REVIEW OF LITERATURE

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Behavioural changes due to toxic effects of chemicals on nervous system have been known through over exposure of humans and animals since antiquity. The neurotoxic properties of lead, for example, were identified as early as 200 B.C. (Environ Health Perspect, 1996). Chlorpyrifos (CPF) being an organophosphorus compound (OP) exhibits its toxic action on mammals/vertebrates/ insects by inhibiting cholinesterase enzymes. Cholinesterases are one of the major kinds of enzymes responsible for proper functioning of nervous system by maintaining acetylcholine levels. Neuropathy due to exposure to OP compounds was first reported in 1899, i.e., many years before recognition of this class of chemicals for their insecticidal properties. Since 1930s, intentional or accidental exposure of individuals to OP compounds resulting in delayed neuropathy in humans has been identified (Ehrich and Jortner, 2001).

Neurotoxicity

Before 1980s, it was general opinion that chemical-induced changes in the structure of the nervous system is adverse, whereas behavioural abnormalities were not universally considered to be evidence of neurotoxicity. Due to manifestation of behavioural abnormalities in many cases of neuropathological changes or, as precedence to morphological changes, behavioral tests serve as more sensitive indicators of chemical's neurotoxicity. Behavior of organisms represents a functional integration of the nervous system and, the capacity of the nervous system cannot be assessed in neurohistological or physiological studies independent of behavioral analyses. Due to significant potential of behavioral tests in the study of deleterious effects of chemicals on the nervous system, during

1980s there was large number of studies focused on behavioral procedures to investigate the effects of chemicals on the nervous system (Tilson, 2000; Fiedler *et al.*, 1996).

Neurobehavioral techniques used in animal behavioral toxicology, measure neurobiological functions (including alterations in sensory, motor, autonomic and cognitive function) similar to those measured in humans. Neurobehavioral techniques can be used to study onset and duration of effects of chemical exposure, tolerance following repeated exposure or recovery of functions that can occur following cessation of exposure. Therefore, neurobehavioral procedures serve as valuable tools for research designed to reduce major uncertainties associated with the risk assessment process, such as, animal to human extrapolation and dosing regimens (i.e., high-to-low dose or acute vs. repeated dosing).The ability to link chemically induced behavioral changes to alterations at the neurophysiological, neurochemical, and neuroanatomical levels will lead to a greater acceptance of the validity and reliability of neurobehavioral endpoints in defining adverse effects of chemicals on the nervous system (Tilson, 1993)

Behavioral toxicology is a discipline which has evolved out of the need for data on the effects of toxic agents on the function of the central nervous system (Wenger, 1990). Behaviour is a dynamic process, since it reflects changes in the interaction of an organism with its environment. In the experimental analysis of behaviour, the primary focus is on defining the functional relationships between an organism's behaviour and its environment. The use of neurobehavioral techniques in toxicology has increased dramatically over the past several years. Several national and international groups have recommended that neurobehavioral tests be

included in the initial stages of hazard identification, and regulatory agencies have responded by preparing testing guidelines for premarket approval of environmental and pharmaceutical chemicals. In addition, neurobehavioral data have been used to set exposure limits in the workplace (WHO, 1986).

The basic units of behaviour are termed responses. Aspects of the external or internal environment that affect behaviour are termed stimuli. Behavioural responses have been typically divided into two classes based on the functional relations that control their occurrence. One class of behaviour is controlled mainly or exclusively by the prior occurrence of an event (stimulus) in the environment. Such responses are referred to as elicited or respondent behaviour. The events are called eliciting stimuli, and the responses are called respondents. The other class of behaviour is controlled mainly or exclusively by its consequences and is referred to as operant or emitted behaviour. Behaviour may be either unconditioned (unlearned) or conditioned (learned). Conditioning refers to the modification of a response result from an organism's interaction with its environment (WHO, 1986; Weiss and Cory-Slechta, 1994).

Respondent behaviour

Respondent behaviours are those that are reliably elicited by a specific observable stimulus. Two major features of a respondent behaviour are: (a) its occurrence depends on the frequency of occurrence of the eliciting stimulus; and (b) its consequences do not affect its frequency, or affects it only to a minor extent. Respondents frequently take the form of simple or complex reflexes and typically involve smooth muscles, glandular secretion, autonomic responses, or environmentally-elicited effector responses.

Examples are the auditory startle response, and olfactory responses. Various types of respondent behaviour, both unconditioned and conditioned, have been used in behavioural toxicology (WHO, 1986).

The use of unconditioned respondent behaviour has generally been focused on 2 response classes:

(a) reflexes, in which the response is limited to a specific effector system, such as skeletal muscle (motor response) or smooth muscle (autonomic response); and

(b) taxis, in which the whole animal orients itself towards or away from a particular stimulus.

Reflexes have been more extensively studied. An example is the acoustic startle response, which occurs following an intense auditory stimulus. The acoustic startle response has been used widely in the study of drugs and more recently in the study of other neurotoxic substances. Since movement is the basic measurement of behaviour, the majority of conditioned reflex procedures evaluates motor responses and is useful in the study of behavioural toxicity (WHO, 1986).

Operant behaviour

Behaviour that appears to occur in the absence of an eliciting stimulus is referred to as an emitted or operant response. Operant responses are movements of the organism that operate on the environment. Although these responses may occur in the presence of many environmental stimuli, they are not readily associated with an identifiable eliciting stimulus, and their occurrence is controlled mainly by their consequences. However, some responses are known to include both respondent and operant components.

The best-known example is provided by bird pecks, which are controlled partly by eliciting stimuli and partly by response consequences, apparently in relation to their consummatory and non-consummatory functions respectively (WHO, 1986).

Emitted behaviour generally occurs with a close temporal relationship to the deprivation and presentation of particular environmental conditions, regardless of whether the deprivation produces an obvious physiological change. For example, an animal given access to a novel environment will show a characteristic temporal pattern of "exploratory" activity, with initial high levels of activity diminishing to low levels. Availability of the novel environment is associated with motor activity, but the novel environment is not an eliciting stimulus. Under these conditions, operant behaviour can be studied by observing all or part of the animal's behaviour during a specified period of time (WHO, 1986).

Functional Observation Battery (FOB)

The FOB is a type of neurological examination in which wide range of neurobiological functions such as posture of animals in home cage, careful hand held and open field examination for predetermined end points like muscle tone, tremors, abnormal movements, autonomic function (e.g., pupillary function, lacriamtion, salivation) stereotypic and bizarre behaviors, reactivity to sensory stimuli (e.g., touch, sound, tail pinch), forelimb and hind limb grip strength, landing hind limb foot splay, air righting reflex and motor activity etc are measured. The battery of tests of FOB developed by Moser *et al.* (1988) include array of measures of both unconditioned operant and respondent behaviors. These tests have been useful for screening

neurotoxicity of potential chemicals i.e., hazard identification and elaboration (Weiss and Cory-Slechta, 1994).

Many types of screening tests are currently used to assess the effects of neurotoxic agents on motor and reflex functions. The simplest of these include observational assessments of body posture, muscle tone, equilibrium and gait, and righting reflexes (OPPTS 870.6200, 1996).These tests are quantal or categorical at best, and are generally subjective. Larger animals permit a conventional neurological examination similar to that used with human beings (WHO, 1986). The test batteries are designed for the purpose of detecting multiple aspects of nervous system dysfunction in experimental animals (Vorhees, 1996).

Sensory function

Exposure to toxic chemicals can cause a wide range of sensory effects. About 44% of chemicals that possess neurotoxicant effects have an impact on sensory function (Environ Health Perspect, 1996). Alterations in sensory processes, such as paraesthesia or visual or auditory impairment are frequently among the first signs of toxicity in human beings exposed to toxic agents. In animals, "psychophysical" methods are used to arrive at some estimation of differential response in the presence of a stimulus varied across some physical dimension. The great majority of psychophysical studies have been carried out on non-human primates and birds; ideally, such studies should be conducted on species in which sensory function closely resembles that of human beings. There are simple approaches to the study of sensory deficits based on the localization or orientation response. There is battery of observational tests in which a visual, auditory, olfactory, or dermal stimulus is delivered to the organism. The presence or absence of

a localization or orientation response to the source of this stimulus is then recorded. Such techniques have been used to demonstrate sensory inattention as well as hyperexcitability in rats having lesions in various regions of the brain. The data are usually quantal (i.e., the response is scored as either present or absent) or categorical (WHO, 1986).

Hind Limb and Forelimb Grip Strength

Forelimb and hind limb grip strength are measured after animals are held firmly by their respective limbs onto the mesh screen of the instrument. Two main factors influence grip strength measurement of animals. First, the animal must be motivated to grab the mesh screen or bar. Second, physical strength of the animal to grab or hold on to the screen. Toxicity on central nervous system, secondary effects of systemic toxicity (malaise) or repetitive testing can all cause reduced motivation. Altered physical ability may arise from changes in peripheral nerve, neuromuscular junction or muscular dysfunction (Maurissen and Mattsson, 1995). Decreased body weight and food consumption also cause altered physical ability. Gerber and O'Shaughnessy (1986) suggested that grip strength measurement were best among the other behavioral tests in discriminating neurotoxicant and neuroactive agents.

