be hypothesised that GSH peroxidase activity is induced by its substrate, lipoperoxide: when phospholipase A$_2$ activity is decreased, due to say a change in membrane conformation, the concentration of free peroxides is decreased; GSH peroxidase activity is subsequently lowered because of a lack of substrate induction. Inhibitors of phospholipase A$_2$ activity, such as that from cobra venen (Davidson et al, 1986) might be used in future experiments to determine whether phospholipase A$_2$ mediates GSH peroxidase activity.

Lipocortin: Corticosteroids induce the synthesis of a group of phospholipase A$_2$ inhibitory proteins, collectively called lipocortin. The anti-inflammatory effects of steroids may well be mediated through lipocortin which prevents the release of arachidonic acid, the precursor of the eicosanoids, from phospholipid membranes (Wallner et al, 1986; Papa et al, 1986). The stress, associated with tumour burden, might have promoted
an increase in lipocortin which possibly caused the hypothetical inhibition of phospholipase $A_2$ leading to decreased hepatic mitochondrial GSH peroxidase activity.

Lipocortin is inactivated when phosphorylated by protein kinase, such as that of platelets (Touqui et al., 1986). It has been reported that human lipocortin is phosphorylated by the protein kinase activity of epidermal growth factor (EGF) receptor (Pepinsky and Sinclair, 1986). If, as was hypothesised earlier, the neoplastic state is characterized by the abundance of growth factor receptors and a pronounced synthesis of growth factors then the anti-prostanoid activity of lipocortin will be opposed. Growth factors might thus promote the synthesis of eicosanoids through inhibition of lipocortin activity.

Insulin receptors have tyrosine protein kinase activity: EGF and growth hormone do not bind to insulin receptors and insulin will not bind to EGF receptors; other peptides, such as angiotensin II, gastrin and casein however can be phosphorylated at both (Cobb and Rosen, 1984). If lipocortins were also substrates for the protein kinase of the insulin receptor, insulin might competively inhibit lipocortin phosphorylation and thereby prolong lipocortin activity. The anticachexic effects of insulin (see earlier) may thus be mediated in part by lipocortins.

Increased Systemic Lipid Peroxidation

Hepatic lipid peroxidation: The present studies, and others elsewhere have indicated that the livers of tumour-bearing mice may have an increased ability to produce lipoperoxides (Boveris et al., 1985; Pierson and Meadows, 1985; Chapter 4). It is important to establish whether this observation is applicable to other species and other tumour models, and whether these animals have increased hepatic lipid peroxidation in vivo. The measurement of expired alkanes is probably the most suitable method
to determine in vivo lipid peroxidation (Chapter 1). Animals of low body weight have relatively pronounced metabolic rates and correspondingly high tissue oxygen concentrations and short life expectancy (Parke and Ioannides, 1984b). The rate of autoxidation in the liver of various species, has a negative correlation with the maximum potential lifespan (Cutler, 1984). Cancer-induced hepatic lipid peroxidation might thus only be significant in small animals, such as mice, and not be evident in man.

Hepatic lipid peroxidation has been reported to cause loss of activity of microsomal enzymes, such as cytochrome P₄₅₀, NADPH-cytochrome c reductase and UDP-glucuronyltransferase (Younes and Siegers, 1984). The ability of the tumour-bearing host to metabolise anticancer drugs might thus be impaired due to liver damage, caused by lipid peroxidation. Disturbed liver function in cancer patients might falsely suggest that there are hepatic metastatic lesions and thereby cause inappropriate therapeutic decisions.

Anaemia of tumour-bearing mice: The anaemia, associated with cancer, is sometimes greater than that predicted from the size of the neoplastic lesions, or from factors such as decreased haemoglobin synthesis, increased plasma volume or bleeding into hollow viscera (Busch, 1962). A pronounced iron concentration has been observed in and around some rat tumours (Greenfield et al., 1958): human breast tumours have been reported to accumulate iron, and in Hodgkin's disease, iron deposits are typically seen surrounding tumour nodules (Halliwell and Gutteridge, 1985). Experiments, using erythrocytes containing radiolabeled iron, have indicated that tumours are able to localize red cells and degrade their contents, probably for the nutritional benefit of the tumour (Price and Greenfield, 1958; Price et al., 1959). It has been hypothesized in the present studies that the anaemia of tumour-bearing mice might have been caused by oxidative destruction of erythrocytes (Chapter 5). The reactive
oxygen species, possibly responsible for the erythrocyte lysis, may have been generated by the tumour itself or by activated macrophages. The degradation of erythrocytes to provide nutrients was possibly a further benefit for the Lewis lung carcinoma, furnished by the hypothetical production of free radical species.

Anticancer Therapy

Earlier in this chapter, it was suggested that possible anticancer regimens could involve the depletion of GSH concentration prior to targeting reactive oxygen species at the neoplastic tissue. The investigations of the systemic effects of tumours indicate that antioxidant defence in the non-neoplastic tissues of the host might be impaired; therapy with GSH depletors and/or oxygen metabolite-producing regimens would thus be limited due to a comparatively low threshold for systemic toxicity. Conversely attempts to improve the prognosis of the tumour-bearing animal or cancer patient, by supplementation with antioxidants, may be counterproductive as they could possibly promote neoplastic growth or abrogate antineoplastic therapy. It has been shown, however, that the GSH concentrations of human lung adenocarcinoma cells, in culture, are substantially less amenable to chemical manipulation (depletion with BSO; augmentation with OZT) than those of normal human lung fibroblasts (Russo et al, 1986). Pretreatment with OZT, considerably protected non-neoplastic cells from the toxicity of melphalan, cisplatin and bleomycin, but did not affect the sensitivity of adenocarcinoma cells to these drugs (Russo et al, 1986). An investigation to determine whether the GSH concentrations of normal and neoplastic tissues, in tumour-bearing mice, can be selectively modulated would be justified.

The lymphokines are a new class of potentially useful anticancer agents. Tumour necrosis factor, for example has pronounced antineoplastic activity against many human cancers and murine neoplasia, including Bl6
melanoma and Lewis lung carcinoma (Watanabe et al, 1985; Somura et al, 1986). Interleukin 2 (T-cell growth factor) has been reported to cause tumour regression in patients with metastatic disease, and in sarcoma-bearing mice, but this was accompanied by substantial adverse side-effects (Papa et al, 1986). Concomitant therapy with cortisone decreased the systemic toxicity of interleukin 2, but also lowered its tumouricidal activity, mainly due to the suppression of killer cell activation (Papa et al, 1986). It would probably be worthwhile to determine whether the immunosuppressive effects of corticosteroids are mediated by lipocortins: if lipocortins do not have a major influence upon the immune response they may well have a potential role in the treatment of cancer.

