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Interactions of dietary methionine, lead and lindane in mice and rats

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Interactions of dietary methionine, lead and lindane in mice and rats

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INTRODUCTION

Increased industrialization exposes many populations to greater amounts of environmental contaminants. These exposures probably involve several toxicants simultaneously. The lack of public awareness of these contacts with contaminants gives people reason for concern.

Lead is a very common environmental contaminant. This nonessential element is deposited in various quantities in organs of humans (Conrad and Barton, 1978). The level of lead that shows minimal toxic effect is difficult to assess. It is known, however, that nutritional status will affect the response of an organism to lead (Levander, 1979).

Lindane, an organochlorine pesticide, has been used as a soil, foliar and seed treatment on a large variety of crops and has been used on livestock, pets and agricultural premises (NCI Carcinogenesis Technical Report Series No. 14, 1977). Unfortunately, it has residue persistence in soil and foods (Reuber, 1979). This compound has recently had its usage reevaluated because of concern for the safety of humans and the environment (IDA, 1983).

Dietary protein is important in detoxifying many toxicants including minerals, pesticides, drugs and natural toxicants (Hathcock, 1976). Both quality and quantity of protein, especially the quantity of sulfur-amino acids methionine and cysteine, influences the effectiveness of this detoxification. A major importance of these amino acids is in the synthesis of glutathione, which is able to conjugate with electrophilic compounds and metabolites. This conjugation reaction is a de-

toxification process because the result of the conjugation is the formation of a less toxic and more readily excretable compound. Methionine is of particular importance because it is the precursor of cysteine and it is also an essential amino acid that can be supplied only through the diet. These sulfur-amino acids are most abundant in animal protein and considerably less so in plant proteins. There has been a growing trend to decrease the amount of meat consumption in the United States. The use of meat substitutes as alternative protein sources has caused an increase in the use of plant proteins such as soybeans in which methionine is the first-limiting amino acid. The amount of methionine and cysteine in the diet could be reduced significantly depending on how much plant protein is substituted for animal protein.

These considerations suggest that alteration of glutathione levels by environmental contaminants may produce toxicities and toxic synergisms which could cause a potentiating effect on cell damage in individuals consuming marginal or inadequate amounts of methionine. The importance of adequate amounts of sulfur-amino acids in the diet and their relation to glutathione levels and xenobiotic detoxification need to be examined.

The purpose of this research project was to investigate the effects dietary methionine levels have on lindane and lead effects; the possible toxic synergisms that may occur, the involvement of glutathione metabolism in these toxicities and what carcinogenic potential, if any, may develop from the interactions examined in these experimental models.

REVIEW OF LITERATURE

Dietary Methionine and Glutathione

Methionine is an essential sulfur-containing amino acid required for the maintenance of protein equilibrium in humans. The methionine requirement may be somewhat reduced if the sulfur-containing non-essential amino acid cysteine is supplied by the diet (Mitchell et al., 1976). Cysteine and its precursor methionine are found more abundantly in animal protein than plant protein. During digestion, methionine and cysteine are released from protein and absorbed into the portal circulation.

Methionine's functions include methylation reactions where it can be used as a methyl donor, for the synthesis of proteins and as a precursor of the sulfur atom of cysteine and the 3 carbon propylamine moiety of spermine and spermidine (Mudd and Poole, 1975). There is also a complicated interrelationship between the effect of methionine and vitamin B-12 on folate metabolism. The importance of this interrelationship is evident in vitamin B-12 deficiencies (Stokstad, 1976). Methionine also is a potent amino acid in the production of toxic effects. It has been suggested that this toxicity could be due to excessive methyl donation, competitive inhibition of amino acid transport and/or adenosine triphosphate (ATP) depletion (Benevenga, 1974).

Methionine is also used in glutathione (GSH) synthesis. Cysteine, which can be synthesized from methionine, combines in a 2-step process with glycine and glutamic acid producing GSH (Figure 1). Both steps are ATP dependent (Lehninger, 1975). In the production of GSH, the

L-Glutamic acid + L-cysteine + ATP $\xrightarrow[\text{synthetase, } Mg^{2+}, K^{+}]{\gamma\text{-glutamylcysteine}}$

γ-glutamylcysteine + ADP + P_i (1)

γ-Glutamylcysteine + glycine + ATP $\xrightarrow[\text{synthetase}]{\text{glutathione}}$

γ-Glutamylcysteinylglycine (glutathione) + ADP + P_i (2)

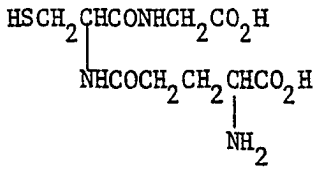

Figure 1. Biosynthesis of glutathione

concentration of cysteine is rate-limiting. Feedback inhibition of γ-glutamyl-cysteine synthetase by GSH normally regulates GSH production (Meister and Anderson, 1983; Maines, 1981).

Glutathione is the tripeptide that can be found in nearly all cells. It usually is the most abundant sulfhydryl compound present in animal tissues and constitutes up to 90 percent of all the nonprotein thiols in mammalian cells (White et al., 1978). It can be found in the reduced form (GSH) and the oxidized form, glutathione disulfide (GSSG). GSH and GSSG are easily interconverted (Javitt, 1961). Intracellular glutathione is found mainly (99%) in the reduced form (GSH) at a concentration of approximately 0.5-10 mM and the extracellular GSH of plasma is at only micromolar concentrations (μM) (Meister and Anderson, 1983). Intracellular GSH is the source of plasma GSH and GSSG. GSH concentrations vary depending on the tissue sampled. The liver of male white mice contained the greatest concentration, followed by the spleen,

kidney, pancreas, lungs, heart, adrenals and blood (Shaker and Soliman, 1966, cited by Chasseaud, 1979). The liver is an important organ for biotransformation and GSH levels are relatively high there at about 170 mg/100 g in rat tissue (Chasseaud, 1976). Intracellular GSH concentrations are susceptible to nutritional status, hormonal balance and growth of the organism (Knox, 1960).

The cellular turnover of glutathione is associated with its transport out of cells in the form of GSH (Meister and Anderson, 1983). The rate of GSH turnover was determined to be the highest in the kidney, followed by liver, pancreas and skeletal muscle when tested in mice (Griffith and Meister, 1979). The metabolism of GSH begins with its release from cells. It is then transported by blood plasma to the kidney or other tissues with substantial transpeptidase activity. GSH in the kidney is degraded into a glutamyl group and cysteinyl-glycine through the action of γ-glutamyl transpeptidase found in the microvilli membranes. The γ-glutamyl moiety is joined to an extracellular acceptor amino acid forming γ-glutamyl amino acid which is transported into the cell. There it undergoes a series of reactions known as the γ-glutamyl cycle. This cycle completes itself by resynthesizing GSH. Thus, GSH serves as a transport of amino acids across cell membranes (Meister, 1983; Siegers and Younes, 1983; Meister, 1982; Meister, 1981; Griffith and Meister, 1979).

All of the functions of glutathione in biological systems are not yet fully understood, but several of its direct and indirect functions include: synthesis of proteins and DNA, enzyme activity (catalyzing disulfide exchange reactions and serving as a

coenzyme for certain enzymes), metabolism and protection of cells (detoxifying peroxides and free radicals) (Meister and Anderson, 1983; Siegers and Younes, 1983). GSH, for example, aids in preventing oxidative damage to erythrocytes. GSH, synthesized in human erythrocytes, can with the aid of glutathione peroxidase (a selenium-containing enzyme) help detoxify hydrogen peroxide through reduction of the peroxide and its oxidation to GSSG. GSSG can then be rapidly reduced to GSH by glutathione reductase. GSH can also help prevent oxidation of the SH groups of hemoglobin (White et al., 1978). Another less direct role liver GSH serves is as a reservoir of cysteine. The cysteine pool used for GSH synthesis is kept separate from the cysteine for protein synthesis through the presence of GSH (Ketterer et al., 1983; Tateishi et al., 1981).

One of the more important functions of GSH involves its participation in the formation of a variety of conjugated organic compounds in the liver. These are excreted in the bile and/or urine (Chasseaud, 1979). Metabolism of foreign compounds with GSH followed by the ultimate production of mercapturic acid involves conjugation of electrophilic compounds with the endogenous nucleophile, GSH. GSH conjugation does not require the initial formation of a high energy intermediate involving ATP (Jocelyn, P. C., 1972). The conjugation is generally catalyzed by glutathione S-transferases but it may occur spontaneously. Enzymes catalyzing the activation, GSH conjugation and removal of the glycine and glutamate moieties occurs predominantly in the liver. The final conversion of the GSH conjugate to a mercapturic acid via N-acetyl cysteine conjugate formation occurs mainly in the kidney

(Moldèus et al., 1978).

The metabolism of many environmental contaminants often involves two phases. Hydroxylation, reduction and hydrolysis of many compounds are some of the common types of reactions occurring in Phase I. The most common Phase I system sometimes is referred to as the microsomal monooxygenase system, mixed-function oxidase system (MFO) or cytochrome P-450-containing monooxygenases. This microsomal enzyme system consists of a membrane-bound multicomponent system localized at high concentrations in the endoplasmic reticulum of liver and less so in the kidney, lung, intestine, brain and skin. Its constituents are NADPH-cytochrome C reductase, cytochrome P-450 and phospholipid. Phase II reactions, often the final detoxification step, takes the product of Phase I and conjugates it by combining it with another compound such as GSH. Some compounds can be conjugated directly without any Phase I reaction. The ultimate result of a conjugation with GSH is the formation of mercapturic acid which is a nontoxic compound that is more excretable (Neal, 1980; Coon, 1978; Williams, 1978). Xenobiotic metabolism depends on the following factors (Neal, 1980):

1. Rate of absorption of the compound;
2. The ease at which the compound crosses cell membranes;
3. Whether proteins have the capacity to bind the foreign compounds;
4. The rate of excretion of the compound;
5. Species and strain differences in rates of metabolism;
6. The effect of age, circadian rhythms and nutritional status;
7. The ability of many compounds to affect hepatic cytochrome

P-450-containing monooxygenase activity.

For example, deficiencies of protein are known to decrease the action of MFO enzymes (Campbell, 1978). Glazenburg et al. (1983) studied what effects methionine deficient diets had on GSH conjugation of acetaminophen. They found that diets with methionine levels as low as 25 percent of the control diet (with no cysteine) lowered the liver GSH concentration in rats to about 20 percent of the controls.