Hind Limb Foot Splay

Hind limb foot splay is usually measured by pressing hind feet on ink pad to make ink impression onto hind feet and then dropping the rat from a height of about 30 cm onto recording sheets. The distance between two ink marks of hind limb is the measure of foot splay. Departure from the normal splay (increase or decrease) has been considered as an indication of impaired

neuro-muscular integration and/or equilibrium/balancing functions (Maurissen and Mattsson, 1995).

Motor activity

Spontaneous motor activity in rodents has been extensively used in behavioural toxicology. There are number of features that make motor activity as a behavioral end point for examining the effects of chemical exposures. Motility is an inherent feature of all animals and it occurs spontaneously without the need for deprivation and pretraining of animals. It is the result of interaction of sensory, motor and integrative processes. Its recording is noninvasive. Further, measures of motor activity have been shown to be sensitive to treatments known to affect central nervous function, including brain (Environ Health Perspect, 1996). Movement within the living space or environment is a high-probability response in animals and can be easily manipulated by environmental changes, including exposure to neurotoxic agents. Although seemingly simple, locomotor activity is a very complex behaviour comprising of a variety of motor acts, such as horizontally and vertically-directed movement, sniffing, and grooming. Rating scales have been developed to fractionate locomotor activity into its relative components. The measures used most often in behavioural toxicology are horizontally- and vertically-directed activities. A large variety of devices, automated and nonautomated, have been invented to measure motor activity (WHO, 1986). Positive results in a motor activity test usually require further testing to identify the precise function affected. Activity is not a unitary measure and a change in the frequency of this behaviour can reflect toxicant-induced changes in one or more sensory or motor functions, alterations in reactivity (excitability) or motivational states, or perturbations of a variety of regulatory states (e.g., diurnal cycles, energy

balance of the animal). For example, a decrease in activity might mean that the animal is paralysed or, perhaps, that it suffers from "general malaise". Thus, if a change (increase or decrease) in motor activity is observed, significance of the observation needs to be assessed with additional tests which are more specific for detection of neurotoxicity (Maurissen and Mattsson, 1989; Meyer, 1998).

The major advantages of behavioral tests are that they can be easily administered and can provide some indication of the possible functional alterations produced by exposure. Subtle changes in behavior are the most sensitive indicators of neurotoxicity, and behavioral techniques are often capable of detecting deficits at doses lower than those producing overt clinical signs of intoxication or structural changes. Behavioural methods are now recognized as having an important role in both the detection of and evaluation of neurotoxicity. Since they are generally non-invasive, these methods can be used to follow the onset of functional impairment and monitor recovery. Nevertheless, they can lack specificity and results can be affected by toxic changes in non-neural organs, by diet or by conditions under which animals are housed. Other potential drawbacks are the wide variations in the normal functioning of the nervous system and that, because of compensatory mechanisms, chemically induced structural damage may not be reflected in functional impairment (Regulatory Affairs Bulletin – 71; WHO, 1986).

Many studies have been conducted to know the relationship between behavior and pathological changes in nervous system. Some chemicals cause transient behavioral alterations for eg., ethanol, amphetamine and physostigmine. Transient effects of chemicals on behavioral changes,

sometimes considered behavioral tests, are more sensitive than neuropathology. Depending on species sensitivity/importance, tests also vary. For e.g., hen are extremely sensitive to ataxia induced by organophosphate toxicity, whereas, behavioral toxicity can be well determined in rats compared to morphological changes that occurs in hen. So many factors like reserve functional capacity of nervous system, duration of exposure (acute and chronic exposure), dose, age and tolerance, make either behavioural tests or neuropathology more sensitive to study the effects of chemicals on nervous system (Broxup *et al.*, 1989).

Chlorpyrifos

Chlorpyrifos is a phosphorothionate organophosphate insecticide. Fundamental toxicity of OP stems from excess acetylcholine (ACh) due to the inhibition of acetylcholinestarase (AChE) and subsequent accumulation of ACh in the target tissues that further leads to neurobehavioral alterations.

The dramatic effects seen in OP intoxication (i.e., at higher acute toxic levels) includes brain activation, epileptiformic convulsions, muscular tremors (which lead ultimately to flaccid paralysis), increased sweating and salivation, profound bronchial secretion, bronchoconstriction, increased activity of the intestine and diarrhea, miosis, hyper tension, lowered body temperature, and hyperglycemia. Early signs of cholinergic poisoning likely involve stimulation of muscarinic neuroeffectors of the parasympathetic system. Symptoms include bradycardia, miosis, diarrhea, urination, lacrimation and salivation. Over stimulation at skeletal nicotinic neuromuscular junctions causes muscle fasciculation and, at higher doses, muscle paralysis. Finally, anti-ChEs affect junctions of the central nervous system producing hypothermia, tremors, headache, anxiety, convulsions,

coma, and death (Wilson, 2001). When the effects of OP compounds are compared, marked differences are evident between them. This is most likely due to difference in their ability to bind with their prime target, AChE, and the differences in the rapidity of ACh accumulation in and close to the targets of ACh. The consequences of excess ACh are primarily mediated via cholinergic muscarinic and nicotinic receptor activation. Cell surface receptors found on cholinergic neurons are activated by muscarine or nicotine and hence, are classified as muscarinic or nicotinic receptors, respectively. There were reports about direct action of organophosphate anticholinesterases on nicotinic and muscuranic acetylcholine receptors (Bakry *et al.*, 1988).

Muscarinic Cholinergic Receptors

Muscarinic receptors (mAChRs) are predominant in the target tissues innervated by the parasympathetic nervous system, endocrine and exocrine glands and blood vessels. Furthermore, muscarinic receptors are the main cholinergic receptors in the central nervous system and are expressed at high concentrations. Activation by ACh of muscarinic receptors is the first step in a G-protein-coupled signal transduction pathway. The cascade of events involves number of macromolecular interactions upon muscarinic receptor activation and, responses of muscarinic receptors are slow compared with those mediated through the nicotinic receptors. The muscarinic receptors are also termed calcium mobilizing receptors, because they are usually coupled with phisohoinositide (PI) signaling and may either excite or inhibit neurons. At somatic neuromuscular junctions, mAChRs also mediate a diverse range of physiological actions, including the regulation of cardiac and smooth muscle contraction, and exocrine gland secretion. These receptors have been shown to be involved in neurologic disorders such as

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inhibit cAMP formation. When expressed in cells, the m4 gene encoded receptor shows a relatively high affinity for pirenzepine and thus resembles the M1 receptor, which is coupled with PI hydrolysis and formation of InsP3 and DAG. The main consideration with ACh –induced stimulation of mAChRs is the subsequent activation of the target cells, which may lead to overt excitation and toxicity, especially in the CNS (Savolainen, 2001; Jett, 1998).

Organophosphorus agents bind directly muscarinic receptors. Many studies, both in vivo and in vitro, indicate that the treatment-induced decrease in muscarinic acetylcholine receptor number is accompanied by a decrease in the mRNA levels of specific muscarinic acetylcholine receptor subtypes. The in-vivo effects of OP exposure on the mRNA levels of the three muscarinic acetylcholine receptor (mAChRs) subtypes (M1, M2, and M3) were studied by Yagle and Costa (1996) in brain tissue and in peripheral mononuclear cells, which express the m3 subtype. Results indicated that OP exposure can differentially regulate mRNA levels for muscarinic acetylcholine receptor subtypes in different brain areas, and suggested that m2 muscarinic receptors in the hippocampus were most affected by the treatment with the OP compound (disulfoton).

Direct interaction of chlorpyrifos with cardiac muscarinic receptors (primarily M2) at relevant exposure levels contribute to cardiac toxicity (Howard and Pope, 2002).

Huff *et al.* (2001) studied the effects of sub-chronic in vivo chlorpyrifos exposure on muscarinic receptors and adenylate cyclase of rat striatum. They used different dosing regimens. The higher dosing regimen inhibited plasma and brain cholinesterase activities by 51 and 70%, respectively, and

resulted in decreased [3H]cis-methyldioxolane ([3H]CD) binding, indicating that the M2 muscarinic receptor subtype to which [3H]CD binds is particularly susceptible to alterations induced by chlorpyrifos treatment. Decreases in M2 receptors occurred with the higher dosing regimen, in the absence of any clinical manifestations. Thus, in the absence of overt clinical signs, perturbations of the muscarinic receptor system did occur as a result of sub-chronic chlorpyrifos exposure. Such alterations may contribute to neurological impairments that develop following chronic organophosphorus exposure.

