

**INTERACTION STUDIES BETWEEN LINDANE (GAM MA LIN -20[®])
AND DIMETHOATE (ROGOR[®]) *IN VIVO* AND *IN VITRO*.**

By

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**A thesis in the department of Pharmacology and Clinical Pharmacy, Faculty of
Pharmaceutical Sciences, Ahmadu Bello University', Zaria, submitted to the
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degree of Doctor of Philosophy.**



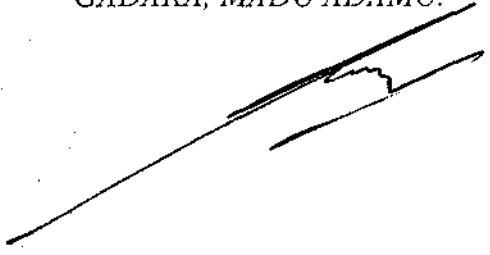
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I hereby declare that the work presented in this thesis was carried out by me and that no previous submission of its has been made for the award of higher degree. However, the works of other researchers are fully acknowledged and referred to accordingly.

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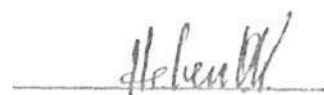
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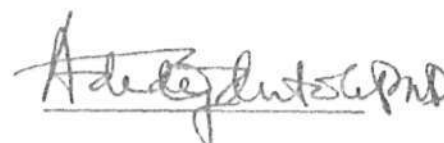
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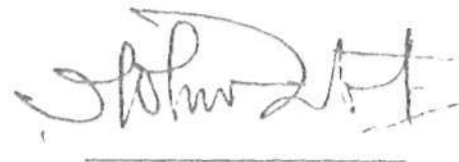
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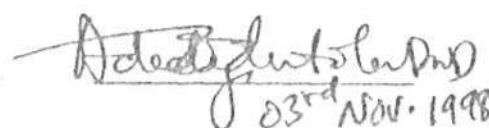
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DEDICATION

This work is dedicated to the loving memory of my late Mother, HAUWA ALII.
ADAMU DODO GADAKA, who passed on during the course of the work (21.7.96).
Adieu, Mama adieu!

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All praise is due to Allah (S.A.W.) my creator and sustainer and to whom I submit myself in total obedience. I thank you O Lord of the universe for bestowing on me the ability to persevere taking me thus far and lifting me this high. I pray you grant me the patience and courage to seek knowledge as long as I live. Amen!

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. ABSTRACT

The toxicity of lindane and dimethoate has been studied individually and in combination in laboratory animals both *in vivo* and *in vitro*. Effects of lindane pretreatment on the toxicity of dimethoate was examined in acute and sub-chronic toxicity studies. The median lethal dose (LD₅₀) for lindane and dimethoate were determined in wistar strain rats by intraperitoneal route as 29.5 mg/kg and 185 mg/kg respectively. Symptoms of poisoning for lindane (hyper-excitation, tremor, convulsions) and dimethoate (swaying gait, respiratory distress, convulsion) were dose-dependent in severity.

Lindane (1mg/kg, i.p) daily administration for seven days increased dimethoate LD₅₀ in rats by 29.5%. The survival time in mice exposed to lethal dose of dimethoate (400 mg/kg) was also increased by 27.5% compared to control. Antidotal therapy trial against acute lindane poison was also carried out in mice using diazepam, phenobarbitone and phenytoin. All three agents produced dose-dependent alleviation of symptoms and as well increased survival rates. The highest trial dose of phenytoin (40 mg/kg), phenobarbitone (20 mg/kg) and diazepam (10 mg/kg) resulted in 50%, 62.5% and 75% survival rates respectively. The control group produced 12.5% survival rate.

EEG studies in rats showed desynchronization of the reticular formation (RF) wave pattern due to lindane which effect was potentiated by dimethoate. Lindane-induced desynchronization was reversed by diazepam and phenytoin.

Twelve weeks sub-chronic toxicity studies in rats investigated the effect of combined exposure to weekly lindane (10 mg/kg, i.p) and daily dimethoate (10, 25, 50, 75 mg/kg feed) oral administration. Control group received normal feed and no lindane treatment. At intervals of four weeks, rats were killed blood samples collected for enzyme assay, serum albumin and total bilirubin were determined. Liver, kidney and intestines were removed for histopathologic examinations.

Serum transaminase (AST and ALT) activities showed general elevation in the dimethoate-fed compared to control animals. Furthermore, AST and ALT activities were slightly higher in lindane-free compared to lindane-treated dimethoate sub-groups throughout the twelve weeks exposure period. Serum AST activities were significantly ($P < 0.05$ - $P < 0.01$) higher in the dimethoate-fed animals in the presence and absence of lindane at four and eight weeks exposure, while activities were insignificantly raised at twelve weeks. Serum ALT activities on the other hand were significantly elevated ($P < 0.02$ - $P < 0.001$) at eight and twelve weeks while activities showed insignificant elevation at four weeks exposure compared to control values. Activities of serum ALT were significantly raised ($P < 0.05$ - $P < 0.001$) at four weeks but dropped significantly ($P < 0.05$ - $P < 0.01$) below control activity at eight weeks. Activity returned to control levels at twelve weeks exposure.

Serum albumin levels showed an insignificant decrease over control value at four weeks. However, significantly lower levels ($P < 0.05$ - $P < 0.001$) were observed at eight and twelve weeks exposure. No clear pattern was however, observed between lindane-free and

lindane-treated groups in the serum albumin levels. Levels for total bilirubin showed a gradual increase with duration of exposure, being insignificantly different from control at four weeks and significantly ($P < 0.02$ - $P < 0.001$) elevated at twelve weeks. Values for total bilirubin were higher in the presence of lindane treatment.

Histopathologic examination after twelve weeks exposure period revealed no lesions in the kidneys and the intestines at all dose levels. However, the liver revealed various degree of fatty degeneration, the occurrence of which showed correlation in extent of damage to period of exposure, rather than dimethoate dose or lindane treatment.

Experiments using isolated rabbits ileum and rat phrenic nerve-diaphragm preparations showed a biphasic response by lindane, with stimulation at low doses and inhibition at high dose. Dimethoate dose-dependently potentiated cholinergic responses at both nicotinic and muscarinic receptors. Dimethoate (8 ng/ml) response was augmented by physostigmine (2.6 ng/ml) and antagonized by atropine (2 ng/ml), while d-tubocurarine (1.3 ng/ml) attenuated both dimethoate (60 ng/ml) and lindane (4.3 ng/ml) response at the neuromuscular junction. Lindane (6.4 ng/ml) attenuated dimethoate (8 ng/ml) response and attenuated both spontaneous rabbit ileum and nerve twitch responses. The effect of lindane was augmented by d-tubocurarine (1.3 ng/ml) and attenuated by lignocaine (16.8 ng/ml). Lignocaine similarly attenuated dimethoate (60 ng/ml) response on the nerve.

The findings of these studies suggest some degree of protection provided by lindane against dimethoate toxicity. The protective effect of lindane was pronounced in acute dimethoate administration and less so in sub-chronic concurrent exposure. The *in vitro* results indicate antagonism of dimethoate response by lindane. Combined pesticides exposure, therefore, need not necessarily imply exaggerated toxicity.

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ABBREVIATIONS AND SYMBOLS

TOCP - TriorthocresylPhosphate.	UN - United Nation
WHO - World Health Organization.	ppm - Parts Per Million
DDT - Dichloro-diphenyl/trichloroethane	IC ₅₀ - 50% Inhibitory concentration
HCH - Hexachlorocyclo hexane	LD ₅₀ - 50% Lethal Dose
Ach - Acetylcholine	GABA - Gamma Amino butyric acid.
PCCH - Pentachlorocyclohexane	DPH - Diphenylantoin
HCCH - Pentachlorocyclohexane	µg - microgram
TCP - Trichlorophenol	ng - nanogram
AchE - Acetylcholine Esterase	mg - milligram
BuchE - Butylcholinesterase	M - molar
KAU - King Armstrong Unit	EEG - Electroencephalography
ALT - Alanine transaminase	EMG - Electromyography
AST - Aspartate transaminase	Hz - Hertz
ALP - Alkaline phosphatase	SEM - Standard error of the mean
SGPT - Serum glutamic pyruvate transaminase	SD - Standard deviation
SGOT - Serum glutamic oxaloacetate transaminase	MFO - Mixed function Oxidase
LIN - Lindane	I.P. - Intraperitoneal
DM - Dimethoate	ICI - Imperial Chemical Industries
EC. - Enzyme Commission	α - alpha
FC - Frontal cortex	ATPase - Adenosine triphosphatase
OC - Occipital cortex	β - beta
RF - Reticular formation	b.p boiling point
Freq. - frequency	Fig. - figure
Amp. - Amplitude	TTX - tetrodotoxin
EC ₅₀ - 50% Exitatory Concentration	STX - Saxitotoxin
	uv - microvolt.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 PESTICIDE USE AND TOXICITY

Since time immemorial mankind had to compete with more than a million other species for the earth's goods essential to survival. Doubtlessly, the struggle has been harsh. The losses inflicted upon man due to these other species ravages are sometimes disastrous.

The use of chemicals to control insects possibly dates back to classical Greece and Rome when sulphur was used to combat many insects and the vapours of the boiling coal tar to control pests of vines and sugar cane. From 1669 and till the second world war, arsenic compounds were used as pesticides against aphids. Towards the end of the 19th Century in 1881, carbon disulphide was in use to control stored grains insects and hydrocyanic gas for fumigating citrus plants against insects. These developments were followed by use of oils and salts for weed control, and copper and mercury compounds for control of certain plant diseases. Another important landmark before the first world war was the introduction in 1913 in Germany of the first organomercury seed dressings (Matsumura, 1975).

In the years between the two world wars, both the number and the complexity of crop protection chemicals increased. Dinitro-orthocresol was patented in France in 1932 for the control of weeds in cereals, and thiram the first of the several important dithiocarbamate fungicides as patented in the USA (Matsumura, 1975).

One of the most remarkable contributions to chemical pest control was the discovery of the insecticidal potential of DDT (dichloro-diphenyl-trichloroethane) ethanol in 1939 by Paul Muller for which he was awarded the 1948 Nobel Prize (Hassal, 1990). This was followed by the discovery of many other chlorinated hydrocarbons, organophosphates and carbamates. A few years later saw the emergence of the so called second generation organophosphorus insecticides which were more selective and less toxic to higher (non-target) animals than the earlier ones. Synthetic pyrethroids were later introduced by Britain and Japan. By 1900 *Paris Green*, used for the control of Colorado beetles was used so extensively as an insecticide that it led to the introduction of the first state legislation governing the use of insecticides in the USA. Ever since, thousands of other chemicals have been tested for their potency against insect pests. One of the most recently discovered is cocaine which was found to function in plants as a natural insecticide with selectivity towards invertebrates (Nathanson, *et al.*, 1993).

The spectacular and successful results so obtained through the use of pesticides in man's control of pest as compared to other methods and means of pest management have encouraged man's reliance upon their extensive and continued use. This resulted from man's demand and pressing needs for high quality food, better crops and fibre, and also better health and protection from insect vectors of diseases. DDT was employed in the control of anopheles mosquito as part of malaria control (W.H.O. 1985). Onchocerciasis control depended entirely upon DDT for the destruction of the vector simuliids for lack of drugs that may be used in mass control programme of the disease (W.H.O., 1986) which practice is still in use in some countries, especially the developing ones. The use of all three major classes of insecticides, namely, organochlorines, organophosphates and

carbamates are still employed in vector control all over the world especially in the developing nations (W.H.O., 1997).

The large-scale pesticides industry dates from the end of World War II with the commercial introduction of the phenoxyacetic acid, selective herbicides and the synthetic organochlorine and organophosphorus insecticides. The total value of all pesticides applied to world crops in 1949 was approximately \$200 million and in 1974, this increased to a market value of about \$500 million of which about 40% was in North America and only slightly less in Europe. Approximately 1,000 pesticides formulations are in use throughout the world today for virtually all forms of agricultural commodities. The annual worldwide agricultural use of pesticides by 1992 has been estimated to be of the order of 5×10^6 tons with a value of about \$16.3 billion (Tardiff, 1992) and has now risen to \$30 billion (Chemical and Engineering News, 1998). The developing countries account for no more than 10% of the total whereas it is precisely in these countries that there is the greatest need for crop protection chemicals. These countries are inhabited by 49% of the world population and make up 46% of the total world's cultivated land area and the greatest losses of crops due to pests (British Agrochemicals Report, 1989). On a world scale, pests destroy about half of the annual crop during growth, harvesting and storage, but in the underdeveloped countries, e.g. India, Africa and Latin America, losses can be as much as 70% of total production (FAO/UN, 1972). In the countries, there is the need to double food production over the next 100 years to feed the increasing population. Estimated losses in storage are 25% and 8% in Africa and India respectively.

It was estimated by the Food and Agriculture Organization (FAO) of the United Nations that cessation of all use of crop protection chemicals in the USA would reduce total

output of crops and livestock by 30% and would increase the price of farm products to the consumer by between 50% and 70% (Green, 1976). In Ghana, the application of insecticides almost tripled the yields of cocoa by effectively controlling the damage to the crops by cuspid bug, and in Pakistan, extensive use of insecticides led to increased yield of sugar crop by 30%. Introduction of pesticides also increased rice production several folds in Japan in 1960 (Fest and Schmidt, 1982). The United Nations Food and Agriculture Organization had estimated that without the use of pesticides some 50% of the total cotton production in developing countries would be destroyed by pests prior to harvest (FAO/UN, 1972).

Annual pesticide use is measured in hundreds of millions of pounds of active ingredients, about 20% of that in Production Agriculture. No other group of chemicals known for their toxicity is so extensively used as part of man's attempt to maintain an advantage over his competitors for food and fibre (Krueger, *et al.*, 1992).

Overall, pesticides use in agriculture in terms of amount applied per hectare has been very much greater in Japan, Europe and the USA, than in the rest of the world. Africa, though witnessed an increase in sales of 18.2% between 1980 and 1984 (Edwards, 1986). In Nigeria, although there is a dearth of statistics on pesticides production and usage, there is evidence of a rise in usage from 1975 to date judging by the increased agricultural activity in the country. Such activities were occasioned by such government programmes as the "Operation Feed the Nation" and "Green Revolution" as well as the advent of large scale farming embarked upon by retired civil and military officers. Recently, the Nigerian Government has embarked upon huge importation of pesticides intended to be supplied to farmers as well as for mass spraying in the event of insects

outbreak. Informed opinion on the subject would agree that it would be difficult to conceive of the world without pesticides which would probably means starvation for millions.

Despite their value to agriculture, pesticides have also in some instances posed direct and indirect threats to the health of humans and to the environment around the world. Their continued massive and indiscriminate use has always been accompanied by adverse effects and create multitudes of problems of grave concern. These problems range from non-target toxicity, residues problems as well as environmental pollution. In recent years, there has been increasing public concern about the safety of chemicals of all types. Pesticides are an important class of chemicals to which everybody is exposed to some extent. However, while it may be possible to detect acute poisoning and thus provide adequate medical remedy, chronic exposure is not readily detected. Some estimates indicates that pesticides account for more than 20,000 fatalities yearly, and most of these would have occurred in the developing countries (Forget, 1991).

It has been estimated that less than 0.1% of pesticides applied to crops reach the target pests, leaving more than 99% having potentials to impact non-target organisms (NTOs) and to become widely dispersed in the environment (Tardiff, 1992). Efforts in finding ways of minimizing pesticides hazards to man has resulted in intensive scientific investigations. At present, the general public is becoming increasingly concerned about the use of pesticides to the extent that some consumers, especially in developed countries, buy "organically"-grown food, free of pesticides. Calculations show that despite that, our diet contains 10,500 times more natural pesticides than synthetic pesticides (Berry, 1990).

Exposure of man to pesticides results from multiple sources. In an agricultural setting, for example, workers may receive dermal and inhalation doses from pesticides sprays application. In the home, exposure can occur through ingestion of unwashed garden produce that contain pesticide residues and from inhalation of indoor pest control sprays. Dietary exposures to pesticides occur for almost all members of the population.

Small children may receive the more significant levels of exposure as a consequence of repeated contact with room perimeters, carpets, and soil. In addition, hand to mouth manipulation are common at this age as in the tendency to taste, chew, and ingest objects (Davis and Ahmed, 1998).

In addition to residues on fresh and processed produce, pesticide residue may occur in meat, milk and eggs as a result of feeds to domestic cattle and poultry being treated with pesticides. One of the most important sources of dietary pesticide exposure in many nations is the drinking water. In agricultural areas of high pesticide use, sampling of ground and surface water supplies has generally revealed the presence of pesticides residues. The problem may become especially serious with sandy soil, such as the northern parts of Nigeria as well as the sub-Saharan Africa in general, which is easily penetrated by pesticides and / or areas with relatively high water tables.

Pesticides, like most other chemicals, have the potential to produce both acute and chronic injury to health. Acute toxic effects are those caused shortly after single dose or a few doses while chronic toxic effects are delayed.

In practice pesticides are usually applied in combinations, either for the purpose of controlling different pests or to fight resistance. These combinations could lead to interactions via various mechanisms to alter the toxic dose of the individual compounds. Frawley, *et al.*, (1957), discovered that *O-p* nitrophenyl phosphorothionate (EPN), an organophosphate, markedly potentiated the toxicity of malathion when the two were given simultaneously to laboratory animals. Since then, mechanism of pesticide toxicity has been reviewed by several authors (Murphy, 1969; DuBois, 1969 and Iyaniwura, 1989).

Public concern about the potential hazards that pesticides could exert on the environment has resulted in the pesticide industry being one of the most stringently regulated in the world. The standards demanded for approval and registration of products have become much more rigorous. In 1980, the tests required by the American EPA involved the assessment of acute toxicity based on the result of 90 day rat and dog feeding tests and similar ones extending over 2 years; rat reproduction studies over three generations and examination for teratogenicity and metabolic studies. Analysis must show that the residues in food crops are not more than 0.01 ppm, in meat 0.1 ppm and in milk 0.005 ppm. In addition environmental studies are also carried out to identify any bio-accumulation problems, as occurs with organochlorine insecticides (Fardiff, 1992).

The use of pesticides is on the increase in both agriculture and vector control in many developing countries including Nigeria. Although, pesticides appear to have come to stay, they are generally toxic by design. Organophosphate and carbamate insecticides are responsible for many poisoning cases. The organochlorines are persistent in soils and the environment generally. Herbicides such as paraquat as well as new pesticides hitherto,

known to be relatively safe to humans (non-target species) are now being implicated in some serious cases of intoxication (Narahashi, *et al.*, 1992).

It is obvious therefore, that the problem at hand is not that of the existence or use of pesticides, but that of safety in their use. The potential hazard of pesticides can be **assessed** scientifically on the basis of toxicological and human exposure data available.

The lack of information at various levels on the safe and rational use of pesticides is often the most important causative factor on chemical intoxication in developing countries. This work is therefore, aimed at providing information on the toxicity resulting from possible interactions of insecticides in particular lindane (Gamalin 20[®]) and Dimethoate (Rogor[®]) following simultaneous exposure. The information so obtained is expected to be useful in understanding the non-target toxicity due to combined exposure to the compounds. It intends also provide ways and means of alleviating/minimizing incidences of intoxication due to these insecticides.

1.2 OBJECTIVES OF STUDIES

The objective of this study, is to carry out the following investigations:

- i) the possible interaction (if any) and the mechanisms of such interaction between lindane and dimethoate,
- ii) proper possible antidotal therapy against lindane poisoning,
- iii) the effect of combined exposure of lindane and dimethoate on some organs in the body.

To carryout the above mentioned investigations, the following protocol were undertaken.

- a) *In vivo* studies.
 - i) acute toxicity studies.
 - ii) subchronic studies
 - iii) antidotal therapy studies
 - iv) EEG studies.
- b) *In vitro* studies
 - i) Muscarinic receptor investigations using, rabbit ileum preparation.
 - ii) Nicotinic receptor investigations using rat phrenic nerve diaphragm preparation.

CHAPTER TWO

LITERATURE REVIEW

2.1 CLASSIFICATION AND USES OF PESTICIDES

The 1990 edition of Chambers Dictionary defines "pesticide" as a substance for killing pests, which is defined as any deadly epidemic disease, plague, anything destructive; any insect, fungus, etc. destructive of cultivated plants, troublesome person or thing. The WHO defined a pesticide as any substance or mixture of substances intended for preventing or controlling any unwanted species of plants and animals and also for use as a plant growth regulator, defoliant or desiccant (WHO, 1976). The term "pesticide" includes any substance used for the control of pests during production, storage, transport, marketing or processing of food for man and or animals or which may be administered to animals for the control of insects or arachids in or on their bodies. It does not, however, apply to antibiotics or fertilizers (Brown, 1980). Pesticides are almost a mixture of different ingredients containing the substances which actually does the controlling (referred to as the active ingredients) and a number of additives. The additives improve the effect of the active ingredients or give the pesticide specific properties. The whole is called the formulation, each formulation having a specific trade name.

Several classifications of pesticide are found depending on their mode of action or simply their nature. Pesticides can be grouped into two major types based on their mode of action, namely, systemic and non-systemic or contact pesticides. However, Arena and Drew (1986), classified pesticides into four major groups based on their nature, namely:

- i. *Synthetic organic pesticides* e.g. chlorobenzene derivatives (DDT); lindane derivatives (aldrin, dieldrin); hexachlorocyclohexane (lindane); carbamates (aldicarb, carbaryl) and organophosphate derivatives (dichlorvos, dimethoate, parathion).
- ii. *Inorganic chemical pesticides*, such as arsenic, thallium and cyanide compounds.
- iii. *Biological pesticides*, such as pheromones, insect specific bacteria and viruses.
- iv. *Pesticides from botanical sources*, such as nicotine and pyrethrin.

The synthetic organic compounds are probably the most commercially useful of the chemical pesticides classes, such as chlorobenzenes and indane derivatives (Hayes 1982; Hassal, 1990). This resulted in their being banned from a range of uses in countries such as USA and the UK (Minton and Murray, 1988).

The major organic chemical pesticides include insecticides, fungicides and herbicides. Others include miticides, nematocides and rodenticides. Herbicides are the major class of pesticides used in the developed countries (temperate weather conditions). The estimated world consumption reported recently shows 44.2% herbicides, 28.8% insecticides, 20.9% fungicides and 6.1% for growth regulators and miscellaneous agrochemicals (Cremlyn, 1991).

Insecticides generally may be classified further into three major groups. These are, the organochlorines, the organophosphates and carbamates. Based on their chemical structures, the organochlorines and organophosphates may be subdivided as follows:

- a) *Organochlorines*
 - i) Oxygenated compounds, e.g. dieldrin.

- ii) Benzenoid, non-oxygenated compounds, e.g. DDT, lindane etc.
- iii) Non-oxygenated, non-benzenoid compounds e.g. aldrin, chlordane etc.

b) *Organophosphates*

- i) Aliphatic derivatives e.g. dichlorvos, malathion, dimethoate etc.
- ii) Aromatic derivatives, e.g. parathion, dicarphon, ronnel, etc. (Hassal, 1990).

Organochlorine insecticides are the most persistent in the environment. In addition, they are known to encourage the development of insect resistance and thus become less effective. Their use is therefore discouraged. The hexachlorocyclohexane (lindane) are useful for their fumigant action, systemic poisoning and high speed action. They are effective against cotton weevils, house mosquitoes, some mites on man and livestock, tsetsefly larvae, army worms, cockroaches, crickets, grain weevils, grasshoppers, leather jackets wireworms etc. They are also especially used for seed dressings (Hassal, 1990).

The organophosphorus insecticides have a wide range of uses covering many crops and effective against a large number of pests. A prototype organophosphorus insecticide, diemthoate, for instance, is especially active against aphids that attack crops like apple, pears, tobacco, tomatoes and beans, among others. It is also effective against mango fly which attack vegetables. Other pests vulnerable to organophosphours insecticides include weevils, stem borer and carrotfly (Hassal, 1990). The majority of insecticides generally elicit their toxicity mostly by attacking the nervous system of their target organism and as absolute insect/mammal selectivity is rarely achieved, the insecticides generally exhibits bio-activity towards mammals at appropriate doses (Hutson, 1981).

An ideal pesticide, is one that posses the following properties.

- i) should be rapid in action
- ii) highly selective for the target organism
- iii) less persistent in the environment (Casarett and Doull, 1975; Hassal, 1982).

2.2 THE NERVOUS SYSTEM: TARGET FOR INSECTICIDES TOXICITY

The nervous system constitute the major, and in many instances, the sole target site for the majority of insecticides which have been developed. It then follows that the ability to measure their effects on this system is critical to establishing the precise mechanisms by which they act. The nervous system is characteristic of mammals and insects and carries signals from the various receptor sites to the brain (Corbett, *et al.*, 1984). Insects have a well-developed central nervous system that is almost comparable to that of mammals. Poisoning the nervous system is the quickest and surest way of chemically upsetting the regular body mechanisms.

Within the context of the mode of action of pesticides, two aspects of the mode of function of nervous system are of great importance. These are:

1. The mechanism whereby a nerve impulse is conducted down an axon so that it eventually leads to a response, such as muscle contraction. This concerns the mode of action of organochlorines and pyrethroids.
2. The way in which the coded message carried by one nerve is conveyed across the synapse or between the last neuron of a chain and the system it innervates e.g. neuromuscular junction in case of muscle cells. This concerns the mode of action of organophosphorus and carbamate insecticides (Hassal, 1990).

Most toxins affecting the nervous system do so by interfering with biochemical systems, some through relatively specific mechanisms. The toxin may be attached to sites of metabolic action, blocking the active sites from access to required compounds or it may for metabolic or physiological activity. A generalized toxic effect may be caused by processes such as protein denaturation or by alterations in acid-base balance or ionic equilibrium. Under some circumstances, there may be interference with biochemical systems, but several added factors may be necessary to produce toxicity.

2.3 MECHANISM OF NERVE IMPULSE TRANSMISSION

Nerve impulse is conducted along the axon and then subsequently over the synaptic gap to the next neuron and finally down to the recipient target organ. Transmission over the gap requires a chemical mediator or transmitter. Impulse transmission is an electrical processes in which the current is carried by ions taking place by means of a series of changes in the membrane's electric potential along the axon. Nerve impulses are transmitted at the synapse by the release of a chemical transmitter, generally acetylcholine and noreadrenaline, although other transmitters such as dopamine, octopamine, L-glutamate and gamma-aminobutyric acid (GABA) are also involved in some synapses. More than twenty other transmitters have been identified in various species of animals (Cremlyn, 1991). Common neurotransmitters in both mammalian and insect nervous system include acetylcholine, noradrenaline, dopamine, GABA and octopamine.

When a nerve impulse arrives at the presynaptic membrane, acetylcholine (ACh) is simultaneously released from the presynaptic cells and the chemical transmitter diffuses across the synaptic cleft to the postsynaptic membrane, where it binds to the acetylcholine receptors sites. The liberated acetylcholine must not persist in the synapse too long, otherwise there would be a continuous chain of nerve impulse generation.

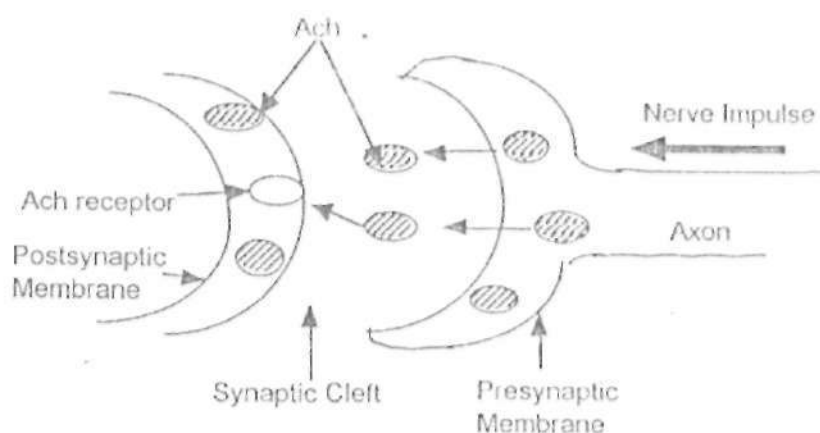
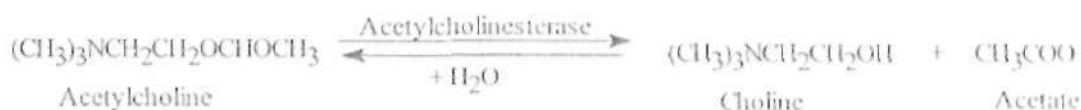


Figure 2.1: Schematic diagram of nerve synapse.



The transmitter is generally eliminated by the enzyme acetylcholinesterase which hydrolyses acetylcholine to choline, a non-transmitter of nerve impulse.

This allows for release of new transmitter to the synapse. The passage of an impulse along an axon is a rapid wave of depolarization occurring at a faster rate in myelinated than in non myelinated (1 - 20 ms) axons. When the stimulus wave arrives at any particular portion of the membrane, depolarization in terms of influx of Na^+ and outflow of K^+ take place. This action changes internal charge of the membrane from being negative instead. Thereafter, the potential inside the membrane rapidly returns to normal.

All cells of the body, whether nerve cells or not, have slight excess of negatively charged molecules on the inside relative to the outside. As a result, a potential difference exists across the plasma membrane in the range of 20 mV - 100 mV, depending on the cell type. This is referred to as resting membrane potential and in the case of neurons, usually about - 70 mV (Ganong, 1987). If for some reason the relative impermeability of the

plasma membrane to Na^+ were to be negated, Na^+ ions would rush into the cell. This is exactly what happens when a nerve or muscle cell is stimulated. Stimulation or depolarization, occurs as a result of specific binding of neurotransmitter to the receptor cell surface. The sudden entry of a small number of Na^+ ions, to which the membrane is now more permeable than to K^+ or Cl^- , causes the cell to become electropositive on the inside relative to the outside at this point of the membrane. That is, changing the potential difference from negative to positive. The result is an electric impulse propagation along the axon by a flow of ions from one point of depolarization to the next. Within less than a millisecond of the membrane increasing permeability to Na^+ , its permeability to K^+ is also increased while that to Cl^- remains the same. This is necessary to maintain ionic equivalence and to restore electrical neutrality on both sides. At this point, however, the permeability “gates” through which Na^+ and K^+ diffuse are “closed” and the cell is repolarized and ready to receive another impulse.

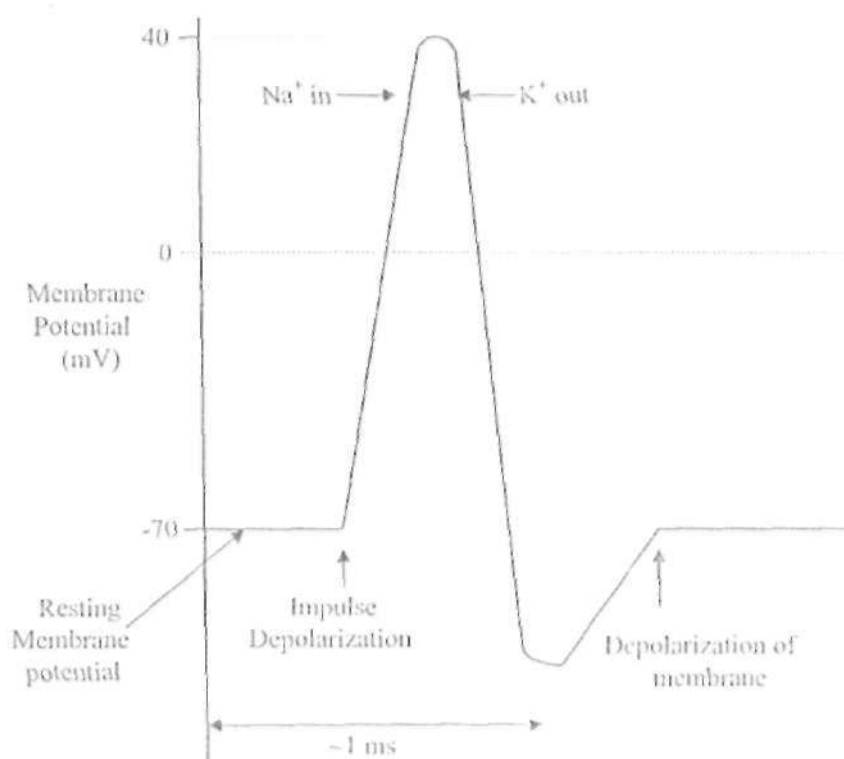


Figure 2.2: Depolarization of nerve cells

These whole processes require energy (in the form of ATP) as they occur against ion concentration gradient. The role of phosphate transfer is closely related to that of Ca^{2+} which plays a critical role in membrane excitation via surface recalcification which is a prerequisite for the restoration of a stable resting potential (Yamaguchi, *et al.*, 1979).

When a stimulus in the form of an electric conduction wave reaches the end of an axon, it triggers an influx of Ca^{2+} into the presynaptic area, which in turn causes the vesicles containing chemical transmitters to migrate to the post-synaptic membrane. The transmitter then combines with receptors at the post synaptic membrane thereby generating an action potential. The generated action potential now travels as a wave across the neuron and down its axon. Upon arrival at the ultimate destination, neuroeffector junction bridged by a transmitter, it crosses to a receptor on the effector cell where it elicits an appropriate response.

2.4 ORGANOCHLORINE INSECTICIDES

The organochlorine insecticides are among the most widely used pesticides owing to their broad spectrum pesticidal action. Their introduction into agriculture and public health is as widely acclaimed as it is controversial. The chlorinated hydrocarbons have a high lipid-water partition coefficient and thus penetrate biological membranes quite easily thereby accumulating in the fatty tissues of organisms (Kenaga, 1972). The lipid solubility of the organochlorine pesticides together with their high resistance to enzymatic attack make them the least biodegradable of all pesticides (Iyaniwura, 1991a). They persist in soils, organic matter including food crops, domestic and wild animals, and man (Hayes, 1982).

Organochlorine insecticides generally exhibit less acute toxicity but possess greater potential for chronic toxicity than the organophosphate and carbamate insecticides (Murphy, 1975). The organochlorine pesticides are the most effective inducers of the mixed-function oxidase, in mammals (Wilkinson, 1976). Several of them have been implicated in cytochrome P_{450} induction including DDT, lindane and chlordane (Fouts, 1971 and Iyaniwura, 1991a). Lindane has been shown to induce both cytochrome P_{450} (Dwivedi and Kumar, 1989) as well as GST in rats (Kraus, *et al.*, 1981).

Structurally, one of the few features which members of the three families of organochlorine insecticides have in common is that the molecules contain one or more chlorinated carbocyclic rings.

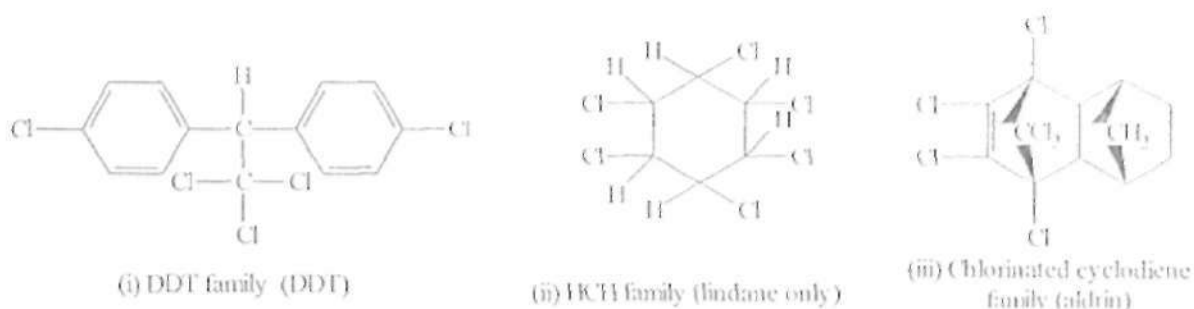


Figure 2.3: The three families of organochlorine insecticides.

Organochlorine pesticides including DDT analogues, the HCH isomers and the cyclodienes are known to be neurotoxic (Narahashi, 1989; Hassal, 1987) and are therefore capable of causing CNS damage by some mechanism associated with Na^+ and K^+ transport across the nerve axon membrane (Narahashi, 1989; Matsumura, 1975; Koch, 1970).

The toxic effects of the members of the three families of organochlorine compounds differ in details but all are neurotoxic substances. Despite some differences, the general effect of destabilization of neural activity manifests in hyper-excitability of nerves and muscles leading subsequently to convulsions. Symptoms of organochlorine poisoning follow a similar pattern and they include, paresthesia, irritability, dizziness, tremor and convulsions (Matsumura, 1975). They are also capable of causing organ damage, notably of the liver and kidneys (Waldbott, 1978; Iyaniwura, 1991a and 1996).

Evidence exist that organochlorines and pyrethroid insecticides interfere with the opening and closing of ion channels in nerve membrane aided by various ATPases, an action which goes a long way in explaining the neurotoxic nature of these compounds (Ecobichon and Joy 1982; Narahashi, 1989; Narahashi *et al.*, 1992). It has been observed that the mere presence of an organochlorine compound near lipoprotein membrane can distort such activities as photosynthesis, oxidative phosphorylation, active transport and nuclear division as well as interference with axonic transmission (Chelurka, *et al.*, 1987). However, for a compound to be neurotoxic as in the case of organochlorine pesticides, it must have a structure capable of destabilizing the membrane which can then trigger the neurotoxic effects (Hassal, 1987). There are two major ways in which channels function may be modified by such chemicals, namely blocking of the channels and modification of gating behaviour resulting in decrease or increase of electrical excitability. For instance, blocking Na^+ channels reduce excitability while agents that affect Na^+ channel blockers such as anesthetics (e.g. lignocaine), anticonvulsants (e.g. phenytoin) and tetrodotoxin and saxitoxin.

