Title of thesis: Biochemical effects of cadmium exposure and the potential pharmacologic significance of cadmium mediated hydrolase inhibition.

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Master of Science
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Abstract

Title of Thesis: Biochemical effects of cadmium exposure and the potential pharmacologic significance of cadmium mediated hydrolase inhibition.

Darrell Jay Nix Master of Science, 1996
Thesis directed by: Brian M. Cox, Ph.D., Professor & Chairman of Pharmacology

The objective of this research was to explore the possible existence of an adverse effect resulting from exposure to cadmium (Cd) within levels allowed or recommended to be “without adverse effect”. The central hypothesis that we tested was that the exposure of Sprague-Dawley rat to Cd (2 mg CdCl₂/kg, administered i.p.) would reduce non-specific serum and tissue hydrolase activity enough to alter the metabolism of an ester compound like methylphenidate. The knowledge that metals affect the function of enzymes was the rationale behind an initial investigation attempting to identify an enzyme that could be used as a biomarker of Cd exposure. It was discovered that Cd was capable of reducing the activity of rat serum hydrolase. The hydrolases are ubiquitous enzymes that are known to be active participants in the hydrolysis of many foreign toxic and therapeutic compounds. However, the physiological role of the esterases is unclear. Therefore, we choose to first demonstrate a significant effect in vitro, then using preliminary data, speculate on the in vivo pharmacologic significance of Cd altered hydrolase activity. In vitro, the substrate phenylacetate was used to measure hydrolase activity in serum and homogenates of various tissues. An IC₅₀ of 0.68 µg Cd/mL (6 µM) was demonstrated in dilutions of control rat serum by
addition of increasing concentrations of CdCl₂ (0-100 µg/mL) to the assay buffer. In our preliminary in vivo investigation one 2 mg CdCl₂/kg exposure was found to inhibit non-specific serum hydrolase as much as 70% over a period of 72 hours after exposure. A kinetic assay, employing the substrate phenyl acetate, was used to measure the in vivo effects on serum hydrolase activity. The inhibition was reversible over time, with full recovery occurring about 96 or more hours after exposure. Methylphenidate (Ritalin®, CIBA-Geigy) was selected for a direct in vivo investigation based on an extensive hydrolase involvement in the metabolic disposition of this ester compound. In our investigation of the effects of Cd on the metabolic disposition of methylphenidate, we found that when rats were pretreated once with Cd (2 mg CdCl₂/kg) there was a three fold increase in the peak plasma (Cₘₐₓ) level. The Cₘₐₓ following a 0.15 mg/kg methylphenidate-HCl dose was 5.0±0.9 ng/ml in controls and 17.1±4.3 ng/ml in the Cd pretreated. Similarly, following a doubling of the methylphenidate-HCl dose the Cₘₐₓ values were 8.1±1.5 and 28.4±9.5 ng/ml in controls and Cd exposed, respectively. The increased Cₘₐₓ resulted in a four fold increase in the total AUC of the plasma time curves of the Cd exposed rats. Neither the time to maximal plasma concentration (Tₘₐₓ) nor the half life (T₁/₂) was affected by Cd exposure. This increase in plasma methylphenidate levels may play a significant role in altering the pharmacologic activity of a dose of methylphenidate, and will require further investigation.
BIOCHEMICAL EFFECTS OF CADMIUM EXPOSURE AND THE POTENTIAL PHARMACOLOGIC SIGNIFICANCE OF CADMIUM MEDIATED HYDROLASE INHIBITION.

By
Darrell Jay Nix

Thesis submitted to the faculty of the Department of Pharmacology
Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of
Master of Science 1996.
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I. Introduction

A. Project summary

Our research addresses a timely issue concerning exposure of the public to cadmium (Cd), a toxic heavy metal. The investigation of toxic heavy metals is an active area in toxicology research, however there are questions that remain unanswered. The focus of this project has been to demonstrate the existence of an adverse pharmacologic event, occurring as an indirect result of exposure to low levels of Cd in the environment. Our investigation found that the metabolic disposition, and hence the pharmacodynamics of methylphenidate were indeed altered following Cd exposure. While the actual clinical significance is unclear, preliminary findings have increased our understanding of the mechanism(s) by which Cd alters physiologic functions and drug metabolism.

Entanglement of biochemistry, physiology, chemistry, and molecular and cellular biology research combine to form the basis for pharmacology research. Further, the study of high dose pharmacologic effects, beyond the level providing therapeutic benefit are the basis of toxicology research. The relationship of these two disciplines is most clearly understood by a single pharmacologic principle for all compounds, that the dose determines the outcome, either beneficial or toxic. Application of toxicology in pharmacology research is common and demonstrates a very important aspect of
pharmacotherapeutics, the line that divides beneficial outcome from toxicity is variable.

Identification of all of the things that will have an affect on the outcome of a compound before it is used therapeutically (in the general population) would be impossible. There are several examples of factors in metabolism and disposition that have been identified, age, race, sex, health (i.e. organ function, liver, kidney, GI etc.), concurrent therapy (i.e. other medications), nutrition and diet, and many environmental factors (ranging from climate to exposure to toxic pollutants, etc.). Relationships have been found that are useful predictors of the increased likelihood of interaction.

The objective of this research was the demonstration of an adverse pharmacologic outcome resulting from altered esterase activity following low level exposure to Cd. This supports the long-term goal of metal toxicology research, which strives to cooperatively develop understanding of Cd toxicity, in order to direct adequate regulatory protection of the human population. Preventative medicine research has established the benefits of attacking the problem, rather than trying to cure the effects. Exposure to environmental pollution is preventable and is the most effective solution to the adverse effects it causes. Toward that goal, the results from this investigation are intended to demonstrate exposure levels currently allowed (or recommended to be “without adverse effect”) must be re-evaluated (Freeman 1989). This research has explored the pharmacologic significance of an inhibitory effect on
hydrolase following exposure to Cd within levels allowed or recommended to be “without adverse effect”. Our central hypothesis was that altered pharmacologic disposition of methylphenidate is the reversible result of exposure to Cd in the Sprague-Dawley rat.

Cd toxicity is most commonly manifested as hepatic or renal damage (Goyer 1991). These tissues are often referred to as the target tissue of Cd toxicity, however this is misleading. Cd is toxic to all tissues, it is the accumulation, the physiologic function of the liver and kidneys, that concentrates absorbed Cd to levels that produce tissue toxicity. Further information on the molecular mechanism is needed. Additionally, there are other adverse effects of Cd exposures, which are unclear (Klaassen 1981; Goyer 1993) The scope of exposure is now considered to be global, as a result of environmental contamination (Mullins and Norman 1994; Burger, Nisbet et al. 1994a). Interest in the effects of Cd exposure should therefore be everyone’s concern, and deserves the attention of the scientists in toxicology research and government regulatory agencies to ensure adequate protective measures exist.

Clinical diagnostic use of blood and urine Cd levels has a limited clinical diagnostic application to recent (24 hrs) acute doses. The knowledge that metals alter or affect the function of enzymes was the rationale behind initial investigation of effects of Cd on various tissue and circulatory enzymes in the rat. The search was directed at locating a useful biomarker enzyme or
protein to develop as a diagnostic tool. It was the serendipitous discovery that rat serum hydrolase was inhibited by Cd, however, that re-directed the research to investigate the effects of Cd on hydrolase activity in the rat. This observation is often missed in large experiments that use a population control activity, rather than the individual control activity, measured in each rat prior to treatment (or time 0 for controls not receiving vehicle). This was avoided by an unplanned shortage of rats when the experiment began.

The esterases are ubiquitous enzymes that are known to be active participants in the hydrolysis of many foreign toxic and therapeutic compounds. However, the physiologic role of esterase enzymes is unclear. Therefore, the significance of altered hydrolase activity will be addressed by evaluation of the role of esterase hydrolysis in drug metabolism. In preliminary experiments, multiple assay problems were responsible for discontinuation of investigation of the compounds nifedipine, nitrendipine and the benzodiazepines. Selection of methylphenidate (Ritalin®, CIBA-Geigy) the next compound was based on the extensive involvement of esterase hydrolysis in it's metabolic disposition. First described in 1954 by Meier and associates (Meier, Gross et al. 1954) methylphenidate (MPH) is a drug that has a long history of clinical use and research, both areas are well represented in the scientific literature. In Figure 1, adapted from Egger, et al. (1981), we've included the metabolic pathways for MPH in the rat that we have analytical methods for detection (Egger, Bartlett et al. 1981).
Methylphenidate Metabolism in the Rat

Major Pathways

Figure 1. Major metabolic pathways common to both human and Sprague-Dawley rats.

The conclusion of this research will be used to demonstrate an adverse effect of Cd exposure on the pharmacologic function of non-specific serum hydrolase enzymes in the rat. From our initial studies, it was seen that the inhibitory effects of Cd on esterase activity resulted in an increase the plasma concentration of MPH. Additional behavioral studies are required to determine the pharmacodynamic significance of the altered MPH pharmacokinetics. It stands to reason, however, that increased serum MPH levels will shift the dose-response plot to the left.

The intention of this project is to determine the effects of Cd exposure on the activity and function of non-specific hydrolase activity. In the absence
of a clear physiologic function for hydrolase enzymes, we have chosen to investigate how Cd affects their role in drug metabolism, leading to altered therapeutic efficacy.

B. Global significance of environmental exposure to toxic heavy metals

Historically, exposure to heavy metals was confined to the “occupational” population, largely limiting exposure to industrial settings, although not entirely inclusive for all the heavy metals. Today, human contact with hazardous materials is often from environmental sources rather than more direct workplace exposures (Elinder 1992; Muntau and Baudo 1992; Thornton 1992). In the last two decades there has been a shift in the trends of exposure to the heavy metal Cd (Cd) reversing the exposure "roles" of the workplace with the environment surrounding the general population (Herber 1992; Iwata, Saito et al. 1993; Freeman 1994; Muller and Anke 1994; Shore and Douben 1994). As a result of the tireless efforts of the Occupational Health Regulators, daily exposure to one or more hazardous substances is now more likely to occur in the general population outside the heavily regulated workplace from broad reaching environmental sources (Elinder 1992; Herber 1992; Thijs, Staessen et al. 1992; Varga, Zsolnai et al. 1993). This demonstrates that in reality there is no separation protecting occupational groups from the effects of environmental pollution (Hall, Dhara et al. 1994; Wenning, Bonnevie et al. 1994).
The scope of toxicity from environmental hazards is variable by compound, however, Cd and other metals are unique persisting over time to acquire an exposure with global reach. Basic elements are not metabolized or broken-down by normal processes like most compounds. Metals are a primary example of persistent contaminants that accumulate in the environment. Entry into the environment, and re-distribution of Cd expands the geographic area of exposure far beyond the initial point source of entry. Often the ground water is responsible for the movement of Cd from the soil into the major aquifers offering almost unlimited passage (Klaassen 1981; Friberg, Elinder et al. 1986a; Goyer 1991). Re-distribution can also be facilitated by plants, animals and human activities, having a combined effect of widening the range of exposure to include the global population.

The heavy metal Cd is known to cause damage to several biological systems. Extensive studies have presented data indicating hepatic and renal tissue as primary targets of Cd toxicity in humans (Kazantzis, Flynn et al. 1963; Nishizumi 1972; Cherian, Goyer et al. 1976; Ellis, Morgan et al. 1981; Goyer 1982; Lauwerys, Bernard et al. 1984; Dudley, Gammal et al. 1985; Guthrie, Chettle et al. 1994; Marshall, Schroen et al. 1994; Sens, Hazenmartin et al. 1994). However, there are additional targets which have not been as clearly identified. The toxic effects of chronic exposure to Cd at trace levels are uncertain in man. The WHO/FAO establishment (1972) of a provisional tolerable weekly intake limit, 0.4-0.5 mg/wk, was re-presented for
support in the early 1980's. It was then discovered that renal function in the elderly is more vulnerable than was postulated, and life long exposure at or even below the safe limit produced serious adverse effects on renal tissue (Roels, Lauwerys et al. 1981a). Further, this limit has been exceeded in many populations, which has promoted Cd pollution into becoming a major environmental priority of the WHO (World Health Organization 1980; Roels, Lauwerys et al. 1989a).

Growing public awareness over this issue has helped to generate an increase in the support for research directed at characterizing the effects of exposure to Cd and other heavy metals. Toxicologists, in an effort to provide information to regulatory agencies, have made significant advances in their understanding of the mechanisms of metal toxicity. However, there are still several important questions that must be investigated, before scientists are able to provide the information that is required to better establish allowable limits of “safe” exposure, with the greatest amount of certainty. The conclusion of metal (Cd) toxicology research is that exposure is a global concern, with an urgent need to acquire further understanding on toxic mechanisms of trace exposure and the effects of multicomponent exposure.

The hydrolase enzymes have been identified in preliminary studies as enzymes that are altered by the metal Cd. In Figure 3 (see Results and Discussion Section III.B.1) the in vitro inhibition of rat serum hydrolase activity is demonstrated with various concentrations of Cd contained in the
incubation buffer, using the substrate phenyl acetate. Further investigations found a similar loss of rat serum hydrolase activity to occur in vivo. Exposure of Sprague-Dawley rats to a single 2 mg CdCl₂/kg (i.p., in normal saline), found that a similar loss of serum hydrolase activity occurred 48-72 hours after dosing. Figure 3 presents data from two experiments where serum hydrolase activity in individual rats is followed for up to 72 hours after CdCl₂ exposure. Using phenyl acetate as a substrate, serum hydrolase activity is determined prior to exposure, then measured daily thereafter, to determine the effects of CdCl₂. Although not immediate, loss of serum hydrolase activity is observed in vivo, between 48 and 72 hours after exposure. Further investigations will be presented in an attempt to characterize this event more completely.

The non-specific hydrolase enzymes are ubiquitous in their tissue distribution. Hydrolase activity is present in the blood, liver, kidneys, GI tract, skin, and even in the brain (Williams 1985; Leinweber 1987; Lockridge 1992). There have been several reports for different tissues that have characterized the hydrolase enzyme content by activity (Lund, Kampmann et al. 1976; Jeffery, Jones et al. 1978; Inoue, Morikawa et al. 1980; Hoensch and Schwenck 1984; Flinois, Chabin et al. 1992). The investigation of blood hydrolase activity in metabolism of therapeutically used drugs has also been reported (Ludden, McNay et al. 1982; Diasio and Harris 1989). Work in this lab has reported the enantioselective esterase hydrolysis of the 1,4-
benzodiazepines (Yang, Liu et al. 1990; Liu, Guengerich et al. 1991; Yang, Hsieh et al. 1992). The non-specific hydrolases are clearly important in the metabolism of both drugs and xenobiotic compounds.