Nicotinic Receptors

Nicotinic receptors are located in autonomic nervous ganglia, in the CNS, in the adrenals, and in the neuromuscular junction, the area specialized for the transmission of neuronal impulses to striated muscles. Nicotinic receptors are ion channels. Their activation leads to influx of sodium into cells. Nicotinic receptors have their most dramatic effects in autonomic ganglia and the neuromuscular endplates. At neuromuscular end plate, OP's induce (1) repetitive activity in response to single nerve stimulation and (2) decremental responses to repetitive nerve stimulation. OPs typically also induce accelerated spontaneous release of ACh, leading to increases in the miniature endplate potential frequency, but at high OP concentrations, the end result is depolarization of the neuromuscular endplate regions (Savolainen, 2001; Jett, 1998).

The nicotinic ACh receptor belongs to the group of ligand gated ion channels and consists of five subunits arranged around a pseudoaxis of symmetry. Structural studies have shown that the subunits are arranged around a central cavity, with the largest portion of the protein exposed toward the

extracellular surface. In the nicotinic receptors of skeletal muscles and electric organs of torpedo, one of the subunits, designated as α, is expressed in two copies; the other three subunits β, ε/γ and δ, are present as single copies. The receptor is thus a pentamer of molecular mass near 280 KDa. However, neuronal nicotinic receptors contain only α and β subunits in different combinations with two α subunits and three β subunits. The central cavity is most likely the ion channel that, in its resting state, is impermeable to ions. Once it is activated, it opens to form a 6.5 A° diameter pore. The open channel is selective for cations, and permeation of the particular cations seems to be controlled by the diameter of the open channel. Both of the subunits have a site for binding ACh. ACh must occupy both sites to permit receptor activation and subsequent channel opening. This then leads to a brief surge of Na^+ ions into the cell. The influx of Na^+ ions causes a change in membrane potential, and thus induces a localized depolarization of one part of the cell membrane. If this local depolarization is sufficiently large, it can trigger action potential that spreads throughout the cell (Savolainen, 2001)

OP anticholinesterases may have direct actions on nicotinic receptors (Katz *et al.*, 1997). There are data to suggest that OP anticholinesterases bind to allosteric sites of the cholinergic nicotinic receptors as identified by inhibition of [^{3}H] phencyclidine binding, but some can also bind to the receptor recognition site because they inhibit [^{125}I]α-bungarotoxin binding. Soman and ecothiopate at micromolar concentrations acted like partial agonists of the nicotinic receptors and induced receptor desensitization. However, the mechanism of this nicotinic receptor –OP compounds interaction remains to be elucidated (Savolainen, 2001).

Organophosphorus (OP) compounds exhibit toxicity differentially depending on the age. Wu *et al.* (2003) studied nicotinic autoreceptor function (NAF) during maturation and aging and evaluated its potential modulation by chlorpyrifos (CPF). They suggested that nicotinic autoreceptor function (NAF) is differentially expressed during maturation and that this neuromodulatory process may be selectively altered by some OP insecticides, potentially contributing to age-related differences in response to AChE inhibitors.

Research of Stanton *et al.* (1994) on time-dependent effects of acute chlorpyrifos administration on spatial delayed alternation and cholinergic neurochemistry in weanling rats reported that the neurochemical effects of acute chlorpyrifos administration are more transient, and that the behavioral effects were shorter-lived compared to adult rats.

Karanth and Pope (2003) studied age related effects of chlorpyrifos and parathion on acetylcholine synthesis in rat striatum. They observed that CPF increased synthesis of ACh in juveniles and decreased synthesis in adults and no effects in neonates. These selective changes in neurotransmitter synthesis may also be responsible for the differential age related toxicity. Won (2001) reported that the functional status of presynaptic processes regulating neurotransmitter release may also contribute to age-related neurotoxicity elicited by high-dose exposures to chlorpyrifos.

The tolerance to organophosphate cholinesterase inhibitors indicates that functional recovery accompanies neurochemical compensations for the inhibited enzyme. It is evident from several studies that a cholinesterase inhibiting organophosphate pesticide like chlorpyrifos induces tolerance to repeated treatment.

Bushnell *et al.* (1993) compared the cholinergic toxicity of chlorpyrifos with the OP compound i.e., diisopropylfluorophosphate (DFP) to study tolerance to prolonged inhibition of cholinesterase. They observed that the degree and time course of ChE inhibition in blood and brain and the down regulation of muscarinic receptors in brain after 125 mg/kg of CPF was closely parallel to the reported effects of 25 daily injections of 0.2 mg/kg of OP diisopropylfluorophosphate (DFP). The functional deficits in working memory and motor function appeared within 2 days after injection of CPF and recovered within 3 weeks, long before ChE activity and receptor density returned to control levels. They observed that the effects of CPF were neither progressive nor as persistent as compared to the daily DFP injections.

Bushnell *et al.* (1994) studied the behavioral, neurochemical and pharmacological indices of tolerance after repeated inhibition of cholinesterase by chlorpyrifos in rats. Weekly injections of CPF (0, 15, 30 or 60 mg/kg s.c.) inhibited ChE activity in whole blood of rats by 60% to 90% after 5 weeks. In highest dose, tremor, working memory impairment and motor slowing in daily delayed matching-to-position/visual discrimination tests were observed. Reduction in CPF injection frequency to every other week relieved the inhibition of whole blood ChE activity (to 50% - 75% of control) and ameliorated all the behavioral deficits. They again restarted weekly CPF injections (0, 15, 30, or 45 mg/kg) for 10 weeks. The whole blood ChE activity was reduced by 75% to 90%, however, tremor was not observed during this period while motor slowing and working memory impairment persisted throughout the dosing period in all treated groups. They also observed pharmacological evidence for tolerance to the muscarinic effects of CPF on trial completion in the daily delayed matching-to-position/visual discrimination task. CPF-treated rats were supersensitive to scopolamine

and subsensitive to pilocarpine. Taken together, these studies indicate that inhibition of ChE activity by repeated injection of CPF produces a constellation of behavioral effects not evident after a single CPF treatment, even though both treatment regimens caused prolonged inhibition of ChE activity and down regulation of central muscarinic receptors.

Pope *et al.* (1992) studied long-term neurochemical and behavioral effects induced by acute chlorpyrifos treatment. A single dose of chlorpyrifos at 279 mg/kg (SC) caused extensive inhibition of cortical and striatal cholinesterase (ChE) activity in adult rats at 2, 4 and 6 weeks after treatment. The inhibition of ChE activity was concomitant to reduction in muscarinic receptor binding sites in cortex and striatum at 2, 4 and 6 weeks after exposure. No changes were observed at 12 weeks after exposure either in ChE activities or in muscarinic receptor density levels. Reduction in locomotor activity was observed in CPF treated animals for the first 2 days after treatment, after which basal activity levels were not different from controls. Following challenge with scopolamine (1 mg/kg, intraperitoneal) at 2, 4, 6, 8, and 12 weeks after treatment, CPF-treated rats showed higher activity relative to controls. They indicated that acute exposure to CPF in adult rats can cause long-term neurobehavioral changes that may persist following the recovery of neurochemical parameters associated with exposure and tolerance to cholinesterase inhibitors.

Nostrandt *et al.* (1997) studied the relationship of oral chlorpyrifos effects on behavior, cholinesterase inhibition, and muscarinic receptor density in rat. Behavioral changes and tissue cholinesterase (ChE) inhibition were examined in animals treated at dose levels of 10, 30, 60, or 100 mg/kg chlorpyrifos. Muscarinic receptor density was assessed as quinuclidinyl

benzilate (QNB) binding in all brain regions, heart, and retina. They observed poor correlations for behavioral and biochemical effects. The low-dose effects on ChE inhibition were not reflected in behavioral signs, and behavioural signs showed recovery at 24 h, whereas ChE activity did not. They also observed >60% of brain ChE inhibition before neurobehavioral effects were evident.

Mattsson *et al.* (1996) conducted a single and repeated dose 13 week neurotoxicity screening study of chlorpyrifos insecticide. Doses of upto 100 mg/kg body weight in corn oil by gavage in the single dose study and upto 15 mg/kg body weight/day in diet for 13 week in the repeated dose study were administered. In single dose study, typical cholinergic symptoms were noticed at 50 and 100 mg/kg body weight. Treatment related effects were most prominent on the day of dosing and clinical and FOB effects were rapidly diminished over the next four days. Effects were more pronounced in females than males. In 13 week repeated dose study, they found out minimal effects and no evidence of accumulation of toxicity during 13 weeks exposure period.

Time of peak effects assessment of chlorpyrifos by Lammers and Kulig (1997) also revealed cholinergic symptoms i.e., gait changes, convulsions, salivation, lacrimation and miosis at single oral dose of 150 mg/kg body weight. The effects were observed after 30 minutes of dosing and persisted up to 24 hours. They have decided that time of peak effect for acute studies with chlorpyrifos be 2 hours.

Lead

Lead is now so ubiquitous that even the most remote human populations probably have body burdens of lead that are orders of magnitude greater than those of our prehistoric ancestors. Although overt lead poisoning is very rare today, subtle dysfunction can be caused by very low level exposure, especially in children. Among all the organs which lead has its toxic effects, the brain is the most sensitive to its effects. Neurobehavioural effects of lead have been observed at blood lead concentrations of 10-15 μg/dL in children, and less than 20 μg/dL in rodents (Audesirk and Audesirk, 1998).