2.4.1 Lindane

HCH, formerly known as benzenehexachloride (BHC) was first prepared by Michael Faraday in 1825. However, its insecticidal property was discovered by Dupire and Raucourt in France, and Slade in England in 1942. There is only one member of the group, namely the gamma isomer which was named lindane in honour of Vander Linden who discovered four isomers of the compound (Matsumura, 1975).

Technical grade HCH consists of 65-70% alpha-HCH, 7-10% of other isomers and compounds. Lindane contains more than 99.8% gamma-HCH (WHO, 1991). Lindane is an almost odourless white solid slightly soluble in water (10 ppm), moderately soluble in kerosine (2 percent) and very soluble in acetone (43 percent). It has a vapour pressure of about 10^{-5} mm Hg at 20 °C and stable to heat and light (Hassal, 1990). Lindane has the chemical formula $C_6H_6Cl_6$ and a molecular weight of 290.8. Its melting point is 122.8 °C, boils at 288 °C and has a density of 1.85 (Fishman and Gianutsos 1988).

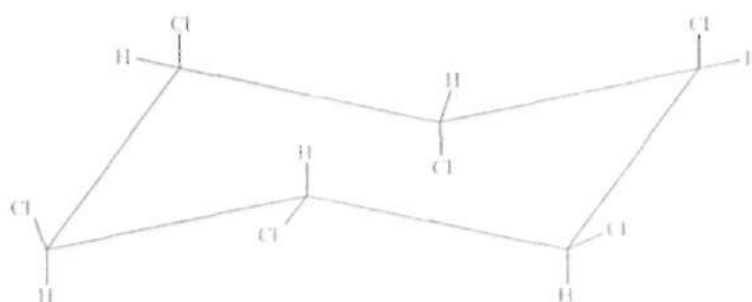


Figure 2.4: Chemical structure of Lindane (1 α , 2 β , 3 β , 4 α , 5 α , 6 β -hexachlorocyclohexane)

Lindane by contact, stomach or fumigant action is about 50-10,000 times more effective than the other isomers (Matsumura, 1975). Lindane has been used as a broad spectrum

insecticide since the early 1950s for the purpose that include treatment of seeds and soil, application on trees, timber and stored materials, treatment of animals against ectoparasites and in public health (WHO, 1991). The allowed daily intake (ADI) for humans is estimated at 0.01 mg/kg body weight or 1×10^{-8} ppm (WHO, 1991).

2.4.2 Mammalian Metabolism of Lindane

In rats, lindane is absorbed rapidly and distributed to all organs and tissues within a few hours. Uptake varies with sex, being higher in females. Skin absorption is, however, low explaining the low dermal toxicity of lindane (WHO, 1991). Lindane was found to accumulate in the adipose tissue of experimental animals. The distribution in the brain was found to be similar in both orally and intravenously administered routes. However, the concentration in the white matter was higher than that of the thalamus, mid-brain, pons and medulla, relative to the mean value for the whole brain (Sanfeliu, *et al.*, 1988).

The metabolism of HCH is a complex process, incompletely understood, and apparently occurs by somewhat different routes in different species (Hassal, 1990). The extensive nature of the subject has been reviewed by Aizawa (1982) and Macholz and Kujawa (1985). Lindane metabolism involves both monooxygenases and glutathione-S-transferase. The monooxygenase enzyme systems are located in almost every tissue, with the far highest concentration found in the liver. These enzymes catalyse the bio-transformation of lipophilic drugs to more polar compounds which are readily excreted by the kidney in the urine. Compared to the parent drugs, these metabolites are normally less active/toxic or even inactive/non-toxic. However, some bio-transformation products exhibit enhanced pharmacological/toxicological activity (Spatzemegger and Jaeger, 1995). All together more than 70 metabolites resulting from lindane metabolism have

been identified in birds and mammals and yet others have been detected in insects and plants (Hassal, 1990). Lindane metabolism is initiated by one of the following four possible reactions.

- i) Dehydrogenation leading to the formation of γ -HCH;
- ii) Dehydrochlorination leading to the formation of γ -PCCl₂;
- iii) Dechlorination leading to the formation of HCH
- iv) Hydroxylation leading to the formation of HCCH.

These initial reactions are followed by a series of further dehydrogenating, dechlorinating, dehydrochlorinating, and dehydroxylating steps. The enzymatic processes take place mainly in the liver (WHO, 1991).

At least two independent pathways were involved in the metabolism of lindane. The first is the possible formation of unstable intermediates, such as hexachlorocyclohexanol, after an initial hydroxylation leading to the main metabolite, 2,4,6-trichlorophenol (2,4,6-TCP) and involving cytochrome P₄₅₀. The second pathway include dehydrogenation of lindane to 1,2,3,4,5,6-HCCH, subsequent hydroxylation and hydrochlorination to 2,3,4,6-tetrachlorophenol (Stein, *et al.*, 1977). In another study, lindane was found to be converted mainly to γ -HCCHG by rat liver microsomes and significant amount of 2,4,6-TPC and 2,3,4,6-tetrachlorophenol were detected.

Human liver microsomes converted lindane into four major metabolites, γ -1,2,3,4,5,6-hexachlorocyclohex-1-ene; γ -1,3,4,5,6-PCCl₂, β -1,3,4,5,6-PCCl₂, and 2,4,6-TCP while

site of action of lindane in both insects and mammals is the Central Nervous System (CNS) (Echobichon and Joy, 1982; Narahashi *et al.*, 1992). Sloley *et al.*, (1985) reported an increase in the dopamine levels and of N-acetyldopamine in the central ganglion of the cockroach following lindane administration. Meanwhile, Abalis *et al.*, (1985) had reported that the GABA receptors in the mammalian brain were the primary target for lindane. GABA is an inhibitory neurotransmitter, present in invertebrates both in the nervous system and in skeletal muscle. It is also the major inhibitory neurotransmitter in the CNS of vertebrates. It is postulated that GABA receptor is a single protein with three sites of activity. One of those binds to GABA, a second controls an ion channel and the third is the site of attachment of certain toxicants e.g. picrotoxin. GABA receptors are of two types: A and B. GABA receptors are a supramolecular complex that gates a chloride ionophore. Drugs which interfere with neurotransmission mediated by GABA have well documented convulsant effects in experimental animals as well as in human e.g. glycine. GABA activation increases chloride conductance and inhibits neuronal activity by hyperpolarization or depolarization block (Ganong, 1980). Lindane and cyclodienes have been shown to antagonize the action of GABA in stimulating Cl⁻ uptake by various nerve and muscle preparations (Ghiansuddin and Matsumura, 1982 and Abalis, *et al.*, 1986). The first patch clamp experiments have clearly demonstrated that the GABA-induced chloride current is suppressed by lindane (Ogata, *et al.*, 1988). Similarly, noise analysis experiments conducted recently by Bermudez, *et al.*, (1991) with cockroach neurons have shown channel openings without changing the mean open time. Lindane was shown to decrease the single channel conductance. It was concluded that lindane and cyclodiene insecticides inhibits GABA induced chloride ion uptake, with the effect that the inhibitory action of GABA is blocked, leading to CNS excitation and convulsion.

Because of its structural similarity to picrotoxin, lindane has a good geometric fit to the picrotoxin binding site at the outer end of the chloride channel. Once bound, lindane is believed to block the action of GABA in the mediation of entry of chloride ions necessary for inhibitory neuronal function (Matsumura, 1975; Hassal, 1990).

Pandy *et al.* (1985) have demonstrated that weekly administration of lindane inhibits Na^+ - K^+ -ATPase activity in skeletal muscle as well as other ATPases notably Ca^+ - Mg^+ , Ca^{2+} and Mg^{2+} ATPases. Since all the ATPases are involved in the maintenance of intracellular Ca^{2+} concentration and have been identified in neural membrane preparations (Yamaguchi, *et al.*, 1979), it is possible that the disruption of homeostatic calcium regulation by lindane contributes to its excitatory action on the CNS as suggested by Cruiswell, *et al.*, (1994).

2.5 ORGANOPHOSPHORUS INSECTICIDES

Work began on organophosphorus compounds during the second world war by German scientists under the direction of Gerhard Schrader. Their work eventually led to the development of the organophosphorus insecticides and the highly toxic nerve gases, tabun and sarin. The organophosphorus insecticides are neutral esters of phosphoric acid or its thio analogues. The phosphonates possess a carbon-phosphorus bond which appears to be biologically very stable (Mein and McBain, 1974). The first organophosphate insecticide was tetraethyl pyrophosphate (TEPP). TEPP, although an effective insecticide, was highly toxic to mammals and was rapidly hydrolysed in the presence of moisture. Further, efforts to find more stable compounds for use in agriculture led to the synthesis by Schrader in 1944, of parathion (*O,O*-diethyl-*O-p*-nitrophenyl phosphorethionate) and its analogue paraoxon (*O,O*-diethyl-*O-p*-nitrophenyl phosphate) (Matsumura, 1975).

It is estimated that some 100,000 organophosphorus compounds have been screened for their possible insecticidal action, and over 100 have been marketed for this purpose (Hassal, 1990). Among the most widely used are dimethoate and malathion.

An important feature of the group is that different members possess very different physicochemical properties. In particular, they have different vapour pressures at room temperature and different solubilities in water. They vary considerably in their chemical stability and their toxicity to mammals.

The pharmacology and toxicology of organophosphorus compounds begin with a reference to their basic chemical structure, which is illustrated below.

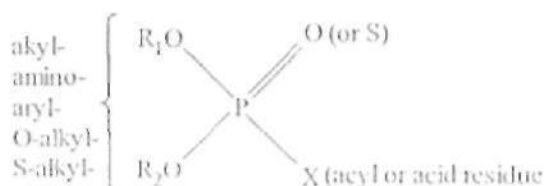


Figure 2.6: Basic chemical composition of organophosphorus insecticides (X = leaving group often joined via an ester or thioester linkage, namely P-O-X or P-S-X).

The characteristic feature is the central phosphorus atom, surrounded by four organic chemical groups. The composition of the surrounding groups vary from one pesticide to another, and with it, the biological activity, the pesticidal value and the toxicological effects. Six major chemical groups of organophosphorus insecticide exist as follows:

- i) Orthophosphate e.g. dichlorvos, phosphamidon etc.
- ii) Thiophosphates (Phosphorothiolates e.g. parathion, diazinon etc.
- iii) Thiophosphates (Phosphorothiolates) e.g. vernidothion etc.
- iv) Dithiophosphates (Phosphorothiolthionates) e.g. dimethoate, malathion etc.
- v) Phosphonates e.g. trichlorophene etc.

- vi) Pyrophosphoramides e.g. schradan etc. (Roberts, 1979).

Organophosphorus compounds have tended to replace the organochlorine pesticides in terms of usage both on tonnage and whole scale value. They appear to have a wide application to many plant crops against an equally wide range of pests.

Organophosphorus insecticides may be classified according to their practical uses into the following sub-groups.

Sub group I: This consists of compounds of low chemical stability soluble in water but more or less rapidly hydrolysed by it. They are used as contact insecticides, e.g. mevinphos, tetrachlorovinphos.

Sub group II: Compounds of moderate to high chemical stability, usually of low solubility in water but soluble in oil. These persistent contact or locosystemic compounds soak into leaves but do not travel around the plant. The sprayed substance is activated before it reaches its site of action in insects e.g. malathion, diazinon.

Sub group III: Compounds of moderate to high chemical stability. Their oil/water partition coefficients are such as to enable them both to enter and translocate within plants. They are systemic compounds activated before reaching their sites of action in insects, e.g. dimethoate, etc.

Sub-group IV: Compounds with sufficiently high vapour pressure and low chemical stability to enable them to be used as fumigants e.g. dichlorvos.

Sub-group V: Organophosphate insecticides used against soil organisms e.g. diazinon, bromophos.

2.5.1 Metabolism of Organophosphorus Insecticides in Mammals

The metabolic pathway of organophosphates in mammals were described by Murphy (1969), Casarrett and Doull (1975) and described by Rang and Dale (1988).

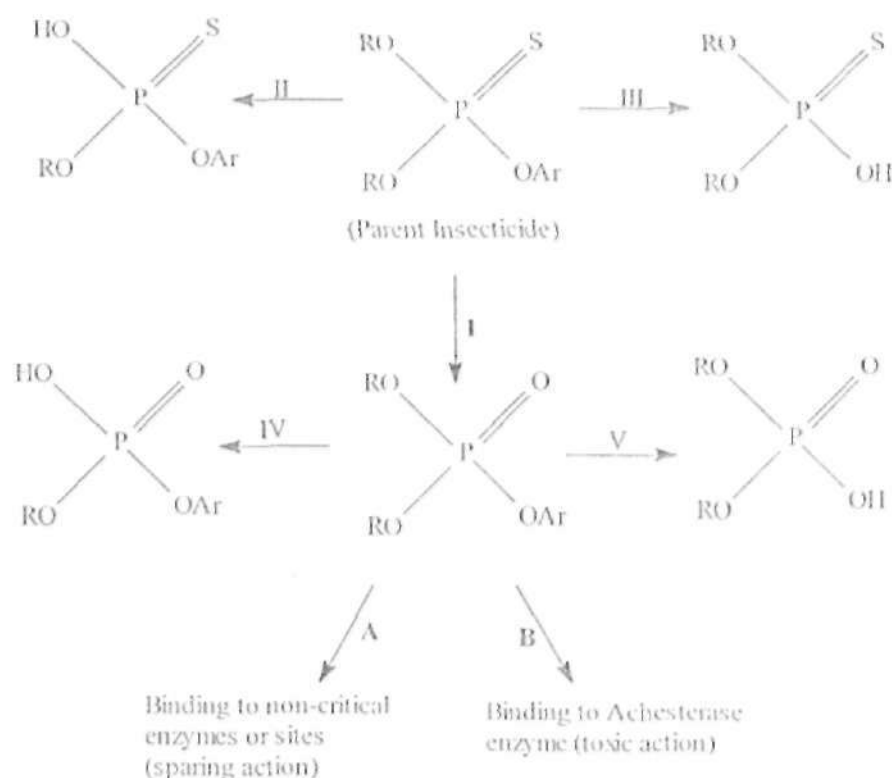


Figure 2.7: General scheme of metabolic pathways of organophosphorus insecticides in mammals (Casarrett & Doull, 1975).

The scheme above (Fig. 2.7) indicates a favoured detoxification in mammals through the rapid formation of inactive metabolites. In insects, toxic/active metabolites are favoured through the rapid formation of the active metabolites and slow formation of the inactive metabolites. Reaction I in the above scheme involves the substitution of -O for -S (desulphuration) and is a prerequisite for toxicity. Reactions II, III, IV and V are detoxification reactions yielding products which do not inhibit acetylcholinesterase (Wilkinson, 1976). However, phosphorothionate insecticides such as parathion, malathion and dimethionates are normally non-toxic as parent compounds but are converted into

toxic/active oxons by hepatic microsomal monooxygenase enzymes (O'Brien, 1967 and Eto, 1974). Products of hydrolysis of the carboxyester (e.g. malathion) or amide group (e.g. dimethoate) do not inhibit cholinesterase, and enzymatic formation of these products have been demonstrated in *in vivo* and *in vitro* studies (Uchida, *et. al.*, 1964; Dauterman, 1971). The activation into toxic forms is normally accomplished by liver microsomal enzymes (Dauterman, 1971). Modification of the alkyl and aryl-groups also influence toxicity. The role of hepatic microsomal monooxygenases in determining the toxicity of insecticides is sometimes complex as, for instance, in the case of certain organophosphates (e.g. dimethoate), where it may have both an activating and a deactivating function (Walker, 1981).

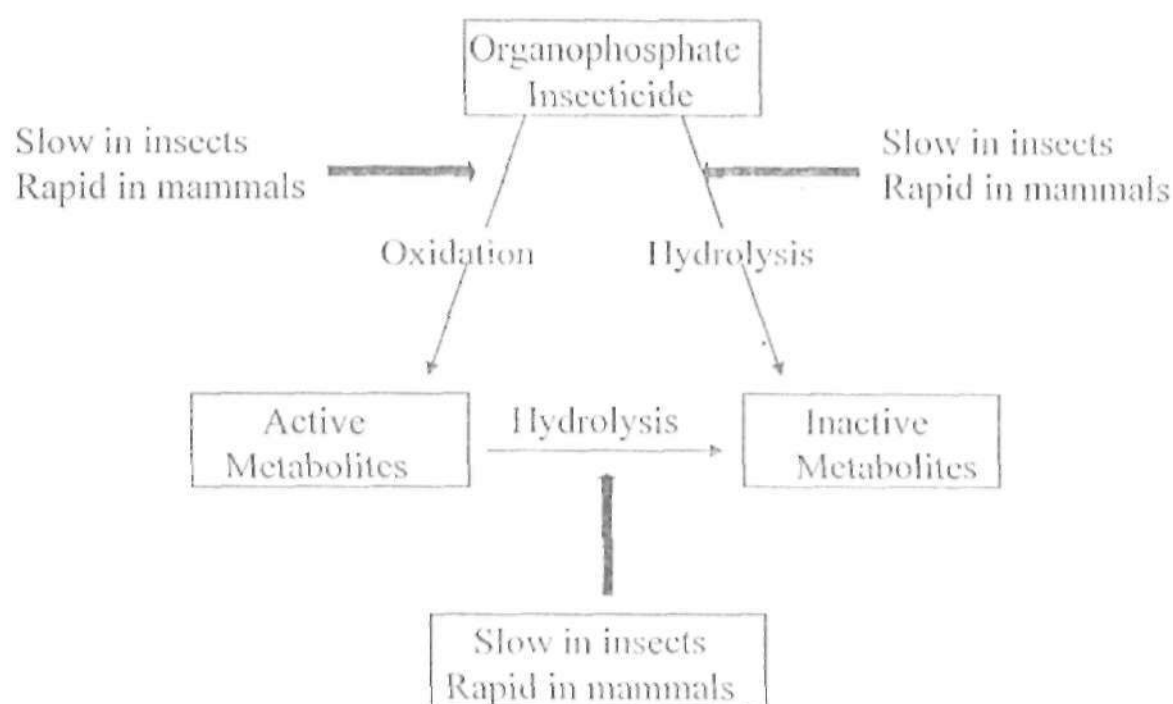


Figure 2.8: Biotransformation pathways of organophosphate insecticides in insects and mammals (Loomis, 1978).

The activities and properties of hepatic microsomal P_{450} -mediated activation (desulphuration) and detoxification (deacylation) of the phosphorothionates as well as of A-esterase-mediated hydrolysis of oxons contribute substantially to understanding the

acute toxicity levels in rats as does the sensitivity of the protective aliesterases to phosphorylation (Chambers and Carr, 1995). Metabolism of insecticides appears to be far more influential in some species than others in determining the toxicity elicited (Chambers and Carr, 1995).

2.5.2 Mechanism of Organophosphorus Insecticides Toxicity

In general, the molecular mechanism of insecticidal action are poorly understood. Well defined biochemical actions have been assigned only to organophosphate and carbamate compounds as powerful inhibitors of acetylcholinesterase (Eto, 1974 and Doherty, 1979).

The primary targets for organophosphorus insecticides in both man and target insects population are the cholinesterase enzyme systems which hydrolyze acetylcholine and various other esters (Roberts, 1979). Acetylcholine is present at the ganglia, parasympathatic neuroeffector and neuromuscular junctions. Cholinesterase enzymes hydrolyze acetylcholine forming choline and acetic acid. Two distinct types of cholinesterase enzymes exist; the true cholinesterase (known as acetylcholinesterase (AChE) and the pseudocholinesterase) (Rang and Dale, 1988).

AChE is a macromolecule that appears to consist of several sub-units; each molecule having a number of active centres where the hydrolysis of ACh takes place. These active centres have two areas that interact with ACh: the anionic site and the esteratic site. The anionic site contains a negatively charged amino group of ACh while esteratic site contains a serine molecule made more reactive by hydrogen binding to a nearby histidine molecule. The nucleophilic oxygen of serine react with the carbonyl carbon of ACh, thereby breaking the ester linkage leading to the liberation of choline and formation of

acetylated AChE which is rapidly hydrolyzed to regenerate free enzyme and produce acetic acid (Donald, 1990). The entire process takes about 100 microseconds and so the turn over is extremely high about 10,000 molecules of ACh hydrolyzed per second by a single active site (Rang and Dale, 1988).

During organophosphate poisoning, acetylcholinesterase is inhibited by dealkylphosphorylation of serine hydroxyl group at its active site. The resulting phosphorylated enzyme may be stable, it may undergo regeneration by spontaneous hydrolysis or may age (by dealkylation) to a very stable permanently inhibited specie. Other esterases, e.g. chymotrypsin, lipases, carboxylesterases and the cholinesterases in plasma and red cells are also inhibited but without acute effects. The inhibitory power of an organophosphorus ester derives from the affinity between its electrophilic phosphorus atom and the nucleophilic serine hydroxyl group of the enzyme (Hutson, 1981). The organophosphate inhibitory compounds having structural similarity to the natural substrate, ACh, also react at the esteratic site of AChE. Generally, however, they are much less selective than the carbamates, inhibiting many enzymes that contain a serine molecule at the active centre.

While the regeneration of most carbamoylated enzymes occurs with a half-time of minutes or hours, the recovery of a phosphorylated enzyme (as obtains with organophosphates) is generally measured in days. These agents are referred to therefore, as irreversible inhibitors (Donald, 1990). The potency of organophosphate insecticides to inhibit cholinesterase has been reported to correlate with sensitivity of the animal to acute toxicity (Pope & Chakarabouiti, 1992) and shows interspecific differences in their neurological hazards (Gordon & Macphail, 1993)

Assessment of the risk of neurotoxicology of organophosphate pesticides generally calls for a thorough understanding of the relationship between tissue cholinesterase activity and changes in the behavioural and autonomic response to organophosphate exposure. In an experiment conducted by Gordon and Fogelson (1993) using Long-Evans strain rats, DFP, was reported to produce a wide range in individual motor activity and colonic temperature responses at doses producing AChE activity inhibition above threshold levels. The same pattern has been reported in other rodents, and observation that may represent a fundamental aspect of chE toxicity.

The organophosphorus insecticides due to their instability are considerably less of a residue problem than the chlorinated hydrocarbon insecticides. No accumulation of organophosphates insecticides has been reported. Obviously, therefore the chronic effects of organophosphates depend upon accumulated physiological effects of frequent exposure to non-acute doses.

2.5.3 Mammalian Toxicity due to Organophosphate Insecticides

The organophosphate insecticides are known to produce a higher incidence of acute toxicity relative to the chlorinated insecticides (Hayes, 1982). The symptoms of organophosphate insecticides intoxication are muscarinic, nicotinic and CNS in nature and are those of excessive cholinergic stimulation. The muscarinic symptoms include hypersalivation, lacrymation, blurred vision, sweating, nasal discharge, difficulty in breathing, headache, nausea and vomiting, diarrhoea and diuresis. The nicotinic symptoms include muscular fasciculation, weakness and paralysis. CNS symptoms include nervousness, ataxia, convulsions (clonic/tonic) and coma. Death is usually due to respiratory failure (Matsumura, 1975, Iyaniwura, 1991b).

Muscarinic effects are symptoms which arise due to actions on postganglionic nerve terminals and to excessive stimulation of autonomic effector cells. Those symptoms include anorexia, abdominal pain, cramps, gastrointestinal hypermotility, respiratory distress, dyspnea, cyanosis and incontinence of faeces and urine. Those symptoms/conditions can be antagonized with atropine.

Nicotinic effects are symptoms which arise due to actions on somatic nerve elements which result in stimulation followed by paralysis of skeletal muscle. Twitching or spasm occur in the muscles of the tongue, eyelids, face and ultimately general musculature followed by weakness, diminished or absent reflexes, flaccidity, rise in blood pressure and paralysis. d-tubocurarine effectively combats these symptoms.

Central nervous system effects are due to direct actions of organophosphate pesticides in the CNS which results from excess acetylcholine at central cholinergic synapse. Death due to organophosphates result mainly from asphyxiation (Iyaniwura, 1991b). Artificial respiration is known to enable animals to survive otherwise fatal doses. Symptoms leading to death include bronchoconstriction and depression of respiratory centre (O'Brien, 1960).

The sequence of events resulting from organophosphate intoxication occur in the following order:

- i) Inhibition of cholinesterase activity
- ii) Acetylcholine accumulation
- iii) Disruption of nerve function centrally and peripherally

- iv) Respiratory failure and death due to asphyxia (Matsumura, 1975; Minton & Murray, 1989).

Incidence of human organophosphate poisoning has been reported globally. Among the early reports was the case of EPN toxicity (Frawley, *et al.*, 1957). Most of the cases of pesticides intoxication involves individuals directly working in agricultural fields. Kaloyanova (1982) reported the death of five workers in Pakistan due to malathion toxicity. Recently, Lopez and Lopez (1993) reported intoxication due to dimethoate and its derivatives in agricultural workers in Mexico, while in Israel, evidence of exposure illness associated with organophosphate pesticides was shown by Richter, *et al.*, (1992). Low AchE levels in the RBC and plasma has been observed as indication of intoxication due to organophosphates in farming communities in South Africa (Rama and Jaga, 1992). Inhibition of serum BuchE and brain AchE have been shown to be biochemical markers of organophosphate intoxication (Johnston, 1995).

The organophosphate insecticides dimethoate and methylparathion have been observed to affect levels of immunological variables in wistar rats following chronic administration of equitoxic doses of the two compounds at 1/75 LD₅₀ dose level (Institoris, *et al.*, 1996). There is therefore the need for public health measures aimed at safer use of pesticides outside the work place.

2.5.4 Delayed Neurological Effects of Organophosphorus Poisoning

Some compounds have been known to cause delayed manifestations of injury after a single large dose. Illustrations of delayed toxicity include neuropathy caused by

organophosphate esters, lung damage by paraquat and sensitization by pyrethroids (Rosenstock, 1989).

The prototype of these processes occurs with over exposure to triorthocresyl phosphate (TOCP) which results in peripheral neurological syndromes and may occur 2-3 weeks following exposure (Murphy, 1986). Chronic effects due to acute organophosphate pesticides poisoning have been reported (Rosenstock, *et al.* 1991; Savage *et al.*, 1983). A retrospective study by Rosenstock and co-workers on agricultural workers in Nicaragua who were admitted for occupation related organophosphate poisoning revealed a significantly less performance in test meant to assess a wide array of neuropsychological functions than a matched control group (Rosenstock, *et al.*, 1991).

EEG abnormalities, due to organophosphate intoxication have been reported by Metcalf and Holmes (1969). EEG disturbance do manifest mostly as an increase in amplitude both human (Duffy, *et al.*, 1979) and animal studies (Beechfield, *et al.*, 1976).

Several hypotheses and theories have been put forward to explain the mechanisms of organophosphate-induced delayed neuropathy (Cassida, *et al.*, 1961; Johnson, 1969 & 1974). AbouDonia and Lapadula (1989) investigated the hypothesis that delayed neurotoxic organophosphorus compounds may interfere with endogenous kinase-mediated protein phosphorylation of neural enzyme or cytoskeletal proteins thereby adversely affecting the regulation of normal neuronal processes resulting in axonal degeneration. In their investigation using triorthocresyl phosphate (TOCP), the authors reported that Ca/calmodulin-dependent protein kinase Type II (CaM Kinase II) may be the enzyme involved in the increased protein phosphorylation with TOCP. CaM-kinase II normally mediates ATP phosphorylation of neural cytoskeletal proteins including α -

and β - tubulin microtubule associated protein (MAP -2), neurofilament triplet proteins, and synapsin I. Others are tyrosine hydroxylase, tryptophan hydroxylase as well as myelin basic proteins (Lin *et al.*, 1987).

2.5.5 Dimethoate

Dimethoate (Rogor®) (*O,O*-dimethyl *S*-methylcarbamoylmethyl-phosphodithioate) was first described by Hoegberg and Cassaday (1951). It belongs to the family of organophosphates which are essentially amides. Dimethoate is used as contact and systemic insecticide and is effective against a broad range of insects and mites on a wide range of crops. It was first introduced in 1959 as Cygon (American Cyanamid Co.) and later as Rogor® (Montedison), Roxion® (Cela) and Perfekthion® (BASF) (Matsumura, 1975). The chemical structure of dimethoate is shown below.

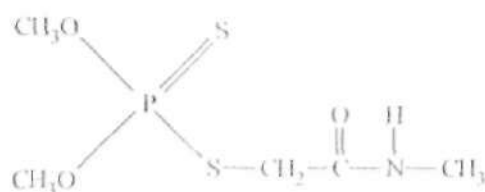


Figure 2.9: Structure of Dimethyl-*S*-(methylcarbamoylmethyl) phosphorothionate.

Dimethoate has a carboxyamide group through which it achieves selectivity. It is a colourless crystal with camphor-like odour and melts at 51.52 °C. It has a vapour pressure of 8.5×10^{-4} mmHg at 25 °C and moderately water soluble, 2.5% (Gunther, *et al.*, 1968), stable in alkali (Matsumura, 1975) and soluble in most organic solvents except paraffin (Charles, 1991).

Dimethoate has been used to control aphids on many crops, including legumes, brassicas, beet, fruits, cane fruits and bush fruits, tobacco and ornamentals etc. It has also found use in farm animal hygiene. The half-life of dimethoate which is relatively short depends on ambient temperature and pH and has been shown to vary between 39 minutes to 206 days at 25 °C (Noble, 1985). The insecticide has a low enough mammalian toxicity as to be used for systemic control of cattle grubs. The highest tolerable doses for humans has been shown to be 0.2 mg/kg body weight (Charles, 1991).

2.5.6 Dimethoate Metabolism in Insects and Mammals

Dimethoate a classical organophosphate insecticide, produces its action via inhibition of cholinesterase enzyme (Gage, 1961). This is accomplished through an activation product (P=O) analogue dimethoxon (Krueger, *et al.*, 1960). Metabolism is important in bioactivation of the phosphorothionate insecticides. Various enzymatic mechanisms of detoxification have been discovered for the organophosphates. These includes: hydrolytic dearylation, glutathione-dependent dearylation, glutathione-dependent demethylation and oxidative de-ethylation.

The activation and degradation of dimethoate in mammals is almost entirely restricted to the biotransforming enzymes of the liver, notably the carboxamidase (Uchida, *et al.*, 1964). The relative lack of carboxamidase enzymes in insects makes them selectively susceptible to the toxicity of dimethoate as compared to mammals (Krueger, *et al.*, 1960; Uchida, *et al.*, 1964). The carboxamidases are more active in mammals than in insects because of the fact that the C-N bond is more vigorous in mammals than in the insects (Hassal, 1990). As a result of oxidation and hydrolysis, various metabolites are formed of which the oxygenated analogue is the most important from purely toxicological

viewpoint. It is more toxic to mammals and also considerably more water soluble than the parent compound (Robert, 1966). In mammalian systems, these metabolites are relatively minor with the primary product reported to be the carboxy-derivatives (Dauterman, *et al.*, 1960, Uchida *et al.*, 1964). Dauterman *et al.*, (1960) reported the presence of dimethoate acid in steer together with other metabolites such as desmethyldimethoate, dimethyl phosphoric acid, dimethylphosphorothioic acid and dimethylphosphorodithioic acid. The mammalian detoxifying pathway of dimethoate is shown below.



Figure 2.10: Metabolic pathway of dimethoate in mammals (Hassal, 1987).

Probably all organophosphorous insecticides with the P→S structure are desulphated by microsomal oxidases of mammals and insects to their corresponding P→O analogues (Dauterman, 1971). Since the P→O analogues are the actual toxic agents and the P→S acid derivatives are often the major degradation products *in vivo*, the balance between these two reactions is of critical importance in determining the action of phosphorothionate insecticides (Wilkinson, 1976).

2.6 PESTICIDE INTERACTIONS AND THEIR TOXICOLOGICAL IMPLICATIONS

There is currently a growing concern over the potential hazard to man of toxic interaction resulting from chronic exposure to low levels of the multitude of drugs, pesticides and

other chemical agents present in our environment. An interaction is said to occur when the effect of one drug or chemical is affected by the presence of another drug/chemical, food, drink or by some environmental chemical agent. The outcome may be harmful if the interaction causes an increase in the efficacy or toxicity of the drug (Stockley, 1994). The increasing use of multidrug therapy (polypharmacy) in human has in recent years provided many examples of both beneficial and deleterious interactions. The recognition of the potential toxicological significance of such interactions has done much to stimulate research on the mechanisms by which they occur. The extent to which one chemical or drug can modify the biological activity of another depends on the site at which the interaction occurs. In other words, it depends on the relative importance of that site in limiting the activity of the first chemical/drug applied alone (WHO, 1981).

Combined exposure to pesticides is of particular interest in view of the common use of pesticide mixture in formulations for agricultural work. In practice, pesticides are often used in combinations in plant protection and public health programmes so as to overcome resistance and/or increase efficiency of the compounds. Agriculturists field workers are often exposed to the combined effects of pesticides as an occupational hazard (Arienes, *et al.*, 1976).

The phenomenon of interaction of several insecticides gains importance, therefore, particularly as it could influence the toxicological profile of the individual insecticides (Dikshith, *et al.*, 1987). Interaction between various kinds of chemicals including drugs and pesticides can be categorized into three types, namely:

- i) Pharmaceutical interactions
- ii) Pharmacokinetic interactions

iii) Pharmacodynamic interactions (Berecky, 1987).

Pharmaceutical interaction arises when the compounds or a mixture of compounds or chemical entities are physically or chemically incompatible (e.g. oxidizing and reducing agents etc.). Pharmacokinetic interaction occurs at the absorption, distribution, biotransformation and elimination routes. Biotransformation route has been reported to play a significant role in pesticide interaction and toxicity (Frawley, *et al.*, 1957; Iyaniwura, 1990). Pharmacodynamic interactions occur between two or more chemical compounds at the site of action. Both compounds could be agonists and / or antagonists at the site thereby competing for the same receptor site.

An understanding therefore, of the mechanisms responsible for potentiation or antagonism of combination of pesticides has become essential as a practical approach to the development of procedures for testing the capacity of one agent to alter the toxicity of another.

The stimulatory action of the organochlorine insecticides on hepatic drug metabolism can markedly influence the acute pharmacological action and toxicity of various drugs and chemicals. Pretreatment of rats with chlordane or DDT afforded protection from gastric lesions caused by phenylbutazone (Welch and Harrison, 1966) and substantially decreased barbiturate sleeping time (Conney, *et al.*, 1967). Furthermore, Salviini and Tomiz (1961) reported malathion toxicity in mammals increased several times by prior administration of *O*-ethyl-*O*-4-nitrophenylphosphorothionate (EPN). Phenobarbitone pretreatment has been demonstrated to offer protection against acute toxicity of paraoxon in mice (Vitarius, *et al.*, 1995). In a similar study however, the hepatotoxicity of carbon

tetrachloride and chloroform were shown to be potentiated by pretreatment with phenobarbitone (WHO 1981). Studies have also reported potentiation of dimethoate toxicity by phenobarbitone (O'Brien, 1967) and the organochlorine insecticides (Menzer, 1970 and 1971) in mice.

2.7 ENVIRONMENTAL HAZARDS OF PESTICIDES

The increase in world population and in demand for food and economic crops has put a pesticides burden on man and the environment. Pesticide residues from vector control and agricultural uses contaminate the terrestrial and aquatic surroundings and contribute to the pollution problem of other environmental contaminants.

In general, insecticides are the most hazardous to the environment followed by herbicides and fungicides respectively (WHO, 1988). The insecticides most frequently involved in environmental pollution are the organochlorines DDT, DDE, endrin, heptachlor, aldrin, dieldrin, toxaphen, and BHC or its gamma isomer, lindane (Nicholson, 1969). In a study conducted to investigate the global distribution of 22 potentially harmful organochlorine compounds, Simonich and Hites (1995) reported high concentrations of organochlorines found in both developing and industrialized countries. Some organophosphate pesticides including dimethoate and methyl parathion have also been found to be present in the environment (Rehana, *et al.*, 1996).

The major source of environment contamination by pesticides is from deposits resulting from applications. Other sources arise from leakage during transportation, distribution or storage. The movement of pesticides from waste disposal sites also presents potential hazard both to the environment and to human health. It is estimated that less than 0.1%

of pesticides applied to crops reach the target pests (Pimentel & Levitan, 1986), thus, leaving more than 99% having potential to impact non-target organism (NTOs) and to become widely dispensed in the environment.

Two aspects of this problem appear to have particularly important effects on man and domestic animals, which may take up pesticides mostly through contaminated foods and feeds and effects on wildlife. Here, species can be severely affected by absorption or accumulation of pesticides through food chain so that the balance of the ecosystem is directly or otherwise disturbed. The levels of contamination of meat, milk and eggs with organochlorine compounds is of importance to man. It was reported that the level of DDT in milk in DDT-fed dairy cows equalled the level in the diet within two weeks (Zweig *et al.*, 1961). A similar observation was made in Indian buffaloes (Kalra *et al.*, 1986). Spindler (1983) provided a comprehensive data based on studies that evaluated residues of organochlorine insecticides in human milk in many countries. Values in 1978 ranged from 0.18 ppm in Germany to 15.6 ppm in Africa. Gunther (1993) has reviewed pesticides residues problems in the environment.

Research in biotechnology has promised new biological controls to replace pesticides. Although these new biological pest controls have some advantages over the use of chemical pesticides, they are not without risks themselves (Pimentel, *et al.*, 1981 and 1989). For example, five vertebrate species intentionally introduced for biological control in the United States have all become pests themselves (Pimentel, *et al.*, 1989). These biocontrol species include the Indian mongoose, English Sparrow and the grass carp. Genetically engineered microbes as bacteria and viruses for biological control may reduce pesticide use, but may also cause environmental problems (Pimentel *et al.*, 1989).

Furthermore, it is true to say that a society which expects to reap the benefits of its technological tools must learn to control the adverse effects of these tools whatever they may be.