Disturbances of GSH and GSSG levels may be induced reversibly or irreversibly. Reversible fluctuation of GSH-GSSG status can be brought about by GSH-peroxidase, certain metabolites, nonenzymatic oxidation of GSH to GSSG, other disulfides, protein and defects associated with GSSG reductase. Irreversible GSH and GSSG disturbances can be produced by failure of GSH synthesis or reactions where GSH forms conjugates as in the case of detoxification reactions (Kosower and Kosower, 1974). Many mercapturic acid precursors detoxified through glutathione conjugation may diminish hepatic GSH stores and that would have important toxicological implications (Chasseaud, 1976).

Glutathione S-Transferases

The conjugation of GSH with environmental contaminants or Phase I biotransformation products is catalyzed by a group of enzymes, the glutathione S-transferases (GSH S-transferases). Other GSH conjugations are with naturally occurring endogenous compounds such as α-β unsaturated compounds (Chasseaud, 1979) or with endogenous substrates such as prostaglandins (Chaudhuri et al., 1978; Cagen et al., 1976) and

Δ^5-3-ketosteroids (Benson et al., 1977). However, many reactions catalyzed by GSH S-transferases also can proceed nonenzymatically.

The GSH S-transferase activity appears dependent on 3 factors: binding of the substrate to the enzyme, presence of a sufficiently electrophilic atom to allow interaction with GSH and increased nucleophilicity of the thiol group of GSH (Kaplowitz, 1980). The enzyme appears to function by lowering the pK of GSH, causing GSH to ionize at physiological pH, thus bringing about a covalent interaction of bound cosubstrates through nucleophilic electrophilic-interactions (Keen et al., 1975). This reaction neutralizes the electrophilic site on the electrophilic substrate and renders the product more water-soluble. Metabolism continues by cleavage of the glutamate and glycine residues followed by acetylation of the resulting free amino acid group of the cysteinyl residue to produce a mercapturic acid (Figure 2) (Chasseaud, 1979).

GSH S-transferases are a family of isoenzymes that appear to be genetically related (Ketterer et al., 1983). Six different GSH S-transferases have been identified (Meister and Anderson, 1983; Habig et al., 1974). They are found in the greatest concentration in the liver but are known to be present in most cells (Kaplowitz, 1980). Rat studies by Kraus and Kloft (1980) showed a surprisingly high level in the steroidogenic organs. Levels were less, equal or greater than those of the liver depending on the substrate and steroidogenic organ tested. Its link here to detoxification is not apparent and is thought to have some other special function. However, Polidoro et al. (1980) identified GSH S-transferase activity in the human placenta cytosol and suggested it to have a detoxifying action against electrophilic compounds.

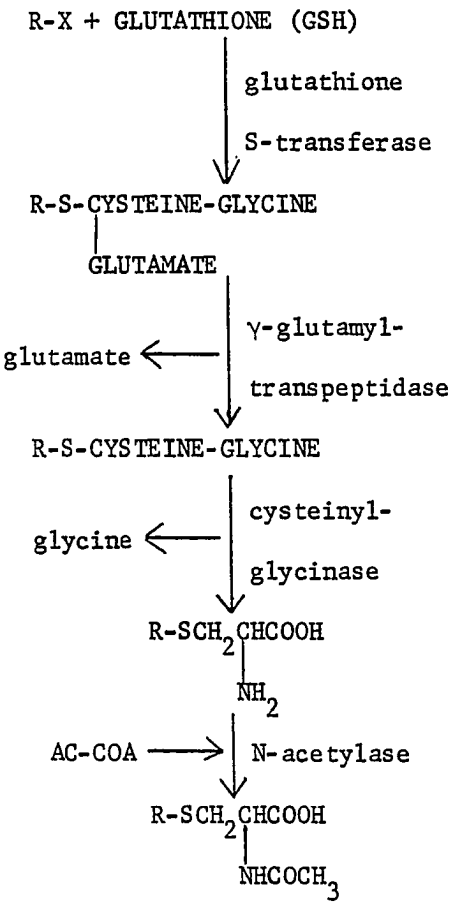

Figure 2. Mercapturic acid formation

Human and rat GSH S-transferases differ in that the human isozymes are closely related in amino acid composition, substrate specificity and immunologic characteristics (Kamisaka et al., 1975), whereas in the rat, GSH S-transferases have overlapping substrate specificities but are, for the most part, immunologically distinct (Kaplowitz et al., 1976; Habig et al., 1976; Habig et al., 1974; Gillham, 1973). Studies with rats showed the transferases differed in activity according to sex, age and substrate (Hayakawa, 1977). Other factors that affect GSH S-transferase activity in rats are fasting, type of inducers, partial

hepatectomy, alcohol pretreatment, experimental diabetes, thyroid dysfunction, cholestasis and liver pre-injury. The effect of each factor may vary depending on the substrate involved (Younes et al., 1980).

The GSH S-transferases are not only found in the cytosol but can also be present in microsomes and mitochondria. Metabolites, some of which become substrates for GSH S-transferase, are produced by the MFO system involved in detoxification and localized on the endoplasmic reticulum. Most of these substrates are hydrophobic and are expected to dissolve in membranes including that of the endoplasmic reticulum (Morgenstern et al., 1980). Kraus and Gross (1979) found decreased concentrations of GSH in liver microsomes and mitochondria when incubated with electrophilic compounds. Microsomes appeared less specific in substance metabolism than the mitochondria. However, both microsome and mitochondrial GSH S-transferase specific activity were lower than in the cytosol. DePierre and Morgenstern (1983) indirectly came to the conclusion that out of the nine organs tested, the largest capacity for conjugation with 1-chloro-2,4-dinitrobenzene was liver cytosol, the liver endoplasmic reticulum and the testicle cytosol. But N-ethylmaleimide-activatable microsomal GSH S-transferase seems only to be found in the liver and is absent from the other organs they examined. Morgenstern et al. (1979) and Morgenstern et al. (1980) found some inducers of the cytochrome P-450 system increased cytoplasmic GSH S-transferase activity but did not affect microsomal activity. N-ethylmaleimide did, however, increase the microsomal activity. Located on the cytoplasmic surface of the endoplasmic reticulum, these membrane-bound microsomal transferases are activated by sulfhydryl reagents and

are presumed to be involved in drug metabolism reactions.

Additional roles have been cited for GSH S-transferase. It appears it can function as a nonselenium glutathione peroxidase (non-Se GSH-Px) (Reddy et al., 1981; Burk et al., 1980; Prohaska, 1980). Ketterer et al. (1983) purified GSH S-transferase B which showed non-Se GSH-Px activity. Selenium requiring glutathione peroxidase catalyzes the breakdown of both organic hydroperoxides and hydrogen peroxide (H_2O_2) but non-Se GSH Px breaks down only organic hydroperoxides not H_2O_2 (Burk et al., 1980). GSH S-transferases also bind to nonsubstrate ligands which has raised questions about their transport and storage capabilities. The binding to nonsubstrate ligands include bilirubin. It has been proposed that the enzymes have 3 binding locations: a glutathione site, an electrophilic substrate site and a nonsubstrate binding site. Another possible role for the GSH S-transferases is transport. By binding to relatively insoluble compounds at the cell membrane and moving them within the cell to sites where biotransformation can occur, these proteins can have a nonenzymatic role in metabolism of foreign compounds. The GSH S-transferases may store compounds by binding them within the cytoplasmic compartments, increasing their apparent solubility and preventing diffusion back into plasma (Kaplowitz, 1980). Bilirubin, a somewhat toxic metabolite of heme, is stored by GSH S-transferases until it can be converted into a less toxic compound (Wolkoff et al., 1978). GSH S-transferase B was found necessary for the release of heme from mitochondria for use by apo-cytochrome P-450 (Ketterer et al., 1983). A GSH S-transferase, mainly the one called ligandin which is identical with GSH S-transferase B, interacts with

active intermediates involved in carcinogenesis by covalently binding and detoxifying them (Smith et al., 1977).

GSH S-transferase activity may be regulated by the presence of an inhibitor. Boyer et al. (1982) found the activity of the GSH S-transferase in rat liver microsomes could be increased five fold by treating the microsomes with small unilamellar vesicles made with phosphatidyl choline (PC). It was suggested that the PC may remove an inhibitor of the GSH S-transferases. Lysophosphatidylcholine, bile acids and palmitic acid decrease enzymatic activity. It is thought that the enzymes' activity is far greater than previously believed and its recognized importance as an enzyme in the prevention of toxic liver injury has increased. Controversies over many of these enzymes' properties still exist with much work remaining in defining their regulation and roles.

Lindane

Benzene hexachloride (BHC) or hexachlorocyclohexane is a chlorinated hydrocarbon insecticide. Characteristics of this insecticide group include presence of carbon, chlorine, hydrogen and sometimes oxygen atoms, the presence of cyclic carbon chains including benzene rings, lack of any particular active intramolecular sites, apolarity and lipophilicity and chemical inertness. Hence, they are very environmentally stable (Figure 3).

Lindane is the gamma (γ)-isomer of BHC. In 1912, it was discovered BHC had four isomers: alpha, beta, gamma and theta. Later, BHC was

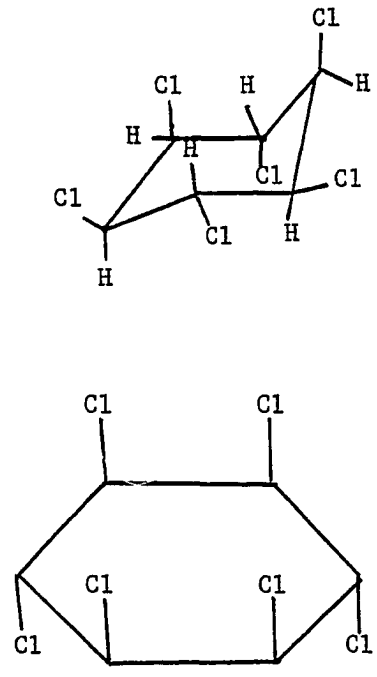

Figure 3. Molecular structure of lindane[a,b]

[a]Matsumura, 1975.

[b]Chemical structure of lindane (Nakajima, 1983).

found to have insecticidal properties and the γ-isomer was isolated and named lindane after Van der Linden. For it to be labelled lindane, the BHC preparation must contain 99 percent of the γ-isomer. Lindane is relatively soluble in water and a hundred times more volatile than DDT, allowing it to have good fumigant action. It is also more effective than the other isomers. The mode of action of BHC as an insecticide is to act as an effector of ion permeability. In mammals, it stimulates the central nervous system (Matsumura, 1975; McNamara and Krop, 1947). Lindane may be said to belong to a similar group of neurotoxicants as DDT, though its mode of action is different from that of DDT. Lindane's

exact mode of action is not yet known; it attacks the nervous system and requires a complete reflex arc to work (Matsumura, 1975).

