HEPATIC AND SERUM ENZYME CHANGES AFTER LINDANE POISONING IN RATS.

BY

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A thesis submitted to the Postgraduate School,
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in partial fulfillment of the requirements for the
award of the Master degree in Pharmacology.

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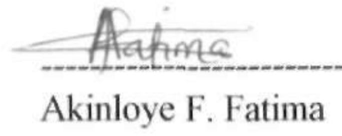
MAY, 1998.

DECLARATION.

I hereby declare that:

(i) the report presented in this thesis is the result of my original research.

(ii) all inclusions from works other than this have been duly referenced and acknowledged.



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CERTIFICATION

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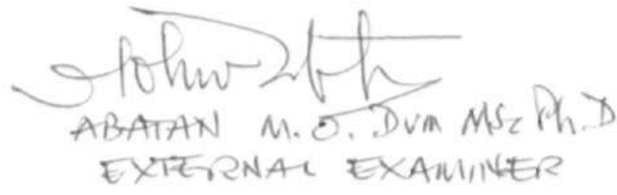

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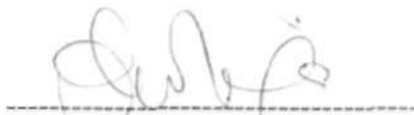

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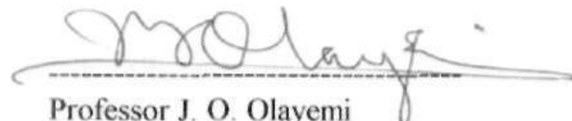

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DEDICATION

Specially dedicated to Shakirat, Rukayyat and Muzammil.

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ABSTRACT

Acute and Chronic toxicity studies of lindane were carried out on rat using biochemical changes as indices of toxicity. The calculated LD_{50} was 25.4 mg per Kg body weight using the arithmetic method of Karber. The symptoms of toxicity were observed and scored by specific points according to the severity of symptoms of poisoning. Symptoms of toxicity observed include tremor, prostration, pallor, convulsion and death of the animal.

Some serum biochemical parameters were analysed to evaluate the effect of lindane on the liver. The parameters includes Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), total protein (TP) and serum albumin (SA). ALT showed the most significant alteration throughout the study period. ALT is more sensitive to liver damage than AST. The other parameters showed no significant alteration.

Histopathological examination of the liver indicates inflammation, congestion of the liver and fatty degeneration after prolonged exposure. The symptoms of poisoning were dose dependent in most cases.

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ABBREVIATIONS

AST:	Aspartate Transaminase
ALT:	Alanine Transaminase
ALP:	Alkaline Phosphatase
TP:	Total protein
SA:	Serum Albumin
IP:	Intraperitoneal
LD_{50}:	Median lethal dose
SEM:	Standard error of mean
SD:	Standard Deviation
Conc.:	Concentration
No. :	Number
IU/L :	International Unit per liter
KAU/dl	King-Armstrong Unit per deciliter
GM/l :	Gram per liter
Gm/l	" " "
Kg	Kilogram
Mg	Milligram
BHC:	Gamma Benzene hexachloro cyclohexane
PCCH:	Pentachlorocyhexene
DDT:	Dichloro-diphenyl trichloroethane
MFO:	Mixed function Oxidase
BSA:	Bovine Serum Albumin
ICI:	Imperial Chemical Industries
ULV:	Ultra Low Volume

CHAPTER ONE

INTRODUCTION

Toxicology was defined by Dubois and Geiling (1959) as that branch of medical science that deals with the nature, properties, metabolism effects and detection via chemical and physical analysis of poisons. It is therefore the science of poisons.

Ariens and Simonis (1976) however defined poison as a substance which causes harmful effects in the user. According to Paracelsus; "The degree of toxicity of a substance depends on the dose". Thus, a substance, even pure water is poisonous when taken in toxic doses (Ariens and Simonis, 1976).

Insecticide toxicology differs from its parent discipline, medical toxicology, in that it does not include diagnosis or treatment of human patients who are affected by insecticides; however, insecticide toxicology does include efforts to determine tolerance levels of insecticides in man and is concerned with establishing a logical basis for selective toxicity in order to kill insects without affecting mammals (Matsumura, 1975).

Pesticides 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 forms of life (Casarett and Doull, 1975). Thus, all pesticides is said to be 'selective' in toxicity if it is highly toxic only to a few related organisms or 'broad spectrum' in toxicity if it has high toxicity to a wide range of organisms. Ideally, the injurious action of pesticides should be highly specific for undesirable target organisms and non-injurious to desirable non target organism. However, most of the chemicals that are used as pesticides are not highly selective but are generally toxic to

many non-target species including many other desirable forms of life that co-inhabit the environment.

Therefore, due to lack of highly selective pesticidal action, the application of pesticides most often are predicted on selecting quantities and manners of usage that will minimize the possibility of exposure of non-target organisms to injurious quantities of these chemicals (Casarett and Doull, 1975).

The biological effect of these chemicals are determined by the dose of the chemical applied (Casarett and Doull, 1975). The ideal study situation would be to evaluate the safety of a specific chemical directly in humans, but since exploration of the biochemical effects of chemicals in human is unethical, initial evaluations must then be performed by laboratory animal experimentation.

Some of the insecticides in common use today include organophosphate, organochlorines, carbamates, pyrethroids, triazines and rotenone (WHO, 1989).

Organochlorines are halogenated hydrocarbon pesticides. Some of the commercially available organochlorine pesticides are DDT groups, cyclodiene group and a group of heterogenous compound that can be called a miscellaneous group (Hatch, 1982). Organochlorine insecticides includes DDT, BHC (Gamma-Benzene hexachloride), aldrin, dieldrin, endrin, chlordane, heptachlor and camphechor.

BHC (Lindane) the insecticide of interest in this study has been used as a broad spectrum insecticide since the early 1950's in the treatment of seeds, soil, application on trees, timber, stored materials, treatment of animals against ectoparasite and in public health.

According to Paget (1970), acute toxicity studies in animals are of value in predicting potential toxic effects of a chemical in humans exposed to near fatal doses while chronic toxicity studies are designed to provide data on the toxic effects of a chemical and to determined the dose level and time -course required for these effects to be produced. Chronic toxicity study also provides a useful insight into the potentially toxic effects of repeated administration of the chemical on the biological system, and because of the frequency of administration, the dose levels are usually reduced from those used in the acute studies, that is, sublethal doses are employed . In this study, the median lethal-dose (LD_{50}) of Lindane was employed for acute toxicity study while biochemical studies on the liver functions was employed for the chronic toxicity study.

In the current study, the laboratory rat serves as a model in which the test material (Lindane) is introduced. Healthy, fairly adult male rats were used to ensure that the enzyme systems are sufficiently developed to metabolize the test material. Acute and chronic toxicity studies were done in the rat to determine the effect of high level one-time exposure to this chemical (acute effect) as well a moderate but prolonged exposure to the compound as would be the case in diet (chronic effect).It was also observed that the rat is also useful in providing information concerning what the adverse response might be when these chemicals are used in excessive amount or when usually high levels are present in the environment.

Biochemical studies were conducted in order to obtain information on the chemical and physico-chemical changes that take place in the living tissue in normal and pathological states. The liver is the major site for the metabolism of chemicals and as such could easily react to their administration (De Bruin, 1976; Wilkinson, 1976). This

study therefore looked into the hepatotoxicity of lindane in mammals and the liver enzymes (Alanine aminotransferase, Aspartate aminotransferase and Alkaline Phosphatase) as well as the total serum protein and albumin assays were selected as the biochemical parameters for monitoring liver dysfunction as could arise from mammalian exposure to this chemical.

Histopathologic analysis were done to determine if there were significant damage that a high or low but prolonged exposure to this chemical may provoke. The liver tissues of selected rats from each of the lindane-treated and control groups were used for this purpose.

In this study therefore, the following parameters have been specifically determined:

i. The median lethal-dose (LD_{50}) of lindane in rats using the intraperitoneal route.

ii. The clinical symptoms and signs of lindane toxicity.

iii. Three months (12 weeks) biochemical studies on the liver functions which includes assays for:

a). AST activity

b). ALT "

c). ALP "

d). TP conc

e). SA "

iv). Histopathologic changes of the liver tissues over the 12 weeks of chronic toxicity study.

CHAPTER TWO

LITERATURE REVIEW

2.1. PESTICIDES.

According to FAO (WHO) report pesticide can be defined as a group of heterogenous substances intentionally used for the prevention or control of any pest which may include vectors of human or animal disease, unwanted species of plants or animal causing harm, or otherwise interfering with processing, production, storage, transport or marketing of food, agricultural commodities, wood and wood products and animal stuff, or they may also be administered to animals for the control of insects, arachnids or other pests in or on their bodies (FA0/WHO 1971b).

Newman (1978) however specifically defined pesticides as biologically active compounds extensively used to control pests of all sort, such as insects, fungi, weeds and rodents that endanger the production, transport and storage of food.

The term pesticide in its ordinary usage would also include insecticides used in eliminating insect pest, herbicides for the control of weeds, fungicides used for the control of mould and fungi on crops, stored foods, timber, and also for external use on fungi growth on human and domestic animal body, rodenticide developed to kill rats and mice, fumigants and defoliants (Iyaniwura, 1990)

Available evidence (Khan, 1973) indicate that insecticides used for eliminating insect pest can be classified into systemic and contact insecticides. Systemic insecticides are first absorbed by the host and then by its parasites and are particularly suitable for the control of those livestock pests that spend part of their life cycle in the body of the

hosts where they are inaccessible to topical treatment, cattle grub, the larval stages of the warble flies are good examples of this type of economically important parasites. Contact insecticides on the other hand are usually applied directly to the surface of livestock and plants either as dusts of sprays destroy the pests as they infest the host.

For a very long time the pesticides in use were primarily compounds of heavy metals and those of plant origin, but since the second half of the 1940's there has been a marked increase in total pesticide usage and a rapid proliferation of synthetic organic compounds. In 1969, there were approximately 900 registered chemicals for sale as pesticides against about 2000 pest species in the United States of America (Secretary`s commission on pesticides, 1969). World production and use of pesticides continue to rise with a 10 (ten) fold increase in production between 1955 and 1990 (Rosenstock et al, 1991).

2.1.1 IMPORTANCE OF PESTICIDES USE.

Pesticides are known to improve the yield of crops and also to prevent the loss of such crops both before and after harvest, hence an increase in usage of pesticides, due mainly to increase in world population and food requirement currently exist especially in the developing countries (Casarett and Doull, 1975). Pesticides have been effectively used to repel unwanted lives which would have drastically reduced food and animal production. Pesticides have over the years gained increasing popularity and the most compelling reason for the continued widespread use is their availability, convenience and drastic effectiveness in dealing with pests on a short term basis (Hassal, 1982). For instance in Ghana cocoa production rose by 110 percent in 1957 when cocoa pods were

protected with pesticides; protection of rice from stem borer also increased rice production in Japan in 1960 (Fest and Schmidt, 1982).

Public health programmes also employ the use of pesticides as a means of controlling vector-borne diseases. It was proved over forty years by the World Health Organisation that the use of pesticides is the most effective way of controlling anopheles mosquito as a part of malaria eradication programmes (W.H.O., 1985). According to Newman (1978), pesticides of various types are also used in the control of insects, rodents and other pests which are involved in the life cycle of vector borne diseases such as filariasis, yellow fever or viral encephalitis thereby substantially reducing their morbidity and mortality rates. In addition, there has also been a large usage of pesticides by individual home owners and gardeners. For example, Casarett and Doull (1975) reported that during a one year period in Salt Lake County, Utah, of the total of 50,288 kg of pesticide used, 46,489 kg were used for domestic or household applications the balance was used by farmers, commercial applications, fruit growers, government agencies, mosquito abatement and on livestock.

Though, all pesticides are of importance in different ways, the ideal pesticide should be rapid in action, highly selective for the target organisms, economically profitable in use and provide a minimum residue in or on the host and must be rapidly destroyed in the environment.

2.1.2 INCIDENCE OF PESTICIDE POISONING.

Toxicologic evaluation of the hazards of handling and use of pesticides have for many years focused primarily on preventing injury to man.

Hazard of pesticides are usually the result of occupational exposure or of careless use, misuse or mishandling of pesticides. Acute cases of pesticide poisoning contribute significantly to cases of morbidity and mortality world wide (W.H.O.; 1986, 1990). Hayes (1969) estimated the mortality rate attributed to poisoning by pesticides at 0.65 per million population in the United States and also 100 non-fatal poisoning for each fatal one. Much of this burden is borne by developing countries where 99% of fatal pesticide poisoning occur and where 25 million episodes of intoxication occur annually among agricultural workers alone (Jeyaratnam, 1988).

Pesticides can come in contact with man and his domestic animal by accidental encounter or as residue in food, water and air resulting often in acute poisoning. Occupational exposure for instance, in the case of pesticide factory workers and pest control operators usually result in chronic poisoning. Man becomes exposed to pesticides via several means ; dermal, respiratory and gastrointestinal absorption. These are all potential routes of intoxication, although absorption via the skin is the most significant usual route of intoxication (Rosenstock, 1987). Occasionally the animal may become intoxicated when their premises are dusted or sprayed or animal may be directly dusted or washed with excessive amount of pesticides (Hatch, 1982).

Environmental contamination by the use of pesticides can result in a lot of hazards (Matsumura, 1975). This environmental contamination might be as a result of translocation of these pesticides from their site of application through the various media of the environment. For example, to control agricultural pest, application to pest causing health problems and industrial waste (Murphy, 1975). Environmental contamination by pesticides may not have a direct effect on man's health and welfare,

but if there is ecological imbalance man now suffers an indirect effects of the pesticides in which case the pesticide affects other species in the environment (White - Stevens, 1973).

Hazard caused by pesticides used in agriculture may be through its application to crops and consumption of the crop before or after harvest. Reports also show (Hoogendam et al., 1965) that acute poisoning do occur during manufacturing of pesticides. In a health survey of 300 workers in a plant manufacturing toxic organochlorine pesticides over a period of nine years, no fatalities or permanent injury was recorded but 17 of the workers, had convulsive intoxication and five out of the seventeen had more then one convulsion.