2.8 BIOCHEMICAL LIVER FUNCTION TESTS

In the course of chronic toxicity tests, it is pertinent that studies are carried out to evaluate the functional integrity of various organ systems especially organs such as the brain, liver, the gastrointestinal tract (GIT) and the kidney (WHO, 1978). The liver holds a central position in intermediary metabolism. It is the major site for metabolism of most compounds or substances that are absorbed through the GIT. The liver carries out the all important function of synthesis, storage and release of various endogenous compounds and nutrients. In addition, it also undertakes detoxification processes of chemicals before their subsequently removal from the body through the kidney (Mathenheimer, 1971). Hepatocellular damage with or without necrosis has been shown to cause acute release of intracellular constituents into the blood stream (Joan and Pannal, 1984). Chronic exposure to chemicals may result in liver injury in a variety of ways, such as degeneration, cell proliferation, necrosis and induction or inhibition of various enzyme activities.

Agents that are selectively toxic to the liver are regarded as hepatotoxins. The liver is the major organ in the body defences against toxic agents as a result of its detoxifying capabilities and excretory role through bile formation. Agents may either be true hepatotoxins or idiosyncratic hepatotoxins. True hepatotoxins cause damage in the liver of most subjects exposed to the agent. The extent of damage is usually dose-dependent. In contrast, idiosyncratic reactions are totally unpredictable, may be triggered by a very

small dose of the chemical, and the reaction needs to be temporarily related to the time of exposure (Iyaniwura, 1996).

Two main classes of methods are available to detect chronic toxicity from these substances in the liver: structural and functional. The structural methods involve histological examination of tissues by microscopic techniques specifically developed to measure the structural or compositional changes associated with exposure to these substances (Tardiff, 1992). Functional methods involve biochemical tests most of these involve an examination of the serum levels of hepatic enzymes that may be released following liver injury (Zimmerman, 1974; Tardiff, 1992).

A transient increase in the activity of serum enzymes or other organ derived constituents may result from a transient change in organ homeostasis that produce no lasting toxic effect (WHO, 1978). Generally however, these methods are not sufficiently standardized or reproducible to detect minor alterations in organ function but they do serve as a useful guide to general clinical status.

In the rat, diagnostic serum enzymology has been mainly directed towards early and reliable identification of liver injury due to toxicants as well as the severity of exposure (Ringhler and Dabich, 1979). The author therefore, concluded that a correlation exists between rises in serum enzymes activity and severity of toxic damage to a particular tissue. Meanwhile, the activity of hepatic enzymes in the serum may be affected by their increased or decreased synthesis, release from cells, release from damage cells, release from extrahepatic tissues and their disappearance rate from plasma (Wilkinson, 1976). Gowenlock (1988) observed that serum enzyme measurements serve in identifying liver

disease, define the nature of the disease process and locate the cellular and intracellular sites of hepatic pathology. The determination of enzyme concentrations therefore, serve as an indispensable diagnostic tool and only those enzymes contained within an organ are released in the event of injury or cell necrosis (Ikiediobi and Ukoha, 1985).

The enzymes related to liver function are the alanine and aspartate transaminases (ALT and AST), alkaline phosphatase (ALP), isocitrate dehydrogenase (ICD), alpha-hydroxybutyrate dehydrogenase (HBD) and lactate dehydrogenase (LDH). However, in the majority of clinical laboratories, clinical request for "Liver Function Tests" are limited to the results of both transaminases and ALP measurements (Wilkinson 1976; Ikiediobi and Ukoha, 1985).

2.9 ELECTROENCEPHALOGRAPHY (EEG) AS APPLIED TO PESTICIDE TOXICOLOGY

The EEG is an objective measure of activity of the brain which provides not only a local, but also global description of the functional state of the brain. The pioneering work of measuring the electrical activity of the brain and its application to clinical experimental purposes was carried out by Hans and Berger between 1924 and 1939 (Berger, 1929 and Guyton, 1976). Electrical phenomena accompanying brain processes can now be detected reliably with sensitive recording devices. Electrical recording from the surface of the brain or from the outer surface of the head demonstrate continuous electrical activity in the brain. Both the intensity and patterns of this electrical activity are determined to a great extent by the overall level of excitation of the brain resulting from functions in the reticular activating system (Guyton, 1976). Naturally a great deal of attention has been focused on the analysis of EEG wave pattern.

The electroencephalogram is composed of a number of electrical rhythms and transient discharges that may be distinguished from one another on the basis of location, frequency, amplitude, form and functional properties (Kenneth, *et al.*, 1978). The complete description of a pattern in terms of all its features is necessary both for its classification and its placement within or outside the range of normal variability (Kenneth *et al.*, 1978). EEG therefore, refers to the study of electrical potential changes that occur in the brain by use of electrodes. The frequency and amplitude give an indication of the state of the brain. Large amplitudes are indicative of deep sleep or anesthesia, while smaller amplitudes indicates wakefulness or stimulation (Bowman and Rand, 1980). Several workers have studied the relationship between the individual neurons and the EEG and have shown correlation between the occurrence of the excitatory postsynaptic potential (EPSP) and the occurrence of surface negative phenomena. It is now possible to know the precise electrical and biochemical processes taking place within some components of the brain. It is also possible to register signals that reflect the global activity with surprising accuracy and one can actually assess, by psychometric methods (Dvorak, 1991), the overall performance of the brain. The traditional description of the EEG within the amplitude and frequency paradigm has since been enriched by various brain-mapping methods (Dvorak, 1991).

EEG has been used in the diagnosis of different types of epilepsies and to localize brain tumour or other lesions of the brain. It has also been employed in the diagnoses of certain types of psychopathic disturbances (Guyton, 1976). EEG recordings using implanted electrodes in specific areas of the brain of experimental animals can be used in the assessment of the effects of toxicants. EEG changes were reported to be the first observed symptoms in some subjects exposed occupationally to pesticides (Jager *et al.*,

1970). Desi *et al.*, (1974) has reported remarkable EEG changes in rats following exposure to several organophosphate compounds. It was concluded that EEG was the most sensitive test for the detection of early and mild changes due to pesticides poisoning (Desi, 1983).

2.10 ANTICONVULSANT AGENTS

Drugs which interfere with neurotransmission mediated by GABA have well documented convulsant effects in experimental animals as well as humans. In the vast majority of inhibitory synapses in the mammalian brain, GABA is the neurotransmitter responsible for mediating inhibition. As a neurotransmitter in the CNS, GABA is ubiquitous and most type of neurones can be shown to respond to exogenously applied GABA or GABA agonist agents (Curtis and Watkins, 1965). Drugs that interfere with GABA transmission lead to severe hyperexcitation of neural circuits and ultimately, to convulsive seizure discharges.

At the same time that GABA antagonists act at certain sites to trigger seizures, they can exert anticonvulsant actions at other sites in the brain. Consequently, the convulsant action of systemically administered GABA antagonists probably reflects the net effect of a combination of opposing (convulsant and anticonvulsant) influences exerted at different sites in the brain (Gale, 1992). In addition to direct blockade of GABA transmission, other cerebral mechanism through chemical agents can provoke convulsive seizure activity. Excessive stimulation of central muscarinic cholinergic receptors, as obtains with some organophosphate insecticides, or of various glutamate receptors, also give rise to convulsive seizures. Accordingly, convulsions precipitated by one mechanism is often susceptible to suppression by another. Consequently, an

understanding of mechanisms involved in the generation of seizure activity, as well as the ability to select effective strategies for preventing chemically-induced seizures, depends upon a knowledge of the functional and neurochemical anatomy of pathways responsible for the initiation and propagation of different types of seizure manifestations (Gale, 1992).

GABA receptors are of two types: A and B. GABA_A receptors are part of a supramolecular complex that gates a chloride ionophore, while GABA_B is predominantly associated with calcium and potassium ions via a GTP-binding proteins (Enna and Karbon, 1987). GABA_A receptors are activated by agonists like GABA and muscimol, inhibited by bicuculline and picrotoxin, and facilitated by benzodiazepines and barbiturates (Olsen and Venter, 1986; Dailey, 1990). Activation of GABA leads to the opening of specific Cl⁻ channels which mediates the fast inhibiting postsynaptic potential (IPSP) response. In contrast, GABA_B receptors are activated by (-) baclofen and GABA whose actions are blocked by phaclofen (Ticku, 1991). These receptors are coupled presynaptically to voltage-gated Ca²⁺ channels, and postsynaptically they activate K⁺ channels, which results in the slow IPSP response (Bowery *et al.*, 1984; Dutar and Nicoll, 1988). Both pre-synaptic and post-synaptic GABA_B receptors appear to be coupled to G-proteins.

The GABA_A receptor is now recognized as the molecular target for many prescribed drugs e.g. sedative barbiturates and the anxiety-relieving benzodiazepines (Nutt, 1991) as well as drugs of abuse e.g. alcohol (Suzdak and Paul, 1987). It has also been shown that GABA_A receptors are also targets for different insecticides including organochlorines (e.g. lindane and cyclodienes) (Gant *et al.*, 1987a) as well as a few organophosphates

(e.g. EPN and ceptophos) (Gant *et al.*, 1987b) in addition to pyrethroids type I & II (e.g. cypermethrin) (Eldefrawi and Eldefrawi, 1989). Voltage-dependent Cl^- channels may prove to be primary molecular targets for insecticides such as lindane and secondary target for certain organophosphates and chlordecone. It should be feasible to utilize the differences in the molecular structures of the GABA receptors of insects and vertebrates to determine insect-selective toxicants.

2.11 GABA AGONISTS AS ANTICONVULSANTS

The oligomeric GABA receptor complex has been demonstrated to possess modulating binding sites. These include benzodiazepine/ β -carboline sites (Mohler and Okada, 1977), picrotoxin sites (Ticku, 1986) and barbiturates sites (Maksay and Ticku, 1985). These sites on the oligomeric receptor complex are coupled and bear an allosteric relationship to each other (Ticku, 1991). All the modulatory sites except the picrotoxin site are involved in agonistic influence. Among the GABA_A agonists which have been tested as anticonvulsants, include muscimol and progabide (Löscher, 1982).

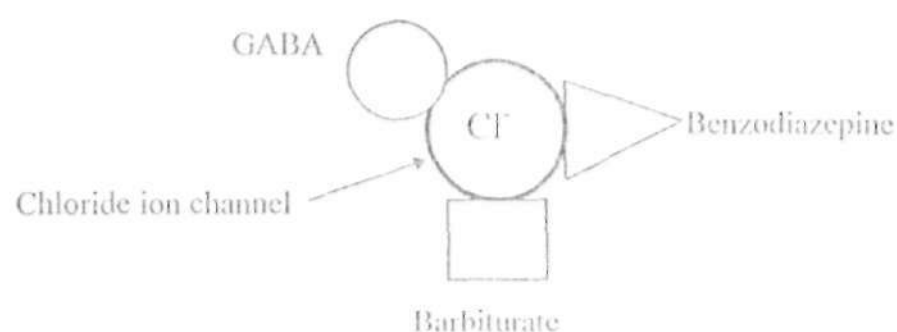


Fig. 2.11: Schematic representation of GABA_A benzodiazepine and barbiturate receptor-ionophore complex, depicting potential sites of drug modulation (Ticku, 1991).

2.11.1 Barbiturates

Barbiturates, the phenobarbitone and mephobarbitone have been used effectively as anticonvulsants for decades. The barbiturates are primarily effective against partial and generalized tonic-clonic seizures (Schleifer, 1985). Barbiturates generally produce their CNS depressant effects in a concentration-dependent manner; lower doses producing muscle relaxant/anticonvulsant effects, while higher doses produce anaesthetic effects. They bind to GABA chloride ionophore and appear to prolong the GABAergic effect (Dailey, 1990).

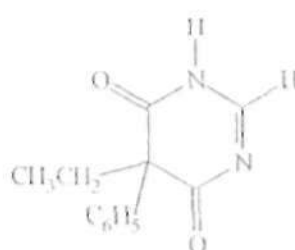


Fig. 2.12: Chemical structure of Phenobarbitone (Mol wt. 254.22; mp 273 °C)

Electrophysiological data support the notion that barbiturate enhance both the presynaptic and postsynaptic inhibition mediated by GABA. Both phenobarbitone and pentobarbitone enhance GABA-mediated responses at lower concentration and activate GABA-gated Cl^- channels directly at higher concentrations with pentobarbitone being more potent than phenobarbitone (Simmonds, 1981). This has been shown to reduce the potency of picrotoxin as a muscimol antagonist (Simmonds, 1981). A study by Macdonald, *et al.*, (1989) has demonstrated that barbiturates like phenobarbitone potentiate the GABA receptor current by increasing mean channel time and prolong bursts, without altering burst frequency.

2.11.2 Benzodiazepines

The benzodiazepines are yet another class of anticonvulsant drugs that appear to produce their effect via the GABA_A receptor complex. They include diazepam and clonazepam which are effective against several forms of epilepsy, including status-epilepticus and primary generalized and partial seizures. Diazepam was shown to enhance GABA-mediated pre-and postsynaptic inhibition in cuneate (Pole and Haefely, 1976). Electrophysiological studies have demonstrated that benzodiazepine receptor agonists increase the frequency of opening of GABA_A-gated CF channels (Chan and Farb, 1985; Dailey, 1990).

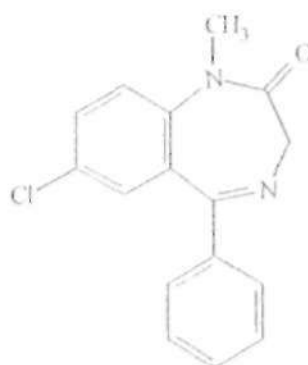


Fig. 2.13: Chemical structure of Diazepam (Mol. Wt 284.76; mp 125-126 °C).

One major problem with the benzodiazepines is the development of pharmacodynamic tolerance to their anticonvulsant effect (Rosenberg *et al.*, 1989) furthermore, there also exist cross tolerance between benzodiazepines and other drugs that facilitate GABA_A ergic transmission, e.g. ethanol and barbiturates (Little, 1988). However, diazepam has recently been demonstrated to be as effective as phenobarbitone in preventing drug-induced convulsive seizures and lethality in mice (Alldredge, 1997).

2.11.3 Phenytoin

Phenytoin is one of the most widely used anticonvulsant drugs. It has no sedative activity and has demonstrated lack of generalized CNS depression seizures by reducing the spread of the process from an active, abnormally firing epileptic focus into adjacent normal brain tissue. It has no apparent effect on the seizure threshold (Craig, 1990).

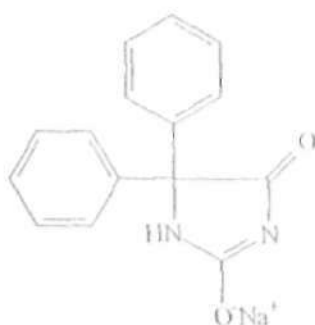


Fig. 2.14: Structure of Phenytoin sodium (Mol. Wt. 274.25; mp > 300 °C)

The major mechanism of anticonvulsant action of phenytoin in excitable tissues involves a decrease in sodium influx into neurons, which in turn, results in a decrease in the intracellular concentrations of both sodium and calcium, the net effect of which is a blockade of neurotransmitter release (Craig, 1990). Phenytoin appears to have a stabilizing effect on all neuronal membranes and probably on all excitable and non-excitable membranes as well.

CHAPTER THREE

MATERIALS AND METHOD

3.1 MATERIALS

3.1.1 Animals

For the entire aspects of experiments reported in this work, three animal species were used, namely, rats, mice and rabbits. Adult albino rats (wistar strain) weighing between 0.15 kg and 0.25 kg and mice weighing between 0.016 kg and 0.028 kg were obtained from the National Veterinary Research Institute, Vom via Jos, while rabbits weighing between 1.1 kg and 1.4 kg were obtained locally from breeders in Samaru, Zaria. All the animals were kept for a period of two weeks pre conditioning time prior to the commencement of study. Animals found exhibiting ill-health were excluded from the study.

3.1.2 Animal Feeds

Commercial feed pellets Pfizer (Livestock Feed, Lagos, Nigeria) or other standard diet was used to feed the rats and mice through out the period of the studies.

3.1.3 Test Chemicals and Drugs

Lindane (Gammalin-20[®]) - Imperial chemical Industries (ICI), England.

Dimethoate (Rogor[®]) - Fisons Agrochemicals Division, Cambridge, England.

Lignocaine 2% - Fantex Pharm. GmbH, Germany. BN-M-104, MD - 01/94; ED - 12/96.

Phenobarbitone injection ampoules Astrapin, Germany. BN-61993; MD-01/94; ED-01/97

Halothane (Hoechst[®]) - Hoechst AG Germany. BN - W483.

Diazepam injection ampoules - Starlab - PVT Ltd., Lahore, Pakistan. BN - HB 1131; MD - 12/94; ED-12/97

Diphenylhydantoin (Phenyton sodium) Capsules-Biomedicine.

All other reagents and chemical used in the experiments reported were of analytical grade obtained from British Drug House (BDH), UK.

3.1.4 Drug Administration.

All the animals used as normal control were administered with the vehicles used in preparing the drug solutions or test chemicals. Administration of drugs were by intraperitoneal route (i.p.) for the acute toxicity studies and by incorporation in the diet (p.o) for chronic toxicity studies. Maintenance doses of halothane anaesthetic (following i.p. pentobarbitone) was administered by inhalational route.

3.2 ACUTE TOXICITY STUDIES

3.2.1 Median lethal Dose (LD₅₀)

Among the studies carried out in this section are the median lethal doses (LD₅₀) of the test chemicals lindane and dimethoate as well as short term (24 hours) investigation involving the two drugs singly and in combination. The LD₅₀ is the dose of any (toxic) substance or drug that would produce 50 percent death in a randomly selected number of animals within a short period, usually 24 hours. Determination of LD₅₀ was carried out by i.p. route of administration and using the standard procedures of the arithmetic method of Karber as adapted by Aliu and Nwude (1982).

Initially, pilot studies were undertaken to determine the minimum doses of lindane and dimethoate separately, that produced 100 percent death and the maximum doses that

produced zero percent death, i.e. no death at all in a group of randomly selected animals. The final acute (LD₅₀) test was then carried out using healthy adult albino rats of average weight of 0.2 kg. The animals were divided into various equally spaced dose groups; 10 mg/kg dose difference for lindane and 50 mg/kg dose difference for dimethoate. Symptoms of toxicity were observed and scored for each rat in the various dose groups according to the method of Iyaniwura, *et al.*, (1991a). Arbitrary numbers (scores) were ascribed to the various symptoms based on their severity (Table 3.1).

The number of animals that died per dose group within 24 hours were noted. The LD₅₀ value was finally calculated using the formula below:

$$LD_{50} = \left(\text{Lowest dose that produced 100\% death} \right) - \left(\frac{\sum (\text{Dose difference}) \times (\text{Mean dead})}{\text{No. of animals per group}} \right)$$

Table 3.1: Score of symptoms as observed in poisoned animals

Symptoms	Score
Micturition	+1
Defaecation	+2
Restlessness	+3
Swaying gait (Muscle weakness)	+4
Respiratory Distress/Tremor	+5
Pallor and Prostration	+6
Convulsions	+7
Death	+8

3.2.2 Effect of lindane pre-exposure on LD₅₀ of dimethoate in rats

Rats were pre-exposed to a daily dose of lindane (1 mg/kg, i.p.) for a period of seven days. They were then used in the determination of LD₅₀ of dimethoate.

3.2.3 Effect of lindane pre-exposure on the survival time of rats challenged with a lethal dose of dimethoate

Adult male and female rats were used for this investigation. One group of animals were pre-exposed to a daily dose of lindane (1 mg/kg, i.p.) for seven days. Another group served as control and did not receive lindane pretreatment. Rats in both groups labeled A and B respectively comprised of eight rats of matched for sex and weight. On the eighth day, the animals were then challenged with a lethal dose of dimethoate (400 mg/kg, i.p.). Survival time for each rat in the two groups were then compared.

3.2.4 Effect of diazepam, phenytoin and phenobarbitone administration in mice pre-exposed to 400mg/kg lindane

A pilot study was carried out to determine the dose of lindane that produced 100 percent death in mice (50 mg/kg). Dose groups were selected for phenobarbitone (5, 10, and 20 mg/kg body weight), phenytoin (10, 20, 50 mg/kg) and diazepam (Valium[®]) (2.5, 5.0, 10 mg/kg). Each group comprised of 8 mice matched for sex and weight. Animals in the control groups received 40 mg/kg lindane alone. The other groups all received 40 mg/kg lindane immediately followed by administration of diazepam, phenobarbitone and phenytoin respectively. The effectiveness of the antidotal therapy was then assessed by calculating percent survival of animals in each group within 24 hour period.

3.2.5 Implantation of Electro cortical and Electromyograph (EMG) Electrodes And Recording of the EEG

Rats were used for this aspect of the work and were anaesthetized with pentobarbitone sodium (45 mg/kg) i.p. Following sufficient depth of anaesthesia, the head of each rat was held firmly in a stereotaxic instrument. It were then surgically implanted with stainless steel screws. The sites in the brain implanted were the frontal Cortex (FC), occipital cortex (OC), and mid-brain reticular formation (RF).

The stereotaxic coordinates for the above mentioned areas in the brain were as follows:

FC	anterior-posterior	+1.9mm
	Lateral	+1.5mm
OC	anterior-posterior	+9.2mm
	Lateral	-2.8mm
RF	anterior-posterior	-3.9mm
	Lateral	+2.0mm
	Vertical	-8.2mm

The implanted electrodes were held firmly in place using dental cement and the neck muscle was similarly implanted for EMG recording. The rat was then allowed 24 hours to recover fully from anaesthesia, under an angle poised lamp, before recording commenced.

The rats were placed in a screened cage and allowed to settle down in their new environment before the commencement of the recording. The electrodes from the rats were connected to the terminals from the polygraph using amphenone connectors. At the

end of each recording the calibration was again checked to ensure optimal response from the instrument during subsequent recordings.

3.2.6 Effects of Diazepam, Phenytoin and Dimethoate on the EEG activity in rats pre-exposed to lindane

The implanted rats were administered with lindane (10 mg/kg, i.p.) and divided into two groups. The three sets of animals were then immediately followed up with administration of diazepam (5 mg/kg i.p.), phenytoin (50 mg/kg i.p.) and dimethoate 50 mg/kg, i.p.) respectively. The EEG activities were then recorded and analysed.

3.3 CHRONIC TOXICITY STUDIES.

The chronic toxicity studies was designed to last for 12 weeks. Administration of test drug was by incorporation into the feed. Four dose groups of dimethoate were selected, namely, 10, 25, 50 and 75 mg/kg feed. The animals were grouped into four major groups of ten rats and each group sub-divided into two sub-groups of 5 rats each. A fifth group was included as normal control group. All the rats in the test group were administered dimethoate (DM) in their diet in accordance with the selected doses. In addition, one of the sub-groups in each dose group received a weekly dose of lindane (LIN) (1 mg/kg i.p.) administered in two divided doses. Control rats were given normal feed devoid of dimethoate. Feeds and water were given *ad-libitum*. Daily, feed consumption as well as body weight were recorded.

Classification of the groups and subgroups were as follows:

Group I - Control group - received normal feed.

Group IIA - 10 mg/kg feed DM alone.

IIB - 10 mg/kg feed plus 1 mg/kg LIN.

Group IIIA - 25 mg/kg feed DM alone

IIIB - 25 mg/kg feed DM plus 1mg/kg LIN.

Group IVA - 50 mg/kg feed DM alone

IVB - 50 mg/kg feed DM Plus 1mg/kg LIN.

Group VA - 75 mg/kg feed DM alone

VB - 75 mg/kg feed DM plus 1mg/kg LIN.

At the end of the fourth, eighth and twelfth week of study, five rats from each of the sub-groups and the control group were sacrificed. The liver, kidneys and intestine were removed and kept in 10% formalin solution awaiting histopathological examinations. Whole blood was allowed to clot, centrifuged and the serum collected in Bujior sample bottles awaiting biochemical assay. Mean monthly body weight as well as feed consumption were recorded.

3.3.1 Biochemical Liver Function Tests

Levels of some specific liver enzymes were determined in the serum. Also determined were the levels of total bilirubin and albumin. These biochemical parameters were used as indices to assess the effect of the pesticide, lindane and dimethoate on the functional integrity of the rat liver exposed to dimethoate in the presence and absence of lindane. Serum alkaline phosphatase (ALP) or orthophosphoric acid monoester phosphohydrolase: (EC 3.1.3.1), was determined and the values were reported in King Armstrong Unit per litre (KAU/L). Alkaline phosphatase is a widely distributed enzyme which releases inorganic phosphate from many organic phosphomonoesters and also from pyrophosphates with an optimum pH in the region of 9 - 10 at 37 °C (Kind and King, 1954). The phenol released by enzymatic hydrolysis is estimated colorimetrically. The reaction taking place is expressed below.



The KAU is the concentration of enzyme which will set free 1 mg of phenol in 15 minutes under defined conditions of the test (at pH 10 and 37 °C).

Serum aminotransferases, L-alanine-2-oxoglutarate EC. 2.6.1.2 (ALT or SGPT) and L-aspartate EC. 2.1.6.1.1 (AST or SGOT) amino transferases were determined by the colorimetric method of Reitman and Frankel (1957). Both aspartate transaminase (AST) and alanine transaminase (ALT) are widely distributed intracellular enzymes and their escape into the blood circulation, in which their activity is normally low, is a sensitive index of tissue damage. Measurements of both transaminases is therefore, useful in proving the specificity of enzymic tests for inflammatory liver disease. The reactions catalysed by the two transaminases are shown below:



In both cases enzyme activity is measured from the rate of oxaloacetate and pyruvate formation respectively (Reitman and Frankel, 1957). Activity values for both enzymes are reported in international units per litre (IU/L) which is the amount of enzyme that catalyses the formation of 1 μ mole of product per minute under defined conditions of the test (PH 10 and 37°C).

Serum albumin was estimated using dye-binding method of Bartholomew and Delaney (1966) as modified by Spencer and Price (1977) and values are reported in gram per litre (g/L). Serum total bilirubin level was also estimated by diazo reaction method of Malloy and Evelyn (1937) as modified by Michalison, *et al.*, (1965). Values are reported in grams per litre (g/L).

3.2.2 Histopathological Studies

For the purpose of histopathological studies, fresh tissues were obtained from both control and treated groups as at when due. The liver, kidney and the intestines were excised and rinsed in ice-cold 0.9% NaCl. Fresh specimens of these organs were cut, placed in processing cassettes and fixed in 10% formalin. Tissue slices of 3 cm thickness were cut and put in the automatic tissue processor and fixed in 10% buffered formalin-saline solution for 2 hours. They were then dehydrated for 2 hours in each of the ascending grades of alcohol - 85%, 90% and 100% v/v. The dehydrated tissues were then cleaned in toluene for 2 hours after which the tissues were then embedded in paraffin wax and left to cool.

The blocks were then trimmed and sectioned on the microtome at 5 microns. The ribbon of sections were then floated in a warm water bath. Suitable sections were selected, attached to slides and dried on a hot plate awaiting staining. Haematoxylin and Eosin stain (H & E stain) was used in staining the tissues. The sections were dewaxed in xylene, dehydrated in descending grades of alcohol 100%, 90% and 70% v/v. They were then stained in haematoxylin for about 5 minutes, differentiated in 1% acid alcohol, glued in Scott's tap water and stained in Eosin for 3 minutes. They were then rinsed and dehydrated in ascending grades of alcohol - 70% 90% and 100% and finally cleaned in

xylene and mounted in a pox. The slides were then examined microscopically for pathological lesions.

3.4 *IN VITRO* STUDIES (ISOLATED TISSUE EXPERIMENTS)

Experiments carried out in this section include investigations of the possible involvements of muscarinic as well as nicotinic receptors in the observed effects. Rabbit ileum preparations were used to carry out muscarinic receptor experiments while the rat phrenic nerve-diaphragm preparations were used to carry out nicotinic receptor experiments.

3.4.1 Muscarinic Receptor Investigations

Rabbits of average body weight of about 1.3 kg were used. They were starved overnight (24 hours) before the day of the experiment to get rid of the GIT contents in order to make the tissue very sensitive. Each rabbit was sacrificed by a blow on the head, decapitated and bled, followed by opening of the abdomen. Tyrode's physiological solution (see appendix IV for composition) was then immediately poured into the opened abdomen. The ileum was removed into an oxygenated petri dish containing Tyrode solution. The set up was made according to the method of Kitchen (1984). The microdynamometer 7050 recorder was used with the chart speed adjusted at 24 mm per minute. The organ bath volume was 25 millilitres and temperature was 37 °C. Agonist and antagonist contact times were 30 seconds and 2 minutes respectively. Air was used to aerate the set up. An equilibration time of 30 minutes followed by thorough washing was allowed before the commencement of the experiments.

3.4.1.1 Dose Responses to Acetylcholine, Atropine and Physostigmine using Rabbit Ileum Preparation.

Dose responses of the rabbit ileum to Acetylcholine, Atropine, and Physostigmine were obtained. A range of doses were used to obtain minimum and maximum responses for each of the drugs. Working doses for each drug was then selected for use in subsequent experiments. In each case, the working dose was selected between doses that produced 20% and 80% response respectively.

3.4.1.2 Effect of Increase in Concentration of Dimethoate on Spontaneous Contraction of Rabbit Ileum

Dose responses to dimethoate between 4 ng/ml and 64 ng/ml were obtained. The 50 percent and 75 percent effective concentration (EC_{50} and EC_{75}) were calculated from a plot of log concentration response. A suitable working dose was selected. In each case the tissue was washed several times and allowed to rest before addition of next dose. Results are reported as means of responses $\pm$ S.E.M.

3.4.1.3 Effect of Increase in Lindane Concentration on Spontaneous Contraction of Rabbit Ileum

The procedure employed here was similar to that used for dimethoate and depicted above. Dose response to lindane were obtained between 0.4 μ g/ml and 25.6 μ g/ml. In this case the 50 percent and 75 percent inhibitory concentrations (IC_{50} and IC_{75}) for lindane was calculated as well. A suitable working dose was selected for subsequent interaction studies. In each case, antagonist contact time was 2 minutes and the tissue was washed and allowed to rest before the introduction of the next dose. Finally a working dose of Ach was used to test the viability of the tissue. Results are reported as means of responses $\pm$ S.E.M. Similarly, responses to lignocaine doses between 20.5 mg/ml and

0.333 mg/ml were obtained. The IC_{50} was calculated and suitable working dose selected for subsequent use in interaction studies.

3.4.1.4 Effect of Lindane on Dimethoate Potentiation of Spontaneous Rabbit Ileum Contraction

The working doses for dimethoate and lindane used here were 8 ng/ml and 6.4 µg/ml respectively. The tissue was washed and allowed to rest. Spontaneous normal responses were observed. 8 ng/ml of dimethoate was then added into the organ bath and 30 seconds contact time allowed. Response was observed and then without washing, 6.4 µg/ml lindane dose was added into the organ bath, two minutes contact time was allowed and response observed. The tissue was now washed several times and allowed to rest. Ach (3.2 ng./ml) was added to check viability of tissue before the next dose cycle. The effect of increasing concentration of lindane (1.6, 3.2, 6.4 µg/ml) on dimethoate (8 ng/ml) was also observed. Results are reported as means of responses $\pm$ S.E.M.

3.4.1.5 Effect of Increase in Concentration of Atropine on Dimethoate Potentiation of Spontaneous Rabbit Ileum Contraction

Spontaneous contraction was observed followed by addition of 8 ng/ml dimethoate into the organ bath. Thirty seconds contact time was allowed which was immediately followed by introduction of the first dose of atropine of 2 ng/ml. Two minutes contact time was then allowed. The tissue was washed and allowed to rest before the next dose cycle. The protocol was repeated for 4 ng/ml and 8 ng/ml atropine following dimethoate administration. Results are reported as means of responses $\pm$ S.E.M.

3.4.1.6 Effect of Dimethoate on Response due to Lindane on Spontaneous Rabbit Ileum Contraction

Spontaneous contraction was observed and immediately followed by addition of 6.4 µg/ml lindane into the organ bath. Two minutes contact time was allowed. Without washing the tissue, 4 ng/ml dimethoate was added into the organ bath and 30 seconds contact time allowed. The response produced was noted and the tissue washed and allowed to rest before the next dose cycle. The procedure was repeated for subsequent dimethoate doses (8, 16, 32 ng/ml) following the same dose of lindane (6.4 µg/ml). Results are reported as means of responses $\pm$ S.E.M.

3.4.1.7 Effect of Dimethoate and Physostigmine on Response due to Lindane on Spontaneous Rabbit Ileum Contraction

Spontaneous contraction was observed. This was followed by administration of lindane (6.4 µg/ml) into the organ bath. Two minutes contact time was allowed and response observed. Without washing the tissue, dimethoate (8 ng/ml) was added and 30 secs. contact time allowed immediately followed by addition of physostigmine (1.3 µg/ml). Again 30 seconds contact time was allowed and response obtained. Results are reported as means of responses $\pm$ S.E.M.

3.4.2 Nicotinic Receptor Investigations

The rat phrenic nerve - diaphragm preparation was set up according to Bulbring (1946) as adapted by Department of Pharmacology and Clinical Pharmacy, A.B.U., Zaria (1990). One hundred and fifty millilitre 150ml organ bath volume was used. Aeration of tissue was done using 95% O₂ and 5% CO₂. The same agonist and antagonist contract times of 30 seconds and 2 min respectively were used. Krebs solution was the physiological solution used.

3.4.2.1 Effect of Increase in Concentration of Dimethoate on Direct Electrical Stimulation of Rat Phrenic Nerve Diaphragm

Normal twitch response was obtained by direct stimulation of the nerve and responses due to dimethoate were obtained for doses between 4 ng/ml and 133 ng/ml by cumulative dosing. A suitable working dose (60 ng/ml) was selected based on calculated EC_{50} . Results are reported as means $\pm$ S.E.M.

3.4.2.2 Effect of Increase in Concentration of Lindane on Direct Electrical Stimulation of Rat Phrenic Nerve-Diaphragm

Dose response due to lindane was obtained by direct stimulation of the nerve following normal twitch response. Responses for lindane dose between 1.3×10^{-1} μ g/ml and 85.1×10^{-1} μ g/ml. A working dose was selected based on the calculated $\pm C_{50}$. Results are reported as means $\pm$ S.E.M.

3.4.2.3 Dose Responses due to Lignocaine and d-Tubocurarine on Direct Stimulation of Rat Phrenic Nerve - Diaphragm

Dose response for lignocaine and d-tubocurarine were obtained following normal twitch response. Suitable working doses were selected for use in subsequent interaction experiments.

3.4.2.4 Effect of Dimethoate on Response due to Lindane on Rat Phrenic Nerve-Diaphragm

Normal twitch response was obtained by direct stimulation of the nerve. Lindane 4.3 μ g/ml working dose was introduced into the organ bath and 2 minute contact time allowed while response was observed. Without washing, Dimethoate 60 ng/ml was added into the organ bath and 30 seconds contact time allowed. The response due to dimethoate

following lindane pretreatment was observed. Results are reported as means of responses $\pm$ S.E.M.

3.4.2.5 Effect of Lindane on Response due to Dimethoate on Rat Phrenic Nerve-Diaphragm

Normal twitch response was obtained by direct stimulation of the nerve and dimethoate 60 ng/ml was introduced into the organ bath and 30 seconds contact time allowed. Without washing, 4.3 μ g/ml lindane was added into the organ bath and 2 minutes contact time allowed. The response due to dimethoate following lindane administration was obtained. Results are reported as mean $\pm$ S.E.M.

3.4.2.6 Effect of Lindane and d-Tubocurarine on Response due to Dimethoate on Rat Phrenic Nerve-Diaphragm

Normal twitch response was obtained by direct stimulation of the nerve. Dimethoate 60 ng/ml was introduced into the organ bath and 30 seconds contact time allowed. Without washing, 2.1 μ g/ml lindane was added. Immediately after the 2 minutes contact time, 2.6 μ g/ml d-tubocurarine was added into the organ bath and two minutes contact time was allowed. Results are reported as means $\pm$ S.E.M. Similarly, the effect of d-tubocurarine on lindane alone was determined using the same concentrations as used in the previous procedure involving dimethoate. Results are reported as means $\pm$ S.E.M.

3.4.2.7 Effect of Lignocaine on Response due to Dimethoate on Rat Phrenic Nerve-Diaphragm

Normal twitch response was obtained by direct stimulation of the nerve. Dimethoate 60 ng/ml was introduced into the organ bath and a 30 seconds contact time allowed. Which was immediately followed by addition of 16.8 μ g/ml. 2 minutes contact time was allowed and response obtained. Results are reported as means $\pm$ S.E.M.

3.4.2.8 Effect of Lignocaine on Response due to Lindane on Rat Phrenic Nerve-Diaphragm

Normal twitch response was obtained by direct stimulation of the nerve. Lindane 4.3 µg/ml was introduced into the organ bath and 2 minute contact time allowed. Which was immediately followed by addition of 16.8 µg/ml lignocaine, 2 minutes contact time was allowed. Responses were obtained and reported as means $\pm$ S.E.M.

3.5 STATISTICAL METHOD

All statistical analyses in this work were carried out using Student's *t*-test and the values are reported as means $\pm$ standard deviation (SD) or means $\pm$ standard error of means (SEM) (Clarke, 1984).

CHAPTER FOUR

RESULTS

4.1 ACUTE TOXICITY STUDY

4.1.1 Median Lethal Dose (LD₅₀) Determination for Lindane in Male Albino Rats using the Intraperitoneal Route

Toxicity symptoms due to lindane poisoning were observed and scored accordingly (see table 4.1). The severity of symptoms were observed to be dose-dependent and are shown in figure 4.1. At doses of 20 mg/kg and above, lindane produced convulsions and death in the animals. (Table 4.1). The LD₅₀ for lindane was then calculated as 29.5 mg/kg using the data in table 4.1.

Table 4.1: Calculation of LD₅₀ of lindane in rats using intraperitoneal route of administration.

