

**AN INVESTIGATION OF THE TOXICITY OF LINDANE
IN VIVO AND IN VITRO**

By

YUSUF BABAYE
B.Pharm (A.B.U.)

A Thesis in the Department of Pharmacology and Clinical Pharmacy

**Submitted to the Postgraduate School
in partial fulfillment of the requirement for the Degree of**

**Master of Science in Pharmacology
Ahmadu Bello University, Zaria - Nigeria**

September, 1997

DEDICATION

2011

DEDICATED TO MY PARENTS

DECLARATION

This is to certify that I carried out the work reported in this thesis in the Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmaceutical Science, Ahmadu Bello University under the supervision of Dr. T.T. Iyaniwura and Dr. (Mrs) H.O. Kwanashie. The work of other investigators is acknowledged and referred to accordingly. I solemnly declare that no part of this thesis has been submitted elsewhere for a degree.



YUSUF BABAYE

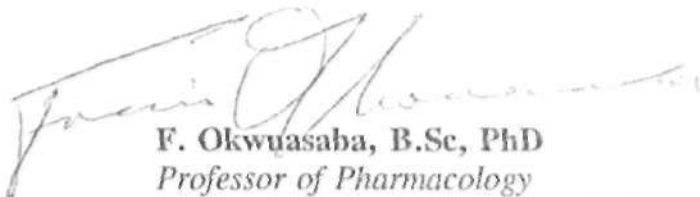
*DEPARTMENT OF PHARMACOLOGY & CLINICAL PHARMACY
FACULTY OF PHARMACEUTICAL SCIENCES
AHMADU BELLO UNIVERSITY, ZARIA.*

CERTIFICATION

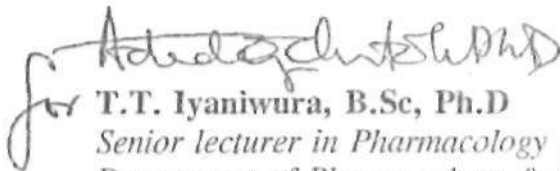
This thesis entitled **AN INVESTIGATION OF THE TOXICITY OF LINDANE IN VIVO AND IN VITRO BY YUSUF BABAYE** meets the regulations governing the award of the degree of Masters of Science in Pharmacology (M.Sc) of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Prof F. Okwuasaba
NAME



SIGNATURE

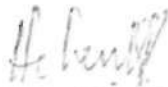


F. Okwuasaba, B.Sc, PhD
Professor of Pharmacology
Department of Pharmacology & Clinical Pharmacy
Faculty of Pharmaceutical Sciences
University of Jos
External Examiner.

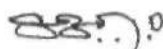


T.T. Iyaniwura, B.Sc, Ph.D
Senior lecturer in Pharmacology & Toxicology
Department of Pharmacology & Clinical Pharmacy
Faculty of Pharmaceutical Sciences
Ahmadu Bello University, Zaria
Supervisor and Internal Examiner.


T. T. IYANIWURA



H.O. Kwanashie, B.Sc, M.Sc, Ph.D
Senior lecturer in Pharmacology
Department of Pharmacology & Clinical Pharmacy
Faculty of Pharmaceutical Sciences
Ahmadu Bello University, Zaria
Supervisor and Internal Examiner.



J.Y. Olayemi, B.Sc, PhD, FRSC, FPI(Nig)
Dean of Postgraduate School, ABU, Zaria

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ABSTRACT

The goal of this project is to study *in vivo* and *in vitro*, the mode of toxicity of lindane (an organochlorine insecticide) at tissue/cellular level with the hope that the information obtained would provide an insight into the non-target toxicity of the compound.

The *in vivo* investigation involved acute and chronic toxicity studies. In the acute toxicity studies, the median lethal dose (LD₅₀) of lindane using the intraperitoneal (I.P.) route was determined as 25.4 mg/kg. The symptoms of toxicity were scored and were found to be dose-dependant and mainly those of central nervous system (CNS).

In the chronic toxicity studies, results of the histopathological examination of sections of the rat brain exposed to feeds contaminated with lindane indicated lesions of different structures. There was vacuolations, necrosis of the oligodendroglial cells and some degree of neuronal degeneration especially in the brain of rats exposed to the contaminated feeds for a long period of time (4 to 12 weeks).

Electroencephalographic (EEG) pattern of rats fed on contaminated feeds showed some degree of desynchronisation as compared to the control. The effect was found to increase with dose and time of exposure. Since desynchronisation of EEG pattern is associated with increased CNS activity, it is possible that chronic exposure to lindane may produce symptoms of CNS stimulation.

The *in vitro* investigation was aimed at evaluating the role of sodium ions (Na⁺) in the neurotoxicity of lindane. It was found that lindane dose-dependently attenuated the effect of lignocaine (P<0.001) on both the rabbit ileum and phrenic nerve preparations. It was suggested that lindane increases the permeability of the membrane to Na⁺ thereby interfering with the generation and propagation of action potential.

The importance of identifying the mechanism by which various chemicals produce their toxicity cannot be overemphasized; in order to design a rational basis for treatment and to make available adequate information necessary in the prevention, diagnosis and management of intoxication.

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CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION TO PESTICIDES

In most parts of the world, there is at one time or the other, the need to chemically control pests and diseases either of livestock or of plants. In this respect, pesticides are quite useful and occupy a rather unique position among the many chemicals that man encounters daily in that they are deliberately added to the environment for the purpose of killing or injuring some form of life (Murphy, 1980).

It has been estimated by the Food and Agricultural Organisation (F.A.O.) of the United Nations that cessation of all use of crop protection chemicals in the U.S.A. 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).

The need for pesticides extends beyond their role in crop protection; substantial livestock and poultry losses occur as a result of diseases, insects and internal parasites. Pesticides are invaluable in the control of vector-borne diseases and have been found to be one of the most effective means of controlling some diseases such as onchocerciasis where pesticides have to be entirely depended upon for the destruction of vector simuliids (W.H.O., 1986). Although a more specific drug which is currently marketed for this disease is Ivermectin.

Overall pesticide use in Agriculture, in terms of amounts applied per hectare has been very much greater in Japan, Europe and the United States than in the rest of the world, although China is also a major user. The fastest growing market however is Africa with an increase in sales of 18.2% between 1980 and 1984 (Edwards, 1986).

In Nigeria, although there is a dearth of statistics on pesticide production and

usage, from 1975 to date, there is evidence of a rise in usage from 1975 to date judging by the increased agricultural activity in the country especially occasioned by such government programmes as the "Operation Feed the Nation" and "Green Revolution" as well as the advent of medium/large scale farms. The economic inclemency in the wake of the Structural Adjustment Programme (S.A.P.) has also turned virtually everyone into at least a part-time farmer. This increased agricultural activity is associated with a rise in pesticide usage and the attendant hazard to man and his livestock. Since pesticide toxicity is not restricted to the target organisms and some of these pests tend to share the environment rather intimately with humans, their chemical control without concurrently injuring the well-being of both humans and other living things has become of great importance.

The F.A.O. defined a pesticide as any substance or mixture of substances intended for preventing, destroying or controlling any pest including vectors of human or animal disease, unwanted species of plants or animals causing harm during, or otherwise interfering with, the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products, or animals feedstuff, or which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies (F.A.O., 1986a).

However, in evaluating the potential health-related effects of all classes of pesticides, it is important to think not only of the active ingredients but also of the carriers, which are often added to enhance delivery. Carriers include talcs, oils, solvents and binding agents. These so-called inert ingredients may themselves have toxic properties explaining some of the acute and persistent health effects; acting as irritants, and less commonly sensitizing agents in exposed individuals (Rosenstock,

1987).

1.1.1 Exposure to Pesticides

It is apparent that there are many sources of exposure of humans and other non-target species to pesticides by direct contact with materials at the site of application. In recent years, however, it has become increasingly apparent that exposures to pesticides far remote from the sources of application are also possible. This results from the translocation of the chemicals from their sites of application through the various media of the environment. The extent to which translocation within the environment occurs will depend to a large degree on the physicochemical properties of the pesticides. Perhaps one of the most important factors is the extent of and time required for degradation of chemicals to simpler non-toxic forms. Since several of the organochlorine insecticides and some of the heavy metals are the most persistent types of pesticides, these compounds have been the object of most concern for problems of translocation and biomagnification.

Exposure of man to pesticide occur in several ways. Occupational exposure occurs during the manufacture, transportation and at the site of application. These exposures could produce acute or chronic symptoms. Accidental poisoning or consumption of crops sprayed with pesticide constitute other means of human intoxication. Exposure may be via inhalation, dermal or oral routes.

While it may be possible to detect acute poisoning and thus provide adequate medical care, chronic exposure is not readily detected. Efforts to find ways of minimizing pesticide hazards to man has resulted in intensive scientific investigations. Toxicological evaluations of the hazard of handling and use of pesticides have for many

years focused on prevention of injury to man and his animals. Laboratory animals serve as experimental models for man's biochemical, physiological and pathological responses to these chemicals.

It is clear that the opportunities for exposure to pesticides are great. The use of pesticides is associated primarily with agricultural operations and in public health for controlling disease vectors e.g. mosquito in malaria. The major concern in these instances, is usually of residual pesticide levels in food crops. However, it is quite possible that less controlled and less regulated uses of pesticides may offer the greatest opportunity for exposure to toxicologically significant quantities. Government must aim at enacting legislation which will minimise these risks without seriously detracting from the benefits (Green, 1976).

1.1.2 Classification of Pesticides

There is as great a diversity in the types of action and primary target tissues of pesticides as there is diversity in their chemistry, physicochemical and toxicity properties which often form the basis of their classification. There are a large number of pesticides whose acute toxicity is manifested through functional or biochemical action in the Central and Peripheral Nervous Systems, but there are others in which nervous system involvement does not occur or is merely secondary to primary effects in other organ systems. The literature on pesticides reveals great disparities in the extent of knowledge concerning specific mechanisms of action. For some groups of compounds the mechanism of toxic action is well understood at the molecular level. For others there is essentially no information concerning mechanisms of toxicity. Similarly, the full gamut of toxic dose-response ranges is represented by pesticide

chemicals. Even within a similar chemical class, individual compounds ranging from extremely toxic to practically non-toxic may be found. Obviously therefore, one cannot generalise either qualitatively or quantitatively concerning the toxicity of pesticides.

Insecticides represent one group of pesticides that are used in large quantities and have a history of causing toxic effects in man. These are chemicals designed to kill insects and other related species that prey on crops before, during or after the harvest. They are further subdivided into organophosphates, organochlorines, carbamates and the pyrethroids. Herbicides are chemicals used to destroy unwanted plants and in terms of quantities needed they are as widely used as insecticides. Fungicides are agents useful in controlling the growth of moulds and fungi on crops, stored foods and timber. Rodenticides are a wide variety of chemicals that have been used in the control of rats and mice which eat and bite off plant items and roots. Fumigants are used in the control of insects, rodents, and soil nematodes. They have in common the property of being in the gaseous form at the time they exert their pesticidal action and are used because they will penetrate to areas otherwise inaccessible for pesticide application.

In the 1970s and 1980s many new pesticides were introduced based on a more thorough understanding of biological/biochemical mechanisms and are often more effective at lower doses than the older pesticides. Examples of these new generation pesticides include the herbicidal sulfonylureas and the synthetic light-stable pyrethroid insecticides developed from the naturally occurring pyrethrins (Hassall, 1982).

1.2 ORGANOCHLORINE INSECTICIDES

The chlorinated hydrocarbon compounds represent an important class of insecticides that enjoyed wide use in agriculture, soil and structure, insect control, and in malaria control programmes (Murphy, 1975).

All compounds which belong to this group are characterised by:

- (1) the presence of carbon, chlorine, hydrogen, and sometimes oxygen atoms including a number of C-Cl bonds
- (2) the presence of cyclic carbon chains (including benzene rings)
- (3) lack of any particular active intramolecular sites
- (4) apolarity and lipophilicity; and
- (5) chemical unreactivity (i.e. they are stable in the environment) (Matsumura, 1975).

The spectrum of pesticidal activity shown by the halogenated hydrocarbon pesticides is remarkably broad. This group of pesticides has a wide range of practical applications against both agricultural pests and pests affecting man and animals (Turner, 1985). There is no doubt that their introduction as agricultural insecticides made a remarkable contribution to the increase in world grain and other crop production. The application of the halogenated hydrocarbon pesticides, especially Diphenyl Dichloro Trichloroethane (DDT) in the areas of public health and veterinary medicine have attracted the most attention (Brook, 1974). DDT is a highly persistent chemical and its exclusive use is highly controversial in developed countries at the moment. Indeed, the very hallmark of these compounds, which characterised them originally as wonder chemicals, was the small amounts needed over a given area to effect complete control of insects (Brooks, 1974).

The interactions between the halogenated hydrocarbon pesticides and the biotic and abiotic elements of the environment, especially as they relate to the possible effects on wild life, are highly controversial and of grave concern to environmentalist (Turner, 1982).

In general, the organochlorine insecticides are most persistent in soils and biological tissues; the organophosphate and carbamate insecticides being less persistent. An understanding of the potential for persistence and translocation, therefore, must take into consideration not only the biological aspects of pesticides but also an analysis of their behaviour under various physical and chemical characteristics of the environment (Murphy, 1975).

As a class, the organochlorine insecticides are often considered less acutely toxic, but of greater potential for chronic toxicity than the organophosphates and carbamate insecticides. There is however, a wide range of acute toxicities of individual organochlorine compounds, from extremely toxic e.g. endrin to slightly toxic e.g. methoxychlor (Murphy, 1975).

The chlorinated hydrocarbons have a high lipid/water partition coefficient and thus penetrate biological membranes quite easily and accumulate in the fatty tissues of organisms. The lipid solubility of chlorinated hydrocarbons together with their high resistance to enzymatic attack make them the least biodegradable of all pesticides (Iyaniwura, 1991a). Organochlorines pesticides persist in soil, organic matter including food crops, man, domestic and wild animals (Hayes, 1982; Hassal, 1987).

The chemical stability of many members of the group (or of their immediate and often toxic metabolites) is high because their molecules are constructed, entirely or largely from C-C, C-H and C-Cl bonds all of which tend to be chemically rather

inactive under normal environmental conditions (Hassall, 1982). With some exceptions (e.g. endosulfan and perthane, which are biodegradable) organochlorine insecticides are very persistent in the environment because they resist chemical or microbial decomposition especially when protected by layers of soil (Brooks, 1974).

DDT is a prototype of the chlorinated hydrocarbon pesticides and intoxication from other organochlorines is of the same pattern as DDT (Iyaniwura, 1991a).

Signs and symptoms of poisoning in man and mammals resulting from high doses of DDT include paresthesia of the tongue, lips, and face; apprehension; hyperirritability; dizziness; disturbed equilibrium; tremor; tonic and clonic convulsions (Hayes, 1982). Motor unrest and fine tremors associated with voluntary movements progress to coarse tremors without interruption in moderate to severe poisoning. Symptoms appear several hours after large doses, and in animals poisoned with fatal doses death occurs in 24 to 72 hours. It has been estimated that a dose of 10mg/kg will cause signs of poisoning in man. Although there are rather marked species difference in susceptibility to acute poisoning by oral ingestion, when the compound is given by intravenous administration, the dose and time required for poisoning are quite similar for a wide variety of species including insects. (Murphy, 1975).

The signs of chronic toxicity are similar in general outline to those of acute poisoning, but usually first appear as tremors in the muscles of the neck and head. These gradually extend so as to involve most of the muscle of the body and increase in intensity so that any purposeful movement becomes difficult or impossible. The tremors give way to convulsions which gradually become more frequent and more severe. Terminally depression appears with respiratory failure and death intervening (Clarke and Clarke, 1975).

Treatment of acute poisoning by the organochlorine insecticides is largely symptomatic. Phenobarbital has been recommended as an antidote to control convulsions produced by DDT and the other compounds. Calcium gluconate has also been useful in controlling convulsions produced by DDT. As with all exposures, attention should be given to removal of unabsorbed poison from the gastrointestinal tract and the skin (Murphy, 1975).

The organochlorine insecticides are broadly classified into chlorinated ethane derivatives of which DDT is the best known example; the cyclodienes, which include chlordane, aldrin, dieldrin, heptachlor, endrin, and toxaphene; and the hexachlorocyclohexanes, such as lindane.

1.2.1 Mechanism of Action of Organochlorine Insecticides

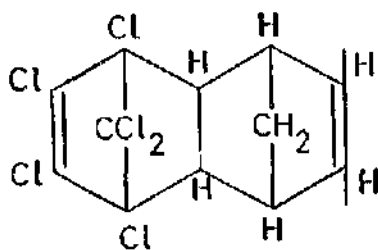
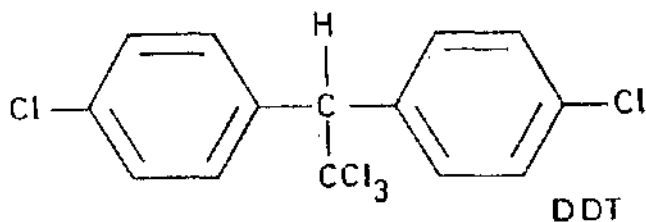
It is generally accepted that the primary target of organochlorine toxicity is the nervous system. There are however, differences of opinion as to exactly how the interference with the nervous system is brought about. A consensus of opinion indicated that DDT acts by prolonging neuronal recovery time through increasing the negative after-potential. This is the time interval after a spike potential, during which the neuron slowly returns to its resting state (Narahashi and Haas, 1968).

The initial effect of DDT is upon the peripheral nervous system whereas lindane and aldrin appear to attack the central nervous system. However, the general effect of all of them is to destabilise neural activity and this is manifested by a hyperexcitability of nerves and muscles (Hassall, 1982).