Cancer treatment is very often more distressing to the patient than the disease itself; cytotoxic anticancer therapy can be self-limiting due to damage to normal tissues, such as the bone marrow or the heart. The prognosis of the cancer patient is so relatively poor that therapy with antioxidants or anti-inflammatory agents to counteract cachexia and the side-effects of drugs, is worthy of consideration. Therapy, designed to restore the function of non-neoplastic tissues to normal, may well improve the quality of life and will possibly prolong life; enhancement of tumour growth by such regimens is arguably an acceptable risk. There is limited evidence that antioxidants, under some circumstances, can cause tumour regression (see earlier). Dexamethasone is a synthetic steroid, used clinically to manage the oedema, accompanying the radiation treatment of brain tumours; dexamethasone has been reported to have no influence upon the sensitivity in vivo of Lewis lung carcinomas to radiation (Agboola et al, 1983).

The interactions between neoplasia and the host in malignant disease are complex. The present studies indicate that antioxidant defence and autoxidation have an important role in these relationships, and will
Overall Conclusions

The studies of this thesis have indicated that antioxidant defence systems of the transplantable murine tumour, the Lewis lung carcinoma, are not substantially different in activity to those of normal tissues: the susceptibility of tumour microsomes to lipid peroxidation was comparatively low; whereas the concentration of malonaldehyde-like material, one of the products of lipid peroxidation, in the carcinoma was pronounced. The relative insensitivity of Lewis lung carcinomas to most conventional anticancer regimens may well be related to its resistance to free-radical mediated injury. It has been further postulated, from these observations, that the Lewis lung carcinoma might use reactive oxygen species to promote the growth and dissemination of the tumour. To remain viable in the presence of oxygen-centred radicals, Lewis lung carcinoma cells must be relatively resistant to autoxidative destruction. Much of the experimental evidence, supporting this hypothesis, was obtained, however, using the TBA test, a method that can produce ambiguous data. Clearly, evidence, based on other methodologies, is required before this theory can be considered to be tenable.

The observations of pronounced Mn-SOD activity and TBA-reactive material concentration in Lewis lung carcinoma tissue conflicted with the levels generally reported for other animal tumour models or for human neoplasms. This indicates that, at the present time, there is insufficient experimental evidence to support any major hypotheses, concerning the role of free radicals in the etiology of neoplastic disease.

Although the cancer-induced diminution of the catalase activities of normal tissues has been known for many years, a systematic investigation
of the major antioxidant defence enzymes of tumour-bearing mice has probably never been attempted before. The present studies have shown that the Lewis lung carcinoma causes several perturbations of antioxidant defence enzymes in the liver, blood and lungs of the host mice. These disturbances may be associated with evidence of increased lipid peroxidation in, at least the livers, of tumour-bearing mice. The causes of the abnormalities in the non-neoplastic tissues of mice, bearing Lewis lung carcinomas, are unknown: several candidates have been suggested; it is considered that the changes in antioxidant defence and lipid peroxidation probably resulted from several factors. Future studies, in which the effects of lymphokines, prostanoids, growth factors, hormones (and chemicals that modulate these substances), upon murine antioxidant defence and lipid peroxidation, are warranted. The possible effects of neoplastic disease upon autoxidative damage and antioxidant defence in the tissues of the host could possibly have profound influence upon the prognosis of the cancer patient and the success of anti-cancer treatment. The studies, described in this thesis, indicate that further investigation of autoxidative injury and its prevention, in other animal tumour models and in clinical cancer, may well provide useful insights towards the successful management of neoplastic disease.
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APPENDICES

Appendix I  Analysis of Animal Diet

Appendix II  Virus Profile of C57BL6 Mice Used in the Study

Appendix III  Tissue Culture Medium

Appendix IV  Histological Staining Procedures

Appendix V  Lists of Figures, Photographic Plates, and Tables
CERTIFICATE OF ANALYSIS

PRODUCT: RAT & MOUSE No. 1 EXPANDED.

BATCH NO: 2199 PREMIX BATCH NO: P289


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<th>Nutrient</th>
<th>Found Analysis</th>
<th>Contaminant</th>
<th>Found Analysis</th>
<th>Limit of Detection</th>
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<td>Fluorine</td>
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<td>Crude Fibre</td>
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<td>Cadmium</td>
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<td>Sodium</td>
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<td>0.001 mg/kg</td>
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<td>Total Viable</td>
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<td>1000/g</td>
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<td>Mesophilic Spores</td>
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N.D. = NONE DETECTED

Signed

Dated 10th April, 1984

Quality Control Manager

Special Diets Services Limited
1 Stepfield
Witham
Essex, CM8 3AB
Telephone: (0376) 513651
**REPORT**

FROM: Abtek Biologicals Ltd
DATE: 31st March 1984
TEST: Viral sero diagnosis
SPECIMEN: 2 serum samples
RECEIVED: 14/3/84

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<td>N</td>
<td>N</td>
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<td>N</td>
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<td>N</td>
<td>AC+</td>
<td>N</td>
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<tr>
<td>Tum</td>
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AC+ = Anticomplementary at 1:10; negative at 1:20

Results recorded...

Results checked...
REPORT

FROM Abtek Biologicals Ltd pp Microbiological Associates, Bethesda, USA
OUR REF: MA-4217; sample ref MVSF-739 & -740 respectively
YOUR REF: ACT-06
DATE 18th May 1984
TEST Whole blood & Tumour passage for LDH virus
SPECIMEN 1 sample each of whole blood & tumour tissue.
RECEIVED 24/4/84

Both whole blood and tumour sample were negative for LDH virus.

Results checked

John A. [Signature]
Dulbecco's Modification of Eagles Medium

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<td>KCl</td>
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<td>10.0</td>
<td>Deoxycytidine</td>
</tr>
<tr>
<td>10.0</td>
<td>Deoxyguanosine</td>
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<tr>
<td>1000.0</td>
<td>Glucose</td>
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<tr>
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<td>Guanosine</td>
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<tr>
<td>0.2</td>
<td>Lipoic acid</td>
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<tr>
<td>10.0</td>
<td>Sodium phenol red</td>
</tr>
<tr>
<td>110.0</td>
<td>Sodium pyruvate</td>
</tr>
<tr>
<td>10.0</td>
<td>Thymidine</td>
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<tr>
<td>10.0</td>
<td>Uridine</td>
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1. Dewax in xylene for 3 min.

2. Bring sections to water:-
   a) 100% w/v ethanol for 1 min.
   b) 70% w/v ethanol for 1 min.
   c) 50% w/v ethanol for 1 min.
   d) Distilled water for 1 min.

