

UNDERSTANDING NEUROMUSCULAR WEAKNESS OF
ACUTE ORGANOPHOSPHATE POISONING: THE
SIGNIFICANCE OF METABOLIC ENERGY, MUSCLE
PROTEIN DEGRADATION AND AMINO ACIDS

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By
MAHALAKSHMI. C

NEUROCHEMISTRY LABORATORY
DEPARTMENT OF NEUROLOGICAL SCIENCES
CHRISTIAN MEDICAL COLLEGE
VELLORE 632 004
INDIA

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ABBREVIATIONS

AChE	- Acetylcholinesterase
ADP	- Adenosine diphosphate
ATP	- Adenosine triphosphate
BuChE	- Butyrylcholinesterase
CK	- Creatine kinase
HPLC	- High Performance Liquid Chromatography
NADP	- Nicotinamide adenine dinucleotide phosphate
NADPH	- Nicotinamide adenine dinucleotide phosphate (reduced)
NMJ	- Neuromuscular junction
OP	- Organophosphate
OPP	- Organophosphate poisoning
TCA	- Trichloroacetic acid

Chapter 1

General Introduction

And

Review of Literature

UNDERSTANDING NEUROMUSCULAR WEAKNESS OF ACUTE
ORGANOPHOSPHATE POISONING: THE SIGNIFICANCE OF METABOLIC
ENERGY, MUSCLE PROTEIN DEGRADATION AND AMINO ACIDS

Abstract

Organophosphate compounds, among the largest class of pesticides manufactured and used worldwide, are common agents for intentional self-harm in agricultural communities of Asia and Africa. Organophosphates inhibit acetylcholinesterase and cause muscle weakness. Delayed paralysis that occurs 24 to 48 hours after severe organophosphate poisoning and which can last for 1 to 3 weeks is the main cause of morbidity and mortality in Indian patients. The pathophysiology that underlies this delayed, persistent muscle weakness is not clear but would be useful to elucidate and improve treatment.

This study examines mechanisms that contribute to skeletal muscle weakness that occurs in acute organophosphate pesticide poisoning. The study specifically explores whether low muscle acetylcholinesterase activity in conjunction with insufficient metabolic energy for contraction and increased degradation of muscle proteins contribute to muscle weakness induced by acute severe monocrotophos poisoning

Rats were subject to acute monocrotophos poisoning (0.8LD₅₀) and the course of poisoning evaluated by cholinergic symptoms and skeletal muscle weakness was correlated to muscle acetylcholinesterase activity, metabolic energy levels and breakdown of muscle proteins.

Metabolic energy available for muscle contraction was estimated by levels of plasma glucose, controlled by gluconeogenesis, insulin, the pancreas and corticosteroids, and by branched chain amino acids in the muscle. Protein degradation in the muscle, in response to metabolic acidosis as a catabolic stimulus, was determined by amino

acids in muscle, blood and urine, ubiquitinated proteins and myosin-actin stoichiometry in the muscle. Plasma creatine kinase was the index of injury to the muscle.

Monocrotophos poisoning rapidly led to muscle injury and progressive muscle weakness in rats accompanied by 75% inhibition of muscle acetylcholinesterase, and hyperglycemia, because of pancreatic injury that lowered insulin secretion. Degradation of muscle proteins through the ubiquitin - proteasome pathway possibly stimulated by metabolic acidosis, with sparing of the actinomyosin complex, characterized muscle weakness in the early phase of poisoning.

Rats recovered from muscle weakness within 24 hours with no treatment. Recovery of muscle strength was characterized by

- a) recovery of muscle acetylcholinesterase activity to 40% of normal,
- b) normoglycemia by recovery of the pancreas and normal insulin secretion,
- c) Decrease in degradation of muscle proteins, although muscle injury persisted,
- d) Recovery of muscle strength was associated with increase of branched chain

amino acids in muscle and sustained increase of plasma glucogenic amino acids.

Severe inhibition of muscle acetylcholinesterase, inadequate supply of metabolic energy substrate to the muscle and degradation of muscle proteins accompany muscle weakness of rats acutely poisoned with monocrotophos. Recovery of muscle strength in these animals required prevention of persistent inhibition of acetylcholinesterase at the neuromuscular junction, normoglycemia and protection of muscle proteins, especially the contractile proteins, from degradation. Interventions that address metabolic energy insufficiency and protein degradation in muscle may reduce the duration and adverse outcome of muscle weakness in organophosphate poisoned patients.

Introduction

Pesticides have developed in response to human requirement, as pests are a human perception, while in nature there are no pests. Early pesticides were elemental. The Romans used sulphur 4500 years ago in Mesopotamia and the Chinese about 1000BC as a fumigant (1). Sulphur continues to be used today especially in the wine industry against yeast. The Chinese also introduced arsenic as an insecticide and in Europe, arsenic, mercury and lead were used as pesticides in agriculture in the fifteenth century (2). The use of pesticides from natural sources continued in the nineteenth century with nicotine, pyrethrum and rotenone extracted from plants (3). Synthetic pesticides followed World War II created from weapons of war. Their use dramatically increased agricultural production and improved health by control of insect borne disease in many parts of the world and as a result they gained wide acceptability. Current day use of synthetic pesticides is inextricably linked to the global economy of agriculture, food security and health. In equal measure, use of synthetic pesticides is linked to increased environmental pollution and health risks to humans and animals. The latter, although of global concern, impact gravely at the regional level.

There are several different classes of synthetic pesticides that include organochlorines, organophosphates, carbamates and pyrethroids. The neurotoxic organochlorines contain one or more carbon-chlorine bonds and were the first pesticides to be synthesized. Dichlorodiphenyltrichloroethane (DDT), aldrin, dieldrin and heptachlor are organochlorine insecticides. Organochlorines were found to be highly effective, cheap and relatively safe for humans and were therefore extensively used around the world. DDT although synthesized in 1874 was widely used as a pesticide only from the 1940s (6,7). However, the lipophilicity and slow metabolism

of organochlorines, due to their carbon-chlorine bond, led to their widespread bioaccumulation in animals and persistence in the environment and in the 1970s their use was banned (8,9). Organophosphate and carbamate compounds which are rapidly metabolized and do not persist in the environment or in exposed individuals have replaced organochlorines as insecticides used worldwide.

Organophosphate and carbamate compounds inhibit acetylcholinesterase and consequently can be extremely toxic to humans. The nerve agents are organophosphates that strongly inhibit acetylcholinesterase. Organophosphates with strong acetylcholinesterase inhibiting properties are also used as pesticides while those with weak inhibiting properties are used in therapy for glaucoma, myasthenia gravis and Alzheimer's disease and in veterinary medicine (10). The major use of both organophosphates and carbamates is as pesticides and they are among the largest classes of pesticides manufactured and used worldwide. The annual global production of organophosphate pesticides exceeds 1.5 million tonnes (11).

Unfortunately, in countries where pesticides are easily available and regulatory control is poor, intentional self-poisoning with pesticides is common. The suicide rate in the general population in rural south India is 92.1/100,000 (152 / 100,000 in young women and 69/100,000 in young men) (12). Following road traffic accidents, suicides are the second most common cause of death in young adults in South India (12). Suicides of significant magnitude occur in Turkey, Iran, Central and South Asia (13,14). A significant number of these suicides are with pesticides, predominantly the organophosphates. The utilization of clinical services reflects the enormity of organophosphate poisoning (OPP). In Sri Lanka organophosphate poisoned patients may at times occupy 40% of medical intensive care beds (6). In India acute severe

OPP is one of the most common causes for admission to the intensive care and for the initiation of mechanical ventilation (7,15).

Acute severe OPP that occurs in the context of suicide frequently results in prolonged neuromuscular paralysis. The neuromuscular paralysis is difficult to treat and is responsible for considerable in-patient morbidity and mortality of poisoned patients. The pathogenesis of persistent neuromuscular paralysis of OPP is not clear. The focus of this thesis is to elucidate mechanisms that may contribute to the development of muscle weakness that occurs in acute OPP, specifically the requirement of sufficient energy for muscle contraction and the role of increased muscle protein degradation.

The review of literature covers the following sections:

- Acetylcholinesterase - molecular forms and catalytic activity.
- Organophosphate structure, mechanism of action and metabolism.
- Acute organophosphate poisoning – clinical profile and treatment options.
- Acute organophosphate poisoning and mechanisms of neuromuscular paralysis.
- Metabolic basis of muscle contraction and mechanisms of muscle wasting

Acetylcholinesterase - molecular forms

Acetylcholinesterase (AChE) is predominantly associated with nervous tissue and erythrocytes, while butyrylcholinesterase (BuChE) is found in the serum, liver and pancreas. The two enzymes can be distinguished not only by their distribution, but also by their substrate specificity and their sensitivity to certain selective inhibitors (163).

Acetylcholinesterase hydrolyses acetylcholine, the most widespread neurotransmitter in the brain and present at all neuromuscular junctions in mammals (164,165). The catalytic activity of the enzyme is one of the fastest among enzymes and is crucial for

rapid termination of acetylcholine neurotransmission in the brain and at the neuromuscular junction (166).

AChE is a polymorphic enzyme. Molecular isoforms of the enzyme arise from a single gene in mammals that is made up of six exons (16). Isoforms are generated by use of different promoters, alternative splicing at the 5' and 3' regions and several polyadenylation sites on the gene. Transcriptional regulation in different cell types is dependent on the promoter used while the polyadenylation site may determine the stability of mRNA. In mammals, the AChE gene produces three types of transcripts through use of different 3' splice acceptor sites. This leads to synthesis of three proteins that possess the same catalytic domain but different C-terminal peptides. These are AChE R (readthrough) subunit, a soluble monomer expressed during development and induced by stress in the mouse brain. AChE H (hydrophobic) subunit that dimerizes and associates with a GPI-anchor and is predominant in erythrocytes. AChE T (tailed) subunit that varies in quaternary structure depending on localization and anchor. It can occur as homo-oligomers : monomer, dimer and tetramer as well as hetero-oligomers in assembly with anchoring proteins ColQ and PRiMA. Subunits H and T dimerize through common interactions but tetramers are only formed through the C-terminal of the T peptides. AChE T subunit forms amphiphilic tetramers with a proline-rich membrane anchor (PRiMA) and presents as a globular form (G4) predominantly in the brain (17-19). AChE T subunit forms non-amphiphilic tetramers; found as A4, A8 and A12 forms, in association with a collagen tail (ColQ) that insert into extracellular basal lamina matrix at the neuromuscular junction (20).

AChE activity

From studies on AChE crystallized from the electric eel (*Torpedo californica*) the active site of AChE appears as a deep gorge lined with 14 aromatic residues at the entrance and down the sides of the gorge (21). The amino acid residues at the entrance of the gorge are referred to as the peripheral site and their anionic charge binds the positively charged choline moiety of acetylcholine. Electronic interactions of the aromatic amino acids in the peripheral site with the quaternary nitrogen of choline direct acetylcholine to the base of the gorge where interaction with a catalytic triad of amino acids, glutamate 334, histidine 447 and serine 203 leads to its hydrolysis (Figure 1.1).

The hydrolysis of acetylcholine by AChE is a multi-step process and is represented in (Figure 1.2). The initial nucleophilic attack of the serine hydroxyl group at the active site of the enzyme by the carbonyl carbon of acetylcholine results in the formation of a reversible enzyme-substrate complex (EAX), followed by acylation of the enzyme at its catalytic site and liberation of choline. This is followed by the rapid hydrolysis of the acylated enzyme, release of acetate and regeneration of active enzyme (22-23).

The rate of acetylcholine turnover (k_{cat}/K_m) is extremely rapid where $k_{cat} = k_2k_3/k_2+k_3$. AChE hydrolyses 6×10^5 molecules of acetylcholine per molecule of enzyme, a turnover time of 150 microseconds and one of the fastest amongst enzymes (22-23). As a result of the rapid rate of hydrolysis, k_1 is the rate limiting step for the hydrolysis of acetylcholine – possibly representing diffusion of the substrate down the active site gorge to the catalytic triad of amino acids at the base.

Organophosphate compounds - structure

Organophosphates are a large class of organic derivatives of phosphoric or phosphonic acid or their sulphur containing analogues in which the phosphorous atom is fully substituted, usually having 2 alkyl substitutes and a 3rd group 'X' more labile to hydrolysis (Figure 1.3) (24). They exhibit lipid solubility to varying extents that is dependent on their side chains. There are at least 13 types of organophosphates most of which are used as pesticides (Table 1.1).

Organophosphates – mechanism of action- inhibition of AChE

Inhibition of acetylcholinesterase (AChE) is the primary toxic action of organophosphate pesticides. AChE is a serine esterase and organophosphates inhibit this class of enzymes by phosphorylating the serine hydroxyl group at the active site of the enzymes (25-26).

Organophosphate compounds bind with high affinity to AChE, their phosphorous reacting with the nucleophilic hydroxyl group of serine at the active site of the enzyme. This phosphorylated enzyme intermediate is highly stable ($t_{1/2}$ = days) as its rate of hydrolysis is very slow unlike the acylated enzyme intermediate that forms with acetylcholine. Bonds of the phosphorylated enzyme intermediate are reversible. However, further dealkylation of the phosphorylated group converts the bond between the phosphorous atom of the organophosphate and hydroxyl group of AChE into a covalent linkage. This results in irreversible inhibition of AChE, referred to as "aging of the enzyme". These kinetics result in the deacylation step (k_3) being rate limiting to the phosphorylating organophosphates (Fig 1.4b) (25-27). A schematic representation of organophosphate reaction with AChE leading to irreversible inhibition of the enzyme, in comparison to acetylcholine reaction with AChE and hydrolysis by the enzyme, is depicted in Figure 1.4.

Organophosphate pesticides – metabolism

Organophosphates are rapidly metabolized, a main reason for their widespread use as insecticides as they do not persist in the environment (28). Organophosphates are lipophilic and on entry into cells are rapidly metabolized by Phase I enzymes, Cytochrome P450 monooxygenases, flavin monooxygenases and hydrolyases especially the A-esterases and carboxylesterases, into more hydrophilic components (29). The CYP450 oxygenases are especially important in the oxidation of the thion group P=S of phosphorothionates to the oxon form P=O. Thions are not effective inhibitors of AChE but oxons are potent inhibitors, often several thousand fold more potent (30). In addition to desulfuration, CYP450 oxygenases de-arylate phosphorothionates, hydrolysing the aryl leaving group that detoxifies the compound. CYP450 oxygenases also dealkylate the alkoxy side groups of organophosphates by oxidation of a carbon to aldehyde resulting in removal of the side chain and leaving a hydroxyl group attached to phosphorus. The desulfuration, dearylation and dealkylation of the organophosphate pesticide, parathion, by the CYP450 system are given in Figure 1.5. The microsomal flavin oxygenases, although more limited in metabolism of the organophosphates, are also important in oxidation of the phorates or phorate-oxon to sulfones (31-32).

Hydrolysis of organophosphates is an important route for their detoxification (33). The Ca^{2+} dependent A-esterases, also known as the paraoxonases, catalyse the hydrolysis of the leaving group of oxon forms of organophosphates, effectively eliminating the anti-AChE activity (Figure 1.6) (34-35). They are among the most abundant organophosphate hydrolyzing systems in the body (36).

Carboxylesterases hydrolyze carboxylic acid esters to their acid form and this prevents bioactivation of organophosphates to active anti-ChE forms (37). The low

vertebrate toxicity of malathion which contains two such esters is due to carboxylesterase hydrolysis (37).

Phase I transformations of organophosphates to more hydrophilic states are followed by Phase II conjugation reactions to sulfate and glucuronide and excretion (38).