Substrate and inhibitor specificity of the hydrolase enzymes has been exploited whenever possible in the development of assay methods and characterization. Esterases exhibit overlapping substrate specificity, and substrates are often hydrolysed by more than a single enzyme (La Du and Snady 1971; Walker and Mackness 1983; Dave, Miller et al. 1993). This has made classification a difficult. Additionally, the lack of agreement on a single system of nomenclature has resulted in confusion and inaccuracy in the literature.

Hydrolysis of therapeutically used drugs is often not limited to one esterase, or location. This reduces the need to attempt positive identification of a specific especially when many are possibly involved. Our goal is to identify a pharmacologic effect that is the indirect result of environmental Cd exposure. The initial finding of Cd inhibition of the hydrolase activity in serum and fractions of hepatic tissue is demonstrated using phenyl acetate as a substrate. Selection of methylphenidate to relate Cd exposure to altered pharmacologic activity, was based on the rapid and nearly exclusive involvement of non-specific esterase in the metabolism, further supported by the long history of use and research.
The goal of this investigation is to demonstrate that Cd exposure is indirectly affecting the pharmacologic activity of methylphenidate. From a larger perspective, it is intended that this information will be in some form added to the overall regulatory understanding of the effects of Cd exposure, and used in establishing adequate exposure limits that assure public safety that technology is capable of providing. In reality, there is also the political attractiveness of an enormous cost-effectiveness of prevention vs. the burden of having to address all unnecessary effects of man's pollution.

Early plans directed the preparation of a database listing drugs containing an ester bond, that was known to be hydrolyzed by esterase; this included the pro-drugs. Compounds were selected for investigation from the accumulating database based on availability, therapeutic application and amount of background information. To accomplish our goal we selected methylphenidate to use as an in vivo “tool” to test the significance of reduced non-specific hydrolase activity. Methylphenidate contains an ester bond that is hydrolyzed in a hydrolase catalyzed reaction. No relationship is intended between the treatment of Attention Deficit Disorder Hyperactivity (ADDH) and Cd or serum and tissue esterase activity. Further evaluation of this compound has noted that the therapeutic use is rather extensive. Public opinion beyond parents and teachers is being driven by considerable attention from the press. While our selection of methylphenidate was based on the chemistry, the current public attention to it’s use has not gone unnoticed.
II. Background

A. Cadmium and other heavy metals

The toxicity of most heavy metals is dependent on the tissue concentration, and not exclusive of any tissue. The apparent specific organ toxicity of some heavy metals is a result of the distribution of metals in the body following absorption. Cadmium and other heavy metals have many important industrial uses, which unfortunately have led to widespread environmental pollution. Cadmium is a toxic substance and a growing environmental problem potentially having effects on the global population. While technological advances have improved man's lifestyle, pollution is too common a side effect. The environment cannot afford to absorb increasing levels of abuse. While this side effect is no longer acceptable, it is an issue that must receive global attention.

Metals are environmentally persistent, not being created or destroyed by normal processes, creating a unique pollution problem. Further, as environmental contaminants, metals are mobile and are easily transported in the atmosphere and by water irrespective of most geographic features or political boundaries. Other forms of pollution that increase the acidity of surface water, create an aquatic environment that enhances the aqueous solubility and bioavailability of metals to the aquatic biota (Dalleska, Honma et al. 1994). Additional contamination of the environment can potentially be
controlled, however the uncertain risks of current levels of exposure represent a different problem.

1. Sources and levels of exposure to metals in the environment

a) Industrial importance

Environmental contamination by Cd has been a result of both its presence as a mineral contaminant and important industrial uses of the metal. The natural levels of Cd in the soil depend on the type of rock from which the soil was derived, ranging from 0.001 to 10 ppm. Soil Cd levels are also affected by contamination from mines (Cannon 1970), metal smelting and refining plants (Obuschowska 1966), electroplating industries (Page, Bingham et al. 1972), and even near roads and highways (Lagerwerff and Specht 1970). Contamination of roadside soil results from the rubber tires more than from fossil fuels. The total emission estimated to result from vehicular tires in the US one year totaled between 5-6 metric tons (Davis and al. 1970; Marchesani, Towers et al. 1970).

The burning of coal and other fossil fuels may also contribute to the contamination of soils. Coal samples from Kentucky and Pennsylvania have been reported to contain 1-2 ppm Cd, and heating oils have been shown to contain 0.4-0.5 ppm Cd (Lagerwerff and Specht 1971).
A major source of Cd contamination is the solid landfill. Materials containing Cd that are deposited in dumps and landfills release the metal into the soil where it is carried by ground water (Lagerwerff and Specht 1971). It also has been found that sewage and the resulting sludge is a significant source of Cd contamination of soil (Page and Bingham 1973). Sludge, is a product of sewage treatment used commercially as fertilizer with no regard for potential metal concentration. The use of commercial phosphate fertilizers with comparatively high Cd levels, results in widespread soil contamination. Levels of Cd in fertilizer ranging from 9-36 ppm (Schroeder and Balassa 1963a) and 50-170 ppm (Caro 1964) have been reported. A single application of such fertilizer that was 10% (V/U) superphosphate will result in an increase in soil Cd from 0.34 to 1.3 ppm (Schroeder and Balassa 1963a).

Industrial use of Cd has not only been responsible for increasing soil levels, but also of contaminating the ambient air in many locations. A survey in 1964 found the range of maximum Cd concentration in suspended air particles from 15 US cities to be from 0 to 0.35 $\mu$g/m$^3$ (US Dept. Health Education and Welfare 1966); the highest value was reported in Newark, NJ (it should be noted that this is the least of that areas concerns). It should also be pointed out that the contamination of air will eventually lead to the contamination of soil, surface water, vegetation and food.
The US has historically been the largest industrial Cd producer and consumer (US Bureau of Mines 1969). Electroplating is the World's largest single industrial Cd consumer. Cadmium has many other industrial applications; pigments in paints, enamels, glazes, textiles, and plastics (National Research Council 1969); stabilizers for polyvinyl chloride plastics; cathode material in Nickel-Cd batteries (Davis and al. 1970); component of low-melting alloys (Weast 1969).

Because Cd is almost always associated with zinc, it has been pointed out that galvanized zinc coatings of containers used for food and beverages can be a significant source of Cd contamination (Stokinger 1969). In all cases, organic acids in foods facilitate Cd solubilization from the walls of containers.

b) Pollution/exposure survey

A significant source of Cd intake is contaminated surface water. The legal safe limit of Cd concentration in water for public use was established by the US Dept. of Public Health at 10 µg/L (US Public Health Service and US Department of Health 1962). The US EPA has set the safe limit at 5 µg/L for Cd in drinking water, however this is only a government recommendation not an enforceable law (US Environmental Protection Agency 1976). A survey reported by Schroeder et al. (1967) showed that some municipal water supplies were contaminated above the then established safe limit, ranging from 0-21 µg/L. Congress later established the first broad based set of water
quality laws in 1972, the Clean Water Act, and later the Safe Drinking Water Act (Safe Water Act) which is currently under revision.

The Safe Water Act expired in 1991, and has been carried on unchanged from year to year under continuing resolutions. According to the EPA many municipal water systems are found to be in noncompliance, meaning that 80% of the time, they are not testing, or not reporting the test results as required [White, 1994]. This may affect as much as 60% of the American population [White, 1994], further addition of contaminants occurs from the pipes that plumb the distance between the entry from the municipal water main to the tap in our kitchen sink.

Pipes are a common, and perhaps most preventable, source of Cd contamination of drinking water. Polyethylene (black), copper and galvanized iron pipes may contain high levels of Cd (Schroeder and Balassa 1961a). Some galvanized iron pipe coatings may contain from 140-400 ppm (US Public Health Service and US Department of Health 1962). In a 1970 survey of surface water near industrialized areas, the following were found to have levels in excess of the safe limit: the Lake Erie basin (near Cleveland, OH), the Allegheny River (near Pittsburgh, PA), and the Niagara River (at Buffalo, NY) (Kopp and Kroner 1970). Water samples from Lake Erie contained 120 µg/L; the average Cd concentration of 47 samples taken from the two rivers in the Lake Erie basin was 50 µg/L. Samples from a river in Idaho
where mining, smelting and ore refining industries are located, show Cd contamination levels near 450 µg/L (Mink and Williams 1971). In New York, waste discharges from electroplating industries brought Cd contamination levels of Long Island ground water to 3200 µg/L (Lieber and Welsch 1954). Water samples from California wells average 23.5 µg/L, demonstrating Cd contamination in the US was a widespread problem (Silvey 1967).

(1) Dietary exposure

Cadmium can form alkyl compounds, however they are very unstable, especially in the presence of moisture (Sidgwick 1950). Generally, inorganic salts are the most common form of Cd in food products. The daily intake of Cd by humans can be estimated from the Cd content in foods. In a 1967 analyses of institutional and hospital diets, Schroeder et al. (1967) found a total daily Cd intake of 470 and 213 µg, respectively. Values were not included for any Cd intake from drinking water. Three subjects in a balanced study by Tipton and Stewart (1969) had a daily intake of 100-220 µg Cd from their diets. Murthy et al. (1971) reported a daily intake of 32 to 158 µg Cd for institutionalized children (ages 9-12 years old) in 28 US cities. The values reported (1969-1980) for the general US population range from 20-100 µg Cd daily (Duggan and Lipscomb 1969; Tipton and Stewart 1969; Murthy, Rhea et al. 1971; Johnson, Manske et al. 1981a), with 50 µg Cd daily being the most recent figure available (Wenning, Bonnevie et al. 1994).
Table 1 shows the Cd content of 12 classes of foodstuffs normally consumed in the US. These values were obtained from a 3-year study of US foods commonly purchased, prepared, and consumed (Manske and Johnson 1977a; Johnson, Manske et al. 1981a; Johnson, Manske et al. 1981b). Various foods show a marked difference in Cd content between classes. All composites of cereal grains and potatoes were contaminated and 90-95% of the leafy vegetables, root vegetables, garden fruits, oils, and fats were similarly contaminated with Cd (Manske and Johnson 1977a; Johnson, Manske et al. 1981a; Johnson, Manske et al. 1981b). Potatoes and leafy vegetables contained the highest levels in the group, notice that all values increased from survey XI to XII.
Table 1 Listing of the levels of Cd in composites of 12 classes of common US foods as reported in four yearly surveys from 1972-76.

<table>
<thead>
<tr>
<th>Foodstuff Classes</th>
<th>(IX) 1972-1973 Range / Mean(^a) (µg Cd/100 gm)</th>
<th>(X) 1973-1974 Range / Mean(^a) (µg Cd/100 gm)</th>
<th>(XI) 1974-1975 Range / Mean(^a,b) (µg Cd/100 gm)</th>
<th>(XII) 1975-1976 Range / Mean(^a) (µg Cd/100 gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dairy products</td>
<td>1-6 trace (5/30)</td>
<td>1-14 1 (4/30)</td>
<td>trace trace (4/20)</td>
<td>1-2 0.2 (3/30)</td>
</tr>
<tr>
<td>Meat, fish poultry</td>
<td>1-6 1 (12/30)</td>
<td>1-6 2 (21/30)</td>
<td>trace trace (11/20)</td>
<td>1-3 1.0 (17/30)</td>
</tr>
<tr>
<td>Grain and cereal</td>
<td>2-5 1 (30/30)</td>
<td>2-5 3 (29/30)</td>
<td>5-8 trace (19/20)</td>
<td>2-5 3.0 (20/20)</td>
</tr>
<tr>
<td>Potatoes</td>
<td>2-12 5 (30/30)</td>
<td>2-13 5 (29/30)</td>
<td>5-12 4 (20/20)</td>
<td>2-9 5.0 (20/20)</td>
</tr>
<tr>
<td>Leafy vegetables</td>
<td>1-28 5 (30/30)</td>
<td>2-14 4 (28/30)</td>
<td>5-14 5 (20/20)</td>
<td>2-10 4.0 (19/20)</td>
</tr>
<tr>
<td>Legumes</td>
<td>1-3 trace (10/30)</td>
<td>1-10 1 (8/30)</td>
<td>trace trace (3/30)</td>
<td>1-7 1.0 (14/20)</td>
</tr>
<tr>
<td>Root vegetables</td>
<td>1-6 2 (24/24)</td>
<td>1-31 3 (24/30)</td>
<td>trace trace (16/20)</td>
<td>1-8 2.7 (19/20)</td>
</tr>
<tr>
<td>Garden fruits</td>
<td>1-6 2 (25/25)</td>
<td>1-10 2 (23/30)</td>
<td>trace trace (17/10)</td>
<td>1-4 2.0 (18/20)</td>
</tr>
<tr>
<td>Other fruits</td>
<td>1-2 trace (4/30)</td>
<td>1-6 trace (3/30)</td>
<td>trace trace (5/20)</td>
<td>1-2 0.3 (5/20)</td>
</tr>
<tr>
<td>Oils, fats</td>
<td>1-6 3 (29/30)</td>
<td>1-7 2 (24/30)</td>
<td>trace trace (17/20)</td>
<td>1-3 1.6 (18/20)</td>
</tr>
<tr>
<td>shortening</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sugars, adjuncts</td>
<td>1-6 1 (13/30)</td>
<td>1-9 1 (12/30)</td>
<td>trace trace (8/20)</td>
<td>1-3 1.1 (14/20)</td>
</tr>
<tr>
<td>Beverages</td>
<td>1-8 trace (5/30)</td>
<td>1-3 trace (6/30)</td>
<td>trace trace (1/20)</td>
<td>0-1 0.2 (3/20)</td>
</tr>
</tbody>
</table>

\(^a\)For numbers in parentheses, numerators represent positive composites, and the denominators represent the total number of composites analyzed.

\(^b\)Superscript numbers represent the number of composites in each category reported to contain trace amounts.
(2) **Regulatory exposure limits**

Before regulations against the use of Cd in food containers were enacted in the US, contamination of foodstuffs after canning was a major problem. Accounts of Cd poisoning from foods stored in such containers was reported almost 50 years ago (Frant and Kleeman 1941). These cases involved acid foods or beverages such as lemonade, raspberry gelatin, homemade punch, lemon-flavored iced tea, and popsicles. In these cases, foodstuff was allowed to remain in cooling containers, such as Cd plated ice cube trays, metal pitchers and popsicle molds. A Cd concentration of 13-15 ppm was found in popsicles which poisoned 29 children, and 300-530 ppm in the lemonade and gelatin, respectively, which poisoned several adults.

