ABSTRACT

PARAQUAT PERINATAL TOXICITY AND A PROPOSED MECHANISM OF ACTION INVOLVING LIPID PEROXIDATION

By

James Stanley Bus

Paraquat, 1,1'-dimethyl-4,4'-bipyridylium dichloride, is a widely used herbicide effective against broad leaf weeds and grasses. The purpose of this investigation was to determine the effect of chronically administered paraquat on the postnatal development of mice and to determine *in vitro* and *in vivo* the mechanism of paraquat toxicity in mammalian systems.

Paraquat, in the drinking water of pregnant Swiss-Webster mice at 50 and 100 ppm at day 8 of gestation and with continued exposure of the pups to 42 days postnatally, did not at any time alter the average litter body weights compared to controls. One hundred ppm, but not 50 ppm, paraquat significantly increased postnatal mortality in a biphasic manner, with an initial 33% increase in mortality reached between days 7 and 28 postnatally. Mortality was 67% at 42 days after birth. Pulmonary lesions consisting of edema, thickening of alveolar septa, and alveolar consolidation were observed in the 100 ppm but not 50 ppm paraquat treated mice at 42 days postnatally. Liver and kidney were not affected.

Concentrations of paraquat in lungs of 100 ppm paraquat treated mice were less than 0.2 μg/g tissue at 42 days postnatally and non-detectable in lungs of 50 ppm paraquat treated mice.

Both 50 and 100 ppm paraquat treated mice were more sensitive to 100% oxygen exposure at 42 days after birth than controls as determined by a significant reduction in the median time to death (LT_{50}) in oxygen. The LT_{50} for control animals in oxygen was 160 hours versus LT_{50}'s of 108 and 40 hours for 50 and 100 ppm paraquat treatments, respectively. Sensitivity to 100% oxygen was detectable in 100 ppm but not 50 ppm paraquat treated mice at days 1 and 28 postnatally. In 42 day old mice, the LT_{50} after 3100 mg/kg ip bromobenzene (LD_{85}) was 4.2 and 3.2 hours in 50 and 100 ppm paraquat treated mice, respectively, compared to the LT_{50} in controls of 20.0 hours.

Under anaerobic conditions *in vitro*, paraquat was reduced to the blue-colored free radical by a single electron reduction reaction catalyzed by mouse lung microsomes and NADPH. The reaction was inhibited by antibody to NADPH-cytochrome c reductase, which suggested that microsomal NADPH-cytochrome c reductase catalyzed the paraquat reduction. Paraquat, when incubated aerobically with NADPH, NADPH-cytochrome c reductase and extracted microsomal lipid, initiated lipid peroxidation in a concentration dependent manner with a maximum 227% increase in malondialdehyde at 10^{-3}M paraquat. Paraquat-induced *in vitro* lipid peroxidation was inhibited by the superoxide radical scavenger superoxide dismutase and a singlet oxygen trapping agent, 1,3-diphenylisobenzofuran. Both agents

together inhibited paraquat-induced lipid peroxidation to a greater degree than either agent alone, suggesting sequential intermediates of superoxide radicals and singlet oxygen in paraquat-induced lipid peroxidation.

Paraquat-induced *in vivo* lipid peroxidation was studied by determination of the paraquat 7-day ip LD_{50} in mice fed diets deficient in the antioxidants selenium or vitamin E. Selenium and vitamin E deficiency significantly reduced the paraquat LD_{50} to 10.4 and 9.2 mg/kg, respectively, compared to 30.0 mg/kg in laboratory chow fed controls. Supplementation of selenium deficient diet with 0.1 and 2.0 ppm selenium and vitamin E deficient diet with 45 and 1500 mg vitamin E per kg diet returned the paraquat LD_{50} to the control value. Liver glutathione peroxidase (a selenium dependent enzyme) activity in selenium deficient animals was 16% of control activity. Vitamin E deficiency was confirmed by the dialuric acid erythrocyte hemolysis test. The paraquat LD_{50} in mice was reduced to 9.4 mg/kg by pretreatment with diethyl maleate at 1.2 ml/kg ip, 30 minutes before paraquat. Diethyl maleate decreased the reduced glutathione (GSH) concentrations in liver and lung. GSH is a substrate for glutathione peroxidase, which detoxifies lipid hydroperoxides formed during membrane lipid peroxidation. Paraquat, 30 mg/kg ip, also significantly reduced liver concentrations of the water soluble antioxidant GSH 21% 24 and 36 hours after treatment and lung concentrations of lipid soluble antioxidants 53 to 59% between 12 and 96 hours after paraquat.

Male Sprague-Dawley rats treated with 100 ppm paraquat in drinking water had a 68% increase in glucose-6-phosphate dehydrogenase

and an 89% increase in glutathione reductase activity in lung tissue. Pretreatment of rats with 85% oxygen for 7 days, which induces tolerance to subsequent 100% oxygen exposure, increased the resistance of rats to a toxic (45 mg/kg ip) dose of paraquat, reflected by a significant increase in the paraquat LT_{50} to 50.0 hours as compared to 26.0 hours in nonpretreated rats.

Mice pretreated with 0.1% w/v phenobarbital in the drinking water for 10 days and with continued exposure after paraquat injection also were protected against paraquat toxicity. The paraquat LD_{50} was 46.0 mg/kg with phenobarbital pretreatment and 30.0 mg/kg in controls. Phenobarbital may compete for electrons necessary for paraquat reduction.

The *in vitro* and *in vivo* experiments suggest that lipid peroxidation of cellular membranes may be involved in paraquat toxicity. Paraquat may undergo a cyclic reduction-oxidation *in vivo*, with subsequent formation of superoxide radicals. Superoxide radicals can nonenzymatically dismutate to singlet oxygen, which reacts with unsaturated lipids associated with cell membranes to form lipid hydroperoxides. The lipid hydroperoxides can initiate the membrane damaging chain reaction process of lipid peroxidation. The result of such a process is loss of cellular function and integrity.

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INTRODUCTION

Objectives

The demand for increased agricultural productivity to support a growing world population has led to the introduction of many pesticidal agents over the last half century. Although many such agents have proved to be effective pest control agents, environmentalists and toxicologists have become increasingly concerned with the problem of pesticide residues in the soil, water, plants and in animals and humans. Of particular concern are the possible consequences to developing organisms of long term exposure to low levels of pesticides. Furthermore, as animals and man become increasingly exposed to a wide spectrum of drugs and chemicals, the potential for toxic interactions between such agents and pesticides in biological systems is significantly enhanced.

Thus, the objectives of this research were twofold, both of which related to examination of the toxicity of the bipyridylium herbicide paraquat (1,1'-dimethyl-4,4'-bipyridylium dichloride). The first objective was to examine the effects of chronic ingestion of paraquat on the perinatal development of mice and to investigate the potential for the existence of subtle toxicity in these animals. The second objective was to determine interactions between acute and chronic paraquat treatment and various agents or experimental diets

that may influence the expression of toxicity. The agents that were examined were those that offered some potential for interaction in an environmental situation and that would yield insights into the mechanism of paraquat toxicity.

The experimental data presented in this dissertation show that paraquat not only has direct toxic effects on postnatal development in mice, but also that more subtle paraquat toxicity is also present, which can be manifested when the mice are exposed to interacting substances such as oxygen. In studies of acute paraquat toxicity in adult mice, the toxicity also is shown to be enhanced in the presence of certain nutrient deficiency states in the animals. Thus, the results of these experiments provide evidence which allows further assessment of paraquat as an environmental hazard, particularly in relation to its effects on mammalian development and potential interactions with other environmental factors.

Over the course of this investigation, however, a second body of information regarding paraquat toxicity became apparent, which may be of greater importance than the results directed to the assessment of the paraquat environmental hazard. This was the evidence obtained through experiments on paraquat interactions, that the mechanism of paraquat toxicity may be mediated through lipid peroxidation. The significance of suggesting a lipid peroxidative mechanism for paraquat toxicity is of threefold importance. First, the elucidation of a mechanism provides a basis for predicting possible interactions with environmental agents not investigated in this study. This is particularly beneficial in the assessment

of paraquat as an environmental hazard. Second, a defined mechanism for paraquat toxicity provides useful information for implementation of a rational therapeutic approach in treating victims of acute paraquat poisoning. Finally, and perhaps most important, the lipid peroxidative mechanism of paraquat toxicity proposed in this study provides *in vivo* evidence for lipid peroxidation as a cause of tissue damage. Therefore it may serve as a model toxic mechanism for a number of other drugs and environmental agents. Furthermore, the techniques used in developing the mechanism for paraquat toxicity may be useful for investigating similar mechanisms of other agents.

Chemistry of Lipid Peroxidation

Lipid peroxidation has been broadly defined by Tappel (1973) as the reaction of oxidative deterioration of polyunsaturated lipids. Peroxidation of lipids involves the direct reaction of oxygen with polyunsaturated lipids to form free radicals and semi-stable hydroperoxides, which then promote free radical chain oxidations (Holman, 1954; Barber and Bernheim, 1967; Tappel, 1973). The free radical chain reaction proceeds in three distinct steps (Pryor, 1973). First is the *initiation* process in which the radicals are generated. Second is a series of *propagation* reactions in which the number of free radicals is conserved. Finally, there is a series of *termination* reactions in which free radicals are destroyed. The 3 steps of lipid peroxidation are depicted below in a simplified scheme (L = polyunsaturated lipid):

Initiation:

$$LH \longrightarrow L^{\cdot} + (H^{\cdot})$$

Propagation:

$$L^{\cdot} + O_2 \longrightarrow LO_2^{\cdot}$$

$$LO_2^{\cdot} + LH \longrightarrow LOOH + L^{\cdot}$$

Termination:

$$L^{\cdot} + L^{\cdot} \longrightarrow \text{nonradical products}$$

$$L^{\cdot} + LO_2^{\cdot} \longrightarrow \text{nonradical products}$$

$$LO_2^{\cdot} + LO_2^{\cdot} \longrightarrow \text{nonradical products}$$

Unsaturated fatty acids are susceptible to peroxidation as the presence of a double bond weakens the carbon-hydrogen bond on the carbon atom adjacent to the unsaturated carbon-carbon bond (Swern, 1961; Demopoulos, 1973a). Thus, these allylic hydrogens are partially "activated" and are liable to abstraction by small amounts of oxidants or initiators (initiation reaction). Molecular oxygen can abstract an allylic hydrogen, but it also must be "activated." The activated oxygen molecule, or singlet oxygen, is an electronically excited oxygen molecule in which a valence electron is shifted from its normal bonding orbital to an orbital of higher energy in which the electron spins are paired (Wilson and Hastings, 1970; Maugh, 1973). The possible role of singlet oxygen as an initiator of lipid peroxidation has been confirmed by several investigators (Howes and Steele, 1971; Dowty *et al.*, 1973; Pederson and Aust, 1973). *In vivo,* singlet oxygen appears to occur from nonenzymatic loss of an electron from the single electron reduced state of oxygen, the superoxide anion, $O_2^{\cdot -}$ (Stauff *et al.*, 1963;

Khan, 1970; Pederson and Aust, 1973), as depicted below:

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + {}^1O_2{}^*$$

Superoxide, which may possibly initiate lipid peroxidation itself, is produced in many biological reactions such as autoxidations of reduced flavins, hydroquinones and catecholamines, and the aerobic actions of the enzymes xanthine oxidase, aldehyde oxidase and many flavin dehydrogenases (Fridovich, 1975).

The lipid hydroperoxides (LOOH) that are generated in the propagation step are unstable and decompose to form additional radical products. The decomposition of lipid hydroperoxides is catalyzed by trace amounts of transition metal ions (Holman, 1954; Heaton and Uri, 1961; Barber and Bernheim, 1967) and is shown in the following reactions (M^{n+} = transition metal ion):

$$LOOH + M^{n+} \longrightarrow LO^{\cdot} + OH^- + M^{(n+1)+}$$

$$LOOH + M^{(n+1)+} \longrightarrow LOO^{\cdot} + H^+ + M^{n+}$$

The catalytic decomposition of lipid hydroperoxides along with propagation reactions previously described are therefore autocatalytic, as more free radicals are the products the reactions. The autocatalytic reactions continue until substrate is depleted, sufficient termination reactions occur, or until intervention by antioxidants, which react with the free radicals and thereby interrupt the chain reaction process (Demopoulos, 1973b).

Biochemical Consequences of Lipid Peroxidation

The membranes of the mitochondria and endoplasmic reticulum have a high proportion of unsaturated fatty acids (Rouser *et al.*, 1968). Consequently, these membranes are highly susceptible to lipid peroxidative damage. Furthermore, some of the most potent catalysts involved in lipid peroxidation, coordinated iron and hemeproteins, are found in close association to these membranes (Tappel, 1973). Thus, the occurrence of lipid peroxidation in biological membranes has profound effects due to the close relationship of the unsaturated lipids with enzymes found in the membrane.

Lipid peroxidative damage of mitochondrial membranes has been demonstrated by Hunter *et al.* (1963) to correlate with swelling and lysis of the mitochondria. Alterations in the activity of NADH-cytochrome c reductase and the succinoxidase system of heart and liver mitochondria also are affected by lipid peroxidative damage (Tappel, 1965; Tappel, 1973). Studies into the mechanism of ethanol induced hepatic damage have attributed lipid peroxidative damage in the mitochondria as a possible subcellular lesion (DiLuzio, 1973).

As with the mitochondria, lipid peroxidative damage also affects microsomes. The most extensively studied agent regarding microsomal lipid peroxidation is carbon tetrachloride. Recknagel (1967) has reviewed the evidence supporting carbon tetrachloride induced lipid peroxidation. More recent evidence has indicated that carbon tetrachloride is metabolized to a trichloromethyl free radical which reacts with unsaturated membrane lipids to initiate

lipid peroxidation (Villarruel, 1973; Recknagel *et al.*, 1974). Carbon tetrachloride has also been shown to decrease microsomal drug metabolism and cytochrome P-450 levels, possibly through lipid peroxidation (Archakov and Kurzina, 1973). In other studies *in vitro* with isolated microsomes, stimulation of lipid peroxidation by addition of NADPH has been demonstrated to decrease the metabolism of acetanilide and pentobarbital (Jacobson *et al.*, 1973). Inhibition of metabolism was also correlated to a destruction of cytochrome P-450 (Jacobson *et al.*, 1973; Levin *et al.*, 1973). Similar observations regarding the *in vitro* inhibition of drug metabolism concurrent with lipid peroxidation have been made by other investigators, such as a decrease in morphine demethylation (Kamataki and Kitagawa, 1973) and a decrease in aminopyrine oxidation and aniline hydroxylation (Wills, 1969).

It is evident from the previous paragraphs that peroxidation of unsaturated membrane lipids has far greater biochemical effects than merely deterioration of lipids. The intimate juxtaposition of unsaturated lipids and proteins in membranes allows for further interaction between the lipids and proteins. Several membrane proteins like succinic dehydrogenase and β-hydroxybutyrate dehydrogenase may derive some of their structure from closely associated membrane lipids. In the presence of lipid peroxidation, and in particular the termination reactions in which two adjacent fatty acids are joined in abnormal bonds, the enzyme structure may be sufficiently altered to affect activity (Demopoulos, 1973a). The lipid peroxy radicals ($LOO^{\cdot}$) that are generated in the propagation

step may themselves abstract hydrogen atoms from neighboring proteins, resulting in protein cross-linking to form polymers (Tappel, 1965). This reaction sequence is shown schematically below (P = protein):

$$LOO^{\cdot} + P \longrightarrow LOOH + P(-H)$$

$$P(-H) + P \longrightarrow P\text{-}P$$

Studies by Chio and Tappel (1969) have demonstrated that sulfhydryl enzymes are most susceptible to inactivation by lipid peroxidation, which may occur either by an alteration of membrane structure or by protein-protein cross-linking. Thus, the biochemical consequences of lipid peroxidation at the membrane level are exceedingly complex and involve not only the unsaturated lipids but also the many different proteins that are an integral part of membranes.

Biological Defenses Against Lipid Peroxidation

Protection Against Superoxide and Singlet Oxygen. Lipid peroxidation, or oxidative deterioration of unsaturated lipids, has been shown to be an exceedingly damaging biological process. Aerobic organisms are continually exposed to many potentially damaging oxidative reactions. As a result, aerobic organisms possess several different mechanisms which provide protection against uncontrolled free radical reactions.

The most general defense of aerobic organisms against lipid peroxidation lies in the structural characteristics of cell membranes. The nature of membranes has been described as consisting of a

hydrophobic midzone between two hydrophilic surfaces. The hydrophobic midzone appears to be richly penetrated by proteins (Rouser *et al.*, 1968). The unsaturated lipids are found for the most part in the hydrophobic inner layer. As many free radicals are cations or anions, they do not readily penetrate the hydrophobic layer where abstraction of allylic hydrogens can occur. Furthermore, the close juxtaposition of proteins and unsaturated lipids hinders free radical reactions. The proteins provide a spatial separation for the unsaturated lipids and thereby can prevent the spread of radical chain reactions through the membrane (Demopoulos, 1973b). However, in the case of oxygen, the structure of the membrane may be disadvantageous. Oxygen is 7-8 times more soluble in nonpolar media and thereby has an affinity for the hydrophobic midzone of membranes, which is where the lipids most susceptible to oxidative damage are located (Demopoulos, 1973a).

Because membrane structure alone is not adequate to prevent oxidation of unsaturated lipids, aerobic organisms also possess other defenses against lipid peroxidation. One specific defense, acting at the level of oxygen attack of unsaturated lipids, is the activity of the enzyme superoxide dismutase. Superoxide dismutase scavenges the toxic superoxide radical by catalyzing the reaction depicted below (McCord and Fridovich, 1969):

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \longrightarrow O_2 + H_2O_2$$

Pederson and Aust (1973) have demonstrated that superoxide may initiate lipid peroxidation, as xanthine oxidase-induced peroxidation

of microsomal lipids was inhibited by superoxide dismutase.

The biological importance of superoxide dismutase as a defense mechanism has been determined by a number of elegant studies conducted with bacteria. McCord *et al.* (1971) examined the distribution of superoxide dismutase among 3 classes of microorganisms: aerobes which utilize oxygen in their metabolism almost exclusively, aerotolerant organisms which have an anaerobic metabolism even when grown in air, and finally strict anaerobes which cannot survive in oxygen. In all cases the aerobic organisms contained the highest activity of superoxide dismutase, followed by intermediate activity in the aerotolerant group. Strict anaerobes contained no superoxide dismutase, which may explain their inability to tolerate oxygen. In other studies (McCord *et al.*, 1973) a mutant strain of *Escherichia coli* was selected that had a temperature sensitive defect in its ability to grow aerobically over a temperature range of 30°C to 43.5°C. Thus, the mutant strain was less able to grow aerobically as the temperature was increased from 30°C compared to the wild type parent strain which could grow at all temperatures. The mutant strain was assayed for superoxide dismutase and found to have a progressive decrease in enzyme activity as the temperature increased. The enzyme activity in the parent strain was unaffected by temperature. Thus, in the mutant strain, the ability to grow aerobically as the temperature increased correlated with levels of superoxide dismutase.

Further investigations have determined that superoxide dismutase is induced in bacteria (Gregory and Fridovich, 1973a,b) and

yeast (Gregory *et al.*, 1974) in response to exposure to elevated oxygen tension. The protective function of superoxide dismutase was also evident in these studies (Gregory and Fridovich, 1973b) since *E. coli* grown under nitrogen were much less resistant to hyperbaric oxygen than *E. coli* grown under 2 atmospheres oxygen. This correlated with superoxide dismutase levels of 3.8 units/mg in the noninduced *E. coli* compared to 21 units/mg extract in the induced *E. coli*. The induction of superoxide dismutase in bacteria, therefore, offered resistance to exposure to elevated oxygen tensions. This resistance was presumably due to the enhanced ability of the bacteria to detoxify the superoxide radical.

Induction of superoxide dismutase by elevated oxygen tensions is not limited to microorganisms, however. Rosenbaum *et al.* (1969) demonstrated that exposure of rats to 85% oxygen for 7 days prolonged the survival time compared to nonpretreated rats when these rats were transferred to 100% oxygen. Superoxide dismutase activity in the lungs of rats exposed to 85% oxygen for 7 days was increased 50% compared to controls. Furthermore, the rate of tolerance development to 100% oxygen for rats pretreated with 85% oxygen closely paralleled increases in pulmonary superoxide dismutase activity (Crapo and Tierney, 1974). Thus, rats show the same parallel in comparing superoxide dismutase activity with resistance to oxygen as do bacteria.

Much of the work done to date regarding all aspects of superoxide dismutase can be found in an excellent review by Fridovich (1974a).

As has been discussed previously, however, superoxide may not be the initiator of lipid peroxidation. Rather, the nonenzymatic dismutation product of superoxide, singlet oxygen, may be the actual initiator of lipid peroxidation (Pederson and Aust, 1973). Experiments conducted by Krinsky (1974a) have indicated a biochemical mechanism protective against the toxic effects of singlet oxygen. A mutant strain of bacteria deficient in carotenoid pigments was observed to be much more susceptible to killing by human polymorphonuclear (PMN) leukocytes than a comparable carotenoid-containing strain. PMN leukocytes have been proposed to destroy ingested bacteria by generation of singlet oxygen which disrupts bacterial membranes (Allen *et al.*, 1972; Maugh, 1973; Allen *et al.*, 1974). Carotenoid pigments have been demonstrated to be quenchers of singlet oxygen *in vitro* (Foote and Denny, 1968) and may function similarly *in vivo* (Krinsky, 1971; Krinsky, 1974b). Thus, the susceptibility of the mutant bacteria to destruction by PMN leukocytes may be due to an inability to quench singlet oxygen. There is some evidence to indicate that carotenoids may also function in animals to quench singlet oxygen. Matthews (1964) was able to protect against the lethal photodynamic effects of intraperitoneally injected hematoporphyrin in mice when the animals were simultaneously injected with β-carotene. The protection afforded the mice to photodynamic lethality may be due to quenching of singlet oxygen, as many photooxidations proceed via a singlet oxygen mechanism (Foote, 1968).

Antioxidant Protection Against Lipid Peroxidation. Whereas superoxide dismutase and the carotenoid pigments appear to prevent the initiation of lipid peroxidation, organisms also possess biochemical defenses against the propagation of free radical reactions. This biochemical defense is centered around the endogenously occurring antioxidants. Two types of antioxidants function *in vivo*: the water soluble antioxidants which include ascorbic acid and glutathione and the lipid soluble antioxidants which mostly consist of the tocopherols (Demopoulos, 1973b). In general, antioxidants function by allowing a hydrogen to be abstracted from themselves rather than from the allylic hydrogen of an unsaturated lipid and thus act by interrupting the free radical chain reactions. A simplified scheme whereby vitamin E (α-tocopherol) acts as a chain breaker is depicted below (Tappel, 1972; αTH = α-tocopherol; αTQ = α-tocopherol quinone):

Propagation: $L^{\cdot} + O_2 \longrightarrow LO_2^{\cdot}$

$LO_2^{\cdot} + LH \longrightarrow LOOH + L^{\cdot}$

Antioxidant action: $LO_2^{\cdot} + \alpha TH \longrightarrow LOOH + \alpha T^{\cdot}$

Termination: $LO_2^{\cdot} + \alpha T^{\cdot} \longrightarrow LOOH + \alpha TQ$

The antioxidant function of vitamin E became apparent when deficiency states of this vitamin were induced. It has been shown that liver mitochondria and microsomes isolated from rats fed vitamin E deficient diets have greater peroxidation rates compared to rats fed basal diets supplemented with vitamin E (Tappel and

Zalkin, 1959; Dillard and Tappel, 1971). Furthermore, evidence of *in vivo* lipid peroxidation, as indicated by the formation of fluorescent lipofuscin pigments in the tissues, has been found in rats fed vitamin E deficient diets supplemented with polyunsaturated lipids (Porta and Hartroft, 1969). Vitamin E deficiency has been reported to increase the susceptibility of mice to oxygen toxicity which was associated with a reduction in lung phospholipids (Mino, 1973). An increased susceptibility to another strong oxidant, ozone, also has been observed in vitamin E deficient animals (Goldstein *et al.*, 1970; Roehm *et al.*, 1972). In humans, erythrocytes from patients with low plasma tocopherol levels have an increased susceptibility to lipid peroxidation (measured by increased malondialdehyde levels) on exposure to hydrogen peroxide vapor. *In vitro* addition of tocopherol reduced the lipid peroxidation (Tudhope and Hopkins, 1975). Dialuric acid has long been known to induce erythrocyte hemolysis in vitamin E deficient animals both *in vitro* and *in vivo* (Rose and Gyorgy, 1952). The hemolysis induced by dialuric acid has been shown to be associated with lipid peroxidation and was reduced by addition of vitamin E (Bunyan *et al.*, 1960; Tsen and Collier, 1960). Recently, Cohen and Heikkila (1974) have demonstrated that superoxide radical is a product of dialuric acid autoxidation and subsequently may initiate lipid peroxidation in the erythrocyte membrane.

Vitamin E deficiency results in several nutritional disease states. The earliest recognized disease state associated with vitamin E deficiency was a degeneration of muscle tissue (Goettsch

and Pappenheimer, 1931). The degeneration of muscle tissue in vitamin E deficiency closely resembles the muscle degeneration associated with human myotonic muscular dystrophy. Consequently, the vitamin E deficient effect has been referred to as nutritional muscular dystrophy (Horwitt, 1965). However, vitamin E administration does not alter the outcome of inherited muscular dystrophy (Tubis *et al.*, 1959). Other nutritional disease states that have been reported with vitamin E deficiency are necrotic liver destruction in rats (Schwarz, 1965) and encephalomalacia in chicks (Dam *et al.*, 1957).

Selenium and Enzymes of the Glutathione Peroxidase System. The antioxidant function of selenium was proposed from early studies which demonstrated that vitamin E deficiency syndromes such as nutritional muscular dystrophy were reversed by addition of small amounts of selenium to the diet (Bieri *et al.*, 1961; Scott, 1962; Scott, 1969). It has also been long known that the enzyme glutathione peroxidase could detoxify hydrogen peroxide and hydroperoxides (LOOH) *in vivo* (Mitts and Randall, 1958; Cohen and Hochstein, 1963; Christopherson, 1969; O'Brien and Little, 1969). The unstable lipid hydroperoxides are enzymatically reduced by gluathione peroxidase to stable lipid alcohols, which results in termination of free radical chain reactions. Recently, Rotruck *et al.* (1973) proposed that selenium was a necessary cofactor for glutathione peroxidase, as selenium deficient rats were unable to prevent hydrogen peroxide induced erythrocyte hemolysis. Purification of glutathione peroxidase from erythrocytes has demonstrated that the

enzyme consists of four subunits, with one gram-atom of selenium per subunit (Flohe *et al.*, 1973; Oh *et al.*, 1974). Other recent investigations have shown that glutathione peroxidase activity is directly related to the levels of dietary selenium in rats (Chow and Tappel, 1974; Hafeman *et al.*, 1974; Reddy and Tappel, 1974; Smith *et al.*, 1974; Tappel, 1974) and in chicks (Noguchi, 1973; Tappel, 1974). Thus, the majority of the antioxidant activity of selenium appears to be mediated through glutathione peroxidase activity.

The importance of gluathione peroxidase activity in detoxifying lipid hydroperoxides has been demonstrated in studies in which animals were exposed to oxidant stress. It has been proposed that glutathione peroxidase may protect against lipid peroxidative damage since the activity of this enzyme was induced in rat lung after exposure to the oxidant gas, ozone (Chow and Tappel, 1972; Chow *et al.*, 1974). It was also observed by these investigators that the enzyme activity of glutathione reductase and glucose-6-phosphate dehydrogenase was induced in response to ozone exposure, in addition to the induction of glutathione peroxidase. The three enzymes were referred to as the glutathione peroxidase system and are proposed to function as a unit in combating lipid peroxidation as follows: the conversion of toxic lipid hydroperoxides to lipid alcohols by glutathione peroxidase is linked to the activity of glutathione reductase and glucose-6-phosphate dehydrogenase which supply reducing equivalents in the form of reduced glutathione (GSH) and NADPH, respectively (Chow and Tappel, 1972).

Activity of the glutathione peroxidase system enzymes appears to be of clinical relevance in humans. Glutathione peroxidase activity is genetically determined in humans and consequently deficiency states of this enzyme in adults have been correlated to drug-induced hemolysis (Steinberg and Necheles, 1971) and to chronic hemolytic anemia (Necheles *et al.*, 1970). Hopkins and Tudhope (1974a) have observed that erythrocytes from glutathione peroxidase deficient individuals had an increased susceptibility to Heinz body formation when exposed to hydrogen peroxide vapor. These investigators also reported increased erythrocyte mechanical fragility with inhibition of glutathione peroxidase by menadione, gentisic acid or potassium chlorate (Hopkins and Tudhope, 1974b). Similar to individuals deficient in glutathione peroxidase, individuals with genetically related deficiencies in either glutathione reductase or glucose-6-phosphate dehydrogenase have been reported to be susceptible to erythrocyte hemolysis by a large number of drugs. The hemolysis has been proposed to be caused by an inability of the erythrocyte to detoxify peroxides that are formed in the cell as a consequence of the presence of the drug. The agents and mechanisms involved in erythrocyte hemolysis in enzyme deficient individuals has been reviewed by Beutler (1969).

Examples of Oxidant Agents that May Induce Lipid Peroxidation

Destruction of Bacteria by Polymorphonuclear Leukocytes (PMN Leukocytes). The importance of oxygen in bacterial killing by PMN leukocytes was first realized with the observation that a large

increase in oxygen uptake by PMN leukocytes occurred during phagocytosis (Sbarra and Karnovsky, 1959). It has been estimated that up to 10% of the increase in oxygen consumption is subsequently excreted from PMN leukocytes as the highly toxic superoxide radical (Babior *et al.*, 1973). The superoxide radicals excreted by the PMN leukocytes may be responsible for phagocytic killing, as *E. coli* deficient in superoxide dismutase are particularly vulnerable to phagocytosis (Fridovich, 1974b). Furthermore, PMN leukocytes isolated from patients with chronic granulomatous disease have been demonstrated to produce only small amounts of superoxide compared to PMN leukocytes isolated from normal individuals (Curnutte *et al.*, 1974). Consequently, patients with such defective PMN leukocytes are not protected against certain species of bacteria such as *Staphylococcus aureus*. Recently Beckman *et al.* (1973) found that cytosol superoxide dismutase was found in high concentrations in a number of human cell types except in the cytosol of PMN leukocytes. Fridovich (1974b) has speculated that PMN leukocytes lack the defense against the superoxide radical in order to be better able to kill bacteria through high levels of superoxide.

The superoxide radical, however, is not the only potential bactericidal agent excreted by PMN leukocytes. Highly reactive singlet oxygen has also been demonstrated to be evolved from PMN leukocytes during phagocytosis (Allen *et al.*, 1972; Maugh, 1973; Allen *et al.*, 1974). That singlet oxygen may be a bactericidal agent is evident from a study in which mutant bacteria deficient in singlet oxygen trapping carotenoid pigments were found to be more

readily killed by PMN leukocytes than the carotenoid-containing parent strain (Krinsky, 1974a).