Group	Dose (mgkg ⁻¹)	Dose Difference	Number Dead	Mean Dead	Dose Diff. x Mean Dead
Control 1	0.9% saline	-	-	-	-
2	10	10	0	0.0	0
3	20	10	2	1.0	10
4	30	10	5	3.5	35
5	40	10	7	6.0	60

(Number of animals per group = 10)

The LD₅₀ was calculated using arithmetic method of Karber as follows:

$$LD_{50} = 40 - \frac{105}{10} = 40 - 10.5 = 29.5 \text{ mg/kg}$$

$$LD_{50} \text{ Lindane} = 29.5 \text{ mg/kg (i.p. Rats).}$$

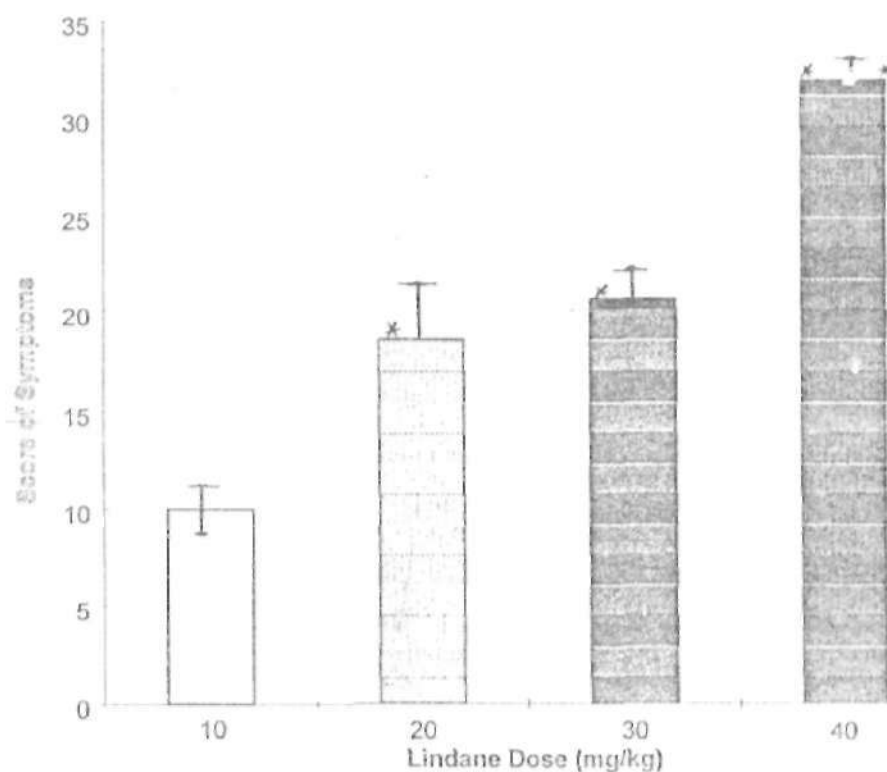


Fig. 4-1: Symptoms score for lindane poisoning in rats.

* significantly different ($P < 0.01$) from 10 mg/kg

** significantly different ($P < 0.001$) from 10 mg/kg.

Values are means $\pm$ SD for seven determinations.

4.1.2 Median Lethal Dose (LD_{50}) Determination for Dimethoate in Rats using the Intraperitoneal Route

Toxicity symptoms due to dimethoate were observed and recorded accordingly. Severity of symptoms were observed to be dose-dependent (See Fig. 4.2). Dose of 100 mg/kg dimethoate and above produced convulsions and death (Table 4.2). The LD_{50} of dimethoate was calculated as 185 mg/kg.

Table 4.2: Calculation of LD₅₀ of Dimethoate in rats using intraperitoneal route of administration.

Group	Dose (mg/kg)	Dose Difference	Number Dead	Mean Dead	Dose Diff. x Mean Dead
1 Control	0.9%	-	-	-	-
2	50	50	0	0.0	0
3	100	50	1	0.5	25
4	140	50	2	1.5	75
5	200	50	2	2.0	100
6.	250	50	4	3.0	150
7	300	50	5	4.5	225

(Number of animals per group = 5)

By the arithmetic method of Karber, the LD₅₀ was calculated as follows:

$$LD_{50} = 300 - \frac{575}{5} = 185 \text{ mg/kg}$$

$$LD_{50} \text{ Dimethoate} = 185 \text{ mg/kg (i.p., Rats)}$$

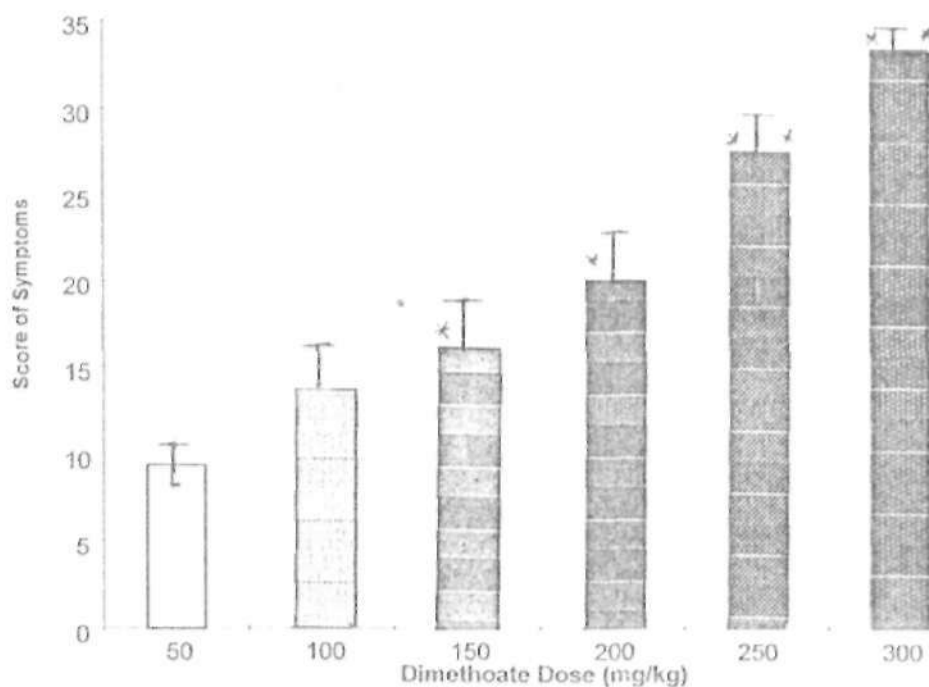


Fig. 4-2: Symptoms score for dimethoate poisoning in rats.

*Significantly different ($P < 0.05$) from 50 mg/kg

** Significantly different ($P < 0.01$) from 50 mg/kg

Values are means $\pm$ SD for five determinations.

4.1.3 The Effect of Lindane Pretreatment on the LD_{50} value for Dimethoate in Rats using I.P. Route

The LD_{50} of dimethoate was determined in rats pretreated with lindane. Symptoms of toxicity were similar to those observed in section 4.1.2. However, the minimum dose that produced convulsion and death was 150 mg/kg as against 100 mg/kg, while 100% death was also recorded at 350 mg/kg as against the 300 mg/kg in section 4.1.2. The LD_{50} was calculated as 255 mg/kg showing a 27.5% rise over the value obtained in section 4.1.2. (table 4.2).

Table 4.3: Calculation of LD₅₀ of Dimethoate in rats pretreated with lindane for seven days.

Group	Dose (mg/kg)	Dose Difference	Number Dead	Mean Dead	Dose Difference	x	Mean Dead
1 (Control)	0.9% saline	-	-	-	-	-	-
2	100	50	0	0.0	0		
3	150	50	1	0.5	25		
4	200	50	1	1.0	50		
5	250	50	2	1.5	75		
6	300	50	3	2.5	125		
7	350	50	5	4.0	20.		

(Number of rats per group = 5)

By the arithmetic method of karber, the LD₅₀ was calculated as follows:

$$LD_{50} = 350 - \frac{475}{5} = 350 - 95 = 255 \text{ mg/kg}$$

LD₅₀ of Dimethoate in rats pretreated with lindane = 255 mg/kg (i.p.).

4.1.4 The Effect of Lindane Pretreatment on the survival time of Mice Challenged with a Lethal Dose of Dimethoate

Survival time of mice following administration of lethal dose of dimethoate was determined in control lindane-free and lindane-treated animal groups. Survival times obtained were 18.8 ± 6.7 minutes and 25.4 ± 10.0 minutes for lindane-free and lindane-treated groups respectively. The result shows a 25.9% increase in survival time over control (See fig. 4-3).

4.1.5 Antidotal Therapy trial against Lindane Poisoning in Mice

Survival rate following 400 mg/kg lindane dose was observed in four groups of animals including the control. The other three groups received phenobarbitone, diazepam and phenytoin immediately following (sub-lethal) lindane dose.

Diazepam (10 mg/kg) provided the highest protection giving a 75% survival rate. Phenobarbitone (20 mg/kg) gave 62.5%, while phenytoin, (40 mg/kg) produced 50% survival rates respectively. The control group gave 12.5% survival rate (table 4.4).

Table 4.4: Effectiveness of phenobarbitone, diazepam & phenytoin used as antidotal agents against lindane poisoning in mice.

Parameter	LINDANE mg/kg	PHENOBARBITONE mg/kg. % Survival		DIAZEPAM mg/kg % survival		PHENYTOIN SODIUM mg/kg % survival		CONTROL % Survival
G-I (Control)	40	-	-	-	-	-	-	12.5
G-II	40	5	25	2.5	37.5	10	25	-
G-III	40	10	37.5	5.0	62.5	20	37.5	-
G-IV	40	20	62.5	10.0	75	40	50	-

(n = 8 mice per group)

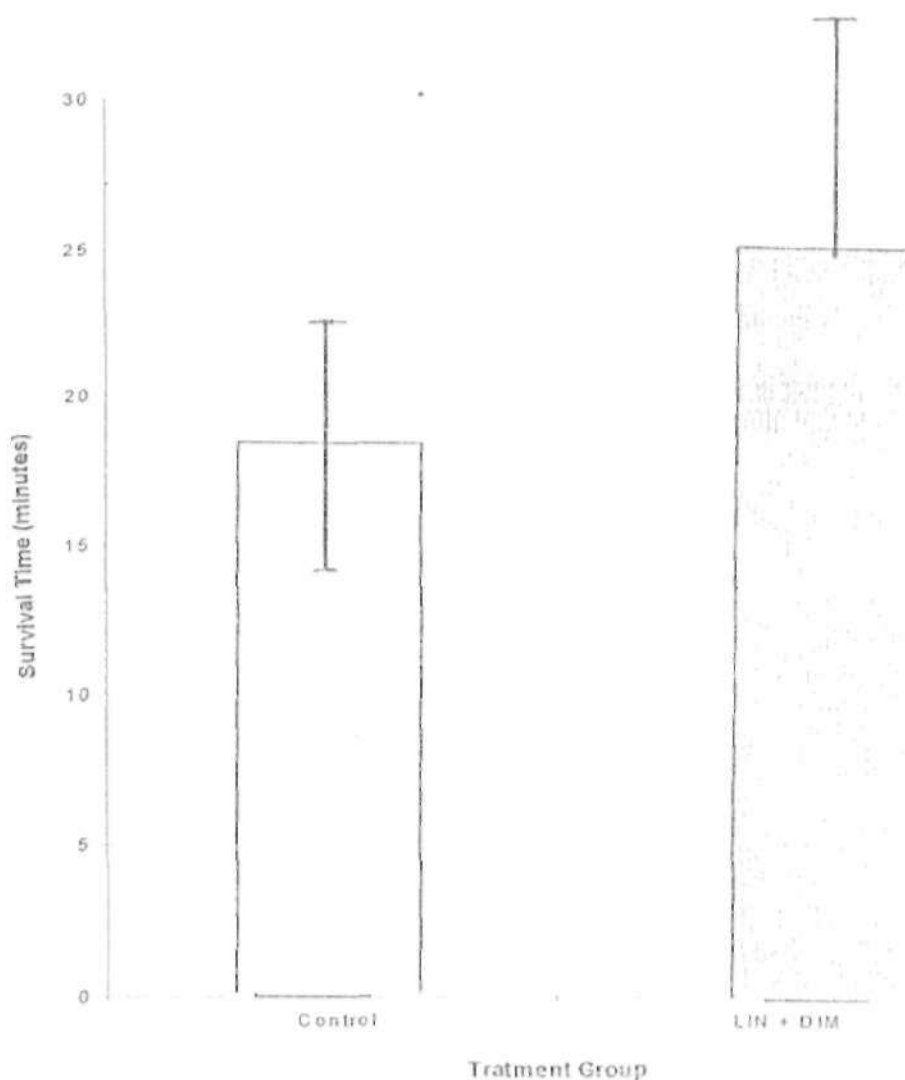


Fig. 4-3: Effect of Lindane pretreatment (1 mg/kg for 7 days: i.p) on the survival time of mice challenged with a lethal dose of dimethoate (400 mg/kg, i.p). Values are means $\pm$ SD for 8 determinations.

4.1.6 The Effect of antidotal trial agents on the EEG activity of Lindane - treated Rats

Diazepam and phenytoin were used as trial agents on EEG activity and their effects were compared to that of dimethoate or lindane. Results of the EEG activity were analyzed based on the measurement of frequency and amplitude of the wave pattern as seen in figure 4.4A, 4.4B and 4.4C. Summary of the mean values for the parameters are shown in figures 4.5A and 4.5B. Significant differences were observed between diazepam and phenytoin on the one hand, as well as dimethoate compared to controls on the other (fig. 4.5A and 4.5B).

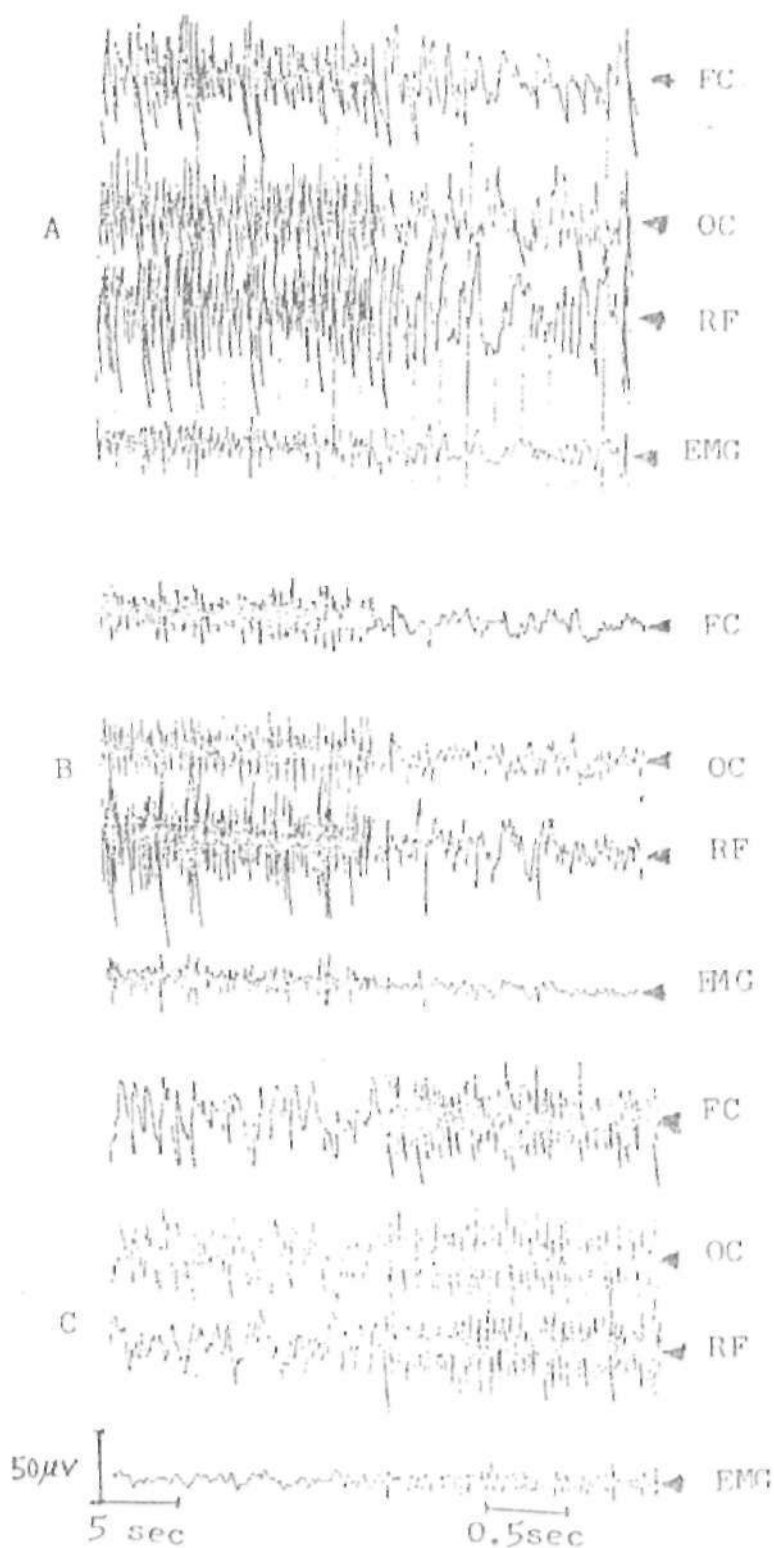


Fig. 4.4A EEG activity of rats administered (B) Lindane (10mg/kg, i.p.), (C) Lindane + diazepam (5mg/kg) and (A) Drug-free control.

FC= Focal cortex

OC= Occipital Cortex

RF= Reticular Formation

EMG= Electromyogram

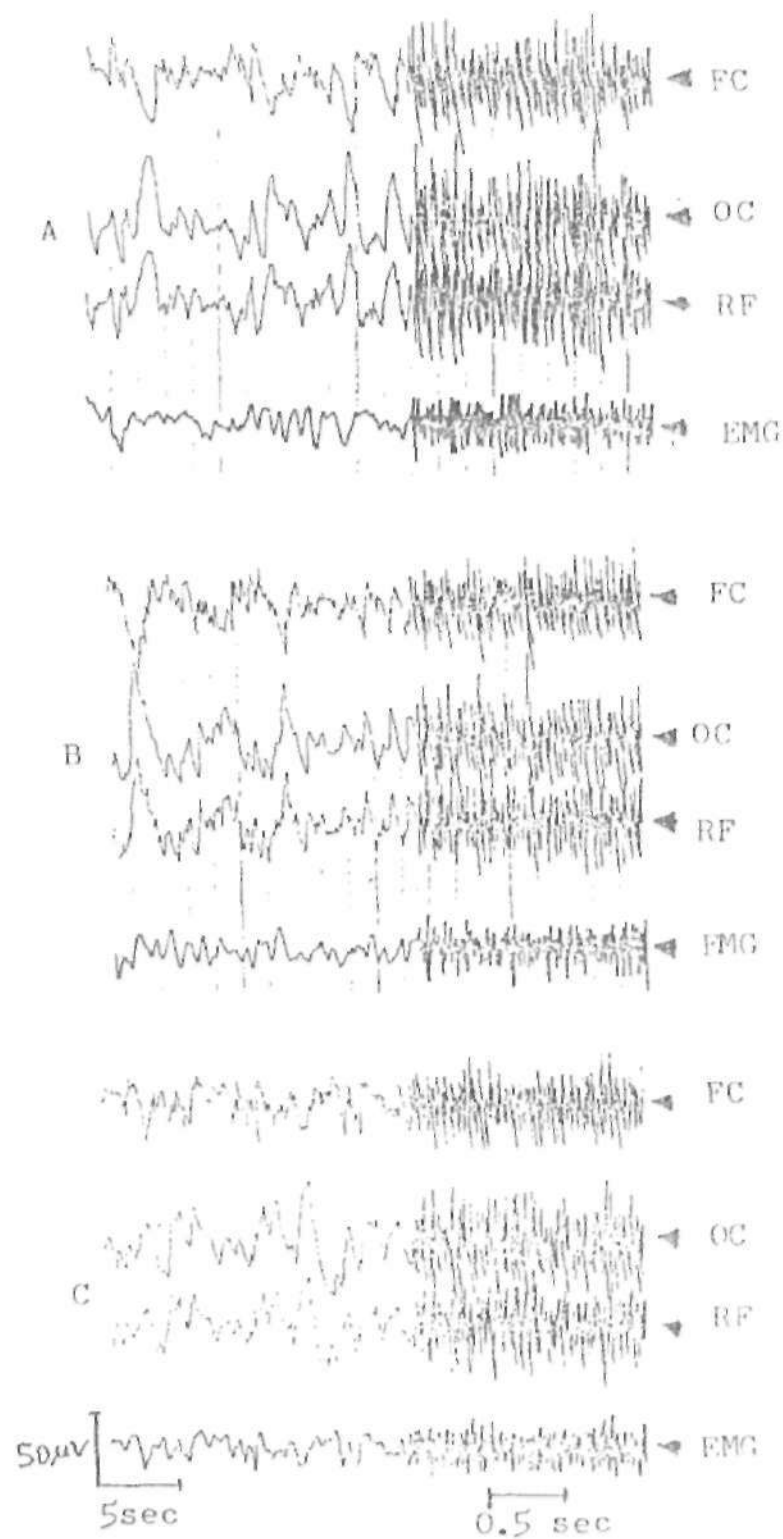


Fig. 4.4B EEG activity of rats administered (B) Lindane (10mg/kg, i.p.), (C) Lindane + Phenytoin (40mg/kg) and (A) Drug-free control.

FC= Focal cortex

OC= Occipital Cortex

RF= Reticular Formation

EMG= Electromyogram

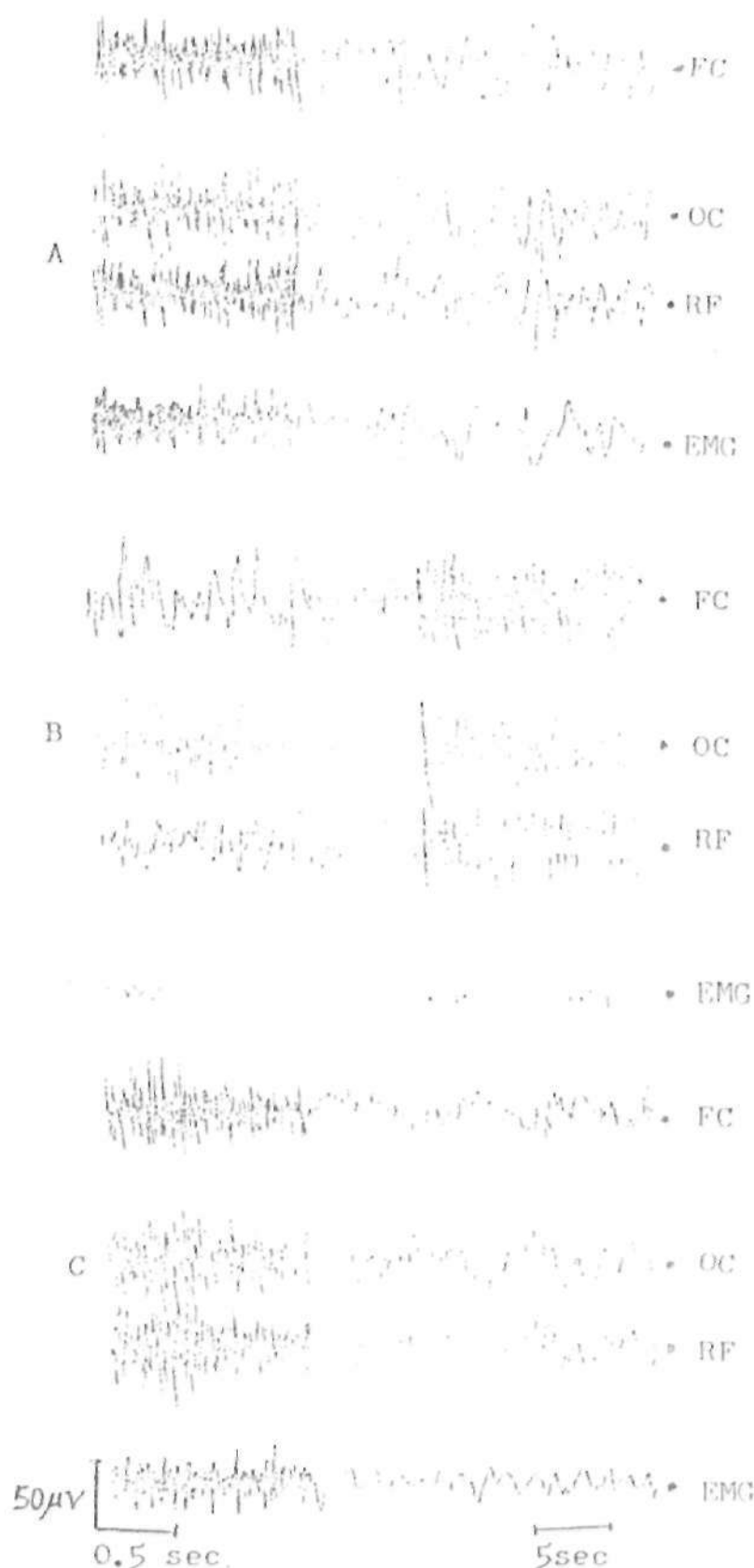


Fig. 4.5C Effect of Lindane and dimethoate on the EEG activity in rats administered (B)

Lindane (50mg/kg), (C) Lindane (10mg/kg) + dimethoate (50mg/kg) and (A) Drug-free

control. FC= Focal cortex OC= Occipital Cortex RF= Reticular Formation

EMG= Electromyogram

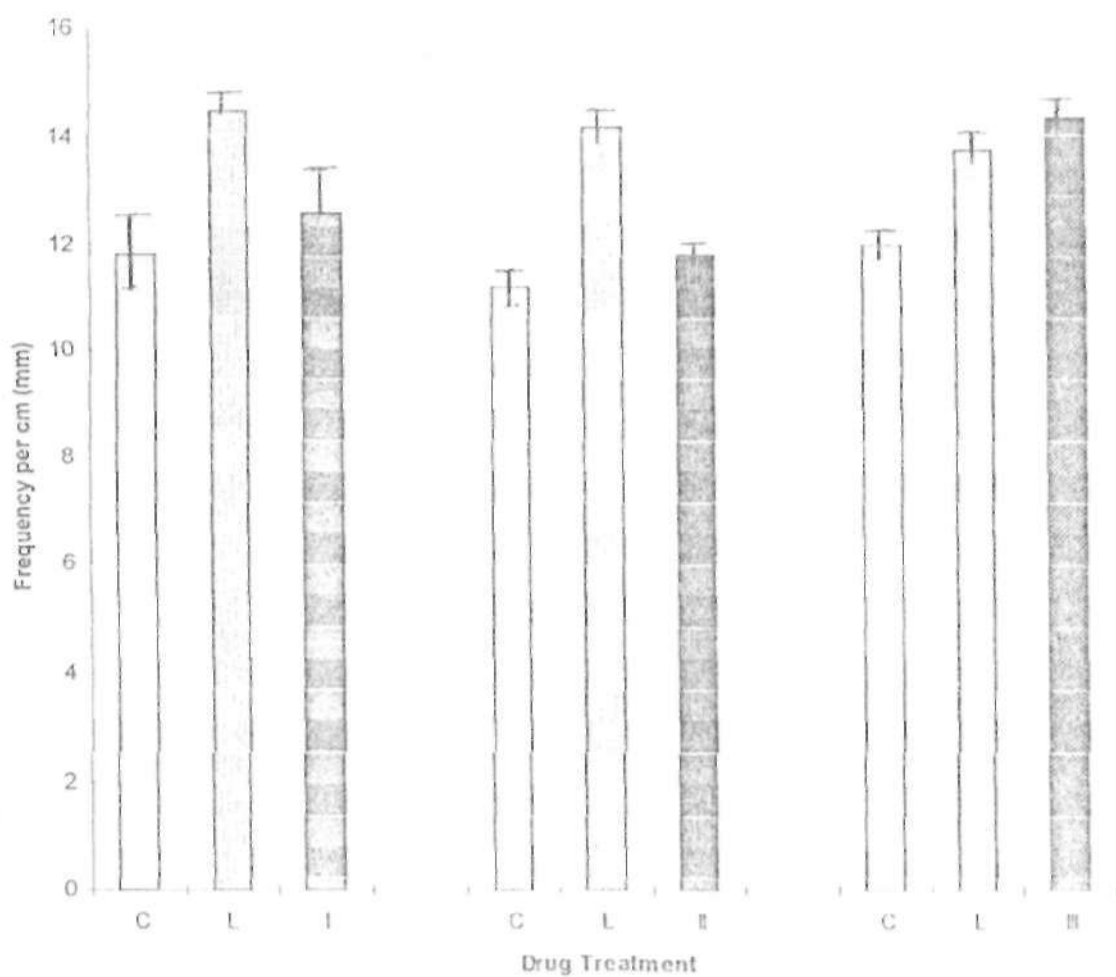


Fig. 4.5A Frequency of EEG activity in (C) normal rats, (L) rats administered lindane (10mg/kg, i.p.) alone, (I) Lindane + diazepam (10 mg/kg), (II) Lindane and Phenytoin (40mg/kg) + (III) lindane + dimethoate (50 mg/kg).

KEY C = Control

L = Lindane (10 mg/kg) alone

I = Lindane + Diazepam (10mg/kg)

II = Lindane + Phenytoin (40 mg/kg)

III = Lindane + Dimethoate (50 mg/kg)

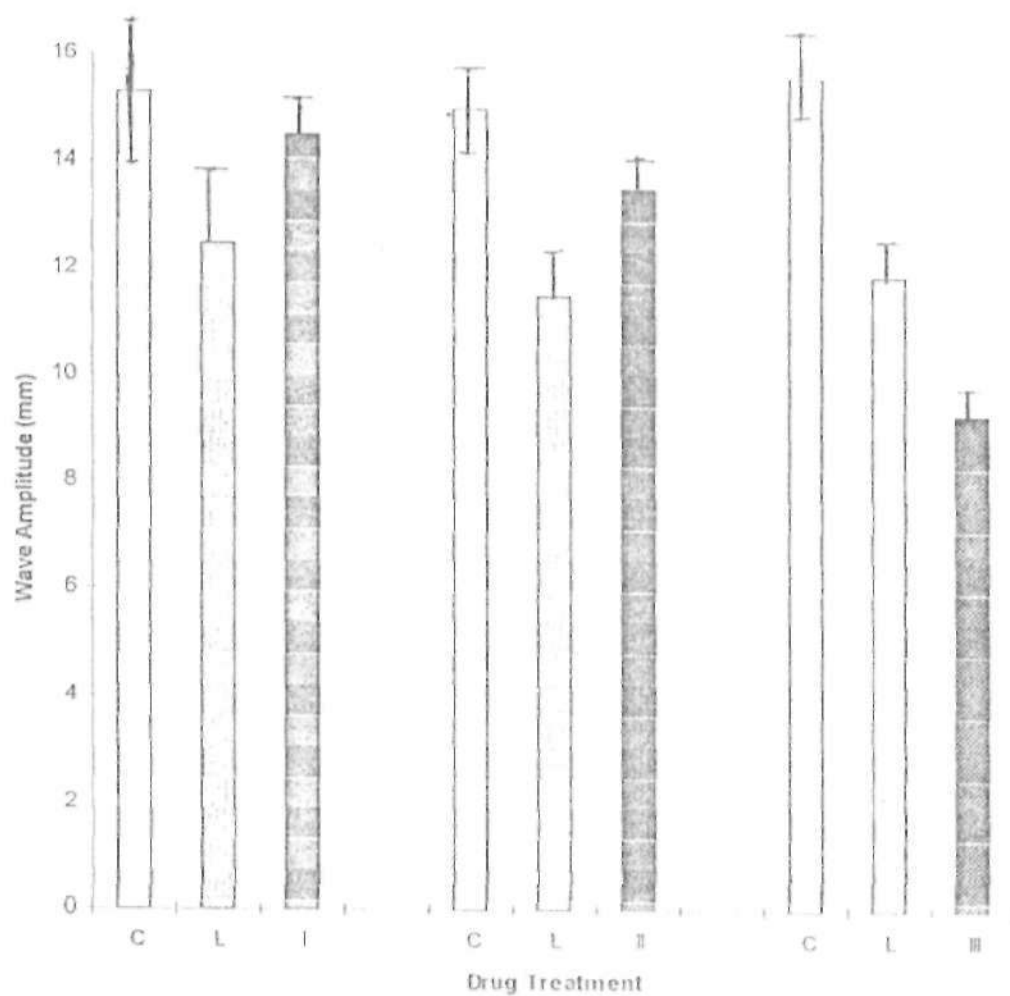


Fig. 4.5B: Amplitude of EEG activity in (C) control rats, (L) rats administered lindane (10 mg/kg) alone, (I) Lindane + diazepam (10 mg/kg), (II) Lindane + phenytoin (40 mg/kg) and (III) lindane + dimethoate (50 mg/kg)

KEY C = Control

L = Lindane (10 mg/kg) alone

I = Lindane + Diazepam (10mg/kg)

II = Lindane + Phenythoin (40 mg/kg)

III = Lindane + Dimethoate (50 mg/kg)

4.2 *IN VITRO* STUDIES.

4.2.1 Effect of Dimethoate on the Cholinergic Receptor using Rabbit ileum preparation

Dose responses of rabbit ileum were obtained for dimethoate concentrations between 1.74×10^{-13} M (4 ng/ml) and 2.78×10^{-12} M (64 ng/ml). Concentrations between 1.74×10^{-13} and 1.39×10^{-12} M produced a dose - dependent augmentation of spontaneous contraction of rabbit ileum (fig., 4. 6A). The 50% and 75% excitatory concentrations (EC_{50} and EC_{75}) were calculated from a plot of log-dose response curve. The values obtained were 2.5×10^{-13} M and 4.36×10^{-13} M respectively (fig.4.6B). Dimethoate concentrations between 1.74×10^{-13} M and 6.96×10^{-13} M were selected for further interaction studies.

4.2.2 Effect of Lindane on the Cholinergic Receptor using Rabbit Ileum Preparation.

Dose response of rabbit ileum were obtained for lindane concentration between 5.5×10^{-11} M (1.6 mg/ml) and 8.8×10^{-10} M (25.6 μ g/ml). Those produced a dose-dependent attenuation of spontaneous contraction of rabbit ileum (Fig 4.7A) 50% and 75% inhibitory concentrations (IC_{50} and IC_{75}) were calculated as 1.25×10^{-10} M (3.63 μ g/ml) and 2.51×10^{-10} M (7.29 μ g/ml) respectively using the log-dose response plot (Fig.4.7A and 4.7B). Dose between 5.5×10^{-11} M and 2.2×10^{-10} M were chosen as working doses for interaction studies.

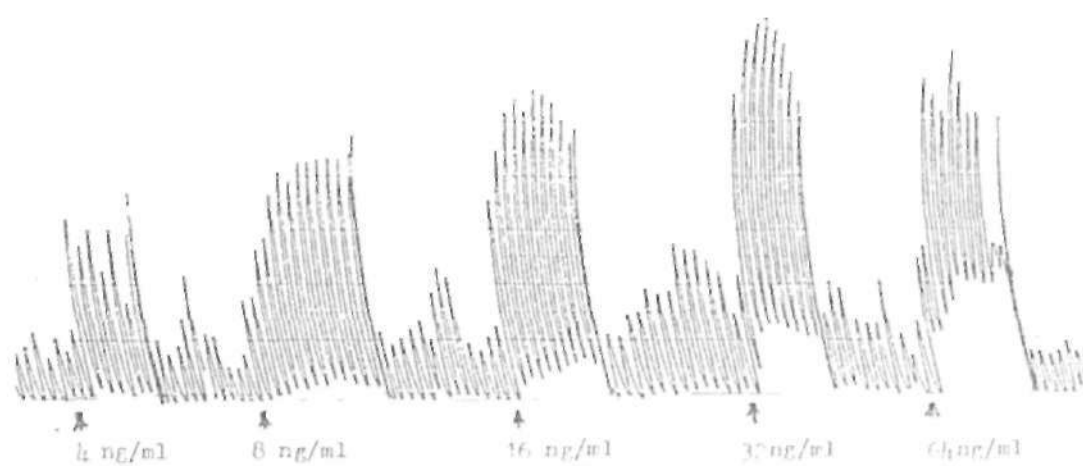


Fig. 4.6A: Effect of dimethoate on spontaneous on spontaneous contraction of rabbit ileum.