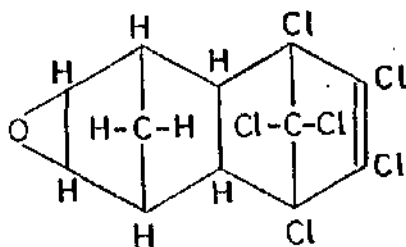
The loci of primary toxic action of DDT is believed to be sensory and motor nerve fibres and the motor cortex.

1.2.2

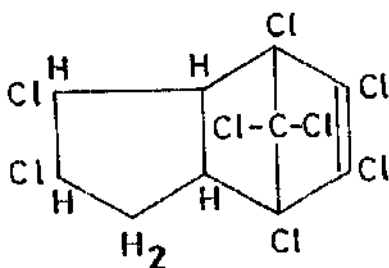
Structures of Some Organochlorine Insecticides



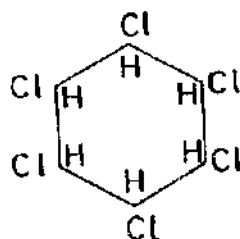
ALDRIN



DIELDRIN



CHLORDANE



LINDANE

Figure 1: Structures of some organochlorine insecticides

The mechanism of action is still incompletely known; however, recent evidence indicates that DDT is capable of altering the transport of sodium and potassium ions across the membranes of nerve axons. Studies on isolated neurons have shown that DDT blocks potassium efflux across the membrane. This action results in an increased negative after potential (O'Brien, 1967).

The organochlorines are well documented for their ability to stimulate liver microsomal enzymes. Induction produces an increase in the levels and activity of the liver microsomal enzymes resulting in an increase in the rate of metabolism of drugs and chemicals that are affected by these enzymes. Induction could produce an increase or a decrease in toxicity of chemicals depending on whether the compound requires activation *in vivo* or not; or whether the component of the microsomal enzyme that is induced, is the activation enzyme or the detoxifying enzyme (Iyaniwura, 1991b).

1.2.3 Uses of Organochlorine Insecticides

For a time, DDT has been outstandingly successful in controlling the vectors of organisms responsible for such mortal and debilitating disease as malaria, river blindness and yellow fever (Hassal, 1982).

DDT is effective against wide variety of pests like hymenoptera, lepidoptera, blossom weevil, plant bugs but not as effective against aphids and spider mites.

The volatility of this group of insecticides is also responsible for some loss of pesticide from exposed surfaces, uptake of pesticide by foliage and exposure of insects, and animals to the pesticide by inhalation (Hatch, 1982).

Lindane has a vapour pressure fifty times that of DDT and is therefore preferable to DDT where a fumigant action is desirable. This high pressure is very

useful for soil organism.

Aldrin and its analogues are useful for short non-persistent foliage action, for insects like mealy bugs, scale insects, wire worms e.t.c.

1.3 ROLE OF SODIUM IONS IN THE GENERATION OF NERVE ACTION POTENTIAL

In nerves, as in other tissues, sodium ion (Na^+) is actively transported out of the cell and potassium ion (K^+) is actively transported into the cell. K^+ diffuses out of the cell down its concentration gradient through K^+ channels, and Na^+ diffuses back in, but because the permeability of the membrane to K^+ is much greater than it is to Na^+ at rest, the passive K^+ efflux is much greater than the passive Na^+ influx. In addition, the membrane is impermeable to most of the anions in the cell, therefore, the K^+ efflux is not accompanied by an equal flux of anions and the membrane is maintained in a polarized state, with the outside positive relative to the inside. This is referred to as resting membrane potential and in neurons, it is usually about - 70 mV (Ganong, 1987).

Nerve cells have a low threshold for excitation. The stimulus may be electrical, chemical or mechanical. The two types of physicochemical disturbances produced are: local, non-propagated potentials called electrotonic potentials; and the propagated disturbances, the action potentials or nerve impulses, and these constitute the only responses of the neurons and other excitable tissues (Ganong, 1987).

The first manifestation of the approaching action potential is a beginning depolarization of the membrane. After an initial 15mV of depolarisation, the rate of depolarisation increases. The point at which this change in rate occurs is called the firing level (fig. 2).

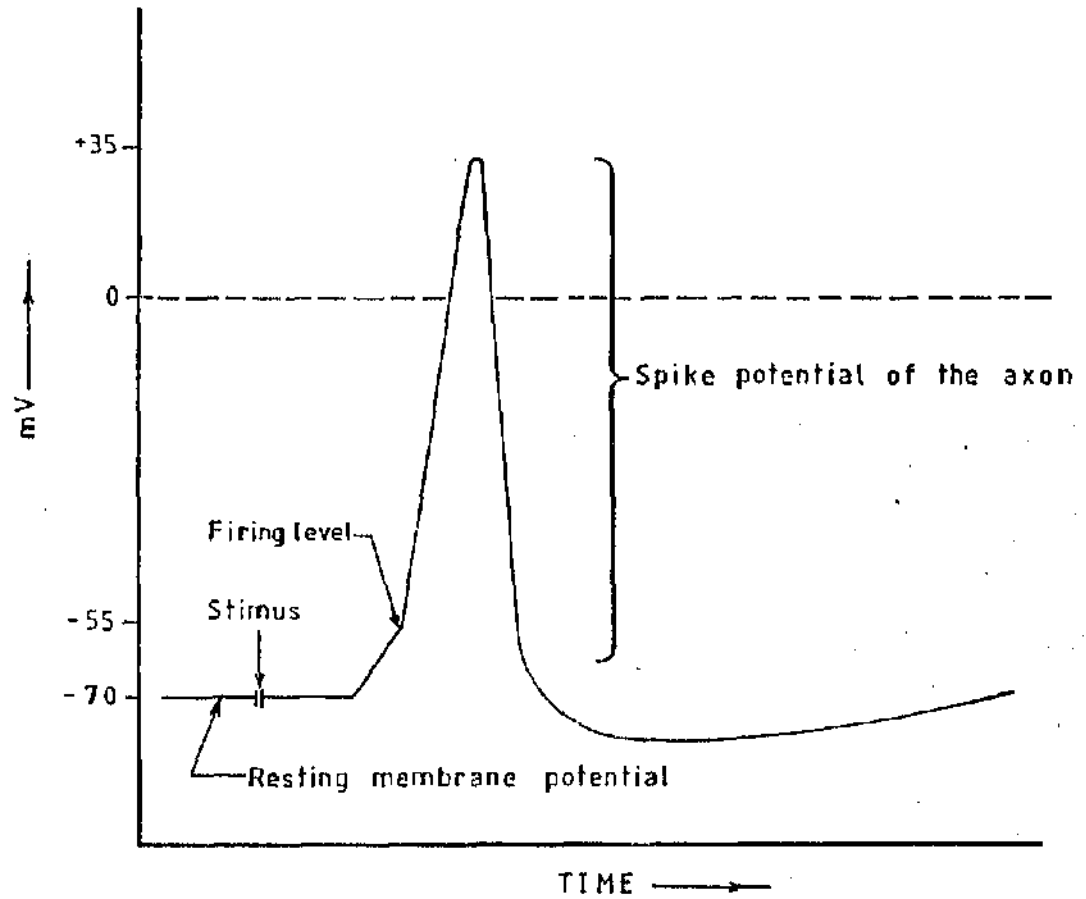


Figure 2: Action potential in a neuron recorded with one electrode inside the cell (Ganong, 1987).

the spike potential of the axon). The process eventually return to normal resting membrane potential (-70mV).

During the initiation of nerve impulse, the first, small depolarisation of the membrane causes the activation of Na^+ channels allowing an inward current of Na^+ ions to flow, which depolarises the membrane further. The process is thus a regenerative one and it is the increase in Na^+ permeability that brings the membrane potential close to what is referred to as sodium equilibrium potential which is a function of the intracellular and extracellular Na^+ concentrations, and is about +40mV under normal conditions (Rang and Dale, 1991).

The spontaneous closure of the channels known as inactivation is an important property of the Na^+ channel. This in itself would gradually cause the membrane potential to return to its normal value, but the process is accelerated by the secondary increase in potassium permeability which becomes appreciable at the peak of the action potential (Bowman and Rand 1980).

Considering, the conduction along normal non-myelinated nerve axons, a nerve axon can be regarded as a cable of aqueous fluid insulated from the extracellular fluid by a lipoprotein membrane, the plasmalemma of the nerve cell. The fluid inside is richer in potassium ions than is the fluid outside indicating that the membrane is relatively impermeable to ions when the nerve is resting.

The passage of an impulse along a non-myelinated axon is a rapid wave of depolarization moving at a speed of 1-20 m/s. As the impulse passes any particular position along the axon, the inside of the nerve membrane at that point rapidly changes from being negatively charged compared to the outside of the memberane and becomes

positively charged instead. This is due to rapid influx of sodium ions. Thereafter, the potential inside the membrane rapidly returns to normal due to the following reasons:

- (a) The membrane's normal low permeability to sodium ions is restored.
- (b) Due to efflux of potassium ions.

There are broadly speaking, two ways in which channel function may be modified, namely block of the channels and modification of gating behaviour. Either mechanism can cause an increase or a decrease of electrical excitability. Thus, blocking Na^+ channels reduced excitability while an agent that affects Na^+ channel gating in such a way as to increase the probability of a channel being open will tend to increase excitability. Examples of Na^+ channel blockers include local anaesthetic (e.g. lignocaine), anticonvulsants (e.g. phenytoin) and two neurotoxins, tetrodotoxin and saxitoxin which are extremely potent and selective Na^+ channel blocking agents.

1.4 LINDANE

Benzene hexachloride (BHC) was first prepared in 1825 by Michael Faraday, who did not recognize its insecticidal properties (Matsumura, 1975). Van der Linden in 1912 discovered four isomers of benzene hexachloride. Furthermore, Dupire and Raucourt in France as well as Slade in England in 1942 discovered the insecticidal properties of BHC. The British group isolated the toxic-isomer and named it Lindane in honour of Van der Linden (Matsumura, 1975).

Technical-grade hexachlorocyclohexane (HCH) consist of 65-70% alpha-HCH, 7-10% beta-HCH, 14-15% gamma-HCH, and approximately 10% of other isomers and compounds. Lindane contains more than 99% gamma-HCH, (W.H.O. 1991).

Lindane can be determined separately from the other isomers of HCH after extraction by liquid/liquid partition, Column chromatography and detection by gas chromatography with electron capture. As these analytical methods are highly sensitive, residues of lindane can be identified at a level of nanograms per kilogram or per litre. (W.H.O. 1991).

The isomers of BHC are relatively stable. They are stable to light, high temperature, hot water and acid, although they are dechlorinated in alkali. Lindane is relatively soluble in water (10ppm; Matsumura, 1975). Lindane is approximately 100 times more volatile than DDT and therefore has a fumigant action.

The toxicity of BHC is proportional to the content of its toxic element. The gamma isomer by contact, stomach or fumigant action is about 50-10,000 times more effective than other isomers (Matsumura, 1975). The gamma isomers of BHC have different actions. The gamma and alpha isomers are stimulants of the central nervous system with principal symptoms being convulsions. The beta and delta isomers are depressant of the central nervous system (Matsumura, 1975). Like DDT, BHC is capable of rapid penetration of the insect cuticle, however the biochemical mechanism of toxicity of BHC has not been clearly defined (Matsumura, 1975).

Lindane has been used as a broad spectrum insecticide since the early 1950s for purposes 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). Lindane is thus distributed all over the world and can be detected in air, water, soil, sediment, aquatic and terrestrial organisms, and food, although the concentrations in these different compartments are generally low and are gradually decreasing. Humans are exposed daily via food, and lindane has been found in blood, adipose

tissue and breast milk. The levels of intake, however, are also decreasing (W.H.O., 1991).

1.4.1 Pharmacokinetics of Lindane

1.4.1.1 Absorption

The uptake of lindane by rats or mice has been studied after oral administration. Direct information on the velocity of uptake from the gastrointestinal tract is available, which can be supplemented by information from studies in which excretion of orally administered radioactive lindane was followed (W.H.O., 1991).

In rats, lindane is absorbed rapidly from the gastrointestinal tract and distributed to all organs and tissues within a few hours. The highest concentrations are found in adipose tissues and skin; in various studies the fat: blood ratio was about 150-200, the liver: blood ratio, 5.3-9.6 and the brain: blood ratio, 4-6.5. The same fat: blood ratio was found in rats exposed by inhalation. These ratios vary with sex, being higher in females. Uptake of lindane through the skin after dermal application is slow and occurs to a very limited extent; this may explain the low toxicity of lindane after dermal exposure (W.H.O., 1991).

Lindane taken up from the intestines is transferred almost exhaustively to the blood. No significant amount was found in the lymph of rats after injection of 0.05 or 0.1 μmol into the loops of the small intestines in vivo. Absorption was rapid: 29-53% of the injected material was absorbed from the intestinal loops within the first 30 minutes (Turner and Shanks, 1980). Uptake of lindane from the intestines of rats given 12.5mg in oil over five days was less effective in animals depleted of their intestinal microorganisms by maintenance under aseptic conditions than in conventional rats.

The asepticed rats also excreted more unchanged lindane in the faeces than conventional animals (Macholz *et al.*, 1983).

1.4.1.2 Distribution

After uptake, lindane is distributed to all organs and tissues in the body of laboratory animals, at measurable concentrations within a few hours.

When lindane was administered orally to rats at doses of 1, 10, or 100 mg/kg diet for up to 56 days, the highest concentrations were found in adipose tissue. The fat: blood ratio in this study was very close to 150 at all times, whereas the liver: blood ratio was only 3.4-3.5. Lindane concentrations in organs reached a maximum after 2-3 weeks and slowly decreased thereafter. The authors did not differentiate between males and females (Oshiba, 1972).

One day after intraperitoneal injection to rats of a mixture of ^{14}C - and ^{36}Cl - lindane in rape seed oil, the highest contents were those of skin and fat - 15.7% and 10.7%, respectively. Less than 1% was found in all other organs, including the central nervous system (Koransky *et al.*, 1963). When lindane or deuterated lindane was administered intraperitoneally to rats at a dose of 10mg/kg body weight, about 40mg lindane/kg fat were found after one day in both males and females. At that time, the blood concentration in males was 0.2mg/litre, 1-2mg/kg were present in brain and 0.7mg/kg in skeletal muscle. Deuterated lindane was found at 110mg/kg in depot fat of males, and the levels in brain and muscle were about twice those of undeuterated lindane (Stein *et al.*, 1980).

The distribution of lindane in brain after oral administration at 30mg/kg or intravenous administration at 0.3mg/kg was studied using autoradiography-imaging

analysis and dissection-liquid scintillation counting techniques. The two routes of administration gave similar results. A heterogeneous distribution of label in brain regions was observed: the radio label concentration in the white matter was higher than that in thalamus, mid brain, pons and medulla at different times relative to the mean value for whole brain. The affinity of lindane for white matter and myelinated structures was related to its lipophilic behaviour (Sanfeliu *et al.*, 1988).

1.4.1.3 Metabolism

Lindane is converted by enzymatic reactions, mainly in the liver. One group of enzymes involved in the biotransformation of lindane is microsomal, i.e. cytochrome P-450-dependent monooxygenases. Five groups of male Wistar rats were injected intraperitoneally with gamma-HCH at 25mg/kg body weight on four consecutive days to investigate the induction of cytochrome P-450 in liver microsomes. Gamma-HCH was found to be a "mixed type" inducer which mediates the induction of cytochrome P-450 b/e, c and d forms (Kumar and Dwivedi, 1988). These enzymes are involved in hydroxylation, dehydrogenation, and dechlorination. Other hepatic cytosolic enzymes are involved in the dehydrochlorination reaction.

Stein *et al.*, (1977) found that 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 P-450. The second pathway includes dehydrogenation of lindane to 1,2,3,4,5,6-Hexachlorocyclohexane (HCH), subsequent hydroxylation and dehydrochlorination to 2,3,4,6-tetrachlorophenol.

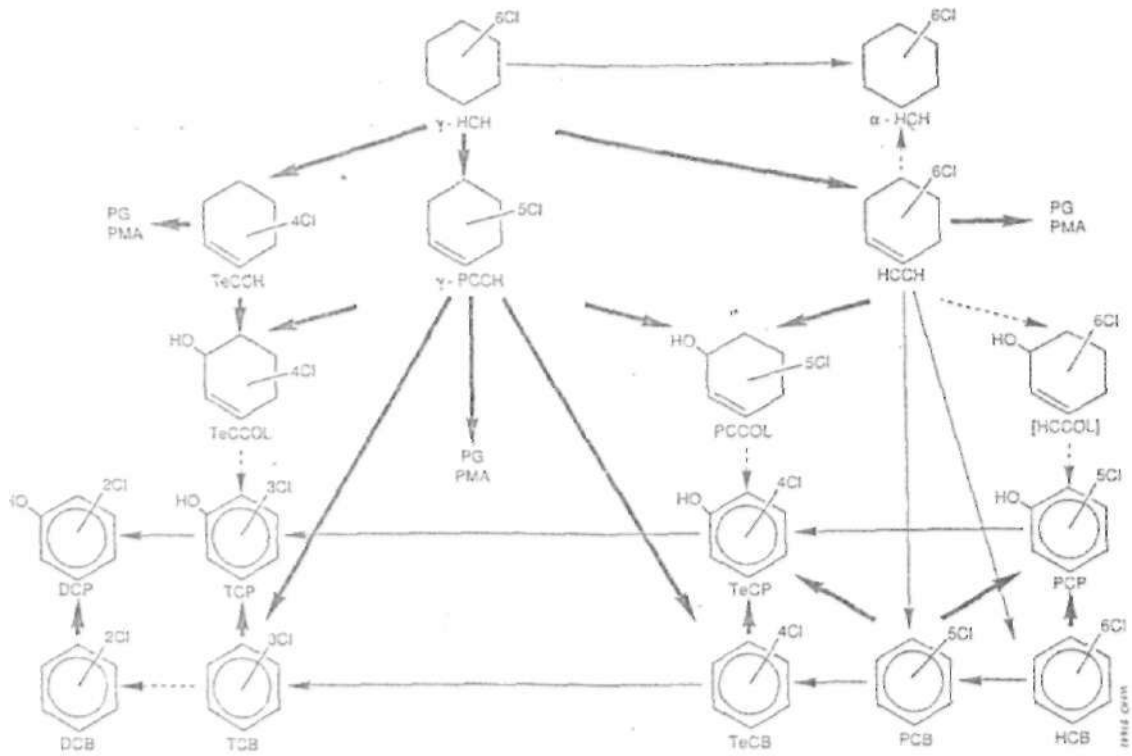


Figure 3: Pathways of Lindane Metabolism (WHO 1991)

TCP and tetrachlorophenol are formed *in vitro* in a ratio of about 2:1.