Streptonigrin. Streptonigrin is a paraquinone antibiotic which has been shown to undergo a single electron reduction to a parahydroquinone in *E. coli* cultures (White and Dearman, 1965). The parahydroquinone can be spontaneously reoxidized back to the quinone by molecular oxygen with formation of superoxide radical, which may be the bactericidal agent (Misra and Fridovich, 1972). The generation of superoxide due to the cyclic reduction and reoxidation of streptonigrin may account for the decreased toxicity of streptonigrin to *E. coli* in the absence of oxygen and its enhanced toxicity in the presence of oxygen (White and White, 1968; White *et al.*, 1971). Further evidence that superoxide mediates streptonigrin toxicity is found in experiments in which *E. coli* containing high induced levels of superoxide dismutase were resistant to streptonigrin (Gregory and Fridovich, 1973b).

Oxygen. Both animals and man develop pulmonary lesions after exposure to elevated oxygen tensions. Exposure of monkeys to 90-100% oxygen resulted in sequential pulmonary changes characterized by interstitial edema, destruction of Type I alveolar cells, endothelial cell thickening, proliferation of Type II alveolar cells, and finally infiltration by fibroblasts. Rats were also observed to undergo similar pulmonary changes in 100% oxygen, although most died before reaching the proliferative stage (Kistler *et al.*, 1967; Kapanci *et al.*, 1969; Kaplan *et al.*, 1969; review by Winter and

Smith, 1972). Lipid peroxidative damage may result from oxygen exposure as vitamin E deficiency enhanced oxygen toxicity (Mino, 1973) and vitamin E administration protected against hyperbaric oxygen (Kann *et al.*, 1964). Tierney *et al.* (1973) also observed that rats made tolerant to oxygen by exposure to 85% oxygen for 7 days had increased pulmonary levels of glucose-6-phosphate dehydrogenase. The tolerance development may be in part associated with a response of the glutathione peroxidase system enzymes to lipid peroxidative damage.

Ozone and Nitrogen Dioxide. Ozone and nitrogen dioxide are oxidant gases that are found in air pollutant gas mixtures. The pulmonary lesions resulting from exposure to ozone or nitrogen dioxide have been characterized as an initial destruction of epithelial Type I cells (Stephens *et al.*, 1972; Stephens *et al.*, 1974) followed by a proliferation of Type II pneumocytes (Evans *et al.*, 1972; Stephens *et al.*, 1974). Most of the lesions occur in the area of the terminal bronchiole and proximal alveoli (Stephens *et al.*, 1974). As with oxygen, exposure of animals to sublethal levels of ozone or nitrogen dioxide resulted in the development of tolerance to subsequent exposure to previously lethal doses of the gases (Stokinger and Scheel, 1962; Fairchild, 1967). The mechanism of tolerance development has been linked to induction of the glutathione peroxidase system which indicated lipid peroxidation as the mediator of toxicity of the two gases (Chow *et al.*, 1974). Other investigators have also provided evidence that the *in vivo* pulmonary toxicity

of ozone and nitrogen dioxide may involve lipid peroxidation (Thomas *et al.*, 1968; Goldstein *et al.*, 1969; Fletcher and Tappel, 1973).

Paraquat

Historical Background and Uses. In the mid-1950's a series of bipyridylium compounds was found by Imperial Chemical Industries to possess herbicidal activity (Boon, 1965). The compound paraquat, 1,1'-dimethyl-4,4'-bipyridylium dichloride, was introduced for commercial use in 1962 in England and in the United States in 1964 (Kimbrough, 1974) as a broad spectrum herbicide effective against both broad leaf weeds and grasses. Paraquat is marketed outside the United States as a 20% concentrate, Gramoxone, and in a 5% granular form, Weedol. It is available in the United States as a 29.1% solution, Orthoparaquat, a 42% solution, Ortho dualparaquat, and as a 0.44% solution, Ortho Spot.

The broad spectrum herbicidal activity of paraquat has resulted in its wide use throughout the world. Paraquat has been used to kill weeds and grasses in orchards and between rows of crops and vineyards, to defoliate and desiccate crops for easier harvesting, to renew pastures overgrown with weeds and grasses, and for aquatic weed control. Calderbank (1968) has extensively reviewed the herbicidal uses of paraquat and other bipyridylium herbicides.

Application of paraquat to crops appears to result in only low levels of residue contamination. Minute residues of paraquat have been detected in potatoes which were defoliated prior to harvest (Boon, 1967) while seed harvested from previously desiccated crops

contained paraquat residues which varied from nondetectable up to 10 ppm (Calderbank, 1968). Paraquat has been reported to be firmly bound when it contacts most types of soils and upon adsorption can be degraded by several microorganisms present in the soil. When paraquat is used for aquatic weed control, residues in the water decline fairly rapidly. However, up to 112 ppm paraquat has been found in aquatic weeds when the surrounding water was at an initial concentration of 1 ppm (reviewed by Calderbank, 1968).

Human Toxicity of Paraquat. One reason for paraquat's rapid acceptance as an all-purpose herbicide was its apparent lack of any severe toxicity to workers or animals exposed to spray during field application. The only reports of toxicity to field workers exposed to paraquat spray were those of nail damage (Samman and Johnston, 1969; Swan, 1969; Hearn and Keir, 1971) and irritation of the nasal mucosa, skin and eyes (Swan, 1969). One case of severe eye injury resulted from splashes of the concentrate (Cant and Lewis, 1968).

Soon after paraquat was introduced, however, cases of paraquat ingestion began to be reported. The major route of paraquat ingestion is oral, either by accident or intention, and in many of these cases death was a result (Bullivant, 1966; Clark *et al.*, 1966; Duffy and Sullivan, 1968; Fennelly *et al.*, 1968; Oreopoulos *et al.*, 1968; Masterson and Roche, 1970; Beebeejuan *et al.*, 1971; Malone *et al.*, 1971; Copland *et al.*, 1974). In one suicide case, death resulted from a subcutaneous injection of 1 ml of 20% paraquat concentrate (Almog and Tal, 1967). Accidental ingestion of paraquat has also resulted in a number of deaths in children (Campbell,

1968; McDonagh and Martin, 1970). In one nonlethal case in a child, paraquat was detected in the urine after the herbicide was spilled over the skin, suggesting percutaneous absorption (McDonagh and Martin, 1970). A 1971 report indicated that the total number of deaths attributable to paraquat intoxication has reached 124 (Editorial, 1971). The fatal dose of paraquat in humans has been estimated to be from 4 mg/kg subcutaneously (Almog and Tal, 1967) to perhaps up to 50 mg/kg orally (Murray and Gibson, 1972). The overall mortality rate, encompassing the entire range of amounts ingested, appears to be approximately 33-50% (Editorial, 1971).

The clinical symptoms that develop after ingestion of a toxic dose of paraquat proceed from an initial nausea and vomiting, ulceration of the mouth and pharynx, oliguria, albuminuria and an increase in blood urea nitrogen, which then after a latent period progress to dyspnea, cyanosis and death due to massive pulmonary edema and interstitial fibrosis associated with a proliferation of alveolar epithelium (Bullivant, 1966; Almog and Tal, 1967; Campbell, 1968; Pasi and Hine, 1971; Toner *et al.*, 1971; Anonymous, 1972). In some cases both renal and centrilobular hepatic necrosis was observed (McDonagh and Martin, 1970). In most cases victims of paraquat poisoning survive for at least one week with some surviving up to four weeks after ingestion. The protracted development of lung lesions has been associated with a prolonged excretion of paraquat in the urine (Beebeejaun *et al.*, 1971).

The treatment of individuals poisoned with paraquat has utilized a number of therapeutic approaches, all with varying

degrees of success. Adsorption of paraquat cation in the stomach with Fuller's earth, activated charcoal and bentonite gel has been recommended as a nonspecific antidote for paraquat (Browne, 1971; Anonymous, 1972). Hemodialysis (Grundles *et al.*, 1971; Galloway and Petrie, 1972), peritoneal dialysis (Fisher *et al.*, 1971) and forced diuresis (Kerr *et al.*, 1968; Beebeejaun, 1971; Tompsett, 1970) have all been used with variable results. As the pulmonary symptoms developed oxygen therapy has been used (Copland *et al.*, 1974), although evidence exists that oxygen may enhance paraquat pulmonary toxicity and is therefore contraindicated in paraquat poisoning (Flenley, 1971; Nienhaus and Ehrenfeld, 1971; Fisher *et al.*, 1973a). Other investigators have recommended either a combination of steroids and cyclophosphamide (Malone *et al.*, 1971) or of steroids, d-propranolol and superoxide dismutase (Anonymous, 1973; Davies and Davies, 1974). In an extreme case a lung transplantation was attempted without success (Matthew *et al.*, 1968). Because of the wide variation in the amount of paraquat ingested among these cases, however, it is difficult to assess the effectiveness of any one treatment compared to another.

Animal Toxicity of Paraquat. The toxicity of paraquat has been studied in several animal species including rats (Kimbrough and Gaines, 1970; Murray and Gibson, 1972; Robertson *et al.*, 1971; Sharp *et al.*, 1972), mice (Brook, 1971), rabbits (Butler and Kleinerman, 1971), guinea pigs and monkeys (Murray and Gibson, 1972). The pulmonary lesions in rats and monkeys have been observed to be similar to the lesions in humans with initial pulmonary edema,

congestion and intra-alveolar hemorrhage followed by the onset of interstitial fibrosis (Murray and Gibson, 1972). Examination of the ultrastructure of the pulmonary lesion in rats (Smith, 1971; Kimbrough and Linder, 1973) and mice (Brook, 1971) revealed early damage to Type I pneumocytes, increased numbers of fibroblasts, and a later proliferation of Type II pneumocytes. Vijeyaratnam and Corrin (1971) reported that such paraquat induced pulmonary damage resembled that of oxygen toxicity. Not all species are similarly affected by paraquat toxicity, however, as guinea pigs developed pulmonary edema which did not progress to interstitial fibrosis (Murray and Gibson, 1972) and rabbits did not develop any type of pulmonary lesions (Butler and Kelinerman, 1971).

The toxicity of paraquat in tissue sites other than the lung has also been examined. Paraquat induced centrilobular and tubular necrosis of rat liver and kidney, respectively (Murray and Gibson, 1972). Necrosis of the cells of the proximal convoluted tubule in the kidneys of mice exposed to paraquat was associated with the appearance of lipid lamellate cytosomes (Clark *et al.*, 1966; Fowler and Brooks, 1971). Atrophy of the thymus has been noted in rabbits (Butler and Kelinerman, 1971). Paraquat was also toxic *in vitro* to rat alveolar and peritoneal macrophages (Styles, 1974). Paraquat has been reported to induce a slight increase in costal cartilage malformations in rat embryos (Khera *et al.*, 1968) and to have a low teratogenic potential in mice (Bus *et al.*, 1975). Furthermore, Pasi *et al.* (1974) observed that paraquat was not mutagenic in mice, although it had an antifertility effect.

Paraquat absorption is rapid and complete when administered subcutaneously in rats (Daniel and Gage, 1966). However, absorption after oral administration in rats appears to be poor as significant amounts of paraquat have been detected in the feces (Daniel and Gage, 1966; Murray and Gibson, 1974). The poor oral absorption of paraquat is reflected in the 6- to 8-fold increase in the oral LD_{50} in rats compared to the parenteral LD_{50} (Calderbank, 1968; Howe and Wright, 1965). Paraquat also has been reported to be sufficiently absorbed from the eye (Sinow and Wei, 1973) and skin (McElligott, 1972) to result in death in rabbits.

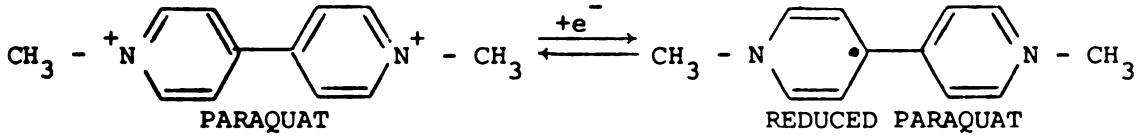
Once absorbed, paraquat is distributed to most tissues except for the brain and spinal cord (Sharp *et al.*, 1972; Litchfield *et al.*, 1973; Murray and Gibson, 1974). Several investigators have reported that paraquat rapidly disappears from most tissues in rats and mice except for the lung, where retention of the herbicide occurs (Molnar and Hayes, 1971; Sharp *et al.*, 1972; Litchfield *et al.*, 1973; Illett *et al.*, 1974; Murray and Gibson, 1974). This observation has resulted in the suggestion that retention of paraquat in lung tissue may account for its pulmonary toxicity. No metabolism of paraquat has been demonstrated upon absorption (Murray and Gibson, 1974; Bus *et al.*, 1975) although paraquat may be metabolized by the gut bacteria after oral administration (Daniel and Gage, 1966). Paraquat is largely excreted by the kidney (Daniel and Gage, 1966; Murray and Gibson, 1974) and only small amounts are excreted in the bile (Daniel and Gage, 1966; Hughes *et al.*, 1973). Elimination of paraquat by the kidney

appears to be by an active secretory process in the proximal tubule (Ecker, J. E., unpublished observation).

A number of different antidotal regimens have been attempted in animals in order to suggest possible therapeutic approaches for treatment of paraquat poisoning in humans. Staiff *et al.* (1973) reported that the Amberlite CG-120 resins, either 100-200 or 200-400 mesh, lowered the tissue levels of paraquat and may have provided protection against poisoning. Treatment of rats with a stomach wash followed by four administrations of bentonite plus purgatives (0.5 ml castor oil, 250 mg magnesium sulfate/kg) at 2- to 3-hour intervals was effective as an antidote even if treatment was delayed up to 10 hours after paraquat administration (Rose, M. S., personal communication). Administration of expectorants prevented paraquat induced decreases in lung surfactant although no measurements were made as to alterations in survival (Cambar and Aviado, 1970). Exposure of paraquat poisoned mice to lowered oxygen tensions (Rhodes, 1974) as well as administration of superoxide dismutase in rats (Autor, 1974) has also been able to alter paraquat lethality. Recently, repeated administration of propranolol has been shown to protect against paraquat lethality in rats (Maling *et al.*, 1975).

Mechanism of Action of Paraquat. The mechanism of paraquat's herbicidal activity has been extensively studied. As early as 1933, it was shown that paraquat (which was used as an oxidation-reduction indicator with the name methyl viologen) was capable of undergoing a single electron reduction to a blue-colored free radical form with a redox potential of -446 mv (Michaelis and Hill, 1933a,b).

This reduction reaction is depicted below:



PARAQUAT REDUCED PARAQUAT

The free radical that is generated is apparently stabilized due to the complete delocalization of the added electron over the coplanar paraquat molecule (Boon, 1965). The ability of isolated plant chloroplasts to form the blue-colored paraquat free radical under anaerobic conditions has been demonstrated (Dodge, 1971). Anaerobic conditions were necessary in order to prevent the immediate reoxidation to the colorless parent compound. Early work by Mees (1960) showed that oxygen was necessary for the herbicidal activity of bipyridylium compounds. These studies demonstrated that such agents could not kill plant leaves when there was no oxygen present, despite the continuation of photosynthetic reactions capable of generating the free radical. Later experiments suggested that the paraquat free radical may transfer its extra electron to molecular oxygen to form superoxide radical (Farrington, 1973). There was evidence that the superoxide radical may persist long enough to diffuse to cell membranes where membrane damage may be initiated. Hydrogen peroxide has also been proposed as the membrane damaging agent (Conning *et al.*, 1969; Thorneley, 1974). The possibility that lipid peroxidation of cell membranes might be the damaging lesion caused by paraquat was indicated by experiments which found

increased levels of malondialdehyde (a by-product of lipid peroxidation) in plant leaves six hours after paraquat exposure (Dodge *et al.*, 1970).

In animals, the pulmonary lesion caused by paraquat has been postulated to be a result of disruption of pulmonary surfactant, and thereby to closely resemble human respiratory distress syndrome (Manktelow, 1968; Robertson *et al.*, 1971). Both groups of investigators found decreased pulmonary surfactant activity associated with the formation of alveolar hyaline membranes after acute paraquat administration. Further investigations have shown that paraquat induced a specific decrease in the lecithin fraction of lung surfactant, which led to the suggestion that the pulmonary atelectasis associated with paraquat poisoning may be due to an increase in alveolar surface forces (Fisher *et al.*, 1973b; Malmqvist *et al.*, 1973). In other reports, however, no changes after paraquat were observed in pulmonary phospholipid fractions (Fletcher and Wyatt, 1970) or in the synthesis or destruction of lung dipalmitoyl lecithin (Fletcher and Wyatt, 1972) indicating that paraquat does not interfere with phospholipid metabolism.

In 1968, Gage demonstrated the production of the blue-colored paraquat free radical when paraquat was incubated *in vitro* with rat liver microsomes and NADPH. It was further shown that the cyclic reduction-oxidation of paraquat was associated with an increase in malondialdehyde production in microsomal phospholipids. Recently, the link of oxygen to the herbicidal activity of paraquat has likewise been demonstrated to the animal toxicity of paraquat.

Paraquat induced lethality in rats was significantly enhanced by simultaneous exposure to 100% oxygen (Fisher *et al.*, 1973a) and was significantly decreased in mice with exposure to 10% oxygen (Rhodes, 1974). Davies and Davies (1974) demonstrated that paraquat incubated with rat liver microsomes and NADPH stimulated the oxidation of epinephrine to adrenochrome, which was an indication of the production of superoxide radicals. Furthermore, the oxidation of epinephrine was inhibited with the addition of superoxide dismutase. *In vivo* evidence for involvement of superoxide has been suggested from experiments in which paraquat induced lethality in rats was delayed by administration of superoxide dismutase, an enzyme which detoxifies superoxide radicals (Autor, 1974). The hypothesis that paraquat may cause pulmonary damage through oxidant reactions is further supported from observations in rats that paraquat stimulates the activity of lung glucose-6-phosphate dehydrogenase (Witschi and Kacew, 1974) and that sensitivity to paraquat is directly related to lung glucose-6-phosphate dehydrogenase activity (Ayers and Tierney, 1971). Glucose-6-phosphate dehydrogenase activity has been linked to the activity of glutathione reductase and glutathione peroxidase in combating oxidant stress (Chow and Tappel, 1974).

Other biochemical parameters have also been measured in order to further investigate the mechanism of paraquat toxicity. Krieger *et al.* (1973) demonstrated that paraquat inhibited the *in vitro* epoxidation of aldrin, causing a 50% inhibition at a concentration 7×10^{-4}M. Because of paraquat's ability to accept electrons, the

inhibition was proposed to be due to an interruption of microsomal electron transport processes. Paraquat has been shown to decrease rat but not rabbit lung cytochrome P-450 concentrations and bromobenzene metabolism by rat lung microsomes (Ilett *et al.*, 1974). A significant increase in protein synthesis has been reported in rat lung, liver and kidney after a toxic dose of paraquat, while DNA synthesis was depressed in the three organs (Van Osten and Gibson, 1975). The biochemical changes showed a time correlation with histopathological changes induced by paraquat. Paraquat also increased plasma corticosteroids in rats for 24 hours after administration while ACTH (adrenocorticotrophin) was elevated for only 4 hours, suggesting that paraquat may increase the response of the adrenal cortex to ACTH (Rose *et al.*, 1974a).

Recently, experiments *in vitro* have shown that lung slices accumulated paraquat by an apparent energy-dependent process. The lung slices were able to accumulate paraquat but not an analog, diquat, which correlated to the *in vivo* retention of these compounds by rat lung (Rose *et al.*, 1974b). Uptake studies of paraquat into the isolated perfused rabbit lung did not appear to be by an energy-dependent mechanism. Little or no uptake of paraquat was observed initially and the subsequent uptake of paraquat in the perfused lung paralleled the development of edema, indicating that paraquat may be trapped in the damaged lung tissue (Orton *et al.*, 1973). However, the rabbit has been described as unusually resistant to the development of the paraquat pulmonary lesion (Butler and Kleinerman, 1971) and thus account for the differences between the two studies.

Purpose

The purpose of this investigation was twofold: first, to determine the effects of chronically administered paraquat on the development of mice and to investigate subtle toxicities of paraquat that may exist in these animals; and second, to conduct experiments both *in vitro* and *in vivo* which will provide data for the formulation of a mechanism of toxicity of paraquat in mammalian systems.

METHODS

Animals

Animals used in all experiments were purchased from Spartan Research Animals, Inc., Haslett, Michigan. Female Swiss-Webster mice, 30-35 grams body weight, were used as breeding stock to obtain timed pregnancies in developmental studies. Female Swiss-Webster mice, 25-30 grams body weight, and male Sprague-Dawley rats, 150-200 grams body weight, were used in other experiments.

Toxicity of Chronically Administered Paraquat in Developing Mice

Mice were mated by placing 1 male in a cage of 5 females for 1 hour starting at 8 a.m. The day vaginal plugs were found was designated day 1 of gestation. Paraquat dichloride (paraquat concentrate, 240 mg cation/ml, Chevron Chemical Co., Richmond, California) was placed in the drinking water of pregnant females at concentrations of 0, 50, 100 and 150 ppm (parts per million, 1 mg paraquat per 1000 ml solution) beginning on day 8 of gestation, which is the onset of organogenesis in mice (Rugh, 1968). Pregnant females were housed individually in clear plastic shoebox cages and allowed access to food and the paraquat treated water *ad libitum*. Following delivery of pups on day 20 of gestation, litters were normalized to 10 mice. The total number of live pups born in

each treatment group was recorded. Total litter body weights and mortality were recorded at weekly intervals from day 1 postnatally to termination of the experiment at 42 days postnatally. Water consumption was also measured up to 42 days postnatally. All litters were weaned on day 28 postnatally and segregated by sex. Exposure of mice to paraquat treated water was continuous from day 8 of gestation to 42 days postnatally.

In other experiments, litters were either exposed to 100 ppm paraquat from day 8 of gestation to 28 days after birth (weaning) and then transferred to nonparaquat treated water until 42 days postnatally or placed on 100 ppm paraquat only between days 28 and 42 postnatally. Litter mortality rates were recorded at weekly intervals for each treatment group.

The stability of the paraquat solutions was confirmed by colorimetric assay (Sharp *et al.*, 1972). Two milliliters of an appropriate dilution of the paraquat solutions was added to 0.5 ml of sodium dithionite (sodium hydrosulfite, 0.2% in 1N sodium hydroxide), mixed and the absorbance immediately read at 395 nm in a Beckman DB-GT spectrophotometer (Beckman Instruments, Fullerton, California). Paraquat dichloride (methyl viologen, Sigma Chemical Company, St. Louis, Missouri) was used to prepare the standard curve. No change in paraquat concentrations was observed for up to 4 weeks after preparation.

Assay of Lung Tissue for Paraquat. Concentrations of paraquat in the lungs of 42 day old 50 and 100 ppm paraquat treated mice were determined by a colorimetric modification of the method of Ilett

et al. (1974). Lung tissue was homogenized in 4 ml distilled water with a Polytron^{R} homogenizer (Brinkman Instruments, Westbury, New York) followed by addition of 0.1 volume 10N hydrochloric acid. The acid-precipitated homogenate was centrifuged at 5000 xg for 20 minutes and the resultant supernatant transferred to a Dowex ion-exchange column made from a 1.0 ml slurry of a 1:1 mix of water-Dowex 50W-X8 cation ion resin (100-200 mesh, Bio-Rad Laboratories, Richmond, California). The previous precipitate was washed once with 2.0 ml 1N hydrochloric acid, centrifuged, and the supernatant added to the ion-exchange column. The column was subsequently washed with 20.0 ml distilled water and the paraquat eluted with 5.6 ml of 5M ammonium chloride. The paraquat eluate was brought to 6.0 ml with 1N sodium hydroxide with a final eluate pH of 10. A 2.0 ml aliquot of the eluate was assayed colorimetrically for paraquat by the method previously described.

Assay of Tissue for Reduced Glutathione (GSH). Liver GSH was measured in 42 day old 50 and 100 ppm paraquat treated mice by the fluorometric method of Cohn and Lyle (1966). Approximately 100 mg of liver tissue was homogenized (Polytron) in 4.0 ml of cold 30 μM EDTA (Versene, Dow Chemical Co., Midland, Michigan) to which 1.0 ml of cold 25% w/v metaphosphoric acid (HPO_3) was added and followed by centrifugation in the cold at 5000 xg for 10 minutes. An 0.01 ml aliquot was removed from the supernatant and added to 0.99 ml of cold distilled water and 0.5 ml of 0.1M sodium phosphate buffer, pH 8. To the previous solution, 0.1 ml of freshly prepared o-phthaldialdehyde (0.1% w/v in absolute methanol, Sigma) was added, the

solution allowed to set at room temperature for 15-20 minutes, and then transferred to quartz cuvettes and the fluorescence measured at 420 nm resulting from activation at 350 nm in an Aminco^{R} fluorometer (American Instrument Co., Silver Spring, Maryland). A standard curve was prepared from reagent GSH (Sigma).

Assay of Tissue for Malondialdehyde. Malondialdehyde was assayed in lung, liver and kidney tissue of 42 day old 100 ppm paraquat treated mice by a modification of the method of Placer *et al.* (1966). Tissues were homogenized (Polytron) in 20 volumes of cold normal saline and centrifuged in the cold for 5 minutes at 400 xg. An 0.2 ml aliquot of the supernatant was added to a 25 ml tube containing 1.3 ml of 0.2M TRIS-maleate buffer, pH 5.9, 1.5 ml of thiobarbituric acid reagent (0.8% w/v in 7% perchloric acid), and 0.15 ml ADP-Fe^{+++} (40 mM disodium adenosine diphosphate, Sigma, and 2.4 mM ferric ammonium sulphate) and placed in a boiling water bath for 10 minutes. Marbles were placed over the top of the tubes to prevent evaporation. After cooling for 10 minutes, 1.0 ml 1N sodium hydroxide and 3.0 ml of a 3:1 pyridine-butanol solution was added with a subsequent 15 second mix on a vortex blender. The absorbance of the resulting clear solution was read at 548 nm in a Spectronic 20^{R} (Bausch and Lomb, Rochester, New York). Calculations of malondialdehyde concentrations were made using an extinction coefficient of 1.52×10^{5}.

Histopathology. At 42 days, control, 50 and 100 ppm paraquat treated mice were sacrificed and lung, liver and kidneys fixed in

10% formalin. Five micron paraffin sections were prepared, stained with hematoxylin-eosin, and examined by light microscopy.

Exposure of Chronically Paraquat Treated Mice to Oxygen. Developing mice exposed to 0, 50 and 100 ppm paraquat beginning at day 8 of gestation were examined for sensitivity to 100% oxygen at 1 atmosphere on days 1, 28 and 42 postnatally. Oxygen exposure was accomplished by placing both control and treated mice in a clear plastic cage approximately 12 liters in volume (12x24x44 cm) with a plexiglass top and supplied with animal bedding, food and tap water. The one day old group was normalized to 10 newborns per mother and the mothers replaced after 48 hours of oxygen exposure with mothers which had recently littered to insure adequate nursing of the newborns. One hundred percent oxygen, which was humidified by flow over a water source prior to entry into the chamber, was maintained at a flow rate of 1.65 liters/min into the chamber. Oxygen concentrations in the chamber were measured to be 95 to 100% by a blood-gas analyzer (Radiometer, Copenhagen, Denmark).

The sensitivity of the 28 and 42 day old paraquat treatment groups to 100% oxygen was measured by determination of the LT_{50}, or median time to death after initiation of oxygen treatment as calculated by the method of Litchfield (1949). The interaction of oxygen with newborn mice was determined by recording the number of mice dead after 120 hours of oxygen exposure.

In other experiments, one day old mice obtained from timed pregnancies were injected with paraquat (methyliologen, Sigma), 5 mg/kg sc, and placed in 100% oxygen along with saline injected

controls. Other paraquat injected one day old mice were exposed only to room air after injection. The number of mice that died in either oxygen or room air was recorded after 30 hours exposure. Other one day old mice obtained from dams treated with 100 ppm paraquat were injected with vitamin E (d-α-tocopherol in soybean oil, type I, Sigma), 25 IU per mouse sc, and placed in 100% oxygen along with other 100 ppm paraquat treated and nonparaquat exposed newborns injected with oil carrier. The number of mice dead after exposure to 100 hours of 100% oxygen was recorded.

Interaction of Bromobenzene with Chronic Paraquat Treatment in Mice. The toxicity of bromobenzene in 42 day old 50 and 100 ppm paraquat treated mice was measured by determination of the bromobenzene LT_{50} (Litchfield, 1949). Bromobenzene (Aldrich Chemical Co., Milwaukee, Wisconsin) dissolved in peanut oil was administered at 3100 mg/kg ip [LD_{85}, based on the curve of the calculated LD_{50} of 1800 mg/kg (95% Confidence Interval, C.L.; 1333-2430)] and the time to death recorded.

Distribution of ^{14}C-Paraquat in Prenatal and Newborn Mice. Distribution of ^{14}C-paraquat in organs of prenatal mice was examined after a 3.35 mg/kg ip dose. Paraquat (methyl-^{14}C-paraquat, 36 mCi/mmole, Amersham-Searle, Arlington Heights, Illinois) was diluted with nonradioactive paraquat (Sigma) for a final specific activity of 60 μCi/mg and administered to pregnant mice on day 16 of gestation. At various times the dams were sacrificed and fetal tissues prepared for liquid scintillation counting by solubilization in

Soluene^{R} (Amersham-Searle) followed by addition of 15 ml of toluene counting solution [5 g of 2,5-diphenyloxazole (PPO) and 200 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl]benzene (dimethyl POPOP) per liter of toluene. For the purpose of comparing the distribution in late fetal and neonatal animals, ^{14}C-paraquat (36 mCi/mmole, Amersham-Searle, diluted with nonradioactive paraquat for a final specific activity of 10 µCi/mg) in one day old mouse organs was determined at various times after a 4.5 mg/kg sc dose of paraquat. Tissues were prepared for liquid scintillation counting as described above. Radioactivity was determined in a Packard 3380 liquid scintillation counter (Packard Instrument Company, Downers Grove, Illinois) and was converted to microgram levels of paraquat through use of external standard quench correction and assuming no *in vivo* metabolism of paraquat.

Paraquat *In Vitro* Oxidation-Reduction

Microsomes were prepared by cold homogenization of lung tissue from mice in 4 volumes of 1.15% potassium chloride containing 0.2% nicotinamide with 4 strokes of a loose fitting Teflon-glass Potter-type homogenizer (Gram, 1973). The homogenate was centrifuged at 15,000 xg for 20 minutes in the cold, the supernatant removed, poured through gauze and recentrifuged at 105,000 xg for 90 minutes. The supernatant was discarded and the microsomal pellet resuspended with 3 strokes of a Potter-type homogenizer in 0.05M tris buffer, pH 7.5, containing 50% glycerol. The final protein concentration was 4.6 mg/ml. The microsomal suspensions were divided into several

small vials, flushed with nitrogen, and stored at -20°C. Protein was assayed by the method of Lowry *et al.* (1951).