Acute organophosphate poisoning – clinical profile

The primary event responsible for the pathophysiology of acute organophosphate pesticide poisoning is the inhibition of acetylcholinesterase in the central and peripheral nervous systems and at neuromuscular junctions. Inhibition of AChE leads to acetylcholine overstimulation of muscarinic and nicotinic receptors throughout the nervous system and of nicotinic receptors at the neuromuscular junction (NMJ). Clinical effects of OPP are a manifestation of these receptor activations.

Acute organophosphate poisoning with intention of self-harm may vary in its severity (mild, moderate or severe) depending on the toxicity of the compound, the amount ingested and the rapidity with which decontamination measures are instituted (39). The major route of poisoning in self-harm is oral ingestion. However, absorption through skin may contribute to toxicity in some patients. The cholinergic crisis or autonomic storm, which occurs in minutes to hours after poisoning, is characterized by salivation, lacrimation, urination, defecation, emesis, blurred vision, bronchorrhea, bradycardia and sweating. This reflects an activation of muscarinic receptors on secretory glands and cardiac muscle that is effectively controlled with the muscarinic antagonist, atropine (40-41).

In addition to the cholinergic crises OPP can cause three types of neuromuscular weakness, **TYPE I, II and III paralysis** (40-42) (Figure 1.7). Type I paralysis is a generalized muscle weakness with hyperreflexia, pyramidal signs and fasciculations accompanying the cholinergic crisis within the first 48-72 hours after poison

ingestion. Type I paralysis is a result of AChE inhibition and acetylcholine over-stimulation of nicotinic receptors at the neuromuscular junction (42).

Type II paralysis or intermediate syndrome is a delayed muscle weakness occurring 48-72 hours after ingestion of the poison, affecting the cranial, proximal limb, trunk and respiratory musculature associated with hyporeflexia, lasting for a variable period between 1-3 weeks. A variable proportion of patients develop Type II paralysis varying from 16-70%. Type II paralysis requires prolonged mechanical ventilation in the ICU. Therefore it is the main cause of morbidity, mortality and high cost of treatment of acute OPP in India (40-41,43-44).

Type III paralysis or organophosphorus-induced delayed neuropathy (OPIDN) is a polyneuropathy occurring 1-4 weeks after ingestion of specific organophosphates and not commonly seen in India (45). It is due to the inhibition of neuropathy target esterase and not AChE (46).

CNS effects seen in OPP are those of confusion, ataxia, tremors, seizures and coma (167, 168).

Acute organophosphate poisoning – treatment – oximes and bioscavengers

The medical management of acute organophosphate poisoning includes in addition to atropine, oxime therapy and mechanical ventilation for patients who are hypoxic or who have respiratory muscle weakness (47).

As given earlier organophosphate pesticides inhibit AChE by phosphorylating the enzyme at its active site. Phosphorylation is initially reversible but progresses to irreversible binding by molecular re-arrangement of the phosphoryl moiety and permanent inhibition of the enzyme. Spontaneous reactivation of AChE occurs by hydrolysis of the phosphoryl group reversibly bound to the enzyme. This is a slow

process and insignificant in maintaining appreciable levels of the active enzyme in humans (39).

Oximes were discovered 50 years ago to facilitate nucleophilic displacement of phosphate from the OP-AChE complex to the oxime, resulting in a phosphoryl-oxime and re-activation of AChE (48-49). Oxime reactivation of an OP-AChE complex can only occur when the organophosphate is still reversibly bound to AChE, before the interaction becomes a covalent bond and the enzyme has aged and is irreversibly inhibited. Pralidoxime (pyridine 2-aldoxime), the first oxime to be used as a cholinesterase re-activator, does not cross the blood brain barrier to reactivate AChE in the brain (50). It does not efficiently reactivate AChE inhibited by several of the nerve gases (51). In the context of severe OPP with intent of self-harm irreversible inhibition of AChE has invariably occurred by the time the patient accesses medical care. In this situation recovery of AChE activity sufficient for normal function is through de novo synthesis.

Most organophosphate pesticides are dimethyl or diethyl compounds and give rise to dimethoxy or diethoxy phosphoryl-AChE. Rates of spontaneous or oxime-induced re-activation and aging of organophosphate bound AChE are dependent on the nature of the alkoxy group and not on the leaving group (52). Studies done *in vitro* and in rodents indicate the rate of spontaneous re-activation is greater for dimethoxy than for diethoxy phosphoryl AChE and thus diethyl organophosphate compounds are considered more toxic than the dimethyl compounds. The toxicity of organophosphates observed in rodents may differ from that which occurs in humans as spontaneous reactivation of OP-inhibited AChE is not of significance in humans.

The different rates of AChE inhibition by different organophosphates influence the ability of an oxime to re-activate an OP-AChE complex. Specific phosphoryl-oximes

re-inhibit cholinesterases while others are too large to be discharged from the catalytic gorge of AChE (53-54). In addition, oximes are toxic, affecting acetylcholine release, nicotinic receptors and allosterically modulating muscarinic receptors in peripheral and central cholinergic synapses (55). Oximes may be less useful for the treatment of patients poisoned with dimethyl organophosphates that age AChE rapidly but may benefit patients poisoned with diethyl organophosphate compounds that do not rapidly age the enzyme. This is exemplified in a study of organophosphate self-poisoned patients from Sri Lanka that found oxime reactivation of dimethoate and fenthion inhibited AChE was poor and the compounds were more toxic than chlorpyrifos (56).

It is therefore not surprising that there has been considerable controversy regarding the use of oximes in the treatment of acute organophosphate poisoning. Three randomized controlled trials showed that oximes were not beneficial in the treatment of acute OPP. These studies have been criticized for the delay in arrival of patients to the hospital, the greater proportion of patients who had taken dimethyl organophosphate compounds and use of pralidoxime in inadequate doses (40,57-58). A study from Maharashtra showed that high dose pralidoxime (1g/hr) was highly beneficial in treatment when given early to moderately poisoned patients (59). The biquaternary oximes, HI-6 and obidoxime and their derivatives, have proved more efficient cholinesterase re-activators than pralidoxime especially of AChE inhibited by sarin and other nerve gases (51).

Scavenging organophosphates with AChE and butyrylcholinesterase is a treatment modality that is under study (60-61). An uncontrolled treatment trial of fresh frozen plasma was associated with a reduction in the rate of Type II paralysis in a series of 11 patients (62). This was explained by the ability of plasma butyrylcholinesterase to

bind to organophosphates and prevent further poisoning. The cholinesterases bind organophosphates in 1:1 stoichiometry and extremely large volumes of plasma would be required to effectively scavenge organophosphates in severe poisoning. The benefit of this study with small volumes of plasma, suggest that additional mechanisms may be involved, such as organophosphate binding to albumin (63). Further studies are required to examine the role of fresh frozen plasma. Recombinant cholinesterases are effective in scavenging organophosphates (64). Recombinant human butyrylcholinesterase expressed in milk of transgenic goats was effective in protecting guinea pigs against nerve gas poisoning (65). In other studies polyethylene glycol coated recombinant human butyrylcholinesterase was effective in protecting mice against nerve agents, sarin and VX, indicating rapid release of sufficient concentrations of the enzyme in response to the organophosphates (66).

Catalytic hydrolysis of organophosphates is another approach to treatment of organophosphate poisoning (169). Paraoxonases hydrolyze organophosphates although at a rate too slow for treatment of poisoning. Site directed mutagenesis of amino acids at the active site of the enzyme and stereospecificity studies using nerve agents as substrates indicate possibilities of engineering mutant forms of paraoxonase 1, with enhanced hydrolytic activity, suitable for treating organophosphate poisoning especially nerve gas exposure (67).

The current trend in developing treatment for organophosphate poisoning appears to be towards bioscavenger prophylactic therapy because of the threat of chemical warfare. Prior prophylactic therapy cannot be a treatment option for suicide related OPP but bioscavenging treatment regimens for suicidal OPP could be appropriately developed.

Acute organophosphate poisoning and mechanisms of neuroparalysis

The morbidity and mortality of acute severe OPP are associated with muscle weakness that occurs in Type II paralysis (83). Type II paralysis is characterized by prolonged neuromuscular weakness that can lead to muscle loss (83). Muscle loss may be an important reason for the poor outcome of Type II paralysis because, although the initial loss of muscle maybe slow, once degradation sets in, the process is difficult to reverse.

The mechanisms that underlie the development of Type II paralysis are not clear and this lack of understanding prevents the development of specific treatment for the neuroparalysis. Improved medical management of patients would occur with a better appreciation of the pathophysiology underlying Type II paralysis.

Severe, persistent inhibition of AChE is postulated to underlie the development of Type II paralysis (68). This may partly be through, down regulation of nicotinic receptors on the post synaptic muscle due to prolonged acetylcholine over stimulation. This does not explain the near absence of muscarinic signs in Type II paralysis although muscarinic receptors are not down regulated in acute OPP. Further all patients with severe and persistent inhibition of AChE do not develop the paralysis, which suggests that in addition to AChE inhibition other mechanisms also contribute to the development of Type II paralysis.

The clinical features of Type II paralysis mimic an acute myopathy and electrophysiological studies, support reversible lesions at the neuromuscular junction and possibly at the anterior horn cell (40). Type II paralysis was first recognized by Wadia *et al.* (41) in 1974 and associated with a myasthenic response. In 1987, Senanayake and Karalliedde (40) coined the term intermediate syndrome to indicate paralysis that occurs between Type I and Type III paralysis. They also suggested,

from electromyographic studies in their patients, that a postsynaptic block occurs at the neuromuscular junction in intermediate syndrome. Reversible subclinical changes were noted in single fibre electromyographic studies of subjects voluntarily exposed to sarin. These were suggestive of a non-depolarizing neuromuscular block due to reduced AChE receptors or less acetylcholine release. Clinical and electrophysiological studies now suggest that Type I and Type II paralysis are a clinical continuum and argue against a strict compartmentalization of these two syndromes (69)

Several EMG studies together with repetitive nerve stimulation at 20 to 30Hz found decrements of the common muscle action potential (CMAP) during Type II paralysis which returned to normal on recovery of muscle strength. Jayawardene *et al.* (70) found that patients who do not develop Type II paralysis but manifest muscle weakness exhibit decrement / increment even at high frequencies (20-30Hz) while those with Type II paralysis exhibit severe decrement. Although Type II paralysis is recognized as a disorder of the neuromuscular junction it is not clear whether the pathology is occurring at the pre-synaptic junction of the motor neuron or at the post-synaptic junction of skeletal muscle or both.

The severity and course of muscle weakness in acute OPP are determined by the severity of poisoning. Mild / moderately poisoned patients manifest cholinergic symptoms, may develop Type I paralysis but infrequently develop Type II paralysis. Severe OPP results in a severe cholinergic crisis and Type I paralysis that often leads to Type II paralysis. Studies by John *et al.* (71) indicated that muscle injury, as evidenced by elevation of the muscle isoenzymes, creatine kinase and lactic dehydrogenase, occurred in acute organophosphate poisoning and correlated to the severity of poisoning. Patients with Type II paralysis had a greater degree of muscle

injury. The recovery of muscle power was associated with decline in muscle enzymes. These findings suggested that Type II paralysis was due to an AChE induced myopathy (71).

Other postulates regarding Type II paralysis include nicotinic signs manifested later than muscarinic signs and the inhibition of serine esterases other than AChE (41). The excess acetylcholine seen in OPP stimulates pre and postsynaptic acetylcholine receptors resulting in prolonged receptor associated channel opening and influx of Ca^{2+} in neurons and in muscle cells (72). In animals, this led to muscle necrosis with diaphragm being the most affected muscle while in muscle cultures removing Ca^{2+} from the medium prevented cell death (73). Muscle necrosis of a magnitude sufficient to explain the muscle weakness of Type II paralysis does not occur in patients and muscle lesions are similar in patients with and without the paralysis (68,72).

Oxidative and nitrosative stress are also implicated in organophosphate induced muscle weakness. In rodents organophosphate induced fasciculations produced oxidative muscle injury which was prevented with muscle relaxants (74). Oxidative stress produced in mice severely poisoned with fenthion was reversed with N-acetylcysteine and led to significant survival, when administered either as a prophylactic or therapeutic drug (75-76).

Increased nitric oxide production, associated with the development of myopathy was noted in rats injected with paraoxon (77-78). Co-administration of non-specific or neuronal specific nitric oxide synthase inhibitors with paraoxon, decreased muscle cell degeneration in these animals, suggesting that increased nitric oxide mediated the myopathy.

Oxidative stress is well documented in OPP patients arising from induction of the pro-oxidant enzymes, xanthine dehydrogenase / xanthine oxidase, and insufficient ROS scavenging by anti-oxidant enzymes, catalase and glutathione reductase (170). Ranjbar *et al.* (2005) have shown oxidative stress to occur in acute OPP, arising from depletion of anti-oxidant substrates. Although oxidative stress occurs early in OPP patients and contributes to muscle injury it does not underlie the development of Type II paralysis (79).

Disruption of energy metabolism of muscle cells may play a role in the etiology of prolonged muscle weakness that occurs in OPP patients. Cholinergic hyperstimulation of OPP leads to a high rate of ATP consumption, which coupled with inhibition of oxidative phosphorylation compromises the ability of cells to maintain energy levels. This has been noted in the brain with different organophosphates. Soman poisoning leads to low ATP levels in the rat brain because of the inhibition of glycolysis (80). Mevinphos intoxication is related to inhibition of respiratory complexes I, III, IV and reduced ATP levels in the rat rostral ventrolateral medulla that leads to cardiotoxicity and hypotension of the poisoning (81). In muscle, dimethoate has been shown to disrupt intracellular homeostasis and energy metabolism by increasing the activity of Na^+K^+ ATPase and inhibiting Ca^{2+} -ATPase (82). Reduced ATP levels from inhibition of Complex V occur in the muscle following monocrotophos poisoning (83). The inhibition of energy metabolism in muscle would suggest that insufficient ATP levels might contribute to muscle weakness of OPP.

Metabolic basis of muscle contraction

The skeletal muscle is the largest organ in the body responsible for generating force to support and move the skeleton. The contractile proteins of muscle myosin, actin, tropomyosin and troponin powered by ATP, generate muscle force through contraction. Contraction follows motor neuron excitation of skeletal muscle and the release of acetylcholine at the neuromuscular junction between the neuron and muscle fibre. Acetylcholine initiated action potential at the motor end plate of the muscle results in release of Ca^{2+} from the sarcoplasmic reticulum. Increased sarcoplasmic Ca^{2+} binds to troponin to displace tropomyosin from its binding to actin that permits the binding of the myosin head or cross bridge to actin. This binding results in conformational change of the myosin head, the power stroke, which pulls the actin filament. Actin and myosin dissociate following the power stroke. Contraction occurs when the cycle of myosin-actin binding and dissociation is repeated many times. The myosin cross bridge also possesses an ATPase site that binds ATP. Prior to myosin-actin binding ATP is hydrolyzed and the site is occupied by ADP and Pi. The chemical energy of ATP is absorbed by myosin and powers contraction. Following the power stroke, ADP and Pi are released and a fresh ATP molecule binds to the ATPase site, which leads to detachment of the myosin cross bridge from actin, and the system, is ready to start another cycle. Fresh ATP must be bound to the ATPase site for myosin - actin detachment although ATP is not hydrolysed during this process. ATP is therefore crucial for muscle contraction for two reasons: in providing chemical energy that transforms to mechanical energy and in dissociation of myosin-actin at the end the power stroke to permit a new power stroke cycle (84). Muscle contraction is affected when the supply of ATP is absent or depleted (85).

Disruption at any stage of the force generating ability of the muscle can lead to muscle weakness. Numerous pathologies affect force generation of the muscle from genetic absence of proteins in neuromuscular disorders, infections, toxins to inflammatory myopathies and aging (171, 172). These pathologies affect muscle function through various molecular mediators that influence fibre size, architecture, composition, metabolism and mitochondrial homeostasis, all of which need to have an effect on the contractile apparatus to induce muscle weakness. This includes disruption of ATP necessary for force generation.