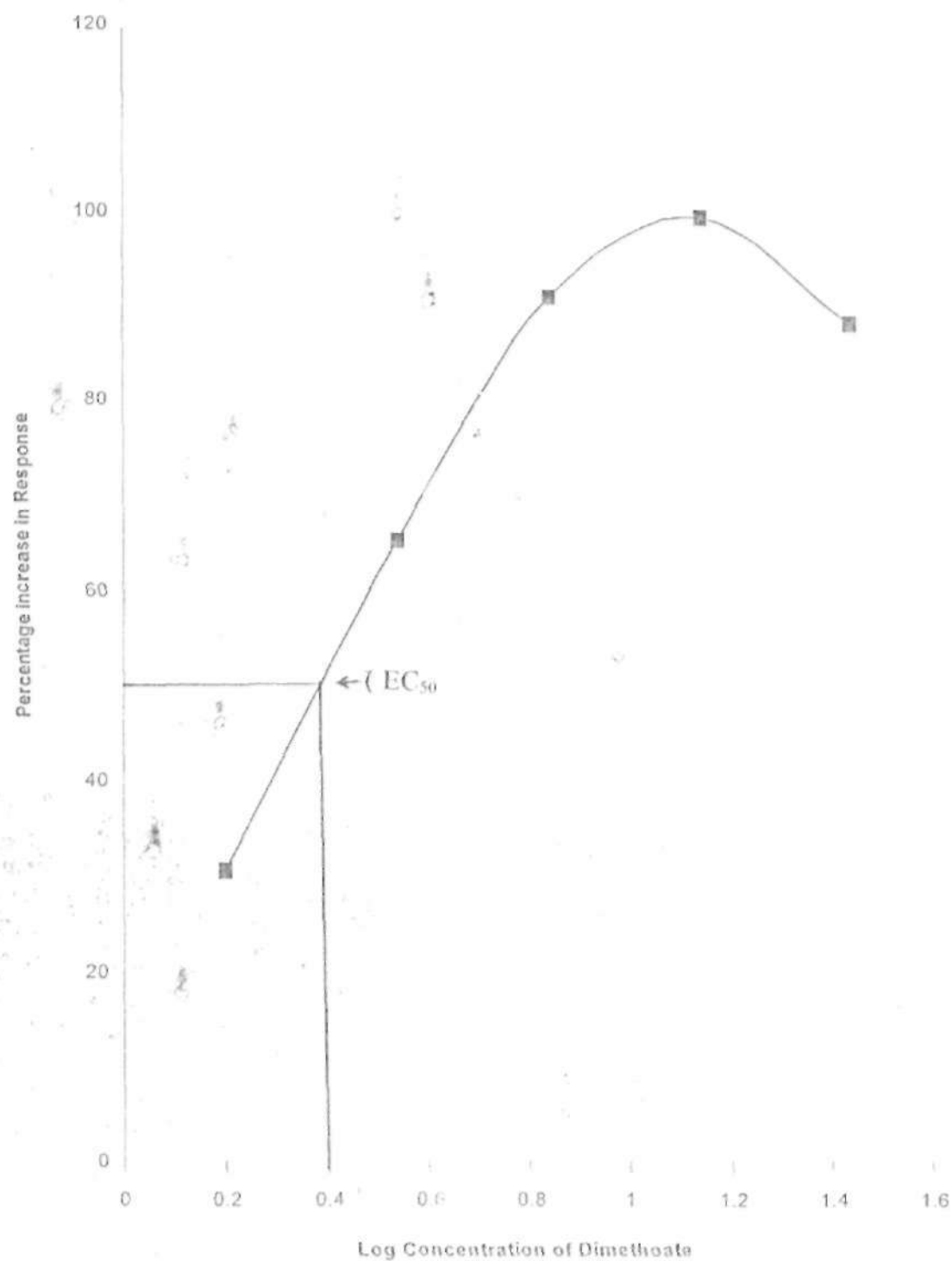


Fig. 4.6B: Log concentration curve for dimethoate using rabbit ileum preparations

($EC_{50} = 2.50 \times 10^{-13}$ M; $EC_{75} = 4.36 \times 10^{-13}$ M).

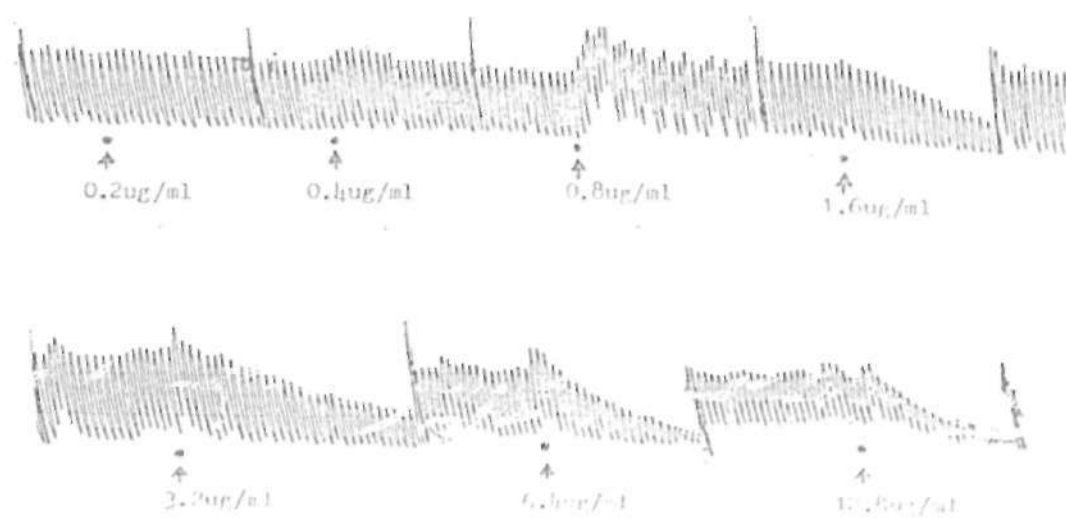


Fig. 4.7A: Effect of Lindane on spontaneous on spontaneous contraction of rabbit ileum.

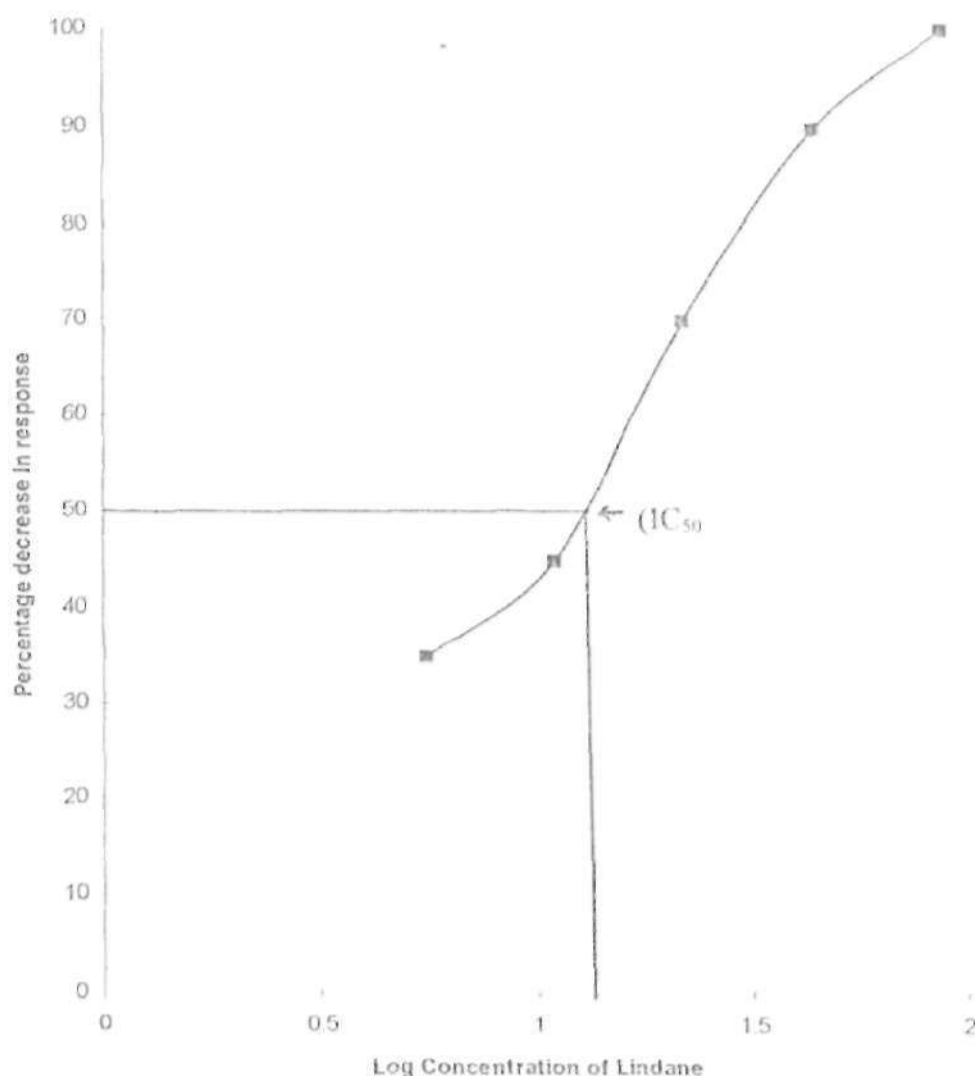


Fig. 4.7B: Log-concentration curve for lindane using rabbit ileum preparation ($IC_{50} = 1.2 \times 10^{-10}$ M; $IC_{75} = 2.51 \times 10^{-10}$ M)

4.2.3 Effect of Atropine on Response due to Dimethoate on Spontaneous Contraction of Rabbit Ileum

Responses due to 8.0 ng/ml dimethoate was obtained which was followed by introduction of atropine. Responses due to atropine concentrations between 2.0 and 8.0 ng/ml were obtained following introduction of 8 ng/ml dimethoate in each case. Atropine at those concentrations significantly ($P < 0.001$) attenuated (antagonized) responses due to 8 ng/ml dimethoate. 8.0 ng/ml atropine reduced dimethoate response by 57.4% (Fig. 4.8).

4.3 CHRONIC TOXICITY STUDIES

4.3.1 Effect of combined Exposure to Lindane and Dimethoate on the Functional Integrity of the Rat Liver

The parameters determined in this section of study included the serum activity levels of transaminase (ALT and AST), alkaline phosphatase, serum albumin and total bilirubin. The summary of mean values for these parameters in the various dimethoate dose groups and subgroups as a well as the period of exposure are shown in appendix II and III statistical analysis and comparisons between dose groups and subgroups and successive months are contained in appendix V and VI respectively.

4.3.1.1 Serum Transminases and Alkaline Phosphotase Activities

Activity levels for the serum transminases, ALT and AST were observed to be elevated for all the dose groups and subgroups when compared to control values. ALT activity at 75 mg/kg in the 12 week period showed a value lower than the control value. Generally, activity levels were high in the lindane-free compared to the lindane-treated subgroup for all the done groups for the entire period of study. These elevations were, however, not statistically significant (fig. 3.17, 3.18 and 3.19).

At four weeks exposure period, AST activity showed a dose-dependent elevation which were observed to be significant ($P < 0.01$) compared to control values. A comparison between the 10 mg/kg and 75 mg/kg subgroups, however, showed no significant differences. Activities of ALT on the other hand showed no significant difference at all the dose groups compared to control as well as between 10 mg/kg subgroups (fig. 4.17)

At eight weeks study period, AST activity was within the control range at 10 mg/kg and 25 mg/kg dose groups with no significant different. At 50mg/kg dose group significant

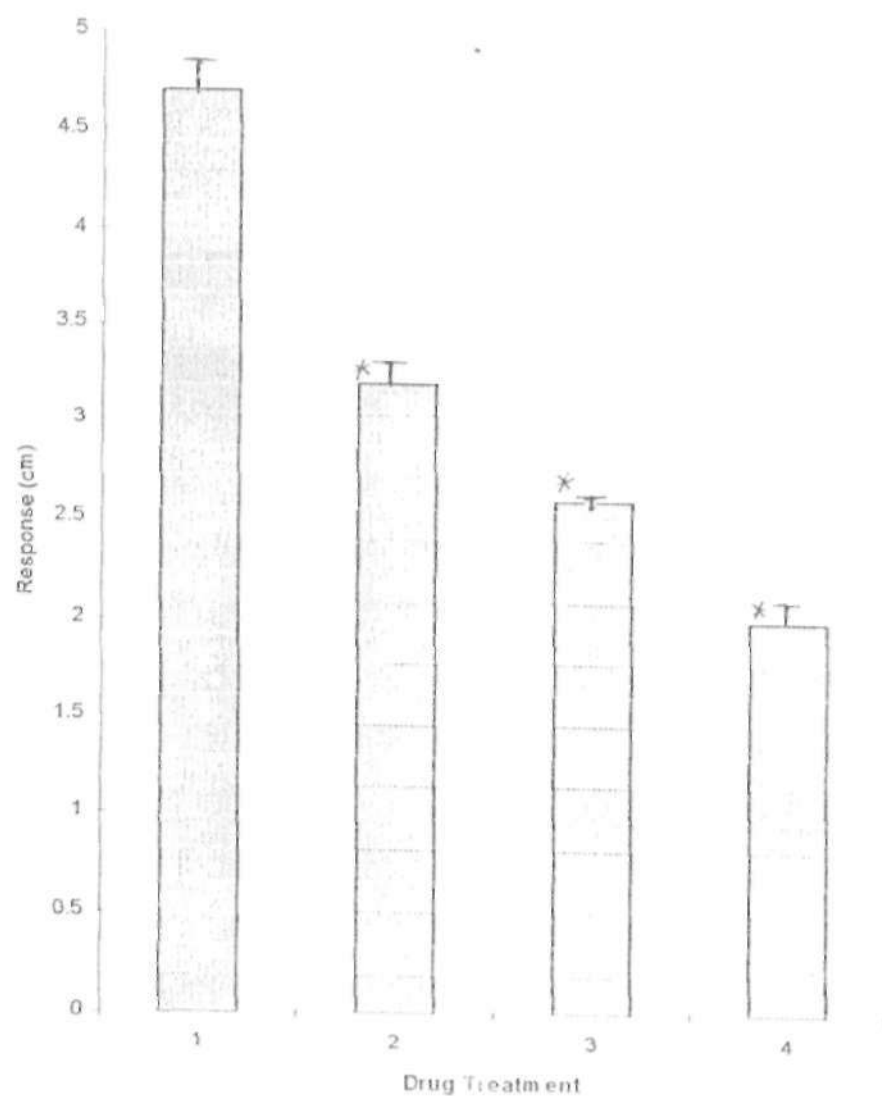


Fig. 4.8: Effect of increase in concentration of atropine on response due to dimethoate on rabbit ileum ($n = 5$; mean $\pm$ SEM)

KEY 1 = 8.0 ng/ml DM

2 = 8.0 ng/ml DM + 2.0 ng/ml Atropine.

3 = 8.0 ng/ml DM + 4.0 ng/ml Atropine.

4 = 8.0 ng/ml DM + 8.0 ng/ml Atropine.

* Significantly different from 1 ($P < 0.001$).

4. 2.4 Effect of Lindane on Dimethoate Response on Rabbit ileum

8.0 ng/ml (3.48×10^{-13} M) dimethoate concentration was used as working concentration. Lindane concentrations between 1.6 and 6.4 ng/ml dose-dependently attenuated dimethoate response (fig. 4.9). 3.2 and 6.4ng/ml lindane doses significantly ($P < 0.01$ and $P < 0.001$ respectively) reduced responses due to 8.0 ng/ml dimethoate. At 6.4 ng/ml concentration, lindane produced 22.4% decrease in response due to dimethoate (Fig. 4.9).

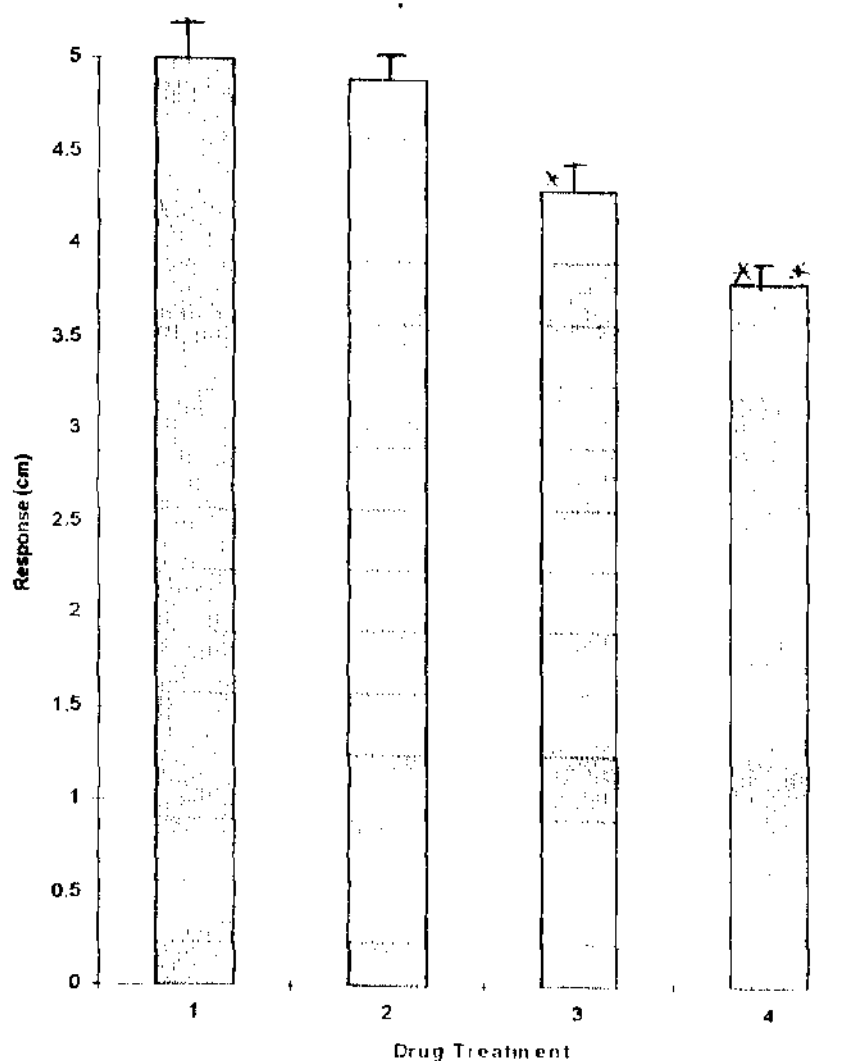


Fig. 4.9 : Effect of increasing concentration of lindane (LIN) on response due to dimethoate (DM) on rabbit ileum ($n = 5$; Mean $\pm$ SEM)

KEY 1 = 8.0 ng/ml DM

2 = 8.0 ng/ml DM + 1.6 μ g/ml LIN

3 = 8.0 ng/ml DM + 3.2 μ g/ml LIN

4 = 8.0 ng/ml DM + 6.4 μ g/ml LIN

*Significantly different ($P < 0.01$) from 1.

** Significantly different ($P < 0.001$) from 2.

4.2.5 Effect of Lindane pretreatment on Dimethoate response on Rabbit ileum

Lindane 6.4 ng/ml and dimethoate 4.0-16.0 ng/ml concentrations were selected as working doses. Dimethoate, dose-dependently increased prior responses due to 6.4 ng/ml lindane. Responses were significantly ($P < 0.001$) increased following introduction of 8 and 16 ng/ml dimethoate (fig. 4.10). Furthermore, 16 ng/ml dimethoate produced 48.5% increase in response.

4.2.6 Effect of Lindane pretreatment on Physostigmine Potentiation of Dimethoate response on Rabbit ileum

Lindane 6.4 ng/ml, dimethoate 8 ng/ml and physostigmine 1.3 µg/ml were selected as working concentrations. Spontaneous (Control) response was obtained followed successively by introduction of lindane and then physostigmine (Fig 4.11A) 6.4 µg/ml lindane significantly ($P < 0.001$) reduced control response by 52.9%, and further addition of 8.0 µg/ml dimethoate significantly ($P < 0.001$) increased lindane response by 36.8%. Dimethoate response was, however, potentiated significantly ($P < 0.001$) by 1.3 µg/ml physostigmine, producing a 55.5% increase in response (fig. 4.11B.)

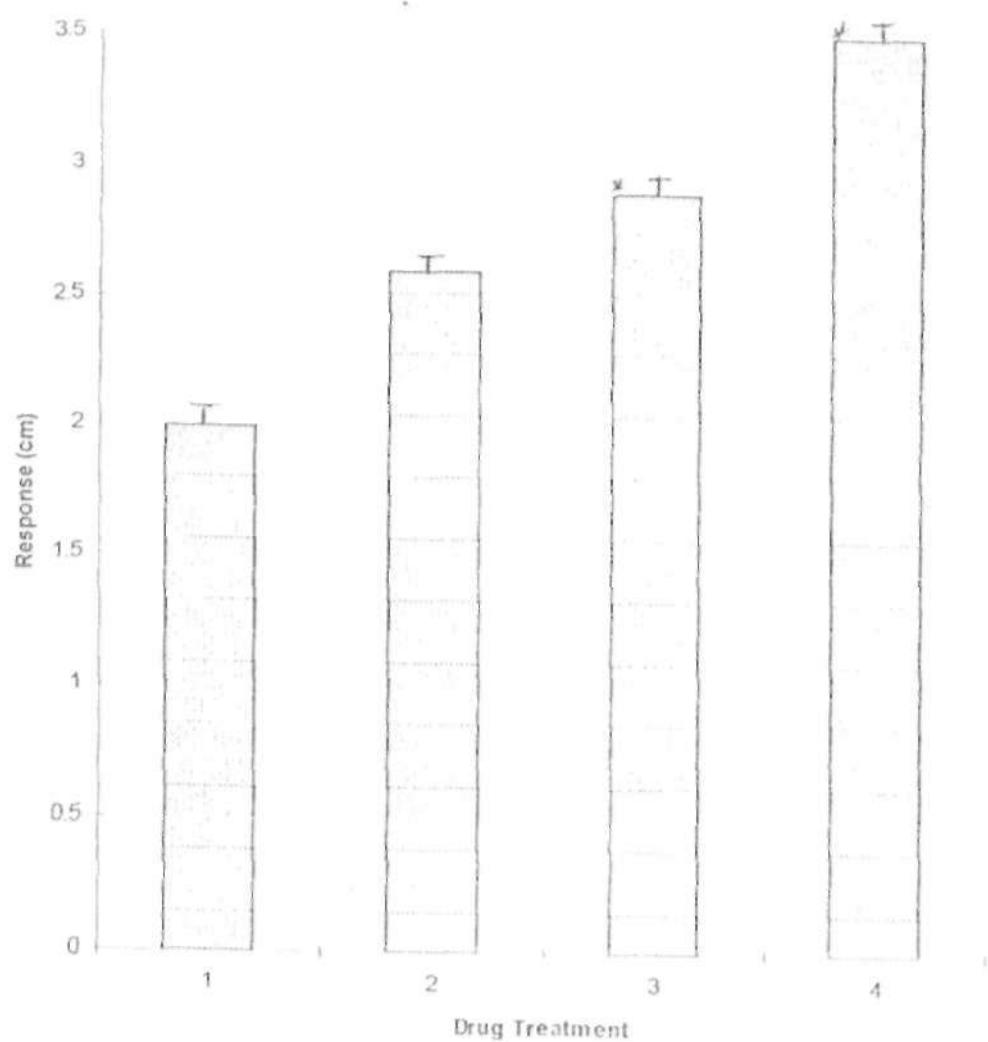


Fig. 4.10: Effect of increasing concentration of dimethoate (DM) on response due to lindane (LIN) on rabbit ileum, ($n = 5$; Mean $\pm$ SEM).

KEY 1 = 6.4 $\mu\text{g/ml}$ lindane

2 = 6.4 $\mu\text{g/ml}$ LIN + 4 ng/ml DM

3 = 6.4 $\mu\text{g/ml}$ LIN + 8 ng/ml DM

4 = 6.4 $\mu\text{g/ml}$ LIN + 16 ng/ml DM

*Significantly different ($P < 0.001$) from Lindane response (1).

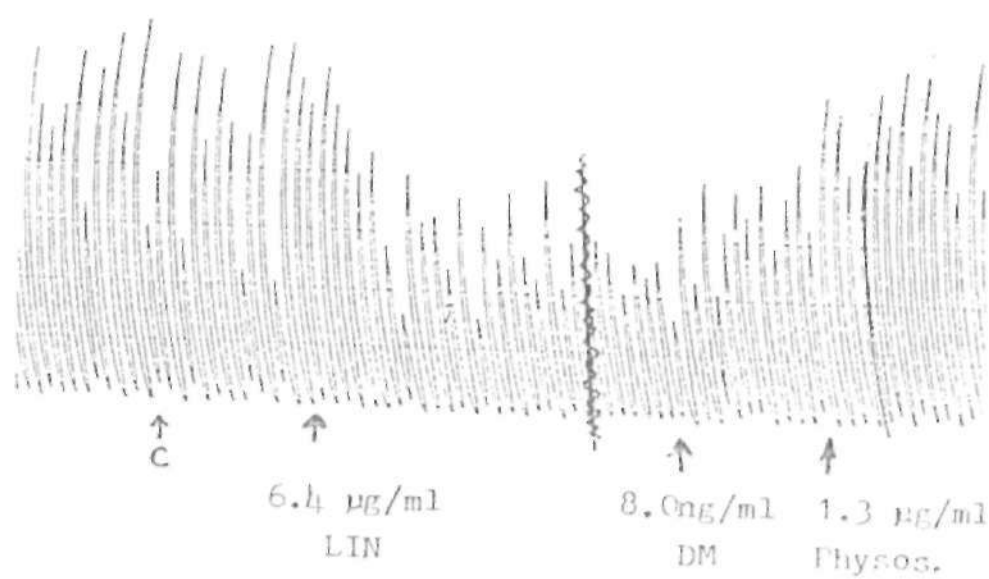


Fig. 4.11A: Effect of lindane pre-treatment on physostigmine potentiation of dimethoate response on rabbit ileum.

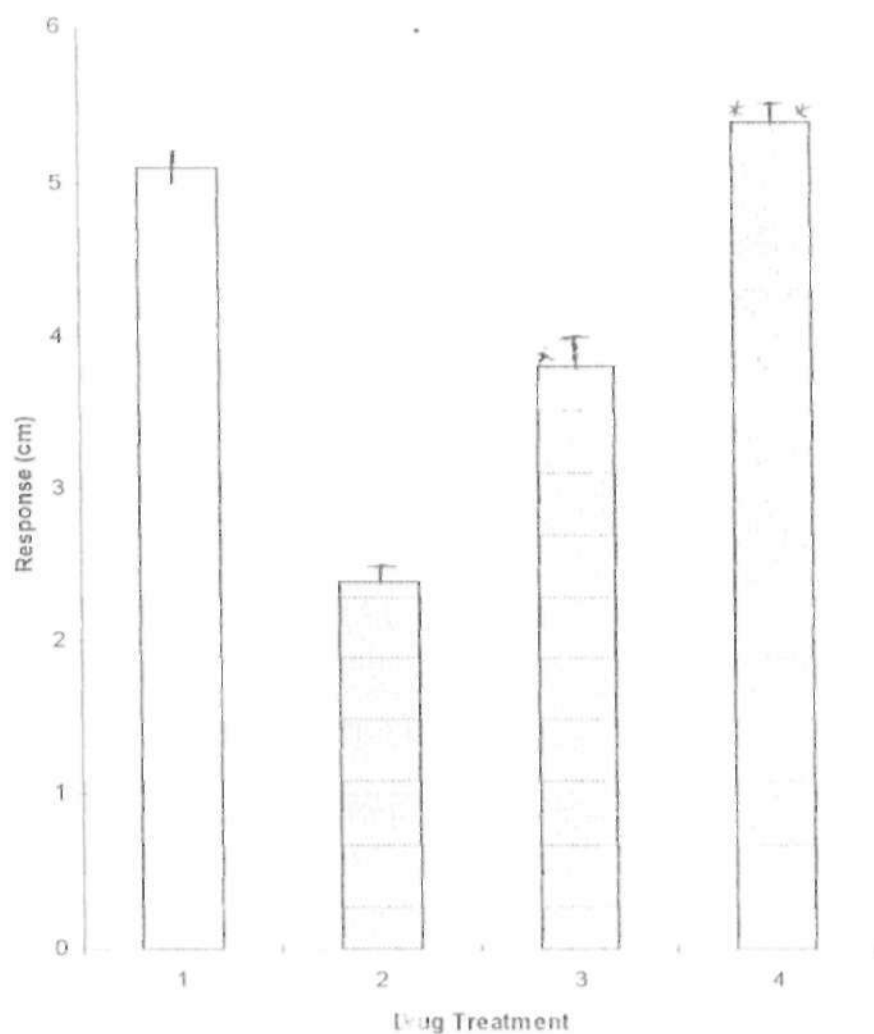


Fig. 4.11B: Effect of lindane pre-treatment on physostigmine potentiation of dimethoate response on rabbit ileum ($n = 5$ mean $\pm$ SEM).

KEY 1 = Control Response

2 = 6.4 $\mu\text{g/ml}$ LIN.

3 = 6.4 $\mu\text{g/ml}$ LIN + 8.0 ng/ml DM

4 = 6.4 $\mu\text{g/ml}$ LIN + 8.0 ng/ml DM + 1.3 $\mu\text{g/ml}$ Physostigmine.

* Significantly different ($P < 0.001$) from lindane (2) and Control (1) response.

** Significantly different ($P < 0.001$) from (2) and (3).

4.2.7 Effect of Lindane on the Nicotinic Receptor using Rat Phrenic Nerve Diaphragm preparation

Normal twitch (control) response was obtained by direct stimulation of the nerve. Responses were obtained for lindane doses between 1.3 µg/ml (4.47×10^{-13} M) and 172.8 (5.72 $\times 10^{-11}$ M). Dose-dependent attenuation of control response was observed with 172.8 ng/ml lindane producing 100% reduction, thus producing zero response (fig. 4.12A). 50% and 75% inhibitory concentration (IC₅₀ and IC₇₅) were obtained as 14.56 ng/ml (5×10^{-12} M) and 148.27 µg/ml (1.51×10^{-11}) respectively (Fig 4.12B). 2.1 µg/ml and 4.3 µg/ml lindane doses were selected as working doses for interaction studies.

4.2.8 Effect of Dimethoate on the Nicotinic Receptor using Rat Phrenic Nerve-Diaphragm preparation

Normal twitch control response was obtained for stimulation of the nerve. Responses were obtained for dimethoate doses between 4 x 180 ng/ml (1.74×10^{-13} M - 7.8×10^{-12} M). Dose-dependent augmentation of control responses was observed with doses between 4 and 133 ng/ml (fig. 4.13A) ED₅₀ and EC₇₅ values were obtained from the log-dose response plot as 9.12 ng/ml (3.98×10^{-13} M) and 19.96 (8.71×10^{-12} M) respectively (fig. 4.13B). 60 ng/ml (4.58×10^{-12} M) dimethoate was selected as working dose for interaction studies.

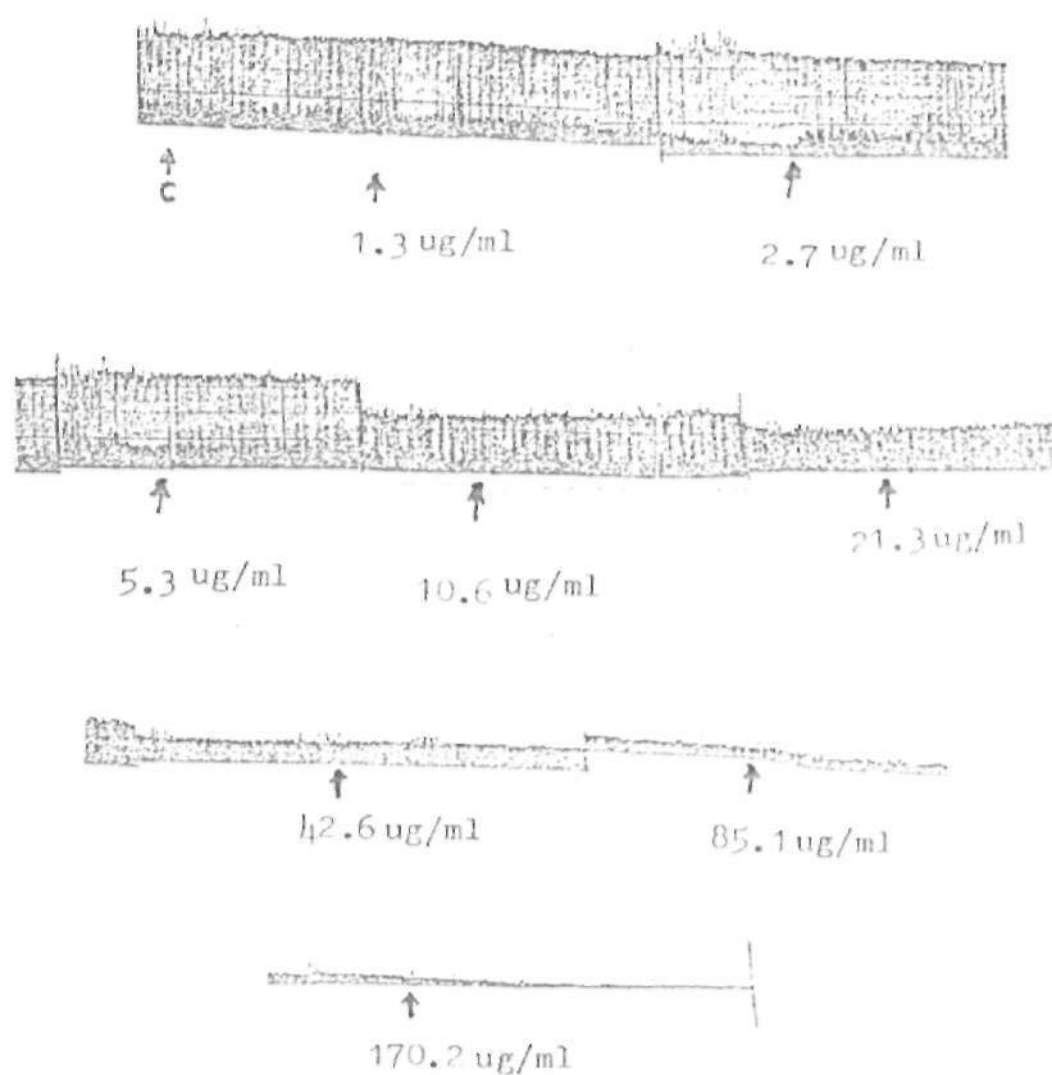


Fig. 4.12A: Effect of Lindane on direct stimulating rat phrenic nerve - diaphragm preparation.

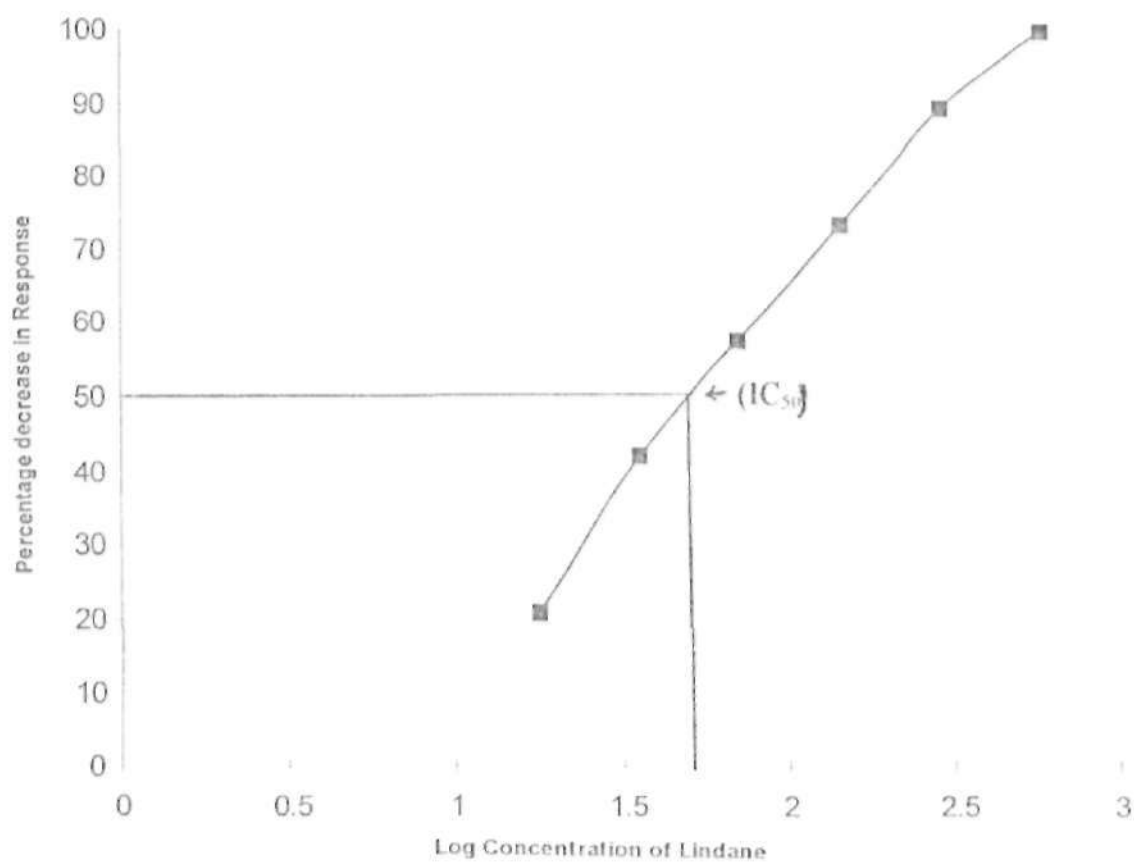


Fig. 4.12B: Log concentration curve for Lindane using rat phrenic nerve - diaphragm preparation. ($IC_{50} = 5.01 \times 10^{-12}$ M; $IC_{75} = 1.51 \times 10^{-11}$ M).