The Joint FAO/WHO Expert Committee on Food Additives estimated that the average human Cd daily intake was 50-150 µg (World Health Organization 1972). The Committee further proposed a “provisional tolerable” weekly intake of 400-500 µg per person. Several of the studies cited above show dietary Cd levels in the US that exceed the proposed FAO/WHO tolerable weekly intake. In the Fuchu-cho area of Japan, individuals had intake levels up to 600 µg/day from industrially contaminated foods (Yamagata and Shigematsu 1970). This is more than 8 times the proposed weekly intake. Mining operations contaminated the Jintsu River, the main source of water used to irrigate local farmland. The contaminated grains
contained 0.35-3.35 ppm Cd, daily consumption of about 300 gm rice/day was equivalent to a Cd intake of up to 1000 mg/day (Yamagata and Shigematsu 1970).

The 1972 WHO/FAO provisional tolerable weekly intake limit of 400-500 µg per wk, was further supported by data in an early 1980’s report. Researchers discovered that renal function in the elderly is more vulnerable than was known, and life long exposure at or even below the limit still produce serious adverse effects on renal tissue (Roels, Lauwerys et al. 1981a). Further, this limit has been exceeded in many populations, which has promoted Cd pollution to become a major environmental priority of the WHO (World Health Organization 1980; Roels, Lauwerys et al. 1989a).

2. Heavy metal toxicity
   a) Dietary absorption

Our primary defense against toxicity from dietary metals is simply limited absorption by the GI mucosa. Estimates of the amount of Cd absorption range from 5-75% of the ingested amount (Schroeder, Nason et al. 1967; Tipton and Stewart 1969; Fassett 1972; World Health Organization 1972). There are individual variations, as well as nutritional status and dietary factors that can affect GI absorption. Many references are in agreement that GI absorption of Cd is between 5-10% of the ingested amount (Goyer 1991).
Calcium deficiency can increase Cd absorption from the intestines (Larson and Piscator 1971; Itokawa, Abe et al. 1974; Pond and Walker 1975). Pyridoxine, vitamin B₆, is believed to be essential to GI absorption of Cd, deficiency of the vitamin decreases the absorption (Stowe, Goyer et al. 1974). Absorption is also influenced by the intake of cholecalciferol, protein and other trace minerals (World Health Organization 1972). Increased Cd absorption, for example, is the result of iron deficiency (Hamilton and Valberg 1974). In rats, a milk diet increased Cd absorption by 14-18% (Kello and Kostial 1977).

Age also influences the GI absorption of Cd. Newborn rats retained 80 times more dietary Cd than the adults, showing no difference in the amount retained when injected intraperitoneally (Kello and Kostial 1977). Results from another study indicate that the body burden of Cd increases more than 200 times during the first 3 years of life in humans, concluding that by this time 1/3 of the total body burden is accumulated (Henke, Sachs et al. 1970).

In contrast to GI absorption, respiratory absorption of Cd is high. As much as 90% of the total soluble Cd deposited in the lung are absorbed and enter the general circulation (Oberdorster 1986). Most reports for respiratory absorption are lower, where 15-30% is probably a more accurate level (Goyer 1991). Respired Cd is not accumulated in the pulmonary tissue. Nearly all respired Cd is absorbed from lung tissue and re-distributed in the general
circulation to other target organs (Oberdorster and Kordel 1981).

Nephrotoxicity related to chronic inhalation of Cd demonstrates the lung's limited role (Oberdorster 1986). The importance of respiratory absorption of Cd is significant in persons exposed to cigarette or tobacco smoke. A single cigarette contains 1-2 µg Cd on average, of which 0.1-0.2 µg enter the lungs (Elinder, Kjellstrom et al. 1983). Smokers consuming 1 or more packs per day, double their body burden of Cd.

**b) Retention and turnover of cadmium**

The body retention, or burden, of Cd also affects the resulting toxicity. According to Schroeder et al (1967), only 1.8-3.6 µg of the total Cd ingested daily is retained in the human body. This estimate is based on the observed total accumulated dose of 30-60 mg over 45 years. It was shown that 20.9-21.8% of the 200 µg Cd ingested daily is absorbed. Since urinary excretion of Cd accounts for 20% of the daily intake, only 0.9-1.8% of the dietary Cd is retained (Schroeder, Nason et al. 1967). The results suggest a very low retention, however, as in absorption, individual differences as well as other factors can affect retention. Retention values of 11% and 36% were observed in two subjects, and a negative Cd balance in a third subject demonstrates this point (Tipton and Stewart 1969). Studies using animals have shown that calcium deficiency increases the incorporation and retention of Cd in the bones (Itokawa, Abe et al. 1974; Pond and Walker 1975).
Levels of retention and accumulation or burden of Cd can be misleading. The bulk of ingested Cd is reported to be recovered in the feces with the appearance of no resulting toxicity. Transit of ingested Cd through the alimentary canal is slowed by uptake into luminal mucosa cells. Accumulation of Cd in the cell results in toxicity and the sloughing of the cell, which returns the metal to the luminal compartment (Shaikh and Smith 1980). This is the cause of GI distress reported in humans following acute Cd exposure (Waalkes, Wahba et al. 1992a).

The rate of Cd turnover in human tissues is remarkably low. Exact biological half-life of Cd in the body is unknown, however, there is agreement that it is extensive and reported as high as 30 years (Goyer 1991). Analyses of Cd accumulation in kidneys obtained from a cross section of Japanese, American, and Swedish populations was used to estimate the biological half-life of Cd in this tissue, the resulting range of 17.6-33 years is less than conclusive (Kjellstrom, Friberg et al. 1971; Tsuchiya and Sugita 1971).

Gross analysis of various tissues (American) at autopsy has shown that the kidney and the liver are the principal organs where Cd accumulates (Klaassen 1981; Nordberg, Kjellstrom et al. 1985; Goyer 1993). Retention of Cd in the kidneys increases with age, with none found in the kidneys of newborn or stillborn infants (Koizumi, Hatayama et al. 1994). The number of kidneys with detectable amounts of Cd increases with age in children. The Cd content of the kidneys in older children and adult subjects increases linearly
with age up to a maximum at age 40-50 years, after which the level declines. Several factors have been shown to affect this decline, data supporting a clear explanation has not been presented. Studies in rats and dogs demonstrate a similar avidity of renal and hepatic accumulation of Cd (Decker and al. 1958; Anwar and al. 1961; Lauwerys, Bernard et al. 1984; Goyer 1991).

Toxicity from Cd, as stated earlier, is the result of tissue accumulation to a tissue specific “critical-level” (Goyer 1982). In several reports of Cd effects targeting hepatic and/or renal tissue for toxicity, an incorrect overlay implies that these are the only tissues susceptible to toxicity. Cadmium is toxic to all tissue, however at different tissue dependent levels (Timmermans, van Hattum et al. 1989; Goyer 1991; Koizumi, Hatayama et al. 1994).

The development of Cd toxicity is enhanced up the food chain through a phenomenon called bioamplification (Burger, Nisbet et al. 1994a). In this process, tissue concentrations multiply with each step up the food chain, creating a non-linear scale of toxicity most dramatically in the uppermost positions.

Damage will occur when the tissue concentration reaches the toxic level. Reduction of tissue Cd below the toxic level will prevent tissue damage. Seabirds have the capacity to detoxify from high levels of the toxic heavy metals Cd and mercury (Hg) (Furness and Rainbow 1990), accumulated in the tissues of their prey. Many species accomplish this by excreting the metals
into their feathers during molt to prevent the accumulation (Burger, Nisbet et al. 1994a).

c) **Tissue distribution of cadmium**

Absorbed Cd (~10%) enters the general circulation where it is transported to various tissues. In the blood Cd is found in the free state in plasma or can be associated with red blood cells and other proteins (Nordberg 1972). Blood levels of Cd may be used only as indicators of acute exposure, they are not considered to be an important component of total body burden because it is rapidly cleared from the circulation into various tissues (Johnson and Miller 1970).

Cadmium is distributed to the tissues rapidly and has a high volume of distribution (Klaassen 1981; Friberg, Elinder et al. 1986a; Goyer 1991). Circulating Cd is removed by the liver most significantly, while distribution in testes, spleen, heart, lungs, thymus, salivary glands, epididymis and skeletal muscle tissues has been demonstrated (Waalkes and Klaassen 1985). Renal and hepatic tissue combined is responsible for 50-75% of the total body burden of Cd (Goyer 1991).

Cadmium bound to large plasma proteins, i.e. albumin, is removed from circulation by the liver (Goyer 1991). Deposited Cd ultimately follows a general path to the kidneys, regardless of the initial site of disposition. Several reports inaccurately summarize that renal accumulation of Cd is a
result of re-distribution of the hepatically deposited Cd (Nordberg, Goyer et al. 1975; Cherian, Goyer et al. 1976; Fowler and Vouk 1979; Friberg and Kjellstrom 1981; Klaassen 1981; Goyer 1982; Dudley, Gammal et al. 1985; Waalkes and Klaassen 1985; Clarkson 1986). Re-distribution of the absorbed Cd to the kidneys, from various tissues, is associated with metallothionein, a metal-binding protein with high affinity for Cd (Waalkes and Klaassen 1985).

Originally discovered in equine kidney cortex around 1957, metallothionein has a molecular weight of about 10,000 (Margoshes and Vallee 1957). The liver and kidneys produce most of this protein, however there are many other tissue sources demonstrated (Chen and Ganther 1975b; Goyer 1991). Metallothionein is produced in response to metal exposure (Shaikh and Lucis 1970; Lehman-McKeeman and Klaassen 1987). Induction of metallothionein by Cd and Zn pre-treatments has been shown to have a protective effect on the hepatotoxicity of acute Cd exposure (Goering and Klaassen 1984).

d) Cadmium toxicity

Cadmium is toxic to all tissues, again emphasized, the tissue sensitivity varies widely. The liver and kidneys are the major target organs of toxicity, as a result of Cd accumulation. Toxicity resulting from Cd exposure is a complex event. There are many details in the characterization of this event, the most important and consistent is that the toxicity of Cd is dependent on
the tissue concentration of Cd in a specific organ. The type, acute vs. chronic, and amount of exposure will determine how the toxic effect will result.

(1) **Acute toxicity**

Studies of Cd exposure in experimental animals have shown many toxic effects. Some of these effects are anemia, hypertension, testicular damage and atrophy, and carcinogenesis, as well as many others. The Japan Environment Agency sponsored long-term Cd feeding experiments because of the widespread exposure of the population to high levels of Cd. In the first study 10 primates were fed with food containing 0-300 mg CdCl₂/gm for 1 year. The monkeys that received 300 mg CdCl₂/gm developed tubular dysfunction within 4 months, yet showed no osseous, or bone mass, changes at the end of 1 year (Nomiyama 1980). In a second experiment primates were fed with food containing 0-100 mg CdCl₂/gm for 7 years. The monkeys that received 100 mg CdCl₂/gm developed tubular dysfunction within 12 months, however showed no osseous changes at the end of 7 years (Akahori, Nomiyama et al. 1983). In a third experiment, primates were given a diet that was deficient in calcium, phosphate, vitamin D and protein, with a Cd content of 30 mg/gm. The resulting osteomalacia was not preceded by any renal dysfunction over the entire 6 years (Yoshiki, Tachikawa et al. 1983).

The effects of acute, high dose Cd exposure are documented for both ingested and respired human exposure. Inhalation of Cd will primarily target lung tissue, toxicity is a dose dependent. Acute respired Cd may produce an
acute chemical pneumonitis and pulmonary edema (Goyer 1991). Acute respiratory exposure is commonly limited to industrial settings. Ingestion of Cd is a more common acute exposure, often resulting from contaminated foods. Toxicity from acutely ingested Cd targets the GI tract, causing nausea, vomiting, and abdominal pain (Frant and Kleeman 1941). Complete recovery has been reported in most cases where acute ingestion ranged 10-100 mg (Nordberg 1972). While acute Cd poisoning in humans is significant, it is a rare occurrence in the general population.

(2) Chronic toxicity

Chronic exposure to Cd is of great concern, potentially having global scope. Long term Cd exposure has been implicated to play a role in several toxic effects. The principal effect of chronic Cd exposure is nephrotoxicity, manifested as renal tubule disease (Goyer 1991). Renal dysfunction has been shown to occur only after a certain tissue concentration of Cd is reached in the renal cortex. The cortex concentration was determined to be about 200 \( \mu g \) Cd/gm wet weight tissue and is referred to as the “critical concentration” (World Health Organization 1972). The establishment of the critical concentration value is quite important in the determination of exposure risk. Several kinetic models have been developed to mathematically describe an exposure and predict toxicity. Incorporation of the “critical” value in a model allows an accurate estimation of “life-time” exposure levels producing renal accumulation that is less than the critical concentration for toxicity.
The details describing Cd induced nephrotoxicity are well published in the scientific literature (Kazantzis, Flynn et al. 1963; Nishizumi 1972; Cherian, Goyer et al. 1976; Ellis, Morgan et al. 1981; Goyer 1982; Lauwerys, Bernard et al. 1984; Dudley, Gammal et al. 1985; Guthrie, Chettle et al. 1994; Marshall, Schroen et al. 1994; Sens, Hazenmartin et al. 1994). In man, the onset of renal tubular injury is preceded by increased urinary Cd, proteinuria, aminoaciduria, glucosuria, and decreased tubular reabsorption of phosphate (Goyer 1991). Changes in the tissue morphology are reported to be nonspecific, however, initial tubular cell degeneration moves quickly to interstitial inflammatory reaction that results in fibrosis. Chronic Cd exposure can result in complete renal failure, often without producing clinically useful warnings. As the tissue level approaches the critical concentration, the irreversible damage precipitates a rapid massive event, manifesting clinically as total renal failure (Kazantzis, Flynn et al. 1963; Nishizumi 1972; Cherian, Goyer et al. 1976; Ellis, Morgan et al. 1981; Goyer 1982; Lauwerys, Bernard et al. 1984; Dudley, Gammal et al. 1985; Guthrie, Chettle et al. 1994; Marshall, Schroen et al. 1994; Sens, Hazenmartin et al. 1994).

Additionally, chronic respiratory and pulmonary diseases are linked to occupational exposure to dust and fumes containing Cd. Other effects on cardiovascular and skeletal systems are believed to be the result of chronic exposure (Nomiyama 1980; Friberg and Kjellstrom 1981). Epidemiological
data shows an increase in mortality from cardiovascular disease in humans from areas with high levels of Cd pollution (Nagawa, Kobayashi et al. 1979).

(3) uncertainty of exposure

The complete details of the toxic effects resulting from chronic exposure to Cd remain to be determined. The level of exposure to Cd, and other toxic metals, of the general population is becoming significant.

Japan is a geographic area where the general population has most commonly been cited in the literature for their high level exposure to Cd. This is no longer an area that is unique for this reason. Environmental Cd contamination in Europe and the US is now quite widespread. Most of the former Eastern European Nations have recently been identified to include some of the World's most poisoned environments.

The shadow of pollution over Eastern Europe is extreme and spreads from the Baltic to the Black Sea. Over 50 years of runaway industrialization is responsible for this destruction. The network of European rivers posed a convenient area to dispose toxic industrial waste that broadened the scope of pollution internationally. Recent reports out of the former Soviet Union reveal that these problems reach deep into Russia.