Anaerobic and aerobic metabolic pathways supply energy for skeletal muscle contraction. Short bursts of intense physical activity (60-90 sec) utilize the phosphocreatine shuttle and anaerobic glycolysis for energy (85,86). Thus, glycogenolysis and glycolysis are the first carbohydrate metabolic pathways involved in providing ATP for skeletal muscle contraction. Long periods of muscle activity are fuelled by aerobic metabolism for which blood glucose, synthesized through activated gluconeogenesis, becomes an important energy source. Muscle glycogen and blood glucose are therefore important sources of energy for the contracting skeletal muscle during exercise. Glycogen depletion has been implicated in muscle fatigue following prolonged strenuous exercise (87).

Glucose uptake by the muscle is regulated by insulin and glucagon. Skeletal muscles mainly take up glucose through the membrane glucose transporter GLUT-4 which is insulin dependent (88). Contractile activity however increases the permeability of muscle to glucose even in the absence of insulin (88). Further glucose uptake by muscle occurs only when glycogen is depleted by contractile activity not for instance by an overnight fast. Branched chain amino acids through conversion to acetyl CoA in the muscle and oxidation in the TCA cycle are also a source of ATP for muscle

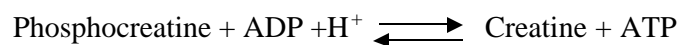
contraction (89). They are important during periods of sustained muscle activity when carbohydrate sources of energy are depleted (90).

During stress, blood glucose levels are also regulated by the corticosteroids, stress hormones released by the adrenal cortex (91). Corticosteroids increase blood glucose by inducing glucose release from hepatocytes and inhibiting glucose uptake by muscle cells through decrease in GLUT-4. Corticosteroids also stimulate gluconeogenesis and glucagon secretion to increase blood glucose (92).

Organophosphate poisoning induces hyperglycaemia and pancreatitis, with the pesticides detected in the pancreas in some cases of poisoning (93). These conditions impose a limitation on the availability of glucose to the muscle. The early phase of severe OPP characterized by strenuous muscle activity is followed by muscle weakness that is mainly attributed to the inhibition of AChE. It is not known if there is a metabolic basis to the muscle weakness and if inadequate substrates of energy to and in the muscle contribute to prolonged muscle weakness of acute OPP.

Markers of muscle injury

Muscle injury often accompanies muscle weakness. Acute OPP rapidly induces muscle injury in humans that correlates to the severity and duration of poisoning (71). Muscle injury invariably results in a damaged sarcoplasmic membrane that leaks cytoplasmic contents into the circulation. Muscle injury can therefore be determined with a high degree of sensitivity by measuring muscle specific enzymes in the blood, most commonly creatine kinase (94). Creatine kinase (CK) (EC 2.7.3.2) catalyzes phosphate exchange between high-energy phosphates ATP and phosphocreatine by the reaction:



It is therefore a key enzyme in maintaining cellular energy homeostasis and is especially important in maintaining a constant ATP/ADP ratio and modulating intracellular pH when ATP utilization exceeds production by glycolysis or oxidative phosphorylation (95). In this function, it is considered to act as a temporal energy buffer. CK may also be important for communication between intracellular sites of ATP production and consumption, acting as a spatial buffer. Phosphocreatine is also a significant source of cellular inorganic phosphate (96,97).

CK, first identified in 1928, exists in several isoforms that differ in their developmental and tissue patterns of expression and subcellular localization (98). The enzyme is found in the cytoplasm as a dimeric protein and in the mitochondria as an octamer. The subunits of the cytosolic dimeric enzyme are the B-type monomer characteristic of the brain and M-subunit specific to the muscle (98). This leads to the formation of three cytoplasmic isoenzymes of CK: CK-MM, CK-MB and CK-BB, each of molecular weight 86kDa. Genes of the M and B proteins are on chromosomes 19q13 and 14q32 respectively.

CK-MM makes up over 95% of total CK in skeletal muscle and CK-BB comprises most of the brain enzyme (99). CK-MB is a useful marker of cardiac muscle infarction even though CK-MM is the most abundant isoenzyme in the myocardium and constitutes over 60% of the enzyme (100). Total CK content is largely centred in skeletal muscle, where it forms almost 10% of total soluble cytoplasmic protein, exceeding the myocardium concentration by as much as twofold. Consequently, serum normally contains CK predominantly from skeletal muscle and almost exclusively as CK-MM (101).

CK-MM predominates in fast twitch muscles (102) and is considered important for energy utilization at sites of high-energy turnover i.e. cross bridges, sarcoplasmic

reticulum Ca^{2+} pumps and sarcolemma $\text{Na}^+\text{-K}^+$ pumps. Mitochondrial CK is considered important for efficient transport and conversion of high energy phosphates in the mitochondria (103).

Serum CK levels represent an index of cellular necrosis and tissue damage following both acute and chronic muscle injury (104). Injury to the muscle either from severe exercise or from pathology that compromises the integrity of the cell will allow for leakage of large molecules. It is in this context that major soluble proteins of the muscle, such as CK, are used as markers of muscle injury (104). Membrane leakiness follows a decrease in membrane resistance as may occur in metabolically exhausted muscle fibres because of a massive influx of Ca^{2+} that promotes activation of K^+ channels (173).

Mechanisms of muscle wasting

Muscle wasting is a consequence of a negative nitrogen balance arising from decreased protein synthesis or increased protein degradation (105). In most acute and chronic pathologies that induce muscle wasting, such as sepsis, cancer, renal failure, muscular dystrophies and immobilization stress, muscle loss is a result of increased protein degradation (106). In these conditions, metabolic acidosis, impaired insulin sensitivity and high levels of circulating cytokines activate protein degradation in the muscle (107).

Similar signalling pathways and protein degradation mechanisms are activated in the muscle in wasting although different events initiate wasting in different pathologies (108). Proteolytic pathways activated in muscle and contributing to wasting include the ubiquitin - proteasome system, Ca^{2+} activated proteases and the lysosomal pathway (109). The ubiquitin - proteasome system is up regulated in several wasting pathologies. A number of signaling mechanisms, especially the NF-kappa B

transcription pathways, cytokines such as TNF alpha and oxidative stress, up regulate these proteolytic systems (110). The suppression of the anabolic growth factors insulin and insulin like growth factor-1 by proinflammatory cytokines also contribute to loss of muscle mass (111). The ubiquitin - proteasome system can also be up-regulated by the transcription factor FOXO (178). Muscle loss can also arise from increased levels of myostatin, an inhibitory muscle growth factor that prevents muscle differentiation and repair through inhibition of MyoD (112) (Figure 1.8).

The lysosomes degrade extracellular proteins that are endocytosed, phagocytosed bacteria, receptor-mediated endocytosed cell surface membrane proteins and cytosolic proteins taken up by autophagic vacuoles (113). In addition, proteins that generate peptides for presentation to the immune system in association with MHC class II molecules are also degraded in the lysosomes (113). In the liver, lysosomal degradation of proteins is enhanced in response to low levels of amino acids and insulin (114).

The calpains are Ca^{2+} activated cysteine proteases that initiate protein degradation in response to cell injury and rise in cytosolic Ca^{2+} (115). Their role in the normal turnover of proteins is not clear although muscle specific calpains are responsible for initiating the degradation of the actinomyosin complex in the muscle by disruption of the secondary structure of the complex (115, 116).

The ubiquitin - proteasome pathway

Most cellular proteins are degraded by the ATP-dependent ubiquitin - proteasome pathway, that is stimulated by catabolic conditions such as fasting, metabolic acidosis, muscle denervation, renal failure, glucocorticoids, insulinopenia (107). The pathway is responsible for the removal of misfolded proteins, short lived regulatory proteins as well as normal longed lived cellular proteins (117). Peptides that are to be

presented in association with MHC Class I molecules are also generated through the ubiquitin - proteasome pathway (117).

Proteins degraded through the ubiquitin - proteasome pathway require selection and processing prior to proteolysis. Signals that identify proteins for degradation through this pathway include :

- The N-end rule whereby N-terminal aspartic acid signals a short lived protein ($t_{1/2} < 3$ minutes) and N-terminal serine is associated with a long lived protein ($t_{1/2} > 20$ hours) (118).
- PEST sequences, short stretches of about eight amino acids enriched with proline, glutamic acid, serine, and threonine, which signal short life and found in several regulatory proteins especially transcription factors (119).
- Domains masked through interactions with other proteins or by modification of amino acid side chains (174).
- Domains that are masked due to conformation and which are exposed on unfolding (174).

The first requirement of proteins degraded through the ubiquitin - proteasome pathway is conjugation to ubiquitin. Ubiquitin is a eukaryotic, highly conserved, 76 amino acid protein, distributed throughout the cell. Protein-ubiquitin conjugation initially requires the activation of ubiquitin by ubiquitin activating enzymes, the E1 enzymes that use ATP to form a high-energy thiolester bond with the C-terminus of ubiquitin. Activated ubiquitin is then transferred to a ubiquitin conjugating enzyme (E2), followed by conjugation to target protein, catalyzed by E3 ubiquitin ligase, resulting in an iso-peptide bond between C-terminal glycine on ubiquitin and ϵ -amino group of lysine on the target protein. Proteins to be degraded are polyubiquitylated and successive transfers of activated ubiquitin to lysine-48 of the

previously conjugated ubiquitin molecule leads to the formation of a polyubiquitin chain (Figure 1.9) (113).

Ubiquitylated proteins are shuttled to the proteasome for degradation. The proteasome is a 26S complex made up of two regulatory components. These include the central 20S proteasome of approximately 700kDa (the 20S core particle, CP) and the 19S regulatory particle (RP: PA700) of 900kDa, attached at one or both ends of the 20S proteasome (120).

The 20S particle is barrel shaped and made up of four stacked rings, two outer α rings and two identical inner β rings. Each α and β ring is made up of seven distinct subunits. The inner surface of the 20S particle possesses chymotrypsin, trypsin, and caspase like proteolytic enzyme activity with chymotrypsin-like activity the most important of the three enzyme activities for proteolysis (121).

Access to the 20S chamber is via a pore too narrow for the quaternary conformation of proteins. Proteins are therefore deubiquitylated and unfolded in the 19S particle, which is made up of several proteins required for the multiple processes, prior to entry and degradation in the 20S particle. The 19S particle also contains six different ATPases that generate the energy required for proteolysis. Within the catalytic core of the 20S particle proteins are degraded to peptides of 3-22 amino acids in size. The ubiquitin moieties are recycled for subsequent reactions (121).

The muscle is composed of myofibrillar, sarcoplasmic and extracellular proteins of which myofibrillar proteins constitute 55 to 60% of the total protein weight (122). The two major myofibrillar proteins, myosin and actin constitute approximately 55 and 25% of muscle proteins by weight (123) and muscle strength and function are critically dependent on the maintenance of their stoichiometry. Muscle weakness

occurs with breakdown of these contractile myofibrillar proteins. The ubiquitin - proteasome pathway is the major route for breakdown of the contractile proteins although the actinomyosin complex requires initial degradation by the calpains (122,124). It is not known if these mechanisms of protein degradation are activated in muscle following acute OPP and contribute to weakness.

Muscle specific amino acids as markers of muscle degradation

Skeletal muscle constitutes approximately 45% body weight of the young adult and muscle loss is associated with release of amino acids characteristic of skeletal muscle proteins. These include the aromatic amino acids, tyrosine and phenylalanine, which are not synthesized or degraded in the muscle (125, 126). Their circulating levels are indicative of net release and / or uptake by the muscle. Muscle proteins, especially the contractile proteins are characterized by 3-methylhistidine, a posttranslational modification of histidine unique to muscle proteins. On degradation of muscle proteins 3-methylhistidine cannot be re-utilized and is excreted. The appearance of 3-methylhistidine in the urine is considered a measure of myosin and actin breakdown and a marker of muscle protein catabolism (127).

Aims and Objectives

Each year two to three million people are acutely poisoned with pesticides and an estimated 200,000 die, mostly in rural regions of the developing world. The majority of these poisonings are due to intentional self-harm. Acute OPP as a result of deliberate self-harm is a common medical emergency in India and accounts for over a third of all pesticide poisonings that present to hospitals in India.

The high degree of AChE inhibition that occurs in acute OPP with intention of self-harm results in an autonomic cholinergic storm that is a medical emergency and responsible for presentation of patients to hospitals. Muscarinic symptoms of the cholinergic crisis are well managed with the muscarinic receptor antagonist, atropine. The electrical activity underlying convulsions that occur in OPP are suppressed with diazepam. There is no effective treatment for the nicotinic symptoms of OPP which especially affect the muscle and therefore muscle weakness is a predominant symptom of OPP. Generalized weakness that occurs early in the course of poisoning (Type I paralysis) is due to AChE inhibition in the brain and at the neuromuscular junction. Delayed neuromuscular weakness that occurs 48-72 hours after poisoning (Type II paralysis) occurs frequently in acute severe OPP and is associated with the morbidity, mortality and high treatment cost of poisoning. Type II paralysis involves prolonged weakness of cranial and proximal limb muscles and respiratory muscles. Weakness of the respiratory musculature often requires the initiation of mechanical ventilation, the reason for the high cost of treatment. Prolonged muscle weakness can lead to wasting from which recovery is difficult that may account for the poor prognosis of Type II paralysis.

Although prolonged muscle weakness is an important symptom of acute OPP and a main cause of morbidity and mortality of the poisoning, it is not treated specifically as there is lack of clarity on its pathophysiology. To improve the medical management of

acute OPP the pathophysiology that leads to the development of prolonged muscle weakness needs to be elucidated.

In acute OPP the cardinal event that leads to muscle weakness is the inhibition of AChE at the neuromuscular junction. OP inhibition of neuronal and neuromuscular junction AChE are a consequence of the amount of blood borne OP reaching these sites after detoxification and removal by binding to plasma proteins such as albumin and carboxylesterases. OP inhibition of neuronal and neuromuscular junction AChE cannot be determined in humans as the tissues are not available for testing. In humans, OPP is determined by inhibition of plasma BuChE and erythrocyte AChE, which reflect blood levels of the pesticide. BuChE is a serine esterase that hydrolyses butyrylcholine with greater affinity than acetylcholine. The enzyme is highly susceptible to inhibition by organophosphates although the inhibition does not induce pathology. The easy accessibility of serum for testing in the poisoned patient makes BuChE the preferred diagnostic marker of OPP. Erythrocyte AChE is considered to reflect the neuronal enzyme in kinetics of OP inhibition and reactivation although not necessarily the extent of inhibition. To understand muscle weakness pertaining to the course and outcome of OPP there is need to study muscle AChE.

Muscle weakness reflects an absence or reduction in contractile ability. Contraction is dependent on sufficient supply of chemical energy, ATP, sourced from metabolic energy substrates, glucose and glycogen. Muscle contraction is also dependent on an intact, functional contractile apparatus, the actinomyosin complex.

Intense muscle activity that follows OPP may rapidly exhaust muscle ATP levels. Low ATP levels in the muscle together with a compromise in the supply of metabolic energy substrates to the muscle at this time will injure the muscle and contribute to muscle weakness.

Prolonged muscle weakness associated with wasting that occurs in acute OPP patients suggests loss of muscle proteins. A study of the most common protein degrading mechanism that occurs in muscle, the ubiquitin - proteasome pathway, may help determine if protein loss contributes to muscle weakness that occurs in acute OPP.

Aim of the study

The aim of this thesis was to examine if low muscle AChE levels together with insufficient metabolic energy for the muscle and degradation of muscle proteins, through the ubiquitin - proteasome system, contribute to muscle weakness that occurs in acute severe OPP. Metabolic understanding of muscle weakness that occurs in acute OPP may help develop treatment strategies that prevent or reduce the weakness.