3. Stain in haematoxylin (Delafield's) for 15 min.
4. Rinse in tap water for 1 min.
5. Differentiate in 1% v/v acid alcohol for 5-10 sec.
6. Blue in tap water for 1 min.
7. Counterstain in 1% w/v eosin.
8. Rinse in tap water.
9. Dehydrate:-
   a) 85% ethanol for 30 sec.
   b) 100% ethanol for 30 sec.
   c) 100% fresh ethanol for 30 sec.
10. Clear in xylene for 30 sec, twice.
11. Mount section in DPX (10g Distrene-80 in 5ml dibutylphthalate and 35 ml xylol).
Appendix IVb - Weigert and van Gieson's Stain for Connective Tissue

(1889)

1. Dewax and bring sections to water (see Appendix IVa).
2. Stain nuclei for 7 min in Weigert's haematoxylin (1:1 mixture of 1% w/v haematoxylin and 1.2% w/v FeCl₃ in 0.1 M HCL).
3. Rinse in tap water.
4. Rinse in distilled water for 1 min.
5. Counterstain with van Giesson's solution (100ml of saturated picric acid plus 10ml of 1% acid fuchsin) for 3 min.
6. Rinse rapidly in distilled water for 1 min.
7. Dehydrate:
   a) 90% ethanol for 30 sec.
   b) 100% ethanol for 30 sec.
   c) 100% fresh ethanol for 30 sec.
8. Clear in xylene and mount in DPX.

Results

Nuclei....................................................brown to blue-black.
Collagen....................................................bright red.
Muscle, cytoplasm, erythrocytes, fibrin...yellow
1. Use plastic slide holders and double-distilled water to avoid contamination by iron.

2. Bring sections to water.

3. Transfer to fresh solution of 1:1 by vol of 2% aqueous potassium ferrocyanide in 0.2 M HCl, for 30 min.

4. Wash thoroughly in several changes of distilled water.

5. Counterstain lightly with 1% neutral red for 20-30 sec.

6. Rinse in distilled water.

7. Dehydrate, clear in xylene, and mount in DPX (see Appendix IVa).

Results

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Nuclei..............................................red.

Cytoplasm...........................................pink.
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<td>b) Lewis lung carcinoma-bearing mouse (x 45).</td>
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</tr>
<tr>
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<td>b) Region containing golden-brown pigment (x 730).</td>
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</tr>
<tr>
<td></td>
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IFOR D. CAPEL* and ANDREW C. THORNLEY
Research Department, Marie Curie Memorial Foundation, The Chart, Oxted, Surrey RH8 0TL, U.K.

Abstract—Superoxide dismutase (SOD) activity, plasma caeruloplasmin activity and the level of whole tissue and subcellular lipoperoxides have been determined in normal and neoplastic tissues from control and tumour-bearing mice, measurements being made nine, twelve and fifteen days after the inoculation of Lewis lung carcinoma cells. SOD activity of host liver and lung tissues did not vary significantly from those of the control animals. Blood SOD activity of the tumoured animals was markedly elevated on the ninth and twelfth days after inoculation, decreasing to control levels on the fifteenth day. Tumor SOD diminished from an activity on the ninth day which was greater than that for control lung to a level significantly lower than that for control lung on the twelfth and fifteenth days after inoculation. The presence of a tumour did not appear to affect plasma caeruloplasmin oxidase levels. The lipoperoxide level of hepatic tissue rose significantly as the tumour progressed. In the lung tissue the lipoperoxides decreased from a level four times higher on the ninth day to one not significantly different from that of the controls. Tumour lipoperoxides were about twice the level of hepatic tissue and of the order of ten-fold greater than those of lung. The level of lipoperoxide in the plasma of tumoured mice did not differ markedly from that of control mice. Assays of lipoperoxide in subcellular fractions of liver, lung and tumour tissue revealed that the elevated lipoperoxide was principally synthesized in the endoplasmic reticulum.

INTRODUCTION

Lipid peroxidation has been implicated as a feature of a large variety of pathological conditions, including atherosclerosis, aging, encephalomalacia, ceroid lipofuscinosis, chronic pancreatitis, haemolytic anaemia, iron toxicity, liver necrosis, lung damage, oleonalyde poisoning, reproductive dysfunction and testicular atrophy[1,2]. Lipid peroxidation could also be implicated in the carcinogenic process since free radicals are formed by the activation of many carcinogens and in the process of photocarcinogenesis[3]. Antioxidants, agents which quench oxygen-derived radicals, have been shown to suppress carcinogenesis in laboratory animals[4,5]. In addition, it has been widely claimed that there is an inverse correlation between the intake of dietary antioxidants such as selenium and the incidence of various forms of cancer in human populations, suggesting that there could be a link between cancer and peroxide formation[6]. The damaging actions of peroxidized lipids upon cell membranes, that is, protein polymerization and cross-linking, phospholipid degradation, polypeptide scission and chemical alterations of amino acids could provide the basis of an hypothesis which would encompass the initiation of carcinogenesis[7] and provide an explanation for many of the characteristic biochemical and physical phenomena of cancer cells. Superoxide dismutase (SOD, EC1:15:1:1), which catalyses the dismutation of superoxide anions to hydrogen peroxide, glutathione peroxidase (GSH-Px, EC1:11:1:9) and catalase (EC1:11:1:6), which reduces hydrogen peroxide and lipoperoxides to water and fatty acids respectively, have low activities in neoplastic tissues[8-12]. In measurements made from eight human colon carcinoma excisions, the mean malonaldehyde level (as an index of lipid peroxidation) was elevated by about $\frac{3}{2}$-fold in...
Hydrogen peroxide, a likely precursor of lipoperoxides in human tissues [17]. Furthermore, it has been shown that hydrogen peroxide is not formed in the mitochondria of non-cancerous tissues [17]. Tissue from the Lewis lung carcinoma has been reported to have a low activity of SOD and GSH-Px, and elevated levels of thiobarbituric acid-reactive material (TARM) [15, 16]. The increase in TARM is associated with elevated lipid peroxidation because the only substrate pool available to support peroxidation of this magnitude is the polyunsaturated fatty acid (PUFA) of the intracellular and plasma membranes of tumour cells.

It has been reported, however, that hydrogen peroxide, a likely precursor of lipoperoxide, is not formed in the mitochondria of neoplastic tissues [17]. Furthermore, it has been claimed that there is no potential in vitro for peroxidation reactions to occur in animal tumours [18], and that most tumour cells are much less susceptible to lipid peroxidation [19]. The presence of a tumour evokes deleterious biochemical changes within non-cancerous tissues of the host [20-24]. Mice bearing the Lewis lung carcinoma have been shown to have significantly raised levels of hepatic lipoperoxide [15].