Glutathione enhanced the conversion of lindane to dichlorophenol (DCP) by a factor of 3-4, but conjugates were formed only in the presence of liver cytosol protein as a source of glutathione transferase. The initial step appears to be dehydrochlorination to 1,3,4,5,6 - Pentachlorocyclohexane (PCCH), followed by conjugation and further dehydrochlorination to DCP-mainly 2,4-DCP. The DCPs found in the urine of rats are a mixture of different isomers (Tanaka *et al.*, 1979).

Two independent investigative groups have reported the formation of trace amounts of chlorinated benzenes from lindane in rats. Hexachloro benzene was found in faeces (Gopaldaswamy and Aiyar, 1984) and pentachlorobenzene in brain (Vohland *et al.*, 1981). The two studies are consistent in so far as the identified amounts of chlorobenzenes are extremely low and near the detection limit; however, they are also contradictory, because hexachlorobenzene was found exclusively in the first study and pentachlorobenzene in the second. It is impossible to clarify whether artefacts were measured in these studies, e.g. enrichment of impurities in the starting material. If indeed chlorinated benzenes are formed from lindane, the amounts obtained are insignificant compared to other metabolites.

1.4.1.4 Excretion

In mammals, including human beings, lindane is excreted very rapidly in urine and faeces after metabolic degradation; only small quantities are eliminated unchanged (Seidler *et al.*, 1975). As lindane is subjected to four types of reaction-dehydrochlorination, dechlorination, dehydrogenation, and oxidation - many intermediate metabolites are found, the nature of which depends on the initial reactions.

Nevertheless, the excreted metabolites are all various isomers of dichlorophenol, trichlorophenol and tetrachlorophenol, which are excreted either free or in conjugated form with glucuronic or sulfuric acid or N-acetylcysteine (Rhone-Poulenc Agrochimie, 1986).

Intraperitoneal administration to rats of lindane in arachis oil at daily doses of 40mg/kg body weight (total, 4g) was followed by urinary excretion of 2,3,5- and 2,4,5-Trichlorophenol, either in free form or as sulfuric and glucuronic acid conjugates (Grover and Sims, 1965).

One day after intraperitoneal injection of a mixture of ¹⁴C- and ³⁶Cl- labelled lindane to rats, 18.97% of the radiolabel was found in the excreta; 7.39% was still not absorbed, indicating again that elimination of lindane begins during the absorption phase. After 4 days, 52% of the total activity was found in the excreta. The resulting half-time was about 4 days (Koransky *et al.*, 1963). An even shorter half-time, of 1-2 days, was seen in depot fat in another study after intraperitoneal injection of 10mg/kg to rats (Stein *et al.*, 1980).

After intraperitoneal administration of 40mg/kg body weight to rats, 20% of the total dose left the body via the faeces and 80% via the urine (Koransky *et al.*, 1963, 1964). In another study, however, the amounts of radiolabel excreted by rats in urine and faeces were about equal (Seidler *et al.*, 1975). Only traces of unchanged lindane were found in faeces and urine. Of the chlorine derived from lindane that is excreted in the urine, about 60% is inorganic and 40% is organic (Koransky *et al.*, 1964).

1.4.2 Neurotoxicity of Lindane

The insecticidal action of lindane is somewhat similar to that of DDT in that the

insect starts to tremble, with telescopic movements of the abdomen. With passage of time, hyperexcitation increases and movements become increasingly uncoordinated. At higher dosage, the insect falls on its back and eventually suffers paralysis. As with other organochlorine insecticides, poisoning causes a pronounced increase in respiratory rate. The overall effect on the nervous system also resembles that of DDT but the primary site of action in both insects and mammals is the central nervous system (CNS). This can be shown by the fact that, if insect nerves are isolated from the CNS so that only sensory impulses can arise spontaneously, it takes sometime before multiple discharges appear unless the concentration of lindane is high (Ecobichon and Joy, 1982).

The precise effect of lindane on the CNS remains uncertain. Sloley *et al.*, (1985) found that lindane led to an increase in the levels of dopamine and of N-acetyldopamine in the cerebral ganglion of the cockroach. On the other hand, Abalis *et al.*, (1985) concluded that, in mammalian brain, receptors for γ -aminobutyric acid (GABA) were a primary target for lindane. GABA is an inhibitory neurotransmitter, present in the CNS of vertebrates. It is also present in invertebrates, both in the nervous system and in the skeletal muscles. It was postulated that the GABA receptor is a single protein with three sites of activity. One of these binds to GABA, a second controls an ion channel and the third is the site of attachment of certain toxicants. It was concluded that lindane and cyclodienes inhibit GABA-induced chloride ion uptake with the effect that the inhibitory neurotransmitter action of GABA is blocked, leading to CNS excitation and convulsions (Abalis *et al.*, 1985).

The work done by O'Brien (1967) as mentioned earlier, further explains the possible involvement of sodium and potassium ions in the mechanism of action of

organochlorines.

The membrane destabilising effects of lindane and dieldrin are similar to those of DDT although the precise mechanism by which these effects are brought about is still obscure. However, two important explanations have been proffered, the first of which is that the insecticides may inhibit one or several enzymes of importance to the mechanism of membrane stability or of nerve impulse transmission. There also exists the possibility that they may initiate some physical change in the structure of the nerve membrane which causes it to misfire (e.g. by alteration of its permeability to ions) (Hassal, 1982).

A single electrical stimulus leads to multiple uncontrolled responses of poisoned nerves, thus muscular spasm occur. At high concentrations, trains of repetitive discharges arise spontaneously such that after initial stimulus, electrical impulses are no longer necessary to evoke succeeding trains of impulses. One report (Hassall, 1987) indicates that the organochlorines (DDT, lindane and aldrin) all have membrane destabilising effect, though not entirely the same. However, the details of the distabilising effect by organochlorines pesticides still remain obscured.

In recent years, the involvement of Na^+ channels in the toxicity produced by organochlorines particularly lindane is becoming more apparent. Organochlorines prolong the falling phase of the spike in isolated nerve fibres. Experiments involving the voltage clamp technique have shown that this effect is due to two actions of DDT; it delays the closing of the Na^+ channels, and it partially blocks the K^+ channels. As a result of these actions, the negative after-potential outlasts the refractory period of the axon, and repetitive firing results (Bowman and Rand 1980).

It is hoped that the *in vitro* aspect of this work will reveal comprehensive data

on the involvement of Na^+ channels in the mechanism of action of lindane.

1.4.3 Interaction of Lindane with Other Pesticides

In practice, pesticides are often used in combinations in plant protection and public health programmes to overcome resistance, as well as to increase the efficacy of the compound. The phenomenon of interaction of several insecticides gains importance therefore, particularly as it could influence the toxicological profile of the individual insecticide (Dikshith *et al.*, 1987).

Interaction between pesticides means that exposure to pesticide simultaneously or in combination has the potential of reacting with each other hence influencing the overall pesticide toxicity.

Toxic compounds may mutually compete with each other for absorption, distribution, biotransformation (metabolic detoxification or activation), target organs, specific receptors, carrier plasma proteins, renal or biliary excretion sites (Iyaniwura, 1989).

Organochlorine combinations have been shown to interfere with the storage and metabolic excretion of one another, e.g. storage of dieldrin and DDT were reduced in adipose tissues when they were fed in combinations. This effect has been attributed to accelerated rate of metabolism and excretion possibly by induction of microsomal enzymes (Street *et al.*, 1969).

Kaloyanova and Tasheva (1983) suggested that resistance and cross resistance in insects after they have been sprayed with various pesticides formulations may be due to the ability of pesticides particularly organochlorines to stimulate their own metabolism and biotransformation of other pesticides.

Combination of lindane, thiuram and heptachlor have been found to exhibit metabolic potentiation in rats and cats (Kagan, 1981). Reports on interaction between lindane and dichlorvos at the muscarinic receptor also showed antagonism of dichlorvos potentiation of acetylcholine response (Iyaniwura *et al.*, 1991).

That pre-exposure of the rabbit ileum to lindane significantly ($P < 0.001$) decreased the *in vitro* toxic effect of coumaphos by attenuating the initial coumaphos-induced potentiation of the cholinergic receptor response, is by extension, consistent with reports of *in vivo* studies by earlier workers, who had observed that pre-treatment with organochlorine pesticides reduced the lethal effects of certain organophosphate and anticholinesterase agents in experimental animals (Ball *et al.*, 1954; Main, 1956; Triolo and Coon, 1966; Triolo *et al.*, 1970; Iyaniwura and Gubio, 1990).

1.5 ELECTROENCEPHALOGRAPHIC (EEG) STUDIES AND PESTICIDE TOXICOLOGY

Electrical recordings 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). The undulations in the recorded electrical potentials are called brain waves and the entire record is called an electroencephalogram (EEG).

Electroencephalographic studies therefore involve the detection of electrical potential changes which occur in the brain by use of electrodes. These are placed at various positions on the scalp and connected through amplifier to a recorder. The potentials occur in the form of waves of differing frequency and amplitude. The frequency and amplitude give an indication of the state of the brain. Large amplitude

is indicative of deep sleep or anaesthesia. Small amplitudes on the other hand indicate wakefulness or stimulation (Bowman and Rand, 1980).

EEG analysis was one of the first forms of electrodiagnosis of nervous system dysfunction (Berger, 1929). In clinical neurobiology, the EEG has been used in the diagnosis of different types of epilepsy, also to localise brain tumours or other space occupying lesions of the brain and to diagnose certain types of psychopathic disturbances (Guyton, 1976).

The normal EEG whether recorded from the scalp or with indwelling electrodes in specific regions, has an amplitude of about 10mV. The useful frequency spectrum of EEG is below 50 Hz though higher frequencies are encountered in certain brain regions (Johnson, 1980). Analysis is commonly by the amount of electrical activity contained within specific frequency bands. EEG recordings using implanted electrodes in specific brain areas can be used to assess the effect of toxicants on these different brain areas and structures.

1.6 SCOPE AND OBJECTIVES

The mechanism of action of organochlorine is not well understood. Though it is widely accepted that the effect of organochlorine toxicity is on the nerves, there are different views as to how these effects are mediated. Lindane is very popular locally. As *Gammalin 20^(R)* it is used in the control of black pod disease in cocoa and as *Fernansan D* (20% lindane) in the control of insect pest of bean seedlings. It thus presents an occupational hazard, and is of environmental and public health concern. It has been shown to be very toxic to non-target species in particular mammals (Iyaniwura, 1991b).

The goal of this project is to study *in vivo* and *in vitro* the mode of toxicity of this insecticide at tissue/cellular levels with the hope that the information thus obtained would provide an understanding of the non-target toxicity of this compound. It is also hoped that based on the data obtained from this study, a rational basis could be formed for the treatment of poisoning from the organochlorine insecticides, a group to which lindane belongs.

Chronic toxicity studies will be conducted over a period of 12 weeks. Determination of symptoms of toxicity and EEG recording will be carried out in rats that have been fed with feeds contaminated with pesticides at various doses. Animals will be sacrificed at the end of the 4th, 8th and 12th week respectively and histopathological studies will be conducted on the poisoned rats. The same procedure will be performed on rats on normal feeds i.e. controls.

In vitro studies are included to identify the effects of lindane on transmission of impulse in the neuron. The role of ions known to be involved in generation and propagation of action potential and nerve impulse will be studied on isolated tissue preparations. Also effects of chemical substances interacting with these ions will be studied.

CHAPTER TWO

MATERIALS AND METHODS

2.1 *IN VIVO* STUDIES

The experiments reported in this section were carried out in an attempt to determine the following:

1. To calculate the median lethal dose (LD_{50}) of lindane and to determine the symptoms of toxicity associated with acute poisoning in rats.
2. To find out if the chronic poisoning by lindane causes the degeneration of any part of the brain, and if the histopathological studies of the brain can be used to explain the mode of toxicity of lindane.
3. To conduct Electroencephalographic (EEG) study on poisoned rats and to determine how the EEG relates to the central nervous system effects of lindane.

2.1.1 Acute Toxicity Study in Rats

Determination of the median lethal dose (LD_{50}) of lindane using the intraperitoneal (IP) routes

The median lethal dose (LD_{50}) of lindane in rats by the intraperitoneal route was determined using standard procedures (Arithmetic method of Karber adapted by Aliu and Nwude, 1982). Male albino rats weighing approximately (1.8 - 2.2 kg) were used.

Pilot studies were carried out to determine the minimum dose that would cause 100 per cent death and the maximum dose that would not cause any death in the rats. These two dose levels were further used as focal points for the determination of dose range used for the subsequent experiment.

The final acute toxicity test was carried out using 30 healthy male rats of 0.20kg average body weight. The animals were then divided into 5 groups of 6 rats per

group. The fifth group served as control (injected with 0.9% saline only). The other 4 groups received 10, 20, 30 and 45mg/kg body weight of lindane respectively.

Symptoms of toxicity were observed and scored for each rat in various dose groups. The number of animals that died within 24 hours were noted for each group. The LD₅₀ value was then calculated using Arithmetic method of Karber (Aliu and Nwude, 1982).

$$LD_{50} = \text{lowest dose that gave 100\% death} - \frac{\text{sum of (dose diff X mean dead)}}{\text{No of rats in each group}}$$

2.1.2 Chronic Toxicity Study in Rats

The chronic toxicity study was designed to last for a period of 12 weeks (3 months). The administration of the drug (pesticide) was by oral routes (incorporated in the diet). Animals were weighed fortnightly to observe any change on weight.

The rats were obtained in 4 group consisting of 21 rats in each group (total of 84 rats were used for the study).

The groups were classified in the following order:

- (a) Control group (0 mg/kg feed)
- (b) 2nd group (5 mg/kg lindane)
- (c) 3rd group (10 mg/kg lindane)
- (d) 4th group (15 mg/kg lindane)

Preparation of drug-treated feeds

Stock solution of lindane (*Gammalin 20*) contains 200g per litre (0.2g/ml). Therefore 1ml of stock solution will contain 200mg of lindane. 1ml of the stock solution was diluted to 200ml and this gave 1mg per ml (1mg/ml).

For 5mg/kg group, every 1kg of feed was contaminated with 5ml of diluted solution, for 10mg/kg group, every 1kg of feed was contaminated with 10ml of diluted solution and for 15mg/kg group, every 1kg of feed was contaminated with 15ml of diluted solution.

Determination of symptoms of toxicity and EEG recordings were carried out in the rats that have been fed with contaminated feeds at various doses. Five rats from each group were sacrificed at the end of the 4th, 8th and 12th weeks respectively and histopathology was conducted on the brain of the poisoned rats.

2.1.2.1 Histopathological Study

Tissue processing and sectioning

Fresh tissue samples of the brain from each of the groups of rats (see next page) were obtained in the first month and subsequently in the second and the third months.

- (a) control group (Rats fed on normal feed)
- (b) 5 mg/kg group (Rats fed on 5mg/kg lindane treated feed)
- (c) 10mg/kg group (Rats fed on 10mg/kg lindane treated feed)
- (d) 15mg/kg group (Rats fed on 15mg/kg lindane treated feed)

The tissue samples were initially fixed in 10 percent formalin. Tissue slices of 1 cm thickness were cut from each organ for processing. The tissue were transferred to the automatic tissue processor where they were further fixed in 10 percent buffered formalin saline for 2 hours, and then dehydrated for 2 hours in each of the ascending grades of alcohol (85%, 90% and 100% V/V). The dehydrated tissues were then cleared in Toluene for 2 hours and the tissues impregnated in molten paraffin for 2 hours, after which the tissue slices were 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 prior to staining.

Staining of sectioned tissue

The sections were dewaxed in xylene and rehydrated in descending grades of alcohol: 100%, 90% and 70%. 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 solution 70%, 90% and 100% and finally cleared in xylene and mounted in pox. The slides were then examined microscopically for lesions.

2.1.2.2 Electroencephalographic (EEG) Study

Implantation of electrocortical and electromyographic (EMG) electrodes was carried out on the rats and the electrodes were subsequently connected to the EEG machine.

The rats were anaesthetized using pentobarbitone sodium (45mg/kg I.P.). The rats were held firmly with ear plugs in a stereotaxic apparatus. They were then

surgically implanted with stainless steel screws (Grass instruments). The sites of implantation include the frontal cortex (FC), optic cortex (OC), and mid brain reticular formation (RF). The implanted electrodes were connected to the male connector as follows:

Monopolar electrodes from upper surface of mouth: No 1

Monopolar electrodes from frontal cortex of mouth: No 2

Monopolar electrodes from optical cortex of mouth: No 3

Bipolar electrodes from brain stem RF: No 4

Two EMG electrodes from the neck: No 5 & 6.