In the past, lindane was used in a great variety of ways on crops and livestock as an insecticide. However, lindane's usage has been limited due to its residue persistance and possible toxic effects. Indoor smoke fumigation, dog dips (other than controlling mites), aerial application and aquatic application are prohibited. Restricted use with certified applicators is allowed for commercial ornamentals, pecans, avocados, livestock, Christmas trees, forestry, dog dusts and shampoos and structural treatments. Moth sprays, uninhabited buildings and storage bin sprays, dog dips to control mites and household products other than above require labels to be modified as appropriate to reduce applicator risks and require child resistant packaging for household products with more than 6.5 percent active ingredient (Iowa Department of Agriculture, 1983).

The entry of insecticides into mammals occurs via the skin, orally as residue in food and water, and inhalation. Lindane has relatively low skin penetration. Once in the body, the insecticide is usually transported and distributed via the bloodstream. After intestinal absorption, lindane is carried by lymph until it enters the blood. Most lipophilic insecticides are carried in the blood in protein- and lipoprotein-associated forms (Morgan et al., 1972). The liver, the primary site of detoxification, stores high levels of lindane. When the body accumulates more than it can detoxify, residues begin to build up. Starvation may cause release of the insecticide residue into the bloodstream from the adipose tissue.

Srinivasan and Radhakrishnamurty (1983) found rats fed diets with 100 and 800 ppm lindane for up to 15 days had accumulation of lindane residues in this general pattern of distribution, in decreasing order: fat, brain, kidney, muscles, lung, heart, spleen, liver and blood. Feeding of lindane at 100 and 800 ppm for up to 15 days did not increase tissue residue levels over that of 10 or 5 day fed animals, respectively. Also, food restriction (one-third of normal food intake) after 15 days of lindane treated diets did not have adverse effects on lindane toxicity. Increased brain residue levels were not sufficient to cause toxic symptoms and death even though fat and lungs did have increases in concentration of lindane.

Elimination of insecticides may occur through degradation in the liver. This organ converts apolar and lipophilic compounds, like the chlorinated hydrocarbons, into polar, water-soluble compounds which are excreted through the renal system (Matsumura, 1975).

Movement of BHC into the environment can occur through plant tissues, most importantly agricultural crops. Lindane is translocated up through the root system. Soil type also influences the rate of insecticide uptake by the plant. The physiochemical characteristics of the insecticide determines how long it will remain in the soil. BHC is relatively stable and lindane remains in the soil for 3-10 years (Edwards, 1966). Lindane's volatility allows it to disappear from the soil through evaporation into the air (Acree et al., 1963, cited by Matsumura, 1975). Stable pesticides such as BHC are only environmentally altered by factors such as high alkalinity and high temperatures (Bradbury, 1963). In the soil, BHC can degrade into isomers of lindane under anaerobic

conditions. Isomerization reactions by microorganisms and selective degradation of lindane results in leaving behind the minor isomers of lindane, particularly alpha and beta BHC (Matsumura, 1975).

Toxicity of lindane in mammals stimulates the nervous system, raising the blood pressure, causing the heartbeat to fall (bradycardia) and produces an irregular encephalogram. Symptoms of BHC poisoning are excitation, tremors, ataxia, convulsions, paralysis, and respiratory failure which results in death. Lindane has an acute LD_{50} in rats of 190 mg/kg (Matsumura, 1975). Gaines (1969) found the acute LD_{50} in rats to be about 90 mg/kg. A single dangerous dose in man is approximately 7-15 g, depending on the individual's size, age and state of health. The total dietary intake of lindane in the U.S. over a 6-year period of 1964-1970 was surveyed and found to average 0.05 μg/kg body weight/day. The Food and Agricultural Organization-World Health Organization (FAO-WHO) acceptable daily intake from food is 12.3 μg lindane/kg body weight/day (Duggan and Corneliussen, 1972). The effects of lindane at the cellular level may result in enlargement of the liver, necrosis, congestion and fatty degeneration of the liver and kidney, bladder congestion, hemorrhages of the GI tract, heart and lungs, and edema of the brain and spinal cord. Antidotes used for lindane include atropine to prevent bradycardia and phenobarbital to counteract rise in blood pressure and convulsions (Matsumura, 1975). Accidental lindane poisonings have occurred when lindane solutions used for treatment of pediculoses and scabies were swallowed or improperly applied externally (Davies et al., 1983; Telch and Jarvis, 1982).

Studies on chronic toxicity of lindane in mammals are not as ex-

tensive as acute toxicity studies. West (1967) suggested a few cases of possible chronic lindane poisonings but Samuels and Milby (1971) (cited by Matsumura, 1975) found no clinical indications in their study of 79 humans exposed to lindane for several weeks to years. Other chronic effects known or suspected to be caused by lindane are oncogenicity, teratogenicity and reproductive effects. Studies by Nobuyuki et al. (1973) induced hepatocarcinogenesis in mice fed a diet containing at least 250 ppm of α-BHC for 24 weeks but no tumors were found in the lindane fed mice. Similar results were reported by Ito et al. (1975). Thorpe and Walker (1973) observed liver enlargement and liver lesions in mice fed lindane (400 ppm) for 2 years. Kashyap et al. (1979) fed technical grade BHC to mice at 100 ppm for 80 weeks. The technical BHC contains 68.7% α-BHC, 6.5% β-BHC and 13.5% γ-BHC. The treated mice had significant increases in incidences of liver tumors and tumors of the lymphoreticular tissue as compared with the control mice. The NCI Lindane Rat Study (1977) fed male and female rats pure lindane. Increased incidences of carcinomas and neoplasms of endocrine organs were found. Thorpe and Walker (1973) fed CF1 mice lindane at 400 ppm for 110 weeks and observed an increased incidence of liver tumors. The NCI Mouse Study (1977) fed B6C3F1 mice lindane at 80 or 160 ppm for 80 weeks. The treatment was carcinogenic for the liver of the male mice and for the spleen of female mice. Other mice and rat studies from independent labs indicated similar results. Hanada et al. (1973) and Goto et al. (1972) found liver neoplasms in mice fed lindane for 32 weeks. An 80-week study by Herbst et al. (1975) found no increased tumor incidence in mice treated with lindane. The low dosages used in

their study offers an explanation for the lack of tumor incidence. Rat studies by Ito et al. (1975) found liver hyperplasia occurred from lindane treatment. Fitzhugh et al. (1950) fed rats lindane and found preneoplastic and neoplastic lesions of the liver, lymphosarcoma of the lung, uterine polyps, and polyarteritis of the mesenteric artery. One positive effect found from lindane treatment was that on reproduction. Wolfe and Esher (1980) found the number of litters produced was higher in lindane treated mice. Welch et al. (1969) suggested this effect could be due to an estrogenic effect which has been reported for other organochlorine insecticides.

The adverse effects due to acute or chronic exposure to lindane can be explained only partially because the exact metabolism of lindane in mammals is not fully understood. Several metabolic pathways have been proposed and there is disagreement as to what intermediate compounds are formed before final formation of the urinary metabolites.

In early studies, Grover and Sims (1965) proposed that lindane was dehydrochlorinated to 2,3,4,5,6-pentachlorocyclohex-1-ene (γ-PCCH) which is further metabolized to either 2,4-dichlorophenyl mercapturic acid or 2,3,5- and 2,4,5-trichlorophenol (TCP). Chadwick and Freal (1972) found no PCCH or chlorinated benzenes in urinary metabolites. Large quantities of 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol (PCCOL) were found in the neutral fraction of the urine. In the acid fraction of urine, various chlorinated phenols were found. In Chadwick and Freal's study, previously unreported metabolites from rats fed lindane include 3,4-dichlorophenol, 2,4,6-TCP, 2,3,4,5- and 2,3,4,6-tetrachloro-

phenol (TTCP) and (PCCOL). Chadwick et al. (1975) proposed another pathway in rats where first the orally administered lindane is dehydrogenated to hexachlorocyclohexene (HCCH) and then to PCCOL. They report the dehydrogenation step is a cytochrome P-450-dependent mixed function oxidase reaction requiring molecular oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH). Other pesticides have been reported to be metabolized via microsomal dehydrogenases but they do not require cytochrome P-450 or NADPH. Chadwick et al. (1975) expressed disbelief in Grover and Sim's theory because they felt the γ-PCCH would need to be converted to PCCOL to account for the presence of PCCOL identified in the urine. This conversion necessitated a hypothesis of an allylic oxidation with a double bond shift. Matsumura (1975) suggests perhaps only hydroxylated products can be excreted into the urine and that PCCH and tri- and tetrachlorobenzenes were retained in the body. This would account for the absence of PCCH and tri- and tetrachlorobenzenes in the urine of rats. Aizawa (1982) incorporated new research work with Grover and Sim's results and was able to demonstrate how both PCCH and PCCOL were intermediates of lindane's metabolism. Fitzloff et al. (1982) compared the metabolites of lindane produced by human and rat liver microsomes. They identified a second form of γ-PCCH not seen before, 3,4,6/5-PCCH. They found 4 major primary metabolites 3,6/4,5-HCCH, 3,6/4,5-PCCH, 3,4,6/5-PCCH and 2,4,6-TCP, and 2 secondary metabolites 2,3,4,6-TTCP and pentachlorobenzene (PCB) in humans. Rat liver microsomes produce 3,6/4,5-HCCH, 2,4,6-TCP and 2,3,4,6-TTCP at rates similar to humans but 3,4,6/5-PCCH was produced at a much lower rate and 3,6/4,5-PCCH and PCB were

not detected. Nakajima (1983) demonstrated the presence of HCCH, PCCOL and PCCH metabolites in rat urine.

Lindane has been shown to induce liver microsomal drug metabolizing enzymes in rats (Mikol et al., 1980; Kolmodin-Hedman et al., 1971). The induction of these microsomal monooxygenases of the liver and the related increased synthesis of cytochrome P-450 occurred when lindane was present in the rat diet at concentrations as low as 20 ppm (Pelissier and Albrecht, 1976). The effects of lindane on antipyrene metabolism led Kolmodin et al. (1969) to conclude that insecticides have the ability to induce microsomal drug metabolism in man. Phenobarbital induces cytochrome P-450 synthesis whereas methylcholanthrene promotes cytochrome P-448 synthesis. Lindane has been demonstrated to be a phenobarbital-like inducer. Both lindane and phenobarbital pretreatment produced the identical benzopyrene (BP) metabolite 4,5-BP-dihydrodiol, which also represents a detoxification process (Mikol and Decloitre, 1979). Other enzyme systems also appear to be affected by lindane. Tiwari et al. (1982a) and Tiwari et al. (1982b) treated rats with lindane orally for 15 days and found several enzymes affected. L-ascorbic acid has been shown to possess protective effects against several toxic compounds. Tiwari et al. (1982a) and Tiwari et al. (1982b) wanted to see if L-ascorbic acid would be effective against lindane. Vitamin C neutralized completely or partially the effects lindane had on many of these enzymes as well as the animals' growth retardation.