Increased control and regulation of pesticide use should prevent exposure to toxicologically significant quantities. Governments must aim at enacting legislation which will minimize these risks without seriously detracting from the benefits (Green, 1976).

In evaluating the potential health - related effects of all classes of pesticides, it is important to think of both the active ingredients and also the carriers which are often added to enhance delivery. Carriers mostly used include talcs, oil, solvents and binding agents. These carriers which are so called inert ingredient may themselves have toxic properties explaining some of the acute and persistent health effects, acting as irritant and less commonly sensitizing agents in exposed individuals (Rosenstock, 1987).

Assessment of the public health impact of agricultural pesticides must include an estimation of the number of cases of severe or minor effects of poisoning, number of fatalities and hospitalization (WHO, 1990).

2.2 ORGANOCHLORINE INSECTICIDES.

Turner (1982) reported that organochlorine insecticides are halogenated hydrocarbon with a wide range of practical applications against both agricultural pests and pests affecting man and animals. Thus, their introduction as agricultural insecticides made a remarkable contribution to the increase in crop and animal production. The use of halogenated hydrocarbon pesticides especially DDT in the area of public health and veterinary medicine have attracted most attention (Brooks, 1974).

The cost effectiveness of these pesticides has remained unequaled since their introduction some four decades ago. Indeed, the very hallmark of these compounds, which characterized them as wonder chemical, was the small amount needed over a given area to effect the complete control of pests. Effective use of these compounds also have some attendant complications such as its influence on the ecology of beneficial insects such as predators and aquatic insects (Brooks, 1974.)

In the early successes of the use of these compounds in insect control, there was development of resistance to them in target species. Emergence of insect resistance has led to major set back in the use of halogenated hydrocarbon pesticides (Brooks, 1974.)

Murphy (1975) reported that halogenated hydrocarbon pesticides have proved to be chemically and biologically stable and thus persist and accumulate in environments, thus discouraging their extensive usage. Interaction between halogenated hydrocarbon pesticides and the biotic and abiotic elements of the environment are of great concern to environmentalist, (Brooks, 1974 ; Turner, 1982). In an early stage of its development DDT tends to be stored in the body fat and is excreted in cow and human milk (Brooks, 1974).

Organochlorine pesticides are of moderate acute toxicity, but of greater potential for chronic toxicity than the organophosphate and carbamate insecticides. The organochlorine insecticides can also be classed as neuropoisons (Murphy 1975 ; Iyaniwura, 1990.)

2.2.1 TYPES OF ORGANOCHLORINE INSECTICIDES

Organochlorine insecticides includes DDT group, cyclodiene group and a group of heterogenous compounds that can be called a miscellaneous group (Hatch, 1982).

Hayes (1982) indicated that chlorinated hydrocarbon insecticides have a high lipid/water partition coefficient, thus enabling them to penetrate biological membrane quite easily and accumulate in the fatty tissue of organisms. The lipid solubility of the chlorinated hydrocarbon insecticides with their high resistance to enzymatic attack render them the least biodegradable of all pesticides. They persist in soil, organic matter, including food crops, domestic and wild animals.

Most organochlorine insecticides are waxy solids at room temperature. Their vapour are low , but not negligible, their volatilization leads to atmospheric contamination for instance Aldrin and gamma Benzene hexachloride (γ -BHC) are volatile compounds and this enables them to be use as soil fumigants.

2.2.2 TOXICITY OF ORGANOCHLORINES

Organochlorine insecticides includes DDT, BHC (Lindane), aldrin, dieldrin endrin, chlordane, heptachlor and camphechlor. They are all toxic compounds and diffuse stimulants of the central nervous system. Expressions of diffuse stimulant is

predominantly neuromuscular. Onset of symptoms may occur few minute or days after application of ingestion depending on dosage (Radeleff, 1964). Jay (1976) reported that affected animal first become apprehensive and hypersensitive or it may become belligerent, other symptoms of organochlorine toxicity include blepharo-spasms and fasciculation of facial and cervical muscles, those of the forequarters and finally of the hindquarters. Poisoned animals sometime become comatose and remain so for several hours before death. These various symptoms may lead to convulsion. Convulsive seizures may be repeated but once it begins it persists till death. Convulsive seizures following accidental exposure have also been noticed in man and the chlorinated hydrocarbon insecticides are especially recognized as human hazards (Gestaut et al, 1969).

Organochlorine insecticides are strong inducers of microsomal enzymes and can interfere with metabolism of various endogenous and exogenous compound. They are capable of causing kidney and liver damage (Waldboth, 1978). They also cause damage of the central nervous system by interfering with the transport of sodium and potassium ions across the nerve axon membrane.

Signs of chronic and acute toxicity are similar in outline, but usually appear as tremor in the muscle of the neck and head. It later extends to involve most of the muscles of the body which leads to difficulty in movement. The tremor leads to convulsion, depressions appears with respiratory failure and death (Clark and Clark, 1975).

Hayes (1969) reported that DDT is poorly absorbed in dermal exposure, this account for its good safety record in spite of its wide and sometimes careless use by

applicators and formulators. Inhalation of organochlorine insecticides dust results in lungs irritation, dizziness and paresthesia.

2.2.3 MECHANISMS OF ACTION OF ORGANOCHLORINE INSECTICIDES

Hassal (1982) noted that organochlorines insecticides are neurotoxic substances. The initial effect of DDT was on the peripheral nervous system while that of aldrin and lindane is on the central nervous system. General effect of all theses pesticides is to destabilize neural activity which is manifested by hyperexcitability of nerves and muscles.

DDT, considered to be a prototype organochlorine causes its destabilizing effect by producing a multiple of uncontrolled responses of poisoned nerves and muscular spasms following a single electrical stimulus. O'Brien (1967) observed that DDT is also capable of altering the transport of sodium and potassium ions across the membrane of nerve axons. It blocks potassium efflux across the membrane resulting in an increased negative after-potential. Membrane destabilizing effect of γ -BHC and dieldrin are similar to those of DDT, although the precise mechanism by which these effects occur is still obscure. Hassal (1982) proposed two hypothesis for the phenomenon. He reported that the insecticide may inhibit one or several enzymes of importance in the mechanisms of membrane stability or of nerve impulse transmission; the other possibility was that they may initiate some physical changes in the structure of the nerve membrane which rather literally causes it to misfire by alteration of its permeability to ions or to other substances (Hassal, 1982).

2.2.4 ENZYME INDUCTION BY ORGANOCHLORINE

Organochlorines are known for their ability to stimulate liver microsomal enzymes. Induction produces an increase in level and activity of the liver microsomal enzymes. Induction could also 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 activating or the detoxifying enzyme.

Studies conducted on DDT and toxaphene (Kinoshita et al, 1966) showed that maximal enzyme induction occurred after these pesticides were administered for one week, with a tendency to reverse these effects in subsequent weeks. Aldrin has been observed to increase liver aryl-esterase and to protect mice against parathion toxicity (Bell et al, 1954 ; Triolo and Coon, 1966). It was further noted that the rate of microsomal detoxification is more responsive to enzyme induction than microsomal activation producing the toxic metabolites.

2.3 DEVELOPMENT AND GENERAL FEATURES OF LINDANE

Chemical name of lindane is Benzene hexachlorocyclohexane (BHC), Figure 2.1. Lindane has been used as broad spectrum insecticide since early 1950s in the treatment of seeds and soil, application on trees, timber and stored materials, treatment of animal against ectoparasite and in public health.

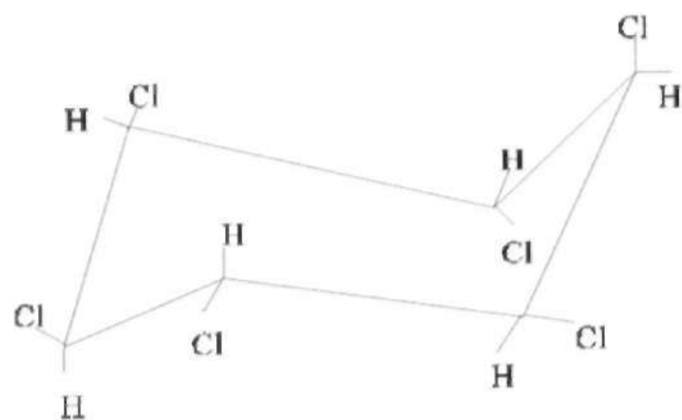

Figure 2.1 Chemical Structure of Lindane

BHC or benzene hexachloride or 1,2,3,4,5,6 hexachlorocyclohexane, empirical formula $C_6H_6Cl_6$ was first prepared in 1825 by Michael Faraday, who did not recognize the insecticidal properties. In 1912, Van-der Linden discovered four isomers. In 1942, Dupire and Raucout in France and Slade in England both discovered the insecticidal properties of BHC. The British group isolated the toxic gamma - isomer and named it Lindane in honour of Van-der Linden.

Lindane is colourless, crystalline solid with either a faint or no smell. BHC are relatively stable to light, high temperature, hot water and acid, and are dechlorinated in alkali. They are also relatively stable in water (10 ppm) (Gunther et al, 1968). Lindane is strongly adsorbed on soils that contain a large amount of organic matter and can move downward through the soil with water from rainfall or artificial irrigation.

Lindane undergoes rapid degradation (dechlorination) in the presence of ultra-violet irradiation, to form pentachlorocyclohexanes (PCCHs) and tetrachlorocyclohexanes (TCCHs). When lindane undergoes environmental degradation under humid or submerged conditions and in field conditions, its half-life varies from a few days to three years, depending on type of soil, climate, depth of application and other factor (WHO, 1991).

In industrialized countries 90% of human lindane intake originates from food. Over the last 25 years, selected food items have been analysed for lindane in a large number of countries using human exposed to lindane daily via food. It was found in blood, adipose tissue, and breast milk. The levels of intake, however, are also decreasing (WHO, 1991).

2.3.1 MECHANISM OF LINDANE TOXICITY

Lindane is a more acute nerve poison than DDT. In rats, lindane is absorbed rapidly from the gastrointestinal tract and distributed to all organs and tissue within a few hours. In various studies the highest concentration was found in adipose tissue and skin (WHO, 1991).

Uptake of lindane through the skins after dermal application is slow and occur to a very limited extent; this may explain the low toxicity of lindane after dermal exposure.

Acute oral toxicity of lindane is moderate, the LD_{50} for mice and rats is in the range of 60 - 250 mg/kg body weight, depending on the vehicle used. Toxicity of lindane was manifested by signs of central nervous system stimulation (WHO, 1991).

Lindane has been investigated for its effects on all aspects of reproduction in rats over three generations and for its embryo toxicity and teratogenicity after subcutaneous and intraperitoneal administration (WHO, 1991). It has no teratogenic effect after oral or parenteral administration. Maternal toxic effects were observed with doses of 10 mg/kg body weight and above (WHO, 1991). Lindane had no effect on reproduction or maturation in the three generation study in rats at doses up to 100 mg/kg diet (WHO, 1991).

In extensive investigation of lindane ability to induce gene mutations in bacteria and mammalian cells and for its capacity to induce sex linked recessive lethal mutation in *Drosophilla melanogaster*, negative results were obtained consistently. This implies lindane has no mutagenic potential.

Studies to define the carcinogenicity potential of lindane have been carried out in mice and rats using dose level of up to 600 mg/kg diet in mice and up to 100 mg/kg diet in rats (WHO, 1991). Hyperplastic nodules and/or heptacellular adenomas were found in mice given doses of 160 mg/kg diet or more (WHO, 1991). Studies in mice with dose levels of up to 160 mg/kg diet and in rats with 640 mg/kg diet showed no increase in the incidence of tumours (WHO, 1991).

The results on studies on initiation promotion of carcinogenity, on the mode of action, and on mutagenicity indicate that the tumorigenic response observed with BHC in mice is mediated by a nongenetic mechanism.

Several cases of poisoning and illnesses caused by lindane have been reported, which were either accidental, intentional (suicide) or due to gross neglect of safety precautions or improper uses of product containing lindane. Symptoms of human poisoning include nausea, restlessness, headache, vomiting, tremor, ataxia, convulsions. All these effects are reversible after discontinuation of exposure or symptomatic treatment (WHO, 1991).

Lindane is known to act as stimulant to the mammalian central nervous system (McNamara and Krop, 1947). This may also be true in the mammalian nervous system, since lindane poisoning can be antagonized by atropine in mammals (McNamara and Krop, 1947).

2.4 METABOLISM OF INSECTICIDES BY ANIMALS AND PLANTS.

Organic compounds apart from fat, carbohydrate, protein, vitamin, steroid, or mineral are considered to be foreign to the body. Such foreign substances are utilized by man as either drugs, pesticides for agricultural use, additive to food and fabrics. It is

thus important to know the fate of these foreign substances in animal, because organisms do eliminate them through various defense mechanisms.

Metabolism of foreign organic compounds, i.e xenobiotics was called detoxification. However, body do convert some compounds into more toxic substance, this was termed activation. Four types of chemical changes occur, these are oxidation, reduction, hydrolysis and synthesis.

There are two major steps of detoxification of xenobiotics ; the primary phase involving oxidative, hydrolytic and other enzymatic processes to produce polar end products is referred to as the non synthetic process while the secondary phase producing water soluble conjugates is refereed to as the synthetic phase. In the non synthetic process, three types of enzyme systems are involved in animals;

1. Hydrolases split the insecticides substrates through hydrolysis- examples are carboxylesterases, amidases, phosphatases, and A-type esterases (O'Brien, 1960).

2. Glutathione s-transferases depend on reduced glutathione (GSH) for their actions. Examples are DDT - dehydrochlorinase.

3. Microsomal oxidases, require NADPH, microsomes and oxygen in vitro for degradation of their substrates.

The most important biochemical reaction involving the initial stages of insecticides metabolism are the NADPH requiring general oxidation system and the hydrolysis of esters.