Objectives

The studies were carried out in rats subject to severe monocrotophos poisoning, an organophosphate pesticide commonly used for intentional self-harm in India. The temporal profile of muscle weakness from development of weakness to recovery of muscle strength was studied in the rats with respect to:

1. Plasma butyrylcholinesterase and muscle acetylcholinesterase to determine the severity of poisoning and duration of weakness.
2. Blood glucose, plasma insulin, corticosterone and glucogenic amino acids, pancreas pathology, liver gluconeogenic enzymes glucose-6-phosphatase and fructose-1,6-bisphosphatase and muscle branched chain amino acids, to determine availability of metabolic energy.
3. Creatine kinase to indicate muscle injury.
4. Amino acids in blood and muscle, 3-methylhistidine in urine and ubiquitinated proteins in muscle, to determine muscle protein breakdown. Blood lactate levels to determine a catabolic stimulus for muscle protein degradation.

Chapter 2

Materials and Methods

Approach to the study of metabolic energy and muscle protein degradation contributing to muscle weakness that occurs in acute OPP

Muscle pathophysiology of acute OPP was examined in an animal model as muscle tissue from patients is not available for study. OPP was studied using monocrotophos, an organophosphate pesticide commonly used for intentional self-harm in India.

Rats were subject to acute severe monocrotophos poisoning and the temporal profile of muscle weakness, from the early phase of poisoning soon after paralysis to complete recovery of muscle strength, was studied. The stages of muscle weakness were correlated to muscle AChE activity, available metabolic energy and protein degradation in the muscle.

Blood glucose levels, controlled by gluconeogenesis, insulin, the pancreas and corticosteroids, were taken as the index of metabolic energy available for muscle function. Branched chain amino acids were studied as substrates for metabolic energy within the muscle.

Metabolic acidosis that occurs in acute OPP was considered the catabolic stimulus of protein degradation in the muscle. Plasma creatine kinase was measured as a marker of muscle injury.

The ubiquitin - proteasome pathway was examined as the mechanism of proteolytic breakdown in muscle, and amino acids in muscle, blood and urine were studied as markers of muscle protein degradation. Muscle myosin-actin stoichiometry was determined as an index of protein breakdown and muscle weakness.

All studies in this thesis were approved by the Institutional Review Board and Animal Ethics Committee of Christian Medical College, Vellore.

2.1 A rodent model of acute monocrotophos poisoning and methods to study plasma butyrylcholinesterase and muscle acetylcholinesterase activity associated with the poisoning.

To understand the contribution of muscle AChE activity to muscle weakness that occurs in acute OPP, the muscle enzyme was studied in rats subject to acute severe monocrotophos poisoning in relation to the course of cholinergic symptoms and muscle weakness. Plasma BuChE, the enzyme estimated to determine OPP in humans, was assayed to examine the correlation to muscle AChE during the course of OPP in rodents.

CHEMICALS

5, 5' Dithio bis (2-nitro) benzoic acid (DTNB) was from Sisco Research Laboratories Pvt. Ltd, India, acetylthiocholine iodide from s.d fine chem. Limited, Mumbai, India. butyrylthiocholine iodide, eserine hemisulfate, bacitracin, pepstatin, aprotinin from Sigma Aldrich Inc., USA. Bovine serum albumin was from National Biochemical Corp., USA. Monocrotophos (36%) containing no other compound was purchased from Syngenta India Ltd. All other chemicals were of the highest purity available. Water used for all studies in this thesis was Milli-Q ultrapure.

METHODS

Monocrotophos poisoning

For all studies of this thesis Wistar rats (150 ± 5 gms) were used and maintained under standard laboratory conditions with free access to food and water. They were fasted for 12 hours prior to administration of 0.8 LD₅₀ monocrotophos in 200µl water by gavage (Monocrotophos LD₅₀ for rats is 18mg/kg body weight (133). Animals were observed after poisoning for cholinergic signs of chewing, tremor, salivation, lacrimation and diarrhea and evaluated for muscle power as given by

De Bleecker *et al.* 1991 (134). Ataxic gait was classified as Grade I power, stretch movements after tail stimulation as Grade II power and no voluntary movements after tail stimulation i.e. paralysis, as Grade III power. In this study monocrotophos was administered to all animals between 8 and 9 am. The study protocol did not include treatment for monocrotophos poisoning. Animals were sacrificed under ether anesthesia at the following times after poisoning:

2.5 hours i.e. soon after paralysis

8 hours i.e. during recovery of muscle power

24 hours i.e. soon after complete recovery of muscle power

7 days, 15 days and 30 days i.e. well recovered muscle power.

Control animals were administered water. Six rats were studied in each group.

Blood (100 μ l) was collected from the tail vein of each rat prior to monocrotophos administration and 2.0ml was collected on sacrifice. Plasma was stored at -70°C until assay.

The gastrocnemius muscle and liver were dissected immediately on sacrifice, washed in ice cold PBS to remove contaminating blood, patted dry and stored at -70°C until assay.

The pancreas was dissected immediately on sacrifice and pancreatic tissue rinsed and fixed in 10% neutral buffered formalin solution. Fixed tissue was embedded in paraffin and 3 μ m sections stained with haematoxylin and eosin (H and E). The sections were viewed under a bright-field microscope.

Urine, (0.2-1.0ml) was collected from animals pre and post monocrotophos administration at each time point of study.

Muscle preparation

Rat muscle was minced and homogenized in a glass Potter-Elvehjem homogenizer in 5 volumes (w/v) of 10mM Tris HCl pH 7.5 containing 1M NaCl, 1% Triton X100, 5mM EDTA, bacitracin (1mg/ml), pepstatin (20µg/ml), aprotinin (20µg/ml) at 4°C (137). The homogenate was incubated at 4°C for 2 hours, vortexed intermittently, centrifuged at 10,000g for 30 minutes and the supernatant estimated for AChE activity and protein content.

Cholinesterase assay

Plasma BuChE and muscle AChE were assayed by the method of Ellman *et al.* (135) using butyrylthiocholine iodide or acetylthiocholine iodide respectively as substrates. In a reaction of 0.5ml, 2mM DTNB, 3mM thiocholine substrate, 0.1M potassium phosphate buffer pH 7.0 and plasma (1: 10 diluted) or 15µl muscle supernatant were incubated for 10 minutes at 37°C. The reaction was stopped with 0.5ml of 2mM eserine sulphate and absorbance read at 412nm. Enzyme units were expressed as µmoles DTNB formed/minute/ml for plasma BuChE and nmoles/ml/min/mg protein for muscle AChE based on the molar extinction co-efficient of DTNB of 13,600M/cm at 412 nm.

Protein was estimated by the method of Lowry *et al.* (136) using bovine serum albumin fraction V as standard.

Statistical analysis

In this thesis all parameters were studied in a minimum of 6 rats for statistical validity. Values are expressed as Mean ± SD. Data were statistically analyzed for differences between control and monocrotophos treated animals by the Kruskal Wallis test using SPSS statistical software version 11.0. A probability of < 0.05 was considered significant.

2.2 Methods to study metabolic energy available for skeletal muscle function associated with acute severe monocrotophos poisoning

This study examined muscle weakness in relation to blood glucose levels and its regulation by gluconeogenesis, insulin, the pancreas and corticosteroids, in rats subject to acute severe monocrotophos poisoning. Gluconeogenesis was studied by estimating the activities of the liver enzymes, glucose-6-phosphatase and fructose-1,6-bisphosphatase, and by the levels of plasma glucogenic amino acids. Branched chain amino acids in the muscle were also determined in the study.

Hyperglycemia in the critically ill patient is associated with poor outcome. If hyperglycemia compromises energy supply and glycemc substrates in the muscle that leads to muscle weakness during severe monocrotophos poisoning, it can be treated.

CHEMICALS

Glucose oxidase, horseradish peroxidase, 4-amino-phenazone, glucose-6-phosphate, fructose-1,6-bisphosphate, corticosterone, HEPES and all amino acids were from Sigma Chemical Company, USA, isooctane was from British Drug House, UK, trichloroacetic acid (TCA), β - mercaptoethanol and *o*-phthalaldehyde were from Sisco Research Laboratories Pvt., Ltd., India. Rat Insulin ELISA kit was from Merckodia AB, Uppsala, Sweden. All other chemicals were of the highest purity available.

METHODS

Estimation of Blood glucose

Plasma glucose was estimated by the glucose oxidase - peroxidase method of Trinder (138). In a reaction volume of 1ml, 100mM phosphate buffer pH 7.0, 1.8 units glucose oxidase, 1 unit peroxidase, 1mg phenol, 0.16 mg 4-amino phenazone and 200 μ l plasma (15 fold diluted) were incubated for 20min at 37°C, cooled to 25°C and

absorbance measured at 510nm. Samples were read against a glucose standard prepared in benzoic acid and linear between 5-50 µg of glucose.

Insulin assay

Plasma insulin was assayed using a commercial ELISA kit specific for the rat hormone following the manufacturer's instructions. Samples were read against an insulin standard linear between 0-3.0µg / lit.

Corticosterone assay

Free plasma corticosterone was assayed fluorimetrically by the method of Glick *et al.* (139). Corticosterone in 100µl plasma was extracted with 900µl isooctane : chloroform (1:2). The lower chloroform layer was collected, 50µl 4N NaOH added, mixed well and the upper layer of NaOH removed. To 500µl of the lower chloroform layer 200µl ethanolic sulphuric acid (1.75ml ethanol + 5.0ml H₂SO₄) was added, mixed well and the upper chloroform layer removed. The lower ethanolic layer was incubated in the dark at 25°C for 90 minutes and corticosterone measured fluorometrically at λ_{ex}462nm/λ_{em}512nm. Samples were read against a corticosterone standard linear between 100-500ng.

Hepatic glucose-6-phosphatase and fructose-1,6-bisphosphatase assays

Liver glucose-6-phosphatase and fructose-1,6-bisphosphatase were assayed by the method of Wallert and Majumder measuring liberated Pi (140,141).

Liver was homogenized in 6 volumes of 250mM sucrose-HEPES buffer pH 7.0, the homogenate centrifuged at 1000g for 10min at 4°C and the supernatant re-centrifuged at 14500g for 1 hour at 4°C. Glucose-6-phosphatase and fructose-1,6-bisphosphatase were assayed in the supernatant.

Glucose-6-phosphatase was assayed in a reaction mixture containing 100µl of 430mM malate buffer pH 6.0, 300µl water, 70µl of 100mM glucose-6-phosphate and 100µl liver supernatant and incubated for 30 min at 37°C. The reaction was stopped by the addition of 1.3ml 10% TCA, spun and the supernatant assayed for Pi by the method of Goldenberg *et al.* (142) using TCA- iron reagent and ammonium molybdate.

Fructose-1,6,-bisphosphatase was assayed in a reaction mixture containing 100mM Tris HCl pH 7.6, 150mM KCl, 0.1mM fructose-1,6-bis phosphate and 100µl of liver homogenate supernatant, incubated at 37°C for 30 min and the reaction was stopped with 250µl of 25% TCA. The supernatant was assayed for Pi as given by Goldenberg *et al.* (142). Pi in the samples was read against a potassium dihydrogen phosphate standard.

Muscle branched chain amino acids and plasma glucogenic amino acids

Skeletal muscle was homogenized in 10 volumes of 6% TCA, centrifuged at 10,000g for 30 minutes, the supernatant diluted 2fold with 67mM sodium citrate buffer pH 2.2, filtered through 0.22µm Millipore filters.

Plasma samples were deproteinized with equal volumes of 5% TCA for 1 hour at 25°C and centrifuged at 450g for 10 minutes. The deproteinized supernatants were diluted 5-fold with 67mM sodium citrate buffer pH 2.2, filtered through 0.22µm Millipore filters. Amino acids were analysed in 10µl of the filtrate by HPLC by adsorption to a strong cation exchanger and elution according to their isoelectric pH over the pH range 3.2 to 10.0 as given by Ishida *et al.* (1981) (143).. The HPLC system used was Shimadzu LC-20AD.

Amino acids were adsorbed at pH 2.2 to a Na⁺ exchanger at 55°C equilibrated in 67mM sodium citrate buffer pH 3.2 and eluted with the same buffer for 23 minutes followed by 59 minutes of 0.2M citrate borate buffer pH 10.0. at a flow rate of 0.3ml/minute. The column was regenerated with 0.2N NaOH for 5 minutes.

α -amino groups of eluted amino acids were derivatized post column with 0.6mM *o*-phthalaldehyde, 30mM β -mercaptoethanol, 1.4% ethanol, 0.04% Brij-35 in 0.384M sodium carbonate, 0.216 boric acid and 0.108M potassium sulphate at a flow rate of 0.3ml/minute. The fluorescent adduct formed was read by excitation at 350nm and emission at 450nm.

Standard graphs of each amino acid, assayed as given, were constructed and used to quantify amino acids in the samples.

Statistical analysis of the data was carried out as given in Section 2.1.

2.3 Methods to study muscle injury associated with acute severe monocrotophos poisoning

This study examined muscle injury, estimated by plasma creatine kinase (CK) levels, in rats subject to acute severe monocrotophos in relation to the development of muscle weakness and recovery of muscle power. This was to understand the role of muscle injury in contributing to the outcome of acute OPP.

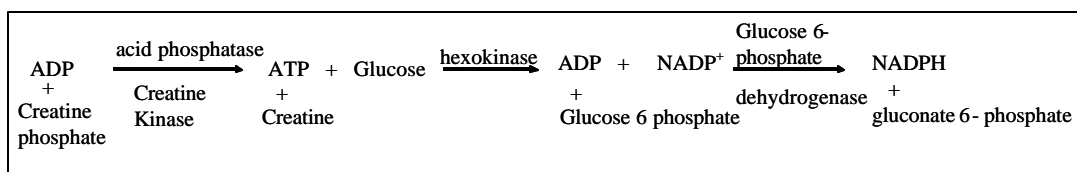
CHEMICALS

Creatine phosphate, adenosine diphosphate (ADP), hexokinase, glucose-6-phosphate dehydrogenase and glutathione were obtained from Sigma Aldrich Inc, USA, nicotinamide adenine dinucleotide phosphate (NADP) were from Sisco Research Laboratories Pvt. Ltd., India. All other chemicals were of the highest purity available.

METHODS

Creatine kinase assay

Creatine kinase was assayed as given by Kavanagh *et al* (144) coupling the reaction of creatine kinase to hexokinase and glucose-6-phosphate dehydrogenase and measuring the reduction of NADP at 340nm, as given :



The assay was carried out by pre-incubation for 5 minutes at 25°C of a reaction mixture of 0.1M Tris HCl pH 6.9, 10mM glutathione, 0.8mM NADP, 20mM glucose, 1mM ADP, 10mM MgCl₂, hexokinase and glucose-6-phosphate dehydrogenase 1unit/ml and 10µl plasma in 1.0ml. The reaction was started by the addition of 20mM creatine phosphate and monitored at 340nm for 5 minutes in a Shimadzu UV 1800 spectrophotometer.

Enzyme units were expressed as mmoles NADP reduced/dl based on the molar extinction co-efficient of NADPH 6,220/M/cm at 340nm.

Statistical analysis of the data was carried out as given in Section 2.1.

2.4 Methods to study protein degradation of skeletal muscle associated with acute severe monocrotophos poisoning

Mechanisms of protein degradation that lead to muscle weakness and loss have not been studied in acute OPP. This study examined protein degradation in skeletal muscle of rodents subject to acute OPP. Severe OPP induces muscle weakness and paralysis in rodents but the animals recover muscle power rapidly. Although muscle weakness is not a major contributor to morbidity of OPP in rodents, the occurrence of

paralysis and severe weakness suggest the possibility of muscle protein breakdown. The severity and period of degradation may be insufficient to promote muscle loss in rodents. We suggest that loss of muscle mass may occur in rodents if protein degrading mechanisms are more severe and sustained. Delineation of these pathways may indicate those responsible for loss of muscle mass in humans.