Glutathione S-transferases are thought to act as the enzyme for the conjugation of lindane. Evidence of this was obtained with rodents producing mercapturic acids when exposed to BHC isomers and also

from GSH conjugation with stereoisomers of PCCH in rat liver cytosol. The exact identities of the intermediate product(s) of lindane that conjugate with GSH are not fully known (Portig et al., 1979). Earlier studies suggest that GSH conjugation of lindane was actually that of GSH-γ-PCCH via GSH S-transferase (Grover and Sims, 1965; Portig et al., 1979; Kraus et al., 1981). Nakajima (1983) found di- and trichlorophenylmercapturic acids (DiCPMA and TriCPMA) were formed either from conjugation of GSH with γ-BHC after dehydrogenation and/or dehydrochlorination of lindane occurred.

Lead

Lead is a ubiquitous element in the environment. Its early use dates as far back as 5000-7000 B.C. (Nriagu, 1978). It was so extensively used during the Roman times (water pipes, tiles, storage containers, paints, etc.) that widespread lead intoxication has been speculated to have been the cause for the decline of the Roman Empire (Gilfillan, 1965). Modern society has eliminated many unnecessary usages of lead but industry still requires lead for batteries, gasoline and other products (Gerber et al., 1980). The health importance of this lead contamination is due to contamination of environmental water and air, from which it enters the animals and plants used for food, and is eventually taken up by humans (Kehoe, 1961). It is fortunate that substantial quantities of lead can be stored by humans in the bone in a relatively inert form. If lead is mobilized or if lead cannot be stored fast enough, lead poisoning occurs (T.G.M.A., 1973). Lead appears to

have no essential biological function, only toxic effects.

The threshold of lead toxicity is difficult to define in man because levels causing toxic symptoms approaches that occurring normally. The usual blood levels are in the order of 20 μg/100 ml. Subclinical changes are noted in sensitive persons at 40-60 μg/100 ml and clinical symptoms can arise at levels greater than 60 μg/100 ml (Gerber et al., 1980). Clinical symptoms are quite diversified because the biochemical changes that occur affect many organs and tissues. Characteristic symptoms of lead intoxication involve the following (Gerber et al., 1980):

(a) hematopoietic system (microcytic anemia from an abnormal and impaired production and an increased destruction of red cells);

(b) nervous system (encephalopathy, mainly in children and peripheral neuropathy, mainly in adults);

(c) kidney (tubular damage in acute intoxication, interstitial-glomerular fibrosis and tubular atrophy in chronic intoxication);

(d) gastro-intestinal tract (colic, constipation and diarrhea);

(e) bone (remodeling of the Haversian system, development of radio-dense "lead lines" and formation of giant multinucleate cells);

(f) liver (altered drug metabolism);

(g) defense mechanisms (bacterial and viral susceptibility increased);

(h) hormones (metabolism of thyroid, pituitary, adrenal and sexual hormones are modified).

Also, lead is known to produce chromosomal abberations but this

effect appears minor compared with that of other mutagenic agents (Gerber et al., 1980). Contradictory results have been published in regard to lead and its effects on chromosomes; thus, its biological significance in humans is unknown (Damstra, 1977). Another effect lead has on mammals including man is its effects on reproduction. It can prevent implantation and delay fetal growth during later stages of pregnancy as well as cause neurological damage. Lead is capable of crossing the placenta and is considered teratogenic in man depending on the blood level of lead in the mother during pregnancy (Gerber et al., 1980).

All of the adverse effects of lead to mammals are due to its

(a) biochemical properties and

(b) metabolism.

Biochemical properties

Lead, from group IVA of the periodic table has valence states similar to some of the alkaline earths, but its halides, hydroxides and phosphates are insoluble. Lead often follows calcium metabolism and can act as an antagonist to calcium. It also can be laid down in the hydroxyapatite structure of the bone. Lead complexes with organic acids, lipids and aromatics (Nriagu, 1978; Venugopal and Luckey, 1978). It also complexes with amino acids and is very stable especially with sulfhydryl groups. It can bind to proteins and nucleic acids. Pb^{+2} disturbs heme synthesis by inhibiting the cytoplasmic zinc-dependent, sulfhydryl-rich enzyme, aminolevulenic acid dehydratase (ALAD) and the mitochondrial sulfhydryl enzyme, ferrochelatase (Hammond, 1977). Lead also binds to

red cell membranes so that most lead in blood is found in erythrocytes (Rosen and Haymovits, 1973).

Metabolism

Food, drink and inhalation allow the entrance of lead into the body. Once ingested, however, a more complicated situation arises as to how much and how the lead is absorbed, distributed, retained or excreted. Conrad and Barton (1978) found that in rats, dietary lead absorption occurs primarily in the duodenum. There appears to be no feedback mechanism because total body content of lead does not affect lead absorption. Lead absorption increases with starvation. Aungst and Fung (1981a) suggested lead intestinal transport included both a carrier-mediated component and passive diffusion and that it may be bidirectional, similar to calcium transport. Minerals such as iron, zinc and calcium decrease absorption of lead but not by reducing its solubility. Competitive inhibition of lead binding to mucosal proteins by calcium has also been demonstrated (Barton et al., 1978), suggesting absorption of lead through the gastrointestinal tract is regulated and perhaps limited by a requirement of binding to transport protein(s) (Aungst and Fung, 1981a). Aungst and Fung (1981a) and Aungst et al. (1981) found that the mechanism of transport for lead through the rat intestine is largely capacity-limited. Aungst et al. (1981) found gavage and drinking water dosing with lead caused blood and organ lead concentrations that were not proportional to the dose of lead in either adolescent or adult rats. Aungst and Fung (1981b) found that intestinal lead absorption in rats was reduced when oral doses of lead

were given in the presence of food perhaps due to decreased lead solubility or dissolution rate or reduced gastric emptying rate. Undernourished rats had similar lead absorption as the controls but food deprivation altered the distribution of lead in rats. Age also is an important factor in lead absorption. Rader et al. (1981) found when immature and adult rats were given drinking water with equal concentrations of lead, the younger rats were exposed to greater quantities of lead relative to body weight than the older animals. Erythrocytes are important in lead transport. Lead is excreted in urine and feces and via the bile where the gut is involved. Excretion of lead is limited because once lead is stored in bone, it becomes relatively unavailable. Lead accumulation in the skeleton accounts for the largest fraction of the total body burden of lead, residing there as long as 30 years whereas lead in blood and tissues turn over rapidly (Barry, 1978).

Nutritional factors such as iron, vitamin D, calcium and protein also can affect lead absorption. Consumption of deficient or excessive amounts of protein can increase the severity of lead poisoning. Baltrop and Khoo (1975) found dietary deficiencies of protein were associated with increased lead concentration in tissue of rats administered lead in the diet whereas Quarterman et al. (1978) found increases of dietary protein above 20 percent also increased lead retention. Anders et al. (1982) studied the effects of different proteins on lead absorption in rats. They found rats fed a casein + lead diet had blood concentrations of lead significantly greater than animals fed soybean meal + lead. Similar effects were found for brain, kidney and liver tissues. Hematocrit and hemoglobin values were also significantly lower in the

casein + lead diet compared to the soybean meal + lead diet. Quarterman et al. (1980) studied the influence dietary amino acids had on lead absorption and found a number of amino acids increased tissue lead concentrations, but these decreased, however, as the rats aged. Some rats were fed lead and methionine supplements in their semi-synthetic diet that already contained casein (22 g/kg). These rats gained more weight and had slightly reduced lead content in most tissues, but to a much greater extent in the liver, than lead treated rats given no methionine supplementation. Other nutritional deficiencies effect lead toxicity. Baltrop (1976) found that lead absorption depended upon both quantity and type of dietary fat. Both low calcium diets and iron deficiency increases lead toxicity. Vitamin E deficiency and lead poisoning together produce an anemia in rats that is more severe than that caused by either treatment alone (Levander, 1979).

A less direct effect lead has on heme synthesis is that on cytochrome P-450, a heme containing component of an important microsomal enzyme system. Chronic 5-9 week studies involving p.o. (150 or 300 ppm) or i.p. (20 mg/kg) administration of lead acetate to rats revealed that cytochrome P-450 became alternately depressed and elevated during the course of treatment (Egan and Cornish, 1973). Similar research was carried out by C. P. Chow and H. H. Cornish (1978). They found that one i.p. injection of lead acetate (56 mg/kg) prevented the stimulation of zoxazolamine metabolism normally induced by phenobarbitol. Lead also delayed phenobarbital induction of hepatic microsomal cytochrome P-450 at 12 and 24 hours after treatment but this inhibitory effect of lead disappeared by 48 hours. Wagstaff (1979) fed

rats diets fortified with lead acetate for 10 days and showed stimulation of microsomal enzymes. Besides microsomal enzyme activity, glutathione is often measured when analyzing for the metabolism of toxic substances including heavy metals such as lead. Dalvi and Robbins (1978) found that 24 hours after an i.p. injection of lead acetate (100 mg/kg) in mice resulted in depressed GSH levels as well as microsomal enzyme activity. Eaton et al. (1980) found when rats were given 2 i.p. injections of the maximum tolerable dose (MTD) of lead (400 μM/kg/day) at 36 and 12 hours prior to sacrificing, this decreased hepatic cytochrome P-450 content. Only at doses lower than the MTD (50 and 100 μM) produced a significant decrease in hepatic GSH content. Lead, however, significantly increased renal GSH content at the 2 highest levels of lead administration (400 and 200 μM). A study of chronic lead treatment of dogs (13 weeks) showed no significant changes in blood GSH concentration, measured weekly, compared with the control animals. The addition of CaEDTA following lead treatment reduced clinical signs of lead toxicosis (Mitema et al., 1980). In vitro studies by Reddy et al. (1981) demonstrated that lead inhibited both the peroxidase and transferase activities of non-Se GSH-Px isolated from calf liver (Reddy et al., 1981). Hsu (1981) studied the effects of lead on rat pups born and nourished by dams receiving commercial laboratory diet supplemented with 0.5 percent lead acetate. Lead decreased hematocrits and increased liver weights and GSH concentrations in erythrocytes, liver and kidney. However, glutathione reductase and glutathione peroxidase activities were unaffected by lead. Lead enhanced the incorporation of glycine-1-^{14}C into kidney GSH and

cystine-^{35}S into liver and kidney GSH. From these results, Hsu (1981) suggested that lead had a stimulatory effect on the biosynthetic pathways of GSH. Lead appeared to disturb protein synthesis by changes in the concentration of several free amino acids in the plasma. These changes reflected impairment of heme and hemoglobin synthesis and interconversion of glycine and serine.