While the US is not an area of such extreme pollution as Eastern Europe, there are widespread areas of excessive levels of metal pollution. Metal contamination of estuarine and marine sediments has been identified in
every major metropolitan area along the entire eastern coast of the US
(O'Connor and Huggett 1988; National Research Council 1989). Included as
sites on the EPA's Super Fund list is every major port from Boston Harbor
extending south along the entire Eastern seaboard (Sagar and Davis 1984;
National Research Council 1989). In 1990, the Agency for Toxic Substances
and Diseases Registry (ATSDR) reported that metals (specifically citing Cd)
are the second most often identified toxic compounds in EPA Super Fund
sites, also referred to as National Priorities List (NPL) Sites (ATSDR 1990).

The National Research Council further reported that sediment
contamination is a widespread problem, yet the general understanding of the
geographical extent and ecological significance of the problem is not well
developed (National Research Council 1989).

Metal contamination in the US is not only a coastal problem, there are
several areas of contamination spread across the continent. These areas
include old mines and mine waste dump sites, industrial areas, farmland and
community rubbish dump sites. The movement of metals through the
environment is a complex problem. Deposited metals usually move through
the environment in water, however, in a recent study it was shown that
windblown dust from mine sites contained significant amounts of metals. The
level of Cd in dust from a mine waste dump site in Butte, Montana, was
111 ppm (i.e.: mg Cd/gm dust) and much higher for lead, copper and arsenic
(Pb, Cu, and As) (Mullins and Norman 1994).
B. Enzyme kinetics and metals

There are nearly 100 naturally occurring elements that constitute the earth's basic building blocks. Only 22 of these elements are known to be essential constituents of the mammalian body. Further classifying 17 of these essential elements into a loosely termed mineral group, due to occurrence as minerals or other inorganic compound forms. These mineral elements are also commonly part of our diet from both plant and animal sources as well as being dissolved in our drinking water. Several elements, such as iron, magnesium, iodine, cobalt, calcium, phosphorus and sulfur also occur as components of various organic compounds in the human and animal body and in foodstuffs. Table 2 lists several enzymes that require the presence of an essential inorganic element in some form for catalytic activity.
Table 1 Examples of enzymes that contain or require inorganic metals as cofactors for their normal function.

<table>
<thead>
<tr>
<th>Metal Cofactors</th>
<th>Enzyme Requiring Cofactor Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe$^{2+}$</td>
<td>Cytochrome oxidase</td>
</tr>
<tr>
<td>Fe$^{3+}$</td>
<td>Catalase and Peroxidase</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>Cytochrome oxidase</td>
</tr>
<tr>
<td></td>
<td>Carbonic anhydrase</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>DNA polymerase</td>
</tr>
<tr>
<td></td>
<td>Alcohol dehydrogenase</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>Hexokinase</td>
</tr>
<tr>
<td></td>
<td>Glucose-6-phosphatase</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>Arginase</td>
</tr>
<tr>
<td>K$^+$</td>
<td>Pyruvate kinase</td>
</tr>
<tr>
<td></td>
<td>(also requires Mg$^{2+}$)</td>
</tr>
<tr>
<td>Ni$^{2+}$</td>
<td>Urease</td>
</tr>
<tr>
<td>Mo</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td>Se</td>
<td>Glutathione peroxidase</td>
</tr>
</tbody>
</table>

Most mineral elements, or metals found in the body, whether essential or non-essential, have high chemical and biological activity, particularly in the form of ions, radicals, or organic complexes. In this form they are potentially toxic, depending on the dose and other conditions. Calcium, phosphorus and magnesium are minerals needed at relatively high daily levels. However, the other essential elements are only required in trace amounts for their specific physiologic function, outside these levels they have adverse effects. Therefore, at levels above those nutritionally required, the
effects of essential elements are not different from the effects of toxic, non-essential elements (Underwood 1977; Goyer 1993).

The effects of metals like Cd on enzyme function may have subtle effects that will develop irreversible damage from chronic exposure. The increased awareness of these effects is required for their prevention. Through research we will increase the understanding of the toxicologic significance, then we can accomplish prevention. There are two broad enzyme groups that are known to be affected by Cd, the sulphydryl enzymes and the zinc containing enzymes.

1. **Catalytic function of essential metals**

Several examples of essential metals have been presented in Table 2 along with their associated enzyme proteins. Critical biological functions cannot be accomplished without the assistance of inorganic materials. Therefore there are limitations on the abilities of some organic materials, namely many of the enzymatic proteins. For example, the mechanical strength of naturally occurring organic compounds is never very great. Organic compounds also are not well suited for catalyzing reactions of the oxidation-reduction type. And as a result inorganic compounds are essential to organisms, these are the essential metals that are presented in Table 2.

In 1978 Ochiai published a set of basic principles or rules that could be used to ascertain the essential nature of such compounds in physiologic
systems [Ochiai, 1978a; Ochiai, 1978b]. The following is a list of these basic rules:

(1) Rule of Basic Fitness (Chemical Suitability).

(2) Rule of Abundance.

(3) Rule of Efficiency.

(4) Evolutionary Adaptation (Evolutionary Pressure).

The Basic Fitness Rule asserts that a certain element(s) has an inherent capacity or potentiality for a specific biological function. Any element for which this is the case obviously becomes essential for the biological system in question. There could, of course, be more than one such element for a particular function. Therefore a second rule, the Rule of Abundance, is applied to dictate which element is selected for the function. Given a simple choice, the more abundant element is utilized because it is more readily available. In the case where the element is unavailable and no alternative element exists, the organism would simply be unable to exist as such or would have to develop a means for securing the needed element. In the Rule of Efficiency, when two or more elements are equally available, the more efficient one would be preferred. Once essentiality is established for a given element, an organism would be expected to try to make the best possible use of it, thus developing a system employing the element as efficiently as
possible. Thereby doing so, the organism would become specifically adapted to the use of that particular element so that an alternative, one that had the potential for being equally effective (basically capable) but that was not chosen because of its more limited availability, would no longer be an effective substitute for the end product of evolution. The whole process of an organism’s adaptation to (or its effort to make the possible best use of) a particular element is what is called the Rule of Evolutionary Adaptation (or the Evolution Pressure Effect Rule).

The important properties of metals in coordinating compounds can be divided into two groups based on their properties, either continuous or discrete. The continuous properties include reduction potential, Lewis acidity, electron transfer rate, and effective charge (of the central metal). The discrete properties include oxidation state, number of electrons, coordination number, shape of orbitals and spin state. It is beyond the scope of this thesis to further describe these properties.

A few of the most notable examples of the biologic function of essential metals would include the following: iron incorporation into hemoglobin, incorporation of copper into cytochrome c oxidase and the plasma protein ceruloplasmin, zinc incorporation in carbonic anhydrase, albumin, and α2-macroglobulin. There are many other known examples of metals that are essential to the proper function of enzyme proteins. In short, manganese,
cobalt, chromium, potassium and calcium are very important to normal biological function.

\section{Examples of metals known to alter the function of enzymes}

Unlike the essential metals, there are metals who are considered non-essential or toxic. These metals have no known physiologic function, instead they interfere with the normal function of many enzyme and membrane systems. The most common group of such toxic metals are the heavy metals. Three toxic elements of major concern in the environment are Cd, lead (Pb), and mercury (Hg).

The significance of low concentrations of toxic metals in foods is, at present unclear. While such concentrations are believed to be without effect in the usual sense, observations in experimental animals have suggested otherwise. In most cases the metabolism of certain essential metals is believed to be altered by these toxic elements. Therefore, the presence of toxic metals at low levels in human food might have biological implications if the essential nutrients with which they interfere are present at low or marginal amounts.

Most studies pertinent to this discussion have been done on experimental animals fed levels of the toxic elements that substantially exceed human exposure. Therefore, the findings must be interpreted with
caution if an attempt is made to apply these findings to humans. Even so, the findings show that toxic elements interact with essential nutrients and disrupt metabolic processes. They also show that specific nutrients have protective properties against specific toxic elements.

In the later part of the 1970's there were several very good reviews on this subject. Many of the nutritional factors that appear to influence the susceptibility of humans and animals to lead have been reviewed by Sanstead (1976), Pond (1975), Goyer and Rhyne (1973) and the National Research Council (1972). Reviews pertinent to Cd include those of Sandstead (1976), Parizek (1976), Pond (1975), Fox (1974, 1976), and Friberg et al (1971). The most comprehensive review on Hg is that of Friberg and Vostal (1972). Parizek (1976) has reviewed some of the more recent findings on interactions of Hg with other trace elements.

3. Non-specific ester hydrolases and the serum esterases

W. N. Aldridge, as part of the requirements for his Doctoral thesis, developed the quantitative methodology to differentiate the activity of two types of esterase that he had identified in serum. Aldridge was investigating the serum esterase hydrolysis of diethyl p-nitrophenyl phosphate (paraoxon, an organophosphate pesticide) when he found that serum contained two types esterase (A- and B-esterase) that hydrolyze p-nitrophenyl acetate, propionate, and butyrate. The two are differentiated by paraoxon inhibition, B-esterase is
inhibited by paraoxon (10 nM). The activity of serum is measured for each of the three p-nitrophenyl substrates, and then repeated after the addition of paraoxon. The difference between the two measurements determines the B-esterase contribution to total activity of esterase in serum. This method is the foundation for the most widely used classification and nomenclature scheme, of the three that are accepted for use in the literature.

Today, this method is still applied to differentiation of serum esterase activity into the A- and B-esterase components. The real significance of this work is the influence it has had on establishing a system of classification, and nomenclature of hydrolase enzymes, based on specific substrates and inhibitors. Interestingly, this is currently accepted to be the “preferred” system used in reporting information on hydrolase research in the scientific literature. Aldridge’s contribution to esterase research if measured only by the application of this method in the investigation and classification of hydrolase, was significant.

It should be noted that the development of this method combined with the application to the characterization of A- and B-esterase in serum, were significant contributions that were not appreciated for several years. Identification of an enzyme in serum that hydrolyzed the organophosphate paraoxon was the primary focus in Aldridge’s research. Characterization of A- and B-esterase in rat, rabbit and horse serum with this method and identification of a serum esterase that hydrolyzes paraoxon is described by Page 40.
Aldridge in two eloquent reports published in 1953 (Biochemical Journal, vol. 53).

Hydrolases are a unique group of enzymes that are ubiquitously distributed throughout the body, yet their physiological roles are not understood, with the exception of acetylcholinesterase (AChE).

Table 2 Six chemical reactions for classification and nomenclature of biochemical enzymes.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Type of reaction catalyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Oxidoreductases</td>
<td>Oxidation-reduction reactions</td>
</tr>
<tr>
<td>2. Transferases</td>
<td>Transfer of functional groups</td>
</tr>
<tr>
<td>3. Hydrolases</td>
<td>Hydrolysis reaction</td>
</tr>
<tr>
<td>4. Lyases</td>
<td>Group elimination to form double bonds</td>
</tr>
<tr>
<td>5. Isomerases</td>
<td>Isomerization</td>
</tr>
<tr>
<td>6. Ligases</td>
<td>Bond formation coupled with ATP hydrolysis</td>
</tr>
</tbody>
</table>

Acetylcholinesterase is a choline esterase found in the nerve endings and striated muscle. The function of AChE is to terminate the action of the neurotransmitter acetylcholine, by enzymatic hydrolysis of the ester bond. However, a physiological role has not been determined for the AChE in red blood cells. Serum cholinesterase enzymes are generally not considered to be
part of the non-specific hydrolase group, and will not be of further interest in this investigation [Quon, 1985; Aldridge, 1954; La Du, 1971].

The importance of establishing a single set of guidelines, accepted for the classification and nomenclature of enzymes is demonstrated by the failure to do so in hydrolase research. The basic scheme for the systematic functional classification and nomenclature of enzymes, adopted by the International Union of Biochemistry (IUB) established a set of guidelines based on chemical reaction. Enzymes are classified and named according to the nature of the chemical reaction they catalyze. There are six classes of reactions, listed in Table 3, that form the framework of this system. The first step in the nomenclature of enzymes, is placement into one of the 6 groups.

Presenting the basic biochemistry detail, is helpful in introducing and understanding the esterases and the confusion caused by inconsistent nomenclature. At this time three systems of nomenclature are accepted for use in reporting research that deals with esterases. The scientific literature reflects the confusion that has developed in esterase research. Failure to accept a system of classification and nomenclature has allowed inaccuracy in the identity of these enzymes, and has ultimately held up progress in hydrolase research. While agreement on a single system has yet to be reached, three systems have gained common acceptance in literature citations and are described in Table 4.
Table 3 The three systems of classification and nomenclature that are most commonly accepted for use in the literature description of hydrolase enzymes.

1. Aldridge (1953) classification based on esterase interaction with organophosphate (OP)

<table>
<thead>
<tr>
<th>A</th>
<th>Esterases hydrolyse OP</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>Esterases are inhibited by OP</td>
</tr>
<tr>
<td>C</td>
<td>Esterases do not interact with OP (group added later)</td>
</tr>
</tbody>
</table>

2. Augustinsson (1961) substrate and inhibitor specificity

<table>
<thead>
<tr>
<th>Esterase</th>
<th>Model Substrate</th>
<th>“Specific Inhibitor”</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholinesterase</td>
<td>Acetylcholine (Ac)</td>
<td>TADP* /High [Ac]</td>
</tr>
<tr>
<td>Cholinesterase</td>
<td>Benzoylcholine</td>
<td>DPP**</td>
</tr>
<tr>
<td>Carboxylesterase</td>
<td>1-Naphthyl acetate</td>
<td>NDGA***</td>
</tr>
<tr>
<td>Arylesterase</td>
<td>Phenylacetate</td>
<td>Metals (Hg, La**)</td>
</tr>
</tbody>
</table>

*TADP bis-(3-trimethyl-ammonium-5-hydroxyphenoxy)-1,3 propane (Williams 1985).  
**DPP 10-(1-dimethylamino propionyl) phenothiazine (Williams 1985).  
***NDGA Nordihydroguaiaretic acid [Sato, 1987].