The aerobic oxidation of NADPH catalyzed by mouse lung microsomes in the presence of paraquat was determined in a room temperature incubation system containing 60 µg/ml microsomal protein, 1 x 10^{-4}M NADPH (Sigma) and varying amounts of paraquat buffered in pH 7.5, 0.15M potassium phosphate. The incubation volume was 2.0 ml. Following the addition of NADPH, changes in optical density were recorded at 340 nm in a Coleman model 124 spectrophotometer (Hitachi, Ltd., Tokyo, Japan). The effect of addition of antibody to liver microsomal NADPH-cytochrome c reductase (IgG, prepared from serum of rabbits immunized with purified rat liver NADPH-cytochrome c reductase by the method of Pederson *et al.*, 1973) on the aerobic oxidation of NADPH was determined in an incubation system identical to the one just described. The paraquat concentration was 2.5 mM. The oxidation of NADPH was also recorded upon the addition of IgG pre-immune serum.

In a related series of experiments, the anaerobic reduction of paraquat to the blue-colored paraquat radical catalyzed by mouse lung microsomes in the presence of NADPH was examined. Incubation conditions were identical to those in the aerobic experiments except that the incubations were carried out in an airtight cell flushed with oxygen-free nitrogen. The appearance of the paraquat free radical was measured at 395 nm in a Coleman 124 spectrophotometer. The effect of addition of antibody to NADPH-cytochrome c reductase or pre-immune serum to the incubation system was also examined.

Paraquat-Induced *In Vitro* Lipid Peroxidation

The ability of paraquat to initiate lipid peroxidation *in vitro* was investigated in an incubation system modified from Pederson and Aust (1973). Incubation mixtures contained 0.25M sodium chloride, 2.0 mM ADP, 0.12 mM ferric ammonium sulphate, 0.5 µmole/ml lipid phosphorus, 0.2 mM NADPH, 60 µg protein/ml rat liver microsomal NADPH-cytochrome c reductase and paraquat in 0.25M TRIS buffer, pH 6.8. The total incubation volume was 5.0 ml. The incubation was conducted at 37°C under room air in an oscillating Dubnoff incubator. Aqueous suspensions of lipid phosphorus were prepared by anaerobic sonication of extracted microsomal lipid isolated from rats by the method of Pederson and Aust (1973). Sonication was accomplished by transferring an aliquot of the lipid stock solution to a plastic tube, removing the chloroform-methanol with a stream of nitrogen, addition of nitrogen saturated buffer, capping of the tube under nitrogen and placement of the tube in an ice-cold water bath near the tip of the sonifier (Branson, model S125, Branson Instrument Co., Danbury, Connecticut) for 5 minutes at a sonication current of 10 amps. NADPH-cytochrome c reductase was prepared by the method of Pederson *et al.* (1973). The incubation reaction was initiated by addition of NADPH after a 2 minute preincubation period at 37°C. At 1, 3, 5 and 8 minutes later, 1.0 ml aliquots of the incubation reaction were removed to 2.0 ml of thiobarbituric acid reagent (15% trichloroacetic acid, 0.375% thiobarbituric acid and 0.25N hydrochloric acid) to which 0.01 volume of 2% butylated hydroxytoluene in ethanol had been added immediately prior to use.

Malondialdehyde was determined by heating the mixture for 15 minutes in a boiling water bath to develop the color followed by cooling for 10 minutes. After centrifugation at 1000 xg, the absorbance of the supernatant of the assay mixture was determined at 535 nm in a Coleman Jr. Spectrophotometer (Coleman Instrument Co., Maywood, Illinois). A malondialdehyde standard curve was prepared from malondialdehyde tetraethylacetal (Aldrich Chemical Co.).

The effect of superoxide dismutase (prepared from bovine erythrocytes by the method of Pederson and Aust, 1973) and 1,3-diphenylisobenzofuran (Aldrich Chemical Co.) on paraquat-induced lipid peroxidation was determined in incubations identical to those described above except for the addition of each agent separately or together to the reaction. Malondialdehyde was assayed in a similar fashion.

Tissue Malondialdehyde Concentrations in Mice after Acute Paraquat Treatment

Malondialdehyde concentrations were determined by the method previously outlined in "Assay of Tissue for Malondialdehyde" in mice after various doses of paraquat. Malondialdehyde was measured in mouse lung, liver and kidney either 3 and 7 days after 30 mg/kg ip (LD_{50}) paraquat or 4, 12 and 24 hours after an ip LD_{90} dose of 44 mg/kg.

Effect of Nutritional Deficiencies on Paraquat-Induced Lethality

The 7-day intraperitoneal LD_{50}, or statistically calculated dose lethal to 50% of the animals 7 days after a single injection

(Litchfield and Wilcoxin, 1949), was determined and used as a measurement of sensitivity to paraquat toxicity in mice. The paraquat (methyl viologen dichloride, Sigma) LD_{50} was determined in 4 groups of mice: control mice which received laboratory animal chow (Wayne Lab Blox, Anderson Mills, Maumee, Ohio); mice fed diets deficient in selenium (General Biochemicals, Chagrin Falls, Ohio) or vitamin E (Draper diet, General Biochemicals) for 5 weeks; and mice treated with diethyl maleate (Aldrich Chemical Co.), 1.2 ml/kg in peanut oil, ip, 30 minutes before paraquat. The paraquat LD_{50} was also determined in other groups of mice fed either the basal selenium deficient diet supplemented with 0.1 and 2.0 ppm selenium (sodium selenite, Alfa Inorganics, Beverly, Massachusetts) or the basal vitamin E deficient diet supplemented with 45 and 1500 mg/kg diet vitamin E (α-tocopherol succinate, Sigma). In each group of mice at least 6 doses of paraquat were used to determine the LD_{50}, with 6 mice per dose. The LD_{50} in the 45 mg/kg vitamin E supplemented group was determined using paraquat dichloride supplied by Dr. M. S. Rose, Imperial Chemical Industries, Ltd., Industrial Hygiene Research Laboratories, Alderley Park, Nr., Macclesfield Cheshire, SK10 4TJ, England. The LD_{50} in the 1500 mg/kg vitamin E supplemented diet was determined using paraquat supplied by Schwarz-Mann, Orangeburg, New York. Mice fed selenium diets were maintained on double-distilled water for the 5 week period. Mouse body weights were recorded at the onset and at the end of the 5 week feeding period.

Supplemented diets were prepared by dissolving appropriate amounts of sodium selenite in water and vitamin E in ethanol such

that 10 ml of solution was added to each kilogram of diet during blending (5 minutes in a Blakeslee diet blender, Blakeslee, Inc., Chicago, Illinois) to give the designated dietary concentration. Assay of diets for selenium found 0.40 ppm selenium in Wayne Lab Blox, 0.01 ppm selenium in the selenium deficient diet and 0.098 ppm selenium and 1.938 ppm selenium in the 0.1 ppm and 2.0 ppm selenium supplemented diets, respectively.

Glutathione (GSH) Peroxidase Assay. Liver and lung GSH peroxidase was assayed in mice fed selenium deficient and supplemented diets for 5 weeks. Soluble liver and lung fractions containing this enzyme were prepared by homogenization (Polytron) of liver in 10 volumes and whole lung in 9 ml of cold 0.05M sodium phosphate buffer, pH 7.0. The homogenate was centrifuged at 750 xg for 10 minutes and then for 65 minutes at 100,000 xg (Chow and Tappel, 1974). The supernatant was assayed for GSH peroxidase by the method of Paglia and Valentine (1967). The assay of GSH peroxidase is coupled to the oxidation of NADPH in that GSH peroxidase reduces hydrogen peroxide with simultaneous formation of oxidized glutathione which, in the presence of an excess of GSH reductase, is reduced back to GSH with oxidation of NADPH. Varying amounts of soluble supernatant were incubated at room temperature with 0.10 ml 0.0084M NADPH, 0.01 ml GSH reductase (100 eu/mg protein in 1.0 ml, Sigma), 0.01 ml 1.125M sodium azide (to inhibit catalase, Sigma), 0.10 ml 0.15M reduced glutathione (Sigma), 0.10 ml 0.0022M hydrogen peroxide, and sufficient volume of 0.05M potassium phosphate buffer, pH 7.6, to bring the total incubation volume to 3.0 ml. The

reaction was started by addition of the hydrogen peroxide and the disappearance of NADPH followed on a Beckman DB-GT recording spectrophotometer at 340 nm. Activity of the enzyme was expressed as nmoles of NADPH oxidized/minute/mg protein from calculations of NADPH concentrations based on an extinction coefficient for NADPH of 6.22×10^6 (Langdon, 1966). Protein was assayed by the method of Lowry (1951).

Tissue Reduced Glutathione after Diethyl Maleate. GSH concentrations were assayed in mouse lung and liver at various times after 1.2 ml/kg ip diethyl maleate by the method outlined previously in "Assay of Tissue for Reduced Glutathione." This was to confirm that diethyl maleate, which reduces tissue GSH by a conjugation reaction and subsequent excretion into the urine (Boyland and Chasseaud, 1970), was reducing tissue GSH at the dose used in the pretreatment regimen.

Erythrocyte Hemolysis by Dialuric Acid. The vitamin E status of mice fed vitamin E deficient or supplemented diets for 5 weeks was assessed through use of the dialuric acid erythrocyte hemolysis assay (Friedman *et al.*, 1958). Blood was obtained by cardiac puncture from an ether anesthetized mouse and a 0.02 ml aliquot transferred to 5.0 ml of saline-phosphate buffer (1:1 mix of 0.1M dibasic sodium phosphate buffer, pH 7.4, and normal saline) in a 6 ml glass tube, mixed by inversion, and centrifuged at 500 xg for 10 minutes. The supernatant was removed and the erythrocytes resuspended in 4.5 ml of saline-phosphate buffer. Aliquots of 1.0

ml of suspended erythrocytes were transferred to each of 3 10 ml glass tubes containing the following: tube 1, 1.0 ml of a 0.01 mg/ml solution of dialuric acid in saline phosphate buffer; tube 2, the same as tube 1; and tube 3, 1.0 ml of saline-phosphate buffer. In the assay of the vitamin E supplemented mice, tubes 1 and 2 contained 0.05 mg/ml dialuric acid in saline-phosphate buffer. Duplicates were run with tube 1. All tubes were incubated at 37°C for one hour in a slowly oscillating Dubnoff incubator followed by incubation at room temperature for one hour. All tubes were inverted once every 15 minutes over the period of the incubation to insure the erythrocytes remained in suspension. Following the incubation, 5.0 ml saline-phosphate buffer was added to tubes 1 and 3 and distilled water to tube 2. Thus, tube 1 served as the variable hemolysis, tube 2 the total hemolysis, and tube 3 the basal hemolysis. The tubes were mixed gently, centrifuged, and the supernatant absorbance read at 415 nm in a Beckman DB-GT spectrophotometer. Erythrocyte hemolysis was calculated by dividing the difference in absorbance of tube 1 and 3 by the absorbance difference of tube 2 and 3.

Histopathology. Sections of lung and liver of mice fed selenium diets were prepared for light microscopic examination as previously described in "Histopathology."

Tissue Reduced Glutathione and Lipid Soluble Antioxidant Concentrations after Acute Paraquat Treatment

The concentrations of GSH in mouse lung and liver were determined at 12, 24, 36 and 48 hours after a 30 mg/kg ip dose

(LD_{50}) of paraquat (Sigma) by the method described in "Assay of Tissue for Reduced Glutathione."

Lipid soluble antioxidants were measured in lung and liver of mice by a modification of the colorimetric method of Glavind (1963). The principle of the assay is based on the decolorization of the violet-colored free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma) upon acceptance of an electron from antioxidants in an assay solution. Lipid soluble antioxidants were measured at various times after a 30 mg/kg ip (LD_{50}) dose of paraquat and 12 hours after ip doses of 25 mg/kg (LD_{25}), 30 mg/kg (LD_{50}) and 44 mg/kg (LD_{90}). Wet lung weights were recorded at the time of sacrifice. In other experiments, percent water content of control and paraquat treated lungs was determined after oven drying. Whole lungs or 300-400 mg of liver were removed to ice-cold saline and subsequently homogenized (Polytron) in 10 ml nitrogen saturated chloroform-methanol (2:1) in the presence of 200-300 mg of anhydrous sodium sulfate. The homogenates were filtered with suction and the filtrate evaporated under nitrogen to a volume proportional to the tissue weight. The homogenates and solutions were kept on ice until the reaction with DPPH, which was done at room temperature. One milliliter of 8 mg% DPPH in chloroform was added to 3.0 ml of tissue extract or solvent blank and after a 20 minute room temperature incubation, the absorbance read at 517 nm in a Beckman DB-GT spectrophotometer. The sample was then decolorized by addition of 2 drops of 0.5% pyrogallol (Sigma) in absolute ethanol and the absorbance redetermined at 517 nm. The difference in the initial

absorbance and the decolorized absorbance of the sample were designated G_s. The difference in the initial and decolorized absorbance of the solvent reference was designated G_r. The decrease in absorbance induced by the sample was calculated from the difference of $G_r - G_s$. Calculations of tissue lipid soluble antioxidants were based on a standard curve prepared with vitamin E standards (d-α-tocopherol, type I, Sigma). Addition of known amounts of vitamin E to homogenates and subsequent assay resulted in a vitamin E recovery of 101.0 ± 3.1%.

Interaction of Paraquat with the GSH Peroxidase System Enzymes

Effect of Chronic Paraquat Treatment on Enzyme Activity. Male rats were allowed access to 100 ppm paraquat *ad libitum* for 3 weeks in the drinking water, with housing in clear plastic cages in groups of four. Rats were killed at the end of the 3 week exposure period by a blow to the head. Lungs were perfused *in situ* with ice-cold saline, removed, and homogenized in the appropriate buffer. Liver was removed with perfusion and homogenized. GSH peroxidase was assayed in lung and liver tissue by the method described previously in "Glutathione (GSH) Peroxidase Assay." Assays for GSH reductase and glucose-6-phosphate dehydrogenase are described below.

GSH Reductase Assay. Rat lung and liver soluble fractions were prepared as described in "Glutathione (GSH) Peroxidase Assay." GSH reductase was assayed by the method of Racker (1955) with an incubation system containing 0.10 ml soluble fraction, 0.10 ml 0.0084M

NADPH, 0.10 ml 3% bovine serum albumin (Sigma), 0.10 ml 6% (w/v) oxidized glutathione (Sigma) and 2.60 ml 0.05M dibasic potassium phosphate buffer, pH 7.6, for a total 3.0 ml volume. The incubation was conducted at room temperature and initiated by addition of oxidized glutathione with rapid mixing by inversion. The oxidation of NADPH was followed at 340 nm in a Beckman DB-GT recording spectrophotometer. The activity of the enzyme was expressed as nmoles NADPH oxidized/minute/mg protein using an extinction coefficient for NADPH of 6.22×10^6. Protein was assayed by the method of Lowry (1951).

Glucose-6-Phosphate Dehydrogenase Assay. Rat soluble lung fractions were prepared as described in "Glutathione (GSH) Peroxidase Assay." Glucose-6-phosphate dehydrogenase was measured by the method of Langdon (1966) in a room temperature incubation system containing 0.10 ml 1M TRIS-hydrochloride buffer, pH 7.5, 0.10 ml 2×10^{-2}M glucose-6-phosphate (Sigma), 0.10 ml 2×10^{-3}M NADP (Sigma), 0.10 ml 0.2M magnesium chloride, 0.10 ml soluble fraction and a sufficient volume of distilled water to yield a final incubation volume of 3.0 ml. The reaction was started by the addition of glucose-6-phosphate and the formation of NADPH recorded at 340 nm in a Beckman DB-GT recording spectrophotometer. The enzyme activity was expressed as µmoles NADP reduced/minute/mg protein using 6.22×10^6 as the extinction coefficient. Protein was assayed by the method of Lowry (1951).

Cross-Tolerance of Oxygen with Paraquat. Male rats were placed in 85% oxygen for 7 days. Oxygen exposure was accomplished by

placing 4 rats in the oxygen chamber described in "Interaction of Oxygen with Paraquat Treated Mice" in which an 85% oxygen concentration was achieved by mixing 99.9% pure oxygen (2.78 liters/minute) and 90% pure nitrogen (0.47 liters/minute) through gas flowmeters. The chamber air was determined to be 86% oxygen by analysis in a blood-gas analyzer (Radiometer). The plastic chamber was cleaned and fresh food added every 36 hours. Control rats were exposed to room air. Following 7 days of exposure, control and 85% oxygen exposed rats were injected with 45 mg/kg ip paraquat (Schwarz-Mann) and the time at death recorded. The paraquat LT_{50} was calculated by the method of Litchfield (1949).

Interaction of Phenobarbital with Paraquat

Mice were maintained in clear plastic shoebox cages with access to food and 0.1% phenobarbital water (0.1% w/v sodium phenobarbital, pH 7.5, Mallinckrodt, St. Louis, Missouri) *ad libitum* for 10 days. The paraquat (Schwarz-Mann) LD_{50} (Litchfield and Wilcoxin, 1949) was determined both immediately after the 10 day exposure with the mice allowed continued access to the phenobarbital and when the mice were transferred to tap water for 24 hours before paraquat administration with subsequent continued access to tap water. In other experiments, the paraquat LD_{50} was determined in mice pretreated with 50 mg/kg ip phenobarbital 30 minutes before paraquat.

Distribution of ^{14}C-Paraquat in Phenobarbital Treated Mice.

^{14}C-Paraquat, 30 mg/kg ip (Amersham-Searle, specific activity of

injected solution 1.3 μCi/mg), was administered to mice pretreated for 10 days with 0.1% phenobarbital in the drinking water. At various times after administration, blood was obtained from ether anesthetized mice by cardiac puncture and plasma separated by centrifugation. Simultaneously, samples of lung, liver and kidney were obtained. All samples and plasma were prepared for liquid scintillation counting as described in "Distribution of ^{14}C-Paraquat in Prenatal and Newborn Mice" and counted in a Packard 3380 liquid scintillation counter with quench correction.

Statistics

Statistical evaluation of the data was by the Student's *t*-test or analysis of variance (completely randomized design) with differences among means analyzed by the least significant difference method (Sokal and Rohlf, 1969). The level of significance was chosen as $p<.05$.

RESULTS

Toxicity of Chronically Administered Paraquat in Developing Mice

Paraquat, when placed in the drinking water of pregnant mice and continued at 50 and 100 ppm from day 8 of gestation to 42 days postnatally, did not alter the average litter body weight compared to controls at any time during the experiment (Figure 1). The average litter body weight on day 1 after birth was 1.69 $\pm$ 0.15 g, 1.69 $\pm$ 0.09 g and 1.95 $\pm$ 0.20 g for the controls, 50 and 100 ppm paraquat treated groups, respectively. At 42 days of age the weights were, respectively, 28.8 $\pm$ 0.4 g, 30.3 $\pm$ 0.8 g and 26.9 $\pm$ 1.7 g. Pregnant mice receiving 150 ppm paraquat died before delivery of newborns occurred (death usually by day 16 of gestation).

Total water consumption was not significantly altered among the 0, 50 and 100 ppm paraquat treated groups when measured over the 14 day period from day 28 postnatally (weaning) to 42 days postnatally (Table 1). The average daily dose of paraquat was calculated assuming an average body weight of 25 g and was 16 and 27 mg/kg/day for the 50 and 100 ppm paraquat treated mice, respectively.

One hundred ppm paraquat significantly increased postnatal mortality compared to 50 ppm paraquat and controls from day 7 after birth, when mortality was 33.3 $\pm$ 8.8%, up to a 42 day mortality of

Figure 1. Effect of 50 and 100 ppm paraquat in the drinking water from day 8 of gestation to 42 days after birth on the postnatal growth of mice. Each point is the mean mouse body weight within litters for three to four litters. There were 10 mice/litter on day 1.

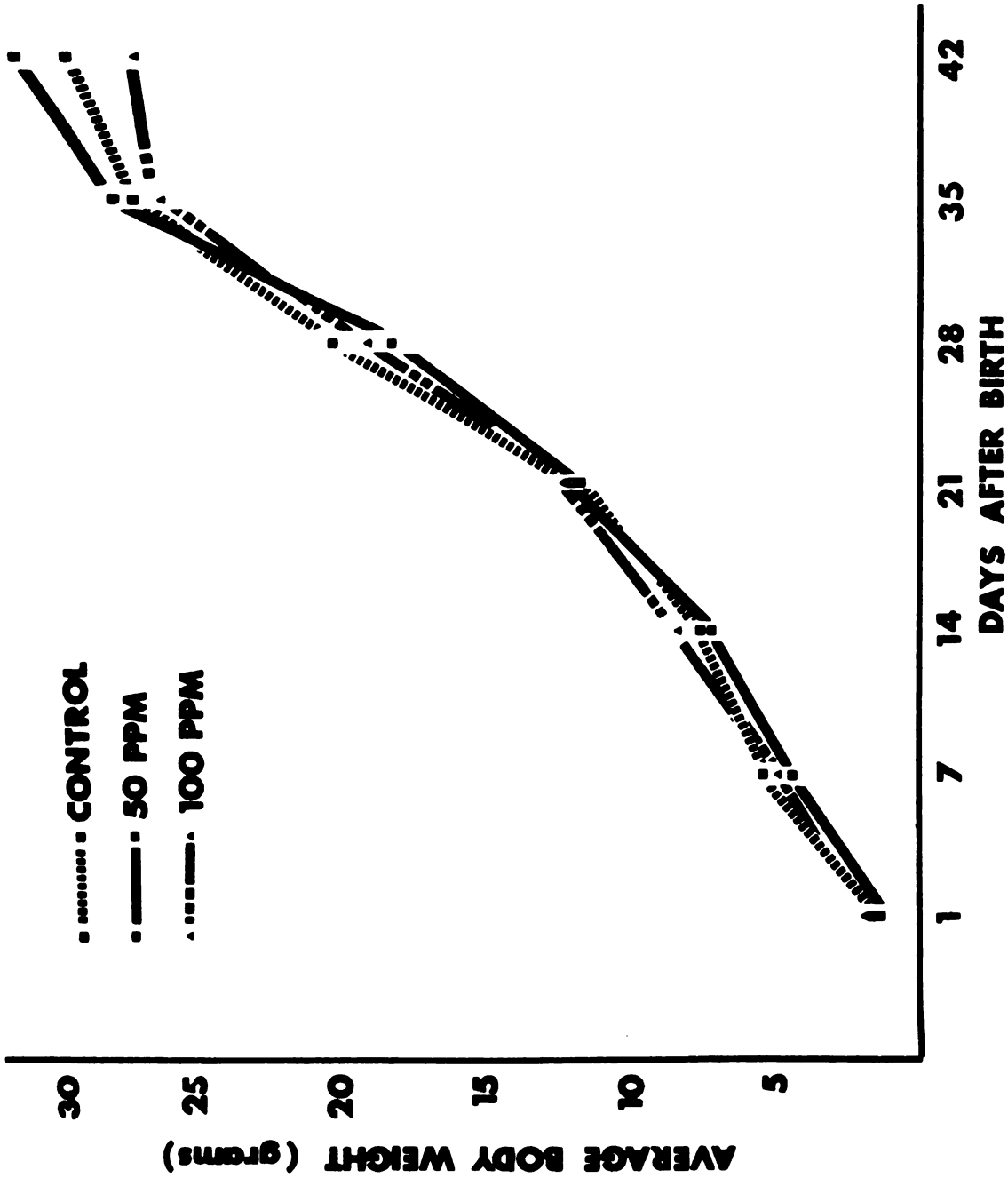

AVERAGE BODY WEIGHT (grams)
30
25
20
15
10
5
1
7
14
21
28
35
42
DAYS AFTER BIRTH
CONTROL
50 PPM
100 PPM

Figure 1

Table 1. Water consumption by developing mice treated with paraquat from day 28 to day 42 postnatally

Treatment	Water consumption: Total consumption[1] (ml/mouse)	Water consumption: Consumption/day (ml/mouse)	Approximate dose[2] (mg/kg/day)
Control	110 ± 5	7.8	---
50 ppm	118 ± 12	8.4	16
100 ppm	95 ± 4	6.8	27

[1]Mean ± S.E. for 3-4 litters; measured from day 28 to 42 postnatally.

[2]Assume average body weight of 25 g.

66.7 ± 3.3% (Figure 2). Mortality among the 50 ppm paraquat group and controls was not significantly different and remained below 7% over the course of the experiment. Mortality induced by 100 ppm paraquat appeared biphasic, with an initial rapid increase to 33.3 ± 8.8% during the first 7 days after birth, which then plateaued up to day 21 when mortality was 36.7 ± 8.8%. The plateau phase was followed by a second increase prior to weaning on day 28, reaching a final mortality of 66.7 ± 3.3% on day 35. Paraquat treatment did not affect the number of live pups born among the treatment groups, with 12 ± 1, 13 ± 1, and 11 ± 2 pups born per litter to the control, 50 ppm and 100 ppm treatment groups, respectively.

Experiments in which developing mice were exposed to 100 ppm paraquat from day 8 of gestation to 28 days postnatally and then transferred to tap water until 42 days after birth resulted in a

Figure 2. Effect of 50 and 100 ppm paraquat in the drinking water from day 8 of gestation to 42 days after birth on the post-natal mortality of mice. Each point is the mean percent mortality within litters for three to four litters. There were 10 mice/litter on day 1.

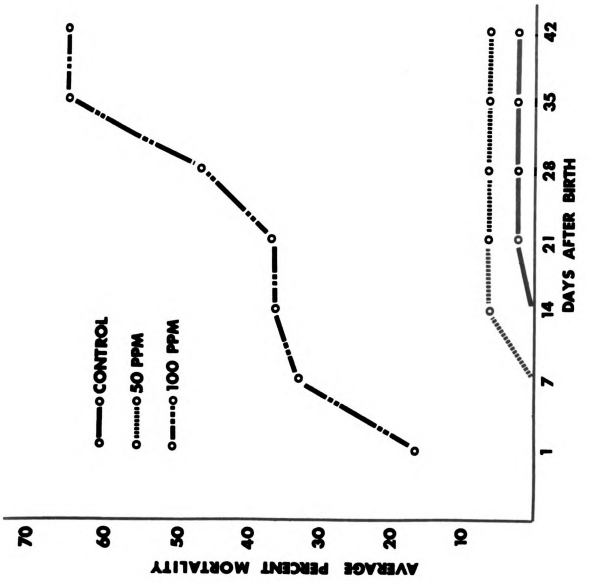

AVERAGE PERCENT MORTALITY
70
60
50
40
30
20
10
CONTROL
50 PPM
100 PPM
1
7
14
21
28
35
42
DAYS AFTER BIRTH

Figure 2

mortality of 26.7 ± 12.0% by day 28 (Table 2). This mortality level was not significantly different from the 28 day mortality (46.7 ± 12.0%) of mice continuously exposed to 100 ppm paraquat throughout

Table 2. Effect of 100 ppm paraquat on postnatal mortality in mice when administered in the drinking water during selected periods of development

Period of paraquat exposure	Percent postnatal mortality[1]	
	Day 28	Day 42
Day 8 of gestation to day 42 postnatally	46.7 ± 12.0	66.7 ± 3.3
Day 8 of gestation to day 28 postnatally[2]	26.7 ± 12.0	26.7 ± 12.0
Day 28 to day 42 postnatally[3]	0.0 ± 0.0	46.7 ± 14.5

[1]Mean ± S.E. of 3 litters, 10 mice/litter or day 1 postnatally.

[2]Tap water from day 28 to day 42 postnatally.

[3]Tap water from day 8 of gestation to day 28 postnatally.

development. Furthermore, transfer to tap water resulted in a 42 day mortality that was unchanged from day 28 and thus eliminated the second rapid increase in 100 ppm paraquat mortality depicted in Figure 2 (Table 2). Exposure of developing mice to 100 ppm paraquat only from days 28 to 42 postnatally resulted in 46.7 ± 14.5% mortality, which was not significantly different from the 30% increase in mortality observed in the second 100 ppm paraquat mortality phase of Figure 2 (Table 2).

Histopathology. Lung sections from 42 day old 100 ppm paraquat treated mice showed extensive alveolar consolidation and collapse, and areas of thickening of intra-alveolar septa. Edema fluid was seen in a few alveoli (Figure 3). Lung sections from 50 ppm and control mice did not show any significant pathological changes. Examination of liver and kidney sections of all treatment groups also did not reveal any significant pathological changes.

Tissue Malondialdehyde of 42 Day Old 100 ppm Paraquat Treated Mice. Assay of lung, liver and kidney tissue for malondialdehyde revealed no significant differences in the lipid peroxidation by-product compared to controls, when measured at 42 days postnatally (Table 3). Malondialdehyde levels were approximately equal in lung

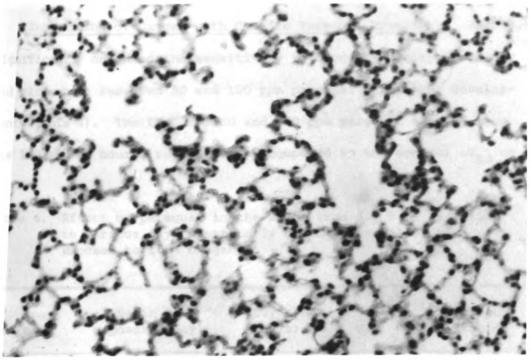
Table 3. Malondialdehyde concentrations in tissues of mice treated with 100 ppm paraquat from day 8 of gestation to 42 days postnatally

Tissue	Malondialdehyde concentrations[1] (nmoles MDA/g wet weight)	
	Control	100 ppm paraquat
Lung	374 ± 38	368 ± 19
Liver	402 ± 60	328 ± 24
Kidney	707 ± 39	776 ± 17

[1]Mean ± S.E. of 4 determinations.

and liver tissue while that in kidney tissue was approximately twice the levels observed in the other two tissues examined.

Figure 3. Lung tissue from 42 day old control mice and mice treated with 100 ppm paraquat from day 8 of gestation to 42 days postnatally. Upper panel is from control mice; lower panel from paraquat treated mice. Original magnification, x100.



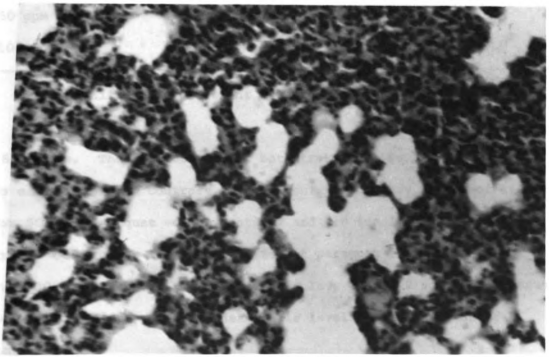


Figure 3

Interaction of Oxygen with Chronic Paraquat Treatment. Paraquat significantly enhanced the sensitivity to oxygen toxicity of 42 day old mice which received 50 and 100 ppm paraquat throughout development (Table 4). The LT_{50} for 50 and 100 ppm paraquat treated mice was 108 and 40 hours, respectively, compared to the control LT_{50} of

Table 4. Effect of paraquat in the water from day 8 of gestation to various days postnatally on the survival of mice exposed to 100% oxygen

Treatment	Begin O_2 exposure (day)	LT_{50} (hours)	95% C.L.	Potency ratio
Control	28	181	(156-210)	---
50 ppm	28	180	(142-229)	1.0
100 ppm	28	121	(108-135)	1.5[1]
Control	42	160	(126-203)	---
50 ppm	42	108	(81-144)	1.5[1]
100 ppm	42	40	(30- 54)	4.0[1]

[1]Significantly different from respective control, $p<.05$.