In this study, protein degradation of skeletal muscle was studied by estimating muscle, blood and urine amino acids as markers of degradation, metabolic acidosis as a catabolic stimulus and the ubiquitin - proteasome pathway as the proteolytic mechanism in rats subject to acute severe monocrotophos poisoning. Muscle myosin-actin stoichiometry was determined as an index of protein breakdown and muscle weakness.

CHEMICALS

o-phthalaldehyde, β -mercaptoethanol, Lactate dehydrogenase, NAD, bisacrylamide and glycine were from Sisco Research Laboratories Pvt. Ltd. India, ethanol from Hayman Limited, England, acrylamide, sodium lauryl sulphate, diaminobenzidine, mouse monoclonal ubiquitin antibody, 3-methyl histidine, lithium lactate, were from Sigma Chemical Co., USA. Amidoblack – 10B and hydrogen peroxide were from Merck & Co., Inc, USA, Tween-20 from Calbiochem, USA, Nitrocellulose membranes from Millipore Corp., USA, biotinylated anti-mouse IgG, streptavidin peroxidase, and protein molecular weight markers (3-205 kDa) were from Genei Pvt, Ltd, Bangalore, India. All other chemicals were analytical grade.

METHODS

Analysis of amino acids in blood and muscle and 3-methylhistidine in urine

Skeletal muscle was homogenized in 10 volumes of 6% TCA, centrifuged at 10,000g for 30 minutes and the supernatant diluted 2fold with 67mM sodium citrate buffer pH 2.2 (149). Ten microlitres of this sample was subject to amino acid analysis by HPLC as given in Section 2.2.

Plasma and urine were deproteinized with equal volumes of 5% TCA for 1 hour at 25°C and centrifuged at 450g for 10 minutes. The deproteinized supernatants were diluted 5-fold with 67mM sodium citrate buffer pH 2.2, filtered through 0.22µm Millipore filters and 10µl subject to HPLC amino acid analysis as given in Section 2.2.

3-methylhistidine in deproteinized urine (10µl) was analyzed by HPLC as given in Section 2.2 with the following modifications : adsorption was at pH 2.2 to the Na⁺ exchanger equilibrated in 0.2M sodium citrate pH 7.0 at 37°C. Elution was with the same buffer and occurred at 21 minutes.

Urinary creatinine was estimated by the method of Jaffe (145) under conditions standardized by Bonsnes and Taussky (1945). Urinary proteins were precipitated by incubation of 25µl urine with 2% sodium tungstate and 0.26N sulphuric acid in a volume of 1.25ml for 10 minutes at 25°C and spun at 1000g for 5 minutes. Five hundred microlitres of the supernatant were incubated with 0.01M picric acid and 0.19N sodium hydroxide in 1.0ml for 15 minutes at 25°C and creatinine picrate measured at 505nm. Samples were read against a creatinine standard that averaged 6 independent experiments and run under similar conditions.

Blood lactate

Blood was deproteinised immediately after collection with an equal volume of 6% perchloric acid and spun at 2000g for 10minutes. Lactate was assayed by following the oxidation of lactate to pyruvate by lactic dehydrogenase and measuring the reduction of NAD at 340nm (146). In a reaction of 1.0ml, 280nM hydrazine, 467mM glycine, 2.6mM EDTA, 2.5mM β -NAD and 50 μ l of deproteinised sample were incubated at 25°C for 5 minutes to stabilize the reaction and the reaction started by addition of 500 Units L-lactate dehydrogenase. The increase in absorbance at 340nm was followed for 5 minutes and samples were read against a lactate standard.

SDS-PAGE of muscle proteins

Skeletal muscle was homogenised in 19 volumes of 0.125M Tris HCl pH 7.4, 10% glycerol, 4% SDS, 4M urea, 10% β -mercaptoethanol and 0.01% bromophenol blue. The homogenate was heated at 100°C for 3 minutes, cooled to 25°C, centrifuged at 3500g for 5 minutes and the supernatant (30 μ g protein) subject to SDS-PAGE (3.5-12% gels) at 100V (147). Molecular weight markers (9 μ g) dissociated with SDS were run in parallel.

Gels were stained for protein with 0.01% Coomassie brilliant blue-R 250 in 50% methanol : 10% glacial acetic acid for 1 hour and destained with 50% methanol : 10% glacial acetic acid for 1 hour (148).

Gels were image analyzed for total protein with software developed in-house that correlated with Bio-Rad Quantity One Quantitation software version 4 ($r = 0.8$), and for actin and myosin heavy chain with Scion software. Intensities were measured as arbitrary units.

Actin and myosin heavy chain were identified by their molecular weights (43 Kda and 200 Kda) determined from molecular weight standards run on the same gels.

Immune blots of ubiquitinated muscle proteins

Following SDS-PAGE, the proteins were electrotransferred to nitrocellulose membranes at 100V for 1 hour at 4°C. Membranes were blocked with 5% non-fat milk powder in PBS/0.02% Tween 20 (PBST) for 1 hour at 25°C. Blocked membranes were incubated with mouse monoclonal anti-ubiquitin antibodies (1:1000 diluted) for 12 hours at 4°C followed by biotinylated anti-mouse IgG (1:1500 diluted) for 2 hours at 25°C and streptavidin - peroxidase (1:2000) for 1 hour. Blots were washed between steps with PBST for 3x10 minutes. Bound peroxidase was developed with 0.024% H₂O₂ / 0.066% diaminobenzidine in Tris buffer saline pH 7.6 for 10 minutes at 25°C (175). Blots were washed with water, dried and image analyzed for ubiquitinated proteins with Scion software.

Post transferred gels were stained with Coomassie brilliant blue -R 250 (148) and image analyzed for myosin heavy chain with Scion software. The ubiquitin intensities of each lane were correlated to their myosin heavy chain intensity to correct for loading.

Statistical analysis

Statistical analysis of the data was carried out as given in Section 2.1.

Urinary 3-methylhistidine levels were analyzed for differences between samples of the same animal at different times post poisoning by the Friedman test using the statistical software package SPSS 11.0. A probability of < 0.05 was considered significant.

Chapter 3

Results

3.1 Plasma butyrylcholinesterase and muscle acetylcholinesterase activity associated with acute severe monocrotophos poisoning

Clinical profile of monocrotophos poisoning in rats

Rats orally administered 0.8LD₅₀ monocrotophos rapidly developed muscarinic and nicotinic symptoms of poisoning and muscle weakness (Table 3.1.1).

The nicotinic symptom of chewing developed within a mean of 8 minutes of poisoning and continued for a mean duration of 15 minutes. Body tremors occurred within a mean of 15 minutes after poisoning and continued for a mean of 125 minutes after poisoning.

Muscarinic symptoms of salivation and lacrimation occurred within a mean of 24 and 29 minutes after poisoning and continued for a mean of 52 and 53 minutes respectively.

The temporal profiles of progression and recovery from these symptoms of monocrotophos poisoning are given in Table 3.1.1.

Skeletal muscle weakness

Ataxic gait (Grade 1 muscle power) was observed in the rats within a mean of 21 minutes after poisoning which progressed to Grade 2 power in 10 minutes. Paralysis (Grade 3 power) occurred in the rats within a mean of 45 minutes of poisoning and continued for a mean of 19 minutes (Table 3.1.2). Eight hours after poisoning, the rats regained muscle power and were normal 24 hours later.

In rodents nicotinic symptoms were the earliest to manifest (chewing) and continued for the longest duration (body tremors) in the early phase of monocrotophos poisoning. Severe muscle weakness was accompanied by both muscarinic and nicotinic effects (Figure 3.1.1).

Cholinesterases

Plasma BuChE was significantly inhibited early in poisoning, in rats administered 0.8LD₅₀ monocrotophos (Table 3.1.3). Two and a half hours and 24 hours after poisoning the enzyme was inhibited 70% and 90% respectively compared to controls (p<0.05). One week later, the enzyme was still significantly inhibited to 35% of normal (p<0.05). Plasma BuChE levels returned to normal 2 weeks after poisoning.

Muscle AChE was inhibited 75% and 63% respectively 2.5 and 24 hours after poisoning compared to non-poisoned controls (p<0.05). One week after poisoning, muscle AChE levels returned to 87% of normal activity and remained active over the following one month (Table 3.1.4).

The rapid inhibition of plasma BuChE and muscle AChE during the first day of poisoning in relation to the slow recovery of enzyme activities following monocrotophos poisoning are depicted in Figure 3.1.2 and Figure 3.1.3 respectively.

3.2 Metabolic energy available for skeletal muscle function associated with acute severe monocrotophos poisoning

Blood glucose, insulin and corticosterone levels following monocrotophos poisoning

Plasma glucose levels increased significantly in rats 2.5 hours after monocrotophos administration and returned to normal 8 hours later (Table 3.2.1).

Insulin levels in plasma decreased significantly in rats 2.5 hours after monocrotophos administration and returned to normal 8 hours later (Table 3.2. 1).

Plasma corticosterone levels in rats subject to monocrotophos poisoning did not differ from normal (Table 3.2.1).

Pancreas pathology

The pancreas of monocrotophos administered rats showed depletion of eosinophilic granules and prominent cytoplasmic basophilia 2.5 hours after poisoning. Eight hours after poisoning an intermediate degree of eosinophilic granules and cytoplasmic basophilia indicated improvement of the pancreas. The pancreas showed normal eosinophilic granules and no significant basophilia similar to control pancreas 24 hours after poisoning (Figure 3.2.1).

The pancreatic duct showed no secretions in the lumina 2.5 hours after poisoning, scanty secretions at 8 hours and normal secretions 24 hours after poisoning. (Figure 3.2.2)

Pancreatic islet cells did not exhibit any pathological change subsequent to monocrotophos poisoning.

Hepatic glucose-6-phosphatase and fructose-1,6-bisphosphatase

Hepatic activities of glucose-6-phosphatase and fructose-1,6-bisphosphatase were not affected by monocrotophos poisoning in rats (Table 3.2.2).

Amino acids

The levels of plasma glucogenic amino acids, alanine, arginine, histidine, proline, methionine, valine, aspartic acid, glycine, serine, isoleucine, phenylalanine and tyrosine were normal in rats over the first 24 hours of monocrotophos poisoning (Table 3.2.3)

Branched chain amino acids valine, leucine, and isoleucine, in the muscle increased significantly 2-fold 8 hours after monocrotophos poisoning and then returned to normal (Table 3.2.3).

3.3 Muscle injury associated with acute severe monocrotophos poisoning

Plasma creatine kinase levels increased significantly in rats treated with 0.8LD₅₀ monocrotophos 2.5, 8 and 24 hours after treatment. Levels increased 3.5-fold 2.5 hours after treatment and were two fold increased 8 and 24 hours after monocrotophos treatment compared to control (Table 3.3.1). Plasma Creatine kinase levels were normal in rats 7 days after monocrotophos treatment.

3.4 Protein degradation of skeletal muscle associated with acute severe monocrotophos poisoning

Temporal profile of amino acids in rats following monocrotophos treatment

Levels of **free tyrosine in the muscle** increased a significant 1.5 fold 8 hours after monocrotophos administration and remained elevated for 24 hours. Levels returned to normal one week after poisoning (Table 3.4.1).

Plasma phenylalanine/tyrosine ratios were 1.8 fold significantly elevated 2.5 hours after monocrotophos administration. The ratios returned to normal within 8 hours of poisoning (Table 3.4.1).

Glucogenic amino acids, arginine, histidine, proline, methionine, valine, aspartic acid, alanine, glycine, serine, isoleucine, phenylalanine and tyrosine, were elevated 1.8 fold **in plasma** 24 hours after monocrotophos administration and remained elevated for one month (Table 3.2.3). The profile of elevation upto one week after poisoning is given in Figure 3.4.1.

Urinary 3-methylhistidine levels were significantly increased 4.8fold 2.5 hours after monocrotophos poisoning and continued to increase for 10 hours after poisoning. Decline in levels but not to normal occurred 24 hours after poisoning (Table 3.4.2).

Temporal profile of plasma lactate in rats following monocrotophos treatment

Plasma lactate increased significantly 2 fold over control levels 2.5 hours after monocrotophos administration, remained elevated for 24 hours and were normal one week later (Table 3.4.3).

Temporal profile of muscle proteins and actin and myosin in rat following monocrotophos treatment

SDS-PAGE protein profiles of muscle homogenates did not differ between poisoned and non-poisoned animals or between muscles at different times after monocrotophos administration as shown in Figure 3.4.2.

Total protein and the myosin heavy chain to actin ratio of muscle, determined from SDS-PAGE Coomassie blue stained protein profiles, did not differ between poisoned and non-poisoned animals or between muscles at different times after monocrotophos administration. (Table 3.4.4).

Temporal profile of protein conjugated ubiquitin levels in rat skeletal muscle following monocrotophos treatment

A representative immune blot of ubiquitinated skeletal muscle proteins from rats subject to acute monocrotophos poisoning (0.8LD₅₀ dose) is shown in Figure 3.4.3. Densitometric image analysis of 6 blots indicated the levels of ubiquitinated proteins in rat muscle increased 2.5 hours after monocrotophos treatment compared to normal controls. The levels increased to a significant 33% 24 hours after monocrotophos administration and further to 65% for 2 weeks after poisoning. Conjugated ubiquitin levels were normal one month after poisoning (Figure 3.4.4).

Chapter 4

Discussion and Conclusion

Plasma butyrylcholinesterase and muscle acetylcholinesterase activity associated with acute severe monocrotophos poisoning

Rats subject to acute, severe MCP poisoning rapidly manifested cholinergic symptoms and muscle weakness indicating rapid inhibition of muscle AChE.

Significant AChE inhibition at the neuromuscular junction was associated with severe muscle weakness and paralysis in the rat and occurred early in the poisoning. The study demonstrated that 40% of AChE activity was sufficient to permit muscle to repolarize and recover from paralysis in rodents. The near normal recovery of muscle AChE activity one week after monocrotophos administration with no further inhibition for a month would indicate the absence of monocrotophos in the rodent.

The inhibition profile of plasma BuChE followed that of muscle AChE in the monocrotophos poisoned rat with significant inhibition early in poisoning, soon after paralysis. There was recovery of BuChE of activity, although not to normal, within a week of poisoning. The progressive inhibition of plasma BuChE during the first day of poisoning may reflect the presence of monocrotophos in the blood. Removal of monocrotophos from circulation by binding to BuChE and other plasma proteins, during the first day of poisoning, may prevent it from reaching the muscle and inhibiting muscle AChE further. This removal of monocrotophos from circulation may avert persistent inhibition of muscle AChE and permit recovery of AChE activity, but is responsible for inhibition of plasma BuChE during this post-poisoning period. The marked recovery of BuChE activity a week after poisoning would imply clearance of monocrotophos from the rodent during this period. These findings would suggest that following acute monocrotophos poisoning in rats, inhibition of plasma BuChE may be used as a surrogate marker for muscle AChE inhibition / activity while plasma BuChE activity reflects pesticide clearance.

Inhibition of AChE that leads to acetylcholine hyper-stimulation and persistent depolarization of the muscle is the primary cause of muscle weakness as a result of severe organophosphate poisoning (150). Muscle power recovers with recovery of AChE activity. In the absence of treatment to restore active AChE, rapid recovery of rodents from monocrotophos induced muscle weakness may occur due to spontaneous reactivation of dimethoxy phosphorylated AChE (151), efficient detoxification of organophosphates (151) and rapid binding of monocrotophos to plasma carboxylesterase that prevent it from reaching the neuromuscular junction (152). In an environment where organophosphates are rapidly removed, *de novo* synthesis of AChE may also contribute to activity, especially with increased time after poisoning (153). Reactivation of inhibited AChE and quick removal of organophosphate in rodents may protect muscle AChE from persistent inhibition, which may be important in preventing prolonged neuromuscular weakness. Catalytic bioscavenging, using recombinant butyrylcholinesterase or paraoxonases, to hasten elimination of organophosphates from the system and prevent severe inhibition of AChE, is being considered in treatment of OPP patients (154,155).