3. International Enzyme Commission (EC) classification of major esterases:

<table>
<thead>
<tr>
<th>EC No.</th>
<th>Systematic name</th>
<th>Trivial name</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1.1.1</td>
<td>Carboxylic ester hydrolase</td>
<td>Carboxylesterase</td>
</tr>
<tr>
<td>3.1.1.2</td>
<td>Aryl ester hydrolase</td>
<td>Arylesterase</td>
</tr>
<tr>
<td>3.1.1.3</td>
<td>Glycerol-ester hydrolase</td>
<td>Lipase</td>
</tr>
<tr>
<td>3.1.1.6</td>
<td>Acetic ester hydrolase</td>
<td>Acetylenzyme</td>
</tr>
<tr>
<td>3.1.1.7</td>
<td>Acetylcholine acetyl hydrolase</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td></td>
<td>(formerly true cholinesterase)</td>
<td></td>
</tr>
<tr>
<td>3.1.1.8</td>
<td>Acylcholine acyl hydrolase</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td></td>
<td>(formerly true pseudocholinesterase)</td>
<td></td>
</tr>
</tbody>
</table>
a) **Tissue distribution of hydrolase enzymes**

Publications reporting the hydrolase characterization based on the use of specific substrates and inhibitors continue to add ambiguous information, primarily by overlooking the ubiquitous nature of these proteins (La Du and Snady 1971; Walker and Mackness 1983; Williams 1985). In Tables 5 & 6, the distribution of esterase activity in rat tissue is presented, using methyl butyrate and paraoxon hydrolysis, and hydrolysis of fluazifop-butyl, respectively (Williams 1985; Tsujita, Miyada et al. 1988). Broad classification

<table>
<thead>
<tr>
<th>Tissue Source</th>
<th>Esterase Activity (µmol/mg/min)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>0.03</td>
</tr>
<tr>
<td>Lung</td>
<td>0.79</td>
</tr>
<tr>
<td>Heart</td>
<td>0.07</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.17</td>
</tr>
<tr>
<td>Intestine</td>
<td>2.54</td>
</tr>
<tr>
<td>Liver</td>
<td>3.29</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.34</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.02</td>
</tr>
<tr>
<td>Testis</td>
<td>1.32</td>
</tr>
<tr>
<td>Adipose</td>
<td>3.02</td>
</tr>
<tr>
<td>Serum</td>
<td>0.14</td>
</tr>
</tbody>
</table>
of hydrolase enzymes, using trivial names like carboxylesterase or arylerase, is problematic and will be avoided as much as possible.

The systems used for nomenclature have been addressed, but will take time to correct. Application of molecular techniques have already had an impact. In a recent report, the primary structure of rat liver "carboxylesterase" was determined by cloning (Alexson, Finlay et al. 1994). The cDNA was found to encode the entire serum enzyme protein, leading to the identification of four alloenzymic forms that were differentiated by their primary structure (Alexson, Finlay et al. 1994). One of the most significant conclusions of this study was the identification of a protein in the serum corresponding to cDNA clone from the liver, demonstrating the role of liver production and secretion of a serum esterase (Alexson, Finlay et al. 1994). This type of work will help correct conflicts by providing individual sequencing to each enzyme protein.
The hydrolase enzymes have an important role in the metabolism of several therapeutic and xenobiotic compounds in man. Several drugs are hydrolyzed to less active compounds by the esterases, aspirin being the best known example, others include: succinylcholine, procaine, atropine, meperidine, acetylated derivatives of morphine, and clofibrate (La Du and Snady 1971; Heymann 1982). Hydrolysis by the esterases is also exploited therapeutically to enhance the effectiveness of compounds referred to as prodrugs. Enhanced absorption following oral administration is the most common benefit of a prodrug. The antihypertensive drug enalapril is an example of a prodrug that is activated by esterase hydrolysis, after absorption

Table 5 Comparison of the kinetic parameters of hydrolyses of the two substrates paraoxon (POX) and fluazifop-butyl (FZB). Activities determined for esterases from various tissues of the Wistar rat (data compiled from Williams, 1985).

<table>
<thead>
<tr>
<th></th>
<th>$V_{max}$ ($\mu$mol-min$^{-1}$gm$^{-1}$ml$^{-1}$)</th>
<th>$K_m$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POX</td>
<td>FZB</td>
</tr>
<tr>
<td>Microsome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.33</td>
<td>5.13</td>
</tr>
<tr>
<td>Lung</td>
<td>0.002</td>
<td>0.38</td>
</tr>
<tr>
<td>Skin</td>
<td>NA*</td>
<td>0.02</td>
</tr>
<tr>
<td>Cytosol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>NA</td>
<td>5.0</td>
</tr>
<tr>
<td>Lung</td>
<td>NA</td>
<td>1.5</td>
</tr>
<tr>
<td>Skin</td>
<td>NA</td>
<td>0.4</td>
</tr>
<tr>
<td>Blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>0.25</td>
<td>2.49</td>
</tr>
<tr>
<td>Erythrocyte</td>
<td>NA</td>
<td>0.032</td>
</tr>
</tbody>
</table>

*NA - no measurable activity.
of an oral dose (Heymann 1982). Extensive reviews of the ester prodrugs are available but there have been few investigations of the biochemical nature of the esterases involved and the importance of genetic variations (Sinkula and Yalkowsky 1975; Notari 1981). Genetic variations are known to alter the pharmacokinetics of many therapeutic compounds (Nebert and Weber 1990). Individual variation in esterase activity can be from genetic or environmental factors, or a combination of the two. Pharmacological significance of this variation can have beneficial as well as adverse or toxic effects for several ester drugs or xenobiotics.

Esterase is a term that will be used to denote a group of the hydrolase enzymes. It is outside the scope of this research to fully characterize the biochemical nature. Investigation of the esterase activity in serum is a reflection of the easy access to serum and plasma from whole blood, the single most exploited source of biological indicators investigated.

C. Methylphenidate

1. Methylphenidate pharmacology

   a) Introduction and summary of therapeutic applications

   Methylphenidate (MPH), is a piperidine-derivative stimulant (shown in Figure 1), predominantly metabolized by esterase hydrolysis in humans (Bartlett and Egger 1972; Faraj and al. 1974; Srinivas, Quinn et al. 1987). Rapid hydrolysis of MPH to ritalinic acid (RA, α-phenyl-2-piperidine acetic acid...
acid) has been demonstrated in many species, including the rat (Faraj and al. 1974; Srinivas, Quinn et al. 1987). Metabolism of MPH, however is not limited to direct hydrolysis to RA. Oxidation and conjugation processes in the rat account for 60% of the metabolites formed from MPH. In man, 90% of the dose is excreted in the urine as either RA or the lactam acid seen in Figure 2 (Egger, Bartlett et al. 1981). Urinary excretion of RA accounts for about 75% of an oral dose of MPH (Bartlett and Egger 1972; Faraj and al. 1974). Minor metabolic pathways produce oxidated, parahydroxylated and glucuronidated products of MPH and RA (Bartlett and Egger 1972; Faraj and al. 1974). Bartlett and Egger (1972) indicate that MPH is more susceptible to hydrolysis of the ester bond, than to direct conjugation in man, continuing that the carboxylic acid (ritalinic acid) produced is more favorably conjugated than the ester parent compound (Bartlett and Egger 1972).
Methylphenidate Metabolism

Main Human Pathway

Figure 2. Human pathway of methylphenidate metabolism. Initially hydrolyzed to ritalinic acid, then oxidized to the lactam acid. Together accounting for ~90% of the urinary metabolites in humans (Bartlett, 1972).

b) Mechanism of action

(1) Pharmacokinetic (PK) profile

Human plasma levels reach their peak concentration 1-3 hr after oral dosing (Faraj and al. 1974). From the urinary excretion profiles it has been determined that the drug is completely absorbed from the lumen after an oral dose. The events preceding the initial passage out of the liver, in orally administered compounds, are combined to create the first pass effect. For MPH, the first pass effect of metabolism is significant. Pharmacokinetic investigations report absolute bioavailability to be 0.31 in children; this is an indication of extensive pre-systemic metabolism [Srinivas, 1991; Srinivas,
1992]. The hydrolysis of >70% of the total dose is believed to occur extra-hepatically [Srinivas, 1992; Isaacson, 1991]. The pre-systemic inactivation is a dramatic portrayal of the presence and function of the non-specific hydrolases. In man the lactam acid and p-hydroxylated MPH are minor metabolites, in contrast to the rat where the opposite is true (Faraj and al. 1974).

The plasma half-life of an oral dose is 2-7 hrs, and 1-2 hrs for an IV dose (Faraj and al. 1974). In children, the plasma clearance is reported to range from 3.1 to 8.5 L/kg/hr (Hungund, Perel et al. 1979). At 2 hrs following 20-100 mg oral doses, plasma levels were 0.02 mg/L, with time (t) to peak level (C_{max}) ranging from 1 to 3 hrs (Faraj and al. 1974). The volume of distribution (V_d) in children appears to vary from 1.1 to 3.1 L/kg (Hungund, Perel et al. 1979).

The extent of distribution of methylphenidate in the human body is now known. Protein binding is below 15%, allowing the majority of the unmetabolized parent compound to be available for penetration into the central nervous system (Hungund, Perel et al. 1979). The brain:plasma ratio of MPH is reported to be ~8 at 30 min after an oral dose and is maintained as the brain amount closely follows the plasma level changes brought about by metabolism and renal clearance [Segal, 1976].
(2) **Pharmacodynamic (PD) profile**

The mechanism of action of methylphenidate is complex. As a stimulant it has many similar pharmacological properties as the amphetamines, with predominantly central activity and minimal cardiovascular effects. Methylphenidate is thought to activate brainstem arousal system and cortex to produce its stimulant effect (Gilman, Rall et al. 1990; Olin 1991). Literature reports on the correlation of serum levels and clinical response are variable. Sprague & Slater (1977) reported differing response curves for cognition functioning and social behavior. The report of peak enhancement of learning followed the administration of 0.3 mg/kg and a decrement in learning at larger doses (Sprague and Slater 1977). Winsberg et al. (1982) found increased improvement in social behavior to be a function of methylphenidate dose. And indicated finding no correlation for dose and plasma levels with cognitive behavior at doses comparable to those used by Sprague and Slater. Shaywitz et al. (1982) reports a positive correlation between methylphenidate serum levels and behavioral response.

There are variable reports in the literature on the existence of a correlation between methylphenidate dose and serum levels with clinical response. Sprague & Slater (1977) saw differing response curves for cognitive functioning and social behavior. Sprague & Slater (1977) found a positive correlation between methylphenidate dose and social behavior with the greatest improvement at 1 mg/kg, the highest dose tested in the study.
Similarly, Winsberg et al. (1982) found increased improvement in social behavior to be a function of methylphenidate dose. Shaywitz et al. (1982) saw a positive correlation between methylphenidate serum levels and behavioral response. Sprague & Slater (1977) found peak enhancement of learning after the administration of 0.3 mg/kg and a decrement in learning at larger doses. Winsberg et al. (1982) saw no correlation for dose and plasma levels with cognitive behavior at doses comparable to those used by Sprague & Slater. Sebrechts et al. (1986) found a positive relationship between dose and school related performance at their highest dose of 0.6 mg/kg. Kupietz et al. (1982) in a small population study, saw a positive relationship between methylphenidate plasma levels and learning performance.

Gualtieri et al. (1984) in a study using the largest sample size among those cited, found no correlation between methylphenidate blood levels and attention or activity. Gualtieri et al. (1984) go on to provide several possible explanations for a lack of correlation. The first hypothesis for the lack of association may lie in a selective affinity of methylphenidate for different tissues. In rat brain, levels of methylphenidate peak earlier and at a higher concentration than serum levels. A second explanation may be the lack of stereochemical specificity of the present methylphenidate assay. Enzyme hydrolysis of esters structurally similar to methylphenidate exhibits extreme stereospecificity. The original racemic mixture may be enriched in favor of a stereoisomer as a consequence of selective enzymatic degradation. The
relative potency of the $d$- and $l$-entantimers has not been examined. Third, methylphenidate response may be a function of neuron sensitivity to the drug, rather than to drug level per se. This could be a function of individual differences or biological subgroups of hyperactive children. Sebrechts et al. (1986) and Winsberg et al. (1982) find a high correlation between methylphenidate concentrations and oral dose. Kupietz et al. (1982) in a very small sample saw no significant correlation between oral dose and methylphenidate plasma concentrations. Peak plasma levels of 11.2 ng/mL were seen 2.5 +/- 0.65 hours after 0.34 mg/kg and 20.2 +/- 9.1 ng/mL at 1.9 +/- 0.82 hours following 0.65 mg/kg (Shaywitz et al., 1982). Swanson et al. (1978) saw maximal effects on performance in a laboratory learning task at 1 to 2 hours post dose. Food does not affect the absorption of methylphenidate when ingested after MPH dosing (Gualtieri et al., 1984). Tolerance does not seem to be a common event in long-term treatment of hyperactive children, given that there is little mention in the literature. There is no apparent decline in methylphenidate blood levels over time (Gualtieri et al., 1984).

Swanson et al. (1978) notes that 30% of children having symptoms of hyperactivity will have impairment of cognitive functioning with methylphenidate. Too much medication in methylphenidate responders had a similar adverse effect [Gualtieri, 1984]. Gualtieri et al. (1984) saw no difference between serum levels of responders and non-responders. Winsberg et al. (1982) noted a higher incidence of side effects with higher
methylphenidate doses. Side effects included gastrointestinal symptoms, anorexia, and insomnia. Sprague & Slater (1977) in monitoring tachycardia found little or no increase in heart rate at low dose but a substantial increase with high dose. Gualtieri et al. (1984) saw no correlation between blood levels and side effects. However, acute side effects such as anorexia, headache, insomnia, and irritability are readily apparent and best treated by a lowering of dose, irrespective of blood levels.

There are no conclusive data on the existence of a correlation between methylphenidate blood levels and clinical response. Most studies have been performed on small numbers of subjects within a cohort group. In addition, tests used for measuring clinical response varied between studies. It is questionable whether any of these varying conclusions can be generalized to address the entire patient population being treated with methylphenidate for attention deficit disorder. The clinical picture of methylphenidate response is complicated by more than one group of target symptoms. Studies suggest that cognitive functioning and social behavior may respond best at different doses [Gualtieri, 1984; McNamara, 1993]. It may be necessary to balance improvement in learning performance against less than optimal social behavior. Methylphenidate serum levels cannot be used to delineate between responders and non-responders. Optimal clinical response to methylphenidate occurs after absorption near peak blood levels even in initial dosing [McNamara, 1993; Solanto, 1982]. The ideal dose for the individual patient
can be titrated from clinical response in a short length of time by clinicians and parents. Most studies indicate a positive correlation between plasma concentration and oral dose. Consequently, plasma concentration of methylphenidate provide little additional information in guiding clinical management of hyperactive children.

Additional response studies have shown that methylphenidate inhibits hepatic drug metabolizing enzymes (Garrettson, 1969) and elevates blood levels of tricyclic antidepressants by inhibiting their hepatic metabolism (Wharton, 1971; Perel, 1969). The clinical significance of this interaction, however, appears to vary from patient to patient. Zeidenberg et al. (1971) have shown that methylphenidate-induced increases in tricyclic antidepressant blood levels occur in some humans but not others, suggesting individual variation of drug metabolizing enzymes to methylphenidate effects. Wharton et al. (1971) reported improved clinical responses to imipramine in several patients when methylphenidate was added to the regimen. This is an interaction between two drugs to cause an adverse reaction. Concurrent therapy with one or more compounds is the most common cause of adverse pharmacologic reactions (Hansten and Horn 1991; Rizack 1991).