160 hours. The LT_{50} curves for both treatment groups were parallel to each other and controls, with calculated potency ratios of 1.5 for 50 ppm paraquat versus controls and 4.0 for 100 ppm paraquat versus controls. The concentration of paraquat in lung tissue of the 100 ppm paraquat treated mice was less than 0.2 µg/g lung tissue (wet weight) and was below detectable levels in 50 ppm paraquat lung tissue.

At 28 days after birth, an increased sensitivity to 100% oxygen exposure was detected only in 100 ppm paraquat treated mice (Table 4). The LT_{50} of 100 ppm paraquat treated mice was significantly reduced to 121 hours, compared to an LT_{50} of 180 hours for 50 ppm paraquat mice and 181 hours for control mice. A potency ratio of 1.5 was calculated for 100 ppm paraquat mice versus controls.

One day old mice, which were exposed to paraquat only by prenatal placental transfer, also were sensitized to oxygen toxicity (Figure 4). Mortality of one day old mice whose mothers received 100 ppm paraquat was significantly increased to 53.5 $\pm$ 7.0% after 120 hours oxygen exposure compared to 25.8 $\pm$ 5.1% and 24.2 $\pm$ 3.2% mortality in 50 ppm paraquat treated and control mice, respectively.

In other one day old mice whose mothers received 100 ppm paraquat in the drinking water from day 8 of gestation to day 19 of gestation, administration of subcutaneous vitamin E resulted in a nonsignificant but definite trend towards decrease in oxygen induced lethality (Table 5). After 100 hours of oxygen exposure, mortality in the nonparaquat treated control mice was 25.0%, in the 100 ppm paraquat treated one day old mice, 71.4%, and 27.3% in 100 ppm paraquat mice which received subcutaneous vitamin E.

One day old mice whose mothers received nontreated tap water during gestation were significantly sensitized to exposure to a 100% oxygen environment when the newborns were injected with paraquat, 5 mg/kg sc, immediately before entering the oxygen chamber (Table 6). Mortality after 30 hours of 100% oxygen was zero in water injected control mice but was 71.4% in paraquat treated newborns.

Figure 4. Effect of 50 and 100 ppm paraquat in the drinking water of pregnant mice from day 8 of gestation to day 19 of gestation on the survival of one day old mice exposed to 100% oxygen for 120 hours. Each treatment represents the mean percent mortality for three to four litters with 10 mice/litter on day 1. * indicates significantly different from control and 50 ppm paraquat, $p<.05$.

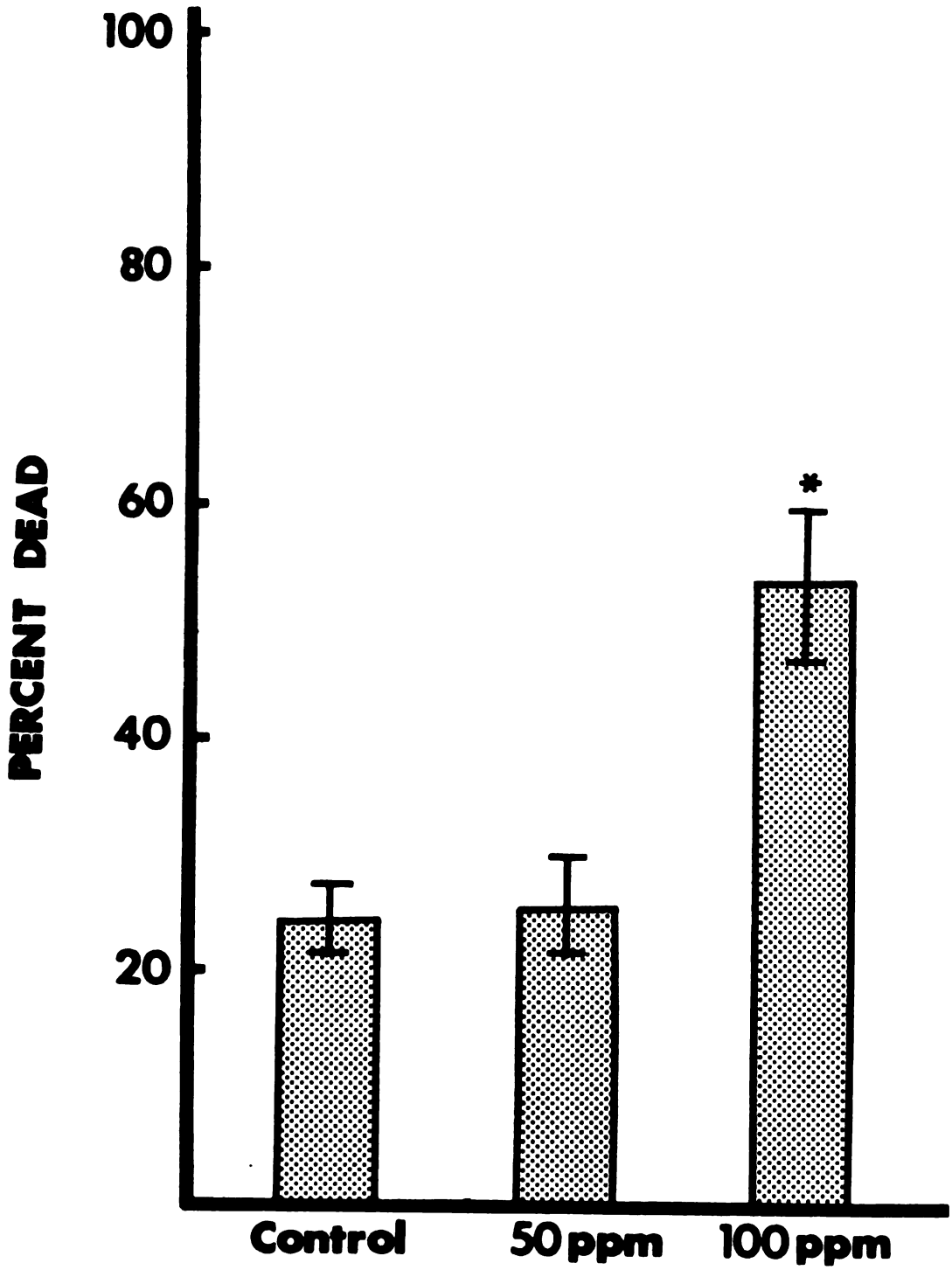


Figure 4

Table 5. Effect of vitamin E on survival of one day old mice pretreated with 100 ppm paraquat on days 8-19 of gestation in 100% oxygen

Treatment	No. mice	No. mice dead after 100 hr of O_2[1]	Percent dead
Control	20	5	25.0
100 ppm	7	5	71.4
100 ppm + vit. E[2]	11	3	27.3

[1] 100% oxygen at 1 atm.

[2] d-α-Tocopherol in soybean oil (type I, Sigma), 25 International Units per mouse, sc.

Table 6. Effect of acute paraquat administration to one day old mice on survival in 100% oxygen

Treatment	No. mice	No. mice dead after 30 hr O_2[1]	Percent dead
Control	32	0	0
Paraquat[2] + O_2	28	20	71.4[3]
Paraquat[2] + air	26	0	0

[1] 100% oxygen at 1 atm.

[2] 5 mg/kg, sc.

[3] Significantly different from controls, $p<.05$.

Newborn mice injected with paraquat, 5 mg/kg sc, and allowed to remain in room air also had no mortality 30 hours later.

Distribution of ^{14}C-Paraquat in Prenatal and Newborn Mice. The elimination of radioactivity from fetal mouse organs following maternal administration of ^{14}C-paraquat at 3.35 mg/kg ip on day 16 of gestation was similar in fetal lung, liver and kidney (Figure 5). No significant retention or elevation of paraquat occurred in fetal mouse lung tissue compared to fetal liver and kidney for 72 hours after paraquat administration. In one day old newborn mice given paraquat, however, radioactivity in neonatal lung was significantly elevated compared to liver and kidney from 8 hours to 96 hours after paraquat administration (Figure 6). Furthermore, detectable levels of paraquat in neonatal lung were observed up to 96 hours after treatment, which was 24 hours after paraquat could no longer be detected in neonatal liver and kidney.

Paraquat *In Vitro* Oxidation-Reduction

Mouse lung microsomes catalyzed the oxidation of 6 nmoles of NADPH per minute per mg protein. In the presence of 1.0 mM paraquat this value increased to 62 nmoles of NADPH oxidized per minute per mg protein. Under aerobic conditions, NADPH oxidation rates (measured at 340 nm) increased linearly with increasing paraquat concentrations, with a maximal oxidation rate reached at 1.5 mM paraquat (Figure 7). Under anaerobic conditions, in which the appearance of the blue-colored paraquat radical was measured at 395 nm, paraquat reduction by mouse lung microsomes and NADPH also

Figure 5. Concentration of paraquat in fetal mouse organs at various times following 3.35 mg/kg ip ^{14}C-paraquat on day 16 of gestation. Each point represents the mean tissue concentration obtained from 3 fetal litters, one pooled litter/determination.

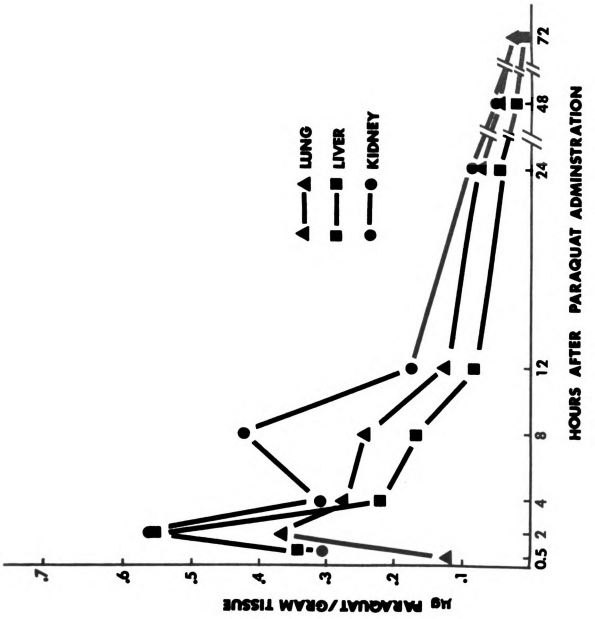

LUNG
LIVER
KIDNEY
μg PARAQUAT/GRAM TISSUE
.7
.6
.5
.4
.3
.2
.1
0.5
2
4
8
12
24
48
72
HOURS AFTER PARAQUAT ADMINISTRATION

Figure 5

Figure 6. Concentration of paraquat in one day old mouse organs at various times following 4.5 mg/kg sc ^{14}C-paraquat. Each point represents the mean of 3 determinations, 3-5 animals/ determination.

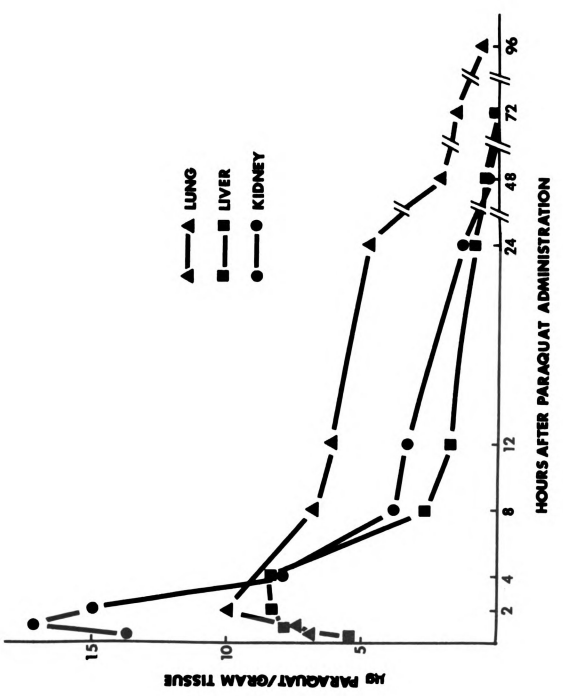

LUNG
LIVER
KIDNEY
15
10
5
µg PARAQUAT/GRAM TISSUE
2
4
8
12
24
48
72
96
HOURS AFTER PARAQUAT ADMINISTRATION

Figure 6

Figure 7. Aerobic oxidation of NADPH by mouse lung microsomes in the presence of paraquat. The incubation mixture contained 1 x 10^{-4}M NADPH, 60 µg/ml lung microsomal protein, and varying concentrations of paraquat.

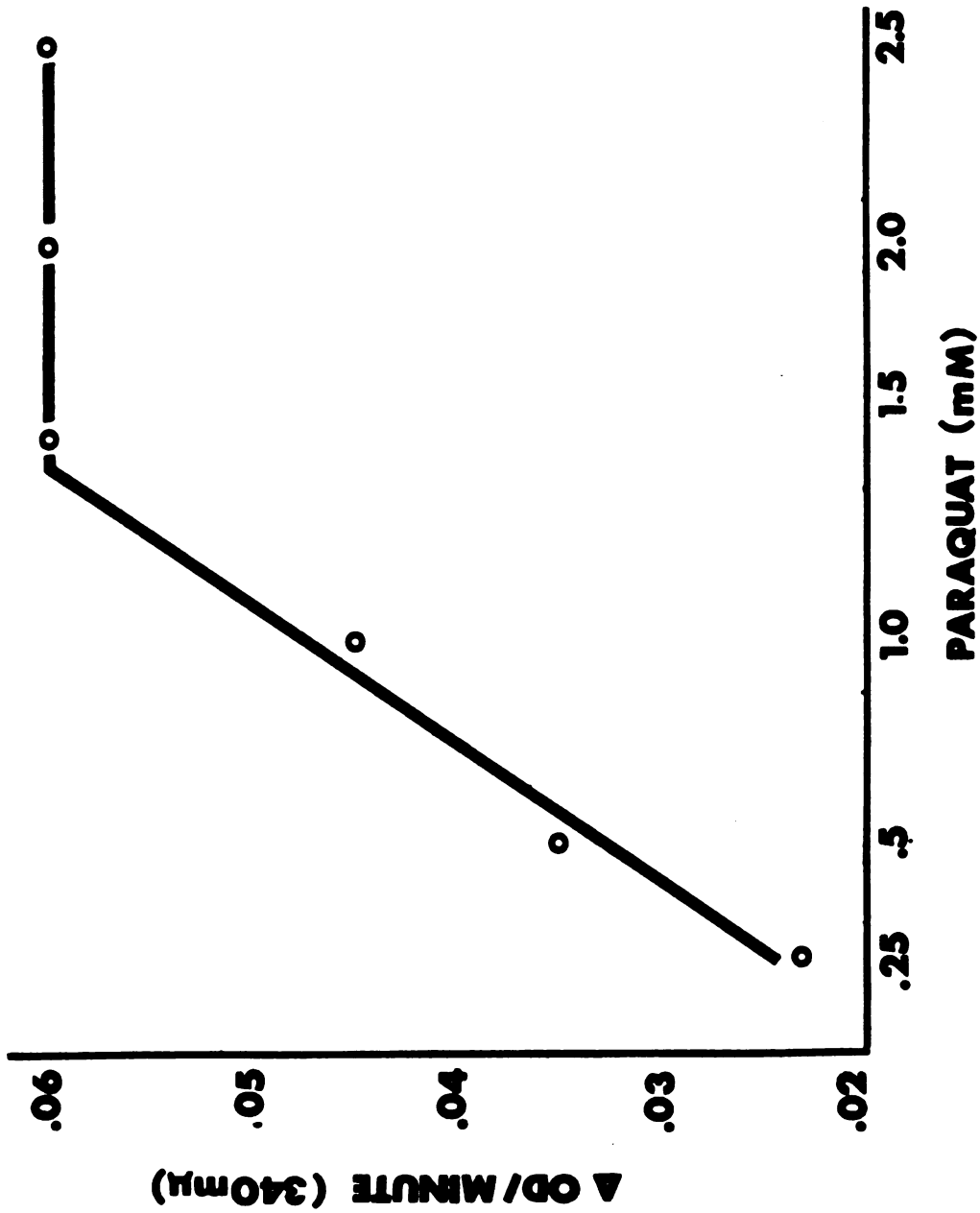


Figure 7

increased linearly with increasing paraquat concentrations, with a maximal reduction rate reached at 1.25 mM paraquat (Figure 8).

In other experiments, antibody prepared against rat liver microsomal NADPH-cytochrome c reductase inhibited the oxidation of NADPH catalyzed by mouse lung microsomes in the presence of paraquat (Figure 9). Under anaerobic conditions, the antibody inhibited the production of the paraquat radical (Figure 10). Substitution of pre-immune serum for antibody in both the aerobic and anaerobic experiments did not inhibit either the aerobic oxidation of NADPH or the anaerobic appearance of the paraquat radical (Figures 9 and 10). This indicated that the added antibody was not causing a non-selective inhibition of the respective reactions, but rather was specific for microsomal NADPH-cytochrome c reductase.

Paraquat-Induced *In Vitro* Lipid Peroxidation

Incubation of paraquat with rat liver NADPH-cytochrome c reductase, NADPH, and microsomal lipid significantly increased malondialdehyde formation in a concentration dependent manner compared to the no paraquat basal rate (Table 7). Malondialdehyde formation was increased 227% at 10^{-4}M paraquat concentration.

Addition of either superoxide dismutase or 1,3-diphenylisobenzofuran, a singlet oxygen trapping agent (Adams and Wilkinson, 1972), to the *in vitro* incubation inhibited malondialdehyde formation in a concentration related manner (Table 8). When superoxide dismutase and 1,3-diphenylisobenzofuran were incubated together *in vitro*, the resulting inhibition of paraquat-induced malondialdehyde formation

Figure 8. Anaerobic reduction of paraquat in the presence of mouse lung microsomes and NADPH. The incubation mixture contained 60 µg/ml lung microsomal protein, 1 x 10^{-4}M NADPH and varying concentrations of paraquat.

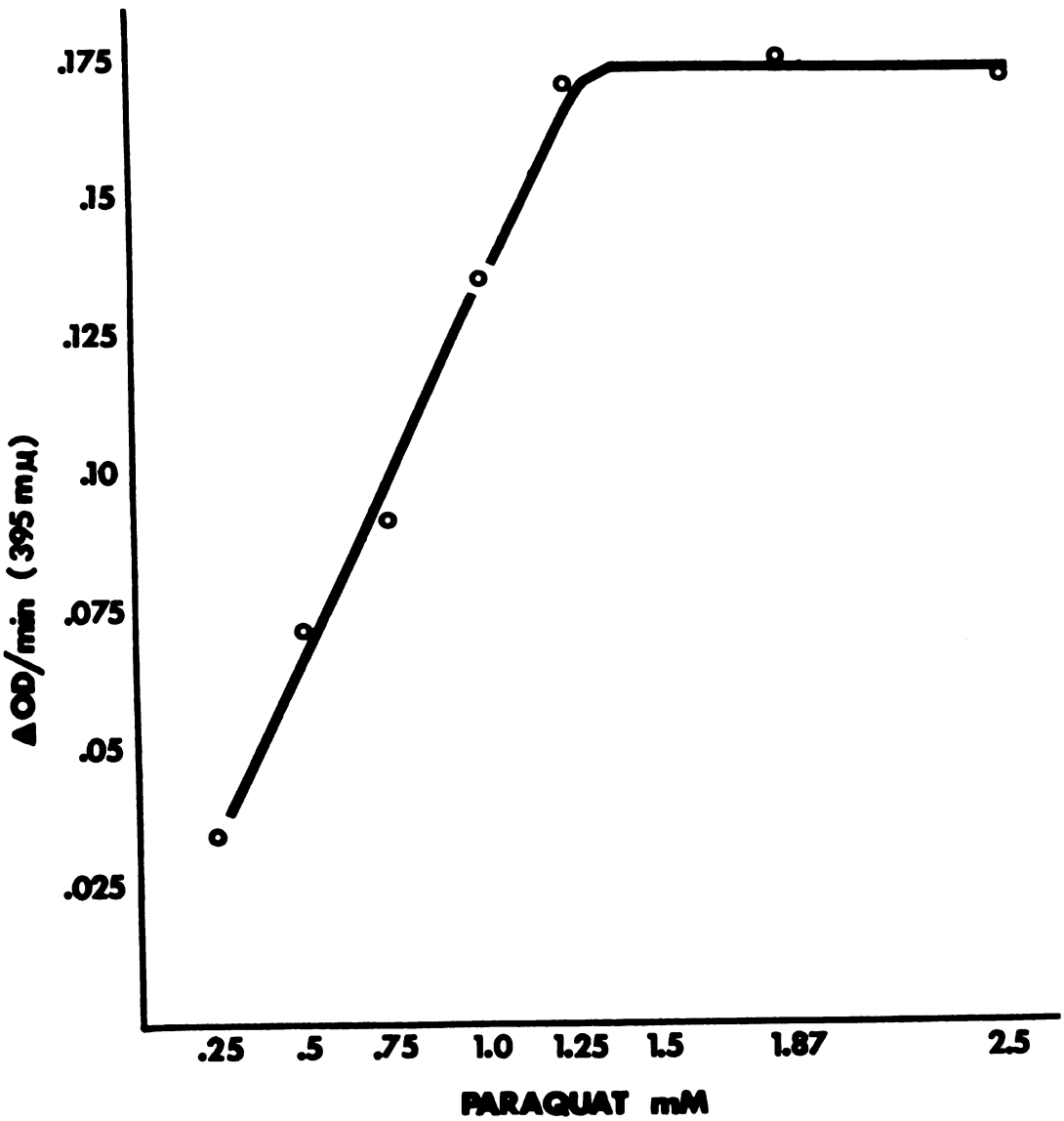

.175
.15
.125
.10
.075
.05
.025
ΔOD/min (395 mμ)
.25
.5
.75
1.0
1.25
1.5
1.87
2.5
PARAQUAT mM

Figure 8

Figure 9. Inhibition of the aerobic oxidation of NADPH catalyzed by mouse lung microsomes and paraquat by antibody to NADPH-cytochrome c reductase. The incubation contained 1 x 10^{-4}M NADPH, 60 µg/ml lung microsomal protein, 2.5 mM paraquat, and varying amounts of either NADPH-cytochrome c reductase antibody or antibody pre-immune serum.

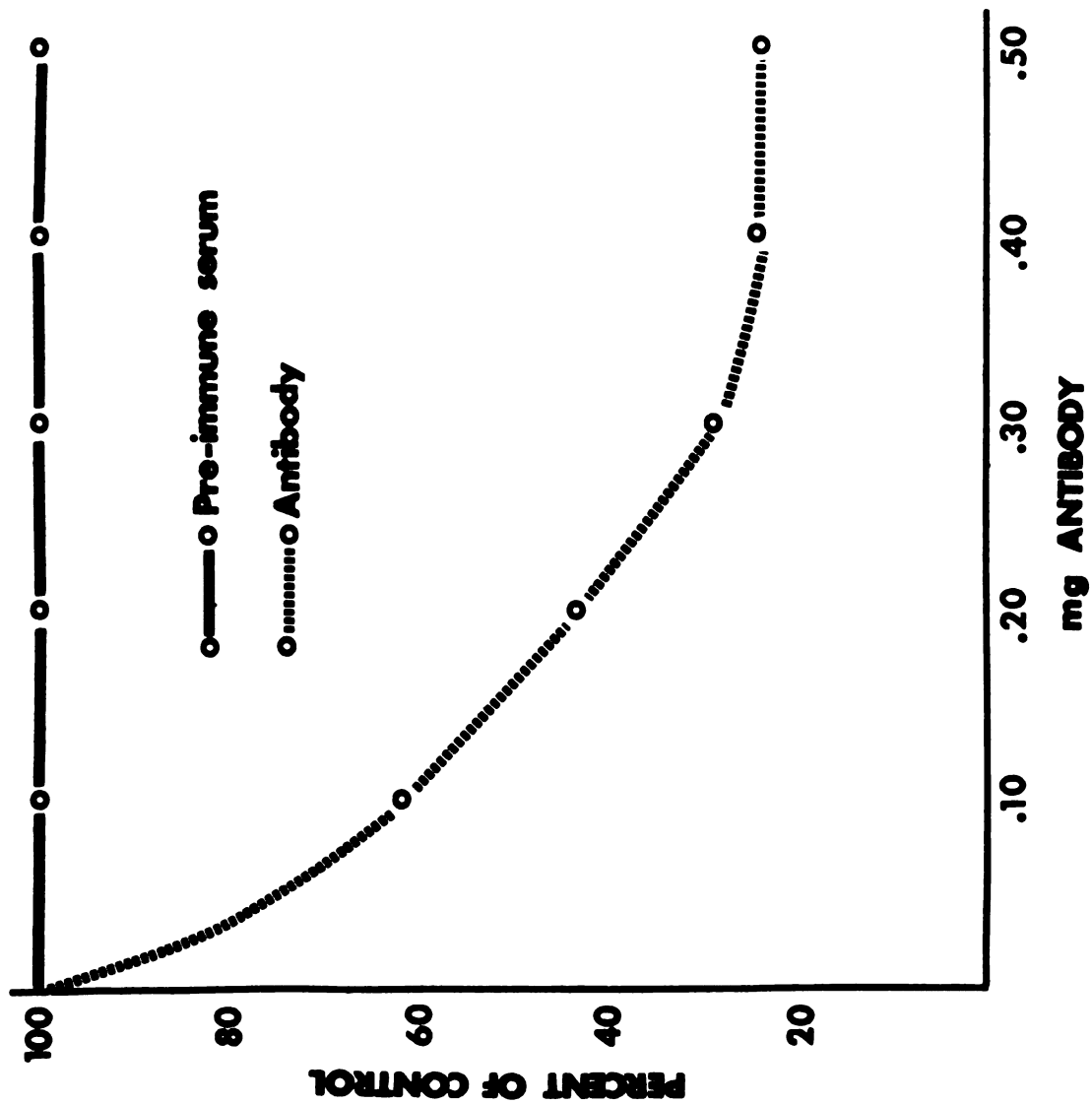

Pre-immune serum
Antibody
PERCENT OF CONTROL
100
80
60
40
20
.10
.20
.30
.40
.50
mg ANTIBODY

Figure 9

Figure 10. Inhibition of the anaerobic reduction of paraquat catalyzed by mouse lung microsomes and NADPH by antibody to NADPH-cytochrome c reductase. The incubation contained 1 x 10^{-4}M NADPH, 60 µg/ml lung microsomal protein, 2.5 mM paraquat, and varying amounts of either NADPH-cytochrome c reductase antibody or antibody pre-immune serum.

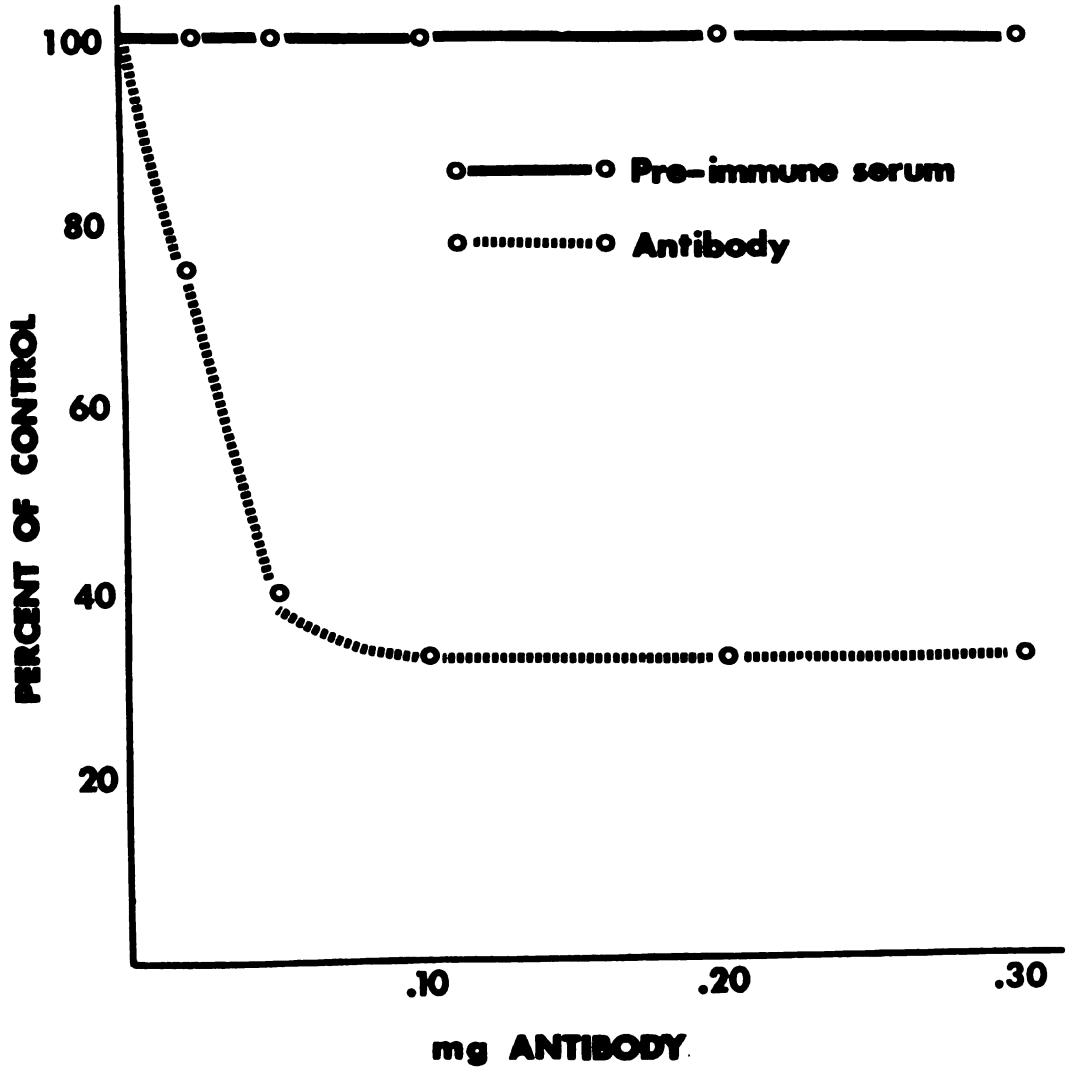

100
80
60
40
20
PERCENT OF CONTROL
Pre-immune serum
Antibody
.10
.20
.30
mg ANTIBODY

Figure 10

Table 7. Paraquat-induced *in vitro* lipid peroxidation*

Paraquat concentration per incubation mixture	Malondialdehyde formed[1] (nanomoles/min/ml)	Percent increase in malondialdehyde formed
0	0.37 ± .01	0
10^{-6}M	0.43 ± .03	16.2
10^{-5}M	0.60 ± .02[2]	62.2
10^{-4}M	1.21 ± .09[2]	227.0

*Incubation mixtures contained 0.25M NaCl, 2.0 mM ADP, 0.12 mM $Fe(NH_4)_2(SO_4)_2$, 0.5 moles/ml lipid phosphorus, 0.2 mM NADPH, 60 µg/ml liver microsomal NADPH-cytochrome c reductase, and paraquat in 0.25M TRIS buffer, pH 6.8. Incubations were conducted at 37°C in an oscillating Dubnoff incubator under air. Total incubation volume was 5.0 ml.