The surface location of these brain areas was first marked before drilling the skull and implanting the electrodes. The implanted electrodes were held firmly in place using dental cement. The neck muscle was similarly implanted for EMG recording. The implanted rats were allowed 24 hours with an angle poised lamp for full recovery from anaesthesia before recordings. And in each case the rat was allowed 1 hour to settle down (accustomed to the new environment) before the commencement of EEG recording.

The recordings were carried out on each of the 4 groups (control, 5mg/kg, 10mg/kg and 15mg/kg) at the end of the 4th, 8th and 12th weeks respectively.

2.2 IN VITRO STUDIES

The experiments reported in this section were carried out in an attempt to determine the following:

1. To identify the effects of lindane on transmission of impulse along the neuron.

2. To determine the involvement of sodium ions (Na^+) in the mechanism of action of lindane using a voltage sensitive sodium channel blocker (lignocaine).

Physiological solutions

(a) Tyrode's solution: (Using Rabbit Ileum Preparation)

A 10 litre Tyrode's solution was prepared by adding the reagents in the following sequence: sodium chloride (NaCl) 90g, 10% potassium chloride (KCl) 20ml, sodium biphosphate ($\text{NaHPO}_4 \cdot 2\text{H}_2\text{O}$) (10%) 5ml, D-glucose 10g, sodium bicarbonate (NaHCO_3) 10g, calcium chloride (CaCl_2 (10%) 20 ml and magnesium chloride $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) 1ml. The volume was then made up to 10 litre mark with distilled water.

(b) Kreb's solution: (Using Rat Phrenic nerve-diaphragm Preparation)

A 10 litre Kreb's solution was prepared by adding the reagents in the following sequence: sodium chloride (NaCl) 69g, 10% potassium chloride (KCl) 35ml, 10% magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) 29ml, potassium biphosphate (10%) 16ml, D-glucose 20g, sodium bicarbonate (NaHCO_3) 21g, calcium chloride (CaCl_2) (molar) 25.2ml. The volume was made up to 10 litre mark with distilled water.

2.2.1 Investigation on Rabbit Ileum Preparation:

Rabbits of 1.2kg average body weight were used for this experiment. Each rabbit was sacrificed by a blow on the head, the throat was cut open followed by opening up of the abdomen. The ileum was removed into an oxygenated petri dish containing Tyrode's solution. The set-up was made in accordance with Kitchen, (1984) using Microdynamometer 7050 recorder (Ugo Basile, Milan, Italy) with a chart speed of 24 mm/min, organ bath volume of 25 ml, organ bath temperature of 37°C and

aerated with air. The drug contact time was between 1-2 minutes.

2.2.1.1 Determination of 50 Percent Inhibitory Concentration (IC₅₀) of Lindane

Three (3) cm strips of rabbit ileum were obtained and set up according to Kitchen (1984). Thirty minutes equilibration time was allowed prior to commencement of the experiment. Serial dilutions of the lindane stock solution was carried out in order to obtain dose responses of the least concentration up to the highest concentration of lindane.

Dose response of lindane from 0.4-26 $\mu\text{g/ml}$ range were obtained. After the addition of every dose and subsequent observation of response, the tissue was washed 3-4 times and allowed to rest for 3 minutes. The concentration at which the decrease in response is 50% (50% inhibitory concentration or IC₅₀) was determined. In each case, n=4 and results reported as means \pm S.E.M.

2.2.1.2 Determination of 50 Percent Inhibitory Concentration (IC₅₀) of Lignocaine

Serial dilutions of the lignocaine stock solution was carried out and dose response of lignocaine from 1-330 $\mu\text{g/ml}$ range were obtained. The IC₅₀ was determined. All other steps were the same as in the determination of IC₅₀ for lindane.

2.2.1.3 Effect of Lindane on Response due to Lignocaine on Rabbit Ileum

The physiological solution (Tyrode's solution) was prepared to contain the IC₅₀ of lignocaine (33 $\mu\text{g/ml}$). The response thus observed was that commensurate to the IC₅₀ of lignocaine. Equilibration was allowed for 30 minutes to obtain control response with the lignocaine-containing physiological solution. The IC₅₀ of lindane

was then used to select suitable concentrations of lindane involving concentrations that are lower and higher than the IC_{50} respectively. The effect of increase in concentration of lindane on response due to pretreatment with lignocaine was observed. In each case $n=4$ and results reported as means \pm S.E.M.

2.2.2 Investigation on the Phrenic nerve-diaphragm Preparation

Male albino rats of 0.2kg average body weight were used for this experiment. Each rat was killed by a blow on the head. The throat was cut and the skin was removed from the middle of the chest. The muscles were freed from the chest wall and the ribs were cut alongside the base of the sternum on both sides of the animal.

The upper part of the thorax was removed completely and the phrenic nerve was displayed from the diaphragm right up to the thymus gland. Furthermore, an incision was made in the abdominal wall just below the diaphragm and two cuts were made through the diaphragm and the ribs were left attached to it. These produced a fan-shaped segment of muscle. The preparation was then transferred into a petri dish containing Kreb's solution.

The rat phrenic nerve-hemidiaphragm preparation was as set by (Bulbring, 1946), and adapted by Department of Pharmacology and Clinical Pharmacy, A.B.U., Zaria, (1990). Microdynamometer 7050 recorder (Ugo Basile, Milan, Italy), stimulator cat. 3000 (Ugo Basile, Milan, Italy), were used. The organ bath volume was 150ml and the tissue was aerated with 95% oxygen and 5% carbon dioxide mixture. The drug contact time was 2-3 minutes.

2.2.2.1 Determination of 50 percent Inhibitory Concentration (IC₅₀) of Lindane

The preparation was set up according to Bulbring (1946), as adapted by Department of Pharmacology and Clinical Pharmacy, A.B.U., Zaria (1990) and described under investigation on the phrenic nerve-diaphragm preparation above.

Initially, the normal twitch responses were obtained following a direct stimulation of the phrenic nerve to give the control readings. The effect of various concentrations of lindane (0.1 - 15 $\mu\text{g/ml}$) was observed and compared with the control. The drug contact time was between 1-2 minutes and after the addition of each concentration the tissue was washed 5-6 times and allowed to rest for about 10 minutes. The IC₅₀ was determined and in each case n=4 and results reported as means \pm S.E.M.

2.2.2.2 Determination of 50 Percent Inhibitory Concentration (IC₅₀) of Lignocaine

The normal twitch responses were obtained following a direct stimulation of the phrenic nerve. The effect of various concentration of lignocaine (2-30 $\mu\text{g/ml}$) was observed and compared with the control. The IC₅₀ was then determined. All other steps were the same as in the determination of IC₅₀ for lindane.

2.2.2.3 Effect of Lindane on Response due to Lignocaine

The physiological solution (Kreb's solution) was prepared to contain the 50% inhibitory concentration of lignocaine (8.5 $\mu\text{g/ml}$). Therefore the control twitch responses obtained due to direct stimulation of the phrenic nerve were commensurate to those of the IC₅₀ of lignocaine. Suitable concentrations of lindane were selected based on the IC₅₀ of lindane as in the case of rabbit ileum experiment. The effect of

increase in concentration of lindane on response due to pretreatment with lignocaine was observed. In each case n=4 and result reported means \pm S.E.M.

2.2.3 Calculations

Responses in the experiments were obtained by adding various volumes of the drug in to the organ bath. To calculate the final concentration therefore, both the volume of the organ bath (25ml for rabbit ileum preparation and 150ml for phrenic nerve-hemidiaphragm preparation) and the concentration of the stock solution of the drug must be put into consideration. The procedure used in calculating the final concentration is demonstrated below:

2.2.3.1 Lindane

50% Inhibitory Concentration (IC₅₀) of Lindane on Rabbit Ileum

The volume of stock solution of lindane that corresponded to the 50% decrease in response was 0.09ml

the stock concentration = 1mg/ml
the organ bath volume = 25ml

Therefore $C_1 = 1\text{mg/ml}$
 $V_1 = 0.09\text{ ml}$
 $C_2 = ?$
 $V_2 = 25\text{ml}$

$$\text{if } C_1V_1 = C_2V_2, C_2 = \frac{C_1V_1}{V_2}$$

therefore the final
(organ bath) concentration
of lindane

$$= \frac{1 \times 0.09}{25} = 0.0036\text{mg/ml} = 3.6 \mu\text{g/ml.}$$

All other concentrations of lindane were calculated in the same procedure.

2.2.3.2 Lignocaine

50% Inhibitory Concentration (IC₅₀) of Lignocain on Phrenic Nerve-diaphragm

The stock solution of lignocain that corresponded to the 50% decrease in response was 0.4 ml

the stock concentration = 0.32%

the organ bath volume = 150 ml

$$\begin{aligned} \text{Therefore } C_1 &= 0.32\% \\ V_1 &= 0.4 \text{ ml} \\ C_2 &= ? \\ V_2 &= 150 \text{ ml} \end{aligned}$$

$$\text{if } C_1V_1 = C_2V_2, C_2 = \frac{C_1V_1}{V_2}$$

Therefore the final (organ bath) concentration of lignocaine = 0.4 X 0.32

$$\begin{aligned} &\frac{150}{150} \\ &= 8.5 \times 10^{-4}\% \\ &= 8.5 \times 10^{-4} \text{ g/100ml} \\ &= 8.5 \mu\text{g/ml} \end{aligned}$$

All other concentrations of lignocaine were calculated using the same procedure.

CHAPTER THREE

RESULTS

3.1 *IN VIVO* STUDIES

3.1.1 Acute Toxicity Study in Rats

Determination of Median Lethal Dose (LD₅₀) Value and Symptoms of Toxicity of Lindane Using I.P. Route

Symptoms of lindane toxicity were observed, scored and recorded in ascending order of severity. They were found to be dose-dependant and at doses of 20mg/kg and above, convulsion and death were recorded.

Scores of Symptoms Observed in Poisoned Rats (I.P.)

<i>Symptoms</i>	<i>Scores</i>
Tremor	+1
Prostration	+2
Palor	+3
Convulsion	+4
Death	+5

The symptoms were quantified based on the scores and reported as means \pm S.E.M.

Mean Scores \pm S.E.M.

<i>Dose (mg/kg)</i>	<i>Mean Score \pm S.E.M.</i>
10	39.2 \pm 0.6
20	80.5 \pm 0.7
30	100.2 \pm 0.8
45	150.5 \pm 0.5

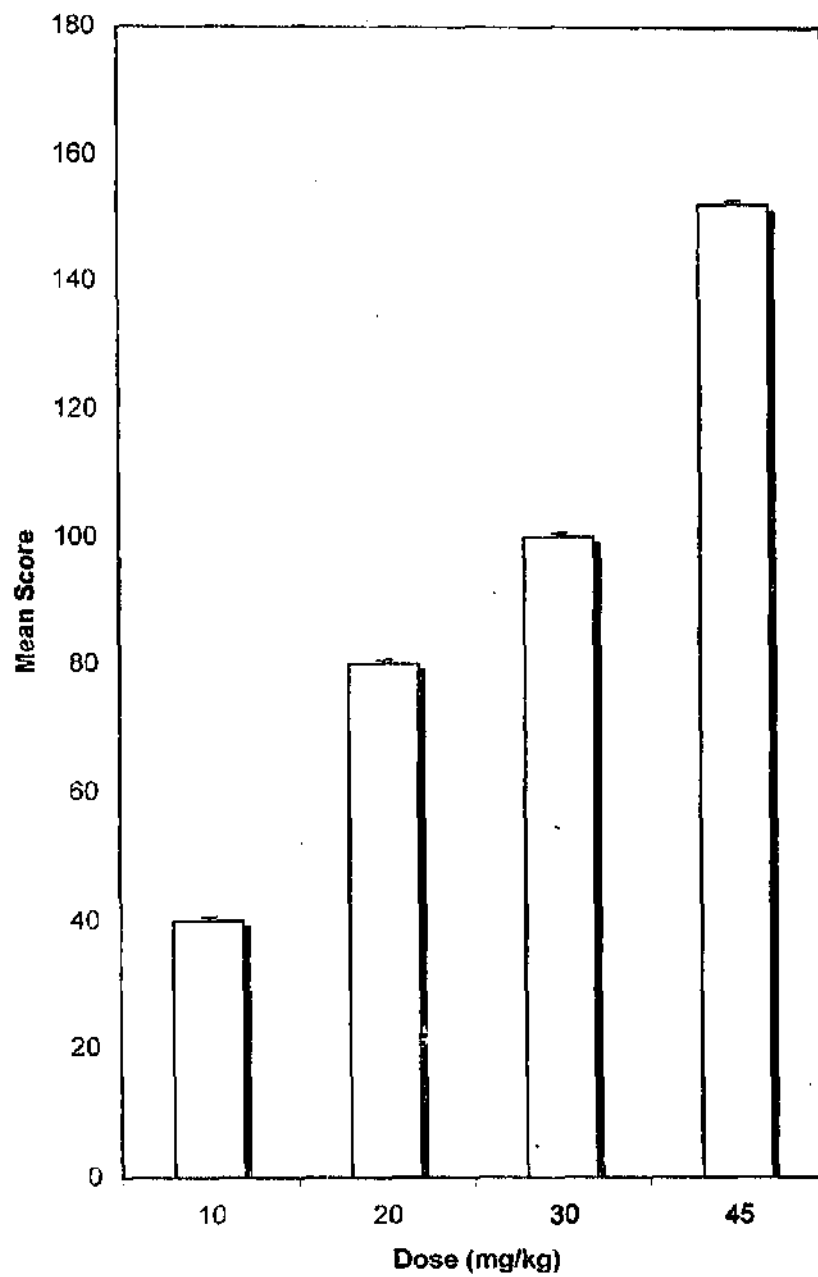


Figure 4: Plot of dose against mean score of symptoms

TABLE 1:

Calculation of LD₅₀ of lindane in Rats using I.P. route

<i>GROUP</i>	<i>DOSE (mg/kg)</i>	<i>DOSE DIFFERENCE</i>	<i>NUMBER OF DEAD</i>	<i>MEAN DEAD</i>	<i>DOSE DIFF x MEAN DEAD</i>
1	10	0	0		
2	20	10	1	0.5	5
3	30	10	5	3	30
4	45	15	6	5.5	82.5
5	CONTROL 0.9% SALINE		0	0	0

Using arithmetic method of Karber:

$$LD_{50} = \text{lowest dose that gave 100\% death} - \frac{\text{sum of (dose diff. X mean dead)}}{\text{no. of rats in each group}}$$

$$LD_{50} = 45 - \frac{117.5}{6} = 45 - 19.6 = 25.4 \text{ mg/kg}$$

The LD₅₀ of lindane in rats using the intraperitoneal route was calculated to be 25.4 mg/kg.

3.1.2 Chronic Toxicity Study in Rats

3.1.2.1 Effect of Contaminated Feeds on the Weights of Rats

The contaminated feeds slightly affected the weights of the rats and this is shown in Table 2 and figure 5.

3.1.2.2 Histopathological study

Vacuolations, congested blood vessels and neuronal degeneration in the brain were observed in group A rats fed with lindane 15mg/kg of feeds. No significant histopathological lesions were seen in the brain of the control rats and in group A rats fed 5mg/kg and 10mg/kg of feeds.

In group B, congested blood vessels in the brain and meninges were observed in all the rats fed 5mg/kg, 10mg/kg and 15mg/kg lindane/feeds. No histopathological lesions were observed in the control rats.

In group C, congested blood vessels in the brain and meninges of the rats treated with lindane at 5mg/kg and 10mg/kg of feeds were observed. Non-suppurative necrosis of oligodendroglia cells, vacuolations, congested blood vessels in the brain and meninges with high degree of neuronal degeneration in the brain of the rats in this group fed with lindane 15mg/kg of feeds were also seen. No significant histopathological lesions were observed in the control rats (see Figure 6).

Table 2

Weights of rats fed on feeds contaminated with lindane taken after every two weeks

	<i>NUMBER OF WEEKS</i>					
	0	2	4	6	8	10
Control	205.3± 5.6	208.7±3.2	219.1±3.3	217.9±2.8	234.1±4.5	249.0±5.0
5mg/kg	200.9± 3.7	205.3±1.9	209.6±2.9	214.6±3.5	221.0±4.8	231.3±5.8
10mg/kg	202.6±4.6	205.0±4.8	204.6±3.1	210.1±2.2	209.6±3.9	209.7±3.3
15mg/kg	205.0± 3.1	204.1±3.7	204.6±3.2	206.4±6.2	202.9±3.7	205.3±4.0

n=7, weight recorded in grams ± SEM.