The conclusion was that methylphenidate appears to elevate blood levels of tricyclic antidepressants in some patients. Patients receiving the combination should be observed for evidence of toxicity from the
antidepressant due to inhibition of hepatic metabolism by methylphenidate. However there is evidence that improved antidepressant responses may occur with combined therapy [Hensten, 1991; Rizack, 1991].

It has already been stated that the incidence of toxicity for MPH is relatively low, information on fatalities with therapeutic was only available for cases of concurrent tricyclic antidepressant interactions presented above [Hensten, 1991]. Neither the Merck Index (11th Ed.) or the Compound’s Information Insert, attached to the bottle of tablets, listed a LD50 for humans. According to the information in the Drug Monographs (Micro Medex, 1995) for methylphenidate, a toxic blood level has also not been determined for humans.

Nervousness, insomnia, hypersensitivity, anorexia, nausea, dizziness, headache and dyskinesia are listed as side effects. Indications were also made for toxicities resulting in psychosis, leukopenia and/or anemia, transient depressed mood, cerebral arteritis and/or occlusion, as the severe toxicities caused by MPH. The advantage that has most likely lead to this is that there is a required parental involvement in the administration of dose, which helps to prevent accidental overdosing.

Addressed in recent clinical studies in hyperkinetic children are the growing concern over potential MPH impairment of learning [Rebec, 1978; Gualtieri, 1982; McNamara, 1993]. At doses up to 0.3 mg/kg, it was reported that performance at various learning tasks improved in a dose
related linear relationship (Gualtieri, Wargin et al. 1982). For the doses above 0.3 mg/kg a decline in performance was recorded. However, the effect on behavior was linear in the sense that higher doses (above 0.3 mg/kg) produced behavior that was “improved” in a linear correlation according to teacher or parent observations (Gualtieri, Wargin et al. 1982). Observations of the intellectual performance at levels above 0.3 mg/kg, is reported by the investigators to be: “sedating” [Gualtieri, 1982; Wargin, 1983; Wetzel, 1981].

2. Comparison to other stimulants

The behavioral effects of MPH are similar to other stimulants. The similarity to amphetamine (AMP) has been reported extensively, and is useful in understanding other compounds and determining their mechanism of action (Cooper, Bloom et al. 1991). The mechanisms and pharmacologic activity have been well established for MPH and AMP [Wargin, 1983; Patrick, 1981; Faraj, 1974; Iverson, 1981; Cooper, 1991]. Agreement on the respective mechanisms and the extent of the dose response relationship in several different labs, is presented here to indicate our grasp of the information and in support for the selection of a behavioral measurement, locomotor activity, as a pharmacodynamic index.

Both AMP and MPH produce a dose dependent sequence of behavioral effects that have been characterized in rats. The increasing dose has an increase in motor activity that is a linear response of stimulation. The increase in dose leads to a change in the motor activity response, to a
multiphasic response pattern [Hungund, 1979; Wargin, 1983]. Initially, linear motor activity, gives way to a phase of focused repetitive behaviors (stereotypies), during which forward locomotion and rearing are absent and post-stereotypy phase of prolonged motor hyperactivity [Segal, 1974]. These effects are well characterized and demonstrated in man and many other animal species (rat, dog, and various primates) [Solanto, 1982; McNamara, 1993].

Methylphenidate and amphetamine act centrally, increasing dopamine (DA) levels outside the nerve terminal, thus enhancing neurotransmission (Cooper, Bloom et al. 1991). Few differences exist between the pharmacodynamics of MPH and AMP, although they possess slightly different mechanisms and more extensive differences in their pharmacokinetics (Volkow, Ding et al. 1995). Amphetamine is an indirect acting sympathomimetic amine in the peripheral adrenergic systems. This means the activity “mimics” the effects of norepinephrine by displacing it from the peripheral adrenergic terminals. Amphetamine is not believed to possess any direct activity in terms of the adrenergic receptor [Cooper, 1991; Goodman, 1990].

Methylphenidate has no similarity to the peripheral effects described for AMP. Entry into the central tissue, or brain, is required for MPH activity. Separation of the brain from the entire rest of the body is accomplished by tight junctions between the cells lining the circulatory vessels. Polar
compounds are prevented from passive diffusion across this boundary. However, MPH is absorbed as the free base, which is non-polar and freely passes into the brain tissue where it actually becomes trapped at a concentration more than 8-fold, from the circulating serum concentration [Patrick, 1984].

Amphetamine is similar, in its entry into the brain. Possessing a larger pKₐ than MPH, AMP is a stronger base, and nearly completely enters the brain after a single pass (Dougan, Wade et al. 1987). Once in the brain, MPH and AMP cause an increase in the DA level in the synaptic cleft of the nerve terminal. Methylphenidate has been shown to be an inhibitor of the DA re-uptake pump, on the presynaptic side of the terminal, where AMP displaces DA from the reserpine sensitive storage pool in addition to possibly interacting with the re-uptake [Goodman, 1990; Cooper, 1991].

The pharmacology of MPH's effects on locomotor activity have been firmly established [Wargin, 1983; Solanto, 1982; Patrick, 1987]. Additionally, there have been many comparisons between AMP and MPH, exploring the similarities of the two compounds [Solanto, 1982; Rebec, 1978; Schweri, 1982; Iversen, 1981]. The completeness of the characterization of MPH in regards to AMP, is useful in establishing characterization studies of new compounds. This is done in vitro with binding studies and in vivo with co-administrations measuring effect in dose response studies.
Complete characterization of MPH, and the comparison to AMP has also been presented to support the use of locomotor measurement in this project as a pharmacodynamic measurement of MPH activity. The use of behavior in psychopharmacology has been extensively reviewed, in order to insure appropriate understanding and application of methods is observed. There is a large body of reference information available on the use of behavioral measurements in research, which has been distilled into a summary of material that relates to this project.

III. Experimental Design and Results

A. Complete Method Detail

1. Non-Specific Serum Hydrolase

   *In Vitro Studies* - Blood is drawn from halothane anesthetized Sprague Dawley rats (male, 200-400 gm) by cardiac puncture. Whole blood is allowed to clot for 15-20 minutes on ice, then separated by centrifugation at 10,000 RPM (10 min at 4°C). Aliquots of 0.1-1.0 mL of serum are placed in labeled tubes and stored at -20°C until thawed for use. Serum is diluted 10-fold in 155 mM NaCl, 20 mM CaCl₂, pH 7.5 buffer before being used experimentally in incubations or prior to being assayed for hydrolase activity.

2. Circulatory (Serum) Enzymes

   *In Vivo Studies* - Whole blood (≤0.5 mL) is drawn from the tail vein of Sprague-Dawley rats (male, 200-400 gm), anesthetized with
halothane. Following the 0 hr blood draw the rats were injected with 2
mg CdCl₂/kg, i.p., (in 0.9% saline). Serum, prepared from whole blood,
as described above, is stored at -20°C. Successive blood draws (≤0.5
mL) are performed at 24 hr intervals (from 0 to 96 hr. after exposure to
CdCl₂). Serum samples are stored frozen at -20°C until the completion
of the study when all samples collected, will be run as a single group,
comparing the effects of Cd on the hydrolase activity over time to the
control (pretreatment) activity.

3. Tissue Fractions

Liver and GI- Microsomal, cytosolic and S9 fractions are
prepared from liver and GI (small intestine) tissue harvested at serial
time points following exposure to Cd or saline vehicle. A group of 3
untreated normal controls will be sacrificed and serum, and tissue
hydrolase activity will establish the baseline (100%) control activity.
Thereafter, control refers to the saline vehicle treated rats (n=3), and
Cd exposed refers to single dose, 2 mg CdCl₂/kg, i.p. group (n=3).

Rats are sacrificed by decapitation and blood sample is collected
into a funnel placed in a glass tube on ice. The liver and entire length
of small intestine are removed and placed in separate beakers of cold
saline (0.9% NaCl, pH 7.5) which are maintained on ice for the duration
of the procedure.
GI tissue refers to the length of small intestine that extends from the proximal end of the duodenum to within 0.5 cm of the distal end of the ileum. The GI tissue is attached to a piece of tubing on a Luer-Lok needle, which can be attached to a syringe. The contents are flushed out with cold saline from the syringe, gently massaging the tissue by hand helps to prevent complete occlusion. Once the bulk of the intestinal content has been cleared the tissue is transferred to another beaker on ice, with fresh saline. Use of a peristaltic pump facilitates the continued rinsing while next tissue sample is being removed and processed to this point. A total of 30-40 mL of saline is sufficient for the second luminal rinse. Tissue is then blotted with paper towel and then carefully inverted to expose the GI luminal mucosae, then blotted with paper towel and weighed. Using a glass microscope slide the intestinal mucosae are scraped from the GI tissue into a cover dish containing 1 volume of 50 mM TRIS-HCl (pH 7.5) buffer with 250 mM sucrose and 12% glycerol (1 vol. is mL equal to the total weight of tissue in gm). Mucosae suspension is then homogenized in a glass tube with a motor driven Teflon pestle, making 5 complete passes.

Liver tissue homogenate is similarly prepared as follows: Whole liver (3 lobes) is removed and placed in a beaker of saline on ice. Fat, connective tissue and any vessels are trimmed from tissue, and it is rinsed of any blood with saline, alternatively a 1.15% KCl buffer
perfusion can be done with a syringe. Liver is then blotted, weighed and then placed in the mL volume equal to 4 times the gm weight of the tissue (25% prep). Scissors are used to mince the tissue and then it is homogenized in a glass tube using a motor driven Teflon pestle.

Both GI and liver homogenates are processed in the same manner from this step forward. Homogenate is centrifuged in a Sorvall RC2-B centrifuge at 9000 x g for 20 min (4°C), separating a pellet of the unbroken cells, nuclei, mitochondria, erythrocytes and large fragments from the supernatant (or S9 fraction), containing the soluble fractions and the microsomes. Supernatant is decanted from the pellet into balanced ultracentrifuge tubes and centrifuged in a Beckman L5-50 at 105,000 x g for 60 min (4°C) to sediment the microsomes into a pellet. The 105,000 x g supernatant is the cytosolic fraction which is decanted off the microsomal pellet and saved. Microsomes are carefully rinsed with cold buffer, then suspended in 2-3 mL of cold buffer and re-homogenized in the glass tube with the motor driven Teflon pestle.

The serum, S9, cytosol, and microsomes are divided into 4-5 aliquots, separating a 1 mL sample for measuring hydrolytic activity and storing rest of them at -20°C. At the end of the study an aliquot of each sample is thawed, and it's hydrolytic activity is assayed. This method of running all the samples in a single “group” prevents any potential day to day assay variability from distorting the data. Three
rats are treated with Cd, and three rats receive saline (as a vehicle control group) for each time point from 24-96 hrs. An additional three untreated rats have been used to represent a 0 hr control for both groups to use as a baseline of 100% normal activity.

For each rat there are several samples collected. These include a serum sample, a GI S9, cytosol, and microsomal fraction, and a liver S9, cytosol and microsomal fraction. Since the S9 fraction is composed of the cytosol and microsomes, there should be a consistent influence from each, and therefore will not be reported. However, if variation is found in the composite activity, it will be investigated and the results presented.

4. **Enzyme Activity Determinations**

**Hydrolase Activity** Enzyme activity is determined kinetically, using a modification of the method of Junge & Klees (1984). Briefly, to begin the reaction one volume (typically 1 mL) of 100-fold diluted serum is added to one volume of substrate (8.08 mM phenylacetate solution, aq), and the entire mixture is transferred to a quartz cuvette placed in the spectrophotometer. The production of phenol is followed at 270 nm for 1-3 minutes. Esterase activity was calculated using the molar extinction coefficient of 1.48 x mol⁻¹ x mm⁻¹ for phenol at 270 nm and expressed in International enzyme Units (IU, is the amount of esterase activity that will convert 1 mmol substrate per minute under the conditions described). A 1/10 dilution of serum is prepared in 150 mM
NaCl, 20 mM CaCl₂, pH 7.5 buffer. For the in vitro incubations the buffer contained 0-100 mg CdCl₂/mL at pH 7.6. Further dilution of the 1/10 serum is made in 100 mM Tris-HCl buffer, pH 7.5, to 1/100 for incubation and hydrolase activity determination. The esterase activity calculated from the assay is protein normalized and expressed in kU/g serum protein. Esterase activity of serum drawn prior to metal treatment is used as the control value (100%) for each animal in order to directly compare the relative changes in activity over time of all animals.

**Glutamate pyruvate transaminase (GPT).** GPT activity is determined based on the “optimized standard method” recommended by the German Society for Clinical Chemistry (Kachmar 1976), using a kit from Sigma Chemical Co. (St. Louis, MO). The procedure is carried out at 25°C and described briefly as follows: Two reagents (reagent A & B) are provided in kit form to be reconstituted with deionized water. Reagent A is 104 mmol/L phosphate buffer pH 7.4 containing 1040 mmol/L L-alanine, 0.234 mmol/L NADH and 1560 U/L LDH. Reagent B is the starter reagent containing 234 mmol/L 2-oxoglutarate. While the reagents are equilibrating to 25°C the spectrophotometer is zeroed using a water blank at 340 nm. Mix 2.5 mL reagent A with 0.5 mL sample and allow 1 min. for equilibration at 25°C. To begin the reaction add 0.25 mL reagent B and mix, again allow 1 min. for
equilibration at 25°C. Record the change in absorbance versus a water reference for up to 5 min. (1-3 min. is adequate). The GPT activity is determined by multiplying the mean absorbance change per min. by 1045* to get U/L.

*1045 = (Total vol*1000)/(6.22 mmol absorptivity NADH*1.0 cm Path length*Sample volume)

Glucose-6-Phosphate Dehydrogenase (G-6-PDH) G-6-PDH activity is determined based on the method described by Kornberg and Horecker (1955) (Kachmar 1976), using a kit from Sigma Chemical Co. (St. Louis, MO). The procedure is carried out at 30°C and described briefly as follows: Two Solutions (Assay Reagent & Substrate Solution) are provided in kit form to be reconstituted with deionized water. The Assay Reagent is 100 mmol/L phosphate buffer pH 7.4 containing 12 mmol/L Maleimide and 15 mmol/L NADP. The Substrate Solution is the starter reagent, 1.05 mmol/L Glucose-6-Phosphate, in phosphate buffer with magnesium. While the reagents are equilibrating to 30°C the spectrophotometer is zeroed using a water blank at 340 nm. Mix 1.0 mL Assay Solution with 0.01 mL sample and allow 5-10 min. for equilibration at room temperature. To begin the reaction add 2.0 mL Substrate Solution and mix, and incubate for 5 min. at 30°C. Record the change in absorbance versus a water reference for up to 5 min. (1-3 min. is adequate). The G-6-PDH activity is determined using the change in absorbance at 340 nm. The procedure is standardized on the
basis of the millimolar absorptivity of NADPH, which is 6.22 at 340 nm. The activity is expressed in terms of U/# of RBC's or U/gm Hb (gm/dL).