Extrapolation from animal studies to human beings is extremely difficult, partly because combinations of uncontrolled conditions are a common phenomena with humans and little can be done to eliminate such factors. Usually, the individual is exposed to multiple uncontrolled treatments that were not part of the animal study. Such factors can involve subtle effects such as those in a person who has multiple marginal nutritional deficiencies. There are more obvious effects, such as the cyclic hormonal changes found in women. Unknown inhibitory or synergistic effects may be present and not realized. For example, Maines (1981) found that the inhibitory effects of mercury on GSH metabolism could be reversed by selenium. The concomitant exposure of rats and mice to the pesticides lindane and zineb showed that lindane prevented zineb's inhibitory and stimulatory effects on liver metabolism of aflatoxin B_1 (Decloitre and Hamon, 1980). The biggest problem with extrapolation of animal study results to humans lies in the variations of the methods used in the experiments and the use of different animal models. Over and over again statements such as "The results indicate that inductive effects are dose dependent, vary with route of administration and are selective with regard to different GSH S-transferase activities" (Kulkarni et al., 1980) or "enzymatic activity... varies according to

species, species-dependent response to inducers or inhibitors, dose and duration of exposure to such compounds" (Decloitre and Hamon, 1980), are being made. Making definite conclusions from animal research and applying it directly to humans is difficult but the research does have its importance. Especially where toxicology is concerned, ground work must be laid and preliminary animal testing must be done to prevent avoidable ill-health in humans that could result from lack of basic knowledge.

MATERIALS AND METHODS

Experiment I: Animals and Treatments

Weanling C3H/HEN female mice were randomly assigned to each treatment. The study was conducted with 9 mice per treatment, a total of 72 mice in all. Animals were housed in rectangular plastic mice cages with stainless steel wire tops, 3 mice per cage. The mice were kept in a temperature/humidity controlled room with 12 hours of light and 12 hours of darkness per day.

The statistical design was a 2^3 factorial as seen in Table 1. Mice were fed either a basal diet that was methionine deficient (MD), meeting only 55 percent of the methionine requirement for mice, or a methionine sufficient diet (MS), meeting 100 percent of the mouse requirement. The MS diet was the basal diet supplemented with 0.22 percent methionine to meet 100 percent of the methionine requirement for the mouse. Both diet groups were given 0 or 250 ppm lead as the acetate in drinking water or 0 or 4.6 mg lindane (γ-BHC) orally in 0.05 ml corn oil once a week or they were given both lead and γ-BHC treatments. Mice were fed their respective diets for 10 weeks and received their toxicant treatments (lead and γ-BHC) the last 9 weeks. Body weights and food consumptions (averaged per cage) were measured once a week.

Diets

The composition of the basal diet is shown in Table 2. A soy protein was chosen because it is low in the sulfur amino-acids methionine and cysteine. L-methionine was used to supplement the diet to meet

Table 1. Experimental design of Experiments I and II

Methionine sufficient diet				Methionine deficient diet			
Control	Lead	γ-BHC	Lead γ-BHC	Control	Lead	γ-BHC	Lead γ-BHC
0	250 ppm	4.6 mg	Same doses	0	250 ppm	4.6 mg	Same doses

100 percent of the mouse requirement for methionine/cysteine (NRC/NAS, 1978). The Hurley and Bell (1974) vitamin mix was chosen because it had been used successfully in previous mouse diets. Additional zinc, chromium and selenium were added to Williams and Briggs (modified) salt mixture to meet the mouse requirements for minerals (NRC/NAS, 1978).

Toxicants

Lead acetate was added to the drinking water, of the appropriate treatment groups, at a concentration of 250 ppm lead. The mice were allowed to drink ad libitum. The γ-BHC was purchased from Sigma as hexachloro-cyclohexane, a commercial grade powder. The γ-BHC was mixed in corn oil and given orally at a dose of 4.6 mg once a week for a total of 9 weeks.

Necropsy and Sample Collection

All mice were decapitated at the end of the experiment. The abdominal cavity was exposed by a longitudinal incision on the midline of the abdomen. Examination was made for any abnormalities including

Table 2. Composition of basal diet[a] for Experiment I

Ingredient	gr/Kg
Isolated soy protein[b]	121.2
Corn starch	284.4
Sucrose	284.4
Beef tallow	190.0
Safflower oil	10.0
Cellulose	50.0
Salt mix[c]	50.0
Vitamin mix[d]	10.0
Total	1000.0

[a]The basal diet contains 55% of NRC mouse requirement for methionine and cysteine.

[b]Supro 660, Ralston Purina Company, St. Louis, MO.

[c]William and Briggs salt mixture (modified), Teklad, Madison, WI, with $ZnCO_3$ added to provide 14.3 mg Zn/Kg diet, $CrCl_3 \cdot 6H_2O$ added to provide 2.0 mg Cr/Kg diet and Na_2SeO_3 added to provide 1.25 mg Se/Kg diet.

[d]Amount in 1 Kg diet: vitamin A retinyl palmitate, 1,100 IU; vitamin D (cholcalciferol), 1,100 IU; vitamin E (D-L α-tocopherol), 32 IU; vitamin K (menadione), 18 mg; biotin, 0.2 mg; choline chloride, 750 mg; folacin, 0.45 mg; niacin, 22.5 mg; Ca-pentothenate, 37.5 mg; riboflavin, 7.5 mg; thiamin, 22.5 mg; B_6 pyridoxine, 22.5 mg; B_{12} cobalbamin, 0.023 mg.

tumor development. Brain, kidney and liver samples were stored in formaldehyde for pathological examination. Other liver samples were rinsed in 0.05 M potassium phosphate (pH 7.4) prior to freezing in liquid nitrogen and then kept frozen for GSH S-transferase analysis.

Analytical Methods

Glutathione S-transferase: The activity of this enzyme was measured by a modified method of Booth et al. (1961). For the calculation of the enzyme activity, the adaption of ΔW for 1-chloro-2,4-dinitrobenzene (1-Cl-2,4-DNB) from Habig et al. (1974) was used. The following is the procedure used for the analysis[1]:

1. Homogenize liver tissue on ice in 4 volume cold 0.1 M phosphate buffer (pH 7.4).
2. Centrifuge homogenate at 2,000xg for 30 minutes.
3. Centrifuge supernatant at 95,000xg for one hour.
4. Reaction mixture for the analysis: 1 mM GSH + 1 mM 1-Cl-2,4-DNB + supernatant + cold 0.1 M KH_2PO_4 (pH 7.5).
5. Read cuvettes at 340 nm for approximately 5 minutes.

Experiment II: Animals and Treatments

The experimental design was the same as that in Experiment I except: (a) the diet composition was changed, (b) the diet and lead treatment lasted only 21 days, (c) and a single dose (4.6 mg) of γ-BHC was given on day 21, 24 hours prior to sacrificing the animals.

Diets

The composition of the basal diet was basically the same as that used in Experiment I (Table 2) except for the carbohydrate and oil content as shown in Table 3.

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[1]All solutions were made fresh daily except buffer.

Table 3. Composition of basal diet[a] for Experiment II

Ingredient	gr/Kg
Isolated soy protein[b]	101.2
Corn starch	364.4
Sucrose	364.4
Corn oil	50.0
Safflower oil	10.0
Cellulose	50.0
Salt mix[c]	50.0
Vitamin mix[d]	10.0
Total	1000.0

[a]The basal diet contains 55% of NRC mouse requirement for methionine and cysteine.

[b]Supro 660, Ralston Purina Company, St. Louis, MO.

[c]Same as in Experiment I.

[d]Same as in Experiment I.

Toxicants

The toxicants were prepared the same as in Experiment I.

Necropsy and Sample Collection

All mice were decapitated and blood samples were immediately collected into vials with EDTA and frozen in liquid nitrogen for GSH analysis. Midline incisions exposed abdominal cavities for examination and sampling of liver and kidney. These tissues were immediately

frozen in liquid nitrogen and kept frozen for GSH and GSH S-transferase analysis.

Analytical Methods

Glutathione S-transferase: The procedure used was the same as in Experiment I.

Total Protein: Lowry et al. (1951) procedure was used for protein analysis so activities of enzymes could be expressed per mg protein.

Glutathione: Reduced, oxidized and total glutathione measurements of blood, kidney and liver were determined by the fluorometric method. A modification was used of Hissin and Hilf's (1976) modified methods of Cohn and Lyle's (1966) methods. The following is the procedure used for glutathione analysis:

GSH and GSSG solutions were made in 0.1 M NaH_2PO_4-0.005 M EDTA (PO_4-EDTA) buffer (pH 8.0) fresh daily. The o-phthalaldehyde (OPTH) solution was a 0.1% (w/v) solution made fresh daily in distilled absolute methanol.

1. Homogenize liver and kidney tissues (100 mg) in 3.75 ml PO_4-EDTA buffer on ice.
2. Add 1.0 ml 25% HPO_3 to homogenate.
3. Proteins are removed by centrifugation (cold) at 5,000xg for 10 minutes and then at 100,000xg for 30 minutes.

Blood:

1. Proteins are removed by precipitation with 0.2 ml blood + 2.0 ml H_2O + 0.4 ml 25% HPO_3.

2. Centrifuge in cold the mixture at 100,000xg for 30 minutes.

Glutathione (GSH) reaction mixture:

1. Remove 0.5 ml of kidney or liver supernatant[1].
2. Add 4.5 ml of PO_4-EDTA buffer to the supernatant.

   (Steps 1-2 = ASSAY MIXTURE)
3. Mix 200 µl of ASSAY MIXTURE + 1.8 ml PO_4-EDTA + 100 µl OPTH.
4. Incubate 15 minutes at room temperature.
5. Read fluorescence at 420 nm, determined with activation at 350 nm.

Glutathione (GSSG) reaction mixture:

1. Remove 0.5 ml of kidney or liver supernatant[1].
2. Incubate supernatant at room temperature with 200 µl of 0.04 M N-ethylmaleimide for 30 minutes.
3. Add 4.3 ml of 0.1N NaOH to the incubated supernatant.

   (Steps 1-3 = ASSAY MIXTURE)
4. Mix 200 µl of ASSAY MIXTURE + 1.8 ml 0.1N NaOH + 100 µl OPTH.
5. Incubate at room temperature for 15 minutes.
6. Read fluorescence at 420 nm, determined with activation at 350 nm.

Standard curve:

Concentrations for GSH and GSSG ranged from 0.005 µg to 1.0 µg. The buffers used in the standard curve reaction mixture in place of the ASSAY MIXTURES should be PO_4-EDTA for GSH and 0.1N NaOH for GSSG.

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[1]Use 0.2 ml of blood supernatant and adjust volume with PO_4-EDTA.