The present study investigates the site of the progidious synthesis of lipoperoxides in tumoured animals, and the role of SOD in tumour growth and the protection of host tissues from the tumour-associated lipid peroxidation.

The TARM level in whole tissue, mitochondria, endoplasmic reticulum and cytosol of liver, lungs and tumour of tumour-inoculated mice has been measured and compared with that of saline-inoculated controls. Whole tissues and red blood cells have been assayed for SOD activity, and the plasma for the superoxide anion radical scavenger, caeruloplasmin.

Since tumour growth is a dynamic process it is probable that there are continuous metabolic changes during the progression of neoplastic disease. In the present study, therefore, the tumour and host tissues were monitored on the ninth, twelfth and fifteenth days after tumour inoculation.

MATERIALS AND METHODS

Chemicals

All chemicals were of the highest grade commercially available and, unless stated otherwise, were obtained from Fisons Scientific Apparatus, Loughborough, Leics, U.K. Thio barbituric acid, Coomassie blue G, O-dianisidine dihydrochloride and the haemoglobin determination kit were supplied by Sigma (London) Chemical Co., Poole, Dorset, U.K.; riboflavin by Koch Light, Colnbrook, Bucks U.K.; and potassium cyanide and EDTA (diaminoethanetetra-acetic acid) by Britis Drug Houses Ltd., Poole, Dorset, U.K.

Animals and treatment

Male C-57 BL/10 ScSn mice were purchased from Bantin and Kingman, Hull, Yorks, U.K. and maintained on experimental No. 1 SQC (B P. Nutritional) diet in high density polypropylene cages. Lewis lung tumour cells were main- tained by regular serial intramuscular transplantation into one flank of a syngeneic host every 14 days. When the animals had reached 35 ± 5 g in weight, twenty-four were inoculated with freshly-prepared tumour cells in the manner previously described [25], while a further nine mice were inoculated with phosphate buffered saline (PBS) and retained as control Group consisting of eight tumour-bearing and three control mice were killed by decapitation once, twelve and fifteen days after tumour inoculation.

Blood was pooled into two groups of four experimental animals and one group of three control animals on the day of each killing. Th liver and lungs of the mice were rapidly excised thoroughly rinsed in isotonic saline and weighed before being homogenized at 10-20 w/v in ice cold buffer containing 0.25 M sucrose, 0.1 mM EDTA and 1 mM Tris-HC (pH 7.4). The tumours were treated in a similar manner except that each tumour was divide into approximately two equal parts, one half being homogenized as above and the other in buffer containing a 1% w/v bovine serum albumin (BSA) additive.

A portion of each homogenate was retained for the whole-tissue assays, while the remainder was separated into mitochondrial, microsomal and cytosolic subcellular fractions by means of differential centrifugation [26]. The mitochondrial and microsomal fractions were washed in buffer and respun, and stored at −20°C with the cytosols prior to analysis.

Assays

The protein concentration of the subcellular fractions was determined by the method of Spector [27]. Tumour microsomes and mitochondria were fractionated in buffer containing BSA, present in order to confer extra stabiliz
Lipoperoxides and SOD in Tumour-bearing Mice

TA RM was determined in whole tissue, in microsomal, mitochondrial and cytosolic fractions, and in plasma by means of a fluorimetric assay essentially as described by Satoh [29]. SOD activity was measured in whole tissue and erythrocytes using the photochemical augmentation assay devised by Misra and Fridovich [30]. Ceruloplasmin activity of pooled plasma from control and experim ental animals was assayed by the method of Schosinsky et al. [31].

Statistical analyses

The data obtained from individual animals were analysed statistically using the unpaired Students' t-test. Differences between control and tumoured animals were considered significant when \( P < 0.05 \).

Since the Lewis lung carcinoma originally arose spontaneously as a primary carcinoma of the lung in a C57 BL/6 mouse [32], data derived from tumour tissue was arbitrarily related to that of control lung in order to provide an index of changes in SOD activity and lipid peroxidation as the tumour progressed. Comparisons between changes in tumour SOD activity and tumour growth (as estimated by increasing percentage of body weight) were made by linear regression analysis.

RESULTS

The SOD activity of liver and lung tissue from tumoured mice did not significantly differ from that of control animals. However, the erythrocyte SOD activity was raised about fivefold in the tumour-bearing groups on the ninth and twelfth days after inoculation. Tumour SOD activity decreased from a ninth day mean value slightly greater than that of control lung to activities significantly lower than those of control lung on the twelfth and fifteenth days (see Table 1). Linear regression analysis showed there to be a negative correlation coefficient on 0.94 between SOD activity and tumour growth.

The serum ceruloplasmin levels of the tumoured mice did not differ significantly from those of the controls. The whole-tissue TA RM levels are given in Table 2. The presence of a tumour significantly raised the concentration of

\[
\text{Table 1. Superoxide dismutase activity in tumour tissue, and in lung, liver and erythrocytes of control and Lewis lung carcinoma-bearing C-57 BL/10 mice}
\]

<table>
<thead>
<tr>
<th>Duration after inoculation of tumour cells (days)</th>
<th>Liver</th>
<th>Control</th>
<th>Tumoured</th>
<th>Lung</th>
<th>Control</th>
<th>Tumoured</th>
<th>Tumour</th>
<th>Erythrocyte*</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>7.8 ± 0.5</td>
<td>7.4 ± 1.3</td>
<td>1.8 ± 0.3</td>
<td>1.7 ± 0.6</td>
<td>2.3 ± 0.7</td>
<td>2.2</td>
<td>8.0, 13.3</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>7.7 ± 0.1</td>
<td>7.3 ± 1.1</td>
<td>1.6 ± 0.3</td>
<td>1.6 ± 0.8</td>
<td>0.67 ± 0.18</td>
<td>2.0</td>
<td>10.3, 8.8</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>7.7 ± 0.6</td>
<td>8.3 ± 1.2</td>
<td>1.6 ± 0.3</td>
<td>1.8 ± 0.2</td>
<td>0.29 ± 0.18</td>
<td>2.1</td>
<td>3.9, 3.0</td>
<td></td>
</tr>
</tbody>
</table>

Results represent the mean ± S.D. of individual observations of 3 control and 8 tumour-bearing mice, expressed as units \( \times 10^5 \)/g wet wt. tissue.