[1]Mean ± S.E. of 3 determinations.

[2]Significantly different from no paraquat, $p<.05$.

Table 8. Inhibition of paraquat-induced *in vitro* lipid peroxidation by superoxide dismutase and 1,3-diphenylisobenzofuran*

Incubation	Malondialdehyde formed (nanomoles/min/ml)	Percent of 10^{-4}M paraquat incubation
10^{-4}M paraquat	0.84 ± .09	---
Plus superoxide dismutase:		
20 µM	0.60 ± .06[2]	71.4
60 µM	0.28 ± .04[2]	33.3
Plus 1,3-diphenylisobenzofuran:		
2.0 µM	0.72 ± .02[2]	85.7
10.0 µM	0.45 ± .03[2]	53.6
Plus superoxide dismutase (20 µM) and 1,3-diphenylisobenzofuran (10.0 µM)	0.11 ± .02[2,3]	13.1

*Incubation conditions were identical to those given in Table 7 except as described above.

[1]Mean ± S.E. of 3 determinations corrected for no paraquat control.

[2]Significantly different from 10^{-4}M paraquat, $p<.05$.

[3]Not significantly different from basal rate, $p>.05$.

was greater than that achieved when either agent was incubated alone (Table 8).

Tissue Malondialdehyde Concentrations in Mice after Acute Paraquat Treatment

Administration of an LD_{50} dose of paraquat, 30 mg/kg ip, in mice did not induce any significant alterations in malondialdehyde concentrations in lung, liver and kidney tissues when measured 3 and 7 days after paraquat administration (Table 9). Malondialdehyde concentrations in the kidney tissue of these mice were elevated compared to lung and liver, which was similar to the observations in 42 day old mice found in Table 3.

Table 9. Malondialdehyde concentrations in tissues of mice after acute paraquat treatment

	Malondialdehyde (nmole MDA/g wet weight)[1]			
	3 days after paraquat[2]		7 days after paraquat[2]	
Tissue	Control	Treated	Control	Treated
Lung	483 ± 55	420 ± 34	420 ± 40	581 ± 145
Liver	477 ± 77	523 ± 90	443 ± 106	546 ± 38
Kidney	---	---	632 ± 102	759 ± 87

[1]Mean ± S.E. of 3 determinations.

[2]Paraquat, 30 mg/kg ip (LD_{50}).

When paraquat was administered at the LD_{90} dose of 44 mg/kg ip, however, malondialdehyde concentrations were significantly elevated in liver tissue at 12 and 24 hours after paraquat administration to

371 ± 34 and 278 ± 32% of controls, respectively (Figure 11). No change in malondialdehyde was observed at 4 hours in liver or at 4, 12 and 24 hours after paraquat in lung and kidney. The 12 and 24 hour groups of paraquat treated mice were extremely ill at the time of sacrifice and no attempts at eating or drinking were observed from a short time after injection until sacrifice.

Effect of Nutritional Deficiencies on Paraquat-Induced Lethality

Mice fed diets deficient in selenium or vitamin E for 5 weeks or pretreated with the tissue GSH reducing agent, diethyl maleate, resulted in paraquat LD_{50}'s of 10.4 mg/kg in selenium deficiency, 9.2 mg/kg in vitamin E deficiency, and 9.4 mg/kg with diethyl maleate pretreatment (Table 10). The paraquat LD_{50} in each of the 3 nutritionally deficient states was significantly decreased compared to the 30 mg/kg control LD_{50} (mice fed Wayne Lab Blox). The log-probability plots of the dose-lethality data for the various treatments yielded curves that were parallel to that of control. Supplementation of the basal selenium deficient diet with 0.1 and 2.0 ppm selenium (as sodium selenite) returned the paraquat LD_{50} to the control value (Table 10). Addition of 1500 mg/kg vitamin E to the basal vitamin E deficient diet also returned the paraquat LD_{50} to the control LD_{50} (Table 10). The LD_{50} in the 1500 mg/kg vitamin E supplemented group was determined with paraquat supplied by Schwarz-Mann, while the other previously described LD_{50}'s were determined with paraquat from Sigma. There were apparently no differences in the paraquat from the two suppliers as the Schwarz-Mann control LD_{50} was 30.0 mg/kg (26.3-34.2) and the two control

Figure 11. Tissue malondialdehyde concentrations in mice after acute paraquat treatment. Paraquat was injected at 44 mg/kg ip (LD_{90}). Each bar represents the mean $\pm$ S.E. of 4 determinations. An asterisk indicates significantly different from control, $p<.05$.

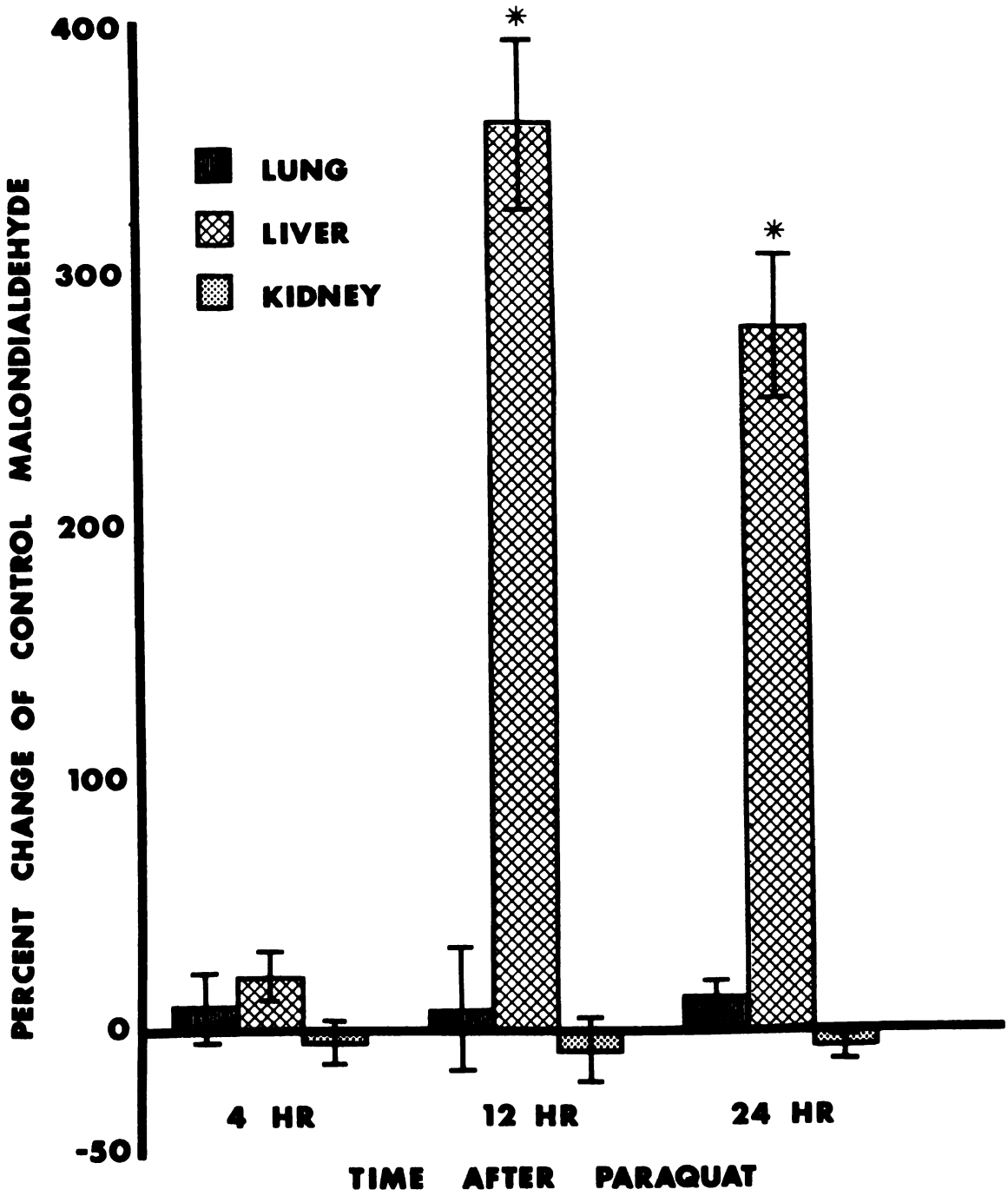

400
300
200
100
0
-50
PERCENT CHANGE OF CONTROL MALONDIALDEHYDE
LUNG
LIVER
KIDNEY
*
*
4 HR
12 HR
24 HR
TIME AFTER PARAQUAT

Figure 11

Table 10. Alteration of paraquat single dose 7-day intraperitoneal LD_{50} in mice by various selenium or vitamin E diets and diethyl maleate pretreatment

Treatment	Paraquat LD_{50} (mg/kg)	95% C.L.	Potency ratio
Control	30.0	(26.5-35.1)	---
Selenium deficient[1]	10.4	(8.9-12.2)	2.88[4]
0.1 ppm Selenium[2]	27.3	(24.8-30.0)	1.10
2.0 ppm Selenium[2]	25.5	(22.2-29.3)	1.18
Diethyl maleate[3]	9.4	(6.5-13.5)	3.20[4]
Vitamin E deficient[1]	9.2	(6.3-13.3)	3.26[4]
1500 mg/kg vitamin E[2]	29.0	(23.8-35.4)	1.03

[1] 5 week exposure prior to paraquat treatment.

[2] Basal deficient diet supplemented with selenium or vitamin E.

[3] 1.2 ml/kg, ip, 30 minutes before paraquat.

[4] Significantly different from control, $p<.05$.

LD_{50} curves were exactly superimposable. The paraquat LD_{50} was also determined in mice fed the basal vitamin E deficient diet supplemented with 45 mg/kg diet vitamin E using paraquat from Imperial Chemical Industries (ICI). The paraquat LD_{50} in this group was 19.4 mg/kg (16.7-22.5) and was not significantly different from the control ICI paraquat LD_{50} of 18.0 mg/kg (14.8-22.0). As the supply of Sigma paraquat was exhausted and the ICI paraquat did not give a biological response similar to Sigma paraquat, Schwarz-Mann paraquat was used in all subsequent experiments.

In mice fed selenium deficient diets, liver and lung GSH peroxidase activity was significantly reduced to 16.8 and 51.7%, respectively, of the comparative tissue GSH peroxidase activity in mice fed 2.0 ppm selenium supplemented diet (Table 11). GSH peroxidase activity was higher in lung tissue compared to liver within each given selenium diet.

Pretreatment of mice with diethyl maleate, 1.2 ml/kg, ip, resulted in a significant reduction in GSH concentrations for a minimum of 12 hours in lung and 4 hours in liver after diethyl maleate administration. The maximal depression of GSH concentrations in both lung and liver was approximately 35 to 40% of controls, occurring between 0.25 and 0.50 hours after injection of diethyl maleate (Table 12).

In mice fed vitamin E deficient diets, vitamin E deficiency was confirmed by the dialuric acid red blood cell hemolysis test, with 86.4% hemolysis in the deficient animals compared to 23.9% hemolysis in control mice. Supplementation of the diet with vitamin

Table 11. Liver and lung glutathione peroxidase activity in mice after 5 weeks exposure to selenium deficient, 0.1 ppm and 2.0 ppm selenium supplemented diets

Tissue and treatment	Glutathione peroxidase activity[1,2]	Percent of 2.0 ppm selenium diet
Liver		
selenium deficient	71.3 ± 3.0	16.8[3]
0.1 ppm selenium	366.7 ± 6.8	86.5[3]
2.0 ppm selenium	424.0 ± 16.0	---
Lung		
selenium deficient	344.7 ± 8.6	51.7[3]
0.1 ppm selenium	616.3 ± 45.0	92.4
2.0 ppm selenium	667.3 ± 100.0	---

[1]Mean ± S.E. of 4 determinations.

[2]nmoles NADPH oxidized/min/mg protein.

[3]Significantly different from 2.0 ppm selenium diet, $p<0.05$.

Table 12. Reduction of reduced glutathione (GSH) in mouse lung and liver by diethyl maleate treatment

Time after diethyl maleate[1] (hours)	GSH (percent of control)[2]	
	Lung	Liver
0.25	41.0 ± 2.1[3]	42.3 ± 5.3[3]
0.50	47.3 ± 3.1[3]	35.1 ± 2.4[3]
1.0	49.9 ± 1.2[3]	37.1 ± 1.6[3]
4.0	80.4 ± 8.1[3]	52.7 ± 3.7[3]
12.0	84.4 ± 5.4[3]	102.4 ± 1.9
24.0	100.9 ± 1.2	100.2 ± 0.5

[1] 1.2 mg/kg, ip.

[2] Mean ± S.E. of 3 determinations.

[3] Significantly different from control, $p<.05$.

E resulted in a dose related protection of the erythrocyte from dialuric acid induced hemolysis compared to control, with hemolysis of 48% of control in 45 mg/kg dietary vitamin E and 6% of control in 1500 mg/kg dietary vitamin E (Table 13).

Over the course of the 5 week feeding period, none of the experimental diets altered the weight gain of the dietary treated mice compared to control mice fed Wayne Lab Blox (Table 14).

Histopathological examination by light microscopy of lung (Figure 12), liver (Figure 13) and kidney of mice fed selenium deficient diet for 5 weeks revealed no pathological alterations. Microscopic examination of lung tissue from selenium deficient mice

Figure 12. Lung tissue from selenium deficient and paraquat treated selenium deficient mice. Upper panel is from selenium deficient mice; lower panel is from selenium deficient mice 24 hours after 11.9 mg/kg ip paraquat (LD_{70} in selenium deficient mice). Original magnification, x100.

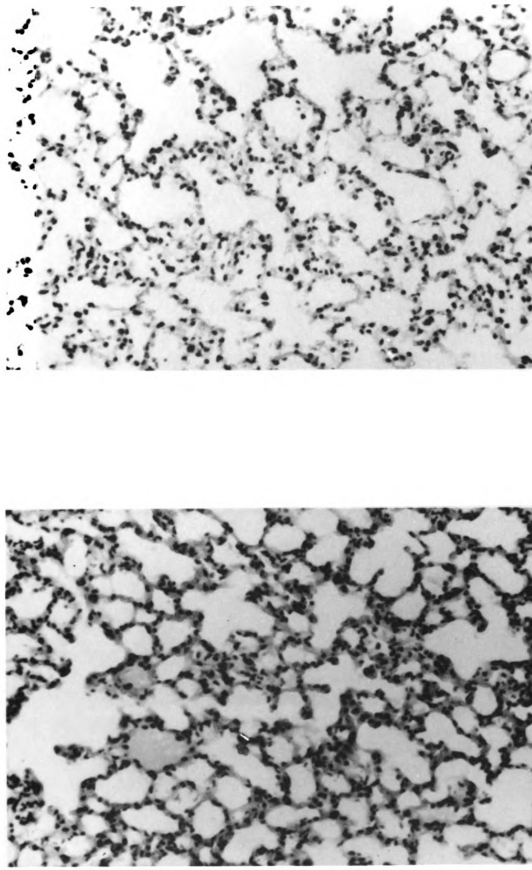
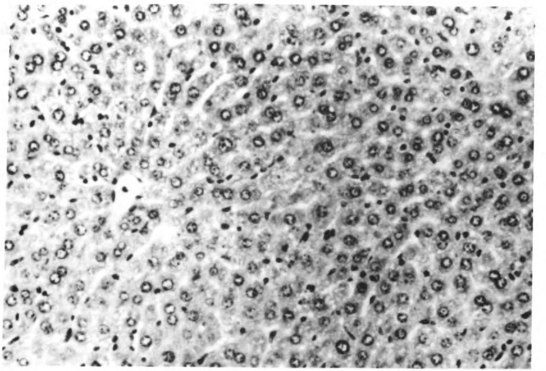


Figure 12

Figure 13. Liver tissue from selenium deficient and paraquat treated selenium deficient mice. Upper panel is from selenium deficient mice; lower panel is from selenium deficient mice 24 hours after 11.9 mg/kg ip paraquat (LD_{70} in selenium deficient mice). Original magnification, x100.



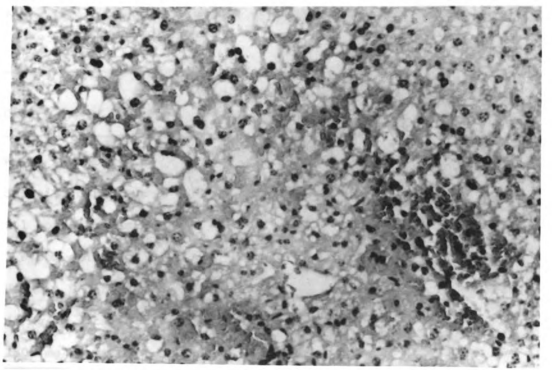


Figure 13

Table 13. Dialuric acid hemolysis of erythrocytes from mice fed various vitamin E diets

Diet[1]	Percent hemolysis[2]	Percent of control
Control (for vitamin E deficient)	23.9 ± 4.8	
Vitamin E deficient[3]	86.4 ± 7.0[4]	361
Control (for vitamin E supplemented)	56.0 ± 3.8	
45 mg/kg diet vitamin E[3]	26.7 ± 12.1[4]	48
1500 mg/kg diet vitamin E[3]	6.5 ± 3.5[4]	12

[1]See Methods for assay of vitamin E deficient or supplemented groups.

[2]Mean ± S.E. of 3 determinations.

[3]5 week exposure.

[4]Significantly different from control, $p<.05$.

Table 14. Body weights of mice fed various selenium or vitamin E diets for 5 weeks

Diet	Initial body weight[1] (g)	Body weight after 5 week diet feeding (g)[1]
Wayne Lab Blox	24.5 ± 0.4	34.5 ± 1.0
Selenium deficient	26.6 ± 0.3	32.8 ± 0.4
0.1 ppm selenium	26.4 ± 0.2	32.2 ± 0.3
2.0 ppm selenium	25.8 ± 0.2	32.0 ± 0.3
Vitamin E deficient	22.3 ± 0.1	31.4 ± 0.4
45 mg/kg vitamin E	24.0 ± 0.2	32.3 ± 0.4
1500 mg/kg vitamin E	23.8 ± 0.1	32.5 ± 0.4

[1]Mean ± S.E.

treated with 11.9 mg/kg ip paraquat (LD_{70} in selenium deficient mice) and sacrificed 24 hours later revealed areas of minor alveolar edema and congestion but no evidence of alveolar congestion and collapse (Figure 12). The liver, however, showed extensive areas of hydropic degeneration or possibly fatty degeneration and appeared extremely congested (Figure 13). Kidney tissue appeared normal in selenium deficiency and with added paraquat treatment.

Tissue Reduced Glutathione and Lipid Soluble Antioxidant Concentrations after Acute Paraquat Treatment

Liver GSH was significantly reduced to 69% of control liver GSH concentrations both 24 and 36 hours after an LD_{50} dose of 30 mg/kg ip paraquat. GSH had begun to return to control concentrations after 48 hours, although recovery was not complete at that time. Lung GSH, however, was not significantly altered from control values up to 48 hours after paraquat administration (Table 15).

The effect of paraquat on lipid soluble antioxidants in mouse lung and liver was the reverse of that observed with the water soluble antioxidant GSH (Table 16). Paraquat, 30 mg/kg ip, significantly reduced lung lipid soluble antioxidants but caused no significant reductions in liver lipid soluble antioxidants at any time after paraquat administration. The reduction in lung lipid soluble antioxidants was very prolonged, remaining at 59.4% of control up to 96 hours after paraquat administration. The reduction in lung lipid soluble antioxidants could not be accounted for by possible increases in lung weight for paraquat toxicity as the control and paraquat wet lung weights were 204 $\pm$ 0.7 mg and 199 $\pm$ .6 mg,

Table 15. Liver and lung reduced glutathione (GSH) after acute paraquat treatment in mice

Time after paraquat (30 mg/kg ip)	GSH (mg/g wet weight)[1]	
	Liver	Lung
Control	4.67 ± 0.27	1.85 ± 0.02
12 hr	3.71 ± 0.33	1.88 ± 0.11
24 hr	3.21 ± 0.17[2]	1.94 ± 0.09
36 hr	3.20 ± 0.45[2]	2.08 ± 0.17
48 hr	3.86 ± 0.88	1.83 ± 0.08

[1]Mean ± S.E. of 4 determinations.

[2]Significantly different from control, $p<.05$.

Table 16. Lung and liver lipid soluble antioxidants after an acute dose of paraquat

Time after paraquat[1] (hours)	Lipid soluble antioxidants (percent of control)[2]	
	Lung	Liver
1	64.0 ± 2.8[3]	72.4 ± 4.4[3]
4	73.6 ± 10.1[3]	95.4 ± 7.9
12	52.7 ± 6.0[3]	103.7 ± 5.8
24	72.7 ± 6.6[3]	94.7 ± 2.8
48	56.4 ± 4.4[3]	103.7 ± 5.0
96	59.4 ± 4.5[3]	87.0 ± 5.6
193	90.7 ± 6.5	88.9 ± 9.5

[1]Paraquat, 30 mg/kg, ip.

[2]Mean ± S.E. of 4 determinations.

[3]Significantly different from control, $p<.05$.

respectively. Total water content was 78.2% for controls and 79.2% for paraquat treated mice.

The decrease in lung lipid soluble antioxidants was observed to be dose related when measured 12 hours after paraquat administration (Figure 14). Both the LD_{50} (30 mg/kg) and LD_{90} (44 mg/kg) doses significantly reduced the lung lipid antioxidants compared to controls.

Interaction of Paraquat with the GSH Peroxidase System Enzymes

Administration of 100 ppm paraquat to rats in their drinking water resulted in a 1.7-fold increase in pulmonary glucose-6-phosphate dehydrogenase activity and a 1.9-fold increase in pulmonary GSH reductase activity, while GSH peroxidase activity was not affected. Liver GSH peroxidase and reductase activities were not altered by chronic paraquat treatment (Table 17).

Pretreatment of rats for 7 days in 85% oxygen was able to significantly delay paraquat induced lethality (Table 18). The paraquat LT_{50} was increased approximately twofold by the 85% oxygen pretreatment.

Interaction of Paraquat with Bromobenzene

The toxicity of bromobenzene, as measured by the LT_{50}, was determined in 42 day old 50 and 100 ppm paraquat treated mice. The LT_{50} for bromobenzene in control mice was 20.0 hours, in 50 ppm paraquat treated mice 4.2 hours, and 3.2 hours in 100 ppm paraquat treated mice. The LT_{50}'s of the 50 and 100 ppm paraquat

Figure 14. Effect of increasing doses of paraquat on lung lipid soluble antioxidants. Paraquat was administered at 25, 30 and 44 mg/kg ip and lung lipid soluble antioxidants measured 12 hours later. An asterisk indicates significantly different from control, $p<.05$. Each bar represents the mean $\pm$ S.E. of 4 determinations.

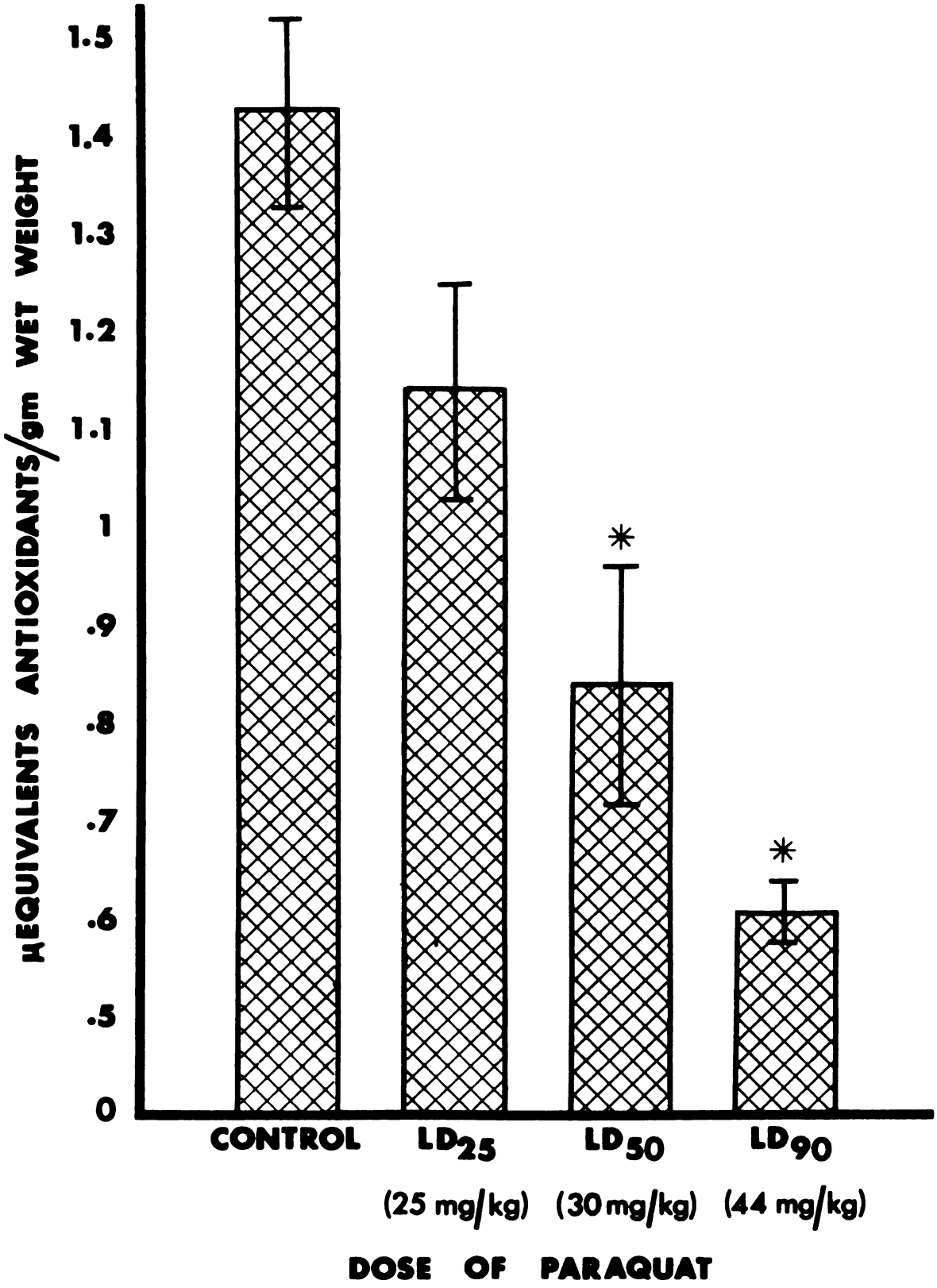

μEQUIVALENTS ANTIOXIDANTS/gm WET WEIGHT
1.5
1.4
1.3
1.2
1.1
1
.9
.8
.7
.6
.5
0
*
*
CONTROL
LD25
LD50
LD90
(25 mg/kg)
(30 mg/kg)
(44 mg/kg)
DOSE OF PARAQUAT

Figure 14

Table 17. Activity of glutathione peroxidase system enzymes in rats after 100 ppm paraquat in the drinking water for 3 weeks

Enzyme	Enzyme activity[1]			
	Lung		Liver	
	Control	Paraquat	Control	Paraquat
GSH peroxidase[2]	1.48 ± 0.14	1.53 ± 0.05	3.03 ± 0.18	2.97 ± 0.01
GSH reductase[3]	36 ± 5	68 ± 1[5]	37 ± 1	40 ± 1
Glucose-6-phosphate dehydrogenase[4]	47.0 ± 2.1	78.2 ± 11.7[5]	---	---

[1]Mean ± S.E. of 4 determinations.

[2]µmoles NADPH oxidized/min/mg protein.

[3]nmoles NADPH oxidized/min/mg protein.

[4]µmoles NADP reduced/min/mg protein.

[5]Significantly different from control, $p<.05$.

Table 18. Effect of 7-day 85% oxygen pretreatment on survival of rats after a toxic dose of paraquat

Treatment	Paraquat LT_{50} (hours)	95% C.L.	Potency ratio
Control[1]	26.0	(21.0-32.2)	
7-day 85% oxygen pretreatment[1,2]	50.0	(38.5-65.0)	1.92[3]

[1]Paraquat, 45 mg ip.

[2]Paraquat administered immediately after removal from 85% oxygen.

[3]Significantly different from control, $p<.05$.

mice were significantly different from controls, but not different from each other (Table 19).

Table 19. Effect of paraquat in the water from day 8 of gestation to 42 days postnatally on the survival of mice treated with bromobenzene

Treatment	Bromobenzene[1] LT50 (hours)	95% C.L.	Potency ratio
Control	20.0	(12.6-31.6)	---
50 ppm	4.2	(2.6- 6.7)	4.8[2]
100 ppm	3.2	(1.9- 5.4)	6.2[2]

[1]Bromobenzene, 3100 mg/kg ip (LD_{85}).

[2]Significantly different from control, $p<.05$.

Liver GSH was assayed in 42 day old 50 and 100 ppm paraquat treated mice (Table 20). No significant differences were observed in the liver GSH of these mice compared to controls.

Interaction of Phenobarbital with Paraquat

Pretreatment of mice with phenobarbital in the drinking water for 10 days significantly increased the paraquat LD_{50} to 46.0 mg/kg (Table 21). This group was continued on phenobarbital after paraquat injection. Transfer of phenobarbital pretreated mice to tap water for 24 hours prior to paraquat injection decreased the paraquat LD_{50} to 39.0 mg/kg, which was elevated but not significantly

Table 20. Liver GSH concentrations in mice administered paraquat in the water from day 8 of gestation to 42 days postnatally

Treatment	Liver GSH[1] (mg GSH/g wet weight)
Control	5.08 ± 0.31
50 ppm	4.80 ± 0.30
100 ppm	5.14 ± 0.63

[1]Mean ± S.E. of 3 to 6 determinations.

Table 21. Effect of phenobarbital (PB) pretreatment on the paraquat single dose 7-day intraperitoneal LD_{50}

Treatment	Paraquat LD_{50} (mg/kg)	95% C.L.	Potency ratio
Control	30.0	(26.3-34.2)	
PB 10 days prior and continued after paraquat[1]	46.0	(41.4-51.4)	1.53[2]
PB 10 days prior plus 1 day tap water[1]	39.0	(33.0-46.0)	1.30
PB, 50 mg/kg ip, 30 min before paraquat	26.8	(22.7-31.6)	0.89

[1]Phenobarbital, 0.1% in water.