Cognitive and neuropsychological deficits are not usually the focus of studies in adults, but there is some evidence of neuropsychological impairment and cognitive deficits in lead workers with blood levels of 41-80 μg/dL. Although similar effects occur in adults and children, children are more sensitive to lead exposure than are adults. Irreversible brain damage occurs at blood lead levels greater than or equal to 100 μg/dL in adults and at 80-100 μg/dL in children; death can occur at the same blood levels in children. Children who survive these high levels of exposure suffer permanent severe mental retardation. Irreversible severe brain damage (overt encephalopathic symptoms) occurs after exposure to high concentrations of lead. In adults, lead encephalopathy occurs at blood lead concentrations of 120 μg/dL or more, but it has also been known to occur at concentrations of only 100 μg/dL in some individuals. The onset of encephalopathic symptoms is very rapid; convulsions, coma, and death can occur within 48 hours in individuals appearing to be asymptomatic. Overt symptoms of subencephalopathic central nervous system (CNS) and peripheral nerve damage are seen at blood lead concentrations ranging from 40-60 μg/dL. Peripheral nerve dysfunction, detected by a slowing of nerve conduction

velocities, occurs at blood lead concentrations ranging from 30-50 μg/dL : this effect has shown no clear threshold ((Davidson, 1994 ; ATSDR, 1999; ATSDR, 2005).

Age-dependent association or differences in the expression of the neurotoxic effects of lead exposure have been observed in many species, including human. Perinatal or developing organisms are the most sensitive. There are some possible explanations for this sensitivity. Greater retention of lead by younger organisms, greater accessibility to lead of the central nervous system in the younger, and a difference in neural tissue makes them more susceptible to lead. Since lead is more sensitive to young children; relatively more studies have been concentrated on the effects of lead on children and relatively fewer studies have concentrated on the effects of lead on adults. It was reported that when compared with non-exposed controls, an early lead exposure is linked to a learning and behavioral dysfunction that could persist for 20 years through adolescence after the cessation of lead exposure in childhood (Neurochemistry, 388).

Experiments on neurotoxic effects of lead on specific neurotransmitters reported that lead blocks the stimulated release of acetylcholine from perjunctional or pregangiolic neurons. Exposure to lead affects the acetylcholine transmission probably by its actions on presynaptic uptake of choline, synthesis or turnover, and release of acetylcholine (Neurochemistry, 388).

As mentioned above, human studies have revealed neurological effects of lead acetate, but this has not been well correlated with effects obtained in animal studies. Screening of lead acetate for neurobehavioral toxicity in different laboratories revealed that lead acetate both in single and repeated

exposures cause considerable general (systemic) toxicity rather than neurobehavioural toxicity. Reduction in both motor activity and body weight were common findings in both the studies. Decrease in motor activity was not shown to correspond to decrease in rearing or compromised motor function which would suggest neuronal effects. High doses of lead acetate produce neurological effects, such as tremors, ataxia and muscular weakness. These signs were observed in both the single and repeated dosing studies, but only in rats that subsequently died or were moribund (Moser *et al.*, 1997).

Few studies have addressed the neurotoxicity of lead acetate in adult rats; the vast majority of studies focus on the known cognitive deficits produced by lead exposure in developing /immature animals. There are several reports about effects of lead on cognitive functions and neurobehavioral discrepancies in neonatal and weanling animals. Kishi *et al.* (1983) observed disturbances in reflex development and behavioral changes at blood lead concentration of approximately 59 µg/100 mL in male rats during first three weeks of life. However, at lower dose levels, they could not observe behavioral changes. The effect of lead on neurobehavioral functions in developing animals (neonates and weanlings) suggests that severity/identification of behavioral effect depends on exposure concentration (Geist and Praed, 1982; Nation *et al.*, 1983; Kishi *et al.*, 1983; Chiodo *et al.*, 2004).

The changes in operant behavior of rats exposed to lead at the accepted no effect level by Gross-Selbeck and Gross-Selbeck (1981) suggest that the dose level of 20 µg/100 mL blood (i.e., accepted no effect level for human being before 1990s) may result in sub-clinical functional changes in the

central nervous system in man. They fed a daily diet containing 1 g lead acetate/kg food to post weaning male and female Wistar rats to achieve blood lead concentration of 20 μg/100 mL blood. Behavioral testing performed between 3 and 4 months of age did not reveal changes in open field tasks including locomotion, local movements and emotionality. However, in the more complex programs (DRH = Differential Reinforcement of High Rates) slight changes in DRH performance were observed. This study gives additional support for EPA conclusion of no clear threshold for neurotoxic effects of lead in children.

Since lead is an element, its neurotoxic actions might be due to interactions with other essential elements such as Ca^{2+}, Mg^{2+}, Na^{+}, and Fe^{2+}. Inorganic lead inhibits calcium influx through voltage sensitive calcium channels at low micromolar concentrations in a variety of cell types. Normal rates of Ca^{2+} influx through voltage – sensitive calcium channels and receptor- operated channels promote neutrite development; so levels of Pb^{2+} sufficient to block these calcium channels would be expected to alter neurite growth (Audesirk and Audesirk ,1998).

Calcium is a divalent cation just like lead. Calcium plays important role in release of neurotransmitters, regulation of some rate-limiting enzymes of neurotransmitter synthesis, storage of transmitters in presynaptic vesicular compartments, and regulation of hormone-sensitive cyclases. Lead acts competitively, but reversibly, with calcium. Lead decreases the uptake of calcium by voltage-dependent calcium channels. If the calcium concentrations inside presynaptic terminals were decreased, calcium-dependent release of neurotransmitters would be inhibited. It was also

observed that micromolar amounts of lead require millimolar increases in external calcium to counteract this effect of lead (Neurochemistry, 388).

Some of the effects of lead can be counteracted by the addition of its competitive substrates. There are evidences that support the interactions between lead and calcium, lead and sodium, and lead and magnesium, and each one offers plausible explanations of some of the actions of lead. Lead-calcium competition might explain the inhibitory effect of lead at acetylcholine synapses. On the other hand, lead-sodium might explain the stimulatory effect of lead at dopamine neurons. In addition, lead might also act on calcium/calmodulin-dependent kinase II with the presence of calcium. This further explains the actions of lead on a neuron. And this reversibility of the effect of lead suggests the strength of the binding of lead is not tight in some cases. As a result, lead might act more potently on certain type of neurons (Neurochemistry, 388).

In -Vivo Effects of Inorganic Lead on Neurite Development

A large body of evidence suggests that chronic exposure to Pb^{2+} in vivo causes abnormal neuronal development. Exposure of fetal and neonatal rodents, usually rats, to Pb^{2+} causes a variety of changes in the fine structure of neurons and in their synaptic connections. Administration of Pb^{2+} to neonatal rats via milk of dams maintained on a diet of 4.0 % $PbCO_3$ until postnatal day 25 caused changes in the morphology of pyramidal cells in the sensorimotor cerebral cortex. A reduction in the number of dendritic branches occurred at distances of 80- 100 μm away from cell body. Various studies suggest neurite abnormalities such as decrease or increase in dendrite branching and decrease in dendritic spine number (Audesirk and Audesirk, 1998).

In-Vitro Effects of Inorganic Lead on Neurite Development

The effects of in-vitro exposure of cultured neurons to Pb^{2+} vary considerably, depending on the species, cell type, and parameter of neurite development measured. The effects of Pb^{2+} exposure in-vitro on neuronal differentiation have been studied in rat dorsal root ganglion cells or explants, IMR32 human neuroblastoma cells, embryonic chick brain neurons, embryonic rat cortical neurons, embryonic rat hippocampal neurons, N1E 115 neuroblastoma cells, and B-50 neuroblastoma cells. Generally, in cortex and hippocampal neurons, mid to high micromolar Pb^{2+} concentration inhibited initiation and enhanced branching, with a low micromolar concentrations having little effect on any parameter of neurite development. The results of numerous studies summarizes that 1) immortal cell lines appear to be relatively insensitive to Pb^{2+} effects, as they required high concentration, 2) neuronal survival is also relatively resistant to Pb^{2+}, even in primary neurons, 3) myelination, neurite initiation, and perhaps, branching appear to be the most sensitive parameters of neuronal differentiation, with inhibition or enhancement occurring at concentrations of 1 μM or less (Audesirk and Audesirk, 1998).