Metabolic energy available for skeletal muscle function associated with acute severe monocrotophos poisoning

Severe muscle weakness following cholinergic hyperactivity in the early phase of monocrotophos poisoning in rodents was accompanied by elevated blood glucose levels. High blood glucose levels were possibly a result of decreased insulin production and not due to increased gluconeogenesis or an effect of glucocorticoid hormones. Although pathological abnormalities of islet cells could not be demonstrated in monocrotophos poisoning, degranulation of the pancreas may have led to low insulin secretion as circulating insulin levels returned to normal with

recovery of the pancreas from pathological abnormalities. This was associated with lowering of blood glucose to normal and recovery of muscle power. This also indicated that the poisoning did not induce insulin resistance in rats.

It is important to remember that rodents do not exhibit prolonged muscle weakness and that they recover spontaneously from monocrotophos poisoning with no treatment. We suggest that this recovery of muscle strength is due to the ability to maintain critical levels of active muscle AChE that enable repolarization of the paralyzed muscle. Our studies also suggest that recovery may be aided by the rapid reversal of pancreatic pathology induced by monocrotophos poisoning and normalization of insulin secretion that ensures adequate glucose uptake by muscle. Abundant basophilia 2.5 hours and 8 hours after poisoning indicates resynthesis of proteins (176, 177).

Acute pancreatitis is described as a complication of OPP resulting from cholinergic stimulation in the pancreas and ductular hypertension (156,157). There are several reports that show pancreatitis leads to hyperglycemia in patients severely poisoned with organophosphates and manifests early in poisoning (158,159). However, in a study of 79 OPP patients, Singh *et al.* (160) did not find evidence of acute pancreatitis or hyperglycemia in the majority of patients although serum amylase was mildly elevated in over 46% of the patients. In none of the studies was hyperglycemia contributing to muscle weakness of OPP explored.

If acute severe OPP induces pancreatic pathology and hypo-insulin secretion that limits glucose uptake by the muscle, especially during periods of muscle exhaustion, this may in part contribute to (prolonged) muscle weakness that occurs in humans. The inability to replenish necessary concentrations of glyceic substrates in the

muscle, required for ATP production and muscle contraction, would be important in the pathophysiology of prolonged muscle weakness that occurs in OPP.

Depletion of glucose in the muscle leads to the activation of alternate pathways of energy production, exemplified by the oxidation of branched chain amino acids. In rodents, the increase of branched chain amino acids during recovery of muscle strength may indicate a role for their oxidation in providing ATP to the muscle subsequent to monocrotophos poisoning.

Inhibition of muscle AChE is the primary event that leads to muscle weakness of OPP (83) but factors that accompany inhibition of the enzyme may characterize the varying phases of weakness, seen in different muscle groups as well as in different species. Depletion of glycemic energy substrates in the muscle may contribute to muscle weakness in rodents. Regain of muscle strength may follow from improvement in several factors in OPP rats. Our studies suggest that in rodents recovery of muscle strength is governed by preservation of critical levels of active muscle AChE, rapid reversal of pancreas pathology that enables normal insulin secretion and glucose uptake by the muscle, and possibly by energy supplied by branched chain amino acids.

Muscle injury associated with acute severe monocrotophos poisoning

In rodents as in humans (71), acute severe monocrotophos poisoning rapidly induced muscle injury and the severity of injury correlated to the severity of muscle weakness. Greatest injury in rodents was associated with muscle soon after paralysis when muscles were still very weak. Muscle injury decreased as rodents recovered muscle power although significant injury persisted during the early phase of recovery of muscle power. Complete recovery from muscle injury occurred in rodents only after

the muscle had returned to normal for a substantial period (i.e. 7 days after monocrotophos treatment).

It would appear that rodents, unlike humans, effectively defend against organophosphate toxicity. Events that occur early in poisoning limit the extent of muscle injury and prevent the development of prolonged muscle weakness in rats. This would contribute to prevention of muscle wasting. It is necessary to delineate events that occur in the early phase of OPP in rodent muscle, as they clearly differ in degree or nature from those that occur in humans, who manifest severe injury and prolonged muscle weakness that can lead to wasting.

Protein degradation of skeletal muscle associated with acute severe monocrotophos poisoning

Rodents do not undergo muscle loss when subject to monocrotophos poisoning at a dose of $0.8LD_{50}$. However, they rapidly manifest cholinergic symptoms and severe muscle weakness at this dose of monocrotophos and recover spontaneously.

Metabolic acidosis with raised glucocorticoids signal protein degradation in chronic renal failure (161,162). In monocrotophos poisoning, a combination of insulin insufficiency and metabolic acidosis, may stimulate muscle protein degradation in the early phase of poisoning although glucocorticoid levels are normal. Metabolic acidosis that accompanies muscle weakness in rodents subject to monocrotophos poisoning may contribute to protein breakdown that continues beyond the period of weakness.

In OPP, molecular events that occur in the early phase of poisoning are considered to affect the course and outcome of poisoning (71). The greater the magnitude of adverse early events, the lower the ability to recover from them, and the worse the outcome of poisoning. This is taken to partly explain the higher incidence of Type II paralysis

(intermediate syndrome) in severely poisoned patients. The nature of these events is not clear. The ability of rodents to recover from severe monocrotophos poisoning would suggest that early events in poisoning are limited in magnitude.

In rodents, the cellular milieu of the skeletal muscle that occurs in tandem with muscle weakness suggests that early events of acute severe monocrotophos poisoning support a catabolic state of protein breakdown. Protein degradation through the ubiquitin - proteasome pathway, elevated free muscle tyrosine, increased phenylalanine to tyrosine ratio in circulation and increased excretion of 3-methylhistidine, characterize the catabolic state. These catabolic reactions and parameters, although significantly increased during muscle weakness, were limited in their increase. Similar muscle protein profiles maintained through the course of poisoning may reflect the restriction of catabolic reactions in the early phase of poisoning.

Maintenance of myosin actin stoichiometry through the course of poisoning in rats indicates the myofibrillar proteins are protected from degradation. This may be an important consequence of restricted catabolism that enables recovery from muscle weakness induced by monocrotophos poisoning. Prolonged muscle weakness of OPP may arise when degradation of the actinomyosin complex occurs. The inability of patients to recover from organophosphate induced muscle weakness may occur when degradation of contractile proteins takes place.

Recovery of muscle power in rodents subject to monocrotophos poisoning was characterized by enhanced protein synthesis, as indicated by the decline in muscle free amino acid levels, despite continued protein degradation through the ubiquitin - proteasome pathway. Rise in circulating glucogenic amino acids during recovery of

muscle power that were sustained for considerable periods, was further indication of increased protein synthesis following monocrotophos poisoning.

In summary, the studies suggest that in rodents, acute monocrotophos poisoning leads to up-regulation of the ubiquitin - proteasome pathway possibly stimulated by metabolic acidosis and insulin insufficiency. Degradation of skeletal muscle proteins may contribute to muscle weakness of the poisoning. Spontaneous recovery of muscle strength may occur if contractile proteins are spared during poisoning.

Conclusion

Prolonged severe muscle weakness of Type II paralysis is a serious consequence of acute OPP that results in increased morbidity, mortality and high cost of treating patients. Type II paralysis may last for 1 – 3 weeks and is postulated to result from persistent inhibition of AChE at the neuromuscular junction. Paralysis from AChE inhibition is a result of a depolarized muscle membrane.

Muscle loss is a feature of acute severe organophosphate poisoned patients that may account for the slow reversibility of Type II paralysis. Immobility is well known to lead to muscle loss and weakness. In addition to weakness arising from the inhibition of AChE, the extended periods of immobility that accompany Type II paralysis may also contribute to muscle loss and weakness of acute OPP. Muscle loss is initially a slow process. However once degradation sets in, the process may be difficult to reverse. Muscle loss mainly occurs by the degradation of muscle proteins particularly through the ubiquitin - proteasome pathway.

Muscle weakness of acute OPP could therefore be a result of acetylcholine-induced depolarization of the muscle and immobility stress induced muscle wasting. Studies of muscle pathophysiology of acute OPP have mostly examined the effect of AChE inhibition. Only a few studies have addressed the cellular and metabolic basis of muscle weakness following acute organophosphate poisoning.

It is not known if muscle paralysis due to cholinergic overstimulation is in part due to depletion of ATP and the inability of the weak muscle to replenish sufficient ATP. It is also not known if ATP production in the muscle is affected by inadequate supply of glycemic substrates. The contribution of protein degradation to muscle weakness of acute OPP has also not been explored.

In these studies muscle weakness following acute severe monocrotophos poisoning was examined in rodents with regard to availability of glyceimic substrates and protein degradation in the muscle. The salient findings of the studies were:

1. Significant AChE inhibition at the neuromuscular junction was associated with severe muscle weakness and paralysis in the rat and occurred early in acute monocrotophos poisoning. Recovery of muscle power correlated to regaining enzyme activity. The prevention of persistent severe inhibition of AChE at the neuromuscular junction was important in preventing prolonged muscle weakness.
2. Acute monocrotophos poisoning rapidly injured the muscle and complete recovery from injury extended beyond recovery from poisoning.
3. Acute monocrotophos poisoning led to hyperglycemia because of pancreatitis and hypo-insulin secretion. Recovery of the pancreas enabled normoglycemia.
4. Muscle protein degradation, possibly stimulated by metabolic acidosis, may contribute to muscle weakness that occurs in acute monocrotophos poisoning. Sparing the contractile proteins from breakdown may have aided the rapid recovery of muscle power.

The overall conclusions of these studies are: (a) prolonged muscle weakness following acute OPP maybe prevented if AChE at the neuromuscular junction is protected from severe persistent inhibition. (b) Inhibition of AChE and immobility stress may contribute to muscle wasting and weakness of acute OPP. (c) Addressing metabolic insufficiency of glyceimic substrates and protein degradation in the muscle may prevent muscle weakness. The role of such interventions in reducing the duration and adverse outcome of Type II paralysis in acute OPP need to be explored.

Chapter 5

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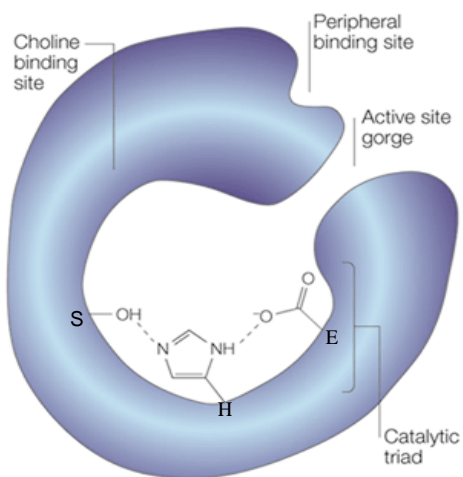
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Figure 1.1

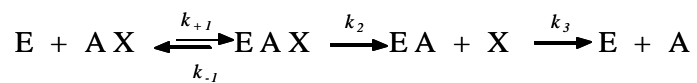
Schematic representation of the active site of acetylcholinesterase
(Modified from Ref 128)



The catalytic triad consists of the amino acids serine-203 (S), histidine-447 (H) and glutamic acid-334 (E). The acetylcholine moiety fits in the catalytic triad.

Figure 1.2

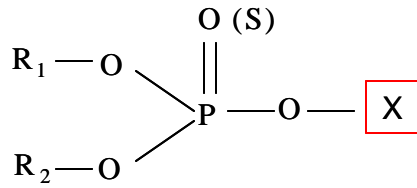
Mechanism of hydrolysis of acetylcholine by acetylcholinesterase (from Ref 129)



E - Enzyme, AX - Acetylcholine, EAX - Reversible enzyme complex, X - Choline,
A - Acetate

Figure 1.3

General structure of organophosphates



R1 and R2 – Alkthio, Alkoxy, Aryloxy

X – Halogen, Cyanide, Thiocyanate

Figure 1.4

The interactions of acetylcholine and organophosphates with the active site of acetylcholinesterase (From Ref 28)

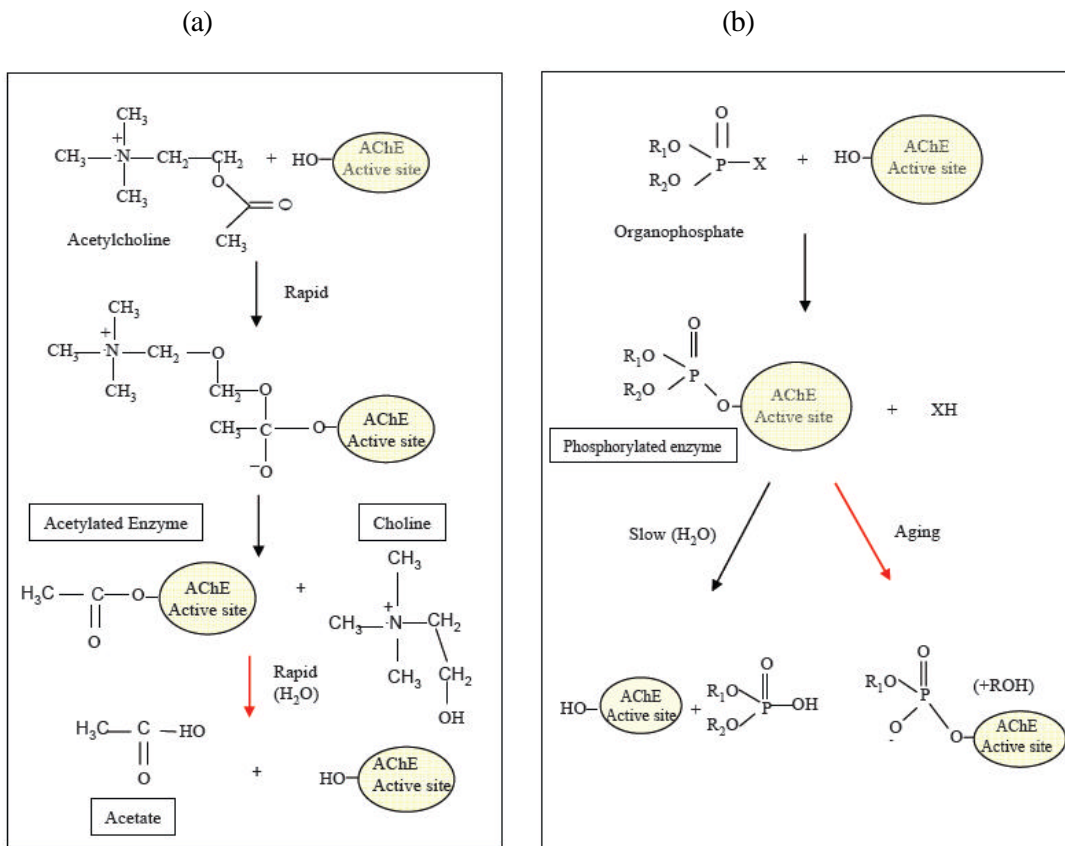


Table 1.1

Types of organophosphates (From Ref 24)