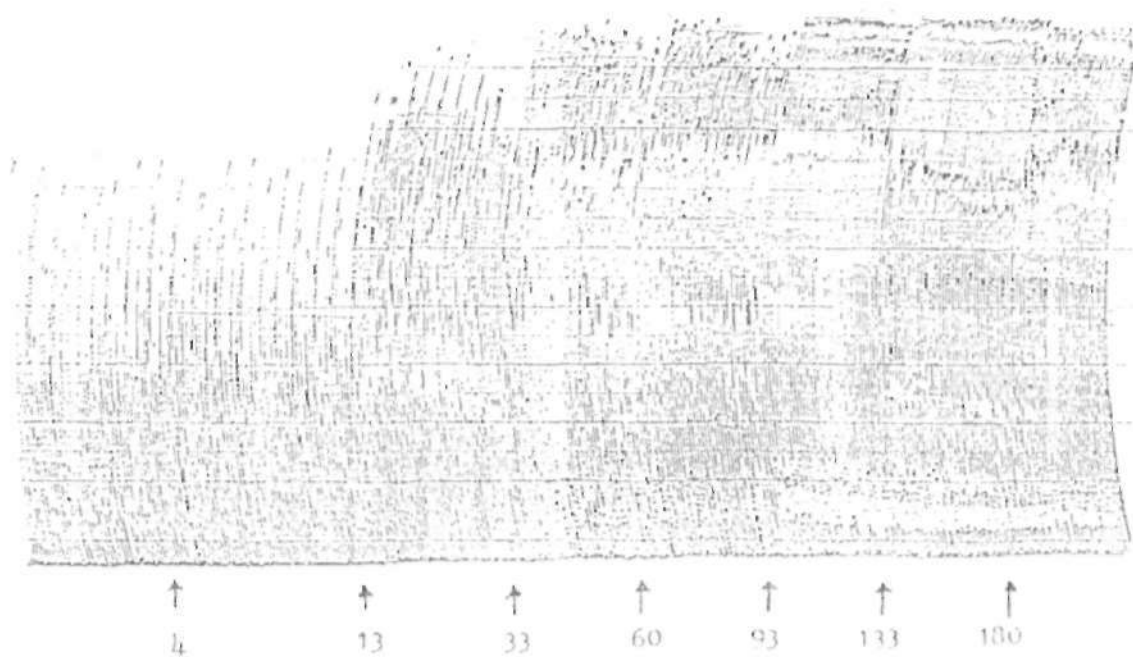


Fig. 4.13A Effect of dimethoate on direct stimulation of rat phrenic nerve diaphragm (Cumulative Dosing ng/ml).

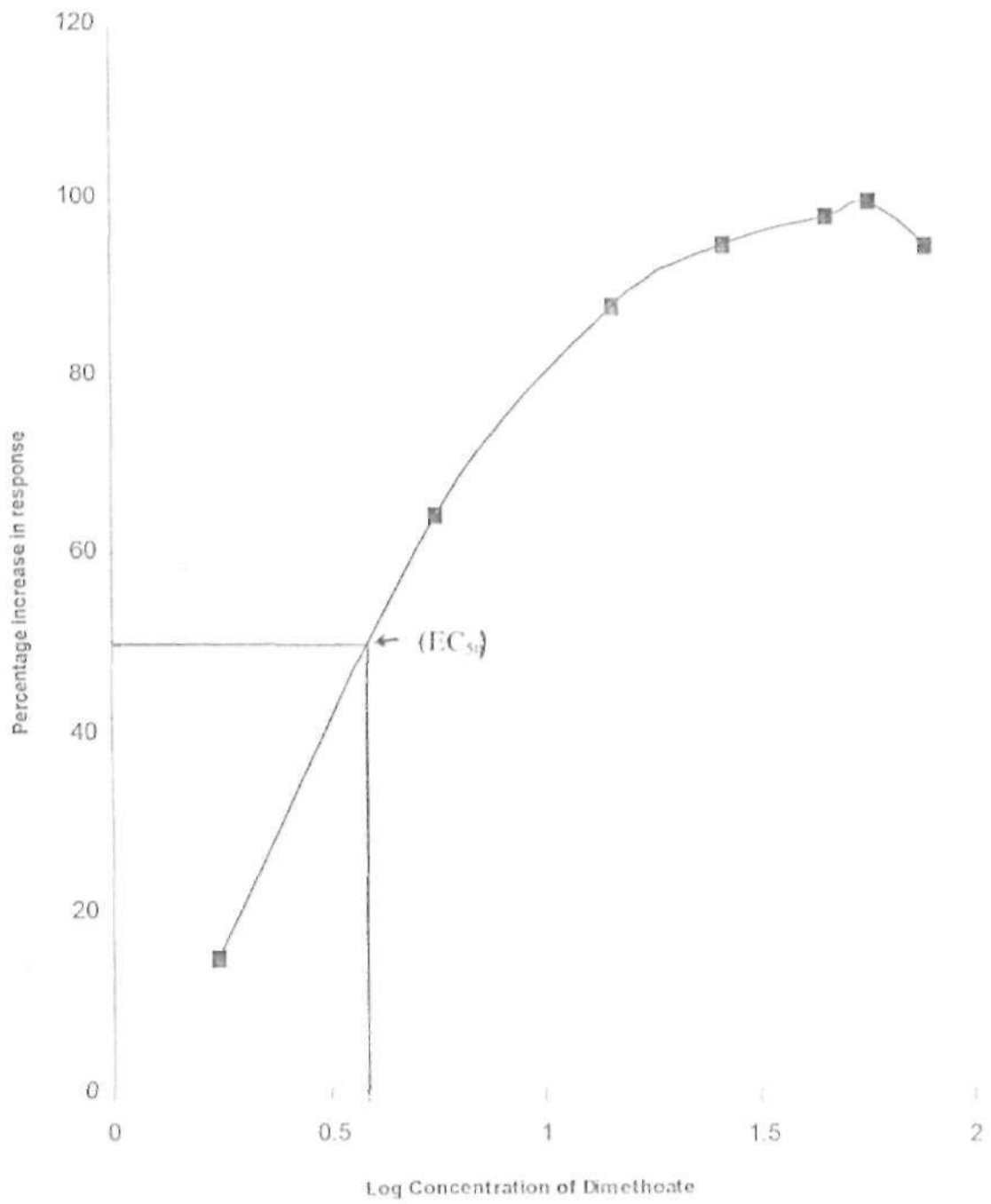


Fig. 4.13B Log-Concentration curve for dimethoate using rat phrenic nerve diaphragm preparations. ($EC_{50} = 3.98 \times 10^{-13}$ M; $EC_{75} = 8.71 \times 10^{-12}$ M).

4.2.9 Effect of Lindane and d-tubocurarine on Response due to dimethoate on Rat Phrenic Nerve-Diaphragm preparation

Normal twitch (control) response was obtained with 60 $\mu\text{g/ml}$ dimethoate produced a significant ($P < 0.001$) increase in twitch response amounting to 14.75%. Introduction of 2.1 $\mu\text{g/ml}$ lindane reduced by 6.6% twitch response produced by dimethoate. The increase was, however, not significant. 2.6 $\mu\text{g/ml}$ d-tubocurarine was observed to further significantly ($P = 0.001$) reduce twitch response following 2.1 mg/ml lindane by 50.8% (fig 4.14A and 4.14B)



Fig. 4-14A: Effect of lindane and dimethoate on response due to lindane on rat phrenic nerve-diaphragm.

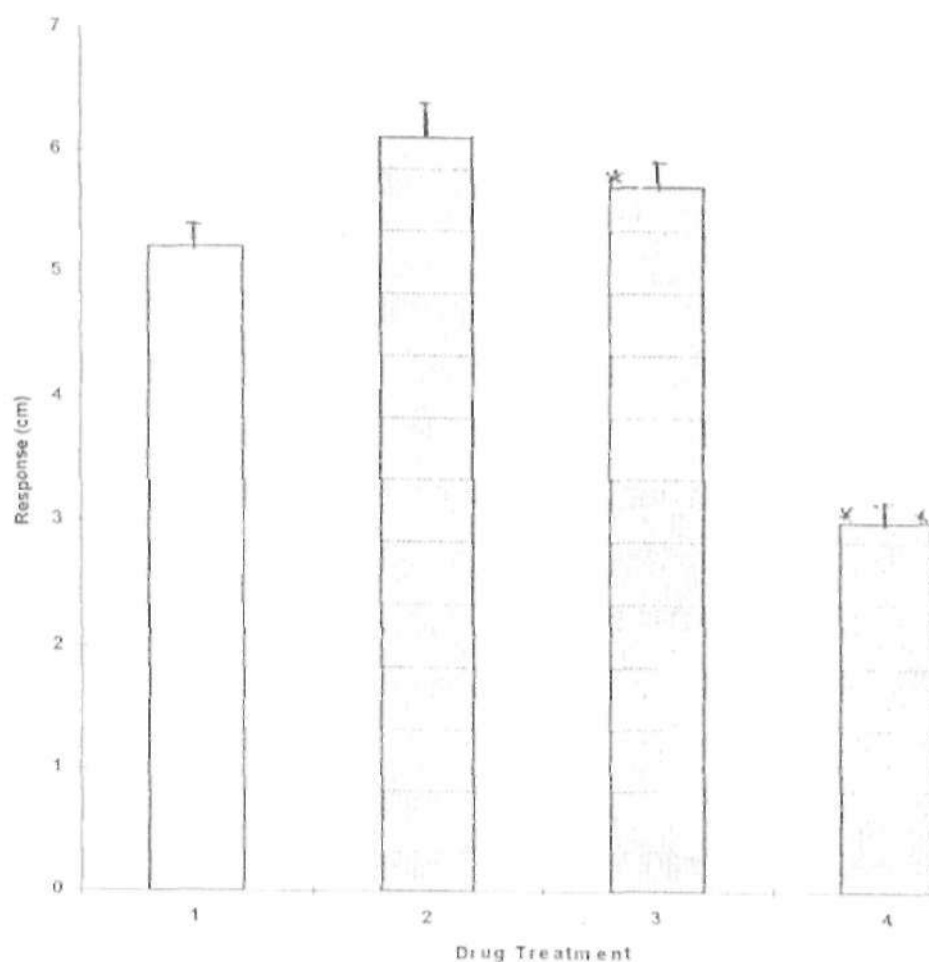


Fig. 4-14B: Effect of lindane and d-turbocurarine on response due to dimethoate on rat nerve-diaphragm. (n = 5; mean $\pm$ SEM).

KEY 1 = Control Response

2 = 60 ng/ml DM

3 = 60 ng/ml DM + 2.1 μ g/ml LIN

4 = 60 ng/ml + 2.1 μ g/ml LIN + 2.6 μ g/ml d-turbocurarine.

*significantly different ($P < 0.001$) from control (1) response.

**significantly different ($P < 0.001$) from control, (1), (2) and (3).

4.2.10 Effect of d-tubocurarine on Lindane Response on Rat Phrenic Nerve-Diaphragm preparation.

Lindane 2.1 $\mu\text{g/ml}$ significantly ($P < 0.01$) reduced control twitch response by 8.6%. 2.6 $\mu\text{g/ml}$ d-tubocurarine introduction following lindane, however, attenuated very significantly ($P < 0.001$) lindane (2.1 $\mu\text{g/ml}$) response by 63.8% (Fig. 4.15A and 4.15B).

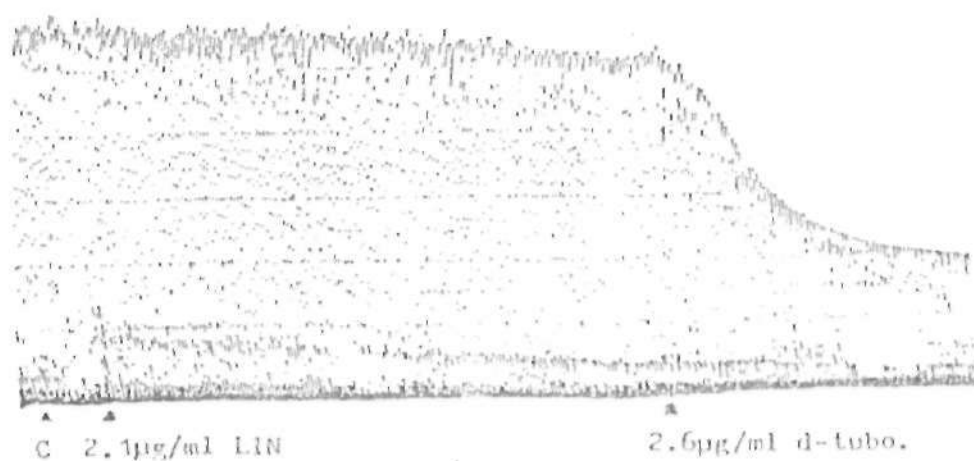


Fig 4-15A: Effect of d-tubocurarine on response due to lindane or rat phrenic nerve-diaphragm.

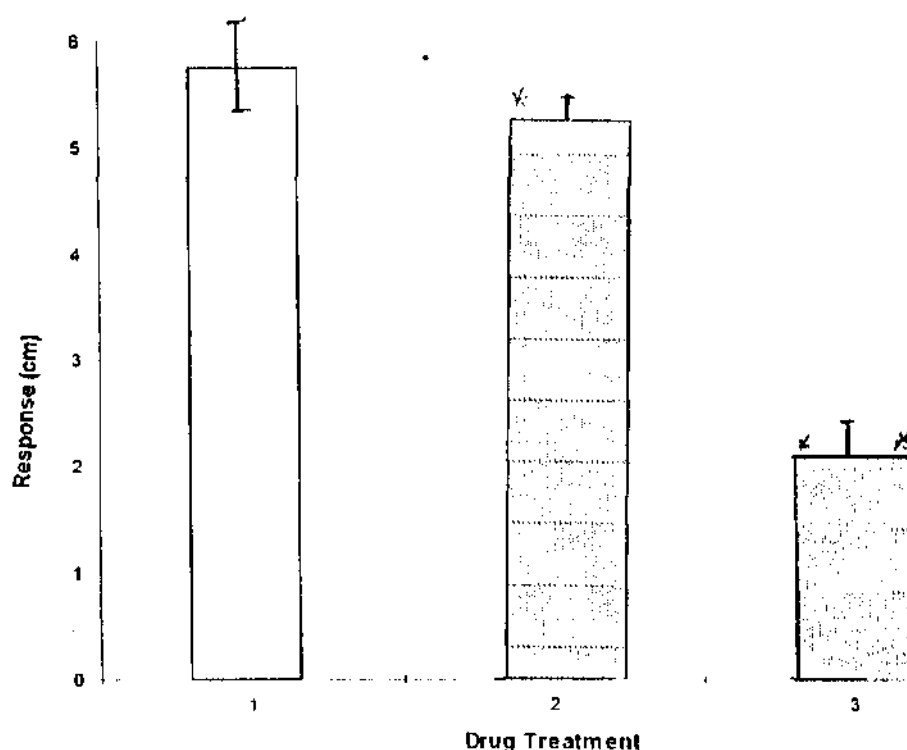


Fig 4-15B: Effect of d-tubocurarine on response due to lindane on rat phrenic nerve-diaphragm (n = 5; mean $\pm$ SEM).

KEY 1 = Control Response

2 = 2.1 µg/ml Lindane

3 = 2.1 µg/ml LIN + 2.6 µg/ml d-tubocurarin

*significantly different ($P < 0.01$) from control (1) response.

**Significantly different ($P < 0.001$) from (1) and (2).

4.2.11: Effect of Lignocaine on Responses due to Dimethoate and Lindane on Rat Phrenic Nerve-Diaphragm preparation

Dimethoate (60 µg/ml) significantly ($P < 0.001$) augmented control twitch response by 20.1%. Further introduction of 16.8 µg/ml lignocaine following dimethoate reduced response significantly ($P < 0.001$) by 36.9%. 16.8 µg/ml. Lignocaine further reduced response significantly ($P < 0.001$) by potentiating lindane (4.3 µg/ml) response (fig. 4.16A and 4.16B).

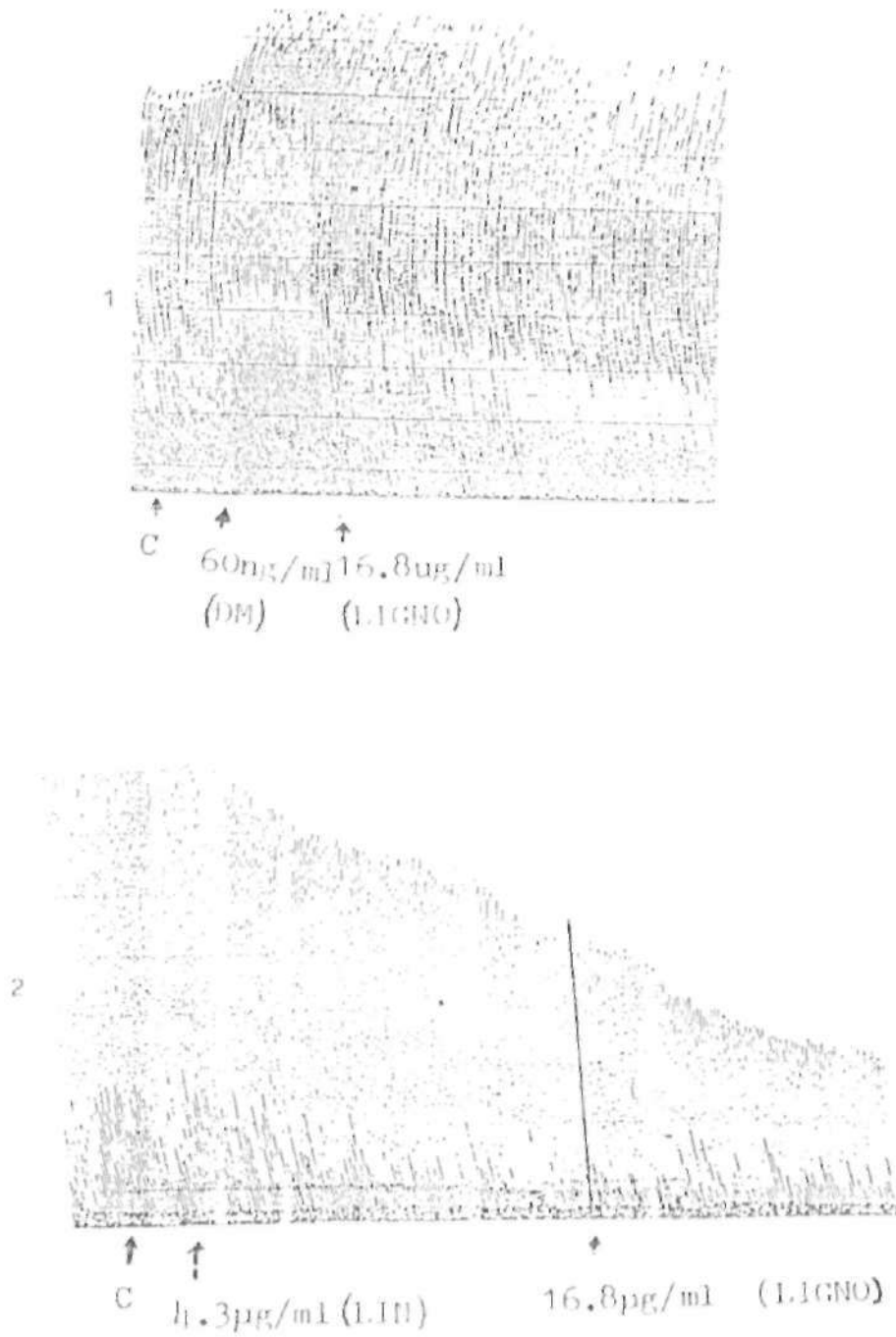


Fig. 4-16A: Effect of lignocaine on response due to (1) dimethoate and (2) lindane on rat phrenic nerve-diaphragm.

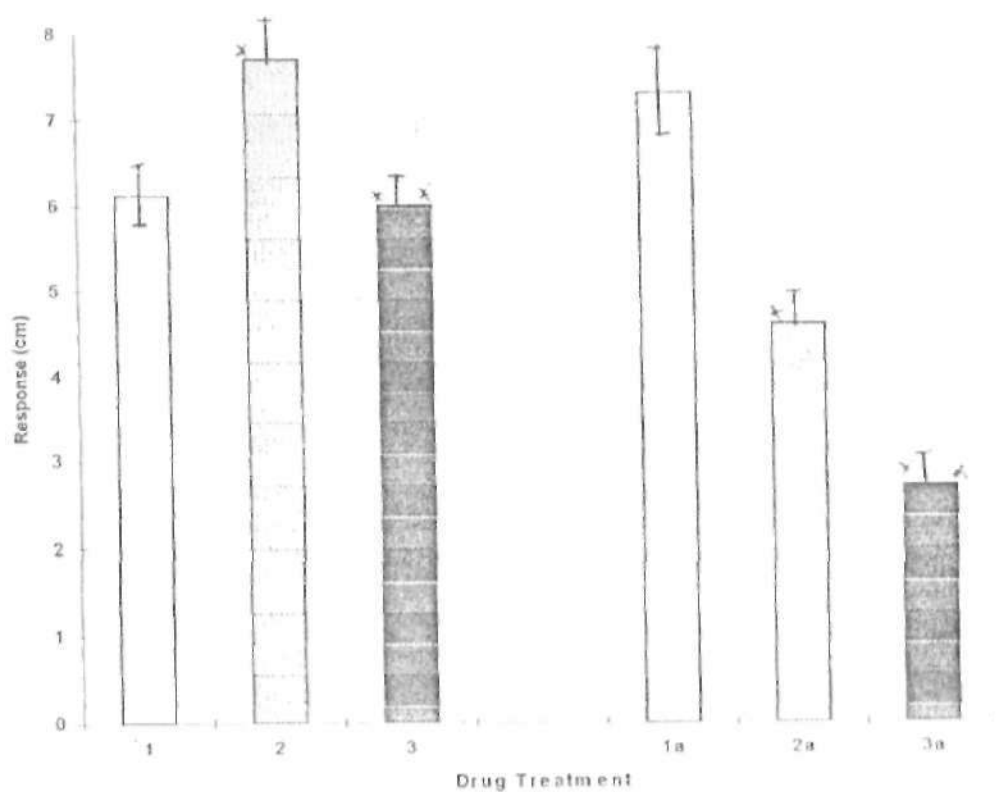


Fig. 4-16B: Effect of lignocaine (LIG) on response due to dimethoate (DM) and lindane (LIN) on rat phrenic nerve-diaphragm ($n = 5$; mean $\pm$ SEM)

KEY 1 = Control Responses

2 = 60 ng/ml DM

2a = 4.3 μ g/ml LIN

3a = 4.3 μ g/ml LIN + 16.8 μ g/ml LIG

3 = 60 ng/ml DM + 16.8 LIG

*significantly different ($P < 0.001$) from control response (1 and 1a)

**significantly different ($P < 0.001$) from 2a and 2b respectively.

4.3 CHRONIC TOXICITY STUDIES

4.3.1 Effect of combined Exposure to Lindane and Dimethoate on the Functional Integrity of the Rat Liver

The parameters determined in this section of study included the serum activity levels of transaminase (ALT and AST), alkaline phosphatase, serum albumin and total bilirubin.

The summary of mean values for these parameters in the various dimethoate dose groups and subgroups as a well as the period of exposure are shown in appendix II and III statistical analysis and comparisons between dose groups and subgroups and successive months are contained in appendix V and VI respectively.

4.3.1.1 Serum Transaminases and Alkaline Phosphatase Activities

Activity levels for the serum transaminases, ALT and AST were observed to be elevated for all the dose groups and subgroups when compared to control values. ALT activity at 75 mg/kg in the 12 week period showed a value lower than the control value. Generally, activity levels were high in the lindane-free compared to the lindane-treated subgroup for all the dose groups for the entire period of study. These elevations were, however, not statistically significant (fig. 3.17, 3.18 and 3.19).

At four weeks exposure period, AST activity showed a dose-dependent elevation which were observed to be significant ($P < 0.01$) compared to control values. A comparison between the 10 mg/kg and 75 mg/kg subgroups, however, showed no significant differences. Activities of ALT on the other hand showed no significant difference at all the dose groups compared to control as well as between 10 mg/kg subgroups (fig. 4.17)

At eight weeks study period, AST activity was within the control range at 10 mg/kg and 25 mg/kg dose groups with no significant different. At 50mg/kg dose group significant

elevation ($P < 0.05$) was observed in the lindane-free subgroup compared to control, while no such differences were found in the lindane-treated subgroups. In the 75 mg/kg dose group, however, significant ($P < 0.01$) elevations in AST activity were observed in both lindane-free and lindane-treated subgroups compared to control values. Highly significant ($P < 0.001$) elevations in the ALT activity were observed in all dose groups compared to control values. No significant difference was observed between 10 mg/kg and 75 mg/kg subgroups when compared (fig. 4.18).

Twelve weeks exposure period revealed slightly raised levels of AST at all the dose groups compared to control values. The differences were, however, not significant. ALT activity between 10 mg/kg and 50 mg/kg, were significantly ($P < 0.02 - P < 0.001$), when compared to control values. The 75 mg/kg lindane-free subgroup showed a value below control activity. The drop was not significant. Similarly, no significant differences were seen in the lindane-treated 75 mg/kg subgroup when compared with the control (fig. 4.19).

At four weeks period of study, alkaline phosphatase activities at all dose groups were significantly higher than control values, except for lindane-treated 10 mg/kg subgroup. A comparison between 10 mg/kg and 75 mg/kg subgroups revealed very significant ($P < 0.01$) rise in the activity in the 75 mg/kg over the 10 mg/kg dose group (fig. 4-17). Values at eight and twelve weeks in the 10 mg/kg and 25 mg/kg groups showed significant decreases ($P < 0.02$) in the 8 weeks compared to control values (fig. 4.18). At twelve weeks both the 10 and 25 mg/kg dose groups did not show any significant differences when compared to control (fig. 4-19). ALP activities remained significantly ($P < 0.05$) lower than control values even at 75 mg/kg at eight weeks period of exposure.

Activities were also significantly ($P < 0.05$) elevated at 75 mg/kg in the lindane-free subgroup while the lindane-treated subgroup was not significantly different compared to control values (fig. 4-20). The activities in that 75 mg/kg subgroups at twelve weeks period showed very significant ($P < 0.01$) elevation when compared with the 10 mg/kg subgroups. No any significant difference, in ALP activity were observed between the subgroups of various dose groups throughout the period of study (fig. 4.20).

A comparison of AST activity levels between months in the 10 mg/kg dose group did not show significant difference throughout except at four and eight weeks. In this case very significant ($P < 0.01$) decreases was observed at eight weeks compared to the fourth week. Similar trend was observed throughout the exposure period at 75mg/kg dimethoate dose group and subgroups. No significant differences was seen in the AST activities between the months (fig. 4.17, 4.18 and 4.19): ALT activities, however, showed highly significant ($P < 0.001$) elevation in the eighth week compared to the fourth week and the twelfth week in both lindane-free and lindane-treated subgroups. Similar significant ($P < 0.05$) rise in activity was observed in the twelfth week compared to fourth week month in both subgroups. Activities were very significantly ($P < 0.01$) higher in the twelfth week compared to eighth week month in both subgroups of the 10 mg/kg dose groups.

At 75 mg/kg dose group, highly significant ($P < 0.001$) elevation in the ALT levels were observed in the eight weeks month compared to the forth week. Differences between the twelfth and forth week, however, were not significant. ALT activity at twelve weeks showed highly significant ($P < 0.001$) elevation compared to the eight weeks (fig. 4-17, 4-18, 4-19).

ALP activities at 10 mg/kg dimethoate dose group in the eight weeks and twelve weeks showed very significant ($P < 0.001$) decreases compared to the fourth. Similarly, levels were significant ($P < 0.05$), lower at eight weeks compared to twelve weeks in the 10 mg/kg subgroup. However, no significant difference was seen between the lindane-free subgroup. The 75 mg/kg dose group showed a highly significant ($P < 0.001$) decreases at eight and twelve weeks compared to the fourth week. On the other hand, a highly significant ($P < 0.001$) higher at twelve weeks compared to levels at eight weeks (fig. 4-20). Generally, for the entire duration of study period, the ALP activity in the lindane-treated subgroups showed lower values compared to the lindane-free subgroups.

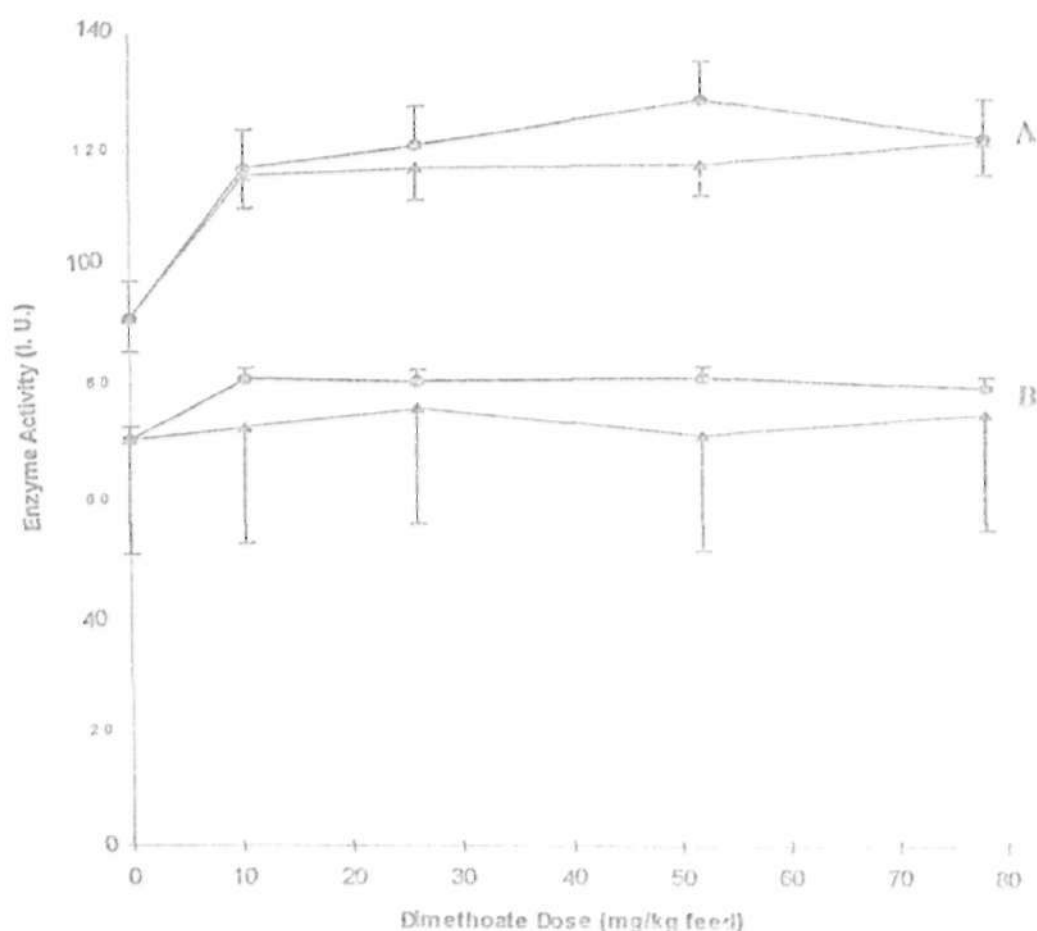


Fig. 4.17: Serum activity levels of (A) AST and (B) ALT in rats fed dimethoate alone (●) and lindane plus dimethoate (▲) for a period of four weeks.
($n = 5$; mean $\pm$ SEM).

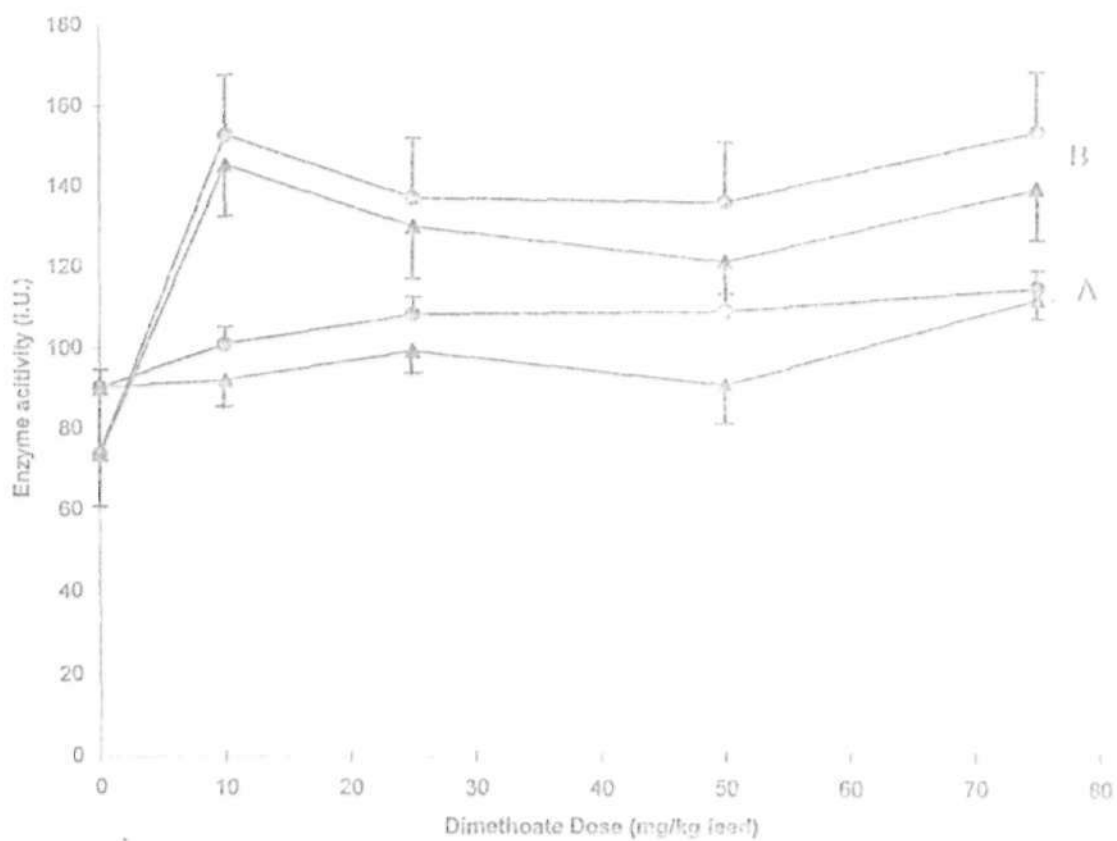


Fig. 4.18: Serum activity levels of AST (A) and ALT (B) in rats fed dimethoate alone (●) and lindane plus dimethoate (▲) for a period of eight weeks.
(n = 5; mean $\pm$ SEM).

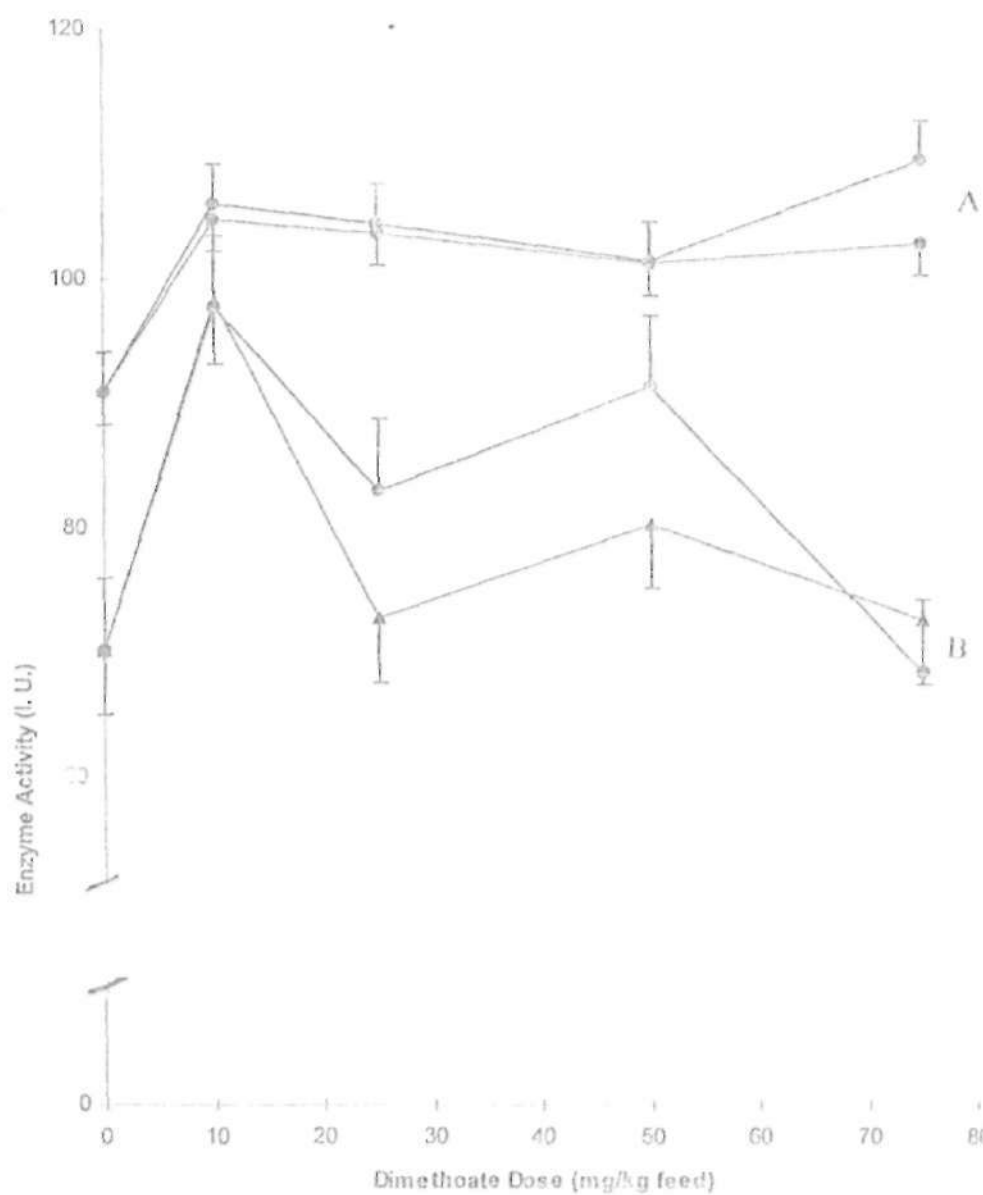


Fig. 4.19: Serum activity levels of AST (A) and ALT (B) in rats fed dimethoate alone (●) and lindane plus dimethoate (▲) for a period of twelve weeks.
(n = 5 per group mean $\pm$ SEM).