Experiment III: Animals and Treatments

(Part A)

Weanling Harlan Sprague Dawley male rats were randomly assigned to each treatment. Animals were housed separately in galvanized metal rat cages and were kept in a temperature/humidity controlled room with a 12-hour light and dark cycle.

Treatment and diet design consisted of 3 main groups as shown in Table 4. Rats were fed either a basal diet that was methionine deficient (MD), that met 60 percent of the methionine requirement for the rat, or they were fed a methionine sufficient (MS) diet. Both diets were also supplemented with lead acetate at 10,000 ppm lead. The MS diet consisted of the basal diet supplemented with methionine to meet 100 percent of the methionine requirement for the rat. The control group was fed the MS diet without lead acetate. The animals were fed ad libitum. The rats were fed their respective diets a total of 3 weeks. They were fasted 24 hours on the last experiment day. One dose of γ-BHC was administered orally to the treatment groups 18 hours prior to the end of the experiment. Body weights were measured weekly and food consumption was measured daily in all groups.

Diets

The composition of the basal diet is shown in Table 5. Soy-protein was chosen as the protein source for its low content in methionine and cysteine. The diet was supplemented, where indicated, with 0.25 percent L-methionine to meet 100 percent of the rat requirement for methionine (NRC/NAS, 1978).

Table 4. Experimental design of Experiment III

MS diet	MS diet	MD diet
Control	Lead + γ-BHC	Lead + γ-BHC
0	Lead 10,000 ppm	Lead 10,000 ppm
0	γ-BHC 25% LD_{50}	γ-BHC 25% LD_{50}
6 rats[a]	10 rats	10 rats

[a]Part B of Experiment III had 7 rats in the control group.

Toxicants

Lead acetate was added to the diets at a concentration of 10,000 ppm lead. γ-BHC from Sigma was mixed in corn oil and given orally at a dose equal to 25 percent of the LD_{50} for rats (88 mg/kg) as reported by Gaines (1969).

Necropsy and Sample Collection

All rats were decapitated and blood was immediately collected. Hematocrits were measured with fresh blood and the remaining blood was stored in vials containing EDTA and stored for determination of blood lead levels. A midline incision exposed the abdominal cavity for examination. Livers were weighed, then immediately frozen in liquid nitrogen and stored at -80°C for GSH and GSH S-transferase analysis.

Table 5. Composition of basal diet[a] in Experiment III

Ingredient	gr/Kg
Isolated soy protein[b]	152.0
Corn starch	305.0
Sucrose	310.0
Cellulose	88.0
Lard	50.0
Corn oil	35.0
Salt mix[c]	50.0
Vitamin mix[d]	10.0
Total	1000.0

[a]The basal diet contains 60% of the NRC rat requirement for methionine and cysteine.

[b]Supro 660, Ralston Purina Company, St. Louis, MO.

[c]William and Briggs salt mixture (modified), Teklad, Madison, WI with $NaSeO_3$ added to provide 0.1 mg Se/Kg diet.

[d]AIN vitamin mix 76, U.S. Biochemicals Corp., Cleveland, OH.

Experiment III: Animals and Treatments

(Part B)

Experiment III, Part A, was repeated except the MS (lead and γ-BHC) treated rats were pair-fed to the MD (lead and γ-BHC) treated rats.

Analytical Methods

Glutathione S-transferase: The procedure used was the same as in Experiments I and II.

Total Protein: The Lowry et al. (1951) procedure was used.

Liver Glutathione: Reduced glutathione (GSH) was determined with a modified Ellman's reaction (Jollow et al., 1974), with modifications as follows:

Solutions:

1. 0.1 M K_2PO_4 (pH 7.4).
2. 0.5% $NaHCO_3$*.
3. Ellman reagent – 40 mg 5,5'-Dithiobis-(2-Nitrobenzoic Acid)/ 100 ml $NaHCO_3$*.
4. 4.0% sulfosalicylic*.
5. GSH standard made in 0.1M K_2PO_4 (pH 7.4)*.

All solutions with asterisk (*) were made fresh each day.

Procedure:

1. Weigh out 0.1-0.15 g liver.
2. Add 4 times volume of 0.1 M K_2PO_4 buffer.
3. Homogenize 10 times with Potter Elvejehm.
4. Remove 0.5 ml aliquot and add equal volume of 4.0% sulfosalicylic acid.
5. Centrifuge in cold for 30 minutes at 10;000xg.
6. To 0.4 ml aliquots of supernatant add 4.5 ml of Ellman reagent[1].

[1]Be sure sample supernatant and Ellman reagent are at room temperature before reading. Prior to addition of sulfosalicylic acid, tissue sample should be on ice.

7. Read at 412 nm immediately.

Standard curve:

Concentrations for GSH range from 0.0 μg to 80.0 μg. Use 0.1 M K_2PO_4 buffer (pH 7.4) in place of the supernatant.

Blood Lead: Model 170-70 Hitachi Atomic Absorption (AA) machine was used to determine blood lead levels. A stock lead solution containing 998,000 ppb was purchased from Sigma and used to make a standard curve. Actual blood samples were analyzed by mixing 0.05 ml blood to 1.2 ml 0.1% HNO_3. This 1:25 diluted sample was put directly into the Hitachi AA machine which dried, ashed and atomized the sample at instrument settings specific for the element lead.

Experiments I, II and III Statistical Analysis

The data gathered were analyzed by SAS programming using factorial analysis of variance. Program results of the data were compared by use of the F-Test (Snedecor and Cochran, 1980) to determine the significance levels. However, the data for the mice survival rates (Table 6) were statistically analyzed using the Chi-square Test for determining significance levels for the proportions dead or alive.

RESULTS

Experiment I

Lead and lindane (γ-BHC) treatment of the mice fed the methionine sufficient (MS) diet had a greater survival rate than those fed the methionine deficient (MD) diet (Table 6). The combination of lead + γ-BHC had the most negative effect on survival. Only 5 out of 9 mice were still alive at the end of the experiment in the MD (lead + γ-BHC) treatment group whereas 8 out of 9 survived in the MS (lead + γ-BHC) treatment group. Lead alone had no effect but γ-BHC alone did have a significant effect in the MD group.

Body weight gains of the mice over the 10-week experiment were affected most significantly by lead treatment, regardless of the methionine status of the diet (Table 7). Lead decreased the overall growth rate of the mice.

The diet had an effect on the total amount of food consumed by the mice. The MD fed mice of all 4 treatment groups consumed a greater amount of total food than the 4 treatment groups in the MS fed mice (Table 8).

The GSH S-transferase activity, expressed per gram of liver, was elevated by the γ-BHC treatment. Mice fed the MD and MS diets had increased GSH S-transferase activity levels when they were given γ-BHC and lead + γ-BHC (Table 9).

Histopathologic evaluation of the mice tissue indicated no significant microscopic lesions in either the brain or the kidney. Lesions observed in the liver were nonspecific and consisted of the presence

Table 6. The effect of dietary methionine, lead and lindane on survival rate[a] (No. survived/starting No.) in mice

Methionine deficient diet				Methionine sufficient diet			
Toxicant				Toxicant			
None	Lead	γ-BHC	Lead + γ-BHC	None	Lead	γ-BHC	Lead + γ-BHC
9/9	9/9	7/9	5/9	9/9	9/9	9/9	8/9

[a]Significant effect of methionine ($p < 0.05$) and lindane ($p < 0.0005$).

Table 7. The effect of dietary methionine, lead and lindane on the total body weight gain[a] (grams) in mice (mean ± SE)

Methionine deficient diet				Methionine sufficient diet			
Toxicant				Toxicant			
None	Lead	γ-BHC	Lead + γ-BHC	None	Lead	γ-BHC	Lead + γ-BHC
11.17 ±0.97	8.11 ±0.95	9.21 ±1.58	8.90 ±1.98	11.28 ±1.12	9.83 ±0.81	12.94 ±0.53	8.13 ±0.74

[a]Significant effect of lead ($p < 0.001$).

Table 8. The effect of dietary methionine, lead and lindane on the total food consumed[a] (grams/cage) by mice (mean ± SE)

Methionine deficient diet				Methionine sufficient diet			
Toxicant				Toxicant			
None	Lead	γ-BHC	Lead + γ-BHC	None	Lead	γ-BHC	Lead + γ-BHC
31.20 ±0.72	27.56 ±1.07	30.80 ±0.50	31.87 ±2.30	27.03 ±0.66	26.43 ±0.61	26.27 ±0.20	26.17 ±0.19

[a]Significant effect of methionine ($p < 0.0001$).

Table 9. The effect of dietary methionine, lead and lindane on the liver GSH S-transferase activity[a] (IU/g liver tissue) in mice (mean ± SE)

Methionine deficient diet				Methionine sufficient diet			
Toxicant				Toxicant			
None	Lead	γ-BHC	Lead + γ-BHC	None	Lead	γ-BHC	Lead + γ-BHC
0.0528 ±0.0014	0.0551 ±0.0029	0.0558 ±0.0033	0.0600 ±0.0047	0.0548 ±0.0023	0.0556 ±0.0026	0.0605 ±0.0027	0.0607 ±0.0034

[a]Significant effect of lindane ($p < 0.0203$).

of fine to large vacuoles in hepatocyte cytoplasm, probably an indication of fatty changes. These histopathological findings appear not to be related to toxicant treatments because they were observed in the MS control animals as well as in the toxicant treated animals.

Experiment II

Weight gain was affected by lead alone regardless of whether the diet was MD or MS. The lead decreased body weight gain of both diet groups and in the absence or presence of γ-BHC treatment (Table 10).

Liver GSH S-transferase activity was significantly affected by the methionine status of the diet. Those mice receiving the MD diet, with or without toxicant treatment, had increased transferase activity per milligram of liver protein (Table 11).

Oxidized (GSSG), reduced (GSH) and total glutathione (TGSH) measurements of blood, kidney and liver were not always consistent with one another in regard to toxicant and/or diet effect. GSSG was not affected

Table 10. The effect of dietary methionine, lead and lindane on the total body weight gain[a] (grams) of mice (mean ± SE)

Methionine deficient diet				Methionine sufficient diet			
Toxicant				Toxicant			
None	Lead	γ-BHC	Lead + γ-BHC	None	Lead	γ-BHC	Lead + γ-BHC
3.50 ±0.25	1.61 ±0.33	3.33 ±0.49	2.94 ±0.23	3.56 ±0.47	2.56 ±0.24	3.61 ±0.47	3.00 ±0.44

[a]Significant effect of lead ($p < 0.0006$).