Type	Chemical structure	Examples
Phosphates	$\begin{array}{c} \text{O} \\ \\ \text{RO-P-OR} \\ \\ \text{OR} \end{array}$	Chlorfenvinphos, Dichlorvos Monocrotophos, Tri-o-cresyl phosphate
Phosphonates	$\begin{array}{c} \text{O} \\ \\ \text{RO-P-R} \\ \\ \text{OR} \end{array}$	Trichlorfon
Phosphinates	$\begin{array}{c} \text{O} \\ \\ \text{R-P-R} \\ \\ \text{OR} \end{array}$	Glufosinate
Phosphorothioates (S=)	$\begin{array}{c} \text{S} \\ \\ \text{RO-P-OR} \\ \\ \text{OR} \end{array}$	Bromophos, Diazinon Fenthion, Parathion Pirimiphos-methyl
Phosphonothioates (S=)	$\begin{array}{c} \text{S} \\ \\ \text{RO-P-R} \\ \\ \text{OR} \end{array}$	EPN Leptophos
Phosphorothioates (S=substitued)	$\begin{array}{c} \text{O} \\ \\ \text{RS-P-OR} \\ \\ \text{OR} \end{array}$	Demeton-S-methyl Echothiophate
Phosphonothioates (S-substitued)	$\begin{array}{c} \text{O} \\ \\ \text{RS-P-R} \\ \\ \text{OR} \end{array}$	VX Azinphos-ethyl Azinphos-methyl
Phosphorodithioates	$\begin{array}{c} \text{O} \\ \\ \text{RS-P-SR} \\ \\ \text{OR} \end{array}$	Dimethoate, Disulfoton
Phosphorotrithioates	$\begin{array}{c} \text{O} \\ \\ \text{RS-P-SR} \\ \\ \text{SR} \end{array}$	DEF (tribufos)
Phosphoramidates	$\begin{array}{c} \text{O} \\ \\ \text{RO-P-N} \begin{array}{l} \swarrow \text{R} \\ \searrow \text{R} \end{array} \\ \\ \text{OR} \end{array}$	Fenamiphos
Phosphoramidothioate	$\begin{array}{c} \text{S} \\ \\ \text{RO-P-N} \begin{array}{l} \swarrow \text{R} \\ \searrow \text{R} \end{array} \\ \\ \text{OR} \end{array}$	Methamidophos Isofenphos
Phosphorofluoridates	$\begin{array}{c} \text{O} \\ \\ \text{RO-P-F} \\ \\ \text{OR} \end{array}$	Diisopropylphosphorofluoridate (DFP)
Phosphonofluoridates	$\begin{array}{c} \text{O} \\ \\ \text{RO-P-F} \\ \\ \text{R} \end{array}$	Cyclosarin, Sarin, Soman

Figure 1.5

CYP 450 Oxidation pathways for parathion. (Ref 130)

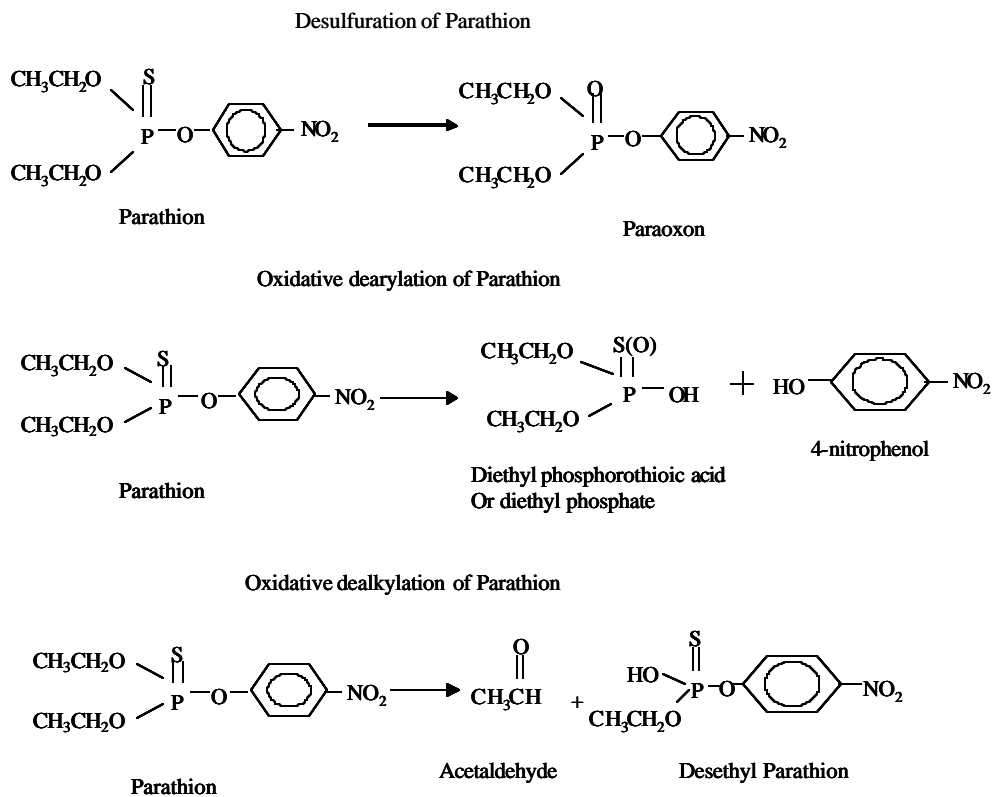


Figure 1.6

Hydrolysis of paraoxon by A-esterases (Ref 131)

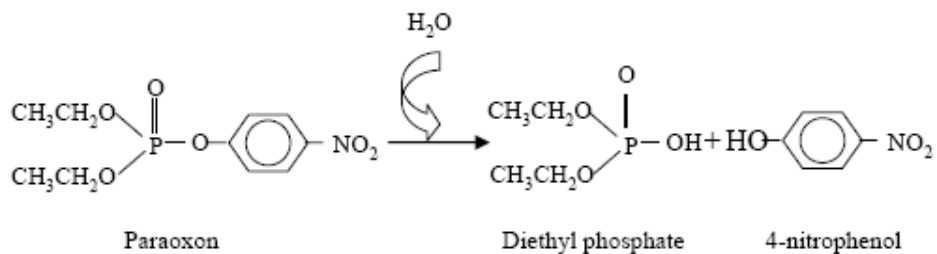
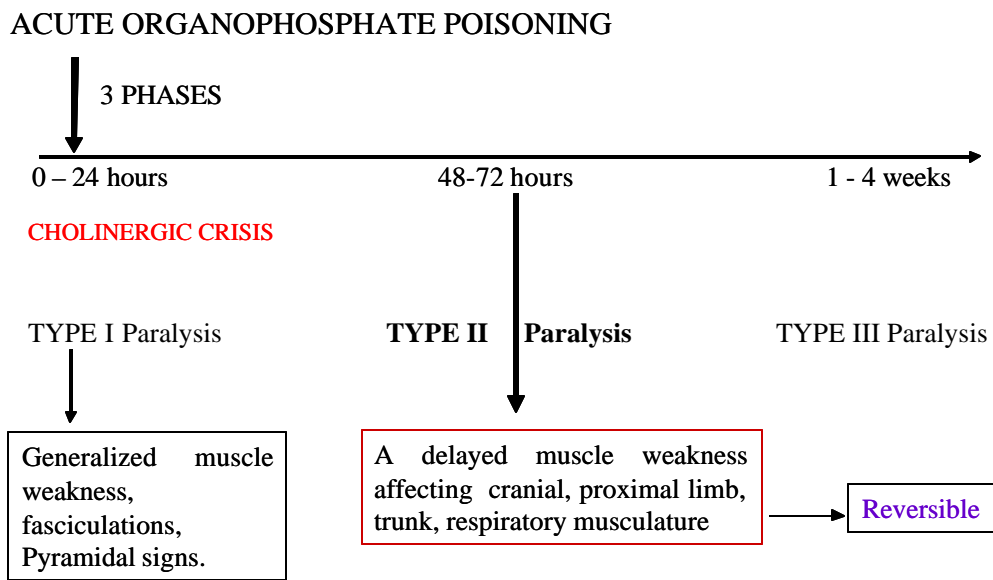


Figure 1.7

Temporal profile of acute organophosphate poisoning



Type II paralysis is the main cause of morbidity, mortality and high cost of treatment of acute organophosphate poisoning in hospital patients in India.

Figure 1.8

Muscle cells proliferation and differentiation (modified from Ref 132)

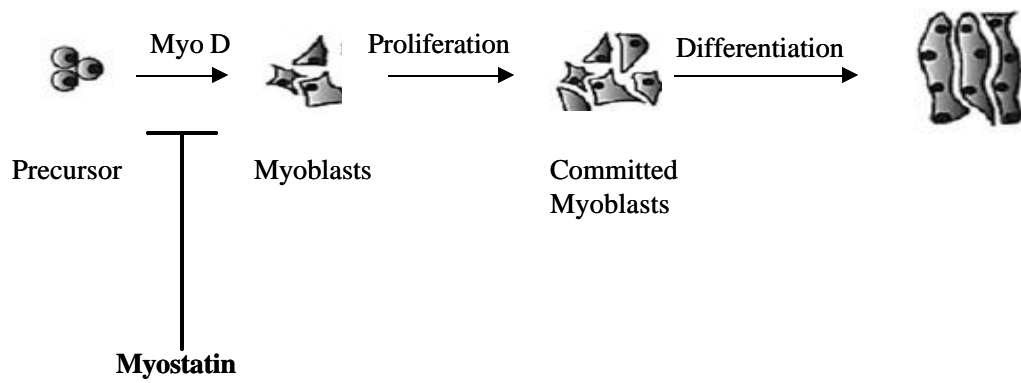
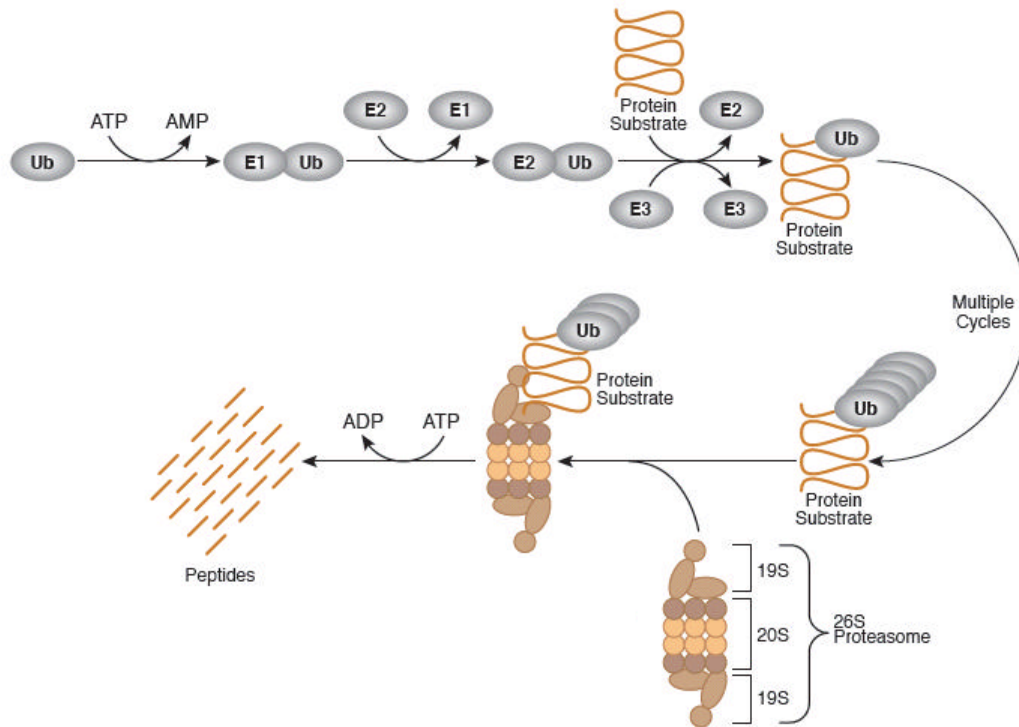


Figure 1.9
Ubiquitin-proteasome system of protein degradation



Ub - Ubiquitin, E1 - Ubiquitin activating enzyme, E2 - Ubiquitin conjugating enzyme, E3 - Ubiquitin ligase

A description of protein-ubiquitin conjugate and degradation of poly ubiquitinated proteins through the proteasome is given in the text.

Table 3.1.1

Progression and recovery from nicotinic and muscarinic symptoms of rats subject to monocrotophos poisoning.

Symptoms	Time of Symptom	
	Start (min) Mean \pm SD	Mean Duration (min)
n=18		
Nicotinic Symptoms		
Chewing	8 \pm 3	15
Body tremors	15 \pm 3	125
Muscaranic Symptoms		
Salivation	24 \pm 6	52
Lacrimation	29 \pm 10	53

Table 3.1.2

Progression of muscle weakness in rats following monocrotophos poisoning

Muscle power	Time (min)	
	Start (min) Mean \pm SD	Mean duration (min)
Grade I	21 \pm 4	10
Grade II	30 \pm 7	13
Grade III	45 \pm 10	19

Figure 3.1.1

Temporal profile of cholinergic symptoms and muscle power in rats following monocrotophos administration.

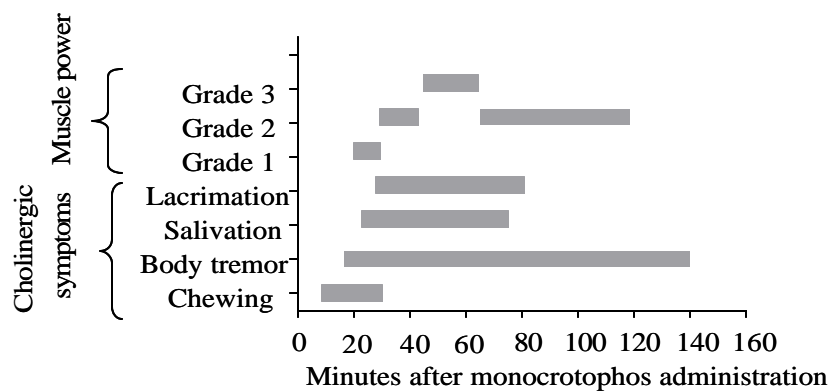


Table 3.1.3

Temporal profile of plasma BuChE activity following monocrotophos administration in rats

		Time after monocrotophos administration					
		0 hours	2.5 hours	24 hours	1 week	2 weeks	1 month
Plasma BuChE	Units/ml Mean	0.91	0.28	0.10	0.66	1.07	1.16
	± SD	± 0.12	± 0.02	± 0.07	± 0.12	± 0.06	± 0.20
n=6	p Compared to 0 hours		p< 0.01	p<0.001	p<0.05	NS	NS

Table 3.1.4

Temporal profile of muscle AChE activity following monocrotophos administration in rats

		Time after monocrotophos administration					
		0 hours	2.5 hours	24 hours	1 week	2 weeks	1 month
Muscle AChE	Units/mg protein Mean	8.66	2.33	3.71	7.03	6.68	7.86
	± SD	± 2.52	± 1.53	± 1.68	± 1.55	± 1.67	± 2.20
n=6	p Compared to 0 hours		p<0.01	p<0.05	NS	NS	NS