Due to importance of the NMDR receptor in cognitive function and in models of synaptic plasticity, the effect of Pb^{2+} on this receptor has been one focus of attempts to define the bases of lead induced cognitive impairments seen in young animals/children. Chronic Pb^{2+} exposure also alter NMDA channel and/or receptor density. Chronic exposure in-vivo has been found to increase or decrease NMDA receptor density, apparently depending on the brain region studied and the exposure regime. Several studies have been done using [^{3}H]-MK-801. Guilarate (1997) revealed that number of [^{3}H]-MK-

801 binding sites associated with high and low affinity sites of Pb^{2+} inhibition in the hippocampus increased as a function of age, peaking at postnatal day 28 and 21, respectively. Cory- Slechta *et al.* (1997) studied the effects of Pb^{2+} on NMDA receptors in adult rats using [^{3}H]-MK801 sensitivity. MK-801 sensitivity was studied in adult rats at three time points: after chronic exposure to 0, 50, or 150 ppm lead acetate; again after exposure levels were increased to 500 and 1000 ppm; and 6 months after termination of lead exposure. They observed decreased MK-801 sensitivity after lead exposure levels were increased, but only at 500 ppm. This suggests biphasic effects and lack of correspondence between behavioral changes and biomarkers of exposure. Though adult lead exposures do produce changes in NMDA function (as indicated by changes in MK-801 sensitivity), vulnerability to such effects is more pronounced in exposures occurring earlier in development.

Effect of Lead on Muscarinic and Nicotinic Receptors

Studies from several laboratories have shown that chronic in vivo exposure to lead during development caused region specific decreases in number of muscarinic receptors in the rat brain. One possible explanation for the effects of lead on muscarinic receptors is by direct interaction i.e., at higher concentration. Several key elements within signal transduction pathways linked to muscarinic and other neurotransmitter receptors are affected by lead. These include adenyl cyclase activity, phosphoinositide turn over and protein kinase C (Jett, 1998).

The nicotinic receptor is one of the most sensitive neuronal targets to the effects of lead at the molecular level. In N1E-115 neuroblastoma cells, 1 nM to 3 μM lead reduced nicotinic ion conductance by 26 - 90%. The

mechanism of the inhibitory effect of lead has been characterized in hippocampal cell culture as noncompetitive, voltage independent, and primarily acting on the fast-desensitizing nicotinic current, associated with α7 subunit bearing receptors. The α7 bearing sub unit is believed to be the presynaptic receptor that is extremely permeable to Ca^{2+} and may contribute to synaptic plasticity. Thus the inhibitory action of lead at presynaptic nicotinic receptors may contribute to impairment of behavioral processes believed to be mediated by post synaptic cholinergic and glutaminergic receptors (Jett, 1998).

Biochemistry (Systemic Effects)

Clinical pathological parameters have been extensively studied by scientists and used in human clinical medicine for decades. The precision and accuracy of these data in diagnosing animal diseases, physiological state, and pathological conditions have been adapted from human to the animal diagnostician (Suber, 1994). Clinical chemistry has a key role in toxicology studies since it can provide advance warning of adverse effects that may be anticipated (e.g.,by histopathologically). By employing combination of tests, it is possible to identify and also evaluate functional status of major organs. In the conventional toxicology studies, biochemical studies consists of usually encompassed hematology and clinical chemistry tests. The majority of tests are the same as those used in human medicine to establish a minimum database.

Hematology

The hematology tests routinely performed during toxicology studies evaluate erythrocytes, Hb, hematocrit, total and differential (% types of different types of leucocytes) leucocytes, platelets and coagulation. The calculated values

MCV, MCH and MCHC are also estimated. Hematological tests are used to study effect of chemicals on blood and its components.

Anemia

The most common findings observed in toxicological studies are mild decrease in erythrocyte count, Hb concentration and hematocrit. Values below their reference limits condition in these tests are considered indicative of anemia. The RBC count, hemoglobin concentration and hematocrit will parallel each other as long as cell size and hemoglobin content do not change. The erythrocyte associated indices MCV, MCH and MCHC measure cell size and hemoglobin concentration.

Iron Deficiency Anemia

This is a kind of nonregenerative anemia occurring during lead poisoning. This usually occurs in long term exposure to chemicals. This iron deficiency anemia occurs because of decrease in transfer of iron to developing erythrocytes. During this condition, cells appear hypochromic and microcytic (Goyer, 1996).

Total Leucocyte Count and Differential Leucocyte Count

The quantitative estimation of total and differential leucocyte count can identify toxicity associated to particular type of WBC cell or decrease in overall population in all types of WBCs i.e., action on stem cells. Differential leucocyte count is helpful in differentiating different types of WBC lines.

Neutrophils and lymphocytes are the principal cell types found in peripheral blood and toxicological effects on leucocytes usually involve these two cell lines. The normal cell counts for basophils and monocytes are so low that decreases are difficult to recognize (Hall, 1992). The lymphocyte is the most

predominant leucocytic cell in the peripheral circulation of mouse and young rats. Increased total leucocyte counts are most often the result of an increase in neutrophils (Suber, 1994). Relative counts for the different types of leucocytes, obtained by performing differential leucocyte count, are of little significance without the knowledge of the total WBC count.

Platelets

Almost immediately following vascular injury, platelets adhere to exposed collagen and begin to aggregate, forming a primary platelet plug that is sufficient to control bleeding from minor injuries of small vessels. Thrombocytopenia is very common in toxicological studies. Thrombocytosis is very rarely a primary effect of a chemical as compared to incidences of thrombocytopenia. Thrombocytosis is generally associated with iron deficiency (Hall, 1992).

Clotting Time

The clotting time is a measure of coagulation mechanism. The majority of clotting factors are synthesized by the liver. Liver injury and dysfunction must be quite severe in order to cause depletion of clotting factors sufficient to prolong the coagulation assays. Statistically significant differences occasionally observed in toxicology studies gives little toxicological significance. However, in some cases it gives early indication of a potential problem (Hall, 1992).

Clinical Chemistry

The results of clinical pathology tests are used to identify general metabolic and pathological processes. Clinical chemistry tests used in toxicology studies generate information concerning carbohydrate, lipid and protein metabolism, renal function, liver function, hepatocyte injury and electrolyte

balance (Hall, 1992). As the liver and kidneys are frequently the target organs in toxicity studies, parameters pertaining to them have been used in toxicology studies to detect damage to these organs. Great emphasis is placed on the use of plasma enzymes as markers of organ damage. Plasma concentration of enzymes depends on 1) concentration 2) intracellular location 3) tissue/organ damage 4) molecular size and 5) rate of clearance from plasma (Stonard and Evans, 1995).

Liver

Hepatic injury is well recognized as a toxicological problem. Xenobiotics may or may not produce functional and structural hepatic impairment. Drugs or metabolites may affect hepatic metabolizing enzymes or exhaust enzyme cofactors, resulting in either the inhibition of essential metabolic pathways or enhancement of alternate pathways with possible toxic by-products. Diagnostic tests for the evaluation of hepatic damage or dysfunction may be arbitrarily grouped as 1) plasma enzymes 2) functional or clearance tests which measure hepatic transport, uptake, conjugation and excretion 3) tests to assess hepatic metabolism of proteins, lipids, carbohydrates. (Stonard and Evans, 1995).

Kidney

The primary function of the kidney is in volume regulation, excretion of waste products, regulation of acid –base balance, regulation of electrolyte balance and endocrine functions including the rennin–aldosterone axis, erythropoietin synthesis, 1,25-dihydroxycholecalciferol and synthesis of prostaglandins and kinins. Combinations of screening tests based on quantitative and qualitative measurements can be used to detect nephrotoxicity. However, renal function tests do not ensure identification of

nephrotoxicity in all cases where structural alterations of the kidneys can be demonstrated histopathologically (Stonard and Evans, 1995).

Glucose

Serum glucose concentration depends upon intestinal absorption, hepatic production, and tissue uptake of glucose. The balance between hepatic production and tissue uptake is influenced by a variety of hormones, including insulin, glucagon, corticoids, adrenocorticotropic hormone (ACTH), growth hormone, and catecholamines. Insulin is the primary factor responsible for the uptake of glucose by tissues. Corticosteriods, catecholamines, and growth hormone are called insulin antagonists because they interfere with insulin's action on cells. Furthermore, hepatic gluconeogenesis is stimulated by glucagon and glucocorticoids, and glycogenolysis is stimulated by glucagon and catecholamines (Hall, 1992; Suber, 1994).

Cholesterol

Cholesterol is required for the biosynthesis of bile acids, corticosteroids, and sex steroids. The liver, via the biliary system, is the major excretory pathway for cholesterol. Biliary stasis either intrahepatic or extrahepatic, and or other forms of liver diseases can increase serum cholesterol concentration. Effects on serum cholesterol concentration are relatively frequent findings in toxicology studies. Both increases and decreases are observed. While the changes are usually small and generally believed to represent minor alterations in lipid metabolism, the exact mechanisms have rarely been identified (Hall, 1992). Hypercholesterolemia has been reported in renal disease, liver disease, hypothyroidism and diabetes mellitus.

Hypocholesterolemia has been reported in hyperthyroidism, severe or chronic hepatitis, heart disease and arteriosclerosis (Suber, 1994).

Total Protein

Total serum protein concentration is a measure of all the different proteins in plasma with the exception of those that are consumed in clot formation such as fibrinogen and the clotting factors.

Albumin

Albumin is the most abundant individual protein and is largely responsible for maintaining intravascular oncotic pressure. Albumin as a transport protein, binds most plasma constituents that do not have specific transport protein. Albumin has been reported to be decreased with cirrhosis, nephritic disease, and malnutrition (Suber, 1994).