*Erythrocytes pooled from 3 control and 2 x 4 tumour-bearing mice, the results being expressed as units/mg haemoglobin.
†Significantly different from controls (P.B.S. injected) for \( P < 0.05 \). (Tumour tissue compared with control lung.)

\[
\text{Table 2. Thiobarbituric acid-reactive material in tumour tissue, and in liver, lung and plasma of control and Lewis lung carcinoma-bearing C-57 BL/10 mice}
\]

<table>
<thead>
<tr>
<th>Duration after inoculation of tumour cells (days)</th>
<th>Liver</th>
<th>Control</th>
<th>Tumoured</th>
<th>Lung</th>
<th>Control</th>
<th>Tumoured</th>
<th>Tumour</th>
<th>Plasma*</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>87.3 ± 19.6</td>
<td>114.2 ± 29.5</td>
<td>12.0 ± 1.3</td>
<td>50.6 ± 19.7</td>
<td>133.1 ± 55.8</td>
<td>2.70</td>
<td>1.60, 1.74</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>62.0 ± 26.5</td>
<td>215.7 ± 66.9</td>
<td>13.0 ± 1.7</td>
<td>26.9 ± 9.0</td>
<td>124.9 ± 54.1</td>
<td>1.45</td>
<td>1.88, 1.90</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>64.8 ± 10.7</td>
<td>252.9 ± 96.1</td>
<td>10.3 ± 1.4</td>
<td>11.3 ± 3.1</td>
<td>153.4 ± 43.8</td>
<td>2.17</td>
<td>2.17, 1.42</td>
<td></td>
</tr>
</tbody>
</table>

Results represent the mean ± S.D. of individual observations of 3 control and 8 tumour-bearing mice, expressed as nmol malonaldehyde/g wet wt. tissue.

*Plasma pooled from 3 control and 2 x 4 tumour-bearing mice, expressed as nmol/ml.
†Significantly different from control (P.B.S. injected) for \( P < 0.05 \). (Tumour compared with control lung.)
peroxidized lipid in liver and lung but not in the plasma. Tumour TARM was of the order of ten-fold and two-fold respectively higher than that of lung and liver. The hepatic liperoxide level of the tumour-bearing mice tended to rise as the tumour progressed, whereas pulmonary liperoxide decreased from a significantly elevated level nine days after injection to the control level by the fifteenth day.

TARM from microsomal fractions of tumour, and host liver and lung was consistently and significantly increased (Fig. 1). On the ninth day after tumour cell inoculation mitochondrial TARM was significantly higher in hepatic tissue but not in pulmonary tissue, while tumour mitochondrial TARM was significantly higher than that of control lung (Fig. 1). Cytosolic TARM in the tumour was significantly higher than the TARM level of control lung cytosol at all three intervals of measurement (Fig. 1).

**DISCUSSION**

In the present study the Lewis lung carcinoma had no effect upon liver and lung SOD activity: the erythrocyte enzyme level was raised while tumour SOD decreased as the carcinoma grew in size.

The activity of erythrocyte SOD was presumably raised in response to elevated levels of superoxide anions ('O_2\(^{2-}\)). It is possible that 'O_2\(^{2-}\) and other active oxygen intermediate could attack and lyse red blood cells, resulting in the characteristic anaemia of cancer. Th 'O_2\(^{2-}\) ions could be released either by activate macrophages[33], by the breakdown of oxy haemoglobin or, alternatively, by tumour cell themselves. Single i.v. or i.m. injections of SO have been demonstrated to increase the life expectancy of animals with Ehrlich ascite tumour cells or Sarcoma 180 tumours[8]. Since SOD cannot enter the cell, this effect must be due to the scavenging of extracellular 'O_2\(^{2-}\).

The decreasing tumour SOD which was associated with tumour growth may have resulted from end product inhibition by continually increasing levels of hydroperoxide[94, 35]. Elevated hydrogen peroxide could in turn be the result of the low GSH-Px activity of the Lewis lung carcinoma[15].

The caeruloplasmin level in the plasma of tumoured mice did not appear to differ from that of the control animals. However, the observed level (0.024 ± 0.007 units/ml plasma) is very low in comparison to other species; only one-tenth of the activity normally recorded for human and rat plasma. It is probable, therefore, that in mice caeruloplasmin has a relatively small role as an oxygen radical scavenger.

The present study indicates that there was an increase in the level of TARM in at least two different organs, namely liver and lung of tumour-bearing mice. In addition, the level of TARM in the tumour itself was remarkably high. The elevated TARM was presumed lipoperoxide and appeared to be predominantly synthesized in the endoplasmic reticulum of the tissues concerned. It would thus appear from these observations that the source of the increased formation of peroxidized lipid was the iron-dependent, NADP linked cytoplasmic system of the endoplasmic reticulum[36] rather than a more general non-enzymic process.

The elevated lipoperoxides in the liver and lungs of tumoured mice are not necessarily produced by the same mechanisms as in the tumour, although there were similarities in the subcellular distribution of TARM. The antioxidant enzymes of host tissues, in common with those of tumour tissues, have been reported to have relatively low activities[35, 37, 38]. Hepatic catalase[37, 38] and SOD are particularly depressed in tumoured animals[35]. Thus oxygen-derived radical species, such as superoxide ('O_2\(^{2-}\)), singlet oxygen ('O_2) and hydrogen peroxide (H_2O_2), may be involved in the el
vation of lipoperoxide within both neoplastic and host tissues. Active oxygen intermediates could possibly catalyse the formation of an active iron species, for example perferryl iron, FeO$_2^+$ [36], which could itself initiate an increase in endoplasmic lipid peroxidation.

The results obtained with tumour tissue provide some evidence for a recent hypothetical model for cancer [35] which suggests that a change in DNA, or its expression, in the stem cell results in the loss of SOD and the ensuing increase in $O_2^-$ and $H_2O_2$ produces a rise in the cyclic GMP/cyclic AMP ratio and intracellular glucose, which in turn inhibits or represses catalase and GSH-Px. This reduction in antioxidant defence enzymes of tumour cells and concomitant build-up of active oxygen intermediates would lead to elevated lipoperoxide levels, as was observed.

It was proposed as long ago as 1947 that there is a tendency for the tumour to influence enzyme systems of the tumour-bearing animal to become like those of the tumour itself [21]. Thus, tumours may reduce the capability of the host tissues to combat toxic oxygen-derived species and, as a result, there is an increase in the lipoperoxide levels in the non-neoplastic tissues.

The rise in hepatic and pulmonary lipoperoxide in mice bearing the Lewis lung carcinoma could be the result of other phenomena such as stress [39] or cell-mediated immunity [40], which arise as a consequence of tumour invasion. Furthermore, lipid peroxidation could be promoted by an increase in host tissue polyunsaturated fatty acid (PUFA). A rise in total and neutral lipids in hepatic microsomes of rats with benzo(a)pyrene induced sarcomas has been reported [41].