**Protein measurements**  Serum and microsomal protein content was determined with the BioRad Protein assay kit using bovine serum albumin as a standard (Bradford 1976).

5. **Animal Procedures**

**Cadmium Exposure/Pretreatment of the Rats**  Rats are given i.p. doses of CdCl$_2$ in sterile 0.9% saline solutions. Stock solutions of 10 mg CdCl$_2$/mL are prepared and 1 mg/mL dilution's are made for injection on the day of use. A stock CdCl$_2$ solution is maintained for 1 month maximum shelf life and then is discarded.

**Methylphenidate Dosing of the Rats**  The methylphenidate-HCl (MPH) solution is prepared from 10 or 20 mg Ritalin® (CIBA-Geigy) tablets. Tablets are weighed and the amount of MPH per mg tablet is determined for each lot. The tablets are solubilized in sterile 0.9% saline. The solutions are centrifuged to pellet the insoluble material from the tablet matrix. Rats are pre-treated with a 1 mg/mL MPH aqueous solution substituted for the normal drinking water 10 days prior** to being used in an experiment. The experimental dosage given to the rats is 5-10 mg MPH by i.p. injection in 0.9% saline, on the day serum samples are drawn.
For chronic studies rats receive 1.0 mg MPH (i.p.) twice a day or 1 mg/mL in the drinking water for 1-4 weeks.

(**Predosing is done to improve the reproducibility of the serum MPH levels (Mathieu et al. 1989).**
Methylphenidate and Motor Activity in Rats  Locomotor activity is quantitated according to the method described by Hollister et al. (1974). A rat is placed in the counting chamber, a polypropylene tub (12"Wx18"Lx14"-depth), for a 60 min habituation period prior to the start of the experiment. Black lines drawn on the floor and walls of the counting chamber divided the area into 4 quadrants. One count was recorded when all 4 feet have crossed the plane of the line dividing the quadrants. Two subjects were run simultaneously, using separate chambers, and off-setting times to allow for observation. Experiments were conducted in dimly lit conditions, between the hours of 8:00 p.m. and 2:00 am. The control counts marked the end of the habituation period, with a 10 min count of baseline activity for each animal before MPH or saline treatment by i.p. injection. Counts were totaled every other 10 min for each animal, and presented as % change from the baseline counts/min. A video record of the activity is recorded for each experiment to review and compare total counts tallied during and after each experiment.

6. Spectrophotometric Determination of Cd

Sample Preparation and AA Determination (Skoog & West 1982) All glassware and equipment for this procedure must be acid-washed and carefully rinsed using with doubly distilled water. Liver tissue and blood components were harvested, and cut to desired weights before being stored at -20°C (as indicated by the data points in Figure
7) until the end of the experiment. A 4 gm piece of liver was homogenized in 3 volumes of ice cold 0.1 M Tris-HCl buffer solution, pH 7.4 containing 0.25 M glucose, using a Polytron Homogenizer. The homogenates were centrifuged at 170,000 g for 60 min at 4°C (Beckman LS-80, 50 Ti rotor), to produce a supernatant. Tissue supernatants are then diluted 10-fold with doubly distilled water.

Serum and RBC samples (1 mL) are digested with an equal volume of mixed acid, HClO₄:HNO₃ (1:5 v/v), followed by dilution to 5 mL with doubly distilled water. The Cd content is determined on a Perkin Elmer 603 atomic absorption spectrophotometer with a HGA Graphite Furnace and a single element cathode lamp using deuterium background correction. The Cd concentrations reported are calculated from standards prepared fresh for each instrumental session.

7. **Extraction of Methylphenidate and Metabolites**

**Serum and Urine Samples.** A liquid extraction of methylphenidate from serum is carried out with cyclohexane. And the major metabolite in plasma and urine, ritalinic acid (RA), is extracted with n-butanol. Extraction of RA is done separately before MPH. Adjust the sample pH ≤2.0 with 2 N HCl, and perform 2-3 extractions using 5-10 mL n-butanol and pool. The compound 2-amino-5-chlorobenzophenone (ACBP) is used as an internal standard. Internal standard is added (resulting conc.: 1-5 ng/mL) to each sample (0.3-0.5
mL) then the samples are made alkaline (pH ≥10) by the addition of 1 mL saturated sodium borate solution. Methylphenidate is extracted from alkaline solution with 6 mL cyclohexane. The layers are separated by centrifugation (5 min at 1000 x g), the top organic layer is removed and dried with anhydrous sodium sulfate. An aliquot of the dried organic layer is transferred to a clean tube and evaporated under a gentle stream of nitrogen at room temperature.

**Incubations with Plasma, RBC, and Whole Blood.**
Separation of MPH from major metabolites using solid phase extraction with Bond-Elut C18 columns was also employed for the *in vitro* metabolism studies. The columns were conditioned with one volume (2 mL) methanol then with one volume of borate buffer (pH ≥ 10). Samples were alkanized with one volume saturated borate solution prior to solid phase extraction. Treated samples were then applied to the column and rinsed with one volume of borate buffer. The MPH was then eluted from the column with 1 mL methanol, collected in a glass tube and dried under a gentle stream of nitrogen gas at room temperature.

**8. Chromatographic Assay of Methylphenidate and Major Metabolites.**

**HPLC Method** HPLC separation and analysis of samples was performed on a Hewlett Packard 1090 HPLC system using a Supelcosil
LC-ABZ column with UV detection at 200 nm and a flow rate of 1.5 mL/min. The HPLC assay conditions were as follows: The initial mixture of 80 mM phosphate buffer with 5% acetonitrile was maintained until 2 min. after sample injection. From 2-8 min. a linear gradient was used to increase the acetonitrile to 20%. These conditions were held until 11.5 min. and then returned to the initial values by the completion of the run (12 min.). The column was allowed to re-equilibrate for 3 min. prior to the next sample injection. Calculation of MPH and RA performed using a linear regression from a standard curve based on peak area.

**Sample Derivatization** Following organic extraction, described above, samples were dried under a stream of nitrogen gas and stored at -70°C. Samples were derivatized with heptafluorobutyric anhydride (HFBA) prior to GC analysis. Dried samples were reconstituted with 300 mL ethyl acetate, then dried with a stream of nitrogen to remove any traces of water from the sample before derivatization. A 10 fold dilution of HFBA in ethyl acetate is prepared for each set of samples for the derivatization procedure. Add 200 mL of the HFBA solution to each sample, cap and mix, then place in heating block set at 50°C, after 10 minutes remove samples and allow to cool. Dry samples under a stream of nitrogen gas at room temperature, then washed once with 300 mL of ethyl acetate, and dry to completeness under nitrogen. Each
derivatized sample is reconstituted in 300 mL of ethyl acetate and injected into the GC (1-2 mL) in triplicate.

Gas chromatography (GC) Method. Gas chromatography (GC) was performed on a Hewlett Packard (HP) Model 5890 Series II gas chromatograph equipped with a $^{68}$Ni electron-capture detector. The instrument was controlled by a computer using HP Series II ChemStation software for Microsoft® Windows. Separations were achieved on a 20 m x 0.25 mm ID, DB-5 capillary column (J&W Scientific). Analytical conditions used in the separation of the analytes were as follows: The column oven temperature (175°C) was held for 5 min. and thereafter increased at a rate of 5°C/min. to a final temperature of 260°C, which was held for 5 min. The injection port temperature was 280°C, the detector temperature 300°C, the argon-methane (carrier gas) flow-rate 1 mL/min, the argon-methane (make up gas) flow-rate 60 mL/min, the split vent flow-rate 28 mL/min, the septum vent flow-rate was 2 mL/min. Analyte quantitation was performed using a standard curve prepared with a ratio of MPH to internal standard peak areas.

9. Data Analysis

Statistical Analysis of the Data. A paired two-sample t test is used to analyze the in vivo Cd/esterase data, the statistical significance
is reported at $p \leq 0.05$. The selection of this test is based on the use of individual controls, and repeated measurements allow the data to be grouped as legitimate pairs. (Each animal serves as its own control, at 0 hr, before Cd exposure, the serum esterase activity is assayed and set to be the baseline or 100% of control.) Statistical tests are run using the raw data, unless otherwise indicated. Esterase data is transformed from enzyme Units to % change from control, for presentation.

**t-Test: Paired Two-Sample For Means analysis statistic**

Performs a paired two-sample Student's t-test to determine whether a sample's means are distinct. This t-test form does not assume that the variances of both populations are equal. You can use a paired test when there is a natural pairing of observations in the samples, such as when a sample group is tested twice — before and after an experiment.

The treatment of the *in vitro* Cd or Pb/esterase data is similar to the *in vivo*, in the presentation of transformed data. However, larger sample groups can be used and benefit from an analysis of variance, using ANOVA rather than t tests. Statistical significance is reported at $p \leq 0.05$, if the computed F ratio exceeds the critical F ratio, unless otherwise indicated.

**ANOVA: Single-Factor analysis statistic**

Performs simple analysis of variance (ANOVA) to test the hypothesis that means from two or more samples are equal (drawn from
populations with the same mean). This technique expands on the tests for two means, such as the t-test.

**B. Effects of Cadmium on hydrolase kinetics**

1. *Hydrolysis of phenyl acetate*

Initial results demonstrated the existence of Cd induced loss of non-specific serum hydrolase activity both *in vitro* and *in vivo*. From this initial finding we proceeded to characterize the effects of Cd exposure on the activity of non-specific hydrolase enzymes in the rat. *Figure 3* presents an example of the "direct" effects of Cd on the serum esterases that were found *in vitro*. These so-called direct effects are observed when CdCl₂ is added to the incubation buffer prior to measuring the enzyme activity. The evaluation of non-specific hydrolase activity has been determined spectrophotometrically using the substrate phenyl acetate, for the complete method details see Experimental Methods III.A, Section 1-2.
In Vitro Inhibition of the Hydrolase Activity of Rat Serum by Metals

Figure 3. In vitro incubation of serum with metals showing the effects on esterase activity, assay details are in method section. The esterase activity is normalized for serum protein, and then transformed from kU/g protein to a % of each serum control value. The control esterase value (100%) is determined for each serum sample in order to report and compare the relative changes in activity over time of all animals. The data points represent the mean (n=6) ±SE shown in the error bars. (The mean control value was 2.09 ±0.67 kU/mg protein.)

2. In vitro kinetics

a) Experimental design

Serum prepared from the blood of untreated control rats (male Sprague-Dawley, 150-300 gm) provided a source of non-specific serum hydrolase enzyme activity for the in vitro investigation on the effects of Cd. Rat serum hydrolase activity was first determined for samples diluted in 150 mM Tris,
pH 7.5, buffer. The substrate phenyl acetate was used to spectrophotometrically determine hydrolase activity, as described in the methods section. Dilutions of serum samples were incubated at 37°C for 15-30 minutes prior to assay for hydrolase activity. Then dilutions were made with buffer containing increasing concentrations of CdCl₂ (0-100 µg/ml), and the hydrolase activity was measured. Two points were intended by these studies: (1) to demonstrate the existence of direct inhibition of hydrolase activity by Cd and (2) further characterize the dose response in order to determine the ID₅₀ and compare to the in vivo effects of Cd exposure.

b) Description of results

In Figure 3 the activity of serum hydrolase from several different animals is presented as a mean percent of control (n=6) ±SE. Data transformed in this manner clearly presents the inhibitory effect of Cd on hydrolase activity while reducing the apparent variability in individual baseline activity. However, in order to obtain a more in-depth understanding of the type of inhibition caused by the presence of Cd we must consider the effects individually. The three Lineweaver-Burke plots in Figure 4 demonstrate that the effects of Cd appear to be the result of a noncompetitive inhibition. In Table 7 the mean values of Kₘ and Vₘₐₓ are listed. From the plots it can be seen that there is a decrease in the rate of the reaction with increasing concentrations of Cd. This is summarized in Table 7, where the Vₘₐₓ of the reaction drops from 1.10±0.18 to 0.23±0.04. Noncompetitive
inhibition characteristically cannot be overcome by increasing substrate concentration, therefore the value of $V_{\text{max}}$ is expected to decrease.

**Table 6** Summary of the kinetic parameters $K_m$ and $V_{\text{max}}$, displayed in Figure 4 describing the noncompetitive inhibition of serum hydrolase activity *in vitro*. Values are mean (n=3) ± SEM.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>$K_m$ (-1/x int)</th>
<th>$V_{\text{max}}$ (1/y int)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.96±0.38</td>
<td>1.10±0.18</td>
</tr>
<tr>
<td>12.2 µM Cd</td>
<td>4.10±2.71</td>
<td>0.85±0.15</td>
</tr>
<tr>
<td>24.4 µM Cd</td>
<td>1.71±0.36</td>
<td>0.59±0.08</td>
</tr>
<tr>
<td>36.6 µM Cd</td>
<td>6.23±5.75</td>
<td>0.56±0.12</td>
</tr>
<tr>
<td>48.8 µM Cd</td>
<td>3.36±0.65</td>
<td>0.23±0.04</td>
</tr>
</tbody>
</table>

The *in vitro* data results, displayed in Figure 3, are presented in terms of percent change from the individual control activity value. This data is quite consistent when presented in this way. Actual hydrolase activity is measured in terms of mmol of substrate hydrolyzed by measuring the production of phenol from phenyl acetate over time (min). Additionally, the amount of protein in each sample is determined to normalize the data. The final activity values are determined as rates, mmol/min/mg protein, which are extremely variable between individual rats. In order to compare the effects of Cd exposure between different serum samples it is necessary to normalize the data for variable baseline activity levels. This is accomplished by establishing an untreated or 100% control activity, determined for each individual rat.
Inhibition of Rat Serum Hydrolase 
by Cd In Vitro

\[\frac{1}{V_0} = k \left( \frac{1}{[P]} \right) + \frac{1}{V_{max}}\]

A. 
B. 
C.

Figure 4. Three double reciprocal plots of the inhibitory effects of Cd on non-specific serum hydrolase activity. Each plot represents a single rat serum sample at varying concentrations of Cd (data points are the mean value of triplicate assays of individual rat serum samples).

Additional sampling from that animal is presented as a percentage of the control value, rather than the mmol/min/mg protein rate.