Globulins

The globulins constitute a heterogeneous populaton of proteins, including specific transport proteins, mediators of inflammation, clotting factors, catalysts and inhibitors of biochemical reactions and immunoglobulins.

The liver synthesizes albumin and most of the globulins, with the major exception of immunoglobulins. Mild to moderate hepatotoxicity may be apparent in chronic studies. Because of the reserve capacity of the liver, hepatic injury must be fairly severe before protein synthesis is noticeably diminished. A slight, significant decrease in serum albumin concentration is one of the most frequent findings in toxicology studies. The exact mechanism is usually not apparently identified (Hall, 1992).

Renal Function Tests

Blood urea nitrogen (BUN) concentration and serum creatinine concentration are used to evaluate renal function. Urea is synthesized by the liver from ammonia that is absorbed from the intestine or endogenous protein catabolism. It is freely filtered through the glomerulus and excreted in urine. Decreased glomerular filtration rate causes serum urea nitrogen concentration to increase.

Creatinine

This is a nonprotein nitrogenous waste material that is freely filtered by the glomerulus and unlike, urea, is not reabsorbed by the tubules. It is formed at a constant rate by the breakdown of creatine, a molecule that stores energy in muscle as phosphocreatine. Serum creatinine concentration is influenced by muscle mass and conditioning but is relatively independent of dietary influences and protein catabolism. Glomerular filtration is responsible for removing creatinine from the extracellular fluid and serum creatinine is usually equated with glomerular filtration rate. The increase in serum creatinine usually does not occur until renal function is substantially impaired (Suber, 1994). Creatinine concentration has a greater predictive value than similar changes in BUN.

No single test is superior for detecting liver toxicity, the pattern of abnormal findings in a battery of tests may help to determine the location and severity of lesion.

Serum Alanine Amino Transferase (ALT)

The serum ALT (formerly known as serum glutamic pyruvic transaminase) is the most useful enzyme for identifying the hepatocellular damage. Though it is present in many tissues, its greatest concentration in most species is

within hepatocytes. This enzyme is primarily cytosolic and its concentration within the cells is up to 10000 times greater than that in the serum. ALT may leak into serum in any condition that alters membrane permeability to a sufficient degree (Hall, 1992).

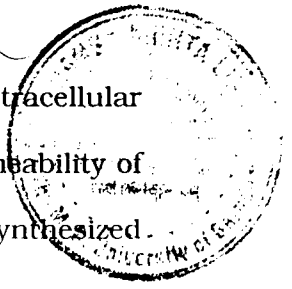
Serum Aspartate Amino Transferase (AST)

Serum AST tends to parallel serum ALT activity with respect to liver damage. Because of high concentration in other tissues, especially muscle, AST is not liver specific. Decreased Serum AST/ALT activity generally observed in toxicology studies may indicate decreased hepatocellular production or release, or an effect on the coenzyme, pyridoxal 5-phosphate (vitamin B6). Decreased serum activity of these enzymes has not been shown to be a pathologically important phenomenon (Hall, 1992).

The diagnostic value of AST and ALT is manifested in the their location. The cellular cytosol contains both AST and ALT, whereas, the cellular mitochondria contain only AST. The majority of AST is found in the cytosol but excessive serum levels of AST with minor or no changes in ALT will allow differentiation of the chemical target site (Suber, 1994).

Serum Alkaline Phosphates (ALP)

ALP that originates from hepatocytes and biliary epithelial cells increase as a result of increased production secondary to intrahepatic or extraheaptic cholestasis and biliary proliferation. The most commonly used enzymes for detection of cholestasis are ALP and gamma – glutamyl transferase (GGT). The mechanism for the cholestasis induced production is uncertain, but bile acids are thought to stimulate enzyme synthesis. Normal serum ALP activity in most adult animals is primarily the liver isoenzyme. Isoenzymes of ALP are produced by the cells of the intestine, kidney cortex, liver, bone, placenta



and myeloid series (Hall, 1992). Since ALP is bound to intracellular microsomal membranes they do not leak out with increased permeability of the cell membranes. This has lead to a postulate that only newly synthesized ALP is released because of cellular damage (Suber, 1994).

Bilirubin

Mononuclear phagocyte system produces bilirubin from heme breakdown through series of biochemical reactions. Macrophage enzymes split hemoglobin into heme and globin, and heme is broken down into biliverdin and iron. Bilirubin reductase converts biliverdin into bilirubin, which is then released into circulation. This bilirubin is known as free, unconjugated, prehepatic, or indirect-reacting bilirubin. In circulation, it binds to albumin. Hepatocytes remove unconjugated bilirubin from plasma and prepare it for removal from the body by a four step process that includes uptake, conjugation, secretion and excretion. Secretion of conjugated bilirubin across the canalicular membrane is the rate limiting step in the process, and small amount of conjugated or unconjugated bilirubin also escapes into plasma.

Prehepatic or unconjugated hyperbilirubinemia occurs as a result of hemolysis. If the hepatocytes are unable to process the large amount of unconjugated bilirubin produced by the mononuclear phagocyte system during a hemolytic problem, there will be an increase in total serum bilirubin concentration consisting primarily of free form. Conjugated hyperbilirubinemia occurs as result of impaired secretion of bilrubin and obstruction of bile outflow. Any disease that damages the hepatocytes can potentially increase serum conjugated bilirubin (Hall, 1992).

Calcium and Inorganic Phosphorus

Serum calcium concentration is affected by parathyroid hormone, calcitonin and vitamin and represents a balance between intestinal absorption, bone formation and reabsorption and urinary excretion. Serum inorganic phosphorus concentration is affected by same hormones, but is more sensitive to dietary intake and urinary excretion. In order to interpret the changes in either of these parameters, it is helpful to know the results of the other. Hypocalcaemia is common in toxicology studies. Signs of hypocalcaemia do not occur because ionized calcium is relatively unaffected. Hypercalcemia is a very rare finding unless the test substance has properties of vitamin D. Serum inorganic phosphorus concentration is very sensitive to glomerular filtration rate and may be increased with prerenal, renal, or post-renal azotemia. Greatly decreased food consumption may cause hypophosphatemia (Hall, 1992).

Sodium, Potassium and Chloride

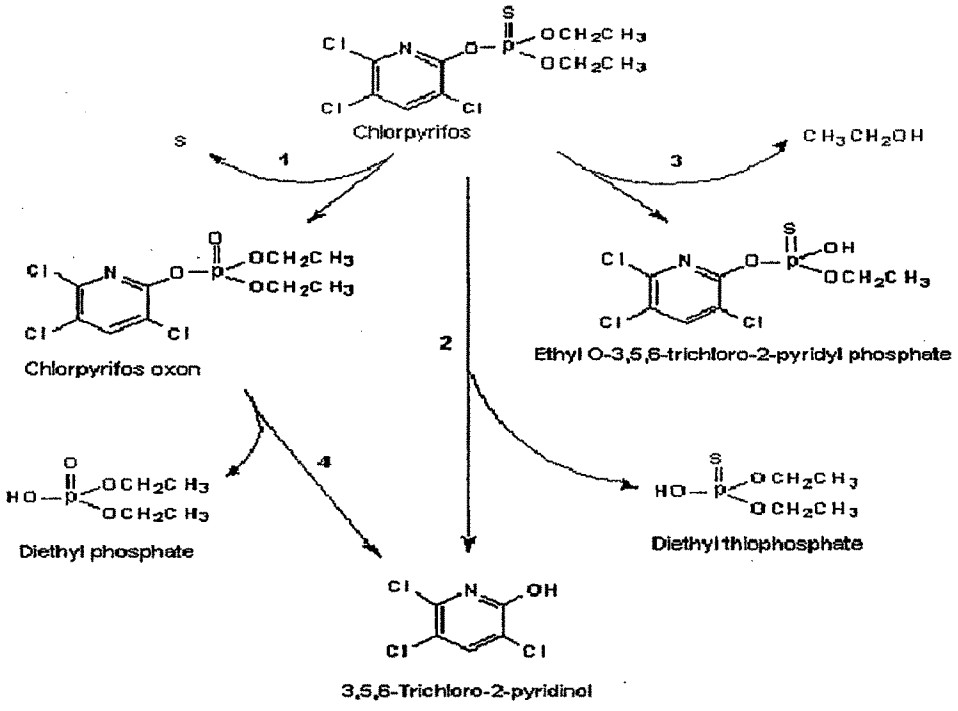
Sodium is the major cation in serum and is the principal determinant of extracellular fluid volume. Potassium is the major intracellular cation and serum potassium concentration is maintained within narrow limits because of its critical role in neuromuscular and cardiac excitability. Chloride is the major anion in serum and serves to support fluid homeostasis and balance cation secretion (Hall, 1992).

Chlorpyrifos

Metabolism and Toxicokinetics

Chlorpyrifos is moderately fat-soluble, and is readily absorbed by oral and inhalation routes but dermal absorption is relatively low (about 2% in humans). Studies in rats, rabbits, chickens, goats, pigs, cows and humans indicated that chlorpyrifos which entered the general circulation was widely distributed throughout the body but was excreted very rapidly in the urine and faeces. In animals, only low concentrations of residues remained in tissues such as fat, lung, liver and brain several days after administration of chlorpyrifos (NRA Review, 2000).