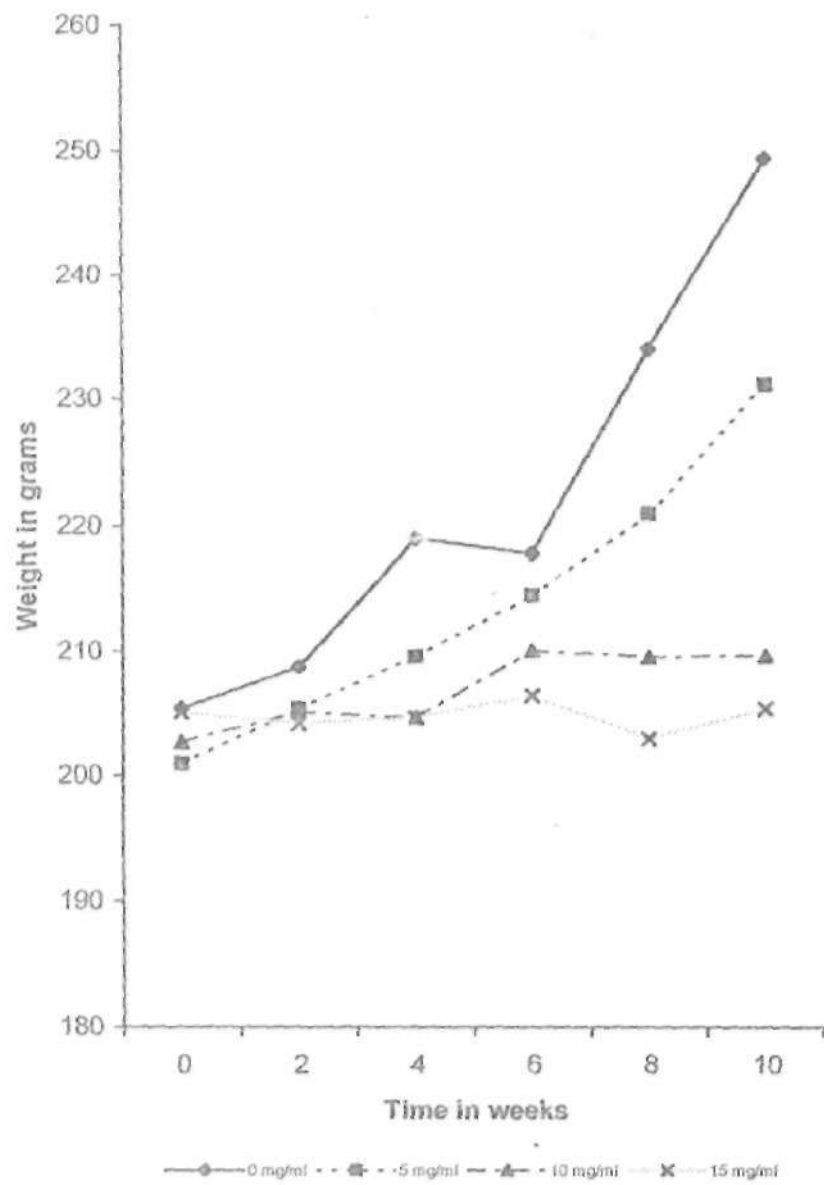


Figure 5: Weight of rats fed on contaminated feeds against time

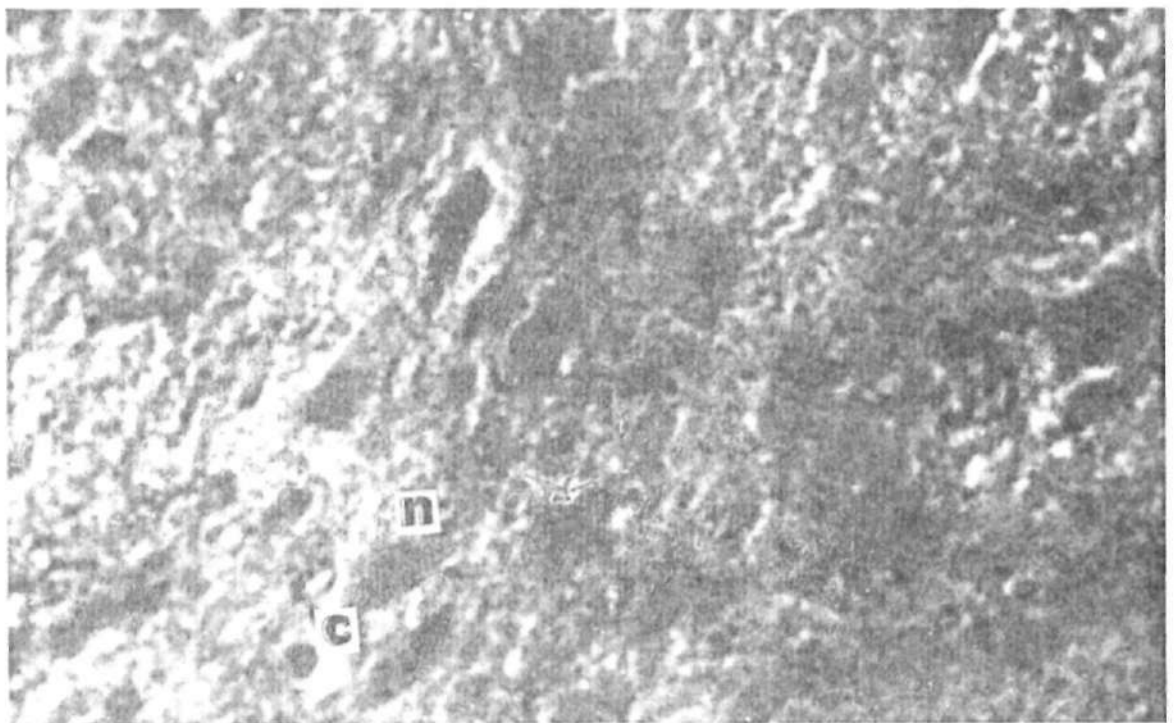


Figure 6a: Note neuronal degeneration (n), vacuolations and congested blood vessel (c). H & E X 400.

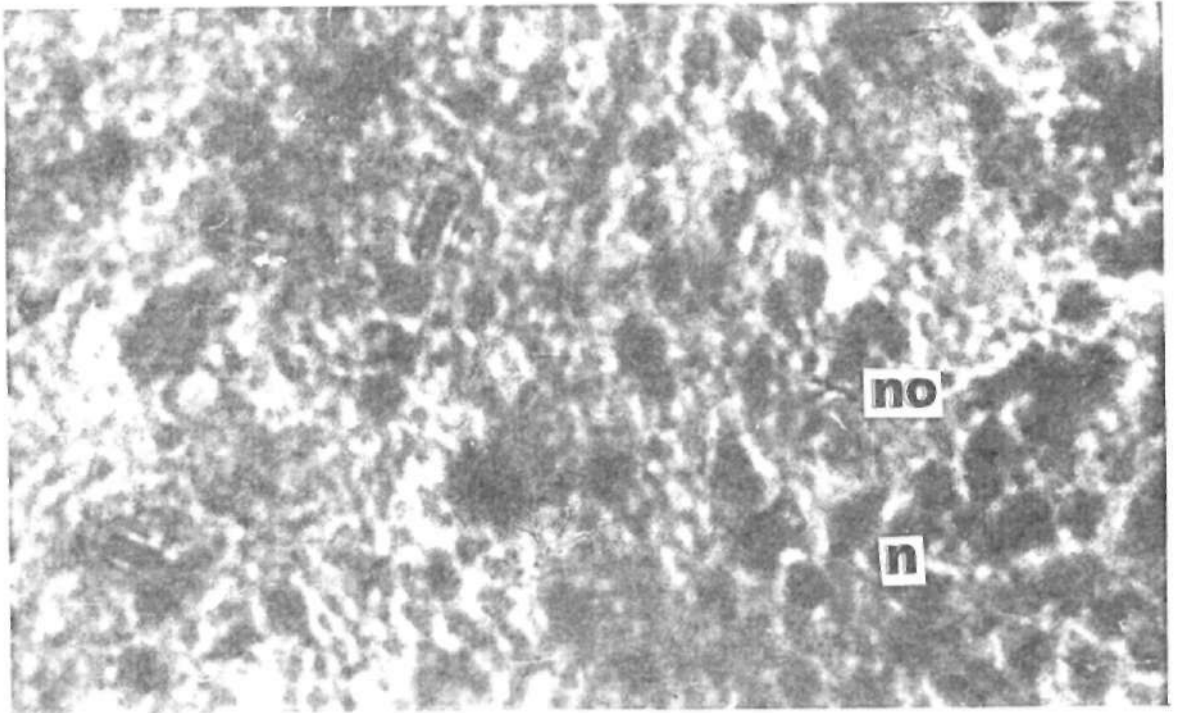


Figure 6b: Non-suppurative necrosis of oligodendroglial cells (no), neuronal degeneration (n) and vacuolations. H & E X 400.

3.1.2.3 Electroencephalographic (EEG) study

The EEG results were extracted by comparing the recordings of the control to that of the test animals. Changes in both the amplitude and frequency of the spikes obtained were put into consideration. Comparison was done on the basis of both the dose difference and period of exposure of the animals to contaminated feeds before the EEG recordings.

In the control, the most prominent components of the EEG gave a regular pattern of waves of about 3 peaks/min and the amplitude was also remarkably similar. This pattern was regarded as the Alpha rhythm - indicating synchronization (i.e the normal characteristics of the EEG).

Desynchronization (which represent a breaking up of the synchronized activity of neural elements responsible for the wave pattern) was recorded for the animals were exposed to 5, 10 and 15mg/kg of feeds. This was characterised by increased frequency and decreased amplitude of the EEG waves. However, the desynchronization was found to increase with dose and time of exposure (see Figures 7a and 7b).

3.2 *IN VITRO* STUDIES

3.2.1 Investigation on Rabbit Ileum Preparation

3.2.1.1 Dose response relationship of lindane on rabbit ileum

Dose response of rabbit ileum to lindane were obtained between the range of 0.4 and 26 $\mu\text{g/ml}$.

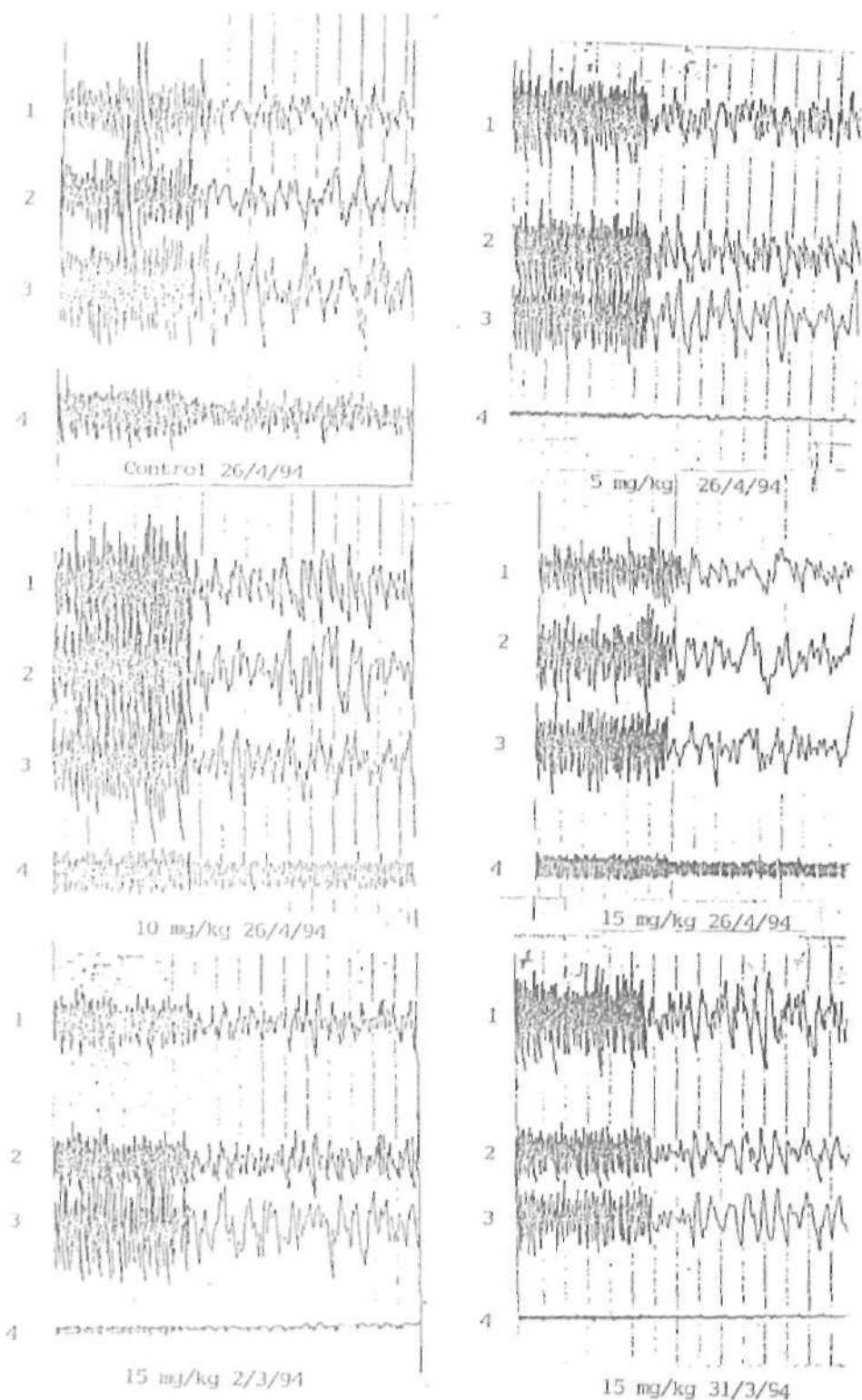


Figure 7a: Some electroencephalographic (EEG) recordings of rats fed on various doses of lindane with dates.

Numbers (1-4) represent recordings as follows:

- 1. Frontal Cortex (FC)
- 2. Optic Cortex (OC)
- 3. Midbrain reticular formation (MBRF)
- 4. Electromyograph (EMG).

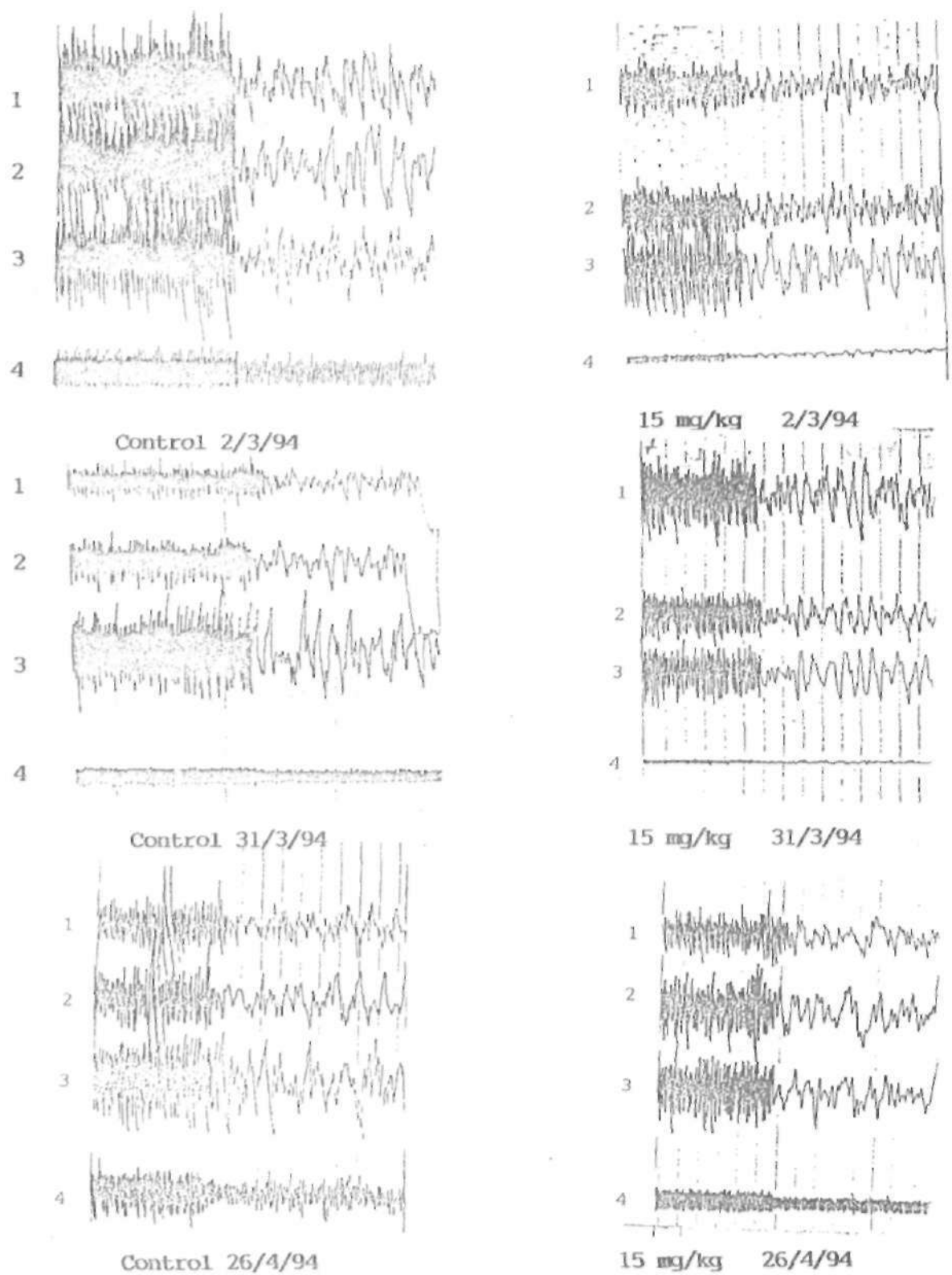


Figure 7b: Some electroencephalographic (EEG) recordings of rats fed on various doses of lindane with dates.

Numbers (1-4) represent recordings as follows:

- | | |
|--|---------------------------|
| 1. Frontal Cortex (FC) | 2. Optic Cortex (OC) |
| 3. Midbrain reticular formation (MBRF) | 4. Electromyograph (EMG). |

The doses of 0.4 and 0.8 $\mu\text{g/ml}$ produced increased response of the spontaneously contracting rabbit ileum. But from 1.6 $\mu\text{g/ml}$ and above there was decrease in response up to about 26 $\mu\text{g/ml}$ when the decrease was 100%. Therefore at lower doses lindane gave increased response and at higher doses it gave decreased response (Table 3; Fig. 8).

3.2.1.2 Determination of 50 percent inhibitory concentration (IC_{50}) of lindane

Lindane (1.6 $\mu\text{g/ml}$ - 26 $\mu\text{g/ml}$) dose dependantly inhibited the spontaneously contraction of the rabbit ileum (Table 4). At this dose range, increase in the concentration of lindane produced a rise in the percentage decrease in response of the rabbit ileum. A plot of log concentration of lindane against percentage decrease in response was carried out (Fig. 9) and the IC_{50} was determined to be 3.6 $\mu\text{g/ml}$.