2.4.1 METABOLIC PROCESSES

(i) Mixed Function Oxidases (MFOs) System:

This is an NADPH- requiring oxidation system. It is located in the microsomal portions of various tissue particularly the liver (Brodie et al, 1958 ; Gillette et al, 1969). MFOs require NADPH as a co-factor. Cytochrome P450 is the electron transport system and is capable of oxidizing different kinds of substrates (i.e. substrate non specificity).

The activity of MFOs in the liver vary according to the nutritional and hormonal states of the animal and also to stimuli arising from the ingestion of some foreign compounds. Some reactions catalysed by MFOs include deamination, demetylation, dealkylation, aromatic ring hydroxylation, alkyl and N-hydroxylation, thioether and side chain oxidation (WHO, 1986).

One of the most important characteristics of MFOs is that they are sensitive to chemicals which contain methylene dioxyphenyl moiety (MDP). Mechanism of MDP inhibition is mainly competitive. Jay (1967) indicates that in housefly, microsome inhibition of naphthalene hydroxylation by sesamex is competitive while epoxidation of aldrin to dieldrin is inhibited by non competitive reaction.

(ii) Reduction:

This is much less common than oxidation, but for some compounds it is a general biochemical reaction. Reduction reactions that occur in the system of higher animals are:-

i Reductive dehalogenation where a halogen atom is replaced by a hydrogen atom, such as in the conversion of DDT to TDE.

ii Saturation of double bond.

iii Sulfhydryl formation from disulfide.

There are two types of reductive reactions that can take place on insecticide - NADPH or NADH- dependent or independent. An example of the NADPH-independent system is the reductive dechlorination reaction on DDT that yields TDE (DDD) as the major end product.

2.4.2 METABOLISM OF CHLORINATED HYDROCARBON INSECTICIDES

(i) Dehydrochlorination

This is the most characteristic reaction of organochlorine. This reaction involves the removal of a chlorine atom from the initial molecule with an hydrogen atom from the adjacent carbon, GSH (reduced glutathione) is required as the co-factor (Dinamarca et al, 1969). The resulting compound is an olefinic compound.

BHC apparently goes through a series of dehydrochlorination steps to yield various chlorinated olefinic and aromatic compounds. Clark et al, (1969), found that GSH plays an important role in BHC degradation in houseflies and grass grubs. There are probably more, substrate for various dehydrochlorination systems such as TDE, some Cyclodiene analogues and toxaphene but details of reaction mechanisms have not been worked out.

(ii) Reductive and Hydrolytic Dechlorination:

This involves replacement of chlorine atom by hydrogen atom and is a very common reaction in microbial world. This system requires a series of dechlorination and/or dehydrochlorination steps followed by hydrogenation steps. There are a number of

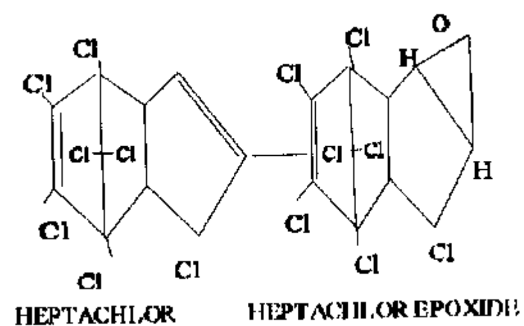

Figure 2.2 Epoxidation of Heptachlor

examples of hydrolytic dechlorination of Cyclodiene insecticides (Brooks, 1969).

In these reactions, chlorine atoms are replaced by - OH groups. Hydrolytic dechlorination reactions have a great susceptibility to pH changes and insensitivity to NADPH and methylenedioxyphenyl inhibitors such as sesamex. This type of reaction occurs only with the chlorinated chemical that are already unstable. For example, N-chlorotiazines such as atrazine, is easily hydrolysed to yield hydroxyatraxone.

(iii) Oxidative Reaction:

There are three types of oxidative attack on chlorinated insecticides. They are epoxidation of double bond, hydroxylation of either direct or substituted carbons or replacement of chlorine atoms and formation of aldehydes, ketones and acids from alcohols.

Epoxidation occurs with various Cyclodiene insecticides. At first it was assumed that only double bonds were attacked, e.g., aldrin to dieldrin, isodrin to endrin, and heptachlor to heptachlor epoxide (Fig. 2.2).

2.4.3 METABOLISM OF LINDANE

Lindane is metabolized mainly in the liver by four enzymatic reactions: dehydrogenation to gamma - HCH, dehydrogenation to gamma-PCCH, dechlorination to gamma - TCCH and hydroxylation to hexachlorocyclohexanol (WHO, 1991). End product of biotransformation of lindane are also said to be di-, tri-, tetra-, penta-, and hexachloro- compounds (Fig. 2.3). These metabolites are excreted mainly via the urine in the free form or conjugated with glucoronic acid, sulphuric acid or N-acetylcystein, (WHO, 1991). Elimination is relatively fast, with 3-4 days half life in rats.

There are two schools of thought regarding BHC metabolism. One group reported that gamma BHC first forms gamma-PCCH (gamma - 2,3,4,5,6 -pentachlorocyclohex-1-ene), which also undergoes various changes. The second group observed/reported that gamma PCCH is not an intermediate, but that gamma BHC is hydroxylated by an efficient system to yield various chlorophenols.

Biodegradation of lindane is faster in unsterilized than in sterilized soils. Anaerobic conditions are the most favourable for its microbial metabolism. Lindane in water is degraded by microorganisms in sediments to form the same degradation products. (WHO, 1991).

Rapid bioconcentration takes place in microorganisms, invertebrates, fish, birds and humans, but biotransformation and elimination are relatively rapid when exposure is discontinued (WHO, 1991).

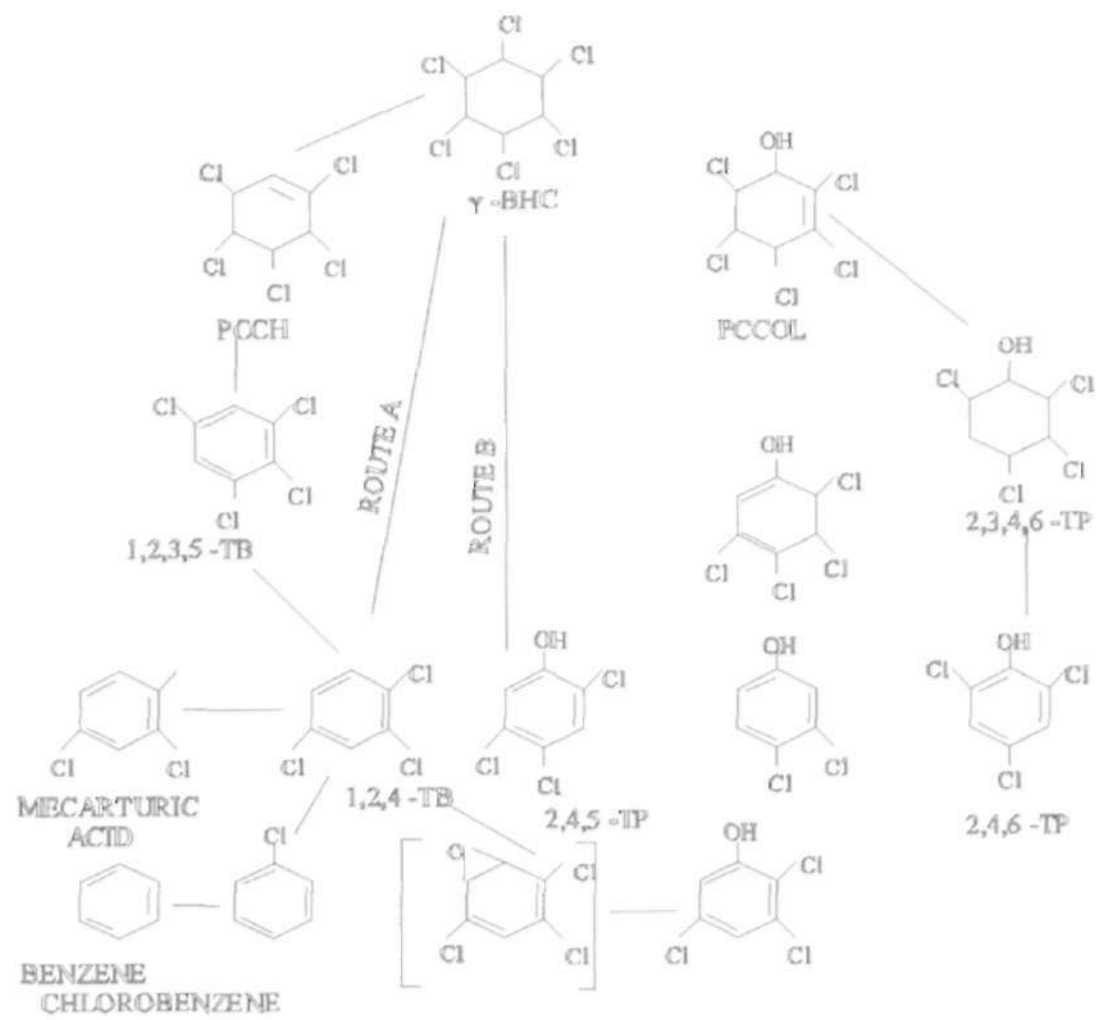

Figure 2.3 METABOLIC PATTERNS OF GAMMA-BHC IN RAT

2.5 LIVER ENZYMES AS INDICES OF CHEMICAL TOXICITY

Liver is the major site for metabolism of most substances or compound absorbed through the gastrointestinal tract (G.I.T). The liver thus holds a central position in the intermediary metabolism.

Mathenheimer (1971) identified the functions of the liver to include the following:

i) storage of nutrients and their release into the blood for example storage of carbohydrates in the form of glycogen and of sugar in the form of glucose into the blood.

ii) synthesis of fat from glucose, galactose and other sugars into glucose and the synthesis of proteins (albumin, some globulins, clotting factors), urea, cholesterol, purines and pyrimidine.

iii) Detoxification of chemical agent by conjugation of toxic substances with glucoronate and sulphate and metabolic conversion of drugs.

iv) Excretion of bile pigments, bile acids and cholesterol.

The toxic effect of a compound is assessed by the change in weight and structural abnormalities of the organ affected. However, Barns and Denze (1954) reviewed the usual test of chronic toxicity and emphasized their low sensitivity and their failure to reveal much about the mode of action of a toxic material. Thus, the function test are now employed for the assessment of organ damage in addition to the commonly used tests (Paget, 1970; Cutler, 1974). Report by Mathenheimer (1971), indicated that the measurement of activity changes of certain enzymes in blood serum is presently the most reliable indicator of liver disease.

Ringler and Dabich (1980) indicated that the clinically useful enzymes are normally found in the serum in relatively low concentrations, thus if there is increase in the serum concentration of a particular enzyme, this indicate damage to the cells that release the enzyme as the enzyme origin is in the cells. These authors further observed that serum enzyme responses usually, vary with the rate and magnitude of absorption of the toxicant and hence may serve as an indication of severity of exposure. It was therefore concluded by Ringler and Dabich (1980) that a correlation exists between rise in serum enzyme activity and severity of toxic damage to a particular tissue. In rat, diagnostic serum enzymology has mainly been directed towards early and reliable identification of liver injury due to toxicants. It has also been observed that the principal reason for an elevated serum enzyme activity in response to pathophysiological conditions was the liberations of enzymes from the damaged organ into the circulation (Wolf, 1972). From later report there could be decrease in serum enzyme activity which may arise occasionally in the case of a specific enzyme deficiency, as a result of blockage of *de novo* enzyme synthesis because of liver damage after prolonged exposure to toxicants (De Bruin, 1976).

The activity of hepatic enzymes in serum may be affected by their increased or decreased synthesis, release from extrahepatic tissues, release from damage cells and their rate of disappearance from the plasma (Wilkinson, 1976). Assay of the activity of serum enzyme is thus a very important approach to detecting and diagnosing toxic, also serve to identify liver disease, to define nature of the disease process and to locate the cellular and intracellular sites of hepatic pathology (Wilkinson, 1976 ; Gowenlock, 1988).

2.5.1 SERUM ENZYME AS AN AID IN THE DIAGNOSIS OF LIVER DISEASE

Liver diseases were among the first disorder to which serum enzyme tests were applied and has proved very useful in diagnosing disease of the liver (Ikediobi and Ukoha, 1985). The determination of enzyme concentration is now an indispensable diagnostic tool and only enzymes contained within an organ are released in injury or cell necrosis. It was indicated by Benirschke et al (1978), that the amount of enzyme released is related to its activity in a particular tissue, although there are exceptions. When cells are injured or disrupted as occurs in acute liver disease, enzymes are released into the serum and their increased level is of diagnostic importance (Wilkinson, 1976).

Liver disorders has been classified into broad categories of hepatocellular (acute and chronic hepatitis and cirrhosis); cholestatic (obstructive disease of the biliary tract); neoplastic and infiltrative disease (Wilkinson, 1976). In hepatocellular disorders, the author observed that the pathological changes are initially prominent in the liver cells (hepatocytes) while in cholestatic disease, they are principally in the biliary tract and in infiltrative and neoplastic disease are in the interstitial tissues.

Mathenheimer (1971) observed that the enzymes used to assess liver function include; the transaminases (AST and ALT), alkaline phosphatase (ALP), isocitrate dehydrogenase (ICD), alpha-hydroxybutyrate dehydrogenase (ABD) and lactate dehydrogenase (LHD). In the majority of clinical laboratories, the results of both the transaminases and alkaline phosphatases measurements are usually reported in response to a clinical request for 'liver function tests' (Wilkinson, 1976; Ikediobi and Ukoha, 1985).

The transaminases are the most important of a group of liver enzymes whose levels in serum are altered in hepatocellular disease, particularly in acute disease. They are therefore referred to as hepatocellular enzymes. Alkaline phosphatase is referred to as cholestatic enzyme because of its pronounced change in cholestalsis (Wilkinson, 1976; Ikediobi and Ukoha, 1985).