Table 11. The effect of dietary methionine, lead and lindane on GSH S-transferase activity[a] (IU/mg protein) in mice (mean ± SE)

Methionine deficient diet				Methionine sufficient diet			
Toxicant				Toxicant			
None	Lead	γ-BHC	Lead + γ-BHC	None	Lead	γ-BHC	Lead + γ-BHC
0.0382 ±0.0031	0.0407 ±0.0054	0.0347 ±0.0026	0.0394 ±0.0033	0.0299 ±0.0026	0.0321 ±0.0023	0.0281 ±0.0027	0.0290 ±0.0024

[a]Significant effect of methionine ($p < 0.0004$).

in the kidney, liver or the blood by toxicant or methionine status. GSH, however, was affected by lead treatment. Lead increased the GSH levels in the kidney, liver and blood. Only in the liver tissue was lindane alone found to decrease GSH levels. TGSH was not affected in the blood but lead increased the level in the liver. In the kidney, TGSH was affected by γ-BHC and diet interaction. Lindane and MD diet decreased TGSH whereas γ-BHC and MS diet increased TGSH (Tables 12-14). There were no deaths of animals in any of the experimental groups.

Table 12. The effect of dietary methionine, lead and lindane in blood glutathione[a] (mg/ml blood) in mice (mean ± SE)

	Methionine deficient diet				Methionine sufficient diet			
	Toxicant				Toxicant			
	None	Lead	γ-BHC	Lead + γ-BHC	None	Lead	γ-BHC	Lead + γ-BHC
GSH	103.77 ±21.70	165.69 ±25.68	82.74 ±13.74	131.61 ±27.38	82.40 ±11.78	130.26 ±20.35	96.66 ±20.04	135.56 ±37.81
GSSG	222.97 ±43.32	192.30 ±25.71	209.98 ±46.80	197.65 ±34.98	178.08 ±31.39	204.72 ±34.11	133.75 ±28.48	157.46 ±21.93
TGSH	326.73 ±57.75	357.99 ±43.69	292.72 ±40.14	329.24 ±54.13	260.47 ±35.73	337.20 ±37.52	230.41 ±30.69	293.03 ±47.16

[a]Significant effect of lead on GSH ($p < 0.0042$).

Table 13. The effect of dietary methionine, lead and lindane on kidney glutathione[a] (μg/g liver) in mice (mean ± SE)

	Methionine deficient diet				Methionine sufficient diet			
	Toxicant				Toxicant			
	None	Lead	γ-BHC	Lead + γ-BHC	None	Lead	γ-BHC	Lead + γ-BHC
GSH	276.73 ±27.78	359.59 ±36.30	253.86 ±61.02	341.20 ±25.06	243.51 ±35.63	293.73 ±26.02	272.79 ±47.54	332.93 ±28.10
GSSG	293.24 ±34.40	257.84 ±47.69	241.00 ±29.10	226.85 ±21.11	211.32 ±30.97	300.04 ±45.84	343.21 ±78.91	275.36 ±48.65
TGSH	569.94 ±35.01	617.39 ±36.16	494.81 ±69.04	568.00 ±40.15	454.80 ±46.72	593.81 ±42.97	615.97 ±73.43	608.24 ±45.76

[a]Significant effect of lead on GSH ($p < 0.0120$) and methionine x γ-BHC interaction on TGSH ($p < 0.0417$).

Table 14. The effect of dietary methionine, lead and lindane on liver glutathione[a] (μg/g liver) in mice (mean ± SE)

	Methionine deficient diet				Methionine sufficient diet			
	Toxicant				Toxicant			
	None	Lead	γ-BHC	Lead + γ-BHC	None	Lead	γ-BHC	Lead + γ-BHC
GSH	2957.41 ±244.86	3424.88 ±232.06	2158.76 ±279.60	3121.88 ±169.74	2553.75 ±124.55	2881.09 ±261.63	2498.53 ±314.28	2464.93 ±282.83
GSSG	1447.69 ±157.71	1902.81 ±185.37	1559.24 ±200.80	1763.46 ±384.33	1568.58 ±134.13	1684.91 ±123.99	1498.65 ±161.27	1761.52 ±200.25
TGSH	4405.10 ±343.14	5327.68 ±305.46	3717.99 ±236.00	4885.35 ±301.10	4122.11 ±208.73	4566.04 ±339.16	3997.18 ±393.14	4226.45 ±250.84

[a]Significant effect of lead on GSH ($p < 0.0105$) and γ-BHC on GSH ($p < 0.0306$) and the effect of lead on TGSH ($p < 0.002$).

Experiment III

By the end of the second week of the experiment for Part A, the MD (lead + γ-BHC) treated rats began to decrease their food intake and did not consume as much as the MS (lead + γ-BHC) treated rats. In Part B, where the MS (lead + γ-BHC) treated rats were pair-fed to the MD (lead + γ-BHC) treated rats, this was a correct pair-feeding study as is indicated by the food consumption data (Table 15). In Part A, both MD and lead treatment decreased the animals' food consumption if compared to the control rats and MS-lead treated rats. When the MS (lead + γ-BHC) treated rats were allowed to eat only the amount the MD (lead + γ-BHC) treated rats ate (Part B), methionine deficiency and lead decreased food consumption levels for both groups when compared to the control animals. No deaths occurred to the rats in any of the treatment groups.

At the end of the experiments (Parts A and B), the final body weights were lower in both diet groups of lead + γ-BHC treated animals in comparison with the control rats. Both lead and MD diets decreased the animals' growth rate. Even when the rats ate the same amount of diet as in the pair-feeding groups (Part B), the MD fed group had lower body weights than the MS fed group (Table 16). The same trends were seen in the average body weight gains per day for diet and lead treatment effects (Table 17).

The effect the methionine status and lead + γ-BHC treatment may have on rat liver sizes was analyzed when liver weights were expressed as percent of body weight. In both Parts A and B, regardless of whether pair-feeding was done or not, MD diets and lead + γ-BHC treatment

Table 15. The effect of dietary methionine, lead and lindane on the average food consumption[a] (g/day) by rats (mean ± SE)

Ad libitum feeding (Part A)			Methionine sufficient pair-fed to methionine deficient (Part B)		
Toxicant			Toxicant		
None	MD diet lead+γ-BHC	MS diet lead+γ-BHC	None	MD diet lead+γ-BHC	MS diet lead+γ-BHC
15.32 +0.34	9.65 ±0.21	11.45 ±0.37	14.30 ±0.57	9.47 ±0.37	9.33 ±0.30

[a]Part A – Significant effect of methionine ($p < 0.0005$) and lead ($p < 0.0001$). Part B – Significant effect of lead ($p < 0.0001$).

Table 16. The effect of dietary methionine, lead and lindane on the final body weights[a] (grams) of rats (mean ± SE)

Ad libitum feeding (Part A)			Methionine sufficient pair-fed to methionine deficient (Part B)		
Toxicant			Toxicant		
None	MD diet lead+γ-BHC	MS diet lead+γ-BHC	None	MD diet lead+γ-BHC	MS diet lead+γ-BHC
150.67 ±6.46	74.20 ±2.52	109.70 ±2.94	138.29 ±3.73	71.08 ±1.97	88.33 ±3.22

[a]Part A – Significant effect of methionine ($p < 0.0001$) and lead ($p < 0.0001$). Part B – Significant effect of methionine ($p < 0.0003$) and lead ($p < 0.0001$).

Table 17. The effect of dietary methionine, lead and lindane on the average body weight gains[a] (g/day) of rats (mean ± SE)

Ad libitum feeding (Part A)			Methionine sufficient pair-fed to methionine deficient (Part B)		
Toxicant			Toxicant		
None	MD diet lead+γ-BHC	MS diet lead+γ-BHC	None	MD diet lead+γ-BHC	MS diet lead+γ-BHC
5.73 ±0.33	1.66 ±0.08	3.52 ±0.14	5.07 ±0.14	1.46 ±0.05	2.15 ±0.14

[a]Part A – Significant effect of methionine ($p < 0.0001$) and lead ($p < 0.0001$). Part B – Significant effect of methionine ($p < 0.0001$) and lead ($0 < 0.0002$).

increased the liver weights of rats when compared to the control animals (Table 18). However, since γ-BHC was given only once (18 hours before the end of the experiment), it is unlikely that γ-BHC had any effect on liver weights or food consumption or body weight data.

Table 18. The effect of dietary methionine, lead and lindane on liver weights[a] (% body weights) of rats (mean ± SE)

Ad libitum feeding (Part A)			Methionine sufficient pair-fed to methionine deficient (Part B)		
Toxicant			Toxicant		
None	MD diet lead+γ-BHC	MS diet lead+γ-BHC	None	MD diet lead+γ-BHC	MS diet lead+γ-BHC
2.85 ±0.05	4.98 ±0.25	3.60 ±0.05	2.94 ±0.04	4.74 ±0.22	3.60 ±0.07

[a]Part A – Significant effect of methionine ($p < 0.0001$) and lead ($p < 0.0001$). Part B – Significant effect of methionine ($p < 0.0002$) and lead ($p < 0.0001$).

Hematocrits of the rats in both Parts A and B experiments were affected by the presence of lead. Lead decreased the hematocrit levels in both MS and MD fed rats. No methionine status effect was evident. Since γ-BHC was administered only once 18 hours before the end of the experiment and it is not known to effect hematocrits, the decreased hematocrit values are probably not related to γ-BHC treatment (Table 19).

Rats consuming both MD and MS lead supplemented diets (10,000 ppm) had very significant increased blood lead levels compared to the controls. Methionine status had no effect on blood lead levels (Table 20).

GSH S-transferase activity of the liver was expressed per milligram of liver protein. Methionine status and/or lead + γ-BHC treatment did not appear to have any effect on the GSH S-transferase activity (Table 21).

Reduced glutathione (GSH) levels of the liver were measured and expressed three different ways. In the event that the change of the liver weights caused by lead and MD diets may have influenced the proportion of

Table 19. The effect of dietary methionine, lead and lindane on the hematocrit values[a] (%) of rats (mean ± SE)

Ad libitum feeding (Part A)			Methionine sufficient pair-fed to methionine deficient (Part B)		
Toxicant			Toxicant		
None	MD diet lead+γ-BHC	MS diet lead+γ-BHC	None	MD diet lead+γ-BHC	MS diet lead+γ-BHC
39.90 ±0.91	29.25 ±0.58	28.12 ±0.59	38.20 ±0.90	30.34 ±0.65	30.21 ±1.00

[a]Part A – Significant effect of lead ($p < 0.0001$). Part B – Significant effect of lead ($p < 0.0001$).

Table 20. The effect of dietary methionine, lead and lindane on whole blood lead levels (μg/dl blood) of rats (mean ± SE)

Ad libitum feeding (Part A)			Methionine sufficient pair-fed to methionine deficient (Part B)		
Toxicant			Toxicant		
None	MD diet lead+γ-BHC	MS diet lead+γ-BHC	None	MD diet lead+γ-BHC	MS diet lead+γ-BHC
16.88 ±2.32	291.00 ±27.15	332.63 ±15.30	14.87 ±1.49	275.14 ±32.83	297.13 ±24.87

[a]Part A – Significant effect of lead ($p < 0.0001$). Part B – Significant effect of lead ($p < 0.0001$).