3.2.1.3 Determination of 50 percent inhibitory concentration (IC_{50}) of lignocaine.

Lignocaine (20-330 $\mu\text{g/ml}$) dose dependant inhibitory response on the spontaneously contracting rabbit ileum was obtained (Table 5). Doses below 20 $\mu\text{g/ml}$ gave no response. A plot of log concentration against percentage decrease in response was carried out (Fig. 11) and the IC_{50} was determined to be 47.0 $\mu\text{g/ml}$.

TABLE 3

Dose response of rabbit ileum to lindane

<i>Lindane conc. ($\mu\text{g/ml}$)</i>	<i>Mean response (mm)</i>	<i>S.E.M</i>
Control	16.5	± 0.30
0.4	19.8	± 0.25
0.8	25.0	± 0.40
1.6	9.0	± 0.00
3.2	7.5	± 0.25
6.4	4.3	± 0.50

(n = 4, mean \pm S.E.M)

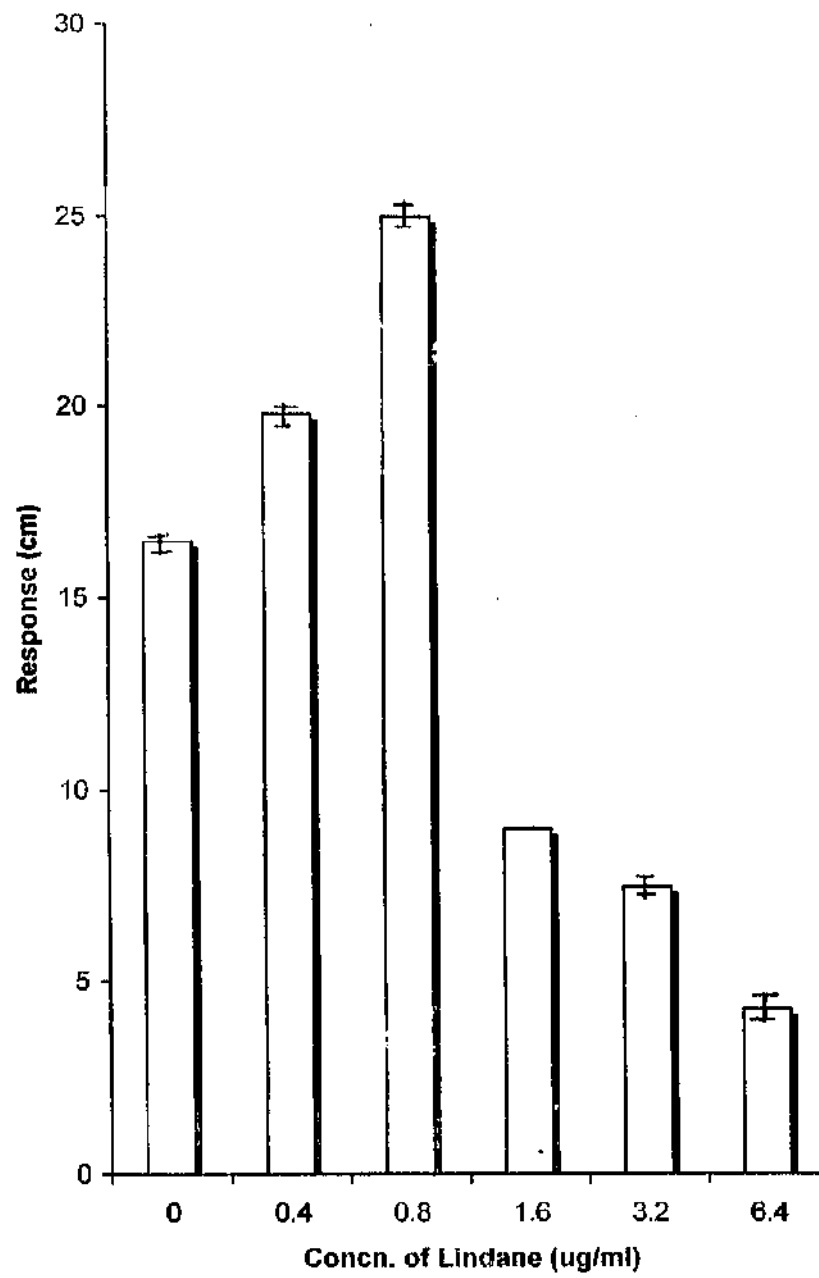


Figure 8: Dose-Response of rabbit ileum to lindane.

TABLE 4

Decrease in response of rabbit ileum caused by increasing concentration of lindane

<i>Lindane conc. ($\mu\text{g/ml}$)</i>	<i>% decrease in response</i>	<i>S.E.M.</i>
1.6	38.9	± 3.9
3.2	47.9	± 3.5
6.4	88.6	± 5.1
12.8	93.1	± 4.1
25.6	100	± 3.8

(n = 4, mean \pm S.E.M)

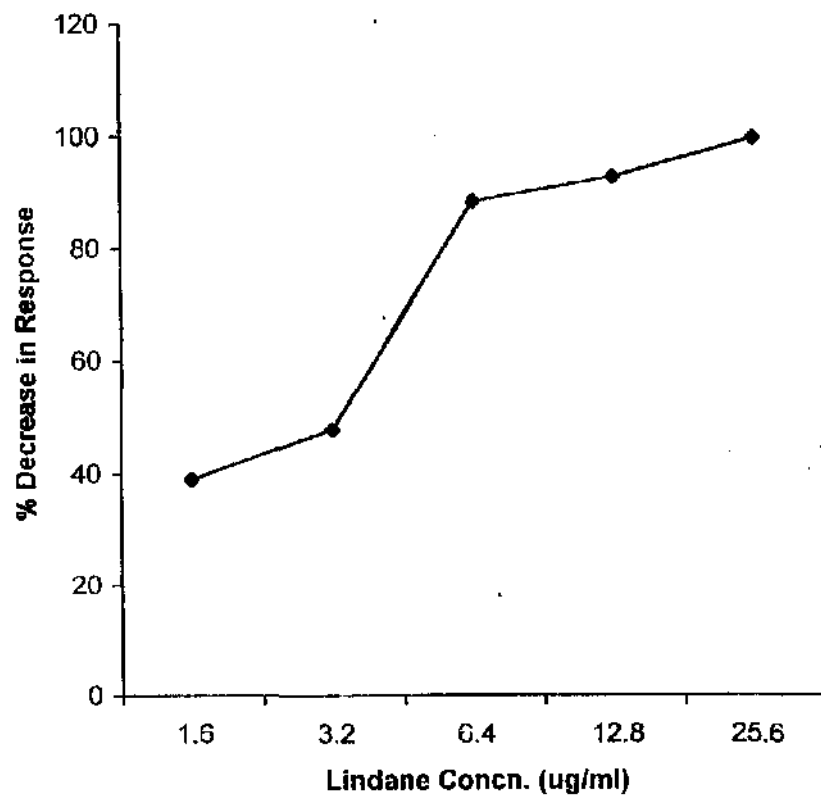


Figure 9: Percentage decrease in response against concentration of lindane using rabbit ileum.

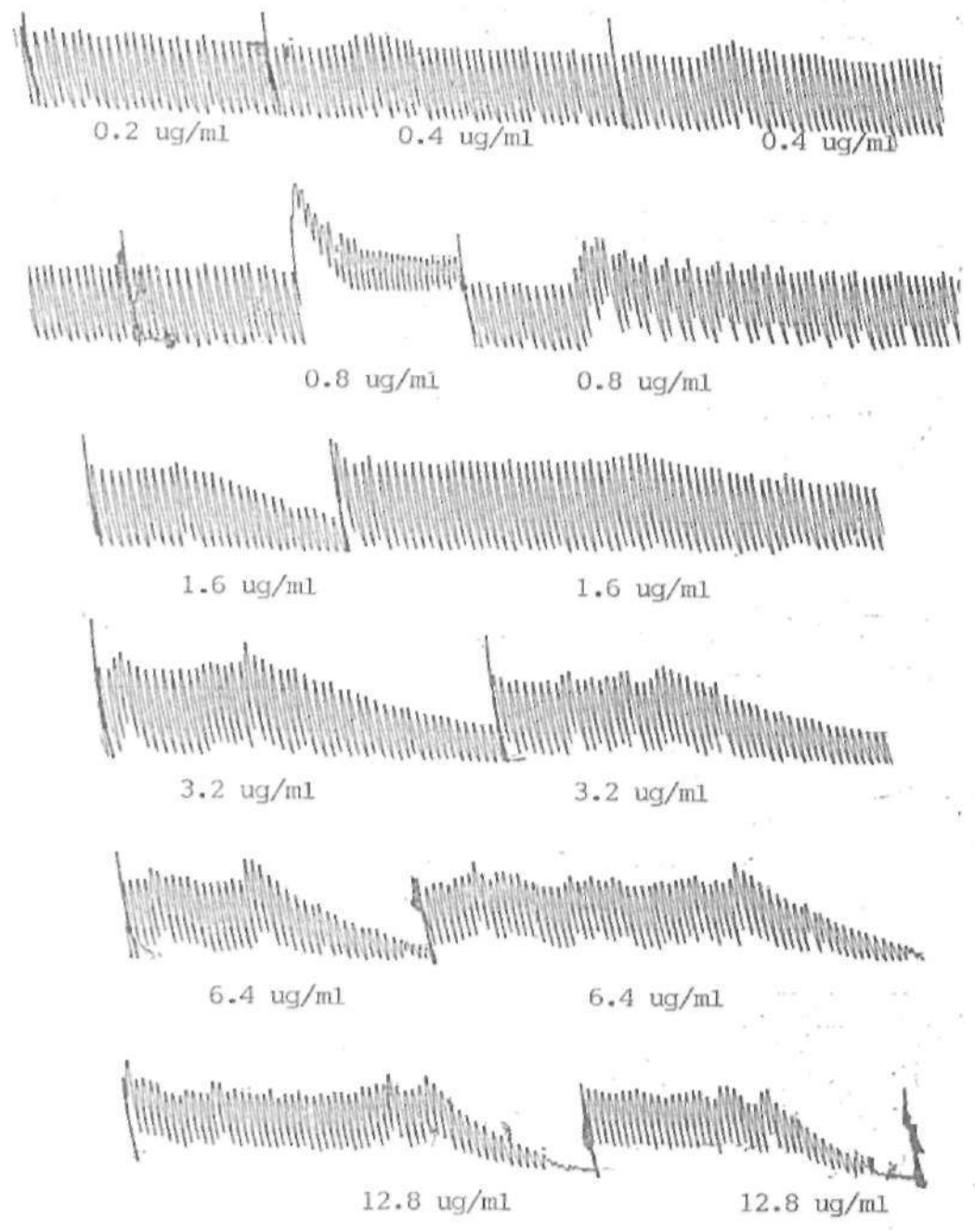


Figure 10: Some tracings of the effect of increasing concentration of lindane on the spontaneous contraction of rabbit ileum.

TABLE 5

Decrease in response of rabbit ileum caused by increasing concentration of lignocaine

<i>Lignocaine conc. (µg/ml)</i>	<i>% Decrease in response</i>	<i>S.E.M.</i>
20.5	29.5	± 2.3
41.0	44.9	± 1.9
82.0	57.4	± 1.9
164.0	72.7	± 1.9
328.0	100	± 2.2

(n = 4, mean \pm S.E.M)

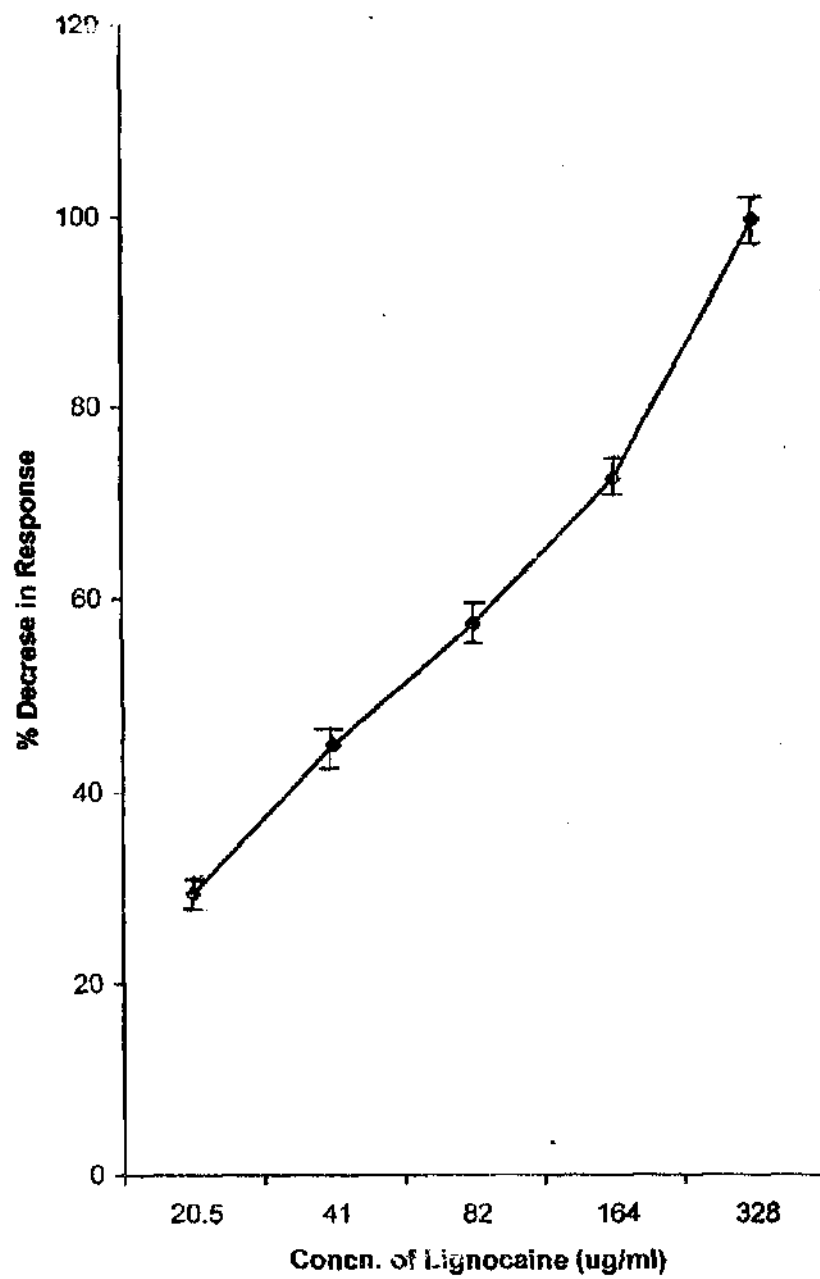


Figure 11: Percentage decrease in response against concentration of lignocaine using the rabbit ileum.

3.2.1.4 Effect of lindane on response due to lignocaine using the rabbit ileum

The control response (response due to physiological solution containing IC_{50} lignocaine) gave 10 ± 0.4 mm. Lindane dose dependently attenuated the inhibitory effect of lignocaine leading to increase in response of the rabbit ileum ($P < 0.001$) (Table 6; Fig.12).

3.2.2 Investigation on the Phrenic Nerve-hemidiaphragm Preparation.

3.2.2.1 Determination of 50 percent inhibitory concentration (IC_{50}) of lindane.

The normal twitch response of the phrenic nerve-hemidiaphragm following a direct stimulation of the nerve was obtained.

Percentage decrease in response for concentrations of lindane within the range of $0.5 \mu\text{g/ml}$ - $8.0 \mu\text{g/ml}$ were obtained and a plot was carried out (Table 7; Fig. 14) the IC_{50} of lindane on the phrenic nerve-hemidiaphragm was determined to be $1.0 \mu\text{g/ml}$.

3.2.2.2 Determination of 50 percent inhibitory concentration (IC_{50}) of lignocaine

A plot of percentage decrease in response against concentration of lignocaine within the range of ($2-34 \mu\text{g/ml}$) was carried out (Table 8; Fig. 15). The IC_{50} of lignocain on the phrenic nerve - diaphragm was determined to be $8.5 \mu\text{g/ml}$. All other steps were the same as in determination of IC_{50} of lindane.