(i) The Transaminases (Aminotransferases)

The transaminases involved in liver function are Aspartate transaminase (AST, EC 2.6.1.1) and Alanine transaminase (ALT, EC 2.6.1.2).

Transamination involves the transfer of an amino group from an alpha-amino acid to an alpha-oxo acid without intermediate formation of ammonia (Wilkinson, 1976; Gowenlock, 1988). These enzymes are also called aminotransferases. All naturally occurring alpha-amino acids can undergo such reaction, since different enzyme are involved (Wilkinson, 1976; Gowenlock, 1988). The only transaminases that have found clinical application are aspartate transaminase (AST), and alanine transaminase (ALT) (Wilkinson, 1976).

The co-factor required by aminotransferases is pyridoxial-5-phosphate. Pyridoxial-5-phosphate is present in adequate amount in most normal sera, but may be deficient in some pathological state leading to decrease in activity of the aminotranferases (Kachmar and Moss, 1976). Transaminase activity has been observed to be widely distributed in mammalian tissue (Bergmeryer, 1974; Kachmar and Moss, 1976). Transamination plays a key role in intermediary metabolism as it provides a means for the synthesis and degradation of amino acids in living cells (Gowenlock, 1988).

With respect to its clinical importance, there is high concentration of transaminases in the liver (Wilkinson, 1976). Significant increase in the activities of the transaminases in serum are observed in acute hepatitis and liver cell injury, with activity of ALT being greater than that of AST, particularly in infective hepatitis (Kachmar and Moss, 1976; Gowenlock, 1988). Gowenlock (1988) observed that with liver cell necrosis or abnormal membrane permeability, the transaminase are released from the cell and their serum level increased. The level of transaminase in serum increases in acute hepatocellular damage and are also increased in chronic hepatocellular and cholestatic disease. AST is located in the cytoplasm and mitochondria while ALT is located only in the cytoplasm (Bergmeryer, 1974; Kachmar and Moss, 1976). The most prominent in mild tissue injury is the cytoplasmic AST and little mitochondria AST. Both AST and ALT are present in the bile but biliary excretion is not an important mechanism of excretion (Mossberg and Ross, 1963).

The picture observed in toxic hepatitis is similar to that found in infectious hepatitis with very high ALT and AST activities in severe cases. Both enzymes are thus excellent markers of liver damage caused by exposure to toxic substances (Kachmar and Moss, 1976). Ratio of AST activity has been used as an aid to the diagnosis of liver disease (De Ritis et al, 1972). In a normal individual this ration is about 1:3 (Hood, 1980). In the myocardium, the activity of AST is higher than that of ALT, thus in myocardial infarction, blood serum AST is seen to increase significantly with a significant rise in the AST/ALT activity quotient. In non liver disease for example heart disease. AST may be increased where as non-hepatic ALT elevation is unusual (Wilkinson, 1976; Gowenlock, 1988). An increased ALT activity is observed in diseased/toxic liver

(cirrhosis and serum hepatitis) and the AST/ALT activity quotient (De Ritis Ratio) is lowered. Thus, Wilkinson (1976) and Gowenlock (1988) observed that ALT elevation is more specific for liver damage than AST and is rarely found in heart or muscle disease, in the absence of liver involvement. AST is widely distributed in all body tissue except bone but has its highest concentration in heart muscle, liver, skeletal muscle and kidney (Kaneko and Cornelius, 1971; Wilkinson, 1976). It was also observed by the authors that erythrocytes contain about one tenth the normal serum level, hence slight haemolysis has little effect on the measurement of serum activity, though the activity may still be elevated.

Activity of serum enzyme is relatively low in normal individual but increase rapidly following traumatic injury or necrosis associated with muscle injury, liver damage, myocardial infarction, metastatic tumors, and acute anaemia (Kaneko and Cornelius, 1971 ; Wilkinson, 1976). Metabolic, infectious and various neoplastic disease has little effect on serum AST level when heart, liver or muscle tissue are not affected (Benirschke et al, 1978).

ALT, like AST is widely distributed in human tissue, but the richest source is the liver, thus measurement of its activity is of diagnostic importance in confirming or excluding liver damage (Wilkinson, 1976). ALT activity is high in liver of man, non-human primates, dogs, cats and rats, but is relatively low in pigs, sheep, horses and cattle (Benirschke et al, 1978).

Heart and liver diseases are the commonest cause of transaminases elevation. Determination of serum AST activity is also useful in myocardial infarction (Gowenlock, 1988). Damage to the liver due to hypoxia may also cause an increase in ALT within a

few days of the onset which is later followed by increase due to congestive heart failure (Gowenlock, 1988).

Bruising and stress may occur when an animal, especially a subhuman primate, is manually caught and forcefully restrained for the withdrawal of blood, and this can increase the transaminases originating from the skeletal muscles (Robinson et al, 1964). Wolf (1972) observed that increased serum levels of AST and ALT, include enzyme release from injury cells and that increased production of the enzyme by various cells are related to various diseases.

(ii) The Phosphatases

Phosphatases are in the class of enzyme known as hydrolases. Hydrolytic enzymes catalyses the scission of compounds containing acyl or phosphoryl (Phosphate) ester bonds as well as compounds containing peptide, amide and similar bonds (Kachmar and Moss, 1976). Phosphatases are hydrolases with low specificity characterized by their ability to hydrolyse a large variety of organic phosphate esters with the formation of an alcohol and a phosphate ion.

Phosphatase activity in serum and biological fluids is measured by determining the rate of hydrolysis of various phosphate esters under specified conditions of temperature and hydrogen ion concentration. Phosphatase is of considerable importance in the transport of sugar and phosphate in the intestine, kidney, placenta and bone (Wilkinson, 1976; Gowenlock, 1988).

The phosphatase of interest is the alkaline phosphatases. Alkaline phosphatase (ALP EC 3 1.3.1; Orthophosphoric acid monoester phosphohydrolase) have optional activity at a pH of 8 to 10. ALP may act as hydrolases whereby inorganic phosphate is liberated

or as phosphotranferases whereby the liberated phosphate radical is transferred to an acceptor molecule.

ALP is widely distributed in most tissues of the body but richest location is the intestinal mucosa, osteoblasts of the bone, biliary canaliculi of the liver, placenta and lactating mammary glands (Kachmar and Moss, 1976; Benirschke et al, 1978; Gowenlock, 1988). In each case the enzyme is located in the cell membrane where it plays a role in transport mechanism involving phosphate.

Several methods can be used for the determination of this enzyme (Kachmar and Moss, 1976). The method of Bondansky, King-Armstrong and Bessy -Lowry -Brook - can be used for the enzyme assay. For the current study, the method of King-Armstrong was adopted. This method involves the use of a substrate of phenyl phosphate at a pH of 9.0, the phenol liberated can be determined by the use of folin reagent. the unit is defined as the activity producing 1mg phenol in 30 minutes per decilitre of serum.

With respect to its clinical importance, serum ALP estimation is used in clinical diagnosis of disease associated with bone and liver (Kachmar and Moss, 1976). In the liver, ALP activity is found at the sinusoidal surface of the liver cells and in the microvilli of the bile canaliculli (Wilkinson, 1976; Gowenlock, 1988), activity may also be prominent in the endothelial cells of the portal and central veins. It was also observed that the highest rise in serum ALP activity occur in post hepatic obstruction and this is said to be related in part to increased synthesis of this enzyme (Wilkinson, 1976; Gowenlock, 1988).

In non-pregnant healthy adult, the liver cells are the major source of ALP, though bone and intestine may also contribute (Hudson et al, 1962). Ikediobi and Ukoha

(1985), observed that impaired secretion of liver ALP into the bile results in an increase in serum ALP of liver cells origin whereas in infectious (viral) hepatitis, ALP will almost not be elevated. Acute cellular necrosis liberates little phophatase into the circulation and serum levels are minimally altered in chronic hepatocellular disease in the absence of concomitant biliary obstruction (Gowenlock, 1988).

Growing bone contains more ALP than mature bone since higher serum value are found among immature animals of all species than among adult (Earl et al, 1971; Kaneko and Cornelius, 1971). However, Gutman (1959) and Young (1967) had earlier observed that pregnancy produces serum ALP levels above the normal adult range as a result of increased placental and bone ALP. ALP was observed to be associated with formation of new bone and destruction of old bone, it is also associated with calcium and phosphorus metabolism (Gutman,1959 ; Young, 1967). The enzyme has also been shown to be increased in nutritional treatment of certain diseases, such as rickets and osteomalacia of dogs and osteodystrophia fibrosa of primates due to vitamin D deficiency, indicating that there was a positive correlation between food consumption and serum ALP levels (Benirschke et al, 1978).

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2.5.2 SERUM PROTEIN

Serum proteins consist essentially of albumin and globulin. The liver produces all albumin and most globulins except for a small amount of gamma-globulin which is produced by the reticulo-endothelial tissue. Albumin is thus the major serum protein in both man and rat (Ringler and Dabich, 1987). Other known serum protein are lipoproteins, glycoprotein, fibrinogen, prothrombin and clotting factors vii, viii, ix and x which are all synthesised by the liver (Benington et al, 1970).

Colorimetric method is the most widely used method for the determination of serum total protein and is based on the biuret reaction. Biuret reaction involves reactions between cupric ions in alkaline solution and a number of compounds containing at least two peptide linkages, as well as a number of simpler compound containing two -$CONH_2$, $-CH_2NH_2$, or simpler groups joined directly or through a C or N atom (Gowenlock, 1988).

With respect to its clinical importance, total serum protein is made up of albumin and globulin fractions. In clinical work, the concentration of protein is given in grams per 100 ml or per litre of serum volume. Total protein concentration normally ranges form 60-80 g/l, serum albumin concentration ranges from 30-50 g/l and globulins from 20-35 g/l (Grants and Kachmar, 1976).

In diseased states, both the total protein and the ratio of the individual protein fractions may change independent of one another (Grants and Kachmar, 1976). Hypoproteimia is characterized by total protein levels below 60 g/l and is encountered in many unrelated diseased states. For instance, parasitism may result in a decreased in total serum protein, albumin and an increase in the concentration of gamma globulin

(Dimopoullous, 1970). Glomerular damage in renal disease allows albumin and some globulin to pass into the urine. When the situation is severe, it may lead to hypoalbuminemia (Benirschke et al, 1978). Significant increases in total protein concentration in disease arises from increase in total globulins, usually gamma-globulins while decrease in total protein is due to fall in albumin or sometimes gamma-globulin (Gowenlock, 1988).

Change in serum protein is associated with may diseased conditions except in case of dehydration. In hepatoxic condition, the liver shows diminished synthetic ability and since most of the serum protein are synthesized by the liver, there will be a reduction in the supply of these protein to the circulation (De Bruin, 1976). Serum protein estimation is therefore regarded as a valid procedure for the estimation of degree of damage to the parenchyma of the liver.

In most infections, serum albumin levels are moderately depressed (32 to 42 g/l) (Gowenlock, 1988). In condition such as liver disease or congestive heart failure, levels of 23 to 32 g/l are common (Grants and Kachmar, 1976). Impaired hepatocellular function or decreased functional mass of the liver usually results in decreased protein synthesis and the concentration of albumin may also decrease as a result (Gowenlock, 1988).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 TEST CHEMICAL.

Lindane (Gammalin 20) ICI Plant Protection Division, England, a synthetic organochlorine insecticide containing lindane in a concentration of 20 percent (ULV)" was used in this study.

OTHER CHEMICALS

These are various reagents used for the assays of

a) Total protein and serum albumin

b) Alkaline phosphatase

c) Aspartate and Alanine transaminase

d) Formalin 10 percent (May and Baker, Dagenham England) was used for fixing the tissue prior to their processing.

Absolute alcohol (Hopkin Williams Chadwell-Health Essex England) was used in tissue processing.

3.2 TEST ANIMALS

Test animals were adult albino rats of both sexes which were purchased from the animal house, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. They were maintained in cages with free access to water and feed. All rats were first observed for about two weeks prior to start of study. Only healthy animals from appearance were used for the study.

3.2.1 ANIMAL FEEDS

Commercial Pfizer feed pellets (Livestock Feeds, Lagos Nigeria) were used to feed the rats before the start of the study, and during the acute toxicity study, For sub-chronic toxicity study, local Grower's mash and cassava flour (as binders) were used to incorporate the drug into the feeds.

3.3 MATERIALS

1) A disposable syringe for measuring the quantity of the test chemical solution for the various dose levels in subchronic toxicity test and also for the acute toxicity study (test). Syringe and needle were used for the intraperitoneal injection of the test animals.

2) 20ml universal bottles to store prepared chemicals for the various enzymes and protein assays and also for storage of tissue specimens, for mixing each dose levels of test chemical with water prior to mixing with the feed.

3) 500ml flasks used for mixing each dose levels of test chemical with water prior to mixing with the feed.

4) Plain/non heparinized bottles for collecting whole blood samples and Boujior bottles for collecting serum.

5) Avery balance (L1 and T. Avery limited, Birmingham, England) for weighing the animals and feed.

6) Non- reactive and water soluble markers to mark individual animal on the tail for identification.

7) Plastic cages to keep the rats according to the various dosage levels of the chemical incorporated into feed.

8) 250ml and 500ml plastic bottles were used to administer drinking water to the animals.

9) Centrifuge machine (International centrifuge, Model K. No. 20130P) for centrifuging the whole blood in order to collect serum.,

(10) Colorimeter (Corning 253).

ROUTE OF ADMINISTRATION OF CHEMICALS

For the acute toxicity study, route of administration of lindane was intra peritoneally while for the chronic toxicity study it was orally.

3.4 ACUTE TOXICITY STUDY (Determination of the Median Lethal Dose Intra peritoneally [IP])

The median lethal dose (LD_{50}) of lindane in rats was determined using standard procedures (Aliu and Nwude, 1982). Pilot studies were initially carried out to determine the minimum dose that would give 100 percent death and the maximum dose that will not produce any death in the rats (Aliu et al, 1982). These dose levels were further used as focal points for the determination of dose range to be used in the subsequent experiments. The acute toxicity test was carried out on 25 healthy albino rats (average weight, 105g). The

animals were divided into 5 experimental groups (six rats per group). The fifth group served as control. Animals in the experimental group were given corresponding dose of lindane viz 5 mg lindane/Kg feed, 10 mg lindane/Kg feed and 15 mg lindane/Kg feed. The control animals received only water. Route of administration was intraperitoneal.