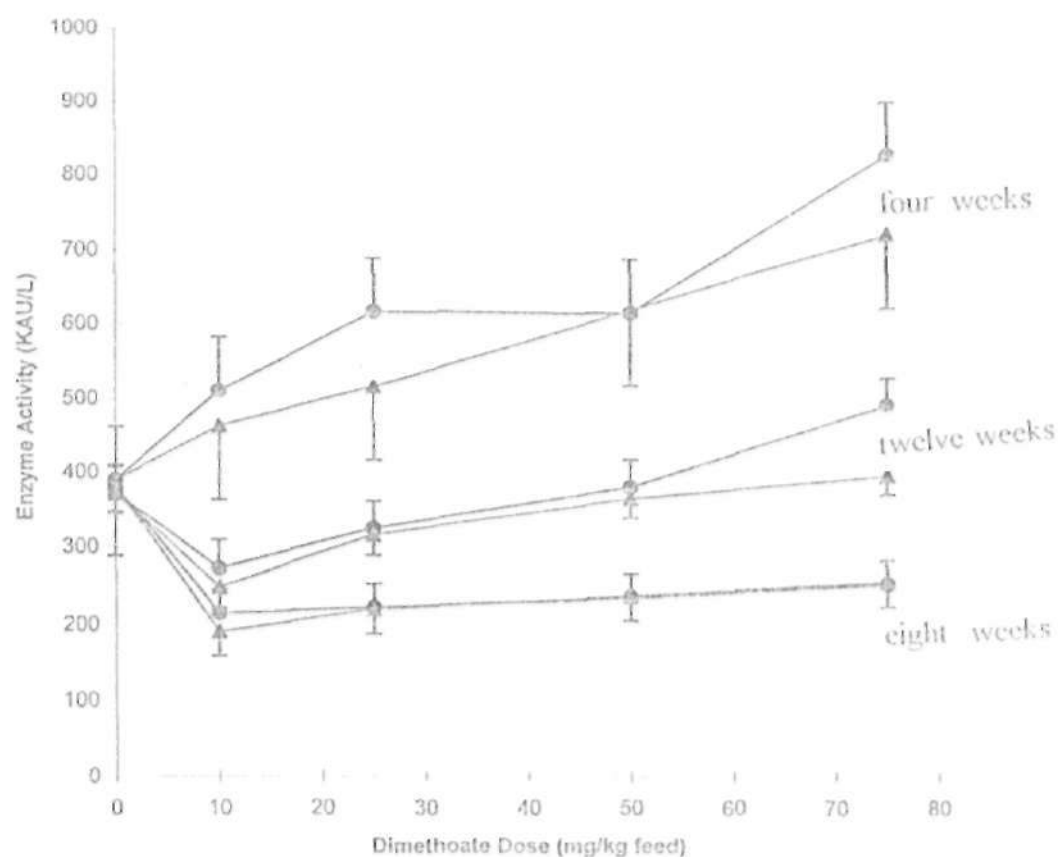


Fig. 4.20: Serum activity levels of ALP in rats fed dimethoate alone (●) and lindane plus dimethoate (▲) for the duration of 12 weeks.
(n = 5, mean $\pm$ SEM).

4.3.1.2 Serum Albumin and Total Bilirubin

Appendix III shows the summary of mean values for serum albumin and total bilirubin in rats receiving dimethoate in the presence and absence of lindane. The statistical analysis and the comparisons of mean values between control and the various dimethoate dose groups and subgroups is shown in appendix V.

Serum albumin levels showed decreases throughout the 12 weeks duration of study in the various dose groups and subgroups compared to control values. At four weeks the no significant differences were observed compared to control except in the lindane-free subgroup of 75 mg/kg ($P < 0.05$, Fig. 4.21A and B).

At eight weeks no significant differences were observed in serum albumin levels between lindane-free and lindane-treated subgroups of 10 mg/kg when compared to control values. Levels in the 25 mg/kg lindane-treated subgroups were however, significantly ($P < 0.05$) lower than control values, while no such difference was observed in the lindane-free subgroup. Similarly significant decreases were observed in the lindane-free ($P < 0.01$) and lindane-treated ($P < 0.05$) subgroups levels in the 75 mg/kg dose groups, while no significant differences were observed between the subgroups.

At twelve weeks, significant ($P < 0.02$) decreases were observed in all the dose groups compared to the control values. The lindane-free subgroup of 75 mg/kg showed a significant ($P < 0.05$) decrease in levels compared to the same subgroup in the 10 mg/kg dose group. No such significant difference was seen between the 10 mg/kg and 75 mg/kg in the lindane-treated subgroup (fig. 4-21A & B).

Summary of the mean values for total bilirubin levels are contained in appendix V and Fig. 4-22 (A - C). Statistical comparisons of these levels are presented in table 3.5. At four weeks exposure period, no significant differences in levels of total bilirubin were observed in all the dose groups when compared to control values. Levels in the lindane-free subgroups were however, higher compared to those of the lindane-treated subgroups (fig 4-21 and 4-22A).

At eight weeks, total bilirubin levels were significantly ($P < 0.02$) higher in the 50 mg/kg lindane-treated subgroup compared to control values. Similarly, significant increase in levels were observed in the lindane-free ($P < 0.02$) and lindane-treated ($P < 0.01$) subgroups compared to the control values in the 75 mg/kg dose group. Comparison between the subgroups in the 10 mg/kg and 75 mg/kg dimethoate dose groups did not show any significant difference. However, values for the lindane-free were slightly higher than those of lindane-treated subgroups in both dose groups (fig. 4-22B).

At twelve weeks exposure period, total bilirubin levels were observed to be very significantly ($P < 0.02$ - $P < 0.001$) higher in all the dose groups compared to control values. Between the subgroups, values for lindane-free were higher than those of lindane-treated. The levels for 75 mg/kg were very significantly higher in the lindane-free ($P < 0.001$) and lindane-treated ($P < 0.01$) compared to the 10 mg/kg subgroups (fig 4-22C).

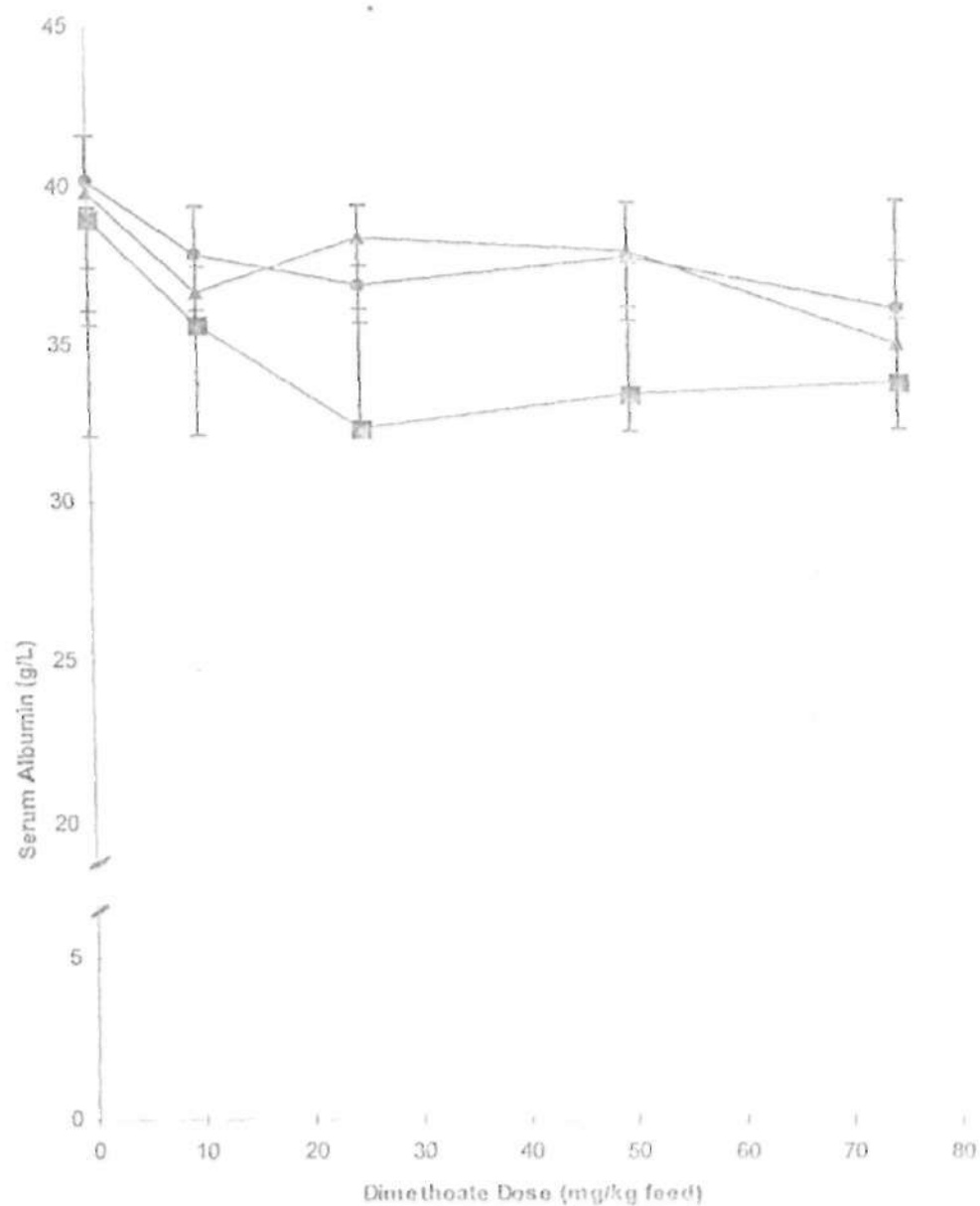


Fig. 4.21A: Profile of serum albumin in rats fed dimethoate alone in the diet for a period of four (●), eight (▲) and twelve (■) weeks. ($n = 5$, mean $\pm$ SD).

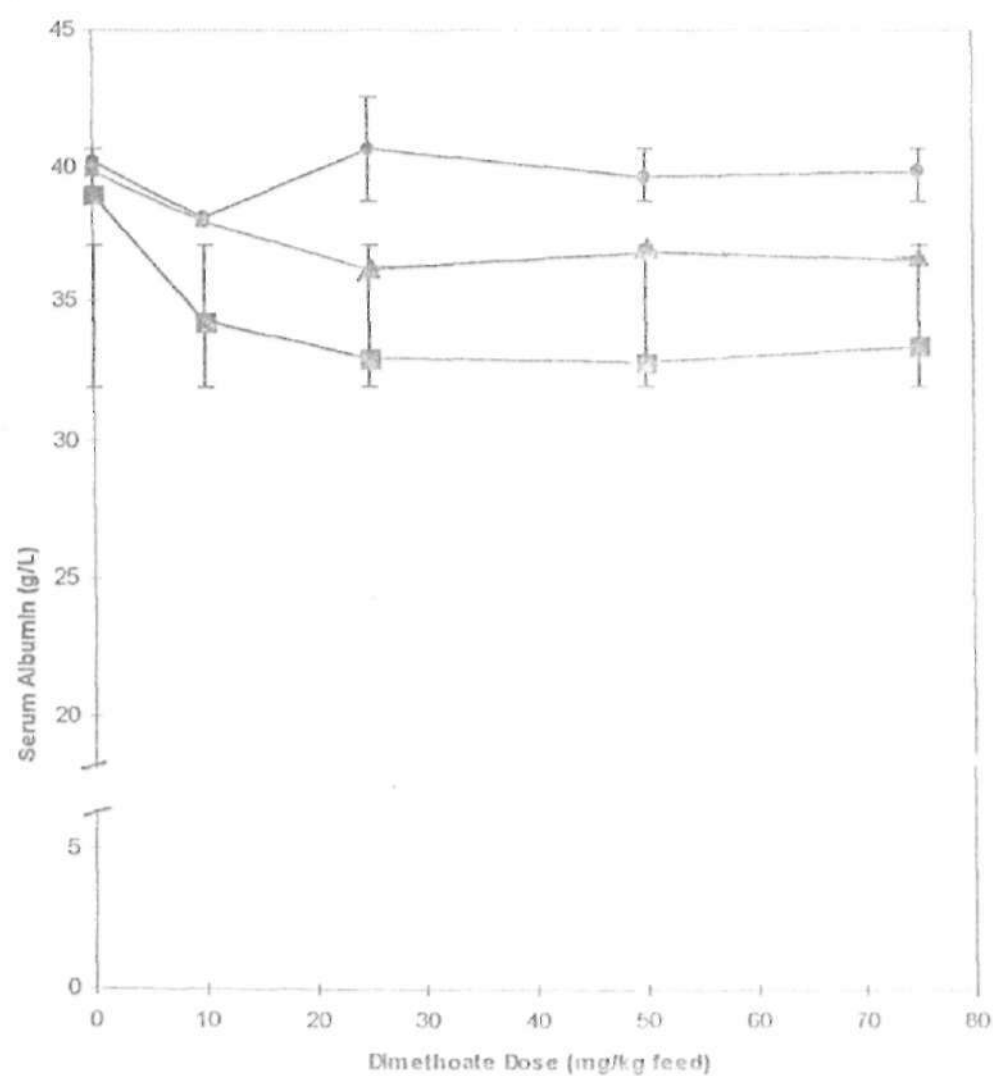
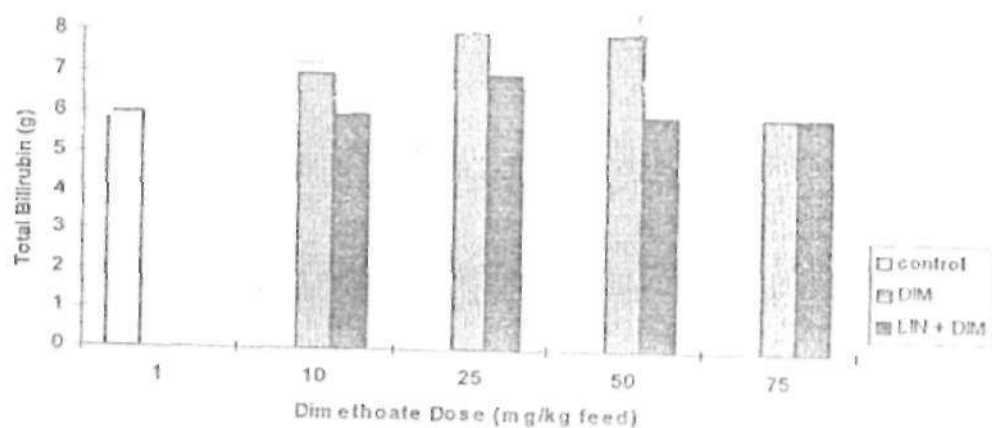
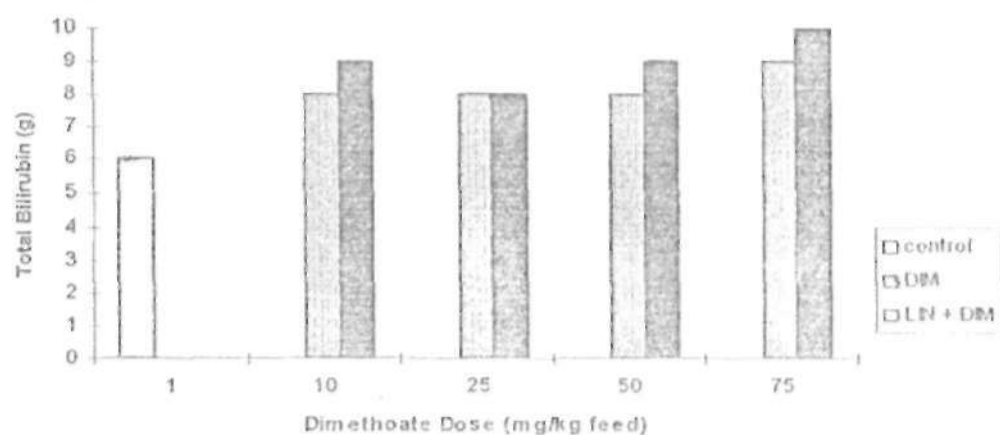


Fig. 4.21B: Profile of serum albumin in lindane - treated rats fed dimethoate for a period of four weeks (●), eight weeks (▲) and twelve weeks (■). (n = 5, mean $\pm$ SD)

(A)



(B)



(C)

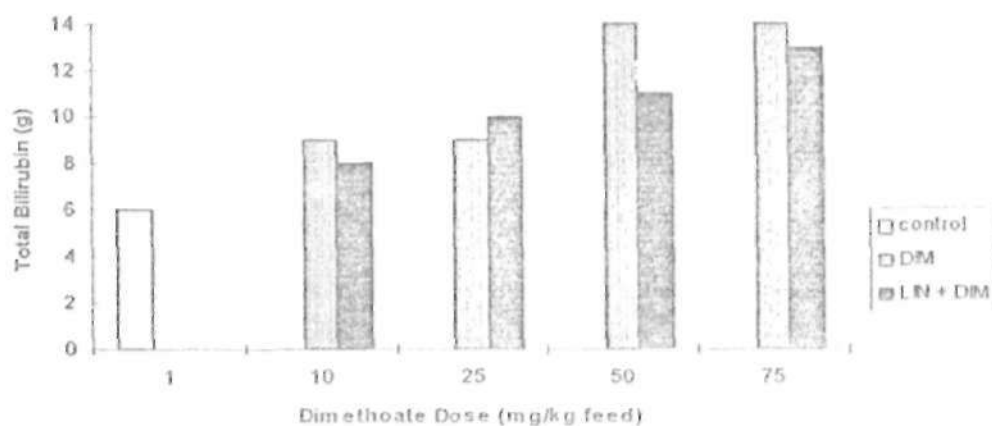


Fig. 4.22: Profile of total bilirubin in normal rats, and rats fed dimethoate in the presence and absence of Lindane for period of (A) 4 weeks, (B) 8 weeks and (C) 12 weeks ($n = 5$; mean $\pm$ SD).

4.3.1.3 Body weight gain and feed Intake

Summary of the mean values for body weight gain and feed intake are shown in appendix IV. A consistent gain in body weight was observed with increase in period of study. Values were significantly higher ($P < 0.02$) at 12 weeks compared to control in both 10 mg/kg and 75 mg/kg dimethoate dose groups. Feed intake also showed a similar trend to that of weight gain. Feed consumption was significantly ($P < 0.05$) higher at 12 weeks study period compared to values at 4 weeks (fig. 4-23 and 4-24).

4.3.1.4 Dimethoate Consumption

Mean values for weekly drug consumption are shown in table 3-8 and presented in Fig. 4.15. In the 10 mg/kg dose group, dimethoate consumption increase in period of study. Values were higher but not significant at 12 weeks compared to 4 weeks. The 75 mg/kg dose group showed a similar trend. Values in this case were significantly higher at 12 weeks ($P < 0.05$) compared to the values at 4 weeks (fig. 4-25).

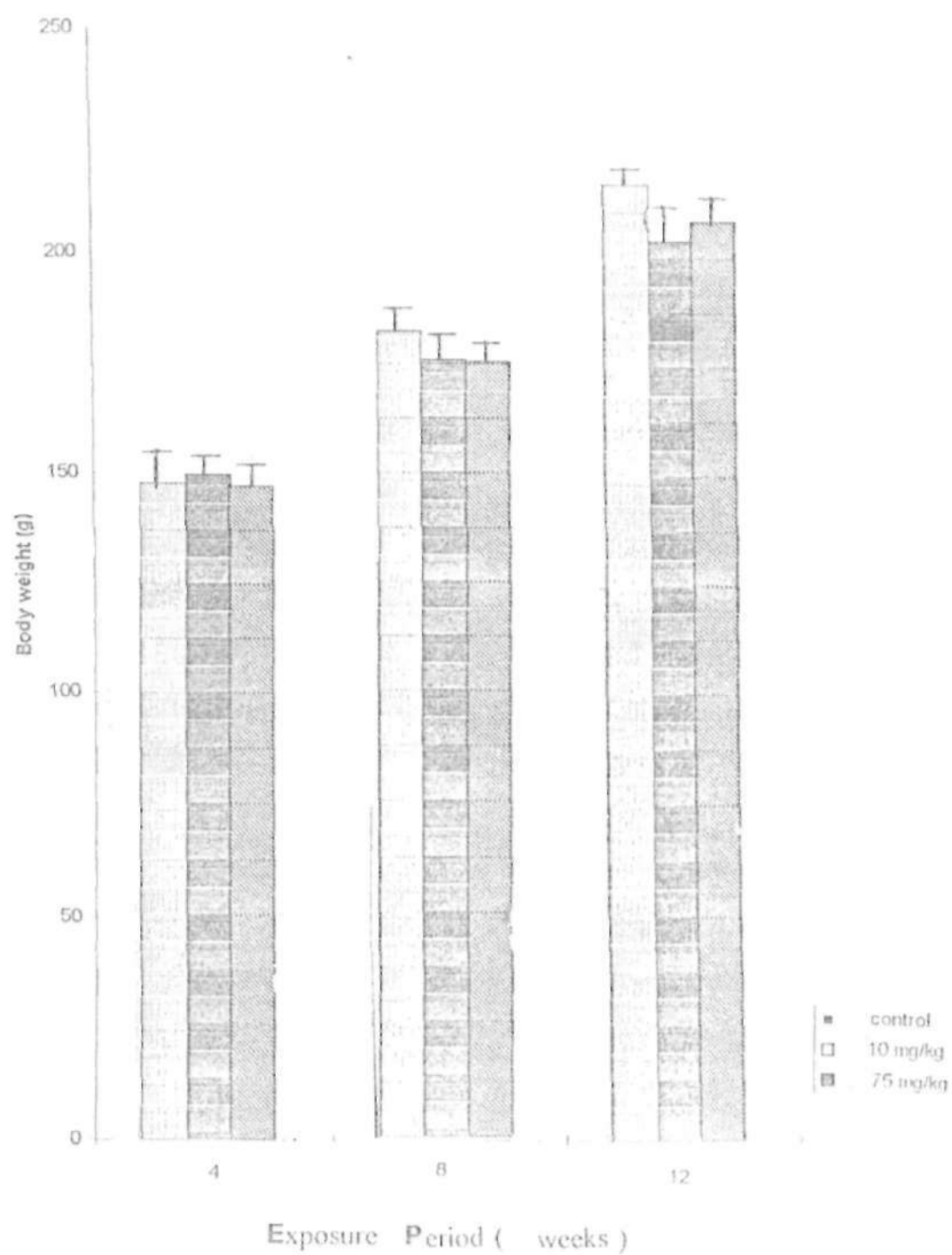


Fig 4-23: Profile of mean values of body weight gain for a period of four, eight and twelve weeks ($n = 4$; mean $\pm$ SD).

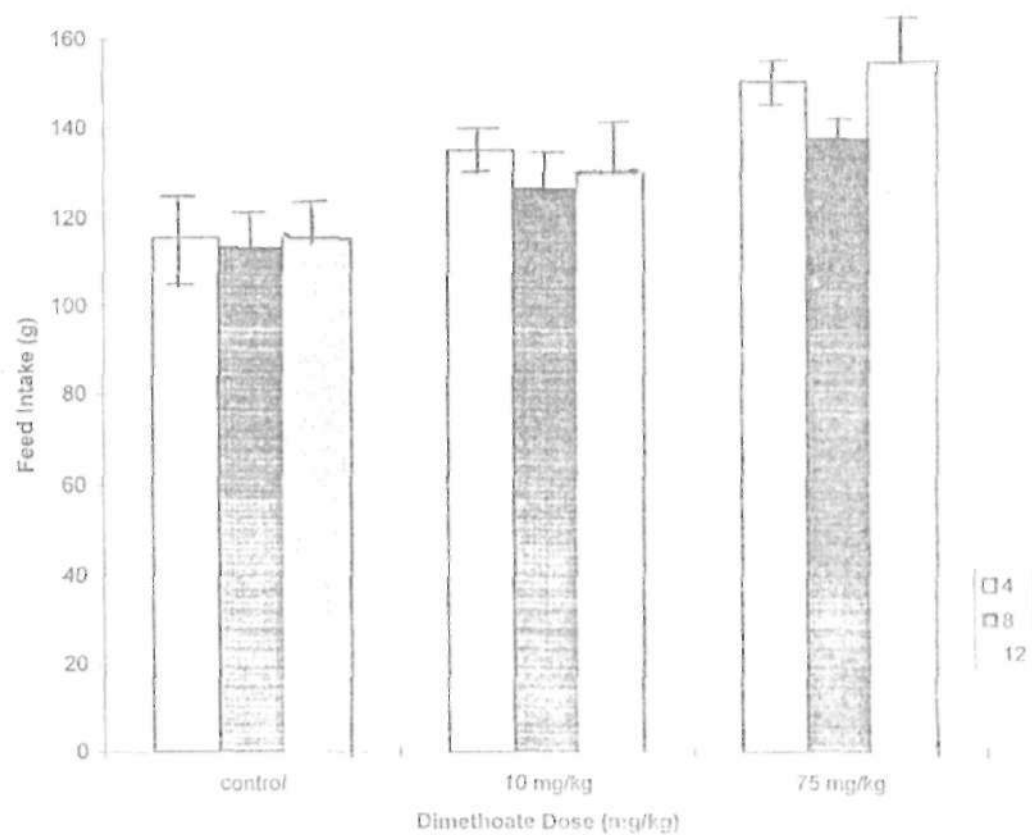


Fig. 4.24: Profile of mean values of weekly feed intake for a period of four, eight and twelve weeks. ($n = 4$; mean $\pm$ SD).

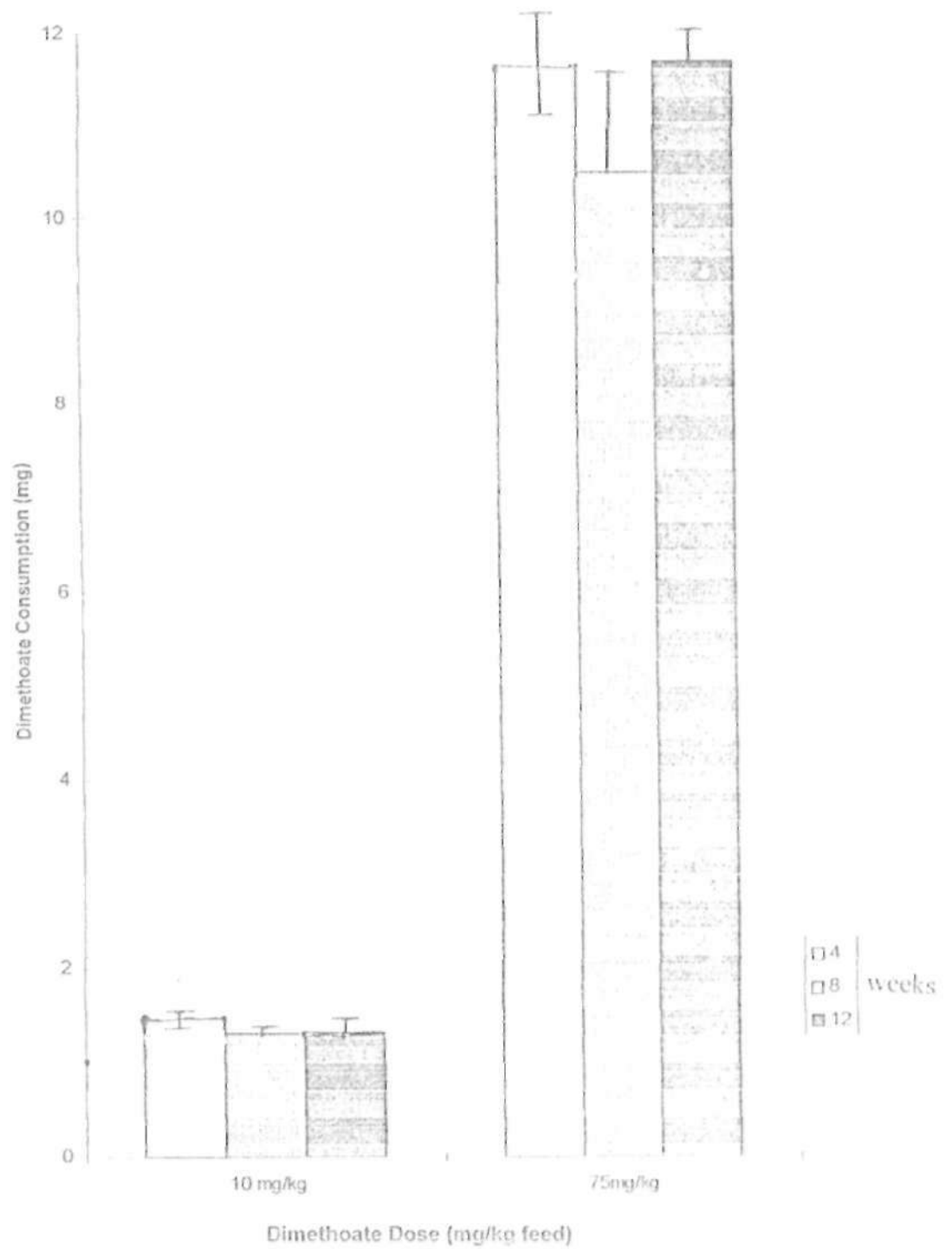


Fig. 4-25: Mean weekly drug (DM) Consumption in rats fed 10 mg/kg and 75 mg/kg dimethoate in their feed for a period of 4 weeks, 8 weeks and 12 weeks respectively ($n = 6$; mean $\pm$ SD).

4.3.2 Effects of combined exposure to lindane and dimethoate on the structural integrity of the rat liver.

This section contains the results of histopathological observations of sections prepared from some organs from the drug treated animals namely, liver kidney and the intestine. In the first month of study the liver the kidneys and the intestines showed no histological lesions in both the lindane-free and lindane-treated subgroups of all the dose groups (10, 25, 50 and 75 mg/kg feed dimethoate) (plates I and II).

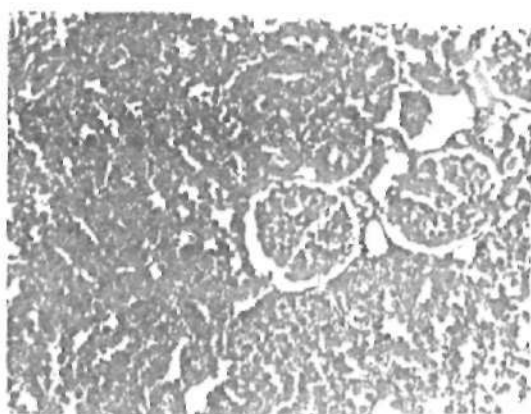
In the second month of study, the kidneys and intestines from all the dose groups were devoid of significant lesions. However, the liver from the 25 mg/kg lindane-free subgroup presented with mild fatty change or degeneration and in the lindane-treated subgroup of the 75 mg/kg dose group. The liver sections from the groups receiving other doses did not show any significant histopathological lesions (Plates IV and V).

In the third month of study, the kidneys and intestines had no histopathological abnormalities, Liver sections from 10 mg/kg dose groups had no lesions. There was a marked fatty change observed, however, in the lindane-treated subgroup of 25 mg/kg dimethoate dose group while the lindane-free presented with mild fatty degeneration. Moderate fatty change appeared in the sections from 50 mg/kg subgroups. Liver sections from the 75 mg/kg lindane-free subgroup demonstrated severe marked fatty change in the lindane-treated subgroup (Plate VI and VII).

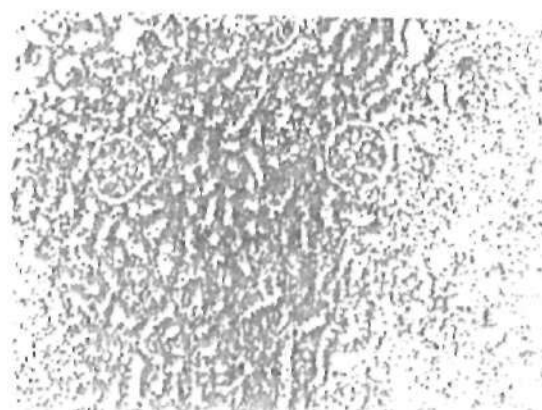


Plate I:

Representative rat intestine histopathology (H & E stain. Original magnification at 160 X). The section from rat in the 75 mg/kg feed dimethoate dose group. Note the lesion-free architecture of the villi.



A (100X)



B (100X)

Plate II:

Representative rat kidney histopathology (H & E stain); sections are from rats in the (A) 50 mg/kg lindane free and (B) 75 mg/kg lindane-treated sub-groups. Note the essentially normal architecture of the glomeruli.

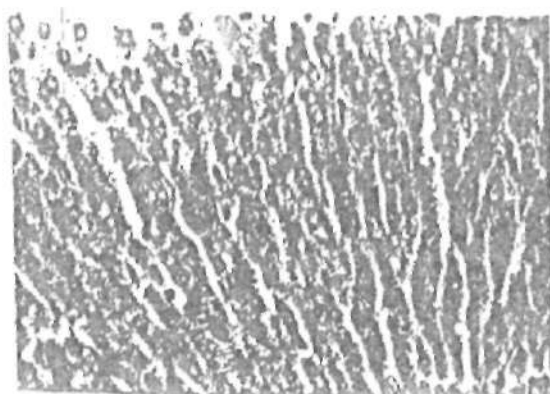


Plate III:

Representative rat liver histopathology (H & E stain) original magnification 160X). Section from control rat that did not receive any drug. Note the normal sinusoidal pattern.

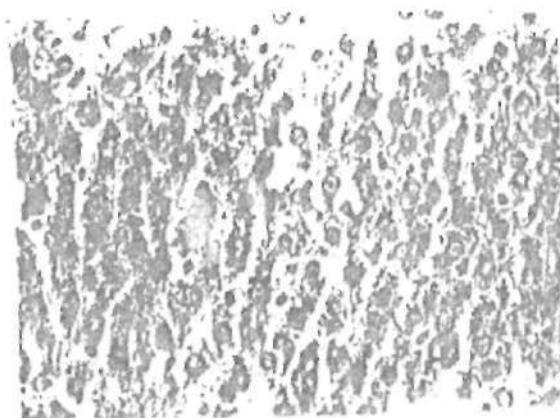
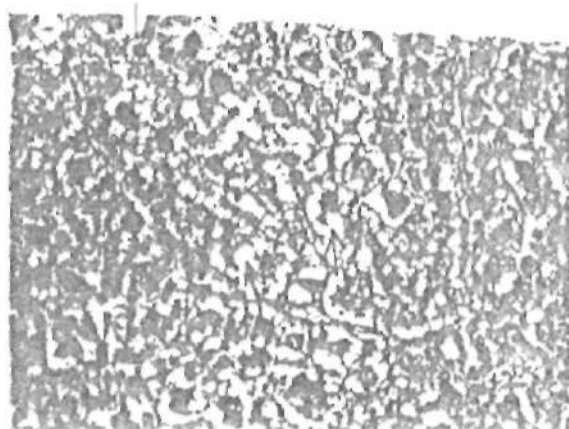
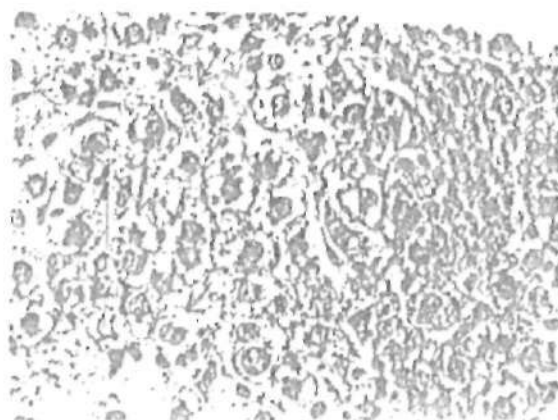


Plate IV:

Representative rat liver histopathology from the 2nd month. (H & E stain; original magnification 160 X). Section from 75mg/kg lindane - free dimethoate subgroup. Note the very slight obliteration of the sinusoids in an otherwise normal architectural arrangement in this plate.



A



B

Plate V:

Representative rat liver histopathology from the 2nd month. (H & E from rat in the (A) 50 mg/kg lindane-treated dimethoate sub-group showing marked fatty degeneration and (B) 25 mg/kg lindane-free dimethoate sub-group showing a mild fatty degeneration.

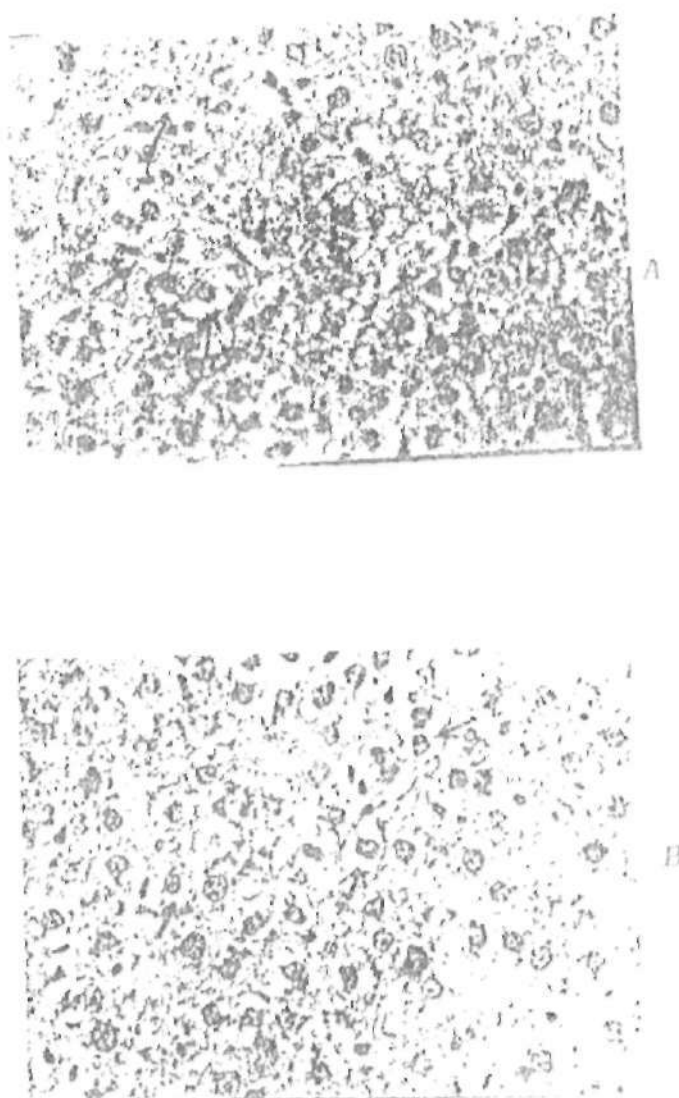
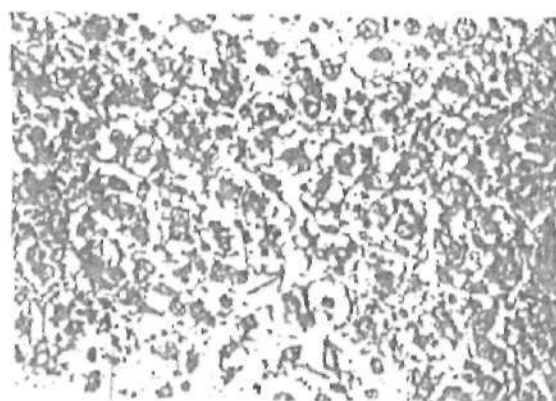
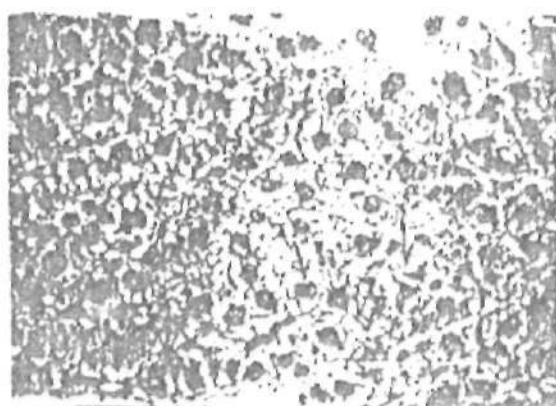


Plate VI:

Representative rat liver histopathology from the 3rd month. (H & E stain, original magnification 160X): Sections from rats in the (A) 50 mg/kg lindane - free dimethoate sub-group showing moderate fatty change and (B) 25 mg/kg lindane -treated dimethoate sub-group showing severe fatty change. Note the total obliteration of the sinusoids.