A generalized metabolic pathway for chlorpyrifos is shown below. Chlorpyrifos, like many of the most commonly used organophosphorus insecticides, is a phosphorothionate. Phosphorothionate insecticides are bioactivated by the microsomal cytochrome P450 system in the bodies of vertebrates and insects to their active oxon (phosphate ester) metabolites, which are about three orders of magnitude more potent as anticholinesterases than the parent compounds. Most bioactivation takes place in the liver, while detoxification takes place in the liver and plasma. Chlorpyrifos is rapidly metabolized by mixed-function oxidases to the highly reactive chlorpyrifos oxon by oxidative desulfuration (step 1). The degradation step occurs by conversion directly to TCP (3,5,6 Trichloro–2-pyridinol) and diethyl phosphate (step 2). The oxon can be deactivated by hydrolysis to diethylphosphate and TCP (step 4). A minor reaction pathway is hydrolysis to monoethyl 3, 5, 6-trichloro-2-pyridyl phosphate (step 3, Wagner, 1999).



Metabolic pathway for chlorpyrifos (Wagner, 1999)

The moderate toxicity of chlorpyrifos, when compared with some other phosphorothionates, may be due to hydrolytic detoxification of the oxon by A-esterases, such as paraoxonase, and high serum concentrations of paraoxonase may protect against poisoning by organophosphorus insecticides with paraoxonase substrates as active metabolites. This effect has been demonstrated in rats directly, in which injection of purified paraoxonase before dosing with chlorpyrifos oxon reduced the inhibition of brain acetylcholinesterase in these animals by 2.5 times when compared with controls. Twelve rats given chlorpyrifos at 5 mg/kg body weight orally by gavage excreted 88% of the administered radiolabel in urine within 48 h. At least six metabolites were present, three of which accounted for 97% of the excreted radiolabel. These were identified as the glucuronide of TCP (80%), a glucoside of TCP (4%), and TCP itself (12%) (Wagner, 1999).

Cholinesterase

Cholinesterases are specialized carboxylic ester hydrolases that break down esters of choline. Two of special concern in the toxicology of pesticides are acetylcholinesterase and butyrylcholinesterase. Cholinesterases are classed among the B-esterases, enzymes inhibited by OP's and possessing a serine catalytic site. Other B-esterases include the broad class of carboxylesterases (CaE), one of which is neuropathy target esterase (NTE), the enzyme associated with organophosphate-induced delayed neuropathy. Organophosphorus -induced delayed neuropathy is a generally progressive, irreversible disorder that causes clinical manifestations appearing days to weeks after humans and certain species of animals are exposed to OP compounds that can essentially irreversibly inhibit most of the available neuropathy target esterase. A different group of enzymes known as A-esterases (eg., paraoxnases, arylesteraes) actively hydrolyze OP's. They represent important means of detoxification (Wilson, 2001). Plasma and tissues of mammals have significant concentrations of the calcium-activated A-esterases, arylesterase (EC 3.1.1.2), and the high-density lipoprotein-associated paroxonase (EC 3.1.8.1). The enzyme activities attributable to A-esterases, B-esterases, and cholinesterases vary widely within populations and are influenced by genetic and environmental factors and disease states. The serum activity of paraoxonase in rabbits is some 40 times greater than that in rats. Most avian, including hens, have relatively low activity of A-esterases, and the mean value of serum chlorpyrifos oxonase activity in humans is 10 times that of rat serum (Wagner, 1999).

There has been immense amount of research on ChEs since 1914. ChEs are widely distributed across animal species. Their presence in insects and other invertebrate pests has made anti-ChE agents popular and effective

pesticides. AChEs in the nervous system regulate excitation by destroying the neurotransmitter ACh. They are found at synapses, neuromuscular and myotendinous junctions, cerebrospinal fluid, central nervous system neuron cell bodies, and axons. AChEs are also present on the surface of erythrocytes of mammals, megakaryocytes, lymphocytes, and platelets. Blood ChE forms are used as surrogates for CNS enzymes in studies of toxicants. The plasma ChE activity of rodents such as the laboratory rat is high in both AChE and BuChE and like A -esterases they also have been shown to protect animals from OP toxicity (Wilson, 2001). Butyrylcholinesterase is the predominant cholinesterase in human serum (>99%), while in rats there is an approximately equal distribution of acetylcholinesterase and butyrylcholinesterase. These enzymes detoxify organophosphorus compounds by sequestering them through binding to or phosphorylation by blood proteins, such as albumin, which stoichiometrically degrades them. These protein-bound organophosphorus esters are secreted into the bile. Erythrocyte acetylcholinesterase is similar to the acetylcholinesterase in nervous systems and can bind to and sequester or hydrolyse organophosphorus compounds (Wagner, 1999). Butyrylcholinesterase and carboxylesterases are inhibited by binding to organophosphorus compounds such as chlorpyrifos oxon but they do not hydrolyse them. While binding to them dose not produces adverse physiological effects and, as such represent detoxification pathway (Timchalk, 2001).

The knowledge of the three dimensional structure of the ChEs has led to a better understanding of the mechanism of action of drugs and chemical agents that inhibit the hydrolysis of choline esters. In general there are three major domains for inhibitors to bind. They are the acyl and choline pockets of the active center and the peripheral anionic site. OP's bind

covalently with the serine at the active center. If the alkyl groups on the OP are methyl or ethyl, spontaneous regeneration may require hours, and may be even longer if the tertiary alkyl groups are involved. Loss of one alkyl group, a phenomenon known as aging, further stabilizes the phosphorylated enzyme, to all intents and purposes permanently inhibiting its catalytic ability (Wilson, 2001).

Electrical switching centers, called 'synapses' are found throughout the nervous systems of humans, other vertebrates, and insects. Muscles, glands, and nerve fibers called 'neurons' are stimulated or inhibited by the constant firing of signals across these synapses. Stimulating signals are usually carried by a chemical called 'acetylcholine. It can be considered an excitotoxic transmitter because when it is present in excess, it readily causes toxicity. The term excitotoxicity implies that a receptor agonist, usually a physiological neurotransmitter or its analog, causes overt excitation of neuronal cells through receptor activation. This overt neuronal excitation then usually leads to elevated levels of intracellular calcium, activation of proteases and endonucleases, and ultimately cell death through apoptosis or necrosis (Savolainen, 2001). Stimulating signals are discontinued by a specific type of cholinesterase enzyme, acetylcholinesterase, which breaks down the acetylcholine. These important chemical reactions are usually going on all the time at a very fast rate, with acetylcholine causing stimulation and acetylcholinesterase ending the signal. If cholinesterase-affecting insecticides are present in the synapses, however, this situation is thrown out of balance. The presence of cholinesterase inhibiting chemicals prevents the breakdown of acetylcholine. Electrical impulses can fire away continuously unless the number of messages being sent through the synapse is limited by the action of cholinesterase. Repeated and unchecked

firing of electrical signals can cause uncontrolled, rapid twitching of some muscles, paralyzed breathing, convulsions, and in extreme cases, death.

Cholinesterases play an important role in architectural development of brain and hence, considered to be important neurotransmitter in the central nervous system. There has been increasing evidence to suggest that cholinesterases have a role in neural development. Transient cholinesterase expression in many neural pathways may be critical in neuronal outgrowth. In vitro exposure to anticholinesterases revealed significant inhibitory effects on neurite outgrowth (Claudio *et al.*, 2000).

Verma *et al.*, (2002) studied chlorpyrifos induced inhibition and reactivation of serum cholinesterase activity in rats. They reported that inhibition of serum cholinesterase was dose as well as exposure frequency dependent. Inhibition of BChE and AChE in blood is the indication for changes occurring in the central nervous system. Cholinergic toxic signs can disappear well before AChE and BChE activities recover.

The activity of cholinesterase's dependent on number of factors, e.g., species, age, interindividual, sex, seasonal changes (birds), etc. (Thompson, 2000).

Young or immature animals are more prone to toxicity of chlorpyrifos. Zheng *et al.* (2000) reported 9 - fold difference in sensitivity to acute –dose lethality of CPF between neonates and adult. There are some evidences to suggest differential sensitivity between young and adult animals depending on the age. Chlorpyrifos and its oxon are detoxified by binding to carboxlyesterases and hydrolysis by A-esterases. Chlorpyrifos, and some other organophosphate (OP) compounds, are detoxified via a two-step pathway involving bioactivation of the parent compound to an oxon by the

cytochrome P450 systems, and then hydrolysis of the resulting oxon compounds by esterases such as liver or serum paraoxonase (PON1). The young animal has minimal activity of these detoxification enzymes compared to adult animals. Detoxification enzyme activities increase with age, the enzymatic profile of newborn rats raises concern that the newborn may be even more sensitive than older neonates to an acute chlorpyrifos treatment. Enzyme levels were compared against the sensitivity of young rats to acute chlorpyrifos exposure at various ages; during development, an inverse relationship between the enzyme activities and sensitivity to chlorpyrifos toxicity was observed. The exact influence of these enzymes on sensitivity to chlorpyrifos treatment has not been properly understood. It was concluded that a lack of these detoxifying enzymes in young rats could at least partially explain their increased sensitivity to chlorpyrifos. Higher systemic toxicity in young rats as compared to adults after acute subtoxic doses of chlorpyrifos has also been reported by Whitney *et al.* (1995). In human, liver carboxylesterase activities of infants and adult vary less in contrast to rodents (Pope *et al.*, 2005).