Symptoms of toxicity were observed and recorded for each rat in the various dose groups. The number of animals that died within 24 hours were noted for each group. The LD_{50} was calculated by Arithmetic method of Karber (Aliu and Nwude, 1982). The surviving rats were observed for 14 days for delayed toxicity symptoms.

$$LD_{50} = D_{min} - \frac{\sum (DOSE_{diff} \times Mean\ Dead)}{n}$$

Where:

LD_{50} = The dose of the chemical that killed 50% of the animal population

D_{min} = Minimal dose that killed all animals

$DOSE_{diff}$ = Dose difference

n = Total number of animal per group

3.5 CHRONIC TOXICITY STUDY

(i) Preparation of Lindane Treated Feed

Administration of drug was by oral route through inclusion in the diet. The sub-chronic toxicity study was designed to last for a period of 12 weeks. Four dose levels were chosen thus 0.00 mg/kg feed which serve the control group, 5 mg of lindane/kg feed,

10 mg/kg feed and 15 mg/kg feed. These dose levels were calculated from the concentration of test drug as follows:

Concentration of lindane in Gammaline 20 is 20% W/V. This implies that 100ml of Gammaline 20 contains 20g of lindane. Therefore 1ml of Gammaline 20 will contain 200 mg lindane. The required test chemical for each dose level will be

Control group; no drugs was added.

5 mg/kg feed group, (5/200) ml/kg feed was added

10 mg/kg feed group, (10/200) ml/kg feed was added

15 mg/kg feed group, (15/200) ml/kg feed was added.

The appropriate volume of Gammaline 20 used for each dose group as obtained from the above calculation were diluted with water and mixed with the weighed out feed (in kg). The well mixed feed was moulded and properly sun dried. Animals were fed ad libitum and weighed at weekly interval throughout the period of study.

(ii) Experimental Design

There were 20 animals per group at the onset of these experiments (sub-chronic toxicity study). Animals were observed for alteration in behaviour and any unusual clinical symptoms resulting from drug effects. At the end of the 4th, 8th and 12th weeks respectively, five animals were selected from each experimental group and sacrificed for liver function tests. Animals were restrained by holding them at the neck region. Blood sample were collected from the neck region and dispensed directly into the centrifuge tubes, sera were harvested after clotting and centrifugation and then stored frozen until assayed for liver function . Liver tissues were collected and fixed in 10% formalin for histopathology studies.

3.5.1 HISTOPATHOLOGICAL STUDIES

(i) Tissue Processing and Sectioning

Fresh liver tissue samples from the treated rats and control rats were fixed in 10 percent formalin. Tissue slices of 3-4 cm thickness were cut from each liver for processing. The tissue slices were transferred into the automatic tissue processor where they were further fixed in 10 percent buffered formal saline for about 2 hours, and then dehydrated for 2 hours in each of the ascending grades of alcohol (85%, 90% and 100% W/V). The dehydrated tissues were cleared in toluene for 2 hours and tissues were impregnated with 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 floated in a warm water bath, suitable sections were selected, attached to slides, dried on hot plate and subsequently stained.

(ii) Staining of Sectioned Tissue (Haematoxylin and Eosin stain- [H $ E stain])

The sectioned tissues 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 percent acid alcohol, blued in Scott's tap water and stained in eosin for 3 minutes. They were subsequently rinsed and dehydrated in ascending grades of alcohol solution 70%, 90% and 100% and finally cleared in xylene and mounted in epoxyl.

These slides were then observed under the microscope for lesions with the assistance of a medical pathologist at the Ahmadu Bello University Teaching Hospital, Zaria.

3.5.2 LIVER FUNCTION TESTS

(i) Determination of Serum Aspartate (AST) and Alanine Aminotransferase (ALT).

The aminotransferases were estimated by the colorimetric method of Reitman and Frankel (1957). Aminotransferase catalyses the transfer of the amino group from aspartate to oxoglutarate. Aspartate was converted to oxaloacetic acid. The oxaloacetic acid formed was relatively unstable subsequently decarboxylates into pyruvate which was measured by its colour reaction with 2.4 dinitrophenylhydrazine to form the corresponding phenyl hydrazone, a coloured compound whose intensity reflects the concentration of oxoacids present.

Alanine aminotransferase catalyses the transfer of the amino group from alanine on to oxoglutarate. Alanine was converted to pyruvate. The pyruvate produced by the transamination activity of ALT reacts with 2,4, dinitrophenylhydrazine (DNPH) to give a brown coloured hydrazone whose intensity reflects the concentration of the oxoacid present. These reactions were carried out at alkaline pH and the brown colour was measured colorimetrically at 510 nm. The procedure used in the preparation of the reagents are explained in Appendix-I, while that of the assay is in Appendix-II.

The calculations of Serum Enzyme Activity (SEA) [I.U/l] were carried out using the following equation:

$$SEA = \frac{T-TB}{S-SB} \times \frac{\text{Conc. of STD}}{\text{incubation time}} \times \frac{1000}{\text{Vol. of complexes}}$$

I.U. (International Unit) of an enzyme is that amount that catalyses the formation of 1umole of product/min under defined condition.

(ii) Determination of Serum Alkaline phosphatase (ALP)

The serum alkaline phosphatase was assayed using King - Armstrong method (Table 3.2.) Phosphatases are enzymes which catalyse the hydrolysis of monophosphate esters.

In this assay, disodium phenyl phosphate is hydrolysed by phosphatase at pH 10 and an incubation temperature of 37°C. The hydrolysed product, phenol then subsequently reacts with alkaline oxidizing chromophores, 4 - amino phenazone to give a red colour, the intensity of which is proportional to the enzymatic activity. of reagents used in assay of alkaline phosphatase (ALP). The procedure used in the preparation of the reagents are explained in Appendix-III, while that of the assay is in Appendix-IV.

Serum Alkaline phosphatase (ALP) activity was calculated using the following equation:

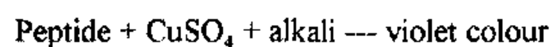
$$SerumALP = \frac{[Test\ Reading] - TB}{[KA\ unit\ /\ 100ml]\ x\ [StandardReading] - B} \ x\ 10$$

The King-Armstrong (KA unit) of phosphatase is the concentration of enzyme which will set free 1 mg of phenol in 15 minutes at pH 10 and temperature of incubation 37°C for the alkaline phosphatase. To obtain unit in i.u, K.A units values is multiplied by 7.1

3.5.3 SERUM PROTEIN TEST

(i) Determination of Serum Total Proteins.

Serum total protein was determined by Biuret method (See appendix-V). This method is based on reaction of copper ion in alkaline solution with the peptide bonds in protein, producing a violet colour which is proportional to the amount of protein present.



Peptide + $CuSO_4$ + alkali --- violet colour

The biuret reaction is positive for proteins and peptides bearing at least two peptide bonds (-C-NH). Total serum protein is determined by comparing the intensity of a coloured solution of dilute protein with that of a known protein concentration (standard solution). The biuret reaction takes its name from the fact that the simple substance, biuret (NH_2, CO. NH, CO. NH_2), gives the same colour as cupric ions. Details of reagent preparation is shown in Appendix-VI. Each copper atoms complexes with four molecules of biuret, the linkages being the central nitrogen atom. The violet colour is due to the formation of a complex with copper ions (Fig. 3.1)

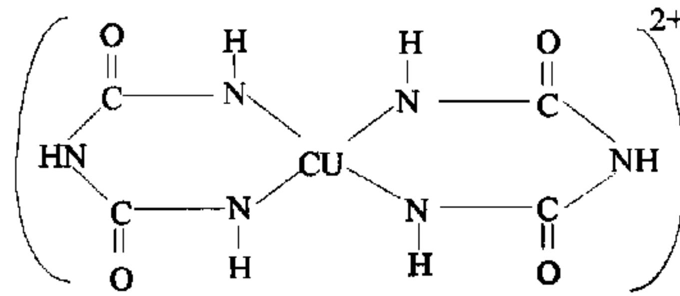


Figure 3.1 Biuret Complex Formation with Copper Ions

The resultant solutions are mixed and incubated in a water bath at 37°C for 10 minutes. After colour development the tubes were cooled to room temperature and the absorbance read on the colorimeter at 540nm using reagent blank to zero the instrument. The absorbance were read off from a standard calibration curve (see appendix-X).

(ii) Standard Calibration Curve

The unknown protein concentration in the serum was compared against a standard solution of bovine albumin. See appendix - IX.

Bovine serum albumin (BSA) of concentration 10g/l in 0.9% NaCl (Normal saline) was used for the standard curve for which subsequent protein concentration of

serum are obtained. Absorbance of the contents in each tube was read at 540 nm against a blank. (See appendix-X for the standard calibration curve).

(iii) Determination of Serum Albumin

Serum albumin was determined by the modified Batholomew and Delaney Method (1966). The principle involves the reaction of Albumin with the dye-Bromocresolgreen which has specific affinity for albumin in acidic solutions to produce a coloured complex which is measured colorimetrically. The intensity of the colour produced is proportional to the concentration of albumin in the sample. (See Appendix VII for the preparation of reagents used in Serum albumin assay)

To an aliquot of 0.02ml serum was added 4.0ml of the working Bromocresol dye solution. The mixture was mixed, and incubated at 37°C for 10 minutes. The absorbance was read at 628 nm after bromocresolgreen was used to set the instrument at zero. Serum albumin concentration was calculated using the formula below:

$$\text{Serum Albumin Level} = \frac{\text{Test Reading}}{\text{Standard Reading}} \times \text{Conc. of Std. } g/100ml$$

3.5.4 STATISTICAL ANALYSIS

Analysis of the biochemical data in this study was carried out using the student t-test. This test deals with an aspect of statistical inference (using statistic to take decision). it is derived thus.

t = [difference between means]/[standard error of difference].

From this test the level of significance for the difference biochemical parameters at the various dose levels were determined.

CHAPTER FOUR

RESULTS

4.1 ACUTE TOXICITY STUDY

Signs of toxicity following the administration of different doses of lindane to the experimental rats by intraperitoneal route were tremor, prostration. pallor and convulsion. In most of the groups convulsion and death were observed at doses of 20 mg/kg and above. All the symptoms (tremor, prostration, falling, convulsion and death) observed were dose dependent.

Symptoms observed in the rats were scored in order of severity and all symptoms are scored as they appeared for each rat in various experimental groups.

Table 4.1: Score of symptoms observed in poisoned rats (IP)

SYMPTOMS	SCORES
Tremor	+1
Prostration	+2
Pallor	+3
Convulsion	+4
Death	+5

The mean of scores of symptoms and standard error of mean for each group were calculated for each dose group (See table 4.2).

Table 4.2: MEAN SCORES + STANDARD ERROR OF MEAN

EXPERIMENTAL GRP	DOSE (MG/KG)	MEAN SCORE + S.E.M
1	10	39.2 ± 0.6
2	20	80.5 ± 0.7
3	30	100.2 ± 0.8
4	45	150.5 ± 0.5

At high doses, convulsion appears to predominate which suggest that there is considerable central nervous system stimulation Lindane had been shown to be neurotoxic. Symptom of toxicity appears to be mainly central (Hassal, 1987). There were no death at doses below 10 mg/kg highest death was recorded for animals given 45 mg/kg body weight, at this dose level, 100 percent death was recorded.

4.1.1 MEDIAN LETHAL DOSE VALUE OF LINDANE IN RATS USING I.P. ROUTE

The median lethal dose (LD_{50}) value of lindane was determined by using the Arithmetic method of Karber adapted by Aliu and Nwude (1982). The product of the mean dead and dose difference divided by the total number of animals per dose group was subtracted from the least dose that killed all the animals in an experimental group to obtain the LD_{50} value.

Results of the acute toxicity test for lindane are as shown in Tables 4.3 and Figure 4.1. The table indicate the various doses of lindane administered per group of the rats and the symptoms of toxicity recorded within 24 hours of dosing. The data in Table 4.3. was processed for the calculation of the median lethal dose (LD_{50}) of lindane using the

Arithmetic Method of Karber (Aliu and Nwude, 1982). Figure 4.1 relate the severity of intoxication with the various dose levels of lindane administered.

Table 4.3: 24 hr Mortality Rate on Lindane Administered

Expt. Group	Dose Admin.	Dose Diff.	No. Dead	Mean Dead	Dose Diff x Mean Dead
1	10	0	0	0	5
2	20	10	1	0.5	30
3	30	10	5	3.0	82.5
4	45	15	6	5.5	-
5	Control	-	-	-	117.5

Dose of lindane administered and the resulting mortality rate in a 24 hour period.

from table 4.3

LD_{50} = 45 - 117.5/6

= 45 - 19.6

= 25.4mg/kg body weight.

Experimental animals in group 5 were given water only. Total number of animals per group is 6.

The least dose that killed all the animals in a group was 45 mg/body weight. Sum of dose difference x mean dead was 117.5

From the results obtained from the acute toxicity studies of lindane using the intraperitoneal route, 25.4mg/kg was obtained as the LD_{50} for lindane.