In the present experiment, pulmonary lipoperoxide levels decreased from an originally elevated level as the tumour grew, thereby indicating that the lung has an antioxidant factor(s) that is either absent or inferior in hepatic tissue. There is a recent report of cytosolic factors, present in the lung but not in the liver, which can inhibit both enzymic and non-enzymic lipid peroxidation [42]. Lung tissue is much more resistant to peroxidation than most other tissues, a resistance that correlates well with the relatively high vitamin E to PUFA ratio [43].

Many agents currently in use for cancer therapy act by raising the levels of active oxygen intermediates within the cell and are presumably selective against neoplastic cells due to their relatively inadequate antioxidant defence systems [8]. Many cytotoxic chemicals raise the content of lipoperoxide in liver and other normal tissues [44]. Should the elevated production of lipoperoxide in both neoplastic and normal tissues be a characteristic common to all or many types of cancer, it is possible that chemotherapeutic agents will exacerbate peroxidative damage in the non-malignant tissues of cancer patients. Elucidation of the relationship between tumour growth and lipid peroxidation could provide an alternative approach to the control of neoplastic disease.

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LIPOPEROXIDE LEVELS, GLUTATHIONE STATUS AND GLUTATHIONE PEROXIDASE ACTIVITY IN LIVER AND TUMORS OF MICE BEARING THE LEWIS LUNG CARCINOMA

IFOR D. CAPEL† and ANDREW C. THORNLEY
Research Department, Marie Curie Memorial Foundation, The Chart, Oxted, Surrey, RH8 0TL, U.K.

Lipoperoxides, glutathione status and glutathione peroxidase activity have been determined in normal and neoplastic tissues of control and tumor-bearing mice, tissues from both groups being assayed 5, 7, 9, 11, 13 and 15 days after inoculation. The ratio of hepatic reduced:oxidized glutathione increased in tumor-bearing animals as the tumor increased in size. This ratio was 2.5-fold higher at 15 days than at 10 days after tumor inoculation. In both tumor and hepatic tissue the alteration in the ratio was the result of both an increase in reduced glutathione and a decrease in oxidized glutathione levels. In tumor tissue the progressively increasing reduced glutathione content correlated closely with tumor growth. The presence of a tumor did not significantly affect hepatic glutathione peroxidase activity and there was no significant difference between tumor enzyme activity assayed at 2-day intervals between 9 and 15 days after inoculation. The livers of tumor-bearing animals had significantly higher lipoperoxides than control mice, the levels increasing progressively with tumor growth. Tumor lipoperoxides were also high, usually in excess of the hepatic level. The lungs of nontumored littermates, which were compared with the carcinoma as reference tissue, showed no significant change in either glutathione peroxidase activity or lipoperoxide levels when monitored over the same period.

INTRODUCTION

Lipoperoxides (lipohydroperoxides, lipid hydroperoxides) are the highly toxic substances whose formation may be induced when tissues are insulted with various xenobiotics (Di Luzio, 1975; Plaa and Witschi, 1976) or as a consequence of the enzymic "detoxication" of highly toxic activated oxygen species (Kellog and Fridovich, 1975, 1977). An obvious toxic consequence of the presence of elevated lipoperoxide levels is the degradation of cellular membranes. Recent reports indicate that hydrogen peroxide and lipoperoxides may catalyze nonenzymatic oxidation of arachidonic acid forming biologically significant "prostaglandin-like" endoperoxides (Carpenter, 1980; Fridovich and Porter, 1981). The cellular antioxidant defense enzymes comprise superoxide dismutase (SOD, EC 1:15:1:1), glutathione peroxidase (GSHPx, EC 1:11:1:9) and catalase (EC 1:11:1:6). The SOD, in reducing superoxide species, forms hydrogen peroxide which is, in turn, detoxified by either GSHPx or catalase. The levels of these enzymes have been determined in a number of human and experimental tumors with conflicting results. Elevated GSHPx levels in human neoplastic tissues have been reported (Baur and Wendel, 1980). The majority of experimental studies suggest that the level of this enzyme is generally low in tumors and that catalase is almost totally absent (Bozzi et al., 1979; Nathan et al., 1980). This experiment examines the levels of GSHPx and its principal substrate, thiobarbiturate-reactive material (lipoperoxides) in a model murine tumor at intervals after implantation. The availability of the coenzyme for GSHPx activity, reduced glutathione (GSH), was also estimated by determination of the ratio of reduced: oxidized glutathione (GSSG) in both tumor and hepatic tissue.

MATERIALS AND METHODS

Chemicals

Malonaldehyde (bis[dimethyl acetyl]) was purchased from Aldrich, Gillingham, Dorset. Oxidized
and reduced glutathione, 2-thiobarbituric acid, 5,5'-dithiobis(2-nitrobenzoic acid), DTNB, Coomassie Brilliant Blue G, glutathione reductase (EC 1.6.4.2) and superoxide dismutase were obtained from Sigma Chemical Co., Poole, Dorset. All other reagents used were of the purest grades available and obtained from either Fisons, Loughborough, Leics., or BDH, Poole, Dorset, U.K.

Animals and Treatment

Male C57 BL/10ScSn mice were obtained from Bantin and Kingman, Hull, Yorks and maintained on R & M experimental No. 1 SQC diet (BP Nutritional). The animals were housed in groups of 20 in high density polypropylene cages on sterilized sawdust bedding at a constant temperature of 21±1°C.

On attaining a weight of 22 g (aged 14 weeks) mice were inoculated intramuscularly with 0.5×10⁶ freshly prepared cells (in phosphate buffered saline 0.2 ml) from a Lewis lung carcinoma which had been maintained by regular passage in syngeneic hosts at 10-day intervals. Control mice were injected with an equivalent volume of saline only. Groups of tumor-bearing and control mice were sacrificed by cervical dislocation 5, 7, 9, 11, 13 and 15 days after inoculation of the tumor-bearing animals. Nontumor ed littermates (saline injected) were sacrificed at the same time intervals in order that the lung tissue could be analysed for comparison with the carcinoma. Lung, liver (and tumors where appropriate) were excised immediately, washed in isotonic ice-cold saline, weighed and homogenized in 1.15% KCl (20% w/v).

Assays

Hepatic, pulmonary and tumor glutathione peroxidase were estimated in the manner previously described (Capel et al., 1980). Tissue lipoperoxides were determined in the manner described by Satoh (1978). Reduced and oxidized glutathione were estimated essentially as described by Griffith (1980) except that the tissues were extracted in 1.67% metaphosphoric acid, the final reaction mixture comprising 700 μl of 0.3 mM NADPH in EDTA-containing 0.125 M sodium phosphate buffer (pH 7.5), 100 μl of 6 mM DTNB, 100 μl of 0.4 M Na₂HPO₄, 100 μl of tissue extract and 10 μl of glutathione reductase (equivalent to 0.5 Sigma enzyme units). Tissue protein content was determined by the method of Spector (1978) with Coomassie blue protein dye.