Figure 3.1.2

Temporal profile of plasma BuChE following monocrotophos administration in rats

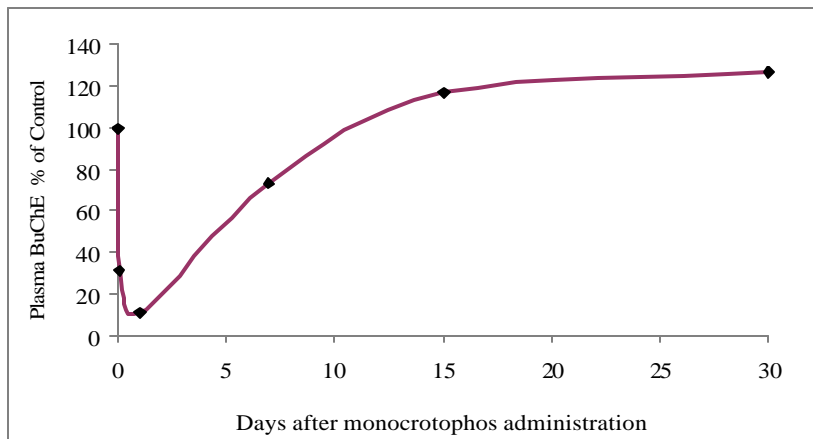


Figure 3.1.3

Temporal profile of muscle AChE following monocrotophos administration in rats

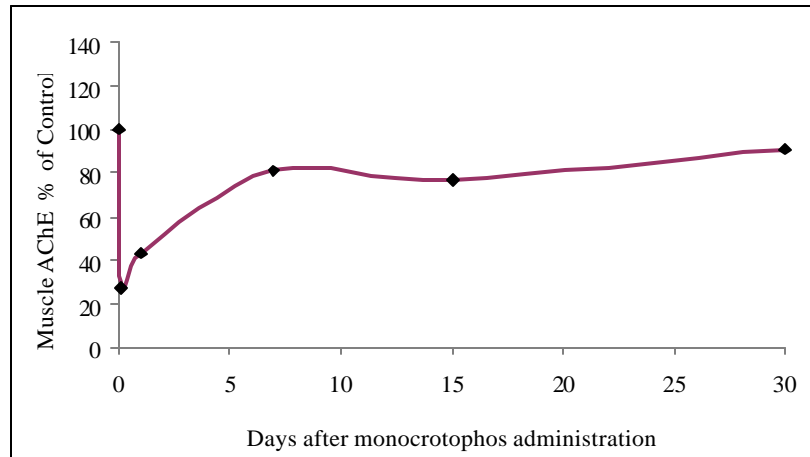


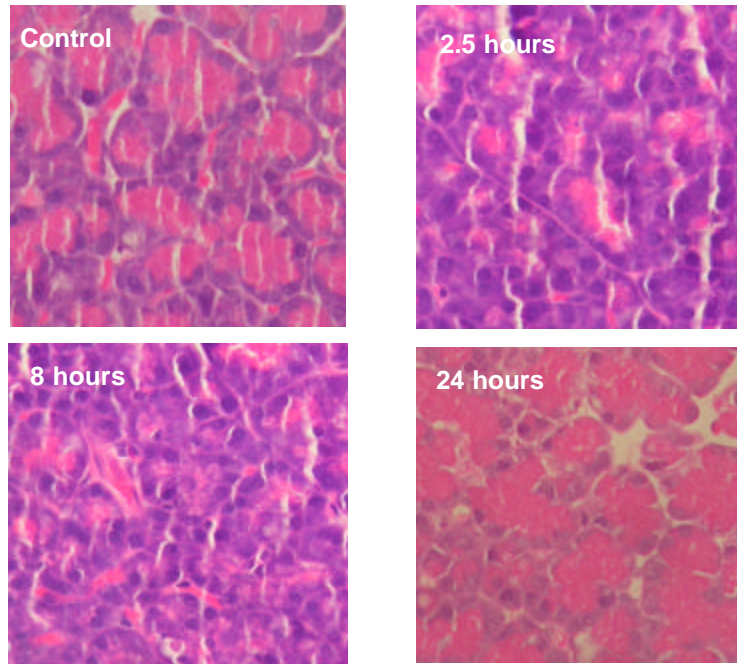
Table 3.2.1

Temporal profiles of blood glucose, insulin and corticosterone following monocrotophos administration in rats.

Time after monocrotophos administration (n=6)	Plasma glucose (mg/dl) Mean \pm SD	Plasma insulin (μ g/l) Mean \pm SD	Plasma corticosterone (μ g/dl) Mean \pm SD
0 hours	111 \pm 17	0.70 \pm 0.44	80 \pm 24
2.5 hours	198 \pm 59*	0.34 \pm 0.16*	97 \pm 44
8 hours	118 \pm 40	0.66 \pm 0.46	72 \pm 35
24 hours	117 \pm 30	0.79 \pm 0.39	85 \pm 64
Compared to 0 hours	* p<0.01	* p<0.05	NS

Figure 3.2.1

Histopathology of rat pancreas following monocrotophos administration

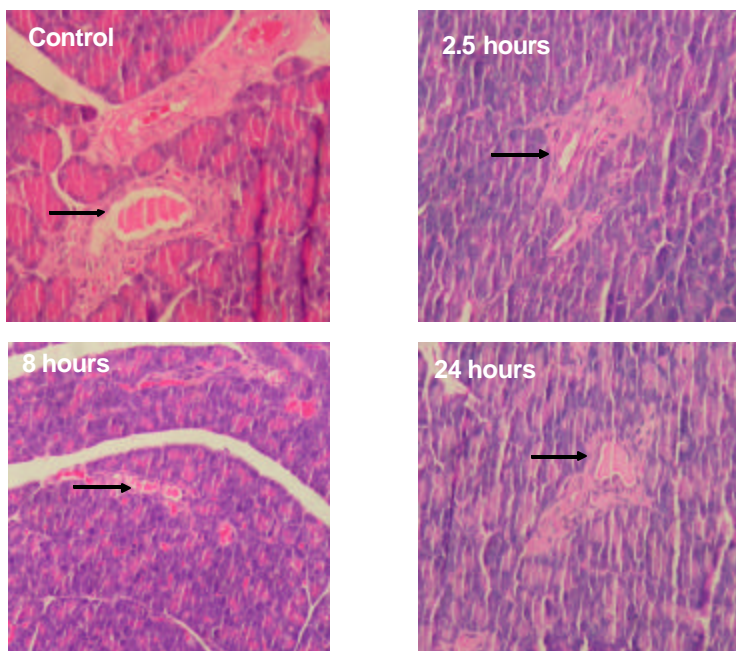


Rat pancreatic sections (3 μ m) stained with haematoxylin and eosin (x40 magnifications). Eosinophilic granules are stained pink and basophilic granules stained purple.

The sections indicate abundant eosinophilic granules and normal levels of basophilia in Controls, depletion of eosinophilic granules and prominent cytoplasmic basophilia 2.5 hours and 8 hours after monocrotophos administration, with recovery of eosinophilic granules and significant basophilia 24 hours after poisoning.

Figure 3.2.2

Histopathology of rat pancreatic ducts monocrotophos administration



Rat pancreatic sections (3 μ m) stained with haematoxylin and eosin (x40 magnification). Arrows indicate pancreatic duct.

Secretions in the lumina of the pancreatic duct are seen in Control pancreas. Absence of secretions 2.5 hours after poisoning, scanty secretions at 8 hours with recovery of normal secretions 24 hours after poisoning are noted.

Table 3.2.2

Temporal profiles of hepatic gluconeogenic enzymes following monocrotophos administration in rats.

Time after monocrotophos administration (n=6)	Glucose-6-phosphatase (Units/mg protein) Mean \pm SD	Fructose-1,6-bisphosphatase (Units/mg protein) Mean \pm SD
0 hours	0.1 \pm 0.02	0.22 \pm 0.05
2.5 hours	0.14 \pm 0.07	0.16 \pm 0.04
8 hours	0.07 \pm 0.06	0.17 \pm 0.02
24 hours	0.09 \pm 0.06	0.18 \pm 0.02

Table 3.2.3

Temporal profiles of plasma glucogenic and muscle branched amino acids following monocrotophos administration in rats.

Time after monocrotophos administration (n=6)	Plasma glucogenic amino acids (μM) Mean \pm SD	Muscle branched chain amino acids (nmoles/gm tissue) Mean \pm SD
0 hours	1240 \pm 444	355 \pm 52
2.5 hours	1095 \pm 348	327 \pm 89
8 hours	1579 \pm 333	615 \pm 105*
24 hours	2190 \pm 536*	308 \pm 67
1 week	1783 \pm 319	319 \pm 110
2 weeks	1740 \pm 320	405 \pm 103
1 month	1825 \pm 348	375 \pm 79
Compared to 0 hours	* p<0.02	* p<0.005

Table 3.3.1

Temporal profile of plasma creatine kinase following monocrotophos administration in rats

		Time after monocrotophos administration				
		0 hours	2.5 hours	8 hours	24 hours	1 week
Plasma creatine kinase	Units/dl Mean \pm SD	12.2 \pm 3.8	42.4 \pm 21.0	18.8 \pm 1.9	21.3 \pm 4.6	17.0 \pm 5.2
n = 6	p Compared to 0 hours		p< 0.05	<0.01	<0.01	NS

Table 3.4.1

Temporal profiles of muscle tyrosine and plasma phenylalanine tyrosine ratio following monocrotophos administration in rats.

Time after MCP administration (n=6)	Muscle tyrosine (nmoles/gm tissue) Mean \pm SD	Plasma Phenylalanine : tyrosine ratio
0 hours	81.5 \pm 35.0	1.34 \pm 0.38
2.5 hours	79.0 \pm 20.1	2.47 \pm 1.16*
8 hours	141.5 \pm 23.8*	1.49 \pm 0.62
24 hours	129.8 \pm 48.4	0.82 \pm 0.15
1 week	89.3 \pm 27.1	0.71 \pm 0.40
2 weeks	115.8 \pm 47.1	0.82 \pm 0.33
1 month	115.8 \pm 24.9	0.68 \pm 0.15
p Compared to 0 hours	*p<0.01	*p<0.01

Figure 3.4.1

Temporal profiles of plasma glucogenic amino acids following monocrotophos administration in rats.

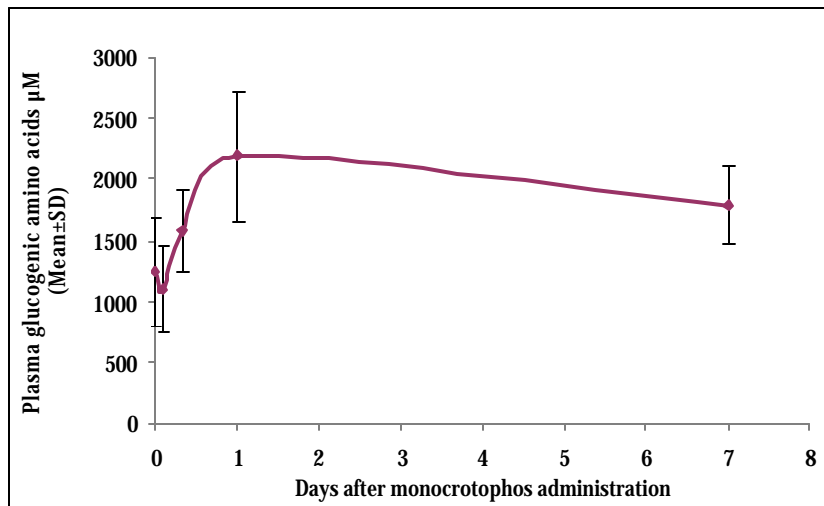


Table 3.4.2

Temporal profiles of urinary 3-methylhistidine following monocrotophos administration in rats

		Time after monocrotophos administration				
Unit		0 hours	2.5 hours	10 hours	24 hours	1 week
Urinary 3-methylhistidine	Mean \pm SD	35.4	171.8	249.7	121.0	50.8
	μ moles/gm creatine	\pm 17.8	\pm 127.1	\pm 166.9	\pm 96.1	\pm 20.9
n=6	p Compared to 0 hours		p<0.05	p<0.05	p<0.05	NS

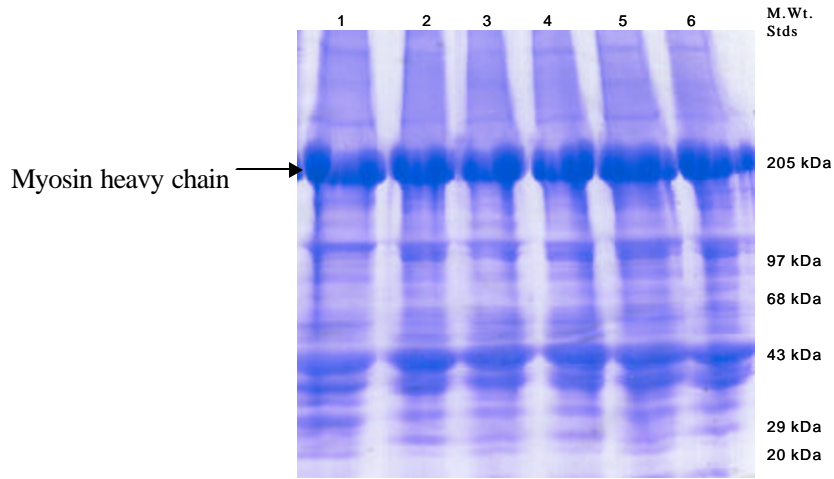
Table 3.4.3

Temporal profiles of blood lactate following monocrotophos administration in rats

		Time after monocrotophos administration				
Unit		0 hours	2.5 hours	8 hours	24 hours	1 week
Blood lactate	mM	1.41	2.78	2.72	2.74	1.48
	Mean \pm SD	\pm 0.12	\pm 0.79	\pm 0.89	\pm 1.3	\pm 0.27
n=6	p Compared to 0 hours		p<0.01	p<0.01	p<0.05	NS

Figure 3.4.2

Representative SDS-PAGE profiles of muscle proteins in rats following monocrotophos treatment.



Skeletal muscle proteins (100µg) of rats administered monocrotophos (0.8 LD₅₀) were subject to SDS-PAGE (3.5 – 12% gels) and gels stained with coomassie blue - R 250 as given in Section 2.4

Lane 1 - 0 hours, Lane 2 - 2.5 hours, Lane 3 - 24 hours, Lane 4 - 1 week, Lane 5 - 2 weeks,

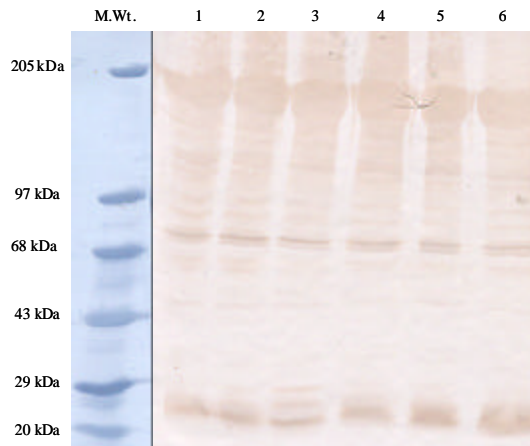
Table 3.4.4

Temporal profiles of total muscle proteins and myosin heavy chain to actin ratios following monocrotophos administration in rats - Densitometric image analysis of coomassie blue stained SDS-PAGE

		Time after monocrotophos administration					
n=6	Unit	0 hours	2.5 hours	24 hours	1 week	2 weeks	1 month
Muscle total protein	Intensity Arbitrary units	1531	1548	1547	1421	1622	1669
	Mean ± SD	± 184	± 251	± 42	± 387	± 367	± 104
Myosin heavy chain : actin	Intensity Arbitrary units	0.64	0.70	0.74	0.65	0.62	0.67
	Mean ± SD	± 0.13	± 0.04	± 0.13	± 0.06	± 0.12	± 0.21

Figure 3.4.3

Representative immune blot of the temporal profile of protein conjugated ubiquitins in rat skeletal muscle following monocrotophos administration.



Skeletal muscle proteins (100µg) of rats administered monocrotophos (0.8 LD₅₀) were subject to SDS-PAGE and immune blots for ubiquitinylated proteins as given in the section 2.4.

Lane 1 - 0 hours, Lane 2 - 2.5 hours, Lane 3 - 24 hours, Lane 4 - 1 week, Lane 5 - 2 weeks, and Lane 6 - 1 month after monocrotophos administration. M.Wt- Molecular weight standard (9µg)

Figure 3.4.4

Temporal profiles of muscle ubiquitinylated proteins following monocrotophos administration in rats-densitometric image analysis of immune blots