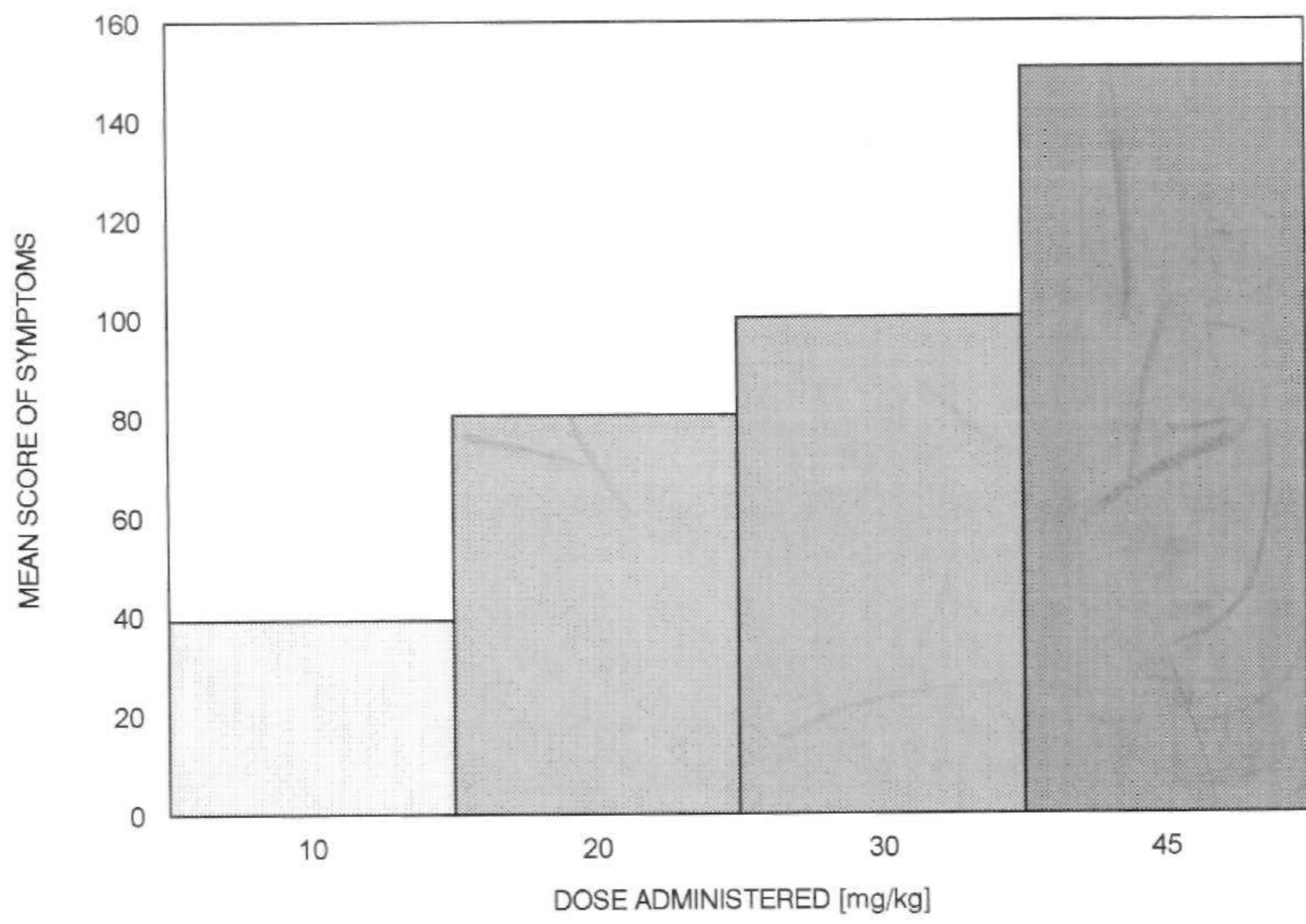


Fig 4.1 DOSE-RESPONSE RELATIONSHIP IN RATS ADMINISTERED WITH LINDANE I/P.

4.2 CHRONIC TOXICITY STUDY

The results of the chronic toxicity study are shown in tables 4.4. to 4.9.

Table 4.4 Shows the various dose levels of lindane incorporated into rat feed throughout the three month study period and mean ± standard error of mean obtained from assays of the various biochemical parameters assessing liver function at month 1, 2 and 3. Five rats per experimental group were killed for each assay.

TABLE 4.4: DATA FOLLOWING ORAL LINDANE ADMINISTRATION THROUGH FOODS FOR THREE MONTHS.

MONTH OF STUDY	MEAN VALUES ± STANDARD ERROR OF MEAN					
	DOSE LEVEL [mg/kg Feed]	AST [i.u/l]	ALT [i.u/l]	ALP [KAu/l]	TOTAL PROTEIN [gm/l]	SERUM ALBUMIN [gm/l]
1st [n = 5]	CONTROL	60.4 ± 0.48	55 ± 1.14	42 ± 0.71	70.2 ± 0.80	32.2 ± 1.43
	5	112.2 ± 1.68	118 ± 1.73	36.4 ± 1.38	85.8 ± 1.68	47.8 ± 2.37
	10	89.8 ± 2.72	106 ± 2.99	61.2 ± 1.49	101 ± 2.45	42.8 ± 1.53
	15	89.8 ± 2.70	105 ± 3.30	85 ± 2.49	75 ± 1.41	39.6 ± 1.75
2nd [n = 5]	CONTROL	60 ± 1.14	57.8±2.33	40.2 ± 1.42	68.8 ± 2.41	31.6 ± 0.87
	5	69.2 ± 9.06	131.6±1.8	22.0 ± 2.91	64.0 ± 3.02	31.4 ± 1.16
	10	54.2 ± 6.16	102±7.42	49.6 ± 1.25	79.2 ± 5.02	35.0 ± 1.92
	15	83.6 ± 4.09	41.6 ±4.15	20.4 ± 3.41	83.2 ± 4.59	36.8 ± 1.93
3rd [n = 5]	CONTROL	68.4 ± 1.69	60.0 ±1.58	40.0 ± 0.63	75.0 ± 1.14	34.6 ±2.16
	5	76.6 ± 2.44	108.6±2.5	26.0 ± 1.0	93.8 ± 2.37	45.0 ± 2.19
	10	64.0 ± 1.48	106.2±2.9	33.2 ± 1.9	103.6±4.94	42.4 ± 1.77
	15	75.4 ± 2.85	109.2±3.2	41.4 ± 2.2	85.2±3.15	41.6 ± 1.6

The level of significance were also compared in table 4.6 and 4.7. The levels of significance of the difference in the mean values between control and experimental groups for each parameter was shown in figures 4.2 to 4.4 using data obtained from table 4.4, while figures 4.5 to 4.9 constructed also from tables 4.4 shows the variation in enzyme levels for each dose level over the 3 months study period.

Table 4.5 shows the reference range of normal values obtained for the various biochemical parameters employed for the current study.

Table 4.5 REFERENCE RANGE OF NORMAL VALUES

PARAMETERS	REFERENCE RANGE (MEAN ± 2SD)
AST	53.35 - 72.51 iu/L
ALT	52.5 - 62.7 iu/L
ALP	38.58 - 49.93 KAU/DL
TOTAL PROTEIN	64.8 - 77.8 Gm/l
SERUM ALBUMIN	29.62 - 35.98 Gm/l

The level of significance of difference in the mean values for successive month intervals of study areas shown in the table.

Table 4.6: COMPARISON OF MEAN VALUES OF SERUM CONCENTRATIONS OF AST, ALT, ALP, TOTAL PROTEIN AND ALBUMIN BETWEEN SUCCESSIVE MONTHS OF STUDY.

Type of Group	Month of Study	AST	ALT	ALP	Total Protein	Albumin
GP2	1st Vs 2nd	P<0.01	P<0.01	P<0.01	NS	P<0.05
	2nd Vs 3rd	NS	P<0.001	NS	P<0.001	P<0.001
GP3	1st Vs 2nd	P<0.001	NS	P<0.001	P<0.01	NS
	2nd Vs 3rd	NS	NS	P<0.001	P<0.001	P<0.05
GP4	1st Vs 2nd	NS	P<0.001	P<0.001	NS	NS
	2nd Vs 3rd	NS	P<0.001	P<0.001	NS	NS

GP2 = 5 mg lindane/kg Feed Group

GP3 = 10 mg lindane/kg Feed Group

GP4 = 15 mg lindane/kg Feed Group

NS = Not Significant

4.2.1 CHANGES IN SERUM PARAMETERS AT 1ST MONTH

During the first month, the mean serum AST, ALT, total protein and albumin in rats treated with 5mg lindane per kg feed was significantly elevated when compared with the control subjects ($P<0.001$), while a significant decrease in ALP was observed when compared with the control group ($P<0.01$) (Fig. 4.2). There was significant elevation in all the parameters in rats treated with 10 mg lindane per kg feed when compared with the control subjects ($P<0.001$). For rats treated with 15 mg lindane per kg feed, the mean serum AST, ALT and ALP were significantly elevated when compared with the control subjects ($P<0.001$), while significant increase in total protein and albumin were observed when compared with the control subjects ($P<0.02$).

4.2.2 CHANGES IN SERUM PARAMETERS AT 2ND MONTH

At the second month, the mean serum ALT in rats treated with 5mg lindane per kg feed was significantly elevated when compared with the control group ($P<0.001$), the mean serum ALP was significantly reduced when compared with the control subject ($P<0.001$), (Fig. 4.3) while for AST, total protein and albumin there was no significant difference in the mean values when compared with the controls. There was no significant difference in mean serum AST, total protein and albumin for rats treated with 10mg lindane per kg, feed when compared with the controls while the mean serum ALT and ALP were significantly elevated when compared with the control group ($P<0.001$ and $P<0.01$ respectively). The mean serum AST in rats treated with 15mg lindane per kg feed was significantly elevated when compared with the control subjects ($P<0.001$), a significant decrease in ALT and ALP was observed when compared with the controls ($P<0.01$ and $P<0.001$ respectively) while a

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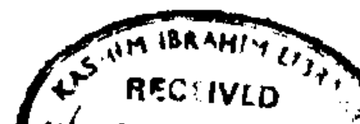

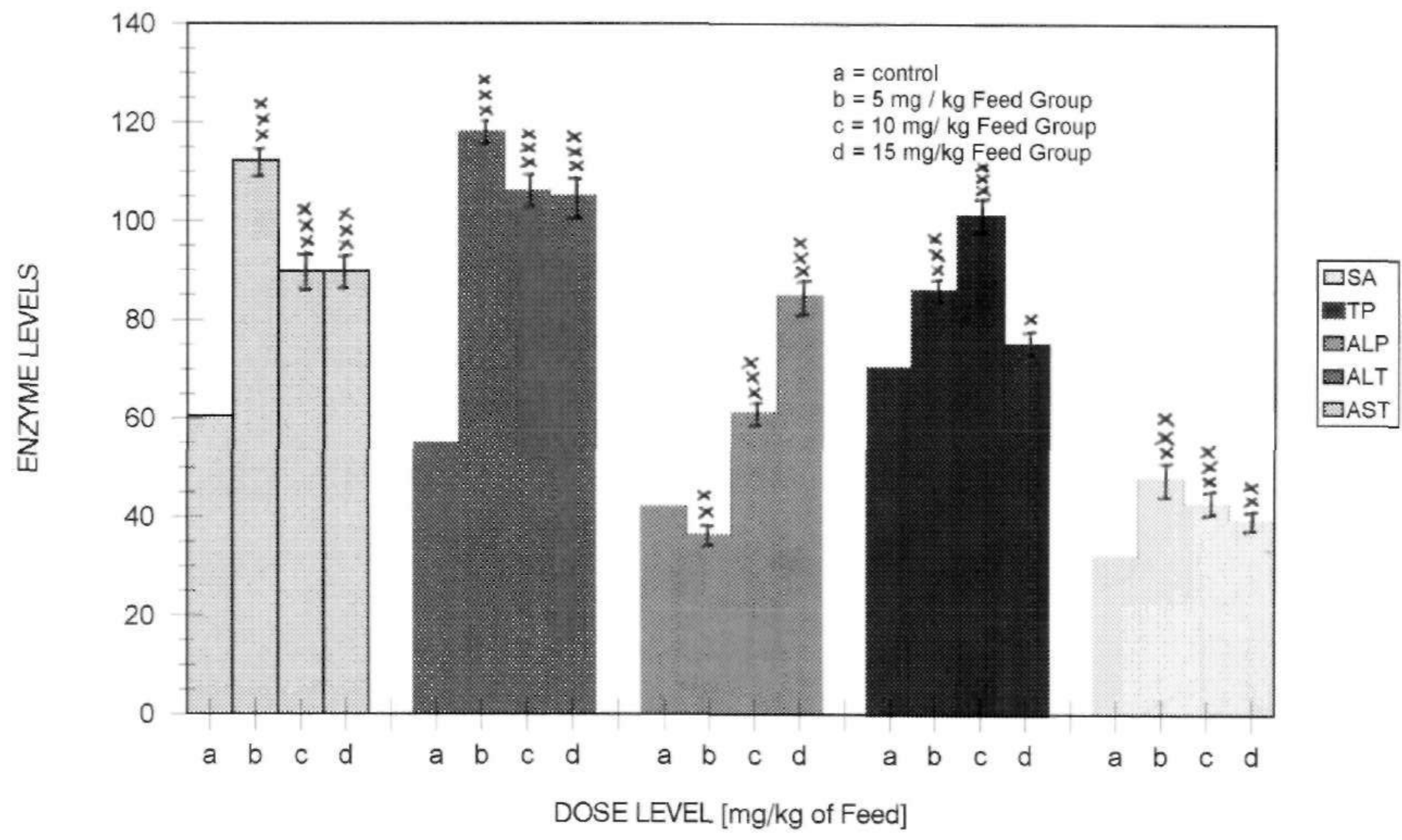

FIG. 4.2 : ENZYME ACTIVITY PROFILE (During the First Month of Study)

TEST OF SIGNIFICANCE [From Control]
* Represents $P < 0.05$
** Represents $P < 0.02$ OR $P < 0.01$
*** Represents $P < 0.001$