[2]Significantly different from control, $p<.05$.

different from the control LD_{50}. Administration of a single dose of phenobarbital, 50 mg/kg ip, 30 minutes before paraquat, had no effect on the LD_{50} compared to control (Table 21).

The elimination of radioactivity after ^{14}C-paraquat from mice pretreated with phenobarbital for 10 days was not significantly different from control mice when elimination of radioactivity was followed in plasma, lung, liver and kidney (Figures 15 and 16). Radioactivity in both control and phenobarbital pretreated lungs was significantly elevated compared to the respective radioactivity in liver and kidney at 24 hours after paraquat administration.

Figure 15. Effect of 10-day phenobarbital pretreatment on elimination of ^{14}C-paraquat from plasma and lung in mice. ^{14}C-Paraquat was administered at 30 mg/kg, ip, to control and 10-day phenobarbital (0.1% in the drinking water) pretreated mice. Each point represents the mean of 3 determinations.

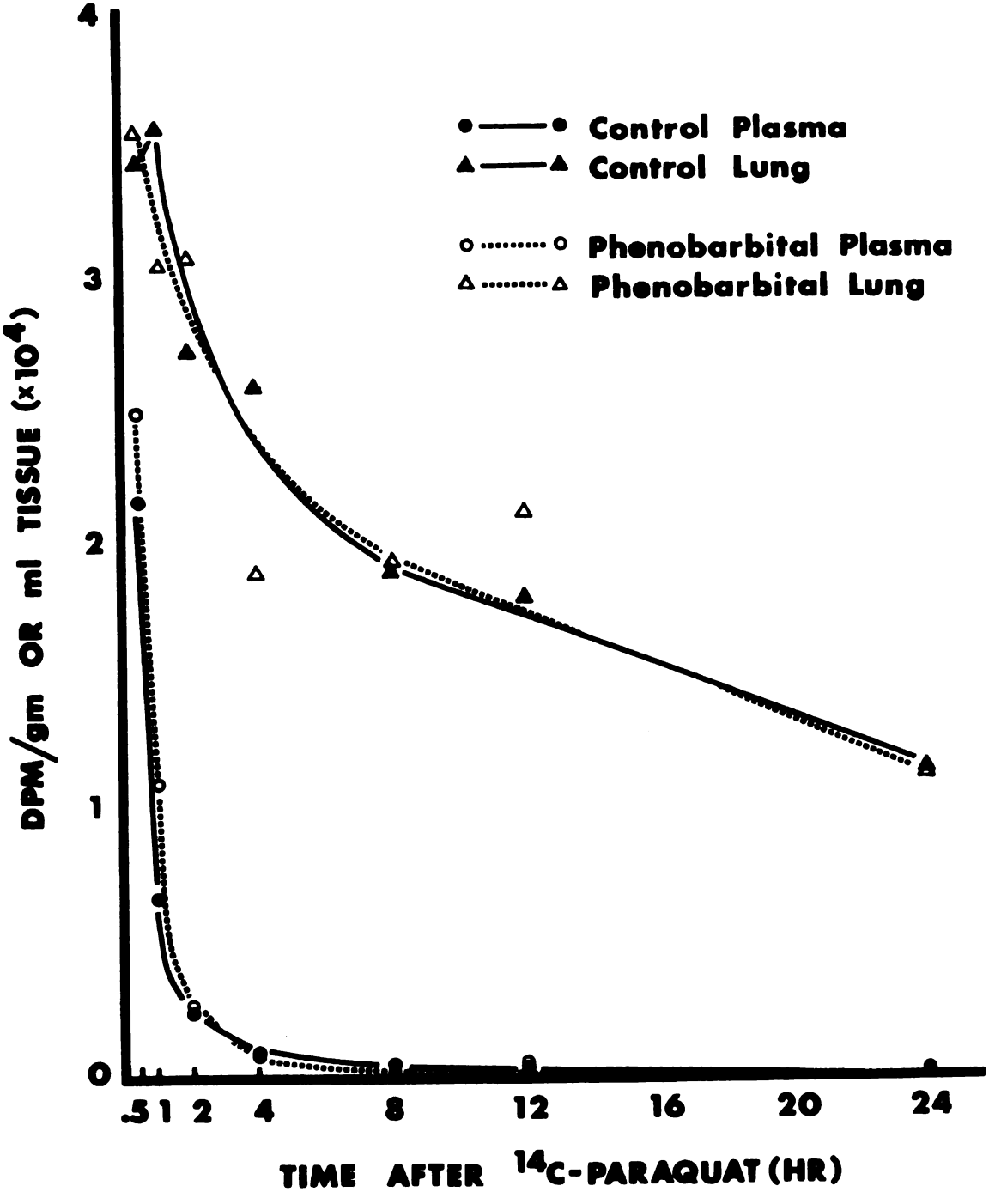

Control Plasma
Control Lung
Phenobarbital Plasma
Phenobarbital Lung
DPM/gm OR ml TISSUE (x10⁴)
0
1
2
3
4
.5 1 2 4 8 12 16 20 24
TIME AFTER ¹⁴C-PARAQUAT (HR)

Figure 15

Figure 16. Effect of 10-day phenobarbital pretreatment on elimination of ^{14}C-paraquat from liver and kidney in mice. ^{14}C-Paraquat was administered at 30 mg/kg ip to control and 10-day phenobarbital (0.1% in the drinking water) pretreated mice. Each point represents the mean of 3 determinations.

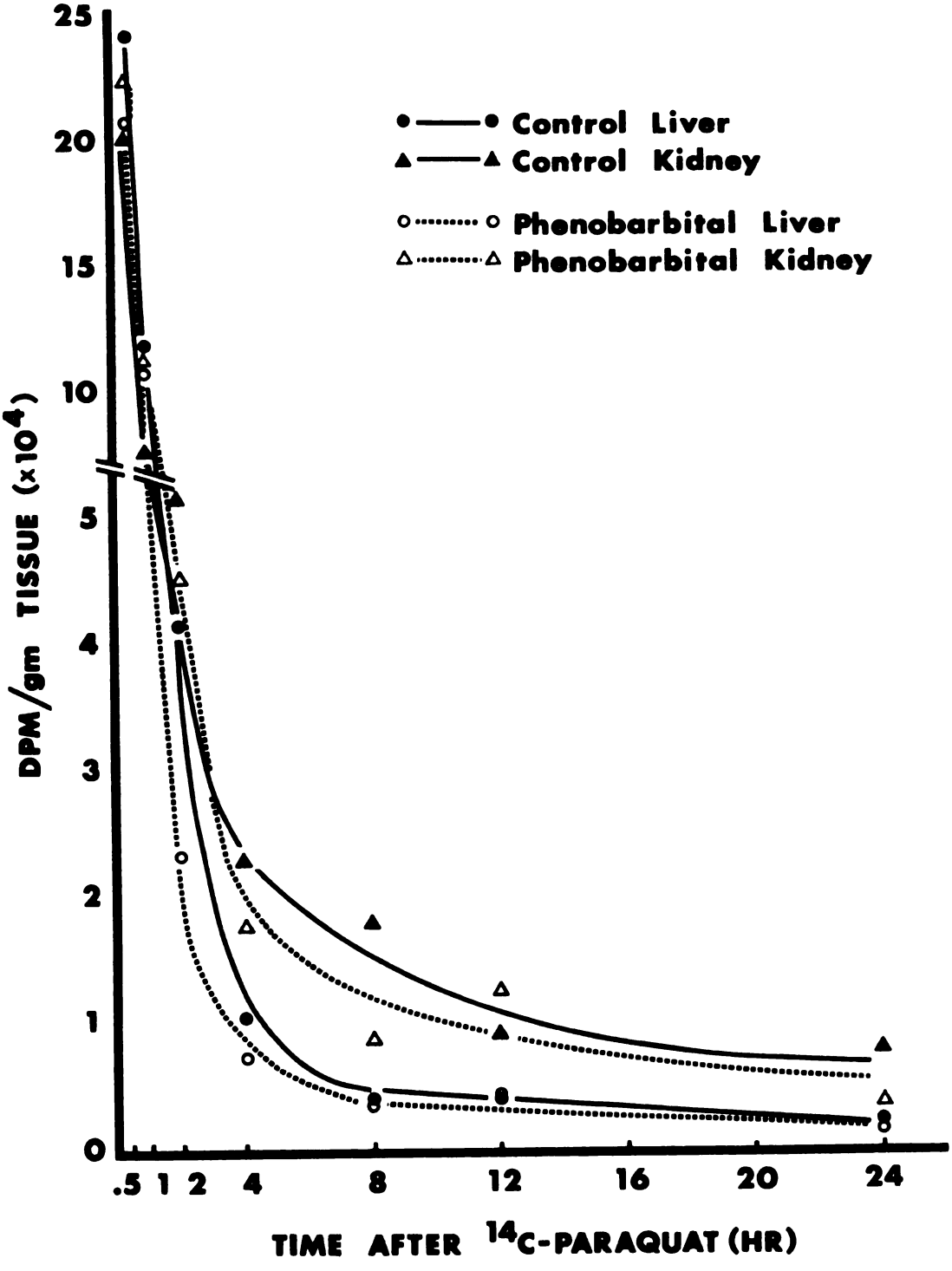

Control Liver
Control Kidney
Phenobarbital Liver
Phenobarbital Kidney
DPM/gm TISSUE (x10⁴)
25
20
15
10
5
4
3
2
1
0
.5 1 2 4 8 12 16 20 24
TIME AFTER ¹⁴C-PARAQUAT (HR)

Figure 16

DISCUSSION

Toxicity of Chronically Administered Paraquat in Developing Mice

Chronic administration of paraquat at 50 and 100 ppm in the drinking water did not alter the development of mice as reflected in the average body weight (Figure 1). Treated mice reaching 42 days of age not only weighed the same as controls, but also did not exhibit any gross malformations. In contrast to the lack of any paraquat effect on postnatal growth rate, 100 ppm paraquat increased postnatal mortality in an apparent biphasic pattern (Figure 2).

The first increase in mortality occurring in 100 ppm paraquat treated animals between days 1 and 7 after birth may have been induced by the presence of paraquat in the newborn. The paraquat in the newborn may have remained from prenatal placental transfer of the herbicide to the fetuses, rather than from paraquat passage to the newborns through the mother's milk. Bus *et al.* (1975) have shown that paraquat administered to pregnant rats during the final 2 days of gestation was detectable in the newborn for up to 7 days postnatally. Furthermore, the mortality in 100 ppm paraquat treated mice plateaued between 7 and 21 days despite continued nursing by mothers exposed to paraquat in the drinking water. The possibility that paraquat ingested from the mother's milk may have been responsible for the initial mortality appears unlikely as

paraquat is poorly absorbed orally (Murray and Gibson, 1974) and thus only minute amounts of paraquat would be expected to be systemically absorbed in the newborn. The immediate postnatal increase in mortality of the 100 ppm paraquat treated newborn may be linked to the initiation of breathing and exposure of lungs to higher oxygen tensions encountered after birth, as increased oxygen tensions have been demonstrated to increase paraquat toxicity in adult rats (Fisher *et al.*, 1973). Furthermore, developing mice exposed to paraquat in the water were more sensitive than controls to oxygen toxicity as early as the first day after birth. The immediate postnatal increase in mortality did not appear to be a continuation of paraquat-induced lethality that might have been initiated prenatally as neither 50 nor 100 ppm paraquat altered the number of live births (see Results).

Young animals begin to ingest water directly from the water supply in the week prior to weaning. This observation may account for the second mortality increase that began in the week before weaning. When other 100 ppm paraquat treated mice were transferred to tap water at weaning, the second increase in mortality was eliminated (Table 2). Furthermore, mortality in developing mice exposed to 100 ppm paraquat only between days 28 and 42 postnatally increased to 46.7 $\pm$ 14.5%, which was not significantly different from the approximate 30% increase that occurred between days 21 and 42 in the continuously treated mice.

Histological examination of tissue from lung, liver and kidney from 42 day old 50 and 100 ppm paraquat treated mice revealed

extensive lung alterations in the 100 ppm paraquat treated group, despite low concentrations of paraquat in lung tissue in these mice (Figure 3). The pulmonary lesions observed with chronic 100 ppm paraquat treatment resembled the pulmonary alveolar congestion and collapse seen in animals and man after acute paraquat intoxication (Toner *et al.*, 1970; Murray and Gibson, 1972). Similar pulmonary lesions were not observed in mice exposed to 50 ppm paraquat, the treatment in which mortality was not significantly elevated. The lung appeared to be the site of chronic paraquat toxicity, as liver and kidney tissue sections were without significant pathological alterations. The threshold dose for the development of pulmonary lesions seen with chronic paraquat treatment in mice appeared to be in the range of 16 to 27 mg/kg/day, or somewhere between the 50 to 100 ppm paraquat exposures (Table 1).

Fisher *et al.* (1973a) reported that adult rats were sensitized to the development of oxygen toxicity after acute treatment with paraquat. The 42 day old 50 and 100 ppm chronically paraquat treated mice in this study also were sensitized to 100% oxygen, as reflected by the significant reduction in LT_{50} values (Table 4). The 50 ppm paraquat treated mice were more sensitive to oxygen toxicity than controls, even in the absence of any alteration in postnatal growth or mortality. Furthermore, the 42 day old 50 ppm paraquat treated mice did not have any evidence of histopathological lesions and had no detectable paraquat in lung tissue before being placed in 100% oxygen. Thus, the 42 day old 50 ppm paraquat treated mice were sensitized to oxygen despite being morphologically indistinguishable from controls.

The 50 ppm paraquat treated mice appeared to develop sensitivity to oxygen toxicity between days 28 and 42 postnatally, as the oxygen LT_{50} of 28 day old 50 ppm paraquat treated mice was not altered from controls (Table 4). One day old mice whose mothers received 50 ppm paraquat during gestation also were not sensitized to oxygen toxicity (Figure 4). In the 100 ppm paraquat exposed group, however, sensitivity to oxygen toxicity had developed by the first day after birth, even though these newborn could have only been exposed to paraquat prenatally (Figure 4).

Pretreatment of adult mice with vitamin E in the diet has been shown to protect against oxygen induced lethality (Mino, 1973). Administration of a single subcutaneous dose of vitamin E to newborn mice whose mothers had received 100 ppm paraquat during gestation resulted in a trend towards decrease in oxygen-induced lethality compared to 100 ppm paraquat, non-vitamin E pretreated, newborn (Table 5). Although the decrease in mortality was not significant, it may be indicative of a slight protective effect of vitamin E against oxygen enhancement of paraquat toxicity in newborn, in that the slight protection resulted from only a single administration of vitamin E.

In adult animals oxygen exposure enhances the acute toxicity of paraquat (Fisher *et al.*, 1973a). A similar enhancement of the acute toxicity of paraquat was also observed to exist in newborn mice in this investigation (Table 6). Thus, exposure of newborn mice to oxygen after injection of a non-lethal dose of paraquat in a room air environment resulted in 71.4% mortality after only 30 hours in 100% oxygen.

The distribution of ^{14}C-paraquat in prenatal mouse organs as compared to the distribution in postnatal mouse organs was investigated in order to determine the possible involvement of organ oxygen tensions in the retention of paraquat in lung tissue that has been observed in several adult animal studies (Sharp *et al.*, 1972; Murray and Gibson, 1974; Bus *et al.*, 1975). Paraquat, when administered prenatally to mice, did not accumulate in fetal lung tissue when compared to fetal liver and kidney (Figure 5). This was in contrast to the significantly elevated levels of paraquat in lung tissue compared to liver and kidney of one day old mice which received the herbicide (Figure 6). The selective retention of paraquat in neonatal mouse lung may be related to higher oxygen tensions in this organ relative to other organs in the body. The enhancement of paraquat toxicity by oxygen in adult rats (Fisher *et al.*, 1973a) and in adult and newborn mice in this study suggests that an organ such as the lung with an elevated oxygen tension might be preferentially susceptible to tissue damage by paraquat. As the pulmonary lesions develop, paraquat may be retained in the lung due to a nonspecific trapping or binding of the herbicide in the lesioned areas. This hypothesis is strengthened by the lack of any retention of paraquat in prenatal mouse lung, which may be due to the relatively equal oxygen tensions among the fetal organs. Thus, the elevated oxygen tensions found in lungs of postnatal animals may be a factor in paraquat accumulation in lung tissue. Furthermore, the differences in pre- and postnatal retention of paraquat by lung may account for the increase in mortality that occurs

immediately after birth in newborn mice whose mothers received 100 ppm paraquat during gestation. When the mice are born, oxygen tension in the lung increases and retention of paraquat may occur as the pulmonary lesions develop. The retention of paraquat may lead to sufficient pulmonary damage to cause death.

Alternatively, retention of paraquat in postnatal mouse lung tissue contrasted to the absence of retention prenatally may be explained by the development of a binding site or active transport process for paraquat in lung tissue. Such a binding site or transport process may not have been functional in mouse fetuses at the times examined in this study and thus would account for the even distribution of paraquat in prenatal organs. Indeed, an energy-dependent process for paraquat accumulation into lung tissue has been proposed from *in vitro* rat lung slice studies (Rose *et al.*, 1974b). The paraquat analog diquat, however, was not found to accumulate in lung slices. The report of Bus *et al.* (1975) that paraquat was retained in both pre- and postnatal rat lung tissue while the analog diquat was not further supports the concept of an active transport process or specific binding site for paraquat in rat lung tissue. The differences in fetal distribution of paraquat in mice seen in this study and rats may have been caused by the difference in time of administration of the compounds during gestation, day 16 in mice and day 21 in rats. Thus, the differences between mice and rats may be indicative of development of binding sites or an active transport process for paraquat during late gestation, particularly since both rats and mice retained paraquat immediately after birth.

In Vitro Investigations of the Paraquat Mechanism of Action

The interaction of oxygen with paraquat toxicity in developing mice suggested that the mechanism of paraquat toxicity in animals might be similar to that in plants as oxygen has also been shown to be a requirement for the herbicidal activity of paraquat (Mees, 1960). In plants, molecular oxygen has been proposed to serve as an electron acceptor in the reduction-oxidation of paraquat with the resultant formation of the superoxide radical (Farrington, 1973). The formation of increased concentrations of malondialdehyde (a by-product of lipid peroxidation) have been detected in plant leaves 6 hours after paraquat exposure (Dodge *et al.*, 1970). Evidence that paraquat could undergo a similar reduction-oxidation in mammalian systems was demonstrated from *in vitro* experiments in which rat liver microsomes were observed to catalyze a NADPH-dependent reduction of paraquat to the blue-colored radical (Gage, 1968). Furthermore, incubation of paraquat with rat liver microsomes and NADPH increased malondialdehyde concentrations in microsomal phospholipids (Gage, 1968). The studies of Pederson and Aust (1973) into the mechanism of xanthine oxidase-induced lipid peroxidation provided a mechanism whereby paraquat might initiate lipid peroxidation. Utilizing superoxide dismutase, which scavenges superoxide radicals, and a singlet oxygen trapping agent, 1,3-diphenylisobenzofuran, *in vitro*, they concluded that xanthine oxidase-induced lipid peroxidation was mediated by the production of superoxide radicals. Singlet oxygen was evolved from superoxide radicals with subsequent reaction with unsaturated fatty acids to form fatty acid hydroperoxides.

Because paraquat has been shown to be reduced *in vitro* by liver microsomes and NADPH (Gage, 1968) and that superoxide radicals may be a product of the cyclic reduction-oxidation of paraquat (Farrington, 1973), a hypothesis based upon the work of Pederson and Aust (1973) was constructed which could be tested *in vitro* as a possible mechanism for the mammalian toxicity of paraquat (Figure 17). Paraquat was proposed to undergo a single electron reduction with NADPH supplying the necessary reducing equivalents. Upon aerobic reoxidation, superoxide radicals are formed which may non-enzymatically dismutate to singlet oxygen. The singlet oxygen then reacts with polyunsaturated lipids with lipid hydroperoxides as products. Thus, through a mechanism as just described, paraquat may initiate lipid peroxidation.

The first requirement of the proposed mechanism, paraquat reduction by mammalian tissue *in vitro*, was demonstrated by both aerobic and anaerobic incubation of mouse lung microsomes and NADPH with paraquat (Figures 7 and 8). Mouse lung microsomes were used as lung tissue is the primary site where the *in vivo* toxicity of paraquat occurs. NADPH-cytochrome c reductase was found to be the microsomal enzyme that catalyzed the reduction of paraquat as addition of antibody to rat liver NADPH-cytochrome c reductase inhibited both the aerobic oxidation of NADPH in the presence of paraquat and the anaerobic production of paraquat radical (Figures 9 and 10). The antibody did not cause a nonspecific inhibition of the reaction as substitution of pre-immune serum for antibody in the incubation system did not inhibit the reaction.

Figure 17. Proposed mechanism for the toxicity of paraquat based upon *in vitro* experiments.

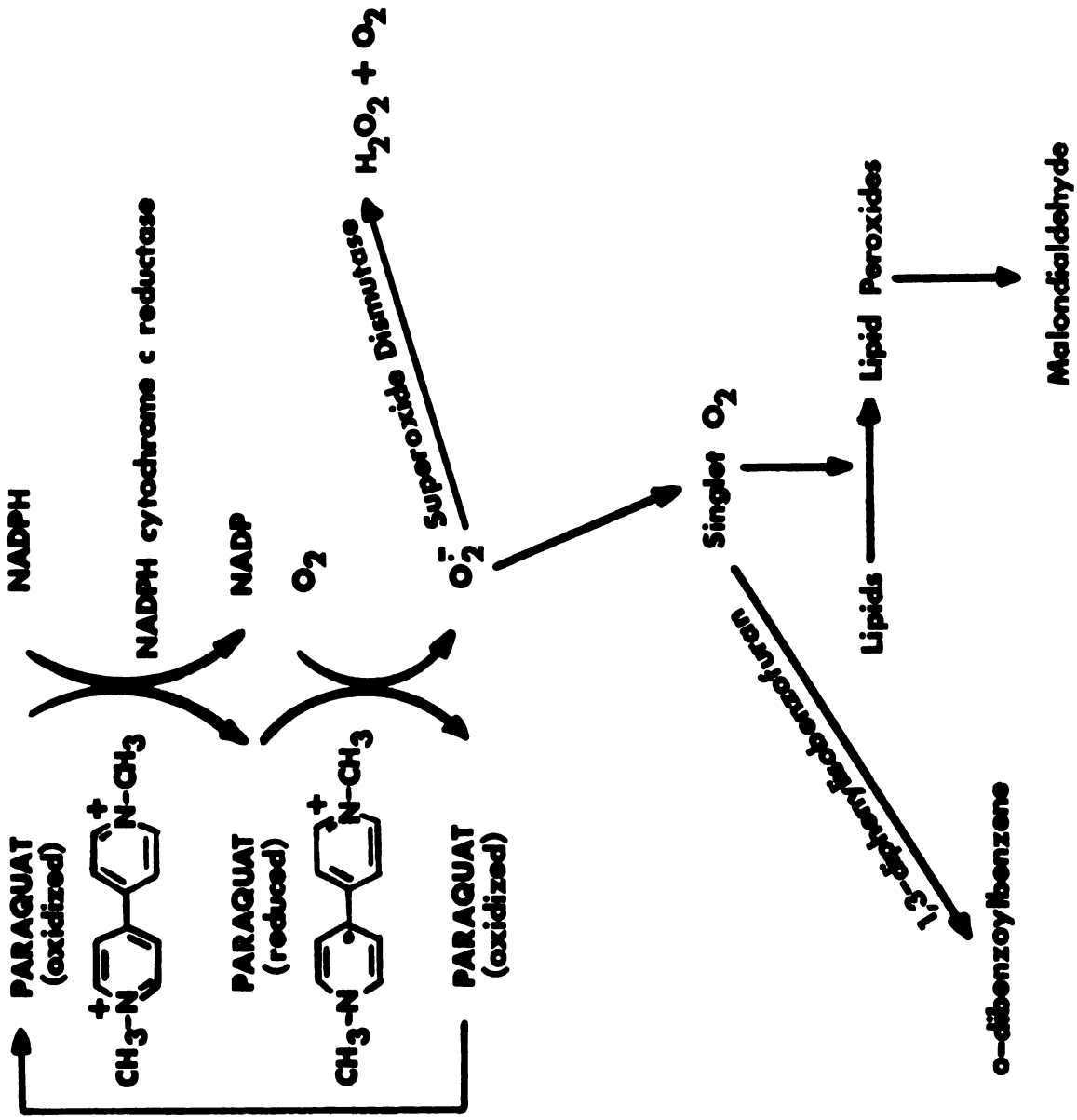

NADPH
NADPH cytochrome c reductase
NADP
O_2
O_2^-
Superoxide Dismutase
$H_2O_2 + O_2$
PARAQUAT (oxidized)
PARAQUAT (reduced)
PARAQUAT (oxidized)
Singlet O_2
Lipids
Lipid Peroxides
Malondialdehyde
1,3-diphenylisobenzofuran
o-dibenzoylbenzene

Figure 17

The second requirement of the proposed mechanism was that the reduced paraquat could initiate lipid peroxidation through subsequent superoxide radical and singlet oxygen intermediates. In a simplified *in vitro* incubation system paraquat promoted lipid peroxidation in a concentration dependent manner (Table 7). Inhibition of paraquat-induced lipid peroxidation by superoxide dismutase and the singlet oxygen trapping agent, 1,3-diphenylisobenzofuran, provides evidence that both superoxide radicals and singlet oxygen are intermediates in the reaction (Table 8). Superoxide dismutase catalyzes the reduction of superoxide radicals to hydrogen peroxide and oxygen while 1,3-diphenylisobenzofuran is rapidly oxidized by singlet oxygen to form o-dibenzoylbenzene. The synergistic inhibition of paraquat-induced lipid peroxidation when both agents were incubated together indicates that superoxide radicals and singlet oxygen might be sequential intermediates. Neither superoxide dismutase or 1,3-diphenylisobenzofuran appears to inhibit paraquat-induced lipid peroxidation by nonspecific antioxidant properties as ascorbic acid-induced lipid peroxidation was not inhibited by these agents in an incubation system identical to these experiments (Pederson and Aust, 1973).

The proposed mechanism for paraquat toxicity, which was supported by *in vitro* experiments, may offer an explanation for the *in vivo* enhancement of paraquat toxicity by oxygen exposure observed in rats (Fisher *et al.*, 1973a) and in chronically paraquat treated mice in this study. Under increased oxygen tensions, higher *in vivo* concentrations of toxic superoxide radicals may be produced

in the presence of paraquat, resulting in a more rapid onset of pulmonary damage. The hypothesis also explains the protective effect of low oxygen tensions against paraquat toxicity (Rhodes, 1974) in that at decreased oxygen tensions fewer superoxide radicals would be generated. Furthermore, if oxygen is involved in the toxicity of paraquat, it may in part account for the specific lung damage caused by paraquat since this organ has the highest oxygen tension in the body. Recently, the generation of superoxide radicals by paraquat has been demonstrated by *in vitro* incubation of paraquat with rat liver microsomes and NADPH (Davies and Davies, 1974). Autor (1974) has provided *in vivo* evidence for the involvement of superoxide radicals in paraquat toxicity in that administration of superoxide dismutase was observed to delay paraquat-induced lethality. Thus, the mechanism proposed for paraquat toxicity is supported both by *in vitro* and *in vivo* observations and provides a basis for further *in vivo* investigations.

In Vivo Investigations of the Paraquat Mechanism of Action

One widely used method for the measurement of lipid peroxidation is the colorimetric assay for malondialdehyde, a by-product of lipid peroxidation. After an ip 30 mg/kg LD_{50} dose of paraquat, however, no changes in mouse lung, liver or kidney malondialdehyde concentrations were observed when determined 3 and 7 days after paraquat administration (Table 9). This was in contrast to the increases in malondialdehyde induced by paraquat in the simplified *in vitro* incubation system. When an ip 44 mg/kg LD_{90} dose was administered,

malondialdehyde was significantly increased in liver but not lung or kidney 12 and 24 hours after injection (Figure 11). The increase in malondialdehyde in liver rather than in lung, which is usually the primary organ affected in paraquat toxicity, may be due to the high dose of paraquat that was given. The increase in liver malondialdehyde may be indicative of a shift in paraquat toxicity to organs other than the lung when high doses are administered. The paraquat treated mice sacrificed at 12 and 24 hours, however, appeared very ill and were not observed to eat or drink from the time of injection until sacrifice. Thus, the poor condition of the animals may have contributed to a general tissue deterioration. The failure to demonstrate an increase in tissue malondialdehyde at the lower LD_{50} dose of paraquat may be indicative of a slow rate of lipid peroxidation occurring within the tissues. This may be reflected in longer survival times after a toxic dose of paraquat, since mice injected with a LD_{50} dose routinely survived for at least 48 hours while those receiving a LD_{90} dose survived a maximum of 24 to 48 hours. If paraquat is inducing a slower rate of lipid peroxidation in the lower range of toxic doses, malondialdehyde increases might not be detectable because this by-product of lipid peroxidation is known to be metabolized *in vivo* (Barber and Bernheim, 1967; Recknagel *et al.*, 1974). Thus, the determination of malondialdehyde levels as an indicator of *in vivo* lipid peroxidation may only be effective when massive amounts of lipid peroxidative damage has occurred. Another technique which has been used as an indicator of *in vivo* lipid peroxidation is the

measurement of an accumulation of fluorescent lipofuscin pigments (Tappel, 1973). Lipofuscin pigments result from the reaction of malondialdehyde with amino groups of protein and phosphatidyl ethanolamine and are thought to be stable *in vivo* (Tappel, 1973). Problems have been encountered, however, in quantitating the *in vivo* concentrations of lipofuscin pigments.

In order to further investigate the proposed involvement of lipid peroxidation in the toxicity of paraquat *in vivo*, therefore, the proposed mechanism presented in Figure 17 was expanded to include several mechanisms which function in mammalian systems to combat the damaging effects of free radical catalyzed lipid peroxidation (Figure 18). First, the lipid hydroperoxides that are formed upon the reaction of singlet oxygen with unsaturated lipids may be enzymatically detoxified to stable lipid alcohols by GSH peroxidase,utilizing reduced glutathione (GSH) as the source of reducing equivalents (Chow and Tappel, 1974). The activity of GSH peroxidase in reducing lipid hydroperoxides has an essential requirement for selenium, which was reflected by significantly reduced activity of this enzyme in rats fed selenium deficient diets (Smith *et al.*, 1974). Second, vitamin E interrupts the free radical chain reactions that are initiated by the decomposition of lipid hydroperoxides (Chow and Tappel, 1974).

The mechanism depicted in Figure 18, therefore, predicts an increased sensitivity to paraquat in selenium deficient animals as these animals would have reduced GSH peroxidase activity and consequently not be able to detoxify the increased levels of lipid

Figure 18. Proposed mechanism of action for the *in vivo* toxicity of paraquat.