In addition to age –dependent differences in A-esterase activity, human and animal genetic polymorphism has been well established. This polymorphism is known to result in the expression of a broad range of A-esterase enzyme activity within a large segment of the human population. Therefore, it is possible that some individuals may be more sensitive to chlorpyrifos toxicity based on genetic factors that regulate serum PON1 activity resulting in a reduced capacity to detoxify chlorpyrifos- oxon. In animals, there is evidence that serum paraoxonase is protective against poisoning by OPs. Animals with low PON1 levels were more sensitive to specific OP compounds than animals with high enzyme levels. For example, birds, which have very low to

undetectable PON1 activity are more sensitive than various mammals to the acute toxicity of oxons for other OPs (paraoxon, diazinon oxon and pirimiphos oxon). Further rabbits, which have a sevenfold higher serum PON1 activity than rats, are more resistant to the acute toxicity of chlorpyrifos (approximately 9 and 25 fold for acute oral and dermal toxicity, respectively). Rabbit paraoxonase hydrolyzes chlorpyrifos-oxon with a much higher turnover number than does rat paraoxonase (Wilson, 2001). Rodent neonatal plasma and liver A-esterase activity were 9 and 50 % of adult activity, respectively. These findings in animals are in agreement with observations in which newborns and children less than 6 months old have lower plasma A-esterase activity than adults (Timchalk, 2001).

Direct evidence of PON1 role in detoxifying OP's was provided by studying mice exposed to chlorpyrifos. They were protected against cholinesterase inhibition and toxicity by administration of purified PON1. Knock-out PON1 deficient mice were more sensitive to chlorpyrifos and chlorpyrifos oxon than genetically unaltered mice (Wilson, 2001).

Lead

Absorption, distribution, and excretion

Gastrointestinal absorption of soluble lead salts in adult humans can be high during fasting (40-50%), but is about 3-15% when ingested with food. On the basis of dietary balance studies, gastrointestinal absorption of lead in children appears to be higher and may account for 40-50% of the ingested dose. Studies in animals also provide evidence that gastrointestinal absorption of lead is much higher in younger organisms. Absorption is strongly affected by nutritional status, with higher absorption of lead in children who are iron deficient. Calcium deficiency also may increase lead

absorption, based on studies in children. Coadministration of calcium with lead decreases lead absorption in adults, and in animal studies. Vitamin D administration has been shown to enhance lead absorption in animal studies. The distribution of lead appears similar across routes of exposure. Initially, lead is distributed to the blood plasma and soft tissues, but under steady state conditions 99% of the lead in blood is found in the erythrocyte, where much of it is bound to hemoglobin. Lead accumulates in blood, such that bone lead accounts for approximately 73% of the body burden in children, increasing to 94% in adults. Inorganic lead ion is not known to be metabolized in the body but it can be conjugated with macromolecules like glutathione. Unabsorbed lead is excreted in the feces and absorbed lead, which is not retained, is excreted through the urine and bile (ATSDR, 1999).

Lead has been shown to affect virtually every organ and system in the body in both humans and animals. The most sensitive effects of lead appear to be neurological (particularly in children), hematological, and cardiovascular (Davidson, 1994).

Lead has multiple hematologic effects. In lead induced anemia, the red blood cells are microcytic and hypochromic and usually there are basophilic stippling, which result from inhibition of the enzyme pyrimidine-5-nucleosidase. The anemia that occurs in lead poisoning results from two basic defects: shortened lifespan and impairment of heme synthesis. Shortened life span of the red blood cells is thought to be due to increased mechanical fragility of the cell membrane. The biochemical basis for this effect is not known but the effect is accompanied by inhibition of sodium and potassium dependent ATPase's (Goyer, 1996)

The key enzymes involved in the synthesis of heme are -aminolevulinic acid synthetase (ALAS), a mitochondrial enzyme that catalyzes the formation of -aminolevulinic acid (ALA), and ALA dehydratase (ALAD), a cytosolic enzyme that catalyzes formation of porphobilinogen. Through a series of steps, coproporphyrin and protoporphyrin are formed from porphobilinogen, and, finally, the mitochondrial enzyme ferrochelatase catalyzes the insertion of iron into protoporphyrin to form heme (Davidson, 1994).

The inhibition of δ-aminolevulinic acid dehydratase (ALA-D) is most sensitive measure of lead exposure. The depression of coproporphyrinogen oxidase caused by lead results in increased coproporphyrin activity. Lead also decreases ferrochelatase activity. Failure to insert iron into the protoporphyrin results in depressed heme formation. The excess protoporphyrin takes the place of heme in the hemoglobin molecule and, as the red blood cells containing protoporphyrin circulate, zinc is chelated at the centre of the molecule at the site usually occupied by iron. Red blood cells containing zinc-protoporphyrin are intensively fluorescent and may be used as reliable indicator of lead exposure/toxicity. Depressed heme synthesis is thought to be the stimulus for increasing the rate of activity of the first step in the heme synthetic pathway. As a consequence, the increased production of delta- aminolevulinic acid by ALAS and decreased activity of ALA-D result in marked increase in circulating blood levels of and urinary excretion of δ-ALA (Goyer, 1996; Davidson, 1994).

Reduced heme synthesis is seen at blood lead levels of 50 μg/dL in adults and approximately 40 μg/dL in children. The threshold for detecting elevated ALAS activity and blood and urinary ALA levels is 40 μg/dL in both adults and children, but evidence indicates that the threshold may be as low as 15-

20 µg/dL. Inhibition of erythrocyte ALAD has been noted at very low blood levels. This enzyme is one of the most sensitive indicators of exposure to lead; the threshold blood lead level for ALAD activity is less than 10 µg/dL in adults and children. Increased coproporphyrin levels are elevated in individuals with blood lead concentrations of 40 µg/dL. Urinary porphobilinogen levels are not elevated in lead-exposed humans. The threshold for detecting elevated zinc protoporphyrin levels is 25-30 µg lead/dL of blood in adults, 16 µg/dL in 15-16 year old children and 15.5 µg/dL in children who were 4 years old. The threshold for elevation of total erythrocyte protoporphyrin (zinc and iron) is 25-30 µg/dL in adult males, 15-20 µg/dL in adult females, and 15 µg/dL in children (Davidson, 1994).

Lead is known to affect calcium-dependent or related processes and result in calcium metabolism disorder and finally in the alteration of the blood calcium level. Competition between lead and calcium occurs at different levels such as intestinal absorption, transport, bone deposition and mobilization and renal excretion. Lead at moderate exposure decreases serum calcium. Disorders of intestinal calcium absorption and also disorders in transport, distribution, deposition and excretion of calcium by the direct action of lead on the cell membranes, but also on the parathhormone, osteocalcine, calcitonine, 1, 25 D dihydroxyvitamine could be responsible for hypocalcaemia (Ossian *et al.*, 1998; ATSDR, 1999).

In occupational exposure, some people showed hypercalcemia. Lead increases the prostaglandine PGE_2 concentration upon moderate exposure, this increased concentration of PGE2 increases calcium mobilization from bones and consecutively may lead to hypercalcemia. (Ossian *et al.*, 1998).

Lead alters carbohydrate metabolism. Stevenson *et al.* (1976) studied effects of subacute and chronic lead treatment on glucose homeostasis and renal cyclic AMP metabolism in rats. They compared the effects of subacute exposure of rats to a 10 mg/kg daily dose of the lead for 7 days with chronic oral ingestion of lead in doses ranging from 20-80 ppm. Irrespective of the treatment regimen they used, lead treatment significantly increased the activities of renal pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose 1, 6-diphosphatase and glucose 6-phosphatase. The enhancement of kidney gluconeogenic enzymes observed in chronically treated animals was associated with a stimulation of the adenylate cyclase-cyclic AMP system, a rise in blood glucose and urea as well as a depression in hepatic glycogen and serum immunoreactive insulin (IRI) levels. Contrariwise, animals exposed to lead for 7 days failed to significantly alter cyclic AMP metabolism and the concentrations of liver glycogen, blood glucose, serum urea or IRI. The insulinogenic index (the ratio of serum IRI to blood glucose concentration) was markedly suppressed in chronically treated rats and this ratio remained within normal limits in sub-acutely treated animals. However, they reported a marked decrease in the insulinogenic index in subacutely treated rats 15 min after the administration of a glucose load. They concluded that increased glucose synthesis as well as suppressed pancreatic function may be responsible for lead-induced disturbances in glucose homeostasis.