Table 21. The effect of dietary methionine, lead and lindane on liver GSH S-transferase activity[a] (IU/mg protein) of rats (mean ± SE)

Ad libitum feeding (Part A)			Methionine sufficient pair-fed to methionine deficient (Part B)		
Toxicant			Toxicant		
None	MD diet lead+γ-BHC	MS diet lead+γ-BHC	None	MD diet lead+γ-BHC	MS diet lead+γ-BHC
0.0264 ±0.0030	0.0269 ±0.0036	0.0285 ±0.0024	0.0211 ±0.0019	0.0243 ±0.0016	0.0248 ±0.0024

[a]Part A – No significant effect from methionine, lead or γ-BHC treatment. Part B – No significant effect from methionine, lead or γ-BHC treatment.

protein to nonprotein composition of the liver, the GSH measurements were expressed as: (a) micrograms per gram of liver tissue, (b) milligrams per 100 grams body weight and (c) micrograms per milligrams soluble protein. Methionine status and lead + γ-BHC treatment both affected

GSH levels regardless of how the measurement was expressed. In Part A only, the lead + γ-BHC treatment increased the GSH level. However, when the rats were pair-fed as in Part B, both diet and lead + γ-BHC treatment affected the GSH levels. Overall, the lead + γ-BHC treatment increased the GSH levels in both MS and MD fed rats. But the MS (lead + γ-BHC) treated rats had higher GSH levels than the MD (lead + γ-BHC) treated rats (Tables 22-24).

Table 22. The effect of dietary methionine, lead and lindane on liver glutathione (GSH)[a] (μg/g liver) in rats (mean ± SE)

Ad libitum feeding (Part A)			Methionine sufficient pair-fed to methionine deficient (Part B)		
Toxicant			Toxicant		
None	MD diet lead+γ-BHC	MS diet lead+γ-BHC	None	MD diet lead+γ-BHC	MS diet lead+γ-BHC
775.50 ±51.06	1137.55 ±87.47	1283.68 ±100.84	919.75 ±53.33	974.13 ±59.89	1856.08 ±162.07

[a]Part A – Significant effect of lead + γ-BHC ($p < 0.0024$).
Part B – Significant effect of methionine ($p < 0.0001$) and lead + γ-BHC ($p < 0.0002$).

Table 23. The effect of dietary methionine, lead and lindane on liver glutathione (GSH)[a] (mg/100 g body weight) in rats (mean ± SE)

Ad libitum feeding (Part A)			Methionine sufficient pair-fed to methionine deficient (Part B)		
Toxicant			Toxicant		
None	MD diet lead+γ-BHC	MS diet lead+γ-BHC	None	MD diet lead+γ-BHC	MS diet lead+γ-BHC
2.21 ±0.16	5.54 ±0.32	4.63 ±0.38	2.70 ±0.17	4.57 ±0.31	6.73 ±0.68

[a]Part A – Significant effect of lead + γ-BHC ($p < 0.0003$). Part B – Significant effect of methionine ($p < 0.0085$) and lead + γ-BHC ($p < 0.0002$).

Table 24. The effect of dietary methionine, lead and lindane on liver glutathione (GSH)[a] (μg/mg soluble protein) in rats (mean ± SE)

Ad libitum feeding (Part A)			Methionine sufficient pair-fed to methionine deficient (Part B)		
Toxicant			Toxicant		
None	MD diet lead+γ-BHC	MS diet lead+γ-BHC	None	MD diet lead+γ-BHC	MS diet lead+γ-BHC
7.15 ±0.50	12.91 ±0.99	12.16 ±1.44	8.00 ±0.44	10.44 ±1.00	15.43 ±1.54

[a]Part A – Significant effect of lead + γ-BHC ($p < 0.0211$). Part B – Significant effect of methionine ($p < 0.0133$) and lead + γ-BHC ($p < 0.0011$).

DISCUSSION

In Experiment I, a 10-week study, the 250 ppm concentration of lead as lead acetate in the drinking water was chosen because of an earlier study by Fowler et al. (1980) who found that rats treated with a nonlethal dose of 250 ppm lead as lead acetate in the drinking water for 6-9 months showed physiological and biochemical effects. Fatalities occurred in Experiment I, however, only when lead treatment occurred simultaneously with lindane treatment or when lindane was the only toxicant used with methionine deficient (MD) diets. The dose of 4.6 mg lindane administered p.o. to the mice is equal to about twice the LD_{50} dose (body weight basis) for the rat. Fatalities were fewer than expected probably because of a species difference. But the patterns of the deaths that did occur in Experiment I indicated that MD fed mice had a lower tolerance level to the lindane and lead + lindane treatment than MS fed mice (Table 6). Protein deficiency has been shown to increase pesticide toxicity. Boyd et al. (1970) (cited by Campbell and Hayes, 1976) found that the toxicity of lindane was doubled when a 26 percent casein diet fed to rats was reduced to 3.5 percent casein. In Experiment II, a 3-week study, where only one dose (4.6 mg) of lindane was given to the mice and Experiment III, also a 3-week study, where only one dose (25% of LD_{50}) of lindane was given to rats, no deaths occurred. Both species difference and a shorter treatment period could account for the absence of fatalities in Experiment II. Even though rats were used in Experiment III, fatalities were not expected because of the dose used.

It is well-known that dietary lead decreases growth rates of rats

(Wagstaff, 1979); the results from Experiments I, II and III confirm this (Tables 7, 10, 16 and 17). Even with increased food consumption by the MD mice in Experiment I (Table 8), the lead + MD fed mice had lower body weights. In Experiment III, where lead was increased to 10,000 ppm, there was also evidence of a MD diet effect as well as a lead effect on growth rates of rats. Lead's suppression of growth was further enhanced by methionine deficiency. Again, a species difference, as well as the fact there was an absence of pair-feeding in Experiments I and II, could account for the fact that MD fed mice in Experiments I and II did not show a statistically significant methionine effect on growth rates. Methionine deficiency increased food consumption of the mice in Experiment I. In Experiment III (Part A), however, the MD fed rats began to reduce their food intake below that of the MS fed rats by the end of week 2 of the experiment. It seems that the increased lead level in the diet of the rats in Experiment III (Part A) depressed the appetite stimulation seen in the MD fed mice of Experiment I. Wagstaff (1979) found in rats as the lead concentration increased in the diet food consumption decreased. So, Experiment III (Part B) was designed for determining the effects of a true pair-feeding study. The MS fed rats were only allowed to eat that quantity the MD fed rats ate.

There was considerable variation evident when examining the GSH S-transferase results in all 3 experiments. In Experiment I where a total of 9 lindane treatments were given to the mice, lindane increased GSH S-transferase activity in both MD and MS fed mice. In Experiment II, where lindane was given once, the MD fed mice had a greater GSH

S-transferase activity. In Experiment III, where lindane was given once, with an increased lead dose and rats were the experimental animal, there were no treatment effects. The variation between GSH S-transferase activity in Experiments I and II can only be partially explained by a difference in the number of lindane doses. The lack of effect that methionine deficiency had on GSH S-transferase activity in Experiment I could possibly be the result of an adjustment and recovery to the methionine deficiency made over a 10-week treatment period. The lack of treatment effects seen in Experiment III (Parts A and B) could be due to a species difference. Kulkarni et al. (1980) found that mice were more responsive to GSH S-transferase enzyme induction than rats.

Glutathione levels in Experiment II were affected by toxicant treatment and less so by dietary methionine. Reduced glutathione (GSH), the form that conjugates with toxicant metabolites, was affected significantly, with lead causing an increase in blood, kidney and liver GSH levels (Tables 12, 13 and 14). Lead increased total glutathione (TGSH) levels only in the liver. Hsu (1981) found that lead had stimulatory effects on GSH synthesis. The direct involvement of lead in GSH conjugation reactions has not been established. Lindane caused a decrease in liver GSH levels, and this was most evident in the MD diet. TGSH of the kidney demonstrated a significant lindane and methionine effect. Lindane with the MD diet decreased TGSH whereas lindane with the MS diet increased TGSH. If enough methionine were present in the diet (MS) to make glutathione, perhaps the stimulation of glutathione can occur in association with the conjugation of lindane with GSH. The overall lack of dietary methionine effect on the toxicant treat-

ments in Experiments I and II could be because the animals in Experiments I and II were not pair-fed. In Experiment III (Part B) where the animals were pair-fed, there was a methionine effect. Lead with lindane increased GSH levels greater in the MS fed group than the MD fed group of rats. The presence of greater quantities of methionine in the diet of the MS group appeared to increase production of GSH: a greater GSH level was observed. Lead stimulation of GSH synthesis perhaps can be enhanced with adequate methionine levels; the conjugation of lindane with GSH could also cause increased production of GSH when sufficient methionine is present in the animals' diet.

Hematocrit levels were significantly lowered by the presence of high lead levels in the diet of rats (Table 19). Lead is known to disturb biosynthesis of heme. Lead inhibits aminolevulinic acid dehydratase (ALAD) required for porphobilinogen (PGB) formation (Hsu, 1981).

In spite of lead causing apparently increased GSH levels, lead's role as a detoxifier is not certain for other substances. Lead has been shown in different experiments to increase and decrease the microsomal enzymes of Phase I reactions in drug detoxification (Eaton et al., 1980; Wagstaff, 1979; Chow and Cornish, 1978; Dalvi and Robbins, 1978; Egan and Cornish, 1973). Variability in these experiments in regard to the lead doses and route of administration could be the cause of the conflicting results.

SUMMARY

When the toxicants lead and lindane were given to mice orally in conjunction with a methionine deficient (MD) diet, more fatalities occurred than in those mice fed a methionine sufficient (MS) diet. Lead supplemented food fed to mice and rats decreased their growth rates. High levels of lead (10,000 ppm) in the diet of the rat decreased their food intake as well as their hematocrits. Lead feeding apparently increased liver glutathione (GSH) levels. And when MS (lead + γ-BHC) treated rats were pair-fed to MD (lead + γ-BHC) treated rats, the MS (lead + γ-BHC) treated rats had a higher level of GSH. The methionine status of the animals appears to be an important factor in determining its liver GSH level.

When studying parameters such as lethal doses of lindane, body weights and methionine status and GSH S-transferase activity, there appeared to be a species difference when comparing rat and mice results. When treated only once, all the mice survived a dose of lindane that was twice the LD_{50} dose for rats. Mice body weights were less affected by methionine deficiency than the rats. GSH S-transferase activity in mice demonstrated treatment effects. In rats, however, no treatment effects were evident for GSH S-transferase activity.

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