A



B

Plate VII:

Representative rat liver histopathology (H & E Stain, original magnification 160X): Sections from rats in the (A) 75 mg/kg lindane - free showing severe fatty change with total obliteration of the sinusoids and (B) 75 mg/kg lindane-treated dimethoate sub-group showing moderate fatty degeneration.

CHAPTER FIVE

DISCUSSION

5.1 ACUTE TOXICITY STUDIES

The purpose of the acute toxicity tests was to determine the symptomatology consequent to administering a single dose of the compounds/toxicants (lindane and dimethoate) and to assess the order of lethality of these compounds. The acute toxicity is defined as the mortality within 14 days after a single dose (Raimo and Jauko, 1994). It is the most significant single test that is conducted on essentially all chemicals of biological interest. The median lethal dose (LD_{50}) is among the acute toxicity tests usually employed to determine the short term toxicity of compounds. In this study, the LD_{50} of lindane and dimethoate were found to be 29.5 mg/kg and 185 mg/kg for lindane and dimethoate respectively (Table 4.1 & 4.2). Based on the classification by Matsumura (1975) lindane belongs to the highly toxic, while dimethoate is categorized among the moderately toxic compounds.

In categorizing toxicants, however, it should be noted that route of administration of a toxicant can modify the toxicity of such an agent. Other factors which may also effect toxicity of compounds include specie and strain differences (Gordon and Mephail, 1993; Festing, 1991) as well as environmental factors such as temperature variation (Narahashi, 1971).

Lindane has been demonstrated to act mainly through GABA receptor channel complex by blocking it, thereby suppressing the inhibitory mechanism leading to hyperexcitation of animals (Narahashi, 1992). The symptoms of lindane poisoning as such resemble those

of caused by GABA antagonists (Joy *et al.*, 1983). Systematic administration of classical GABA antagonists such as bicuculline and picrotoxin to rats results in typical dose-dependent sequence of convulsant seizure responses (Gale, 1992). Lindane has been observed to decrease the frequency of GABA chloride channel openings without changing the mean open time (Bermudez *et al.*, 1991). Symptoms of lindane poisoning in this study were found to be dose dependent in severity (Fig. 4.1) and they were mainly tremor convulsions and death occurred at high doses. This finding is consistent with the observations reported by Doherty (1979).

Exposure to anticholinesterases and some antineurotic esterase compounds culminates in inhibition of these enzymes. Predictably, effects occur at cholinergic synapses in the autonomic nervous system, the peripheral neuromuscular pathways and the CNS. Symptoms observed in this study resulting from acute dimethoate poisoning were dose dependent in severity (Fig 4.2), and may be categorized as muscarinic (e.g. respiratory distress) nicotinic (e.g. swaying gait) and CNS (e.g. restlessness, tremor, and tonic/clonic convulsions). Deaths were recorded at high (≥ 150 mg/kg) doses consistent with observations by Roberts (1979) and Iyaniwura (1996).

5.1.1 Pretreatment with Lindane

Results from the present study demonstrate the hepatoprotective effects of lindane pretreatment on dimethoate toxicity in mice and rats. Lindane (1 mg/kg, i.p.) pretreatment for seven days increased the LD₅₀ value of dimethoate in rats by 29.5% indicating reduction in the toxicity of dimethoate in the animals (Table 4.3). Similarly, the same treatment with lindane remarkably increased the survival time in mice challenged with lethal doses of dimethoate (fig. 3.3). The results in both the LD₅₀ and

survival time could be attributed to the enzyme inducing ability of lindane. (Conney, 1971, Kraus *et al.* 1981).

The organochlorine insecticides have been known to induce hepatic microsomal enzymes (Conney, 1971). Lindane has been demonstrated to induce both the cytochrome P₄₅₀ (Dwivedi & Kumar, 1989) as well as glutathione-S-transferase (GST) (Kraus *et al.*, 1981) enzymes in rats. It has been reported that pretreatment with lindane (2 mg/kg) orally administered caused an increased excretion of gluconic acid conjugates (Chadwick, *et al.*, 1971). In a similar study, lindane administration (15 mg/kg, i.p) and (5 mg/kg, i.p.) decreased hexobarbitone and phenobarbitone sleeping times respectively in FW-49 rats (Schwabe and Wendling, 1967). Results from these previous studies are seen to corroborate those obtained in the present work.

Liver microsomal enzyme inducers like lindane and phenobarbitone possess the ability to influence toxicity of other compounds via enhanced biotransformation process for which these enzymes are involved. Whether this effect as well as the enlargement of the liver that often accompanies it is beneficial or harmful depends on the circumstances. It can result in slower or quicker metabolism of pesticides and other compounds.

Dimethoate belongs to the phosphorothionate insecticides metabolized mainly, in the liver (Uchida, 1964, 1967). Induction by lindane of the mixed-function formation of the toxic oxon analogue could lead to an increased toxicity. Results obtained in the present study however, negates this position, as was seen in decreased toxicity of dimethoate. This could probably be due to the fact that the same enzyme systems can equally result in the direct cleavage of the phosphorothionate, which is a detoxification step as observed by

Matsumura, (1975). It has also been demonstrated that with some organophosphates, the rate of microsomal detoxification is more responsive to enzyme induction than microsomal activation (DuBois, 1969). Similarly, Uchida (1994) has reported that microsomal enzymes induction appears to favour the rate of clearance of dimethoxon *in vivo* much faster than the rate of activation, and thus the net effect is a reduction in dimethoate toxicity as was observed in the present work (table 4.3; fig. 4-3).

Potentiation of dimethoate toxicity has been observed in mice pretreated with phenobarbitone (Menzer and Best 1968) and the organochlorine insecticides (Menzer, 1970). However, the mechanisms of such observations though not immediately obvious, have been associated with changes in tissue carboxyamidase activity (Matsumura, 1975). Furthermore, the induction of activating isozymes may be predominating in such situations, thereby leading to relative accumulation of the toxic metabolites.

Similarly, aldrin pretreatment was observed to increase the rate of inactivation of parathion; whereas by the same mechanism of synthetic stimulation, dieldrin was reported to enhance the acute toxicity of dimethoate (Menzer, 1970).

Exposure to a variety of organochlorine insecticides has been shown to markedly increase the metabolism and excretion of several steroid hormones in both animals and man (Conney *et al.*, 1971 and 1972), with the subsequent implication of influencing several hormonal functions in the body, such as menstrual cycles in females. Main (1956) has also confirmed the protective action of aldrin against orally or intravenously administered parathion and paraoxon in rats. Chlordane and DDT pretreatment were also observed to substantially reduce barbiturate sleeping time in rats (Conney, 1971) and afford protection

GABA while GABA_B receptor is predominantly associated with calcium and potassium ion channels via GTP-binding proteins (Enna and Karbon, 1987).

GABA and other inhibitory amino acids (e.g. glutamate) actively promote an increase in chloride influx into the neural membrane which leads to a membrane hyperpolarization and a reduction in the probability of action potential generation. Interestingly, the chloride channel contains regulatory sites in addition to those specific for GABA. These sites have been shown to possess high affinity for benzodiazepines, picrotoxin, alcohol and the barbiturates (Taylor, 1990).

Results obtained in this study shows a dose-dependent pattern of activity by all the tested agents in alleviating lindane effect. Phenobarbitone (20mg/kg) produced 62.5% survival rate against 12.5% survival rate in the control group. Phenytoin sodium at 40 mg/kg produced 50% survival, while diazepam (10 mg/kg) gave 75% survival (table 3.4). Both benzodiazepine (diazepam) and barbiturate (phenobarbitone) have been implicated with GABA_A receptor complex; the same complex through which lindane mainly exhibits its toxic action (Taylor, 1990). The findings from this work is in line with the report of Alldredge (1997), who recently demonstrated the effectiveness of both diazepam and phenobarbitone in preventing drug-induced convulsions in mice.

The relatively higher performances (efficacy) of diazepam and phenobarbitone may be ascribed to their direct involvement with the GABA_A receptor complex in their mechanism of action. Both agents are classical sedative-hypnotic agents employed as anxiolytics and sedatives. Based on the gross behaviour and observations in the animals, the effects of these agents may be related to their sedative action on the animals. Sedation

might have allowed adequate time for metabolic clearance of lindane through biotransformation at the same time alleviating convulsive seizure symptoms as was reported by Iyaniwura *et al.* (1990).

Phenytoin sodium, a classical anticonvulsant acts by stabilizing the neuronal membranes. In this way, phenytoin decreases sodium influx into neurons resulting in a blockade of neurotransmitter release (Craig, 1990). The relatively low efficacy of phenytoin in both symptoms alleviation and as an antidote, may be explained based on its non-involvement with GABA activity in its mechanism of action.

Iyaniwura (1991b) has demonstrated the effectiveness of diazepam in alleviating symptoms as well as in the treatment of dichlorvos intoxication in mice. Similarly, PK-11195 has been shown to elicit protective effect against the convulsant activity of orally administered lindane (Zisterer, *et al.*, 1995).

5.1.3 Electroencephalographic Experiments

The results of the EEG Activity was based on the differences in the amplitudes and frequencies of the wave obtained (Fig. 3.6A, B and C). The EEG wave pattern examined included the frontal cortex (FC), occipital cortex (OC), reticular formation (RF) and the electromyograph (EMG). The waves of the reticular formation was selected for analysis being the region of the brain mainly concerned with wakefulness and arousal (Guyton, 1976).

The results showed desynchronization of waves in the lindane-treated animals manifested as low amplitude and high frequencies, while the control rats did not exhibit such a

pattern of EEG waves. The arousal reflected as low amplitude was consistent with excitation/hyperactivity observed in the animals (fig. 4-4A, B & C). Waves EEG in RF the region were of significantly ($P < 0.05$) lower amplitude in the rats administered lindane alone (10mg/kg) compared to lindane (10 mg/kg) plus either diazepam (10 mg/kg) or phenytoin (40 mg/kg) as well as the respective controls. Rats given lindane plus dimethoate (50 mg/kg) produced EEG patterns with significantly ($P < 0.02$) lower amplitude when compared with control rats and rats fed lindane alone (fig. 4-5 A & B). This indicates higher state of arousal in animals given lindane plus dimethoate compared with lindane alone. Apparently, dimethoate seemed to potentiate the hyperexcitation state produced by lindane. Studies carried out by Iversen and Iversen (1981) revealed EEG pattern with low amplitudes indicative of arousal produced by physostigmine. Similar observations were also reported by Murphy and Cheever, (1968).

The EEG results in this study shows antagonism of lindane effects by both diazepam and phenytoin by reversing the lindane-induced desynchronization of the EEG waves. This is in support of the antidotal therapy observations made in experiments on both symptoms and survival rates following lindane poisoning.

5.2 SUB-CHRONIC TOXICITY STUDIES

5.2.1 Biochemical Parameters

The results of the simultaneous sub chronic exposure to lindane (i.p.) and dimethoate (in the feed) indicate some relative decrease in hepatotoxicity in rats fed dimethoate alone compared to dimethoate exposure in the presence of lindane in all the dose groups. The hepatoprotective action offered by lindane was symbolised by comparatively lower

elevations in activity levels of the serum transaminase enzymes. This observation was consistent throughout the twelve weeks exposure period.

The most important enzyme implicated in liver damage are the alanine and aspartate transaminases ALT and AST as well as alkaline phosphatase (ALP) in addition to a few others with less significance (Mathenheimer, 1971; Ikediobi and Ukoha, 1985). Both ALT and AST serum activities have been shown to be elevated in minor cellular damage (Wolf, 1972; Wolf *et al.*, 1973). Slight increase in liver function based on raised activities of ALT and AST was observed in groups exposed to organochlorine insecticides residues as compared to control population (Jager, 1970).

The serum activities for AST and ALT indicate relatively higher but insignificant values, in the lindane-free compared to the lindane-treated dimethoate subgroups. This trend was observed throughout the 12 weeks duration of exposure. However increases in serum AST activity was in the first month significant ($P < 0.01$) in all the dose groups when compared to control values. Differences between lindane-pretreated and control groups were not however, significant in all the dose groups. Serum ALT activity in the same period did not show any significant difference from control values. (Fig. 4-17).

At eight weeks exposure period, the ALT activity was very significantly elevated ($P < 0.001$) in all the control groups (fig. 4-18). Wolf *et al.*, (1973) has reported that maximum values for transaminase activities are reached within 7 - 10 days and may persist up to six weeks after which values begin to decline in the case of minor liver damage.

At twelve weeks exposure period, serum ALT activity showed a significant ($P < 0.02$) decrease compared to values in the second month. Activity was, however, significantly

higher than control except in the 75 mg/kg dose group where activity was not significantly different from first and second month as well as control values (Fig. 4-19).

Highly significant decrease ($P < 0.001$) in the serum ALT activity at twelve weeks compared to the eight weeks may indicate tolerance to the insecticides by the hepatocytes. This view is supported by the ascertainment of Joan and Pannal (1984) that rapid or significant fall in serum ALT activity may occur during recovery from damage. The result in this study did not show any significant difference between values at four weeks and twelve weeks in the activity of serum ALT. However, sustained significant elevation above the control values may also indicate continued damage (Joan and Pannal, 1984). Decrease in serum enzyme levels may be attributed to the possible effects of blockage of *de novo* synthesis resulting from damage sustained during prolonged exposure to the toxicants (De Bruin, 1976).

Studies carried out by Iyaniwura (1991a) revealed a decrease in dichlorvos toxicity in mice following joint administration with aldrin. In another study, simultaneous administration of EPN was shown to increase malathion toxicity several times in mammals (Salvini, *et al.*, 1961). The same authors reported that following administration of equitoxic level of malathion and EPN, anticholinesterase inhibition was found to exceed that of either agent given singly.

Results in this study indicate a significant ($P < 0.05$) elevation in the serum activity of ALP at four weeks exposure period (fig. 4-20). This may be due to increased synthesis of the enzyme following stimulation by the insecticide. Activity showed a dramatic fall at eight weeks with values significantly ($P < 0.05$) below the control level (fig. 4-10). The

observed rise in serum ALP activity at four weeks could be attributed to relief of biliary obstruction (Wolf, *et al.*, 1972). Serum activity of ALP returned to levels within control values at twelve weeks and at the same time significant difference ($P < 0.001$) were found in activity between the lowest (10 mg/kg) and highest (75 mg/kg) dimethoate dose groups (fig. 4-20). This may indicate dose-dependence in the activity of ALP with prolonged exposure to the toxicants.

Significant decreases were observed in the levels of serum albumin at twelve weeks ($P < 0.02$) and eight weeks ($P < 0.05$) of exposure compared to the control values (fig. 4-21A & B). De Bruin (1976), had earlier reported decreases in albumin levels and increased globulins to be characteristic damage owing to chronic liver disease. The progressive decrease in albumin levels in this study with the lowest values at twelve weeks may as well indicate equally continued damage to the liver. This is supported by the observation made in the histopathologic investigations of the liver, where damage in the form of fatty liver was found to be severe in the third month as compared to a range of mild to moderate lesions observed in the second month of exposure (Plate V).

Total bilirubin levels as seen in this study were generally higher than the control values with the highest increases occurring at twelve weeks exposure period (Fig 4.12). Values were significantly ($P < 0.02$) higher at the highest dimethoate doses (75 mg/kg) in the second month and equally elevated in the third month at all dose groups. At twelve weeks in particular, increase in the levels of serum bilirubin appeared to be dose-dependent (fig. 4-22). The observation of obvious increase in the magnitude of destruction of haemoglobin with prolonged exposure corroborates the histopathology results.

The results of monthly body weight, feed intake as well as mean dimethoate consumption all showed consistent increase with prolonged exposure period. Compared to the values for their respective controls, however, no significant differences were observed (fig. 4-23, 4-24 and 4-25). Between the months however, increases were observed at eight weeks and twelve weeks compared to the fourth week. The increases in feed intake and dimethoate consumption correlated with the weight gained over the same periods of exposure.

5.2.2 Histopathological Investigations

The histopathologic examinations carried out did not reveal any observable lesions in the kidneys and the intestines (GIT) of rats in all the dose groups throughout the three month study period of dimethoate exposure (Plate I & II).

The result, however, revealed varying degrees of histological lesions from mild lesions to severe ones in the liver from rats in the second and third month of exposure period while livers from the first month at all dose groups were without significant lesions (Plate IV). The main lesions observed were fatty degeneration (lipodystrophy) and obliteration of the sinusoids occasioned by mild vesication.

Chronic exposure to a great number of chemicals and drugs exposes the liver to injuries in a variety of ways such as fatty degeneration, cell proliferation, necrosis as well as induction or inhibition of various enzyme activities (WHO, 1987).

Exposure to pesticides at significantly high doses has been reported to result in accumulation of fat in the liver with the consequent deposition of these compounds within

these lipid depots (especially the lipid soluble ones). These may subsequently lead to alterations of the liver function or some secondary effects that may manifest as a variety of pathological consequences (WHO, 1987). The target organ for the expression of toxicity is however, not necessarily that tissue organ in which the toxicant produces its effect as could be seen in accumulation of lead in bone and accumulation of certain chlorinated pesticides in adipose tissues without producing any local adverse effects (Raimo and Jauko, 1994).

Hepatic lesions in the form of fatty degeneration or fatty liver (lipodystrophy) were mild in the second month, while they appeared moderate and severe in the third. The magnitude of lesions did not seem to correlate with either dose of dimethoate nor the lindane co-administration. This observation arose from obvious lack of lesions in the highest treatment dose (75 mg/kg) dimethoate in both the presence and absence of lindane co-administration. However, mild and moderate lesions were seen at lower dose groups (25 and 50 mg/kg) of dimethoate of exposure at the same period (Plate VA and VB). Apparently, therefore, the degree of fatty degeneration or fatty liver was influenced more by the duration of exposure rather than lindane co-administration or even dimethoate dose. That suggests that longer duration of exposure enhanced the chances of development of fatty degeneration.

Lindane has long been established as an inducer of microsomal mixed function oxidase enzymes (Olsen and O'Brien, 1963; Wilkinson, 1976), and it has also been shown to accumulate in the adipose tissue (Geyer, *et al.*, 1986; WHO, 1991). Results from the three months enzymes assay did show slight influence of lindane on the toxicity of dimethoate probably due to enzyme induction of the dimethoate detoxifying enzymes by

lindane. Observations in the histopathologic examination did not clearly show corresponding effect of lindane on dimethoate toxicity. Meanwhile, results of histopathologic examination does not always corroborate enzyme assays (Cutler, 1974). It has also been observed that at low doses (1-10 ppm) daily dietary exposure, organochlorine insecticides are associated more with induction of enzymes rather than the production of more permanent change such as necrosis (Matsumura, 1975).

5.3 IN VITRO STUDIES

The results in this study demonstrates a biphasic response due to lindane at both muscarinic and nicotinic receptors. Lindane augmented spontaneous rabbit ileum contraction at low (< 1.6 ng/kg) doses while it showed a dose-dependent attenuation of responses at high (≥ 1.6 ng/kg) doses. Similarly, response pattern was observed at the nicotinic receptor as demonstrated by the phrenic nerve-diaphragm preparation (fig. 4-6A and 4-12A). Dimethoate dose-dependently augmented cholinergic responses at both muscarinic and nicotinic receptors (fig. 4-6A and 4-12A).

The initial augmentation of cholinergic responses by lindane observed at low concentration may be due to its effect as depolarizing blocker. Since the activation of sodium ion channels leading to the influx of sodium ions is responsible for the generation of action potential, lindane may be activating these channels in its insecticidal action. At high concentrations, however, lindane may be rendering the sodium channels permanently open in such a way that the resting potential of the channel is not observed as has been reported to occur with other organochlorine insecticides (Narahashi *et al.*, 1989; Narahashi, 1996).

The biphasic lindane response as observed may be isozyme specific. Different isozymes may be involved in producing the dual responses of the same compound as suggested by Hodgson, *et al.*, (1995) in synergistic interactions involving benzodioxole against compounds such as safrarole and piperine.

Dimethoate as an anticholinesterase potentiates the action of acetylcholine by increasing Ach concentration at the synaptic cleft. This is demonstrated in the present study by the dose-dependent augmentation responses of spontaneous rabbit ileum contraction. The effects of dimethoate was dose-dependently antagonized by atropine which also competitively decreased dimethoate response by blocking the muscarinic acetylcholine receptor resulting in diminished response (Fig. 4-8).

Lindane dose-dependently attenuated dimethoate response with significant ($P < 0.01$) decrease observed in dimethoate (8.0 ng/ml) responses following introduction of lindane (6.4 µg/ml) (fig. 3-9B). Conversely, dimethoate (16 µg/ml) significantly increased ($P < 0.001$) responses due to lindane (6.4 µg/ml) (fig. 4-10).

These results demonstrate antagonistic effects of lindane on dimethoate responses which may probably be due to the stimulation of the cholinesterase enzymes which produces reinforcement of the available cholinesterase supply. Responses following lindane (6.4 µg/ml) pretreatment was significantly ($P < 0.001$) increased by dimethoate (8 µg/ml), while physostigmine (1.3 µg/ml) was seen to significantly potentiate dimethoate responses (fig. 4.11A and B).

Both dimethoate and physostigmine are anticholinesterase agents and therefore, are seen to potentiate each others responses. Their action is antagonised through inhibition of the cholinesterase enzyme resulting in accumulation of ACh at the synapse.

Results obtained also shows a significant ($P < 0.001$) potentiation of cholinergic response by dimethoate (60 $\mu\text{g/ml}$) at the neuromuscular junction as demonstrated by increase in normal twitch responses of the rat phrenic nerve-diaphragm produced by direct stimulation of the nerve. Lindane (2.1 $\mu\text{g/ml}$) significantly attenuated responses by dimethoate, while d-tubocurarine (2.6 $\mu\text{g/ml}$) significantly reduced responses by lindane.

d-Tubocurarine is a neuromuscular blocker and acts by combining with a receptor at the motor end-plate to prevent conduction. The effect of lindane on the nerve is suspected to involve the Na^+ channel, where lindane may be blocking impulse transmission through hyperpolarization.

Lignocaine (16.8 $\mu\text{g/ml}$) reduced significantly ($P < 0.001$) responses due to dimethoate (60 ng/ml). Lindane significantly ($P < 0.001$) potentiated lignocaine response. Lignocaine as a local anaesthetic produce its effect through stabilization of the membrane. The local anesthetics produce their effects by interfering with the voltage-sensitive permeability of the membrane to Na^+ conductance (Guyton, 1976). They increase the threshold for electrical excitability of the nerve leading to decreased rate of rise of action potential resulting in turn to decreased impulse conductance which subsequently lead to failure of nerve conduction (Goodman and Gilman, 1990). The attenuation of lindane response by

lignocaine probably result from lignocaine - induced increase in threshold for nerve excitation thereby causing inhibition of nerve conduction.

5.4 CONCLUSIONS AND RECOMMENDATIONS

5.4.1 Conclusions

The acute toxicity studies demonstrates lindane to be over six times more acutely (LD_{50}) toxic than dimethoate in rats. Lindane acute toxicity in mice was remarkably reduced by diazepam, phenobarbitone and phenytoin in decreasing order respectively. These observations strongly points to the involvement of GABA in the mechanism of action of lindane. Apparently, however, pre-treatment with lindane was found to offer some degree of protection against acute toxicity due to dimethoate in mice.

Results of *in vivo* simultaneous chronic exposure to lindane and dimethoate demonstrated the ability of lindane to induce herpatocellular enzymes as manifested by relatively lower serum transaminase enzymes compared to dimethoate exposure in the absence of lindane. Similarly, co-administration of lindane and dimethoate produced fatty liver which progressed with period of exposure. The presence or absence of lindane did not, however, influence the magnitude of the fatty liver formation.

The *in vitro* investigations revealed attenuation and antagonism of dimethoate responses by lindane and atropine respectively, while lignocaine was found to augment lindane response. Results of the EEG studies also demonstrated reversal of lindane-induced desynchronization of EEG wave pattern by phenobarbitone and diazepam, while dimethoate seemed to augmented such an effect.

Result from this study indicate obvious significant interactions between lindane and dimethoate at various levels.

5.4.2 Recommendations

The use of pesticides in our environment seems to be inevitable, at least for now, considering the growing global population and consequent need for more food production. Therefore, the issue at hand is that of the safe use with the aim of minimizing, if not eliminating adverse effects and the non-target organisms. In the light of this and based on the studies carried out in this work, the following observations and recommendations are made.

1. Efforts should be intensified in increasing our basic understanding of the mechanisms of action and by which major interactions can occur, so as to recognize and avoid situations of obvious potential hazards to man and his environment.
2. Field workers should be educated on the safe use of these chemical agents and both preventive measures as well as antidotal agents should be acquired to minimize incidences of accidental poisoning. In this respect, the involvement of GABA through benzodiazepines and barbiturates needs further investigations especially *in vitro*.
3. To achieve a more efficient use of pesticides with the aim of avoiding or at least minimizing non-target toxicity, the actual target needs to be defined in terms of both time and space. The proportion of emitted pesticides that reaches the target in the target in the form available to the pest needs to be greatly increased.
4. There is also the need for accurate analysis of pesticide presence in each step of the food chain. This is because of the high number of these substances, their potential toxicity and the possible presence of amplified toxic effect due to synergistic interaction.

5. It is also necessary, in my view, to have a general evaluation of the environmental effect of the whole pesticide cycle from industrial production to the final disposal of used containers and residuals.
6. Finally, efforts should be directed at obtaining information in studies involving human volunteers directly as against the present use of laboratory animals in studies and the subsequent extrapolations to determine human safety or hazards due to these chemical agents. Animal models cannot be substitutes for human exposure experience.

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• APPENDIX I

Composition of Tyrode's and Kreb's Physiological Solutions.

Tyrode's and Kreb's solution were prepared fresh on each day of the experiments, just before commencement. For a 10 litre solution, the following salts and reagents are required in appropriate amount and in the sequence presented.

Composition of Tyrode's Solution

Reagent	Amount
NaCl	90 g
KCl (10%) solution	20 ml
NaH ₂ PO ₄ . 2H ₂ O (10%) solution	5 ml
D-glucose	10 g
NaHCO ₃	10 g
CaCl ₂ (10%) solution	20 ml
MgCl ₂ . 6H ₂ O	1 ml

Composition of Kreb's solution.

Reagent	Amount
NaCl	698 g
KCl (10%) solution	35 ml
MgSO ₄ . 7H ₂ O	29 ml
KH ₂ PO ₄ (1%) solution	16 ml
D-glucose	20 g
NaHCO ₃	21 g
CaCl ₂ (Molar) solution	25.2 ml

Dionized water was used to make up the volume to 10 litres.

APPENDIX IJ

Profile of serum enzymes (AST, ALT, and ALP) activities in rats that received (A) dimethoate alone and in their diet lindane (1 mg/kg weekly i.p.) in addition to dimethoate in their diet for twelve weeks period of study. (n = 5; mean $\pm$ SEM).

Parameter	G-I (0mg/kg) Control	DIMETHOATE DOSE GROUPS								V (75 mgkg ⁻¹)	
		G-II (10 mg/kg)		G-III (25 mg/kg)		G-IV (50 mg/kg)					
		A	B	A	B	A	B	A	B		
FOUR											
AST	91.2 ± 3.9	117.2 ± 3.9	115.8 ± 4.3	121 ± 4.9	117.2 ± 7.7	128.6 ± 7.9	117.8 ± 6.0	122.0 ± 7.9	121.4 ± 6.4		
ALT	70.8 ± 2.6	81.0 ± 3.9	72.8 ± 2.9	80.6 ± 3.6	76.0 ± 2.5	131.0 ± 3.8	71.2 ± 2.7	79.2 ± 5.3	74.6 ± 5.9		
ALP	389.4 ± 47.1	510.6 ± 18.9	462.8 ± 51.8	616.4 ± 51.7	516.8 ± 46.3	614.6 ± 77.0	618.2 ± 44.3	828.4 ± 59.9	722.2 ± 48.5		
EIGHT											
AST	90.4 ± 3.3	101.2 ± 6.4	92.4 ± 5.4	108.6 ± 9.1	99.6 ± 5.4	109.6 ± 6.6	91.4 ± 7.7	115.6 ± 7.5	112.8 ± 6.3		
ALT	73.6 ± 4.2	153.0 ± 5.6	145.5 ± 3.5	137.4 ± 4.6	130.2 ± 8.8	136.6 ± 8.9	121.8 ± 7.5	154.4 ± 10.3	140.2 ± 9.0		
ALP	379.8 ± 41.1	215.4 ± 32.2	191.4 ± 13.2	223.4 ± 32.1	220.8 ± 11.3	236.0 ± 24.1	238.3 ± 18.6	253.6 ± 18.5	257.0 ± 23.8		
TWELVE											
AST	92.8 ± 5.1	106.0 ± 9.4	104.8 ± 6.1	104.4 ± 6.6	103.6 ± 5.1	101. ± 4 7.4	101.2 ± 6.6	109.6 ± 4.4	102.8 ± 6.5		
ALT	70.2 ± 4.5	97.8 ± 3.7	98.2 ± 3.5	83.6 ± 5.3	72.8 ± 3.8	91.4 ± 4.4	80.2 ± 2.1	68.4 ± 5.7	72.6 ± 3.6		
ALP	371.0 ± 44.7	273.0 ± 23.0	249.2 ± 19.5	324.6 ± 23.9	316.0 ± 27.1	380.6 ± 46.5	364.4 ± 31.6	492.8 ± 27.0	397.2 ± 22.5		

APPENDIX III

Profile of serum albumin and total bilirubin levels in rats that received (A) dimethoate alone in their diet, and (B) Lindane (1mg/kg, i.p) in addition to dimethoate in their diet (n = 5; mean $\pm$ SEM).

Parameter	DIMETHOATE DOSE GROUPS									
	G-I Control (0 mg/kg)	G-II (10 mg/kg)		G-III (25 mg/kg)		G-IV (50 mg/kg)		V (75 mg/kg)		
FOUR		A	B	A	B	A	B	A	B	
Serum Albumin (g/l)	40.2 $\pm$ 1.5	37.8 $\pm$ 0.4	38.1 $\pm$ 1.0	36.8 $\pm$ 0.6	40.6 $\pm$ 0.9	37.6 $\pm$ 0.4	39.6 $\pm$ 0.7	35.9 $\pm$ 0.4	39.8 $\pm$ 0.6	
TOTAL BILIRUBIN (g/l)	6.0 $\pm$ 1.0	7.0 $\pm$ 1.2	6.0 $\pm$ 1.0	8.0 $\pm$ 1.2	7.0 $\pm$ 1.2	8.0 $\pm$ 1.2	6.0 $\pm$ 1.0	6.0 $\pm$ 1.0	6.0 $\pm$ 1.0	
EIGHT										
Serum Albumin (g/l)	39.8 $\pm$ 1.2	36.6 $\pm$ 1.3	38.0 $\pm$ 0.9	38.3 $\pm$ 1.4	36.2 $\pm$ 1.2	37.8 $\pm$ 0.9	36.8 $\pm$ 0.6	34.8 $\pm$ 0.6	36.5 $\pm$ 0.5	
TOTAL BILIRUBIN (g/l)	6.0 $\pm$ 1.0	8.0 $\pm$ 1.2	9.0 $\pm$ 1.9	8.0 $\pm$ 1.2	8.0 $\pm$ 1.2	8.0 $\pm$ 1.3	9.0 $\pm$ 1.2	9.0 $\pm$ 1.0	10.0 $\pm$ 2.2	
TWELVE										
Serum Albumin (g/l)	39.0 $\pm$ 1.0	35.6 $\pm$ 1.2	34.3 $\pm$ 1.4	34.0 $\pm$ 0.8	32.9 $\pm$ 0.9	33.6 $\pm$ 0.4	33.4 $\pm$ 0.7	33.6 $\pm$ 0.4	33.4 $\pm$ 0.7	
TOTAL BILIRUBIN (g/l)	6.0 $\pm$ 1.0	9.0 $\pm$ 1.0	8.0 $\pm$ 1.2	9.0 $\pm$ 1.9	10.0 $\pm$ 1.6	14.0 $\pm$ 1.0	11.0 $\pm$ 1.9	14.0 $\pm$ 1.8	13.0 $\pm$ 1.2	

APPENDIX V

Comparison of the mean values of AST, ALT, ALP serum albumin, and total bilirubin between various dose groups and control group respectively.

ENZYMES		AST			ALT			ALP			Serum Albumin			Total Bilirubin		
DURATION OF EXPOSURE	COMPARED GROUPS	A	B		A	B		A	B		A	B		A	B	
FOUR WEEKS PERIOD (4)	GI vs GII/GIIB	P<0.01	P<0.01		NS	NS		P<0.05	NS		NS	NS		NS	NS	
	I vs III A/B	P<0.01	P<0.01		NS	NS		P<0.02	P<0.05		NS	NS		NS	NS	
	I vs IV A/B	P<0.01	P<0.01		NS	NS		P<0.02	P<0.01		NS	NS		NS	NS	
	I vs V A/B	P<0.01	P<0.01		NS	NS		P<0.001	P<0.01		P<0.05	NS		NS	NS	
	IIA/B vs VA/B	NS	NS		NS	NS		P<0.001	P<0.01		NS	NS		NS	NS	
EIGHT WEEKS (8)	GI vs GII/GIIB	NS	NS		P<0.001	P<0.001		P<0.02	P<0.01		NS	NS		NS	NS	
	I vs III A/B	NS	NS		P<0.001	P<0.001		P<0.02	P<0.02		P<0.05	P<0.05		NS	NS	
	I vs IV A/B	P<0.05	NS		P<0.001	P<0.001		P<0.05	P<0.02		NS	P<0.05		NS	P<0.02	
	I vs V A/B	P<0.01	P<0.01		P<0.001	P<0.001		P<0.05	P<0.05		P<0.01	P<0.05		P<0.02	P<0.01	
	IIA/B vs VA/B	NS	NS		NS	NS		NS	P<0.05		NS	NS		NS	NS	
TWELVE WEEKS (12)	GI vs GII/GIIB	NS	NS		P<0.001	P<0.001		NS	NS		P<0.02	P<0.02		P<0.02	NS	
	I vs III A/B	NS	NS		P<0.02	P<0.001		NS	NS		P<0.02	P<0.01		P<0.01	P<0.01	
	I vs IV A/B	NS	NS		P<0.02	P<0.001		NS	NS		P<0.01	P<0.01		P<0.001	P<0.02	
	I vs V A/B	NS	NS		NS	NS		P<0.05	NS		P<0.001	P<0.02		P<0.001	P<0.001	
	IIA/B vs VA/B	NS	NS		P<0.01	P<0.01		P<0.001	P<0.01		P<0.05	NS		P<0.01	P<0.01	

KEY: GI = Control group, GII = 10 mg/kg DM, GIII = 25 mg/kg DM, GIV = 50 mg/kg DM and GV = 75 mg/kg DM.

APPENDIX VI

Comparison of the mean values of serum enzyme, AST, ALT and ALP in 10 mg/kg and 75 mg/kg Dimethoate dose group between successive months of exposure period.

DM Dose Groups	Compared months	AST		ALT		ALP	
		A	B	A	B	A	B
G-II (10 mg/kg)	1 st vs 2 nd	NS	P<0.01	P<0.001	P<0.001	P<0.001	P<0.001
	1 st vs 3 rd	NS	NS	P<0.05	P<0.05	P<0.001	P<0.001
	2 nd vs 3 rd	NS	NS	P<0.001	P<0.01	NS	P<0.05
G-V (75 mg/kg)	1 st vs 2 nd	NS	NS	P<0.001	P<0.001	P<0.001	P<0.001
	1 st vs 3 rd	NS	NS	NS	NS	P<0.001	P<0.001
	2 nd vs 3 rd	NS	NS	P<0.001	P<0.001	P<0.001	P<0.001

KEY

A = Lindane- free subgroup;

B = lindane treated subgroup.

NS = Not significant