By this method we were able to compare the effects of Cd exposure on a close scale, that was quite reproducible. Otherwise, the differences in the
effects of Cd exposure would be difficult to visualize from the data presented as mmol/min/mg protein. The data presented in Figure 3, compares the effects of Cd inhibition to that of lead (Pb), another heavy metal found to have inhibitory effects on the serum hydrolase activity. The leftward positioning of the Cd curve demonstrates a greater sensitivity of non-specific serum hydrolase activity to inhibition by Cd compared to Pb. According to the indicated ID₅₀ amounts, nearly forty fold difference exists between the effects of Cd and Pb (0.68 and 26.8 µg/ml, respectively).

3. In vivo correlation
   a) Experimental design

The demonstration of Cd altered serum hydrolase activity in vitro, was followed by an investigation of the effects of Cd on in vivo enzyme activity. Each rat’s own initial rate as the untreated, or 100% control activity from which all subsequent measurements were normalized to percent of control activity value. Although there is intra individual variation in the non-specific hydrolase activity rate from day-to-day, the overall percent change is not significant.

In the in vivo experiments the control activity of rats was determined prior to exposure to Cd. The exposure consisted of a single i.p. injection of CdCl₂ (2 mg/ml, in normal saline). The activity of non-specific serum hydrolase was determined at 24 hour increments following Cd exposure.
The rationale initiating this investigation was a search for a sensitive indicator or biological marker of low level exposure to Cd. Chronic exposure to Cd has been known to precipitate renal failure, this can occur with little or no clinical or diagnostic warning. The effects of metals on the function and activity of other enzymes have been previously observed. The effects of Pb on the heme production pathway, is a well known example. With this in mind, the effect of Cd on the activity of enzymes, other than hydrolase, was also explored. Two enzymes selected for this investigation were glutamate-pyruvate transaminase (GPT) and glucose-6-phosphate dehydrogenase (G-6-

![Arylesterase Activity of Serum](image)

Figure 5. Plot of in vivo effects of Cd, followed in individual rats, on the activity of non-specific serum hydrolase over 48-72 hrs. Control (100%) activity value is 3.54 ±0.87 kU/mg protein. Statistical significance determined by Student's paired t test, is indicated for all data points below the asterisks for p≤0.05 (three samples at 24 hrs, and all 48 and 72 hr samples).
PD). The first, GPT, is an important clinical indicator of myocardial infarction [Chinsky, 1956], liver necrosis [Routh, 1970], hepatitis [DeRitis, 1955;DeRitis, 1956], pulmonary infarctions [Wacker, 1960] and poisonings (Waldman and Borman 1959).

The inhibitory effects of Cd on in vivo serum hydrolase activity that was demonstrated in Figures 4 & 5 could have been the result of the halothane used to anesthetize the animals during blood draw as had been suggested in several previous reports [Lunam, 1985;Lind, 1987;Neuberger, 1987;Hughes, 1991]. Briefly, in this experiment we wanted to investigate the possibility that halothane was responsible for the reduction of serum hydrolase activity, as this was the anesthetic employed in our experiments. Two groups of rats (n=6, each group) were used in the experiment. One group received an i.p. injection of normal saline (1 ml/kg body wt.), the vehicle for the CdCl₂ injections. The other group was untreated. Each day (0-72 hrs) rats were anesthetized in a halothane chamber which consisted of a Plexiglass box, 24x9x10 cm (LxWxD), with a tube attached to an entry porthole. The other end of the tube was connected to a halothane vaporizer (Ohio Medical Products, Madison, WI) which delivered 2-2.5% (v/v) halothane in an oxygen base. Rats were placed in the chamber for up to 1 min to obtain optimal anesthesia conditions, then removed for a blood draw (~0.5 ml/day). The results of this experiment are shown in Figure 6. The plot shows serum
hydrolase activity over time and demonstrates that the halothane alone is not

![Graph showing Serum Hydrolase Activity](image)

**Figure 6.** Effects of daily halothane exposure on the activity of non-specific serum hydrolase of male Sprague-Dawley rats. Data points represent the mean (n=6) ± SEM.

responsible for reduction of the enzyme activity.

The effects of Cd on the activity of GPT and G-6-PD were investigated. Results of our *in vitro* investigation were as expected, Cd reduced the activity of both enzymes *in vivo*. The ID$_{50}$ for GPT was 0.356 µg/ml, and nearly ten times greater, 3.865 µg/ml, for G-6-PD (see Figure 8). Further investigation of these enzymes was limited to the GPT determination in serum because G-6-PD is not normally present in serum at detectable levels. The results shown in Figure 9 demonstrate no significant change in the level of GPT in the
serum of rats following a 2 mg/kg CdCl₂ exposure. The conclusion that we have made from these simple studies was that the activity of non-specific serum hydrolase is unique in its measured response to a Cd exposure. And therefore should be further investigated for its potential significance either physiologically or pharmacologically as we will present in the next series of experiments.
Rat Serum Esterase Activity with RBC and Plasma Cd levels
After a 2 mg CdCl₂/kg exposure

Figure 7. In vivo effect of Cd on the hydrolase activity of rat serum. Male Sprague-Dawley rats were administered a single dose (i.p.) 2 mg CdCl₂/kg. The pretreatment serum hydrolase activity (3.06 ± 0.87 kU/mg protein) serves as the baseline (100%) used to express relative change over time. Data points represent the mean (n=10) ± SD. The change in hydrolase activity was found to be statistically significant from day 1 (24 hrs) through day 6 (p<0.05) using the paired two-sample t test.
Cd\textsuperscript{++} Effects on Enzyme Activity

*In vitro*

--- GPT --- G-6-PD

Figure 8. The direct effect *in vitro* of Cd inhibition of glutamate-pyruvate transaminase (GPT) and glucose-6-phosphate dehydrogenase (G-6-PD). Data points represent the mean (n=6) ± SEM.

**b) Description of results**

Figures 3 & 4 presented similar effects in the reduction of non-specific hydrolase activity in two different sets of *in vivo* studies. *Figure 3* presents the initial experimental data that identifies the existence of Cd induced reduction in non-specific serum hydrolase activity. However, in comparison to the *in vitro* data, the *in vivo* data demonstrates reduction in the activity of non-specific serum hydrolase over a 72 hour lag time. This data suggests that the mechanism of the reduced activity is not a direct effect of the circulating Cd, but rather an effect on the actual production of the hydrolase enzyme.
Figure 9. Effects of Cd on the in vivo serum GPT. Data points represent the mean (n=3) ± SEM.

This is further supported by the low levels of Cd that were found to be circulating in the blood over the later part of this 72 hour period (Figure 7).

Investigation of additional sources of hydrolase activity were also carried out in vivo. Male Sprague-Dawley rats were exposed to a 2 mg CdCl₂/kg i.p. administration, and serial hydrolase activity measurements were made from GI (tissue between pyloric sphincter of the stomach and the jejunum) and liver tissue fractions. The results of this study are shown in Figure 10. The hydrolase activity level is presented as mmol of substrate hydrolyzed per minute per mg protein. Since this study required GI and liver tissue samples in addition to serum, it was impossible to maintain each individual animal's own pre-exposure control values and present the data as
% of own control. Therefore the hydrolase data had to be plotted as enzyme activity units (mmol/min/mg). At each time point 6 individual animals were sacrificed to obtain the serum, GI and liver tissue samples. Tissue samples were separated into their respective cytosolic and microsomal fractions for hydrolase activity determinations. The most dramatic effects are seen in the loss of liver microsome and GI cytosolic hydrolase activity. While the reduction in serum hydrolase activity is statistically significant from control at 72 and 96 hours, it is not as dramatic as the transformed data suggested in earlier studies.
Figure 10. Effects of 2 mg CdCl\textsubscript{2}/kg in a single i.p. dose on the \textit{in vivo} activity of non-specific hydrolase in serum and tissue fractions over time. Data points represent the mean (n=6) ± SEM, asterisks indicate statistical significance compared to the control value (not shown) for the sample (either tissue fraction or serum) at each time point.

The conclusion of the data from the \textit{in vivo} experiments is that in summary there is a reduction in the activity of the various tissue and circulatory hydrolase activities, however it's significance requires further investigation. Initially we had presented the idea that hydrolase activity in serum could be a useful biomarker of low level exposure to Cd. Unfortunately, this requires the knowledge of individual pre-exposure hydrolase activity in
order to ascertain with any certainty that there has been a loss in activity. With this in mind we embarked on an investigation to determine what possible significance the reduction of hydrolase activity would have on the metabolism of compounds by the hydrolase enzymes.
A complete investigation on the pharmacologic effects of Cd exposure was not undertaken by our research. Following our demonstration that Cd exposure was responsible for the reduction of non-specific circulatory and tissue hydrolase activity several feasibility studies were undertaken to ascertain if further investigation of this event would produce useful information. These studies investigated the effects of Cd exposure on the in vitro and in vivo metabolism of MPH. The in vitro rate of MPH hydrolysis
was measured with and without Cd in the incubation buffer. **Figure 11** shows that in all three species tested, dog, rat and human, the rate of metabolism of MPH *in vitro* is much slower than it is *in vivo*. Incubations had to be carried out for 8 hours at 37°C, and a significant amount (~40%) of the hydrolysis was not enzyme catalyzed, because blank incubate showed loss of about 40 of the 100 ng/mL. **Figure 12** shows the effect that 20 µM Cd has on the rate of MPH hydrolysis by the same circulatory enzymes *in vitro*. While there appears to be no change in the rate of hydrolysis in the blank incubations, there is a reduction in the total amount of MPH hydrolyzed in all of the fractions tested. This indicates that there is no effect on the autohydrolysis of MPH, while the apparently enzyme catalyzed reactions are altered.
In Vitro Hydrolysis of MPH with 20 µM Cd
100 ng MPH/mL after 8 hrs at 37°C

<table>
<thead>
<tr>
<th>Species</th>
<th>Plasma</th>
<th>RBC</th>
<th>Whole</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
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<tr>
<td>Human</td>
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</table>

Figure 12. *In vitro* hydrolysis of methylphenidate in incubations of plasma, red blood cells (RBC), whole blood, and a buffer blank control, from dog, rat and human sources, all in the presence of 20 µM Cd.

The data from the *in vitro* incubations suggests that Cd may alter the hydrolytic activity of circulatory enzymes and therefore may have an effect on their metabolic activity *in vivo*.

To this point we have demonstrated a reduction in hydrolase activity in the serum and various tissues of the rat following exposure to Cd. However, the significance of the event is not clear. Therefore, the most important questions remains, is there a "clinically" observable significance to reduced hydrolase activity? A complete answer that question is far beyond the scope of
this project, yet must be addressed to some degree. While there were no conclusive results, a single study has demonstrated that this question should be addressed further. In a single study two groups of three rats each were given either a low dose (0.15 mg/kg) or a high dose (0.30 mg/kg) of MPH before and then 72 hours after a single Cd exposure (2 mg CdCl₂/kg, i.p.), and the plasma MPH levels were followed for the 6 hours postdose.

![Rat Plasma Methylphenidate Levels](image)

Figure 13. *In vivo* serum levels of methylphenidate in rats before and after exposure to CdCl₂. Each plot represents mean (n=3) ±SEM serum profiles of rats dosed, with MPH as indicated, once prior to Cd exposure (solid lines), then again 72 hours after a single Cd exposure (dashed lines) of 2 mg CdCl₂/kg, i.p.
The resulting plasma levels of MPH, determined by GC analysis, are presented above in Figure 13. The plot shows a consistent increase in the plasma MPH level following exposure in both groups of animals. While this data is not complete proof (statistically), it indicates some support for claiming the existence of an effect in vivo on the hydrolysis of MPH following exposure to Cd. In Table 7, the pharmacokinetic parameters for this experiment have been estimated using a one compartment model. The important point made by this data is that there appears to be no change in the rate of uptake as demonstrated by the time to maximal concentration, or $T_{\text{max}}$ value, and the apparent half-life, or $T_{1/2}$, also appears to remain unchanged by the Cd exposure. However, there is a three-fold increase in the maximum plasma concentration, or $C_{\text{max}}$, following the Cd exposure, which results in an increased area under the plasma concentration time curve (AUC) of approximately four-fold in both cases.
Table 7  Effects of Cd on the pharmacokinetic parameters of MPH administered as an i.p. dose. Data represents the mean (n=3) ± SEM. Cd exposure refers to a single 2 mg CdCl₂/kg i.p. dose 72 hours prior to MPH dosing.

<table>
<thead>
<tr>
<th>MPH Dose (mg/kg)</th>
<th>Control (mg/kg)</th>
<th>Cd Exposed (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.15</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td>0.30</td>
</tr>
<tr>
<td>Tmax (hr)</td>
<td>0.9 ± 0.1</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>Cmax (ng/mL)</td>
<td>5.0 ± 0.9</td>
<td>8.1 ± 1.5</td>
</tr>
<tr>
<td>T½ (hr)</td>
<td>2.5 ± 0.4</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>AUC (ng/mL*hr)</td>
<td>16.1 ± 1.4</td>
<td>28.9 ± 2.3</td>
</tr>
</tbody>
</table>

The conclusion drawn from this single experiment is that the increase in the plasma levels of MPH from the same dose (either 0.15 or 0.30 mg/kg) following Cd exposure is the result of altered metabolism. Further elaboration of this point is made from the plots in Figure 13, where the event appears to be a pre-systemic one, in that there is no change in the terminal half-life of MPH.

IV. Conclusion

This balance of this report has presented a review of the known effects of Cd exposure and the current levels of exposure found in the various parts of the world. In addition we have reported on the possibility of previously unreported effects on the activity of hydrolase in serum and tissues found in
Sprague-Dawley rats. We have presented data to demonstrate that serum and tissue hydrolase activity is sensitive to inhibition by Cd both in vitro, in incubations, and in vivo, following a single exposure. This is shown in detail in Figure 3, where Cd is found to result in a 50% reduction in the activity level of non-specific serum hydrolase activity (IC₅₀) at 0.68 µg/ml (using the substrate phenyl acetate). Additionally, we have reported that the metabolic hydrolysis of MPH by the non-specific hydrolase enzymes is altered in vitro by the presence of Cd (20 μM).

It is our further inference, that the alteration in the metabolic disposition of MPH may be a significant effect of Cd exposure and requires further investigation. This conclusion is made based on the in vitro demonstrations of the effect of Cd on hydrolase activity in addition to evidence from the single study where plasma levels of MPH were determined in control rats, then evaluated after they had been exposed to 2 mg CdCl₂/kg (Figure 13). The importance of this finding is that low levels of Cd present in the global environment (or variably present) may interfere with the normal hydrolytic metabolism of ester compounds by non-specific hydrolase enzymes, and therefore alter the pharmacologic activity of these compounds. This altered activity may well be responsible for less than optimal, or even dangerous therapeutic outcomes.
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