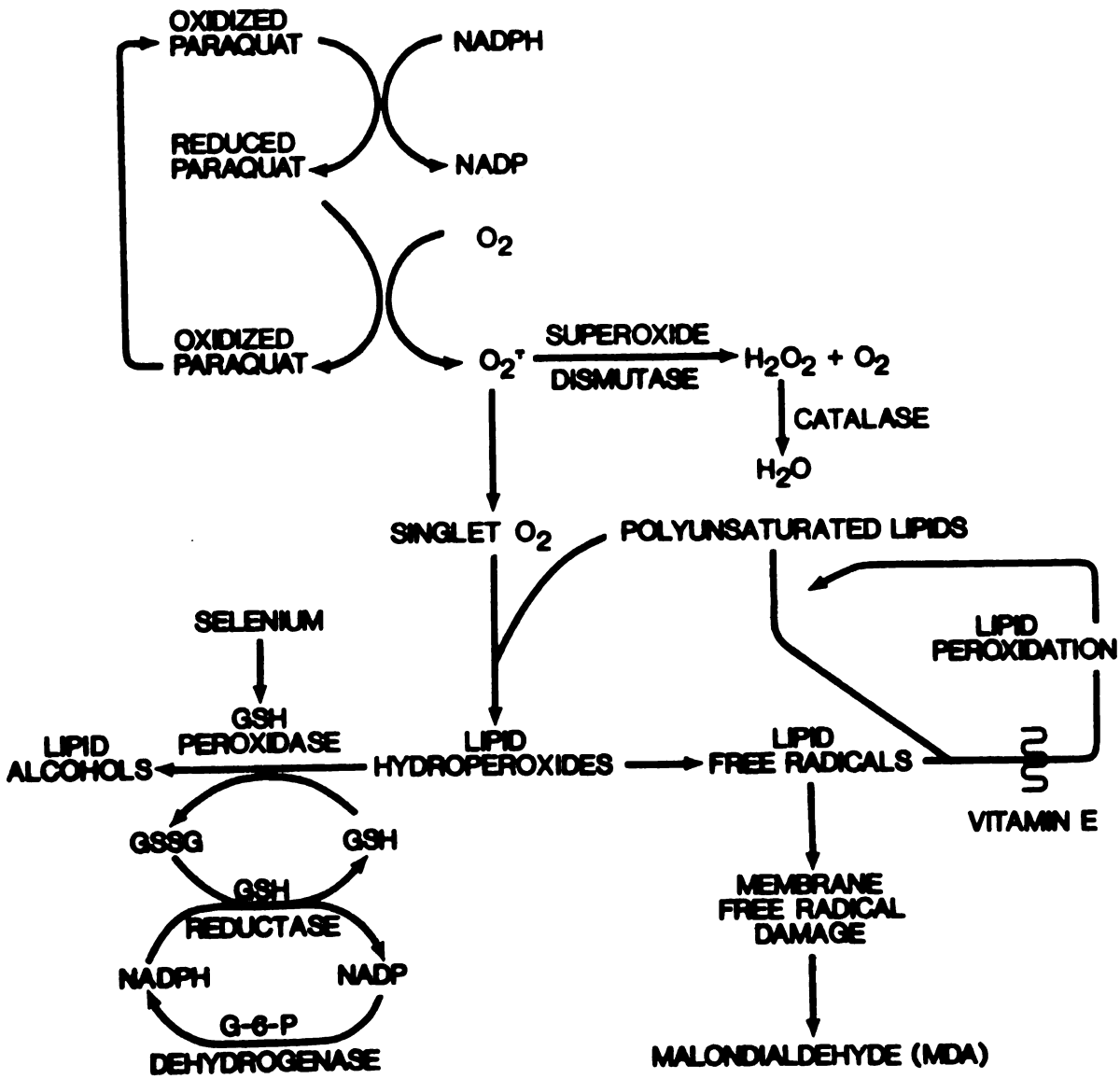

OXIDIZED PARAQUAT
NADPH
REDUCED PARAQUAT
NADP
O_2
OXIDIZED PARAQUAT
$O_2^{\cdot}$
SUPEROXIDE DISMUTASE
$H_2O_2 + O_2$
CATALASE
H_2O
SINGLET O_2
POLYUNSATURATED LIPIDS
SELENIUM
LIPID PEROXIDATION
GSH PEROXIDASE
LIPID ALCOHOLS
LIPID HYDROPEROXIDES
LIPID FREE RADICALS
VITAMIN E
GSSG
GSH
GSH REDUCTASE
NADPH
NADP
G-6-P DEHYDROGENASE
MEMBRANE FREE RADICAL DAMAGE
MALONDIALDEHYDE (MDA)

Figure 18

hydroperoxides. The significant decrease in the paraquat LD_{50} in selenium deficient mice (Table 10) is consistent with the proposed mechanism and thus supports the possibility that paraquat toxicity is mediated through lipid peroxidation. The return of the paraquat LD_{50} to the control value in mice fed basal diet supplemented with selenium indicated that the basal diet caused no effects other than selenium deficiency which could alter the paraquat LD_{50} (Table 10). This was further supported in that body weights and general physical appearances of selenium deficient mice were not altered from mice fed selenium supplemented diets or the control mice (Table 14). Lung and liver GSH peroxidase activity, however, was significantly reduced in mice fed selenium deficient diet compared to mice fed the 0.1 and 2 ppm selenium supplemented diet, confirming that the selenium deficient diet did affect tissue GSH peroxidase activity (Table 11). In mice fed selenium deficient diet, GSH peroxidase activity was reduced to a greater extent in liver than in lung tissue (Table 11). This observation may account for the extensive hepatic necrosis that was seen in paraquat treated selenium deficient mice (Figure 13), since the low liver GSH peroxidase activity may have increased the vulnerability of this tissue to lipid peroxidative damage. Thus, in selenium deficiency, organs other than the lung may be extensively involved in paraquat toxicity.

The proposed role of GSH peroxidase in combating paraquat-induced lipid peroxidation was further strengthened by the observation of a significant decrease in the paraquat LD_{50} after pretreatment with diethyl maleate, a tissue GSH depleting agent (Table 10).

By reducing tissue GSH, the reducing equivalents necessary for GSH peroxidase activity are eliminated and thus diethyl maleate pretreatment accomplishes a result similar to selenium deficiency--an inability of GSH peroxidase to detoxify paraquat-induced lipid hydroperoxides. The dose of diethyl maleate used, 1.2 ml/kg ip, was found to reduce GSH levels in lung and liver (Table 12) and thus would be expected to reduce GSH peroxidase activity in both these tissues.

The significant decrease in the paraquat LD_{50} in vitamin E deficient mice (Table 10) also supports the hypothesis of *in vivo* paraquat-induced lipid peroxidation as such mice lack the lipid peroxidation terminating activity of vitamin E. Vitamin E deficiency was confirmed in the mice fed vitamin E deficient diet for 5 weeks by the significant increase in dialuric acid-induced erythrocyte hemolysis (Table 13). The alteration in the paraquat LD_{50} by vitamin E deficiency did not appear to be caused by nonspecific changes induced by the basal diet because addition of vitamin E to the basal diet at 45 mg/kg diet (see Results) and 1500 mg/kg diet (Table 10) returned the paraquat LD_{50} to the control value. Furthermore, none of the vitamin E diets altered body weights or general physical appearances compared to control animals (Table 14). Supplementation of the basal diet with 45 or 1500 mg/kg vitamin E in the diet was not able to protect against paraquat toxicity compared to control mice (Table 10), despite the observation that both supplemented diets significantly decreased dialuric acid-induced erythrocyte hemolysis (Table 13). This may indicate

that administration of vitamin E may provide protection against lipid peroxidative damage in specific tissues and that the protection afforded other tissues may be limited by the access of vitamin E to the site of lipid peroxidative damage.

Recently DiLuzio (1973) demonstrated that lipid peroxidation results in a reduction in tissue antioxidants because these agents are utilized in terminating the lipid peroxidation chain reactions. Thus, paraquat, which is proposed to act through lipid peroxidation, would also be expected to reduce tissue antioxidants. In agreement with this hypothesis, paraquat was shown to significantly reduce the water soluble antioxidant GSH in liver but not in lung 24 and 36 hours after administration (Table 15). The reduction of liver GSH may have resulted from the activity of GSH peroxidase in reducing lipid hydroperoxides utilizing GSH as the source of reducing equivalents. Alternatively, GSH concentrations may have been reduced by the utilization of GSH in terminating free radicals reactions initiated by paraquat. The lack of any effect of paraquat on lung GSH levels may reflect possible differences in the cellular site of lipid peroxidation between lung and liver. The differential effect of paraquat on tissue GSH may also be the result of an inability of liver tissue to replenish GSH levels through the activity of GSH reductase.

The effect of paraquat on tissue lipid soluble antioxidants was the reverse of that seen with the water soluble antioxidant GSH. Lipid soluble antioxidants were significantly depressed in lung tissue but not in liver for up to 96 hours after paraquat treatment

(Table 16). Furthermore, the decrease in lung tissue was observed to be dose dependent (Figure 14). The decrease in lung lipid antioxidants was not an artifact due to an increase in lung weights caused by paraquat as total lung weights and water content were not altered by paraquat treatment (see Results). The differential decrease in lipid soluble antioxidants in lung in comparison to liver may indicate a different cellular site of lipid peroxidation in lung tissue as compared to liver. This suggestion agrees with the observations made with the water soluble antioxidant GSH. The decrease in lung lipid soluble antioxidants may also reflect possible differences in the available pool of vitamin E in lung and liver, with the vitamin E pool in lung more readily depleted than that in liver. The prolonged depression in lung lipid soluble antioxidants may be indicative of a slow rate of replenishment after an initial depletion or possibly a continual slow rate of lipid peroxidation which prevents the return of antioxidants to control levels. The reduction in lipid soluble antioxidants in lung tissue and the water soluble antioxidant GSH in liver, therefore, provide further support for the hypothesis that paraquat may initiate lipid peroxidation *in vivo*.

Another series of *in vivo* experiments was conducted, however, which also tested the paraquat mechanism hypothesis. The activity of the glutathione peroxidase system enzymes--GSH peroxidase, GSH reductase, and glucose-6-phosphate dehydrogenase--has been observed to be closely linked in their activity in combating oxidative stress to the lung such as caused by ozone (Chow and Tappel, 1972) and

nitrogen dioxide (Chow *et al.*, 1974). Chronic exposure of rats to paraquat in the drinking water also resulted in significant increases in lung GSH reductase and glucose-6-phosphate dehydrogenase activities but not GSH peroxidase (Table 17). The proposed mechanism whereby these enzymes are induced is outlined in Figure 18. The increase in lipid hydroperoxides induced by paraquat results in increased utilization of GSH through the activity of GSH peroxidase, with subsequent formation of oxidized glutathione. Oxidized glutathione is reduced back to GSH by GSH reductase with NADPH as the source of reducing equivalents. Thus, the enzymes glucose-6-phosphate dehydrogenase and GSH reductase may be induced in order to supply the necessary reducing equivalents for the increased activity of GSH peroxidase. Recently, glucose-6-phosphate dehydrogenase has also been shown to be induced in rats after acute paraquat treatment (Witschi and Kacew, 1974). Although induction of all 3 enzymes has been demonstrated in oxidative stress such as ozone (Chow and Tappel, 1972), the lack of any GSH peroxidase induction with chronic paraquat treatment may be due to the sacrificing of the rats at a time when the enzyme had not yet been induced in response to oxidative stress. The induction of 2 of the 3 enzymes of the glutathione peroxidase system by paraquat, however, provides strong evidence for the involvement of lipid peroxidation in paraquat toxicity.

Pretreatment of rats with 85% oxygen for 7 days has been demonstrated to induce tolerance to subsequent exposure to 100% oxygen, since tolerant rats survive the challenge longer than

nontolerant controls (Rosenbaum *et al.*, 1968). Glucose-6-phosphate dehydrogenase (Tierney *et al.*, 1973) and superoxide dismutase (Crapo and Tierney, 1974) activities are elevated in oxygen tolerant rats. The induction in part of the GSH peroxidase system enzymes, as well as superoxide dismutase, may account for the resistance of tolerant rats to further oxidative stress. The mechanism proposed for paraquat toxicity predicts that animals with increased specific activities of these enzymes should be resistant to paraquat toxicity. Indeed, oxygen tolerant rats were found to be more resistant than controls to a toxic dose of paraquat (Table 18), as were rats also administered superoxide dismutase (Autor, 1974). Thus, the proposed mechanism for paraquat toxicity is further validated *in vivo* by the response of oxygen tolerant rats to paraquat.

Interaction of Paraquat Toxicity with Bromobenzene and Phenobarbital

In 42 day old mice chronically treated with 50 or 100 ppm paraquat, bromobenzene toxicity was significantly enhanced as indicated by the reduction in the bromobenzene LT_{50} (Table 19). Bromobenzene is metabolized to a hepatotoxic epoxide by microsomal enzymes (Brodie *et al.*, 1971) and subsequently detoxified by either conjugation with GSH, nonenzymatic rearrangement to phenols, or enzymatic conversion to dihydrodiols by epoxide hydrase (Jollow *et al.*, 1974). In acute studies in rats, bromobenzene toxicity was significantly enhanced by pretreatment with GSH depleting agents due to decreased detoxification of the bromobenzene epoxide (Jollow *et al.*, 1974). Although liver GSH concentrations in the chronically

paraquat treated mice were not altered compared to controls (Table 20), possibly paraquat treatment affected the capability of mice to replenish GSH stores which are depleted by bromobenzene detoxification. Thus, less GSH would be available for epoxide conjugation with the result of increased bromobenzene toxicity.

The evidence that paraquat may catalyze membrane lipid peroxidation, however, suggests a second mechanism whereby paraquat treatment could decrease the bromobenzene LT_{50}. As mentioned previously, paraquat has been postulated to induce the *in vivo* formation of lipid hydroperoxides which are enzymatically reduced to stable lipid alcohols by GSH peroxidase with GSH providing the reducing equivalents (Chow and Tappel, 1974). The availability of GSH appears necessary to combat paraquat toxicity, as pretreatment of mice with the GSH depleting agent diethyl maleate significantly enhanced acute paraquat toxicity (Table 10). Large doses of bromobenzene, like diethyl maleate, also decrease liver GSH for up to 20 hours in rats due to conjugation of the bromobenzene epoxide with GSH (Jollow *et al.*, 1974). Thus, bromobenzene, by depleting GSH, may remove the activity of GSH peroxidase in protecting against paraquat induced lipid peroxidation. Consequently, the decreased bromobenzene LT_{50} in paraquat treated mice may be due to the added expression of paraquat toxicity in conjunction with bromobenzene toxicity.

The possibility also exists that activation of bromobenzene to the epoxide may be affected by chronic paraquat treatment. Paraquat pretreatment in rats has been reported to decrease the

in vitro metabolism of bromobenzene along with a destruction of microsomal cytochrome P-450 which functions to activate bromobenzene (Ilett *et al.*, 1974). Lipid peroxidative damage has been implicated in the degradation of cytochrome P-450 (Levin *et al.*, 1973) with subsequent impairment of the metabolism of pentobarbital and acetanilide (Jacobson *et al.*, 1973). Although 42 day old mice treated with 100 ppm paraquat had no increase in the lipid peroxidation by-product, malondialdehyde, in lung, liver or kidney (Table 3), a slow rate of lipid peroxidation may still have occurred as malondialdehyde is metabolized *in vivo* (Recknagel *et al.*, 1974). Alternatively, paraquat may disrupt the microsomal electron transport system (Ilett *et al.*, 1974; Krieger *et al.*, 1974) and thereby decrease bromobenzene metabolism. The enhancement of bromobenzene toxicity observed in this study, however, may indicate that a possible inhibition of bromobenzene metabolism by chronic paraquat treatment was not sufficient to prevent the formation of epoxide and subsequent reduction in tissue GSH.

Pretreatment of mice with phenobarbital for 10 days significantly increased the paraquat LD_{50} when the mice were continued on the phenobarbital after paraquat administration (Table 21). The maintenance of the mice on phenobarbital after paraquat administration appeared to be necessary to obtain protection since the paraquat LD_{50} was not significantly elevated compared to the control value in mice transferred to tap water for 24 hours before paraquat treatment (Table 21). Furthermore, a single injection of phenobarbital 30 minutes prior to paraquat injection did not alter the

paraquat LD_{50} (Table 21). The alteration of the paraquat LD_{50} by phenobarbital in the water did not appear to be caused by a shift in the distribution of paraquat in the mice as the elimination of paraquat from plasma, lung (Figure 15), liver and kidney (Figure 16) was similar in both phenobarbital pretreated and control mice. The protective effect by phenobarbital may be related to the body levels of phenobarbital that would occur with continued maintenance of the mice on the treated water. The first step in the proposed mechanism for the *in vivo* toxicity of paraquat requires that paraquat undergo a single electron reduction (Figure 18). The microsomal enzyme NADPH-cytochrome c reductase has been shown to reduce paraquat anaerobically (Figure 10). Pretreatment of mice with phenobarbital and their maintenance on it after paraquat treatment may have raised the body concentrations of phenobarbital such that the barbiturate, during its own metabolism, could compete for electrons supplied by NADPH which might otherwise be utilized in the reduction of paraquat. If the reduction of paraquat was decreased in such a way, less superoxide radicals would be generated with a resultant decrease in paraquat toxicity. That such a competition for electrons may exist is evidenced from *in vitro* experiments in which the epoxidation of aldrin was inhibited by paraquat, presumably by an interference with microsomal electron transport (Krieger *et al.*, 1973). The return of the paraquat LD_{50} to the control value when the mice were transferred to tap water for 24 hours prior to paraquat injection may be indicative of a fall in the phenobarbital concentrations over the 24 hour period such that the competition for electrons was

not sufficient to provide protection. The lack of any alteration in the paraquat LD_{50} by a single dose of phenobarbital may indicate that the concentrations of phenobarbital were not high enough or sustained enough to provide protection. An alternative explanation for the protection afforded by phenobarbital pretreatment in paraquat toxicity, or possibly in conjunction with the previous proposal, is that the general effect of phenobarbital on cellular oxidative metabolism (Sharpless, 1970) may in some way interfere with the *in vivo* reduction of paraquat.

Summary and Conclusions

Prenatal and postnatal administration of 50 and 100 ppm paraquat in the drinking water to mice did not cause any significant alterations in body weight compared to controls over the 42 day postnatal exposure period. Mortality in the 100 ppm paraquat treated mice, however, was significantly elevated at from 7 days up to 42 days after birth. The increase in mortality was associated with the development of pulmonary lesions but not liver and kidney lesions. Both 50 and 100 ppm paraquat treated 42 day old mice were significantly sensitized to the toxic effects of oxygen and bromobenzene, even though the 50 ppm group had no physical or histopathological evidence of paraquat toxicity. Furthermore, the significant interaction of paraquat with oxygen was also observed in the 100 ppm paraquat treated group at 28 days postnatally, and as early as the first day after birth.

The observed effects of paraquat on the development of mice are useful in assessing the potential environmental hazard of

paraquat. In the developing mouse, paraquat has been demonstrated to have toxic effects when the herbicide was administered in a way that might be encountered in an environmental situation. An important observation made in these animals was that the expression of toxicity was significantly enhanced by exposure to oxygen or bromobenzene. Thus, animals who are treated with paraquat and yet have no visible expression of paraquat toxicity may have significant toxic interactions with other agents. The interaction of paraquat with oxygen and bromobenzene also has implications for the mechanism of the mammalian toxicity of paraquat, particularly with respect to the generation of superoxide radicals *in vivo* by paraquat and subsequent initiation of lipid peroxidation.

Under anaerobic conditions *in vitro*, paraquat was reduced to the free radical form by a single electron reduction reaction catalyzed by mouse lung microsomes and NADPH. Antibody to NADPH-cytochrome c reductase inhibited the reaction, which suggested that microsomal NADPH-cytochrome c reductase catalyzed the electron transfer reaction. Paraquat, when incubated aerobically with NADPH, NADPH-cytochrome c reductase and extracted microsomal lipid, initiated lipid peroxidation as indicated by the formation of malondialdehyde in a concentration dependent manner. *In vitro* paraquat-induced lipid peroxidation was inhibited by superoxide dismutase and a singlet oxygen trapping agent, 1,3-diphenylisobenzofuran. Both agents together inhibited *in vitro* paraquat-induced lipid peroxidation to a greater degree than either agent alone.

A mechanism for paraquat toxicity was developed from the *in vitro* experiments described above. Paraquat was proposed to undergo

a single electron reduction to the free radical, which would then be reoxidized by transfer of the extra electron to molecular oxygen, resulting in the formation of superoxide radicals. Superoxide radicals nonenzymatically dismutate to singlet oxygen, which attacks polyunsaturated lipids associated with cell membranes to form lipid hydroperoxides. Spontaneous decomposition of lipid hydroperoxides in the presence of trace metal initiates the membrane destructive process of lipid peroxidation.

Although microsomal NADPH-cytochrome c reductase was shown to catalyze paraquat reduction to the free radical, in an *in vivo* situation, a variety of intracellular and extracellular single electron transfer reductases may catalyze this reaction. Thus, the cellular site(s) at which lipid peroxidation is initiated by paraquat may be dependent upon 1) the paraquat concentration at the site, 2) the ability of paraquat to be enzymatically reduced near the site, and 3) the oxygen concentration at the site. The proposed mechanism for paraquat toxicity also accounts for the enhancement of paraquat toxicity by oxygen, as increased oxygen tension may result in increased generation of toxic superoxide radicals.

To determine the possible involvement of paraquat-induced lipid peroxidation *in vivo*, experiments based upon a fourth factor determining the initiation of lipid peroxidation, the ability of mammalian systems to combat lipid peroxidation, were conducted. Selenium deficiency has been shown to reduce the activity of GSH peroxidase, an enzyme which detoxifies lipid hydroperoxides (Smith *et al.*, 1974). Selenium deficient mice were significantly sensitized to paraquat toxicity, as indicated by a threefold decrease in

the LD_{50} of paraquat. Furthermore, the LD_{50} of paraquat was significantly decreased in mice pretreated with diethyl maleate, an agent which reduces the tissue concentrations of GSH. The reducing equivalents utilized by GSH peroxidase to detoxify lipid hydroperoxides are supplied by GSH. Lipid peroxidation is also prevented *in vivo* by the antioxidant activity of vitamin E (Tappel, 1972). Vitamin E deficient mice were sensitized to paraquat toxicity, which was reflected in a threefold decrease in the LD_{50} of paraquat. The significant enhancement of paraquat toxicity by the three treatments described provides evidence for the *in vivo* initiation of lipid peroxidation by paraquat, since all three treatments would make the animals vulnerable to lipid peroxidative damage. Further evidence for *in vivo* lipid peroxidation induced by paraquat was obtained from the observations that the water soluble antioxidant GSH and lipid soluble antioxidants were decreased in liver and lung, respectively, after an acute dose of paraquat. The proposal of paraquat-induced lipid peroxidation *in vivo*, therefore, is consistent with the results of the experiments described.

Rats treated with paraquat in the drinking water for 3 weeks had elevated activities of glucose-6-phosphate dehydrogenase and GSH reductase in lung tissue. The increase in the activities of these enzymes may be a response to combat paraquat-induced lipid peroxidation, as both enzymes supply reducing equivalents required for the activity of GSH peroxidase. Pretreatment of rats with 85% oxygen for 7 days, which has been shown to increase the activity of superoxide dismutase (Crapo and Tierney, 1974) and glucose-6-

phosphate dehydrogenase (Tierney, 1973) in lung tissue, increased the resistance of rats to a toxic dose of paraquat. Thus, the cross-tolerance of oxygen and paraquat supports the proposed mechanism, as animals with elevated superoxide dismutase and glucose-6-phosphate dehydrogenase activities should be resistant to paraquat.

The LD_{50} of paraquat was significantly elevated in mice pretreated with phenobarbital in the drinking water. The protection afforded by phenobarbital may be due to a competition for electrons between its own metabolism and those which might otherwise be utilized in the reduction of paraquat. The shift in the LD_{50} by phenobarbital may thus be an indication of a microsomal site for paraquat reduction. However, the effect of phenobarbital on oxidative metabolism may also contribute to the shift in toxicity.

The *in vivo* toxicity of paraquat, therefore, may involve lipid peroxidation of cellular membranes. Toxicity is the result of a cyclic reduction-oxidation of paraquat with subsequent generation of superoxide radicals, which nonenzymatically dismutate to singlet oxygen. Singlet oxygen reacts with unsaturated fatty acids to form lipid hydroperoxides which results in the initiation of lipid peroxidation.

BIBLIOGRAPHY

BIBLIOGRAPHY

Adams, D. R. and Wilkinson, F.: Lifetime of singlet oxygen in liquid solution. J. Chem. Soc., Faraday Trans. 2, 68: 586-593, 1972.

Allen, R. C., Stjernholm, R. L. and Steele, R. H.: Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. Biochem. Biophys. Res. Communic. 47: 679-684, 1972.

Allen, R. C., Yevich, S. J., Orth, R. W. and Steele, R. H.: The superoxide anion and singlet molecular oxygen: Their role in the microbicidal activity of the polymorphonuclear leukocyte. Biochem. Biophys. Res. Communic. 60: 909-917, 1974.

Almog, C. and Tal, E.: Death from paraquat after subcutaneous injection. Brit. Med. J. 3: 721, 1967.

Anonymous: Touching up the paraquat picture. Fd. Cosmet. Toxicol. 10: 700-705, 1972.

Anonymous: Paraquat attacked. Nature 245: 64, 1973.

Archakov, A. I. and Kurzina, I. I.: CCl_4-induced damage to endoplasmic reticulum membranes. Biochem. Pharmacol. 22: 2095-2104, 1973.

Autor, A. P.: Reduction of paraquat toxicity by superoxide dismutase. Life Sci. 14: 1309-1319, 1974.

Ayers, L. and Tierney, D. F.: High PO_2 and the pentose pathway in rat lungs. Clin. Res. 19: 190, 1971.

Babior, B. M., Kipnes, R. S. and Curnutte, J. T.: Biological defense mechanisms: The production by leukocytes of superoxide, a potential bactericidal agent. J. Clin. Invest. 52: 741-744, 1973.

Barber, A. A. and Bernheim, F.: Lipid peroxidation: Its measurement, occurrence, and significance in animal tissues. In *Advances in Gerontological Research*, ed. by B. L. Strehler, Academic Press, New York, vol 2, pp. 355-403, 1967.

Beebeejaun, A. R., Beevers, G. and Rogers, W. N.: Paraquat poisoning--prolonged excretion. Clin. Toxicol. 4: 397-407, 1971.

Beckman, C., Lundgren, E. and Tarnvik, A.: Superoxide dismutase isoenzymes in different human tissues: Their genetic control and intracellular localization. Hum. Hered. 23: 338-345, 1973.

Beutler, E.: Drug-induced hemolytic anemia. Pharmacol. Rev. 21: 73-103, 1969.

Bieri, J. G., Dam, H., Prange, I. and Sondergaard, E.: Effect of dietary selenium dioxide, cystine, ethoraquin and vitamin E on lipid autoxidation in chick tissues. Acta Physiol. Scand. 52: 36-43, 1961.

Boon, W. R.: Diquat and paraquat--new agricultural tools. Chem. Industry May 8: 782-788, 1965.

Boon, W. R.: The quaternary salts of bipyridyl--a new agricultural tool. Endeavour 26: 27-32, 1967.

Boyland, E. and Chasseaud, L. F.: The effect of some carbonyl compounds on rat liver glutathione levels. Biochem. Pharmacol. 19: 1526-1528, 1970.

Brodie, B. B., Reid, W., Cho, A. K., Sipes, G., Krishna, G. and Gillette, J. R.: Possible mechanism of liver necrosis caused by aromatic organic compounds. Proc. Nat. Acad. Sci., Wash. 68: 160-164, 1971.

Brook, R. E.: Ultrastructure of lung lesions produced by ingested chemicals. I. Effect of the herbicide paraquat on mouse lung. Lab. Invest. 25: 536-545, 1971.

Browne, T. D.: Treatment of paraquat ingestion. Brit. Med. J. 3: 580, 1971.

Bullivant, C. M.: Accidental poisoning by paraquat: Report of two cases in man. Brit. Med. J. 1: 1272-1273, 1966.

Bunyan, J., Green, J., Edwin, E. E. and Diplock, A. T.: Studies on vitamin E. 5. Lipid peroxidation in dialuric acid-induced haemolysis of vitamin E-deficient erythrocytes. Biochem. J. 77: 47-51, 1960.

Bus, J. S., Preache, M. M., Cagen, S. Z., Posner, H. S., Eliason, B. C., Sharp, C. W. and Gibson, J. E.: Fetal toxicity and distribution of paraquat in mice and rats. In press, Toxicol. Appl. Pharmacol.

Butler, C. B., II and Kleinerman, J.: Paraquat in the rabbit. Brit. J. Industr. Med. 28: 67-71, 1971.

Calderbank, A.: The bipyridylium herbicides. In *Advances in Pest Control Research*, ed. by R. L. Metcalf, Interscience, New York, vol 8, pp. 127-235, 1968.

Cambar, P. J. and Aviado, D. M.: Bronchopulmonary effects of paraquat and expectorants. Arch. Environ. Health 20: 488-494, 1970.

Campbell, S.: Death from paraquat in a child. Lancet 1: 144, 1968.

Cant, J. S. and Lewis, D. R. H.: Ocular damage due to paraquat and diquat. Brit. Med. J. 2: 224, 1968.

Chio, K. S. and Tappel, A. L.: Inactivation of ribonuclease and other enzymes by peroxidizing lipids and by malonaldehyde. Biochem. 8: 2827-2832, 1969.

Chow, C. K. and Tappel, A. L.: An enzymatic protective mechanism against lipid peroxidation damage to lung of ozone-exposed rats. Lipids 7: 518-524, 1972.

Chow, C. K., Dillard, C. J. and Tappel, A. L.: Glutathione peroxidase system and lysozyme in rats exposed to ozone or nitrogen dioxide. Environ. Res. 7: 311-319, 1974.

Chow, C. K. and Tappel, A. L.: Response of glutathione peroxidase to dietary selenium in rats. J. Nutr. 104: 444-451, 1974.

Christopherson, B. O.: Reduction of linolenic acid hydroperoxide by a glutathione peroxidase. Biochim. Biophys. Acta 176: 463-470, 1969.

Clark, D. G., McElligott, T. F. and Hurst, E. W.: The toxicity of paraquat. Brit. J. Industr. Med. 23: 126-132, 1966.

Cohen, G. and Hochstein, P.: Glutathione peroxidase: The primary agent for the elimination of hydrogen peroxide in erythrocytes. Biochem. 2: 1420-1428, 1963.

Cohen, G. and Heikkila, R. E.: The generation of hydrogen peroxide, superoxide radical, and hydroxyl radical by 6-hydroxydopamine, dialuric acid, and related cytotoxic agents. J. Biol. Chem. 249: 2447-2452, 1974.

Cohn, V. H. and Lyle, J.: A fluorometric assay for glutathione. Anal. Biochem. 14: 434-440, 1966.