TABLE 6

Effect of lindane on response due to lignocaine using the rabbit ileum

<i>Lindane conc. ($\mu\text{g/ml}$).</i>	<i>Mean response (mm)</i>	<i>S.E.M.</i>
control	10.0	± 0.40
6.4	13.5	± 3.00
12.8	15.8	± 0.25
25.6	18.5	± 0.30
51.2	25.0	± 0.40

(n = 4, mean \pm S.E.M)

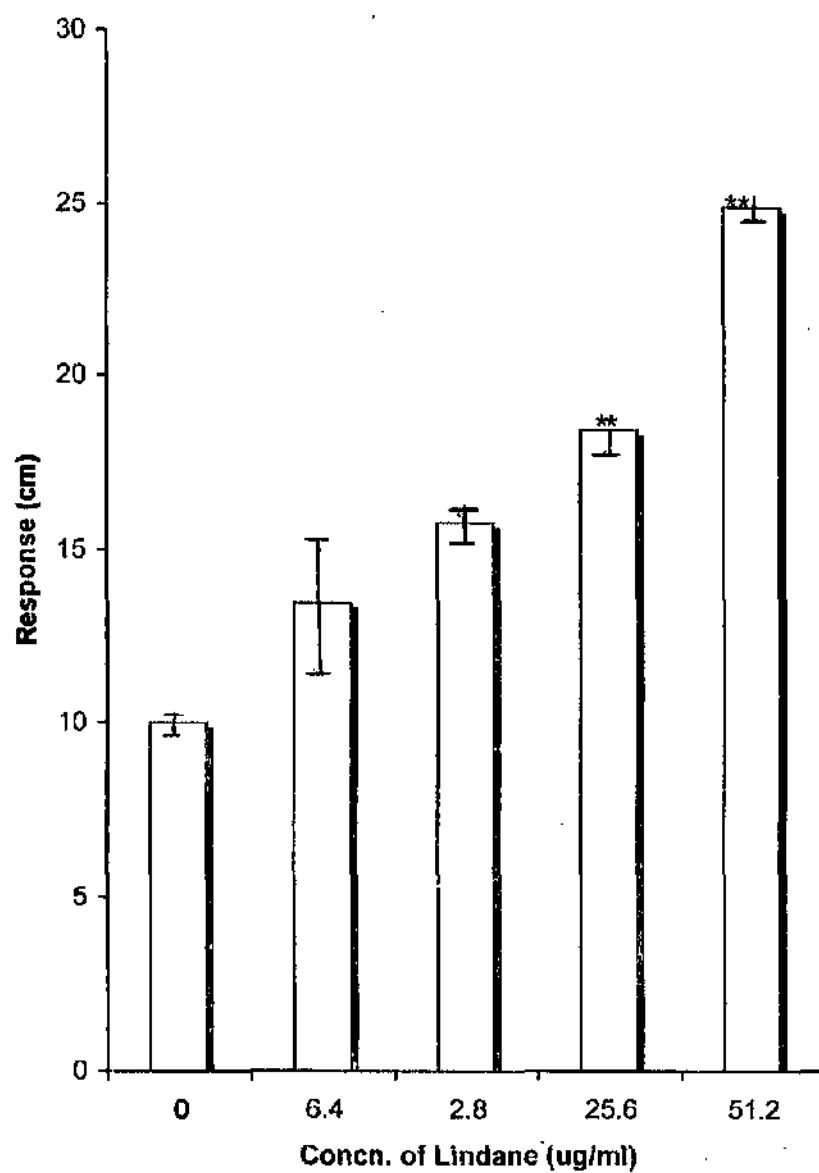


Figure 12: Effect of lindane on response due to lignocaine using rabbit ileum.

** Sig. diff. from the Control ($P < 0.001$; Student's t-test).

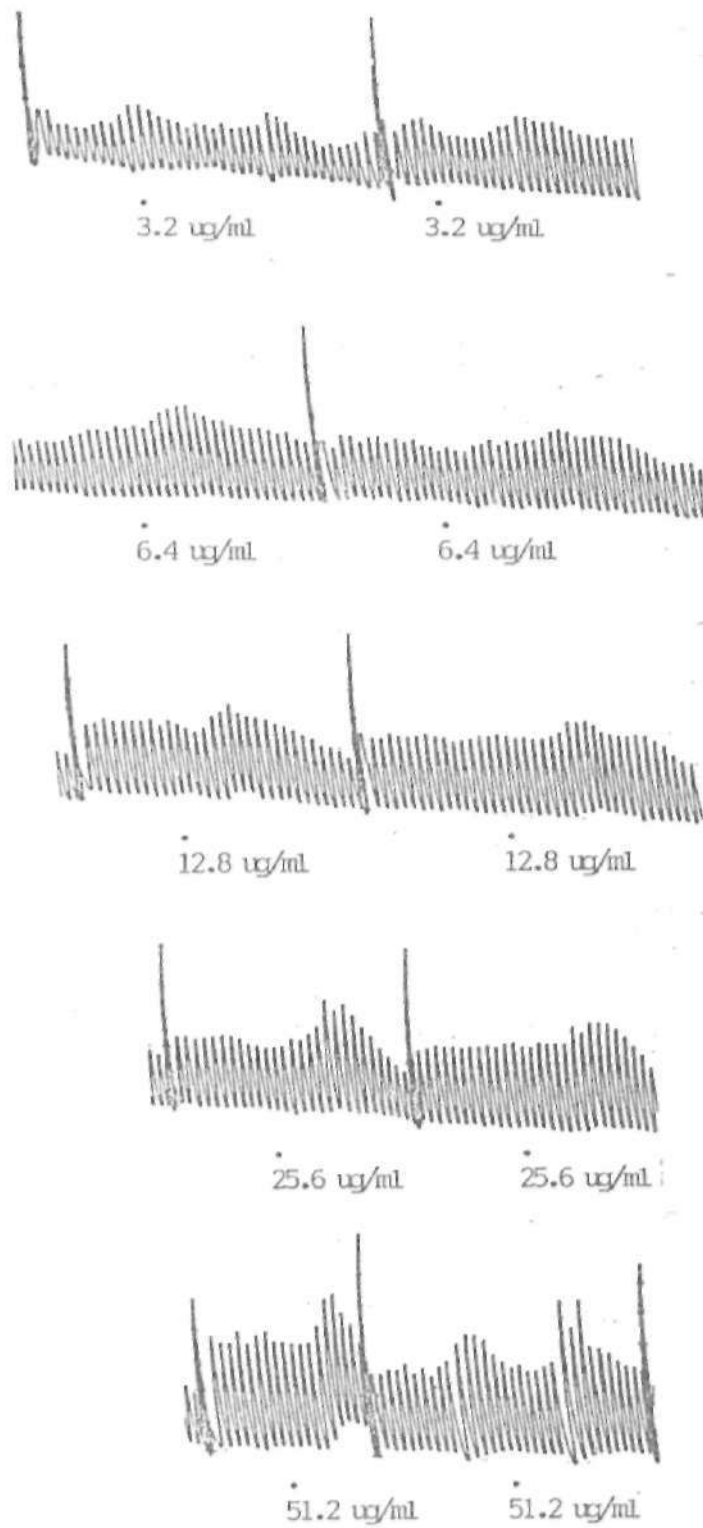


Figure 13: The effects of lindane on response due to lignocaine using the rabbit ileum.

TABLE 7

Decrease in response of the phrenic nerve caused by increasing concentration of lindane

<i>Lindane conc. ($\mu\text{g/ml}$)</i>	<i>% Decrease in response</i>	<i>S.E.M.</i>
0.5	36.8	± 4.4
1.0	59.5	± 3.0
2.0	69.9	± 2.5
4.0	83.1	± 3.0
8.0	100	± 3.5

(n = 4, mean \pm S.E.M)

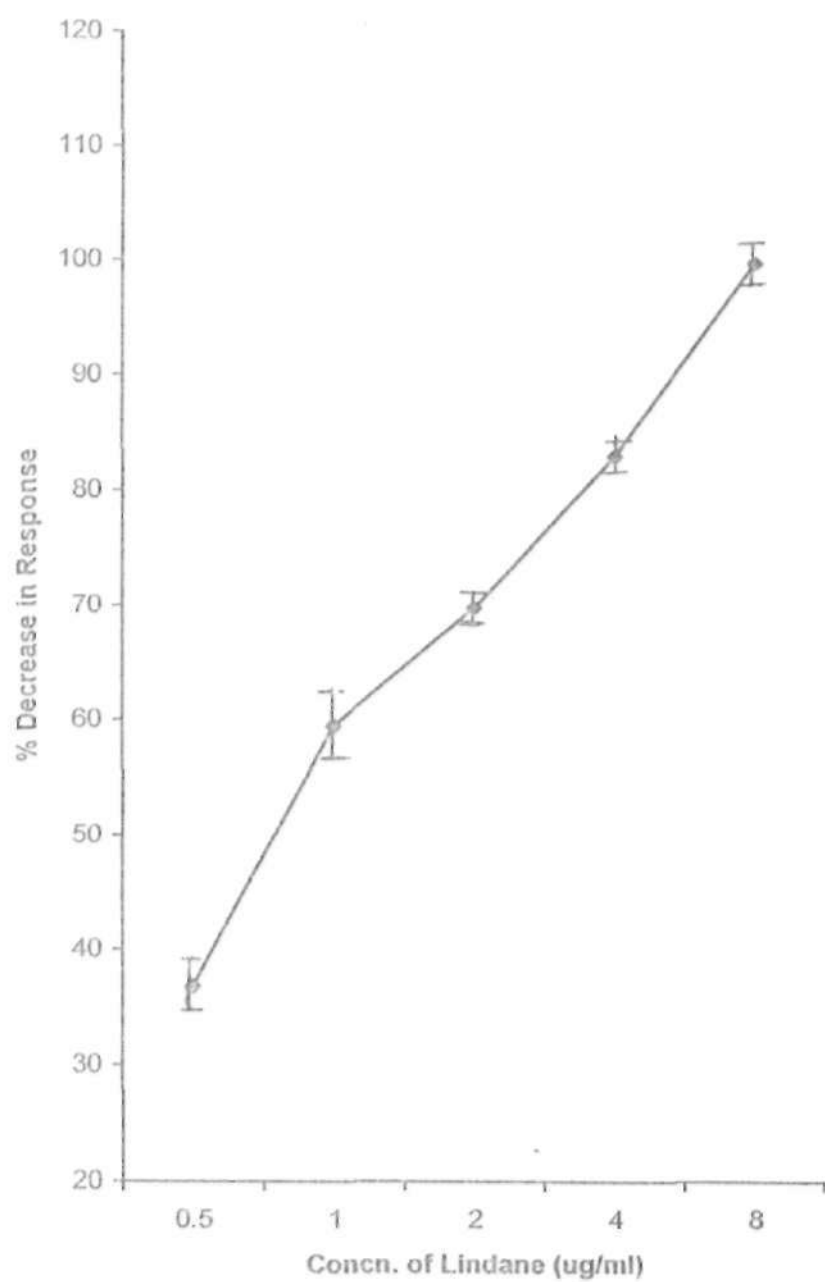


Figure 14: Percentage decrease in response against concentration of lindane using the phrenic nerve.

TABLE 8

Decrease in response of the phrenic nerve caused by increasing concentration of lignocain

<i>Lignocaine conc. ($\mu\text{g/ml}$)</i>	<i>% Decrease in response</i>	<i>S.E.M.</i>
2.13	26.5	± 2.8
4.25	32.4	± 2.8
8.50	49.6	± 4.1
17.00	79.8	± 4.1
34.00	100	± 3.8

(n = 4, mean \pm S.E.M)

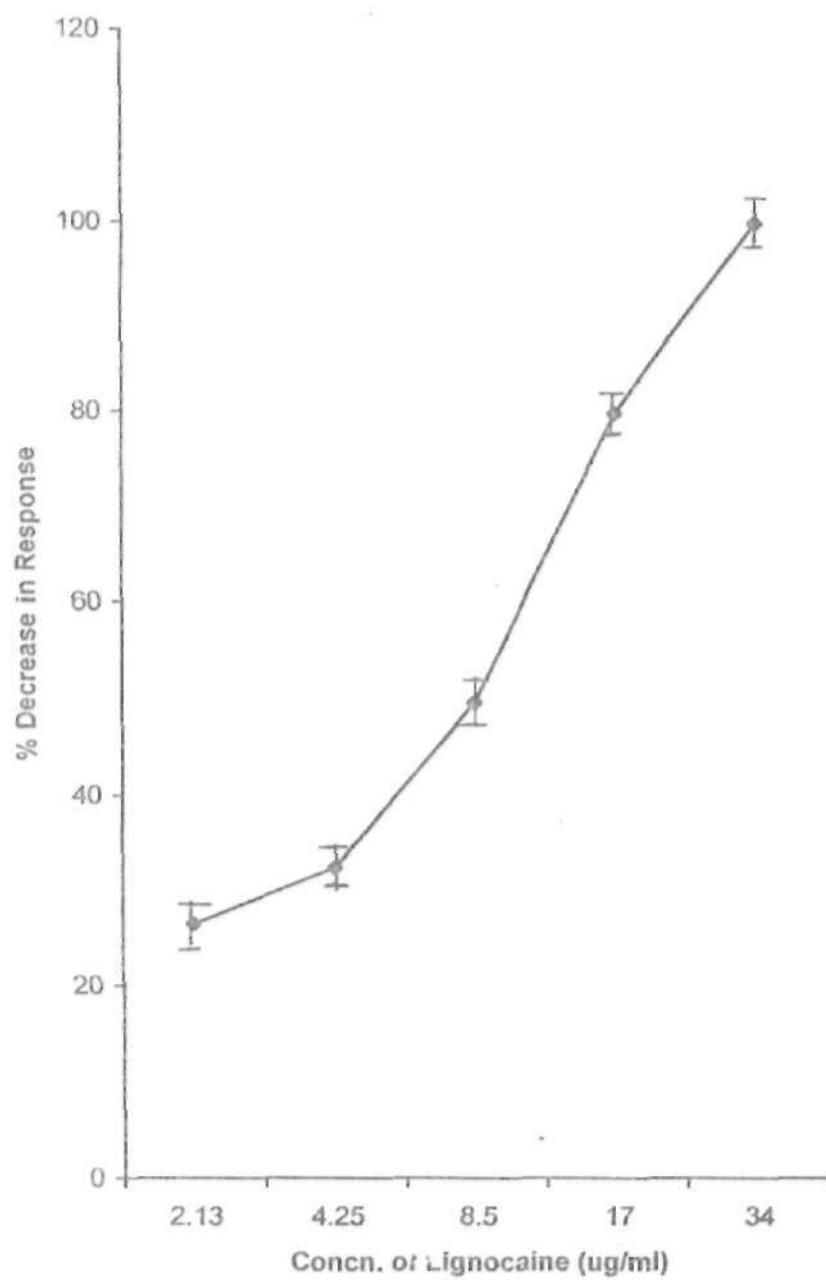


Figure 15: Percentage decrease in response against concentration of lignocaine using the phrenic nerve.

3.2.2.3 Effect of lindane on response due to lignocain using the phrenic nerve

The control twitch response (response due to physiological solution containing IC_{50} of lignocain) gave 24 ± 0.3 mm. Lindane dose dependently attenuated the inhibitory effect of lignocain leading to increase in response of the phrenic nerve ($P < 0.001$) (Table 9; Fig. 16).

TABLE 9

Effect of lindane on response due to lignocaine using the phrenic nerve diaphragm

<i>Lindane conc. ($\mu\text{g/ml}$)</i>	<i>Mean response (mm)</i>	<i>S.E.M.</i>
Control	23.5	± 0.3
64.0	25.3	± 0.25
128.0	28.5	± 0.3
256.0	32.0	± 0.00

(n = 4, mean \pm S.E.M).

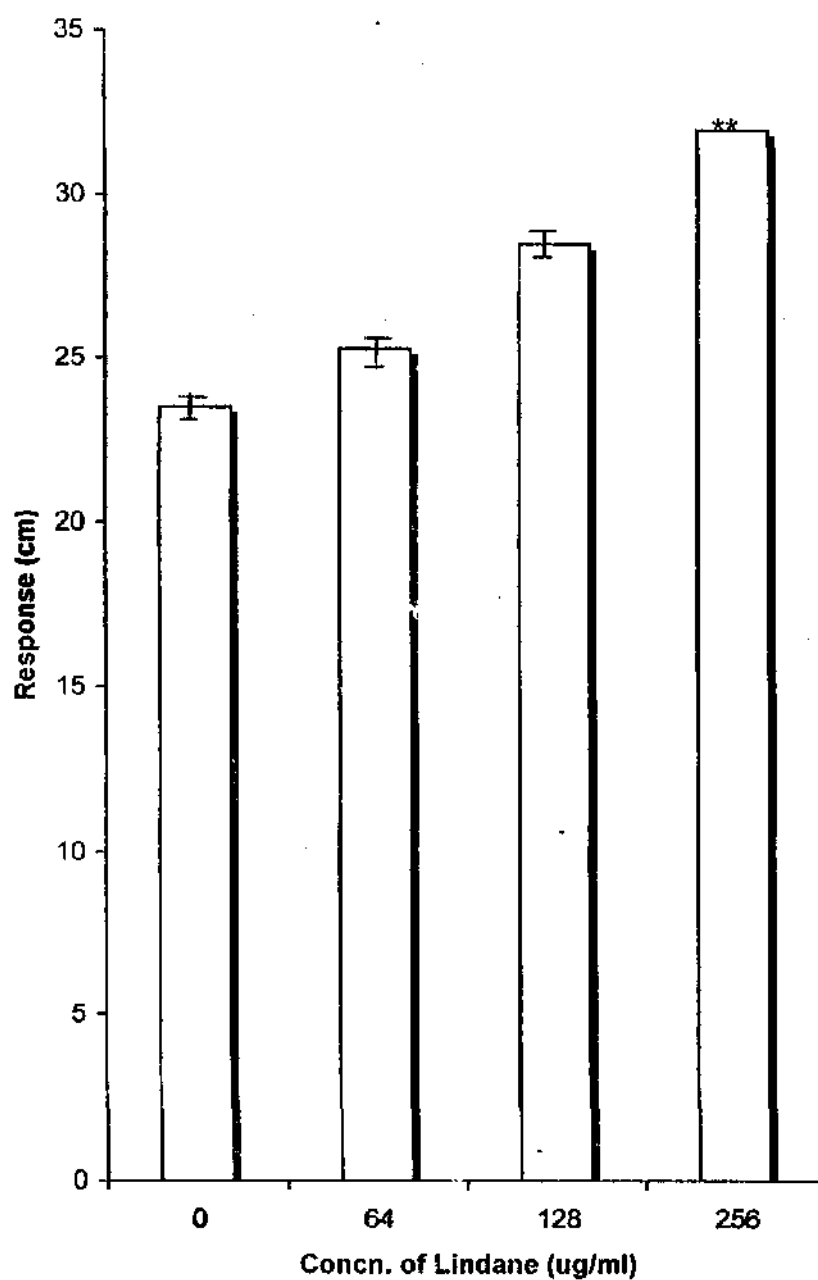


Figure 16: Effect of lindane on response due to lignocaine using the phrenic nerve.