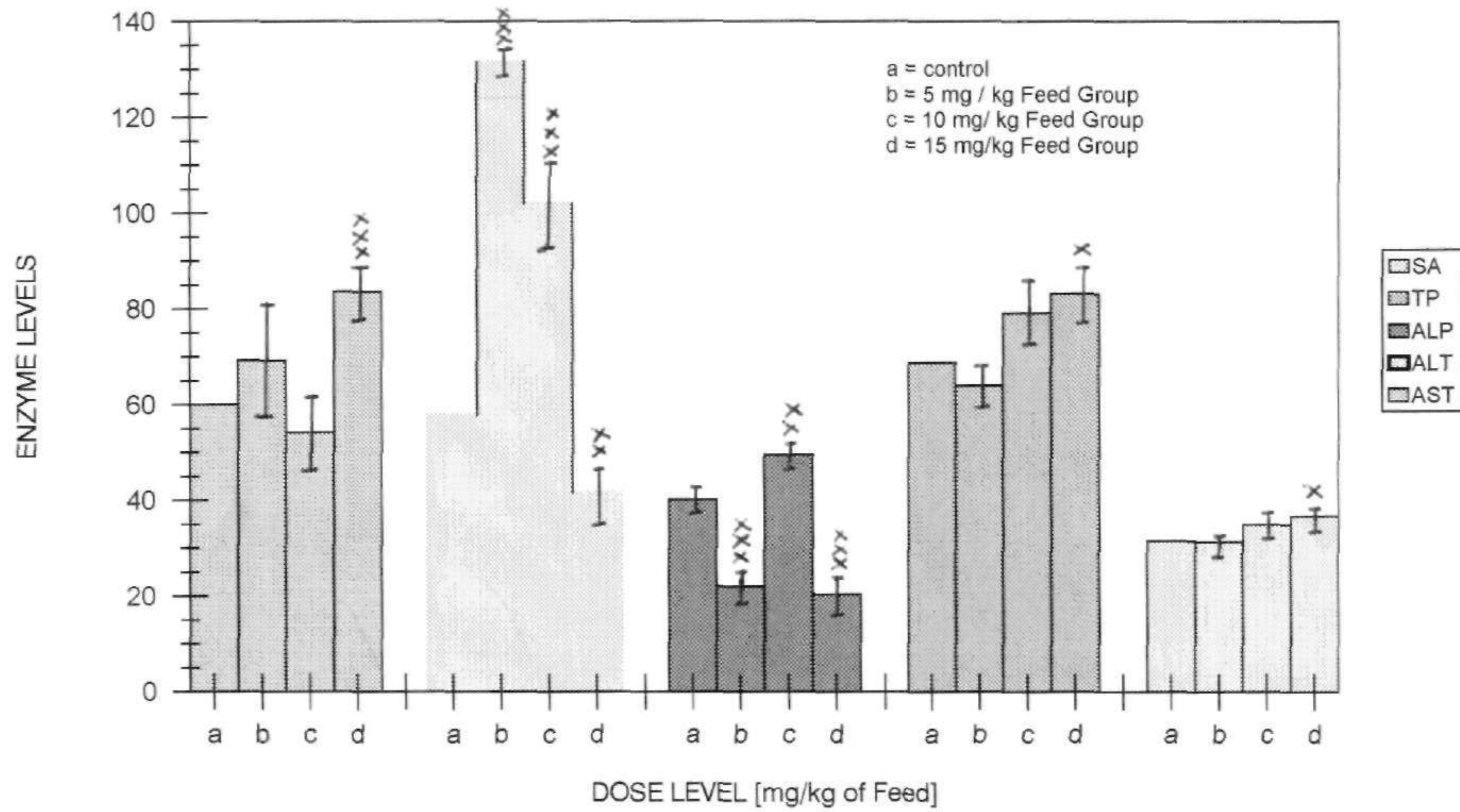

FIG. 4.3 : ENZYME ACTIVITY PROFILE (During the Second Month of Study)

TEST OF SIGNIFICANCE [From Control]

* Represents $P < 0.05$

** Represents $P < 0.02$ OR $P < 0.01$

*** Represents $P < 0.001$

slightly significant elevation was observed in mean serum total protein and albumin when compared with the control animals (P<0.05).

4.2.3 CHANGES IN SERUM PARAMETERS IN RATS AT 3RD MONTH

At the third month the mean serum AST in rats treated with 5mg lindane per kg feed was slightly significantly elevated when compared with the control subjects (P<0.05) (Fig. 4.4), while there was no significant difference in the mean serum AST of rats treated with 10mg and 15mg lindane per kg feed when compared with the control animals. Mean serum ALT in all the treated rats were significantly elevated when compared with the control values (P<0.001). For the mean serum ALP, for both the 5mg and 10mg lindane per kg feed, group there was slight decrease while for the 15mg lindane per kg feed there was no significant difference when compared with the controls. Total protein activity in rats treated with 5mg and 10mg lindane per kg feed were significantly elevated when compared with the control values (P<0.001) and also in rats treated with 15mg lindane per kg feed there was slight elevation in total protein activity when compared with the control subject (P<0.02). In all the treated rats there was slightly significant elevation in the serum albumin when compared with the control subjects (P<0.05).

Table 4.6 further compared the mean values of serum concentration of AST, ALT, ALP, total protein and albumin between successive months of study for each of the drug treated group. This is to give an insight on the activity changes at each dose level throughout the duration of the study.

At 5mg lindane per kg feed, the mean serum AST was significantly reduced at second month compared with first month (P<0.01) while there was no significant difference in serum AST concentration of experimental subjects at the third month when compared

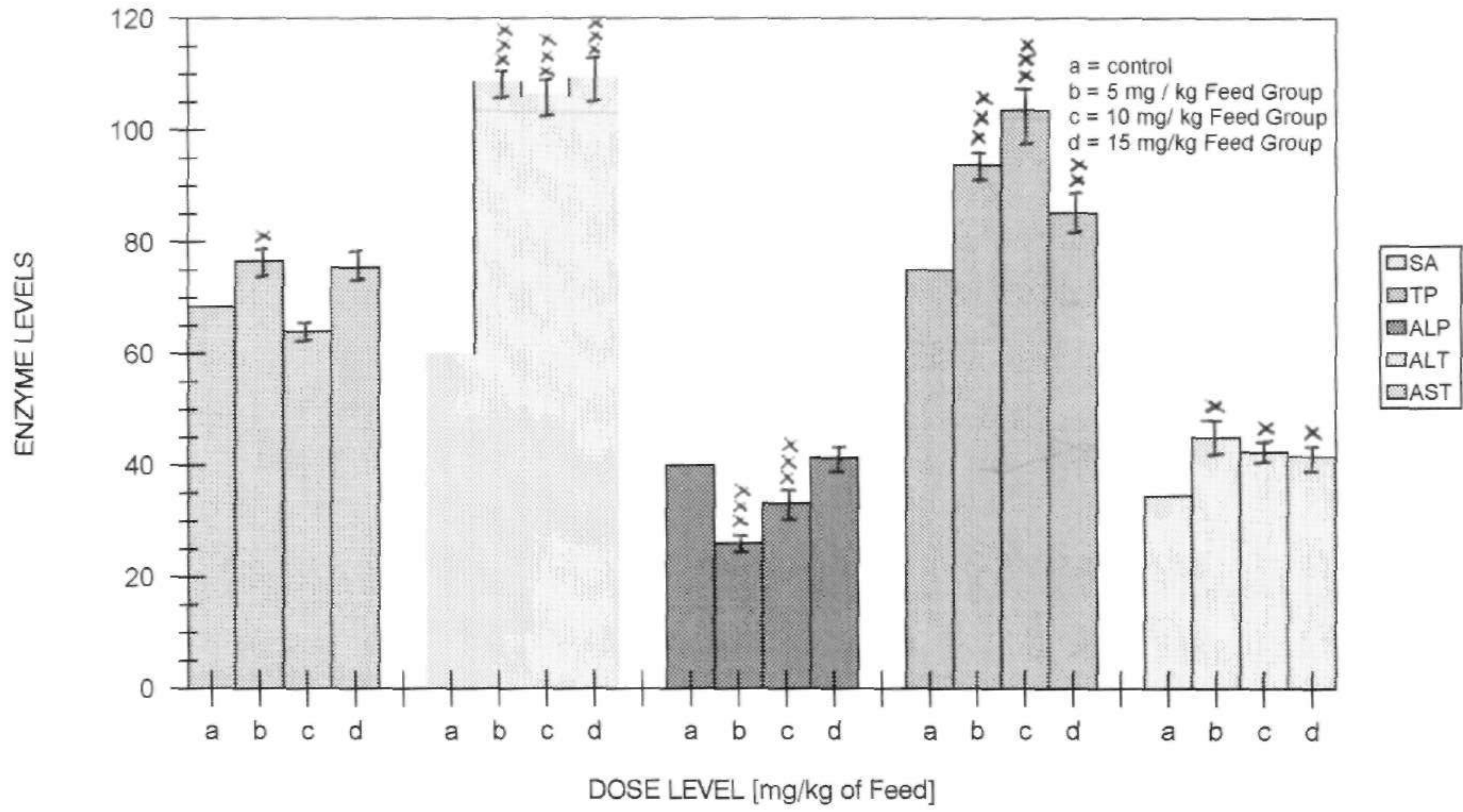

FIG. 4.4 : ENZYME ACTIVITY PROFILE (During the Third Month of Study)

TEST OF SIGNIFICANCE [From Control]
* Represents $P < 0.05$
** Represents $P < 0.02$ OR $P < 0.01$
*** Represents $P < 0.001$

Table 4.7: COMPARISON OF MEAN VALUES OF SERUM CONCENTRATIONS OF AST, ALT, ALP, TOTAL PROTEIN AND ALBUMIN THROUGHOUT THE THREE MONTHS OF STUDY.

Month of Study	Type of Group Study	AST	ALT	ALP	Total Protein	Albumin
First Month	GP1 Vs GP2	P<0.001	P<0.001	P<0.01	P<0.001	P<0.001
	GP1 Vs GP3	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001
	GP1 Vs GP4	P<0.001	P<0.001	P<0.001	P<0.05	P<0.02
Second Month	GP1 Vs GP2	NS	P<0.001	P<0.001	NS	NS
	GP1 Vs GP3	NS	P<0.001	P<0.01	NS	NS
	GP1 Vs GP4	P<0.001	P<0.01	P<0.001	P<0.05	P<0.05
Third Month	GP1 Vs GP2	P<0.05	P<0.001	P<0.001	P<0.001	P<0.05
	GP1 Vs GP3	NS	P<0.001	P<0.01	P<0.001	P<0.05
	GP1 Vs GP4	NS	P<0.001	NS	P<0.02	P<0.05

GP1 = Control Group

GP2 = 5 mg lindane/kg Feed Group

GP3 = 10 mg lindane/kg Feed Group

GP4 = 15 mg lindane/kg Feed Group

NS = Not Significant

with the second month. The mean serum ALT was significantly elevated at second month when compared with first month (P<0.01) and significantly reduced at third month when compared with second month (P<0.001). The mean serum ALP was observed to be significantly reduced at second month when compared with first month while there was no significant difference at third month when compared with second month. The mean serum total protein was significantly reduced at second month when compared with the first month, while there was significant increase in the third month when compared with the second month (P<0.001). The mean serum albumin was significantly reduced in the second month when compared with the first month (P<0.05), significantly elevated in the third month when compared with the second month (P<0.001).

At 10mg lindane per kg feed; the mean serum AST for second month was significantly reduced when compared with the first month (P<0.001), while there was no significant difference in the third month when compared with the second month. Throughout the period of study, there was no significant different in the ALT activity during successive months, ALP was significantly reduced in the second month when compared with the first month (P<0.001) and third month when compared with the first month (P<0.001). There was significant decrease in total protein for second month when compared with the first month (P<0.01) while there was a significant elevation in the third month when compared with the second month (P<0.001). The serum albumin did not show significant difference in the second month compared with the first month while there was slightly significant increase in the third month when compared with the second month (P<0.05).

At 15mg lindane per kg feed; the AST activity did not show significant difference over the three month study period. ALT activity (Table 4.6) was significantly reduced at the second

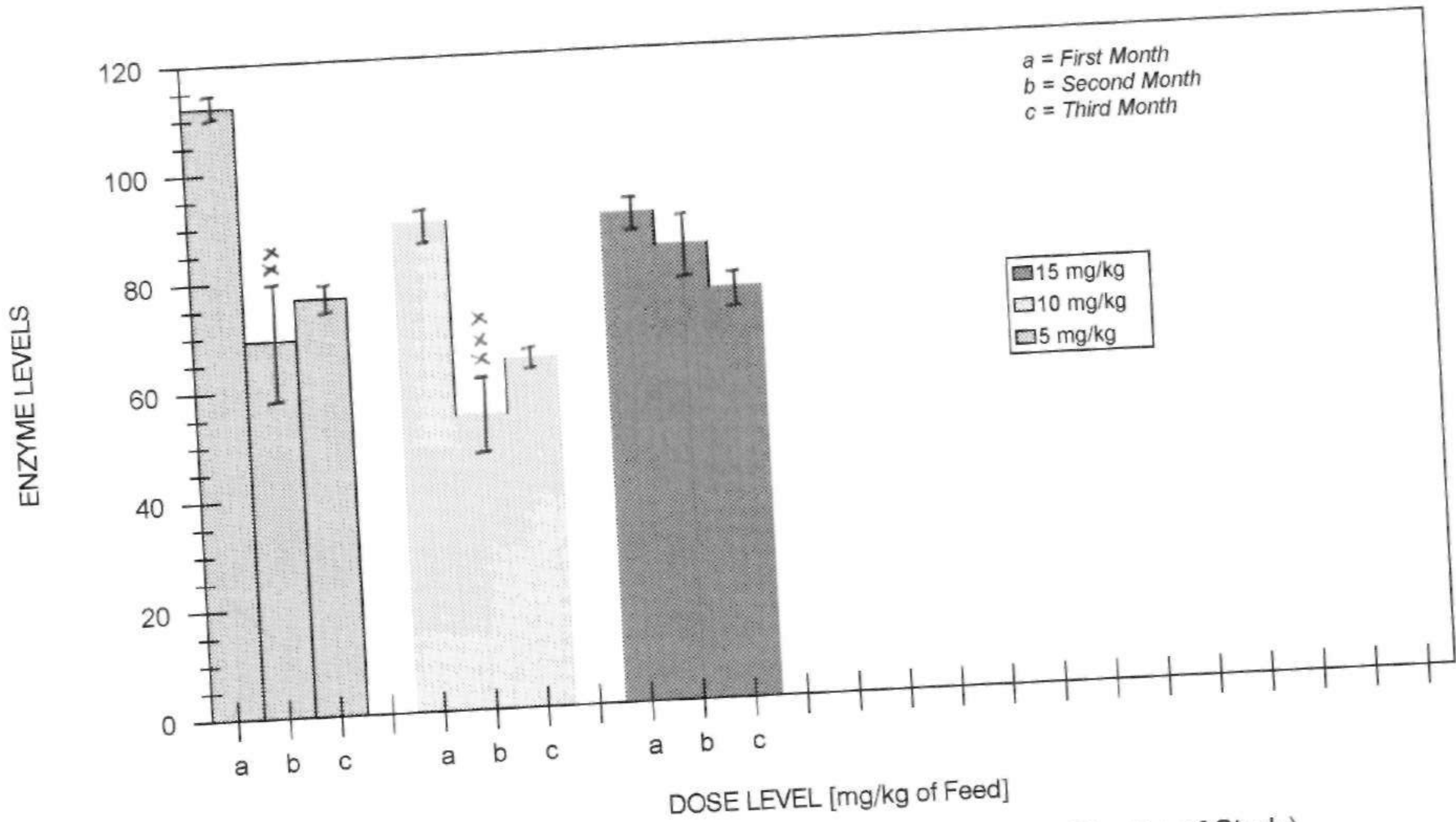

FIG. 4.5 : AST ACTIVITY PROFILE (Between Successive Months of Study)