Conning, D. M., Fletcher, K. and Swan, A. A. B.: Paraquat and related bipyridyls. Brit. Med. Bull. 25: 245-249, 1969.

Copland, G. M., Kolin, A. and Shulman, H. S.: Fatal pulmonary intra-alveolar fibrosis after paraquat ingestion. N. Eng. J. Med. 291: 290-292, 1974.

Crapo, J. D. and Tierney, D. F.: Superoxide dismutase and pulmonary oxygen toxicity. Amer. J. Physiol. 226: 1401-1407, 1974.

Curnutte, J. T., Whitten, D. M. and Babior, B. M.: Defective superoxide production by granulocytes from patients with chronic granulomatous disease. N. Eng. J. Med. 290: 593-597, 1974.

Dam, H., Nielson, G. K., Prange, I. and Sondergaard, E.: The exudative diathesis produced by torula yeast. Experientia 13: 493-494, 1957.

Daniel, J. W. and Gage, J. C.: Absorption and excretion of diquat and paraquat in rats. Brit. J. Industr. Med. 23: 133-136, 1966.

Davies, D. S. and Davies, D. L.: Effect of d-propranolol and superoxide dismutase on paraquat reduction and adrenochrome formation by rat liver microsomes. Fed. Proc. 33: 228, 1974.

Demopoulos, H. B.: The basis of free radical pathology. Fed. Proc. 32: 1859-1861, 1973a.

Demopoulos, H. B.: Control of free radicals in biologic systems. Fed. Proc. 32: 1903-1908, 1973b.

Dillard, C. J. and Tappel, A. L.: Fluorescent products of lipid peroxidation of mitochondria and microsomes. Lipids 6: 715-721, 1971.

DiLuzio, N. R.: Antioxidants, lipid peroxidation, and chemical induced liver injury. Fed. Proc. 32: 1875-1881, 1973.

Dodge, A. D., Harns, N. and Baldwin, B. C.: The mode of action of paraquat. Biochem. J. 118: 43P-44P, 1970.

Dodge, A. D.: The mode of action of the bypyridylium herbicides, paraquat and diquat. Endeavour 30: 130-135, 1971.

Dowty, B., Laseter, J. L., Griffin, G. W., Politzer, I. R. and Walkinshaw, C. H.: Reactions of singlet oxygen with pine pollen. Science 181: 669-670, 1973.

Duffy, B. S. and O'Sullivan, D. J.: Paraquat poisoning. J. Irish Med. Assn. 61: 97-98, 1968.

Editorial: Paraquat poisoning. Lancet ii: 1018-1019, 1971.

Evans, M. J., Stephens, R. J., Cabral, L. J. and Freeman, G.: Cell renewal in the lungs of rats exposed to low levels of NO_2. Arch. Environ. Health 24: 180-188, 1972.

Fairchild, E. J.: Tolerance mechanisms: Determinants of lung to injurious agents. Arch. Environ. Health 14: 111-126, 1967.

Farrington, J. A., Ebert, M., Land, E. J. and Fletcher, K.: Bipyridylium quaternary salts and related compounds. V. Pulse radiolysis studies of the reaction of paraquat radical with oxygen. Implications for the mode of action of bipyridyl herbicides. Biochim. Biophys. Acta 314: 372-381, 1973.

Fennelly, J. J., Gallagher, J. I. and Carroll, R. J.: Paraquat poisoning in a pregnant woman. Brit. Med. J. 3: 722-723, 1968.

Fisher, H. K., Humphries, M. and Bailes, R.: Paraquat poisoning: Recovery from renal and pulmonary damage. Ann. Int. Med. 75: 731-736, 1971.

Fisher, H. K., Clements, J. A. and Wright, R. R.: Enhancement of oxygen toxicity by the herbicide paraquat. Amer. Rev. Resp. Dis. 107: 246-252, 1973a.

Fisher, H. K., Clements, J. A. and Wright, R. R.: Pulmonary effects of the herbicide paraquat studied 3 days after injection in rats. J. Appl. Physiol. 35: 268-273, 1973b.

Flenley, D. C.: Paraquat poisoning. Clinical Pathological Conference. Scottish Med. J. 16: 407, 1971.

Fletcher, K. and Wyatt, I.: The composition of lung lipids after poisoning with paraquat. Brit. J. Exp. Path. 51: 604-610, 1970.

Fletcher, K. and Wyatt, I.: The action of paraquat on the incorporation of palmitic acid into dipalmitoyl lecithin in mouse lungs. Brit. J. Exp. Path. 53: 225-230, 1972.

Fletcher, B. L. and Tappel, A. L.: Protective effects of dietary α-tocopherol in rats exposed to toxic levels of ozone and nitrogen dioxide. Environ. Res. 6: 165-175, 1973.

Flohe, L., Gunzler, W. A. and Schock, H. H.: Glutathione peroxidase: A selenoenzyme. FEBS Letters 32: 132-133, 1973.

Foote, C. S.: Mechanisms of photosensitized oxidations. Science 162: 963-970, 1968.

Foote, C. S. and Denny, R. W.: Chemistry of singlet oxygen. VII. Quenching by β-carotene. J. Amer. Chem. Soc. 90: 6233-6235, 1968.

Fowler, B. A. and Brooks, R. E.: Effects of the herbicide paraquat on the ultrastructure of mouse kidney. Amer. J. Path. 63: 505-512, 1971.

Fridovich, I.: Superoxide dismutases. Adv. Enzymol. 41: 35-98, 1974a.

Fridovich, I.: Superoxide radical and the bactericidal action of phagocytes. N. Eng. J. Med. 290: 624-625, 1974b.

Fridovich, I.: Oxygen: Boon and bane. Amer. Scientist 63: 54-59, 1975.

Friedman, L., Weiss, W., Wherry, F. and Kline, O. L.: Bioassay of vitamin E by the dialuric acid hemolysis method. J. Nutr. 65: 143-160, 1958.

Gage, J. C.: Action of paraquat and diquat on liver cell fractions. Biochem. J. 109: 757-761, 1968.

Galloway, D. B. and Petrie, J. C.: Recovery from severe paraquat poisoning. Postgrad. Med. J. 48: 684-686, 1972.

Glavind, J.: Antioxidants in animal tissue. Acta Chem. Scand. 17: 1635-1640, 1963.

Goettsch, M. and Pappenheimer, A. M.: Nutritional muscular dystrophy in the guinea pig and rabbit. J. Exp. Med. 54: 145-170, 1931.

Goldstein, B. D., Lodi, C., Collinson, M. T. and Balchum, O. J.: Ozone and lipid peroxidation. Arch. Environ. Health 18: 631-635, 1969.

Goldstein, B. D., Buckley, R. D. and Cardenas, R.: Ozone and vitamin E. Science 169: 605-606, 1970.

Gram, T. E.: Comparative aspects of mixed function oxidation by lung and liver of rabbits. Drug Metab. Rev. 2: 1-32, 1973.

Gregory, E. M. and Fridovich, I.: The induction of superoxide dismutase by molecular oxygen. J. Bacteriol. 114: 543-548, 1973a.

Gregory, E. M. and Fridovich, I.: Oxygen toxicity and the superoxide dismutase. J. Bacteriol. 114: 1193-1197, 1973b.

Gregory, E. M., Goscin, S. A. and Fridovich, I.: Superoxide dismutase and oxygen toxicity in a eukaryote. J. Bacteriol. 117: 456-460, 1974.

Grundies, H., Kolmar, D. and Bennhold, I.: Paraquat intoxication. Case report with special reference to hemodialysis. Dtsch. Med. Wochenschr. 96: 588-589, 1971.

Hafeman, D. G., Sunde, R. A. and Hoekstra, W. G.: Effect of dietary selenium on erythrocyte and liver glutathione peroxidase in the rat. J. Nutr. 104: 580-587, 1974.

Hearn, C. E. D. and Keir, W.: Nail damage in spray operators exposed to paraquat. Brit. J. Industr. Med. 28: 399-403, 1971.

Heaton, F. W. and Uri, N.: The aerobic oxidation of unsaturated fatty acids and their esters: Cobalt stearate-catalyzed oxidation of linoleic acid. J. Lipid Res. 2: 152-160, 1961.

Holman, R. T.: Autoxidation of fats and related substances. In *Progress in the Chemistry of Fats and Other Lipids*, ed. by R. T. Holman, W. O. Lundberg and T. Malbin, Pergamon, London, vol 2, pp. 51-98, 1954.

Hopkins, J. and Tudhope, G. R.: Glutathione peroxidase deficiency with increased susceptibility to erythrocyte Heinz body formation. Clin. Sci. Molec. Med. 47: 643-647, 1974a.

Hopkins, J. and Tudhope, G. R.: The effects of drugs on erythrocytes *in vitro*: Heinz body formation, glutathione peroxidase inhibition and changes in mechanical fragility. Brit. J. Clin. Pharmacol. 1: 191-195, 1974b.

Horwitt, M. K.: Role of vitamin E, selenium and polyunsaturated fatty acids in clinical and experimental muscle disease. Fed. Proc. 24: 68-72, 1965.

Howe, D. J. T. and Wright, N.: The toxicity of paraquat and diquat. Proc. N. Zeal. Weed Pest Contr. Conf. 18: 105-114, 1965.

Howes, R. M. and Steele, R. H.: Microsomal chemiluminescence induced by NADPH and its relation to lipid peroxidation. Res. Communic. Chem. Path. Pharmacol. 2: 619-626, 1971.

Hughes, R. D., Millburn, P. and Williams, R. T.: Biliary excretion of some diquaternary ammonium cations in the rat, guinea pig and rabbit. Biochem. J. 136: 979-984, 1973.

Hunter, F. E., Gibicki, J. M., Hofsten, P. E., Weinstein, J. and Scott, A.: Swelling and lysis of rat liver mitochondria induced by ferrous ions. J. Biol. Chem. 238: 828-835, 1963.

Ilett, K. F., Stripp, B., Menard, R. H., Reid, W. D. and Gillette, J. R.: Studies on the mechanism of the lung toxicity of paraquat: Comparison of tissue distribution and some biochemical parameters in rats and rabbits. Toxicol. Appl. Pharmacol. 28: 216-226, 1974.

Jacobson, M., Levin, W., Lu, A. Y. H., Conney, A. H. and Kuntzman, R.: The rate of pentobarbital and acetanilide metabolism by liver microsomes: A function of lipid peroxidation and degradation of cytochrome P-450 heme. Drug. Metab. Disposit. 1: 766-774, 1973.

Jollow, D. J., Mitchell, J. R., Zampaglione, N. and Gillette, J. R.: Bromobenzene-induced liver necrosis. Protective role of glutathione and evidence for 3,4-bromobenzene oxide as the hepatotoxic metabolite. Pharmacol. 11: 151-169, 1974.

Kamataki, T. and Kitagawa, H.: Effects of lipid peroxidation on activities of drug-metabolizing enzymes in liver microsomes of rats. Biochem. Pharmacol. 22: 3199-3207, 1973.

Kann, H. E., Mengel, C. E., Smith, W. and Horton, B.: Oxygen toxicity and vitamin E. Aerospace Med. 35: 840-844, 1964.

Kapanci, Y., Weibel, E. R. and Kaplan, H. P.: Pathogenesis and reversibility of the pulmonary lesions of oxygen toxicity in monkeys. II. Ultrastructural and morphometric studies. Lab. Invest. 20: 101-118, 1969.

Kaplan, H. P., Robinson, F. R. and Kapanci, Y.: Pathogenesis and reversibility of the pulmonary lesions of oxygen toxicity in monkeys. I. Clinical and light microscopic studies. Lab. Invest. 20: 94-100, 1969.

Kerr, F., Patel, A. R., Scott, P. D. R. and Tompsett, S. L.: Paraquat poisoning treated by forced diuresis. Brit. Med. J. 3: 290-291, 1968.

Khan, A. U.: Singlet molecular oxygen from superoxide anion and sensitized fluorescence of organic molecules. Science 168: 476-477, 1970.

Khera, K. S., Whitta, L. L. and Clegg, D. J.: Embryopathic effects of diquat and paraquat in the rat. Industr. Med. Surg. 37: 257-261, 1968.

Kimbrough, R. D. and Gaines, T. B.: Toxicity of paraquat to rats and its effect on rat lungs. Toxicol. Appl. Pharmacol. 17: 679-690, 1970.

Kimbrough, R. D. and Linder, R. E.: The ultrastructure of the paraquat lung lesion in the rat. Environ. Res. 6: 265-273, 1973.

Kimbrough, R. D.: Toxic effects of the herbicide paraquat. Chest 65: 65S-67S, 1974.

Kistler, G. S., Caldwell, P. R. B. and Weibel, E. R.: Development of fine structural damage to alveolar and capillary lining cells in oxygen-poisoned rat lungs. J. Cell Biol. 32: 605-628, 1967.

Krieger, R. I., Lee, R. W., Black, A. and Fukoto, T. R.: Inhibition of microsomal aldrin epoxidation by diquat and several related bipyridylium compounds. Bull. Environ. Contamin. Toxicol. 9: 1-3, 1973.

Krinsky, N. I.: Function. In *Carotenoids*, ed. by O. Isler, Halsted, New York, pp. 669-715, 1971.

Krinsky, N. I.: Singlet excited oxygen as a mediator of the antibacterial action of leukocytes. Science 186: 363-365, 1974a.

Krinsky, N. I.: Carotenoid chromophore length and protection against photosensitization. Photochem. Photobiol. 19: 217-222, 1974b.

Langdon, R. G.: Glucose-6-phosphate dehydrogenase from erythrocytes. In *Methods in Enzymology*, ed. by S. P. Colowick and N. O. Kaplan, Academic Press, New York, vol 9, pp. 126-131, 1966.

Levin, W., Lu, A. Y. H., Jacobson, M. and Kuntzman, R.: Lipid peroxidation and the degradation of cytochrome P-450 heme. Arch. Biochem. Biophys. 158: 842-852, 1973.

Litchfield, J. T., Jr.: A method for graphic solution of time-percent effect curves. J. Pharmacol. Exp. Ther. 97: 399-413, 1949.

Litchfield, J. T., Jr., and Wilcoxin, F. J.: A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Ther. 96: 99-113, 1949.

Litchfield, M. H., Daniel, J. W. and Longshaw, S.: The tissue distribution of the bipyridylium herbicides diquat and paraquat in rats and mice. Toxicol. 1: 155-165, 1973.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J.: Protein measurement with folin phenol reagent. J. Biol. Chem. 193: 265-275, 1951.

Maling, H. M., Saul, W., Williams, M. A., Brown, E. A. B. and Gillette, J. R.: Propranolol treatment of experimental paraquat poisoning in rats. Fed. Proc. 34: 266, 1975.

Malmqvist, E., Grossmann, G., Ivemark, B. and Robertson, B.: Pulmonary phospholipids and surface properties of alveolar wash in experimental paraquat poisoning. Scand. J. Resp. Dis. 54: 4-15, 1973.

Malone, J. D. G., Carmody, M., Keogh, B. and O'Dwyer, W. F.: Paraquat poisoning: A review of nineteen cases. J. Irish Med. Assn. 64: 59-68, 1971.

Manktelow, B. W.: The loss of pulmonary surfactant in paraquat poisoning: A model for the study of respiratory distress syndrome. Brit. J. Exp. Path. 48: 366-369, 1968.

Masterson, J. G. and Roche, W. J.: Another paraquat fatality. Brit. Med. J. 2: 482, 1970.

Matthew, H., Logan, A., Woodruff, M. F. A. and Heard, B.: Paraquat poisoning - Lung transplantation. Brit. Med. J. 3: 757-763, 1968.

Matthews, M.: Protective effect of beta-carotene lethal photosensitization by haematoporphyrin. Nature 203: 1092, 1964.

Maugh, T. H., II.: Singlet oxygen: A unique microbicidal agent in cells. Science 182: 44-45, 1973.

McCord, J. M. and Fridovich, I.: Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244: 6049-6055, 1969.

McCord, J. M., Keele, B. B., Jr., and Fridovich, I.: An enzyme based theory of obligate anaerobiosis: The physiological function of superoxide dismutase. Proc. Nat. Acad. Sci. U.S.A. 68: 1024-1027, 1971.

McCord, J. M., Beauchamp, C. O., Goscin, S., Misra, H. P. and Fridovich, I.: Superoxide and superoxide dismutase. In *Oxidases and Related Redox Systems*, ed. by T. E. King, H. S. Mason and M. Morrison, University Park Press, Baltimore, pp. 51-76, 1973.

McDonagh, B. J. and Martin, J.: Paraquat poisoning in children. Arch. Dis. Childh. 45: 425-427, 1970.

McElligott, T. F.: The dermal toxicity of paraquat: Differences due to techniques of applications. Toxicol. Appl. Pharmacol. 21: 361-368, 1972.

Mees, G. C.: Experiments on the herbicidal action of 1,1'-ethylene-2,2'-dipyridylium dibromide. Ann. Appl. Biol. 48: 601-612, 1960.

Michaelis, L. and Hill, E. S.: Potentiometric studies on semiquinones. J. Amer. Chem. Soc. 55: 1481-1494, 1933a.

Michaelis, L. and Hill, E. S.: The viologen indicators. J. Gen. Physiol. 16: 859-873, 1933b.

Mino, M.: Oxygen poisoning and vitamin E deficiency. J. Nutr. Sci. Vitaminol. 19: 95-104, 1973.

Misra, H. P. and Fridovich, I.: The univalent reduction of oxygen by reduced flavins and quinones. J. Biol. Chem. 247: 188-192, 1972.

Mitts, G. C. and Randall, H. D.: II. Hemoglobin catabolism. The protection of hemoglobin from oxidative breakdown in the intact erythrocyte. J. Biol. Chem. 232: 589-598, 1958.

Molnar, I. G. and Hayes, W. J.: Distribution and metabolism of paraquat in the rat. Toxicol. Appl. Pharmacol. 19: 405, 1971.

Murray, R. E. and Gibson, J. E.: A comparative study of paraquat intoxication in rats, guinea pigs and monkeys. Exp. Molec. Path. 17: 317-325, 1972.

Murray, R. E. and Gibson, J. E.: Paraquat disposition in rats, guinea pigs and monkeys. Toxicol. Appl. Pharmacol. 27: 283-291, 1974.

Necheles, T. F., Steinberg, M. H. and Cameron, D.: Erythrocyte glutathione-peroxidase deficiency. Brit. J. Haemat. 19: 605-612, 1970.

Nienhaus, H. and Ehrenfeld, M.: Pathogenesis of lung disease in paraquat poisoning. Beitrag. Path. 142: 244-267, 1971.

Noguchi, T., Cantor, A. H. and Scott, M. L.: Mode of action of selenium and vitamin E in prevention of exudative diathesis in chicks. J. Nutr. 103: 1502-1511, 1973.

O'Brien, P. J. and Little, C.: Intracellular mechanisms for the decomposition of a lipid peroxide. II. Decomposition of a lipid peroxide by subcellular fractions. Can. J. Biochem. 47: 493-499, 1969.

Oh, S. H., Ganther, H. E. and Hoekstra, W. G.: Selenium as a component of glutathione peroxidase isolated from ovine erythrocytes. Biochem. 13: 1825-1829, 1974.

Oreopoulos, D. G., Soyannwo, M. A. O., Sinniah, R., Fenton, S. A. A., McGeown, M. G. and Bruce, J. H.: Acute renal failure in a case of paraquat poisoning. Brit. Med. J. 1: 749-750, 1968.

Orton, T. C., Anderson, M.W., Pickett, R. D., Eling, T. E. and Fouts, J. R.: Xenobiotic accumulation and metabolism by isolated perfused rabbit lungs. J. Pharmacol. Exp. Ther. 186: 482-497, 1973.

Paglia, D. E. and Valentine, W. N.: Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 70: 158-169, 1967.

Pasi, A. and Hine, C. H.: Paraquat poisoning. Proc. West. Pharmacol. Soc. 14: 169-172, 1971.

Pasi, A., Embree, J. W., Jr., Eisenlord, G. H. and Hine, C. H.: Assessment of the mutagenic properties of diquat and paraquat in the murine dominant lethal test. Mutation Res. 26: 171-175, 1974.

Pederson, T. C. and Aust, S. D.: The role of superoxide and singlet oxygen in lipid peroxidation promoted by xanthine oxidase. Biochem. Biophys. Res. Communic. 52: 1071-1078, 1973.

Pederson, T. C., Buege, J. A. and Aust, S. D.: Microsomal electron transport. The role of reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase in liver microsomal lipid peroxidation. J. Biol. Chem. 248: 7134-7141, 1973.

Placer, A. A., Cushman, L. L. and Johnson, B. C.: Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems. Anal. Biochem. 16: 359-364, 1966.

Porta, E. A. and Hartroft, W. S.: Lipid pigments in relation to aging and dietary factors (lipofuscins). In *Pigments in Pathology*, ed. by M. Wolman, Academic Press, New York, pp. 191-235, 1969.

Pryor, W. A.: Free radical reactions and their importance in biochemical systems. Fed. Proc. 32: 1862-1869, 1973.

Racker, E.: Glutathione reductase (liver and yeast). In *Methods in Enzymology*, ed. by S. P. Colowick and N. O. Kaplan, Academic Press, New York, vol 2, pp. 722-725, 1955.

Recknagel, R. O.: Carbon tetrachloride hepatotoxicity. Pharmacol. Rev. 19: 145-208, 1967.

Recknagel, R. O., Glende, E. A., Ugazio, G., Koch, R. R. and Srinivasan, S.: New data in support of the lipo-peroxidation theory for carbon tetrachloride liver injury. Israel J. Med. Sci. 10: 301-311, 1974.

Reddy, K. and Tappel, A. L.: Effect of dietary selenium and autoxidized lipids on the glutathione peroxidase system of gastrointestinal tract and other tissues in the rat. J. Nutr. 104: 1069-1078, 1974.

Rhodes, M. L.: Hypoxic protection of paraquat poisoning: A model for respiratory distress syndrome. Chest 66: 34, 1974.

Robertson, B., Ehnorning, G., Ivemark, B., Malmqvist, E. and Modee, J.: Experimental respiratory distress induced by paraquat. J. Path. 103: 239-244, 1971.

Roehm, J. N., Hadley, J. G. and Menzel, D. B.: The influence of vitamin E on the lung fatty acids of rats exposed to ozone. Arch. Environ. Health 24: 237-242, 1972.

Rose, C. S. and Gyorgy, P.: Specificity of hemolytic reaction in vitamin E-deficient erythrocytes. Amer. J. Physiol. 168: 414-420, 1952.

Rose, M. S., Crabtree, H. K., Fletcher, K. and Wyatt, I.: Biochemical effects of diquat and paraquat. Biochem. J. 138: 437-443, 1974a.

Rose, M. S., Smith, L. L. and Wyatt, I.: Evidence for energy-dependent accumulation of paraquat into rat lung. Nature 252: 314-315, 1974b.

Rosenbaum, R. M., Wittner, M. and Lenger, M.: Mitochondrial and other ultrastructural changes in great alveolar cells of oxygen-adapted and poisoned rats. Lab. Invest. 20: 516-528, 1969.

Rotruck, J. T., Pope, A. L., Ganther, H. E., Swanson, A. B., Hafeman, D. G. and Hoekstra, W. G.: Selenium: Biochemical role as a component of glutathione peroxidase. Science 179: 588-590, 1973.

Rouser, G. G., Nelson, J., Fleisher, S. and Simon, G.: Lipid composition of animal cell membranes, organelles and organs. In *Biological Membranes*, ed. by D. Chapman, Academic Press, New York, pp. 5-70, 1968.

Rugh, R.: *The Mouse. Its Reproduction and Development*. Burgess Publishing Company, Minneapolis, 1968.

Samman, P. D. and Johnston, E. N. M.: Nail damage associated with handling of paraquat and diquat. Brit. Med. J. 1: 818-819, 1969.

Sbarra, A. J. and Karnovsky, M. L.: The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. J. Biol. Chem. 234: 1355-1362, 1959.

Schwarz, K.: Role of vitamin E, selenium, and related factors in experimental nutritional liver disease. Fed. Proc. 24: 58-67, 1965.

Scott, M. L.: Anti-oxidants, selenium and sulphur amino acids in the vitamin E nutrition of chicks. Nutr. Abstr. Rev. 32: 1-8, 1962.

Scott, M. L.: Studies on vitamin and related factors in nutrition and metabolism. In *The Fat-Soluble Vitamin*, ed. by H. J. DeLuca and J. W. Suttie, pp. 355-368, 1969.

Sharp, C. W., Ottolenghi, A. and Posner, H. S.: Correlation of paraquat toxicity with tissue concentrations and weight of the rat. Toxicol. Appl. Pharmacol. 22: 241-251, 1972.

Sinow, J. and Wei, E.: Ocular toxicity of paraquat. Bull. Environ. Contamin. Toxicol. 9: 163-168, 1973.

Smith, P.: A light and electron-microscopic study of the pulmonary lesions induced in rats by paraquat. J. Path. 104: Pvii, 1971.

Smith, P. J., Tappel, A. L. and Chow, C. K.: Glutathione peroxidase activity as a function of dietary selenomethionine. Nature 247: 392-393, 1974.

Sokal, R. R. and Rohlf, F. J.: *Biometry*. W. H. Freeman and Company, San Francisco, California, 1969.

Sharpless, S. K.: Hypnotics and sedatives. I. The barbiturates. In *The Pharmacological Basis of Therapeutics*, ed. by L. S. Goodman and A. Gilman, The Macmillan Company, New York, pp. 98-120, 1970.

Staiff, D. C., Irle, G. K. and Felsenstein, W. C.: Screening of various adsorbents for protection against paraquat poisoning. Bull. Environ. Contamin. Toxicol. 10: 193-199, 1973.

Stauff, J., Schmidkunz, H. and Hartmann, G.: Weak chemiluminescence of oxidation reactions. Nature 198: 281-282, 1963.

Steinberg, M. H. and Necheles, T. F.: Erythrocyte glutathione peroxidase deficiency. Amer. J. Med. 50: 542-546, 1971.

Stephens, R. J., Freeman, G. and Evans, M. J.: Early response of lungs to low levels of nitrogen dioxide: Light and electron microscopy. Arch. Environ. Health 24: 160-179, 1972.

Stephens, R. J., Sloan, M. F., Evans, M. J. and Freeman, G.: Early response of lung to low levels of ozone. Amer. J. Path. 74: 31-58, 1974.

Stokinger, H. E. and Scheel, L. D.: Ozone toxicity: Immunochemical and tolerance-producing aspects. Arch. Environ. Health 4: 327-334, 1962.

Styles, J. A.: Studies on the effects of paraquat and diquat on cells in culture. Brit. J. Exp. Path. 55: 71-77, 1974.

Swan, A. A. B.: Exposure of spray operators to paraquat. Brit. J. Industr. Med. 26: 322-329, 1969.

Swern, D.: Primary products of olefinic autoxidations. In *Autoxidations and Antioxidants*, ed. by W. O. Lundberg, Interscience, New York, vol 1, pp. 1-54, 1961.

Tappel, A. L. and Zalkin, H.: Lipid peroxidation in isolated mitochondria. Arch. Biochem. Biophys. 80: 326-332, 1959.

Tappel, A. L.: Free-radical lipid peroxidation damage and its inhibition by vitamin E and selenium. Fed. Proc. 24: 73-78, 1965.

Tappel, A. L.: Vitamin E and free radical peroxidation of lipids. New York Acad. Sci. 203: 12-28, 1972.

Tappel, A. L.: Lipid peroxidation damage to cell components. Fed. Proc. 32: 1870-1874, 1973.

Tappel, A. L.: Selenium-glutathione peroxidase and vitamin E. Amer. J. Clin. Nutr. 27: 960-965, 1974.

Thomas, H. V., Mueller, P. K. and Lyman, R. L.: Lipo-peroxidation of lung lipid in rats exposed to nitrogen dioxide. Science 159: 532-534, 1968.

Thorneley, R. N. F.: A convenient electrochemical preparation of reduced methyl viologen and a kinetic study of the reaction with oxygen using an anaerobic stopped-flow apparatus. Biochim. Biophys. Acta 333: 487-496, 1974.

Tierney, D., Ayers, L., Herzog, S. and Yang, J.: Pentose pathway and production of reduced nicotinamide adenine dinucleotide phosphate. Amer. Rev. Resp. Dis. 108: 1348-1351, 1973.

Tompsett, S. L.: Paraquat poisoning. Acta Pharmacol. Toxicol. 28: 346-358, 1970.

Toner, P. G., Vetters, J. M., Spilg, W. G. S. and Harland, W. A.: Fine structure of the lung lesion in a case of paraquat poisoning. J. Path. 102: 182-185, 1970.

Tsen, C. C. and Collier, H. B.: The protective action of tocopherol against hemolysis of rat erythrocytes by dialuric acid. Can. J. Biochem. Physiol. 38: 957-964, 1960.

Tubis, M., Baker, N. and Blahd, W. H.: Vitamin therapy in mice with an hereditary myopathy (*Dystrophia muscularis*). J. Nutr. 68: 595-601, 1959.

Tudhope, G. R. and Hopkins, J.: Lipid peroxidation in human erythrocytes in tocopherol deficiency. Acta Haemat. 53: 98-104, 1975.

Van Osten, G. K. and Gibson, J. E.: Effect of paraquat on the biosynthesis of deoxyribonucleic acid, ribonucleic acid and protein in the rat. Fd. Cosmet. Toxicol. 13: 47-54, 1975.

Vijeyaratnam, G. S. and Corrin, B.: Experimental paraquat poisoning: A histological and electron-optical study of the changes in the lung. J. Path. 103: 123-129, 1971.

Villarruel, M. C.: Carbon tetrachloride target lipids in the liver microsomes. Effect of cystamine administration on their pattern of labeling by ^{14}C-CCl_4. Biochem. Biophys. Res. Communic. 54: 108-115, 1973.

White, J. R. and Dearman, H. H.: Generation of free radicals from phenazine methosulfate, streptonigrin, and rubiflavin in bacterial suspensions. Proc. Nat. Acad. Sci. U.S.A. 54: 887-891, 1965.

White, H. L. and White, J. R.: Lethal action and metabolic effects of streptonigrin on *Escherichia coli*. Molec. Pharmacol. 4: 549-565, 1968.

White, J. R., Vaughan, T. O. and W-Shiang, Y.: Superoxide radical in the mechanism of action of streptonigrin. Fed. Proc. 30: 1145, 1971.

Wills, E. D.: Lipid peroxide formation in microsomes. Biochem. J. 113: 333-341, 1969.

Wilson, T. and Hastings, J. W.: Chemical and biological aspects of singlet excited molecular oxygen. In *Photophysiology*, ed. by A. C. Giese, Academic Press, New York, vol 5, pp. 49-95, 1970.

Winter, P. M. and Smith, G.: The toxicity of oxygen. Anesthesiol. 37: 210-241, 1972.

Witschi, H. and Kacew, S.: Studies on the pathological biochemistry of lung parenchyma in acute paraquat poisoning. Med. Biol. 52: 104-110, 1974.