Statistical Analyses

Statistical comparisons of observed difference between control and tumor-bearing animals were made by unpaired Student’s t test, results were considered significant when P<0.05. Comparisons between the changing ratio of GSH/GSSG and tumor growth (as estimated by increasing weight) was made by linear regression analysis.

RESULTS

Tumor GSHPx activity and also the hepatic level of this enzyme in normal and tumor-bearing animals are given in Table I. The presence of a growing tumor produced no significant change in hepatic GSHPx levels and the tumor level of this enzyme did not change significantly as the tumor increased in size. The GSHPx activity of control lung tissue was approximately 10% of control liver but is approximately twice that of tumor tissue. In control animals there was no significant variation in the GSHPx activity over the same period (15 days) during which the tumored animals were monitored. The average hepatic GSHPx of the tumored animal was always lower than the control mean because of the large experimental variation. Differences did not consistently reach statistical significance. The ratio of GSH/GSSG was increased in the livers of the mice with increasing time after inoculation of the tumor whereas this ratio showed no significant variation in the livers of control animals (Table II). The livers of tumored animals examined 11, 13 or 15 days after tumor inoculation generally had significantly lower GSSG concentration than hepatic tissue examined earlier intervals after implantation. Conversely, the GSH levels were generally higher than those examined 5 days after tumor implantation. The increased ratio of tumor GSH/GSSG is more marked than the change in the hepatic levels (Table III). A direct correlation (r=0.99) between the increasing ratio of GSH/GSSG and the increasing weight of the tumor was observed. Thus, measure at 2-day intervals between 9 and 15 days after implantation, GSSG decreased progressively to 67% of the average 9-day level at day 1 whereas the average GSH level increased to 146% over the same period. Hepatic lipoperoxide leve
GLUTATHIONE IN TUMOR-BEARING MICE

TABLE I

Glutathione peroxidase activity$^a$ in tumor tissues and the livers and lungs of control and Lewis lung carcinoma-bearing mice

<table>
<thead>
<tr>
<th>Days after tumor inoculation</th>
<th>Hepatic tissue</th>
<th>Control</th>
<th>Tumor-bearing</th>
<th>Tumor tissue</th>
<th>Control lung tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>8809 ± 364</td>
<td>7583 ± 345</td>
<td>—</td>
<td>748 ± 67</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>9407 ± 398</td>
<td>7778 ± 212</td>
<td>—</td>
<td>755 ± 75</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>9107 ± 223</td>
<td>7658 ± 599</td>
<td>361 ± 20</td>
<td>695 ± 61</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>8977 ± 316</td>
<td>7829 ± 306</td>
<td>361 ± 22</td>
<td>775 ± 53</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>9209 ± 329</td>
<td>7322 ± 460</td>
<td>379 ± 28</td>
<td>714 ± 73</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>9097 ± 284</td>
<td>8094 ± 512</td>
<td>342 ± 48</td>
<td>765 ± 65</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Expressed as enzyme units/g wet weight tissue.
Results indicate the mean ± S.E.M. for individual assays on groups of 6 control and 6 tumor-bearing mice at each time interval.
— Quantity of tissue too small for assay.
Difference between control and tumor-bearing mice not significant ($P > 0.05$).

TABLE II

The glutathione status of the livers of control and tumor-bearing mice

<table>
<thead>
<tr>
<th>Days after tumor inoculation</th>
<th>Controls</th>
<th>Tumor-bearing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSSG</td>
<td>GSH</td>
</tr>
<tr>
<td>5</td>
<td>143 ±10</td>
<td>5392 ± 328</td>
</tr>
<tr>
<td>7</td>
<td>171 ±13</td>
<td>5395 ± 217</td>
</tr>
<tr>
<td>9</td>
<td>115 ±9</td>
<td>6399 ± 393</td>
</tr>
<tr>
<td>11</td>
<td>156 ±24</td>
<td>5185 ± 295</td>
</tr>
<tr>
<td>13</td>
<td>93 ±13*</td>
<td>5094 ± 215</td>
</tr>
<tr>
<td>15</td>
<td>120 ±9</td>
<td>6136 ± 257</td>
</tr>
</tbody>
</table>

All values expressed as n mol GSH or GSSG/g wet weight tissue.
Results are mean ± S.E.M. for 6 control and 6 tumor-bearing animals at each time interval.
*Significantly different from the respective day 5 value $P < 0.05$.

were higher in tumor-bearing animals and increased progressively with tumor growth to over twice the level in tumor free animals at 13 and 15 days after inoculation. The tumor lipoperoxide level was also consistently high (approximately twice the level of control liver) at the time intervals when it could be measured, but there was no significant variation in the levels measured at successive time intervals (Table IV).
There was no significant variation in control lung lipoperoxides monitored at the various intervals indicated in Table IV. The lipoperoxide
TABLE III
The growth and glutathione status of Lewis lung carcinoma tissue at various intervals after inoculation

<table>
<thead>
<tr>
<th>Days after inoculation</th>
<th>Mean body wt. (g)</th>
<th>Mean tumor wt. (g)</th>
<th>Tumor/body wt. (%)</th>
<th>GSSG</th>
<th>GSH</th>
<th>Ratio GSH/GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>27.4±1.6</td>
<td>0.64±0.17</td>
<td>2.3±0.62</td>
<td>86±6</td>
<td>686±86</td>
<td>7</td>
</tr>
<tr>
<td>11</td>
<td>26.9±1.1</td>
<td>0.86±0.19</td>
<td>3.2±0.73</td>
<td>71±6</td>
<td>749±172</td>
<td>10</td>
</tr>
<tr>
<td>13</td>
<td>25.6±1.1</td>
<td>1.23±0.25</td>
<td>4.8±0.96</td>
<td>64±8</td>
<td>820±106</td>
<td>13</td>
</tr>
<tr>
<td>15</td>
<td>26.2±2.8</td>
<td>1.52±0.31</td>
<td>5.8±1.1</td>
<td>58±11</td>
<td>1003±153</td>
<td>17</td>
</tr>
</tbody>
</table>

Values expressed as nmol/g wet weight tissue. Results indicate mean ± S.E.M. for 6 mice at each time interval. Correlation between tumor growth, expressed as % of body weight and ratio of GSH/GSSG by linear regression analysis (r = 0.99).