** Sig. dif. from the Control ($P < 0.001$; Student's t-test).

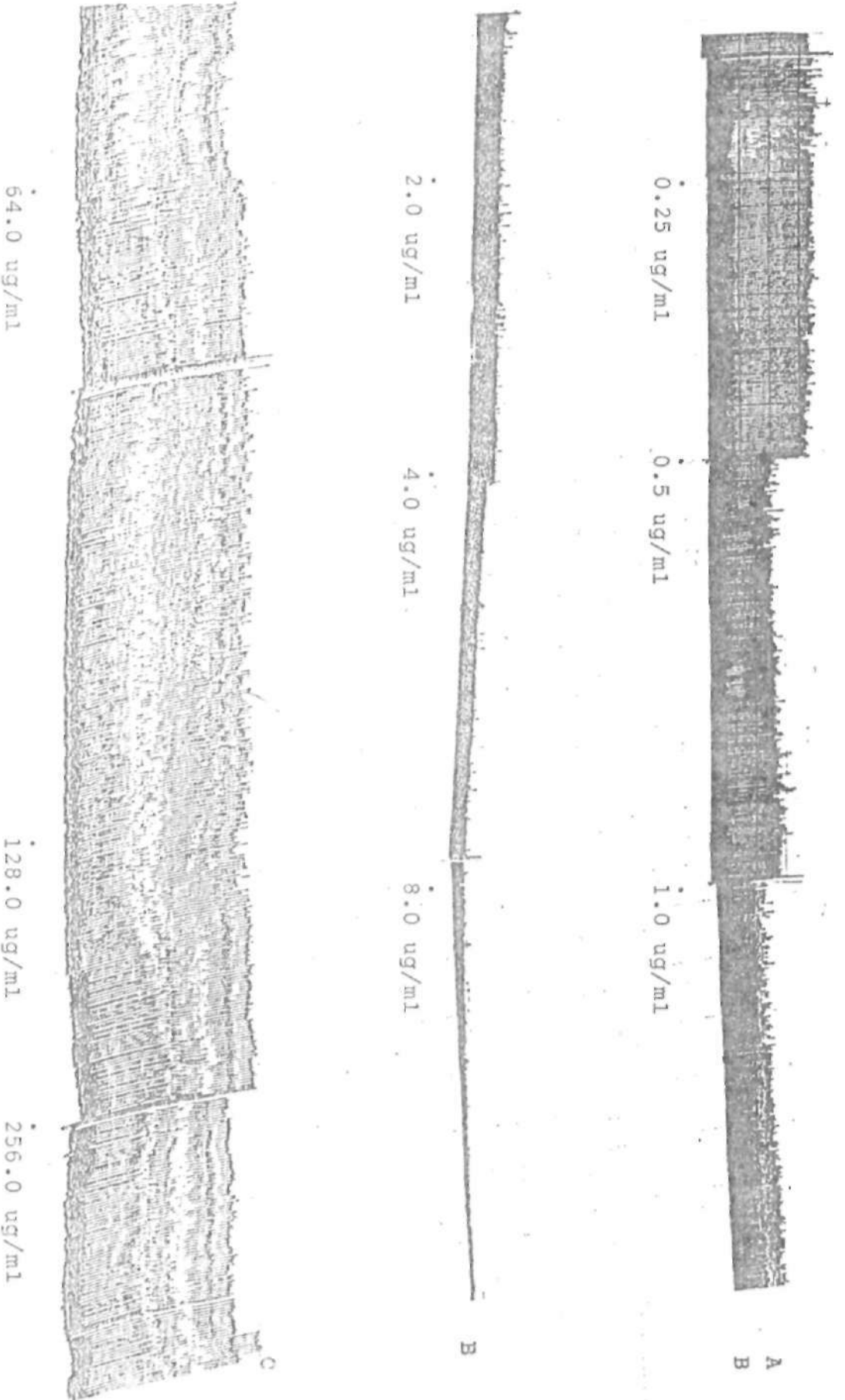


Figure 17: A & B - Effect of increasing concentration of lindane on spontaneous response of the phrenic nerve-diaphragm.
 C - Effect of lindane on response due to lignocaine using the phrenic nerve diaphragm.

CHAPTER FOUR

DISCUSSION

4.1 *IN VIVO* STUDIES

4.1.1 LD₅₀ Determinations in Rats

Acute toxicity study in experimental animals were aimed at detecting the short term effect of high doses of the compound, hence provide basic data for the design of more complex animal studies in order to estimate the safety level for humans. The simplest acute toxicity study usually employs determination of the median lethal dose (LD₅₀) of the compound. This is the dose of any substance that will kill 50 percent of a given population within a specific period of time, usually 24 hours. In this work, the median lethal dose value of lindane by intraperitoneal route which was determined using the Arithmetic method of Karber adapted by Aliu and Nwude (1982), was obtained as 25.4 mg/kg (Table 1).

On the basis of toxicity, Matsumura (1975) classified insecticides as:

1. Extremely toxic (LD₅₀ of 1mg/kg or less)
2. Highly toxic (1-50mg/kg)
3. Moderately toxic (50-500mg/kg)
4. Slightly toxic (0.5-5g/kg)
5. Practically non-toxic (5-15g/kg)
6. Relatively harmless (More than 15g/kg)

While the above classification may be quite useful in grouping various toxicants, an important point to mention here is that route of administration of toxicants strongly influences the LD₅₀ values (Matsumura, 1975). And since the classification given above is via the oral route, it may not have much bearing on the results obtained using

the intraperitoneal route. However, the oral LD₅₀ value for lindane is reported to be 91mg/kg (Matsumura, 1975) and 88 mg/kg (Gaines, 1969). This makes lindane a moderately toxic pesticide.

Sign and symptoms of toxicity were observed following the administration of different doses of lindane intraperitoneally. The symptoms of toxicity were found to be dose-dependant and they were scored according to their order of severity. They included tremor (1), prostration (2), palor (3), convulsion (4) and death (5). The symptoms of toxicity appeared to be mainly of CNS origin (Hassall, 1987).

Although the mechanism of action of the organochlorine insecticides is somewhat obscure, (Hassall, 1982 and Plestina, 1984), the major toxic action of this group of chemicals is on the central and peripheral nervous systems while systemic action in acute overdosage is confined to the CNS and the symptoms are those of CNS stimulation such as headaches, apprehension and excitement accompanied by dizziness, disorientation, vomiting, weakness of the skeletal muscles, tremor and finally epileptiform convulsions (Plestina, 1984). Different reasons have been advanced for these observed effects, the first of which is that the insecticides may inhibit one or several enzymes of importance to the mechanism of membrane stability or of nerve impulse transmission. It may also be due to initiation of some physical change in the structure of the nerve membrane which rather literally causes it to misfire (e.g. by alteration of its permeability to ions involved in transmission).

Investigations in this work suggest that there is an increase in membrane permeability to Na⁺ under lindane. This was such that the resting membrane potential is not restored. The terminal becomes hyperexcitable and depending on the magnitude or concentration, the nerve goes into repetitive discharges or firing. Thus convulsion

was the major symptom of lindane toxicity at higher doses.

Lindane has been shown to inhibit the uptake of chloride ions at inhibitory synapses in the brain (Matsumura & Tanaka, 1984; Abalis et al; 1985, 1986; Fishman & Gianutsos, 1988), and it is this mode of action that is now widely considered to account primarily for the convulsant activity of this insecticide. Because of its structural similarity to picrotoxinin, lindane has a good geometric fit to the picrotoxinin binding site at the outer end of the chloride channel. Once bound, lindane is believed to block the action of the neurotransmitter GABA, which mediates the entry of the chloride ions necessary for inhibitory neuronal function (Matsumura, 1985).

4.1.2 Histopathological Examination and Reports in Rats

The results under this section revealed varying degrees of pathological disorders in the brain which were found to increase with dose and time of exposure of the animals to lindane.

The brain of an average adult is made up of about 1000 billion neurons and is one of the largest organs of the body, weighing about 1,300g. The brain is divided into four principal parts; brain stem, diencephalon, cerebrum and cerebellum. The brain stem consist of medulla oblongata, pons and midbrain. Above the brain stem is the diencephalon consisting primarily of the thalamus and hypothalamus. The cerebrum spreads over the diencephalon. The cerebrum constitutes about seven-eights of the total weight of the brain and occupies most of the cranium. Inferior to the cerebrum and posterior to the brain stem is the cerebellum.

Saufeliu *et al.*, (1988) have studied the distribution of lindane in brain after oral administration at 30mg/kg or intravenous administration at 0.3mg/kg using autoradiography-imaging analysis and dissection-liquid scintillation counting techniques. The two routes of administration gave similar results. A heterogeneous distribution of label in brain regions was observed: the radiolabel concentration in the white matter was higher than that in thalamus, mid-brain, pons and medulla at different times relative to the mean value for whole brain. The affinity of lindane for white matter and myelinated structures was related to its lipophilic behaviour.

Referring to the symptoms of toxicity and EEG of lindane, the histopathology of the brain stem and the cerebellum is of particular importance. Since the brain stem controls consciousness and equilibrium, and the cerebellum controls co- ordination and balance (Gerard and Anagnostakos, 1990), it is possible that these sections are significantly affected by chronic administration of lindane and conditions such as in- coordination may result following long-term exposure to lindane.

Vacuolation and congested blood vessels were reported in the rats exposed to 5mg/kg, 10mg/kg and 15mg/kg lindane. Vacuolation has been shown to be one of the

early morphological changes that leads to hypoxia in the brain (Curan and Harnden, 1972). Neurons require a constant supply of oxygen and glucose, and if this is inadequate they undergo a series of changes termed the ischaemic cell process. The earliest histological feature is microvacuolation and if the neuron is irreversibly damaged, there is gradual transition from the stage of microvacuolation to that of ischaemic cell change leading to impairment of transmission, coma and death.

4.1.3 Electroencephalographic (EEG) Study in Rats

The EEG pattern of the rats fed on 5mg/kg lindane, 10mg/kg, 15mg/kg and control rats (rats fed on normal feed), in the 4th, 8th and 12th weeks were observed for differences in amplitudes and frequencies of waves. The EEG pattern included the frontal cortex (FC), optic cortex (OC), Reticular formation (RF) and Electromyograph (EMG). The brain waves of the reticular formation was chosen for measurement of amplitudes and frequencies since the reticular formation of the brain is the site mainly concerned with wakefulness and arousal.

From the results obtained there was desynchronisation of the EEG waves which was relatively found to increase with dose and time of exposure. This is associated with stimulation of the reticular formation in which the slow waves of the EEG are replaced by waves with a faster and more irregular rate and with a lower amplitude (Brodal, 1992). In line with the *in vitro* findings of this work, lindane appears to cause CNS stimulation by processes involving excitation of the neurons. It is possible that accumulation of lindane in the brain to certain level is associated with increased wakefulness and alertness which at higher dose and/or time of exposure may lead to **mental impairment.**

4.2 *IN VITRO* STUDIES

4.2.1 Effect of Lindane on the Spontaneous Contraction of the Rabbit Ileum and Phrenic nerve-diaphragm Preparation

The effect of lindane on the spontaneous contractions of rabbit ileum preparation was found to be potentiation at lower doses and inhibition at higher doses of lindane (Table 3, fig. 6).

The organochlorines have been shown to cause destabilization of neuronal activity resulting in hyperexcitability on nerves (Hassal, 1987). However, there is great a diversity as to the possible mechanisms by which lindane or indeed the organochlorines in general, produce their neurotoxic effects. The *in vitro* aspect of this work was aimed at gross evaluation of the involvement of sodium ions (Na^+) in the mechanism of action of lindane.

The organochlorines prolong the falling phase of the spike in isolated nerve fibres. Experiments involving the voltage clamp technique have shown that this effect is due to two actions of the organochlorines; it delays the closing of the Na^+ channels and it partially blocks the potassium ion (K^+) channels. As a result of these actions, the negative after - potential outlasts the refractory period of the axon and repetitive firing results (Bowman and Rand, 1980).

Biochemical, biophysical, and molecular biological investigations during the past decade have led to a rapid expansion of knowledge about the Na^+ channel and other voltage-sensitive ion channels (Catterall, 1988; Trimmer and Agnew, 1989). The Na^+ channel of the mammalian brain is a heterotrimeric complex of glycosylated proteins with an aggregate molecular size in excess of 300,000 daltons; the individual sub-units are designated α (260 kilodaltons), β_1 (36 kilodaltons), and β_2 (33 kilodaltons). After

incorporation of the purified polypeptides into phospholipid vesicles Na^+ flux into the vesicles occurs in response to veratridine, a substance known to cause persistent activation of Na^+ channels. Only the α sub-unit is required to reconstitute channel function. Movement of Na^+ into the vesicles is blocked by neurotoxins - tetrodotoxin and saxitoxin, and by local anaesthetics (Tamkun *et al.*, 1984).

Since the activation of Na^+ channels leading to influx of the Na^+ is basically responsible for the generation of action potential (Fig.2), the initial increase in response of the rabbit ileum to lower concentrations of lindane is probably due to activation of the Na^+ channels. As the dose of lindane increases, it tends to inactivate the Na^+ channels in such a way that the resting potential of the channel (which is necessary for reactivation) is not restored.

Analysis of local anaesthetic action (Strichartz 1973, Hille 1977, Strichartz and Ritchie, 1987) has shown that many drugs exhibit the property of "use-dependant" block of Na^+ channels, as well as affecting to some extent, the gating of the channels. Use-dependence means that the more the channels are opened, the greater the block becomes. The channel can exist in three functional states - resting, open and inactivated. Studies by Khodorov and by Hille have shown that many drugs bind most strongly to the inactivated state of the channel. Thus, at any given membrane potential, the equilibrium between resting and inactivated channels will in the presence of such drugs be shifted in favour of the inactivated state and this factor contributes to the overall blocking effect (Rang and Dale, 1991).

Therefore the observation that decrease in response of the rabbit ileum to increasing concentration of lindane progress to a state where no response is recorded may be due to high degree of inactivation of the Na^+ channels. Rang and Dale (1991)

also reported that the main reason for the loss of electrical excitability during a period of maintained depolarization is that the voltage sensitive sodium channels become inactivated (i.e refractory) and no longer able to open in response to a brief depolarizing stimulus.

4.2.2 Effect of Lindane on Response due to Lignocaine on Rabbit Ileum and Phrenic Nerve-diaphragm Preparations

The *in vitro* work was designed to also look at the effect of lindane on response due to lignocaine since local anaesthetics (of which lignocaine is a prototype) interfere with a process fundamental to the generation of action potential, namely, the large transient, voltage dependant rise in the permeability of the membrane to Na^+ conductance which is the basis for local anaesthetic action (Haddox, 1991). This action of local anaesthetics is due to their direct interaction with voltage-sensitive Na^+ channels. As the anaesthetic action progressively develops in a nerve, the threshold for electrical excitability gradually increases, the rate of rise of the action potential declines, impulse conduction slows, and the safety factor for conduction decreases; these factors decrease the probability of propagation of the action potential, and nerve conduction fails (Goodman and Gilman, 1990).

From the results, lindane dose-dependently attenuated the effect of lignocaine both in rabbit ileum and phrenic nerve - diaphragm preparations ($P < 0.001$), (Fig. 9 and 12). This further indicate the involvement of Na^+ in the mechanism of action of lindane. It is possible that lindane and lignocaine compete in an opposite pattern for the Na^+ channels and therefore in presence of lindane, the response due to lignocaine is attenuated. It is also possible that lindane has more affinity for the Resting and Open Na^+ channels than lignocaine such that the action of lindane on the Resting and Open

Na⁺ channels will put the channels in more activated states resulting to reduced magnitude of effect produced by lignocaine. However, more investigations will need to be carried out in this aspect especially using the selective Na⁺ channel blocking agents such as tetrodotoxin and saxitoxin.

4.3 CONCLUSION

The need to chemically control pests and diseases either of livestock or of plants is an absolute necessity. Pesticides have become an ubiquitous part of the environment and occupy a unique position among the chemicals encountered daily by man since they are deliberately added to the environment for the purpose of killing or injuring some form of life (Murphy, 1980).

Therefore the knowledge of the mode of toxicity and mechanism of action would be valuable in predicting and minimizing the human and non-target hazards of pesticides, and would also provide additional help in the treatment of episodes of pesticide intoxication.

From the results obtained in this work, lindane can be regarded as a toxic pesticide with the LD₅₀ of 25.4mg/kg. It has been found to cause pathological lesions of different kinds in the brain following chronic exposure and also to cause desynchronisation of the EEG pattern in rats. Thus the major symptoms of toxicity of lindane were central and the possible sections of the brain that are significantly affected have been suggested.

The *in vitro* investigations have led to the conclusion that lindane interfere with the generation and propagation of action potential along the nerves by increasing the permeability of the membrane to sodium ions. This is because lindane dose-

independently attenuated the inhibitory effect of lignocaine - a Na⁺ channel blocker both in the rabbit ileum and phrenic nerve-diaphragm preparations. Therefore the symptoms of CNS stimulation recorded under the *in vivo* studies were probably due to increased opening of the sodium ion channels which may be associated with increased firing of the central neurons. Bowman and Rand (1980) reported that the organochlorine insecticides cause a delay in the closure of the sodium ion channels leading to prolonged negative after-potential and repetitive firing of the neurons.

In conclusion it can be suggested that research in this and other related areas should be aimed at identifying the mechanism by which toxicity is produced such that antidotal therapy could be designed for various chemical intoxication. Specifically, for the management and/or treatment of lindane poisoning, efforts should be made to regularise the functioning of the Na⁺ channels since they have been shown to be largely involved in the toxicity of lindane. There is also the need for the development of preventive measure; diagnostic tests and treatment facilities in pesticide poisoning (Iyaniwura, 1991a).

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