TABLE IV
Lipoperoxide levels* in tumor tissue and the livers and lungs of control and Lewis lung carcinoma-bearing mice

<table>
<thead>
<tr>
<th>Days after inoculation</th>
<th>Hepatic tissue</th>
<th>Control</th>
<th>Tumor-bearing</th>
<th>Tumor tissue</th>
<th>Control lung tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>79.3±10.4</td>
<td>94.4±9.4</td>
<td>—</td>
<td>11.1±0.4</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>86.0±10.3</td>
<td>88.7±11.8</td>
<td>—</td>
<td>10.9±0.3</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>85.3±9.4</td>
<td>114.8±10.2</td>
<td>180.4±37.4</td>
<td>12.0±0.6</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>86.0±11.5</td>
<td>123.1±11.4</td>
<td>146.6±19.8</td>
<td>13.1±0.6</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>85.6±12.9</td>
<td>156.6±24.3*</td>
<td>150.0±28.7</td>
<td>10.6±0.5</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>86.1±16.4</td>
<td>228.9±37.1*</td>
<td>177.9±24.5</td>
<td>10.3±0.7</td>
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</table>

*Expressed as nmol of thiobarbituric acid reactive material/g wet weight tissue. Results expressed as means ± S.E.M. for 6 animals in each group. — Quantity of tissue too small for assay. *Significantly different from control (P < 0.05.)

Levels of control lung tissue were much lower (approximately 13%) of control liver and represented an even smaller fraction (approximately 7%) of the level of tumor tissue. Comparisons between the carcinoma and control lung tissue were made because the Lewis lung carcinoma originally arose as a spontaneous primary carcinoma of the lung in a C57BL6 mouse. With respect to the parameters investigated in the experiment, however, there is little resemblance between the tumor and the tissue from which it originated. The results obtained with control lung tissue confirm that spontaneous fluctuations in lipoperoxide and GSHPx levels of the ord observed in the tumorred mice do not occur spontaneously in control animals.
DISCUSSION

Rapidly growing normal tissues such as fetal or neonatal and regenerating liver are characterized by the lack of oxidation products such as lipoperoxides and there have been reports of a similar situation existing in tumor cells (Player et al., 1977, 1979). The results of the present experiment contradict this, adding further evidence to the reports that lipoperoxide levels are greatly elevated in neoplastic tissues (Baur and Wendel, 1980; Burbina and Nejafkh, 1970; Burlokov and Molochkina, 1973). The present study differs from those previously conducted in that using a model tumor the lipoperoxides and principal constituents of the glutathione antioxidant defense system are monitored at intervals during growth.

The presence of a tumor resulted in a progressive increase in hepatic lipoperoxide, to a level greater than that of the neoplastic tissue, when the tumor had reached its most advanced state. This is in agreement with previous reports of elevated lipoperoxides in the tissues of tumor-bearing hosts (Burbina and Nejafkh, 1970; Burlokov and Molochkina, 1973). The origin of the elevated lipoperoxides observed in the present experiment is uncertain: these substances are usually the response to oxidant stress, chemical insult or possibly anaphylaxis (Di Luzio, 1973; Plaa and Witschi, 1976; Pelus and Strausser, 1977). The progressively increasing hepatic lipoperoxides could reflect the host's immune response to the presence of the growing tumor or the toxicity of the products excreted by the tumor. It is unlikely that the hepatic lipoperoxides originated from the tumor since no increase in the blood level of these substances has been observed (unpublished result).

The increased tumor lipoperoxides could originate from increased hydrogen peroxide, the result of elevated SOD activity (Baur and Wendel, 1980). Excess hydrogen peroxide can, reportedly, catalyze lipoperoxide production (Kellog and Fridovich, 1977). A number of studies (Bozzi et al., Peskin et al., 1979; Oberley and Beuttner, 1979), including one conducted in this laboratory, using this same model tumor (manuscript in preparation) demonstrates decreased copper- and zinc-dependent SOD and almost negligible manganese-dependent enzyme in this murine carcinoma. The most likely cause of the elevated tumor lipoperoxides is, therefore, the low assayable GSHPx activity. In the present study hydrogen peroxide was used as a substrate to estimate GSHPx activity, indicating the level of the selenium-dependent enzyme (Lawrence and Burk, 1978). Although decreased selenium is often associated with the presence of tumors in the clinical situation (Burk, 1976), such observations are complicated by the tumor treatment as well as its presence (Broghamer et al., 1978; Robinson et al., 1979). Growing tumors concentrate this trace element (Cavaliere et al., 1966), so that it is unlikely that tumor GSHPx activity is limited by selenium deficiency. Since tumors lack appreciable catalase activity (Baur and Wendel, 1980; Bozzi et al., 1979; McCay et al., 1976), the very low GSHPx activity should result in increased hydrogen peroxide and lipoperoxide levels. A number of experiments have tried to exploit this apparent lack of antioxidant defense for tumorcidal therapy. A recent study conducted on a number of differing murine tumors correlated their sensitivity to exogenous hydrogen peroxide with their GSHPx activity (Nathan et al., 1980). Since in the present experiment the tumor lipoperoxides are consistently high even in the first intervals after implantation, it is possible that the degenerate tumor cells have less need of compartmentalization and can thus tolerate the toxic action the lipoperoxides would exert on their membranes. The elevated lipoperoxides might also influence pharmacological actions which could affect tumor growth rate. Hydrogen peroxide and lipoperoxides can catalyze arachidonic acid oxidation and also stimulate the action of cyclo-oxygenase (Kellog and Fridovich, 1975; Carpenter, 1980), the enzyme chiefly responsible for oxidation of this substance. Thus, the lipoperoxides could lead to unscheduled formation of prostaglandins and other biologically significant substances.

The most paradoxical aspect of the low tumor GSHPx is the high level of substrates (lipoperoxides) and coenzyme (GSH) availability. In the present study GSH/GSSG ratio in the tumor correlates directly with tumor growth, indicating an important or even essential role in this process. It is possible that a deliberately low GSHPx, however controlled, prevents usage of GSH and so aids the increasing GSH/GSSG ratio. A high GSH content could influence amino acid uptake (Meister, 1975), and also stimulate protein and DNA synthesis (Kosower and Kosower, 1978), typical of tumor cell metabolism (Costa, 1977). Some indirect evidence for this suggestion is provided by the observation that vincap alkaloid therapy increases tumor GSSG content (Beck, 1980). Further studies are being conducted to determine whether this disturbance in glutathione metabolism is characteristic of tumor...
in general. Should this be confirmed, manipulation of the redox state of this tripeptide could provide an alternative approach to anticancer therapy.

REFERENCES