TEST OF SIGNIFICANCE [Between Successive Months]
* Represents $P < 0.05$
** Represents $P < 0.02$ OR $P < 0.01$
*** Represents $P < 0.001$

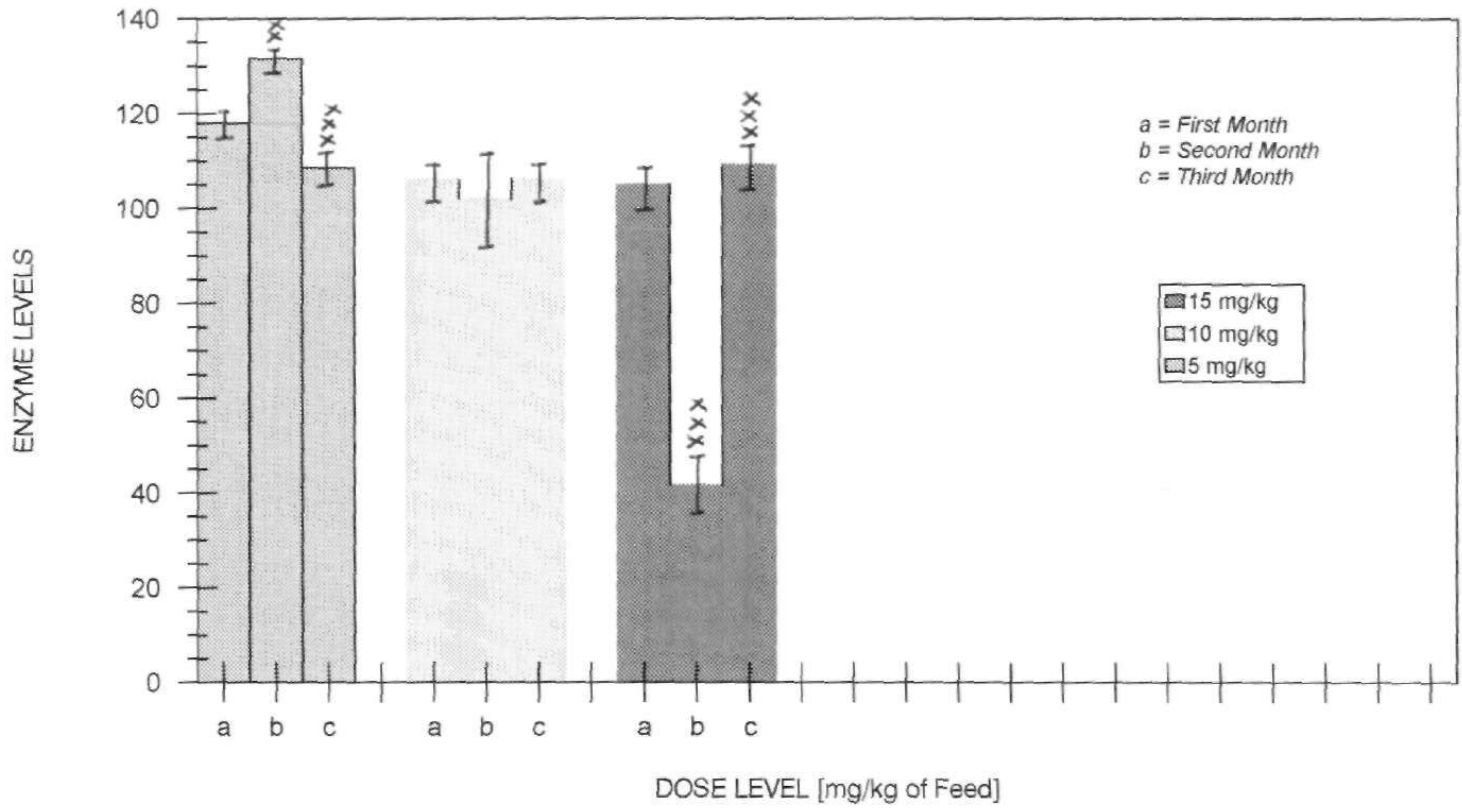

FIG. 4.6 : ALT ACTIVITY PROFILE (Between Successive Months of Study)

TEST OF SIGNIFICANCE [Between Successive Months]
* Represents $P < 0.05$
** Represents $P < 0.02$ OR $P < 0.01$
*** Represents $P < 0.001$

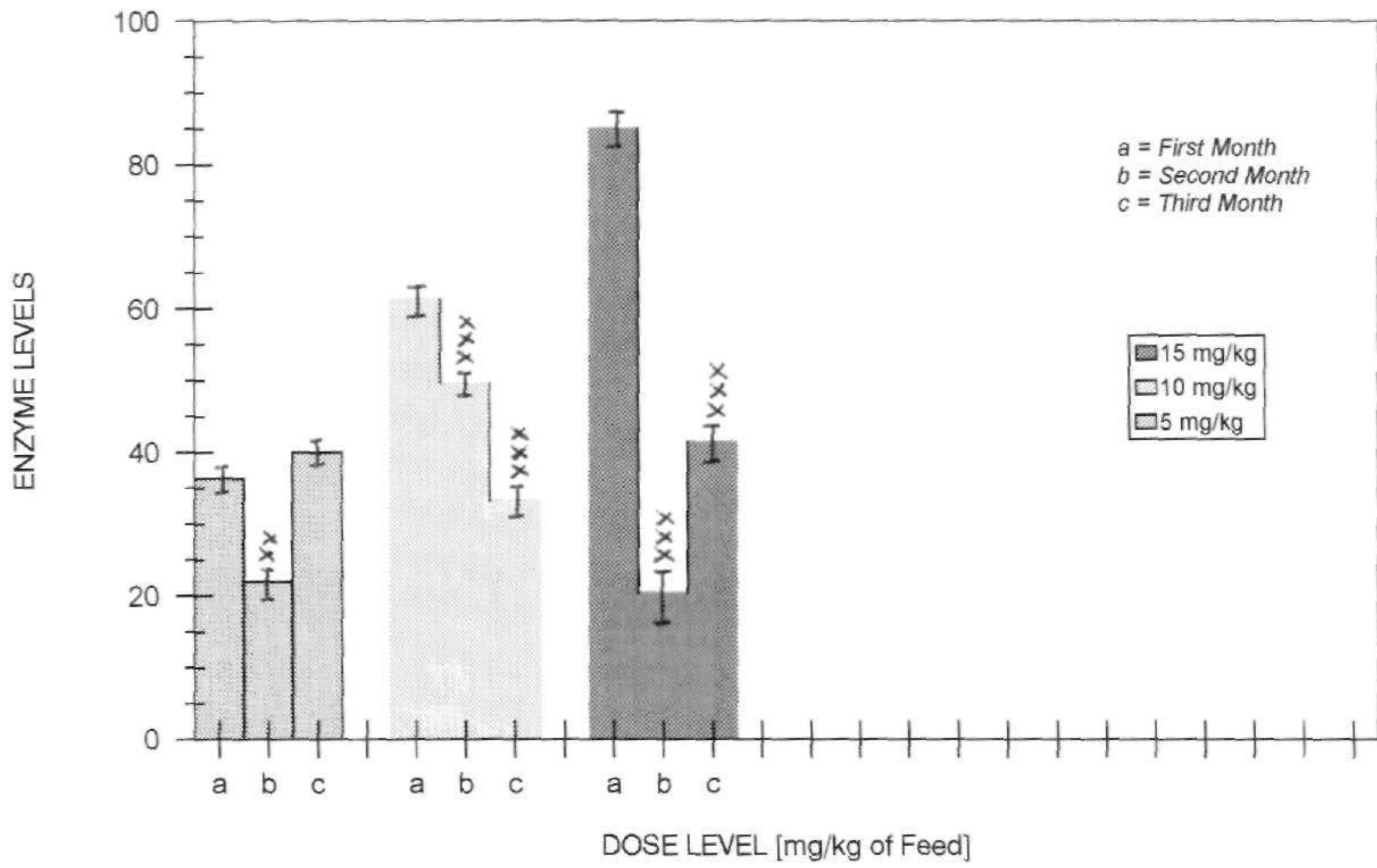

FIG. 4.7 : ALP ACTIVITY PROFILE (Between Successive Months of Study)

TEST OF SIGNIFICANCE [Between Successive Months]
* Represents $P < 0.05$
** Represents $P < 0.02$ OR $P < 0.01$
*** Represents $P < 0.001$

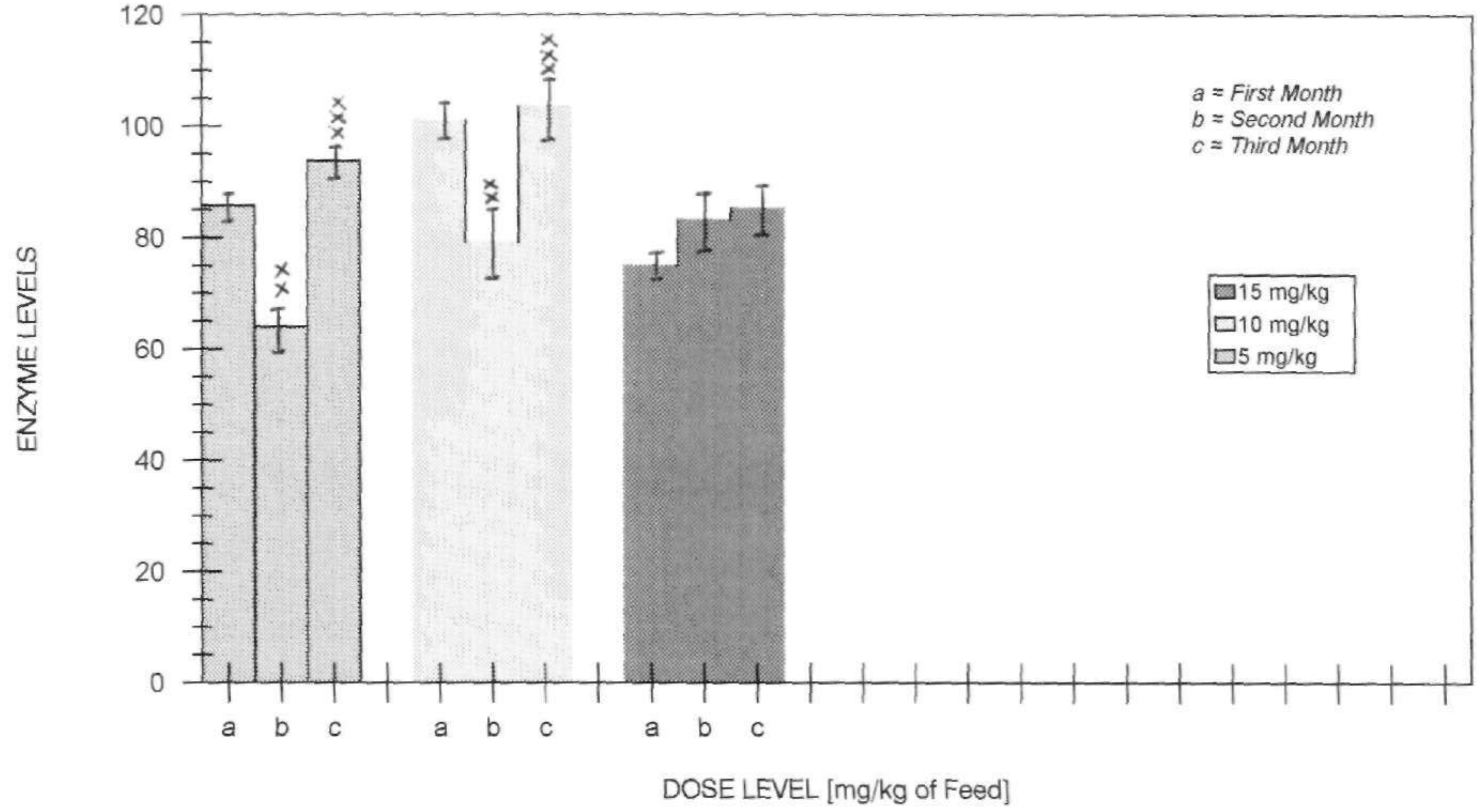

FIG. 4.8 : TP CONCENTRATION (Between Successive Months of Study)

TEST OF SIGNIFICANCE [Between Successive Months]

* Represents $P < 0.05$

** Represents $P < 0.02$ OR $P < 0.01$

*** Represents $P < 0.001$

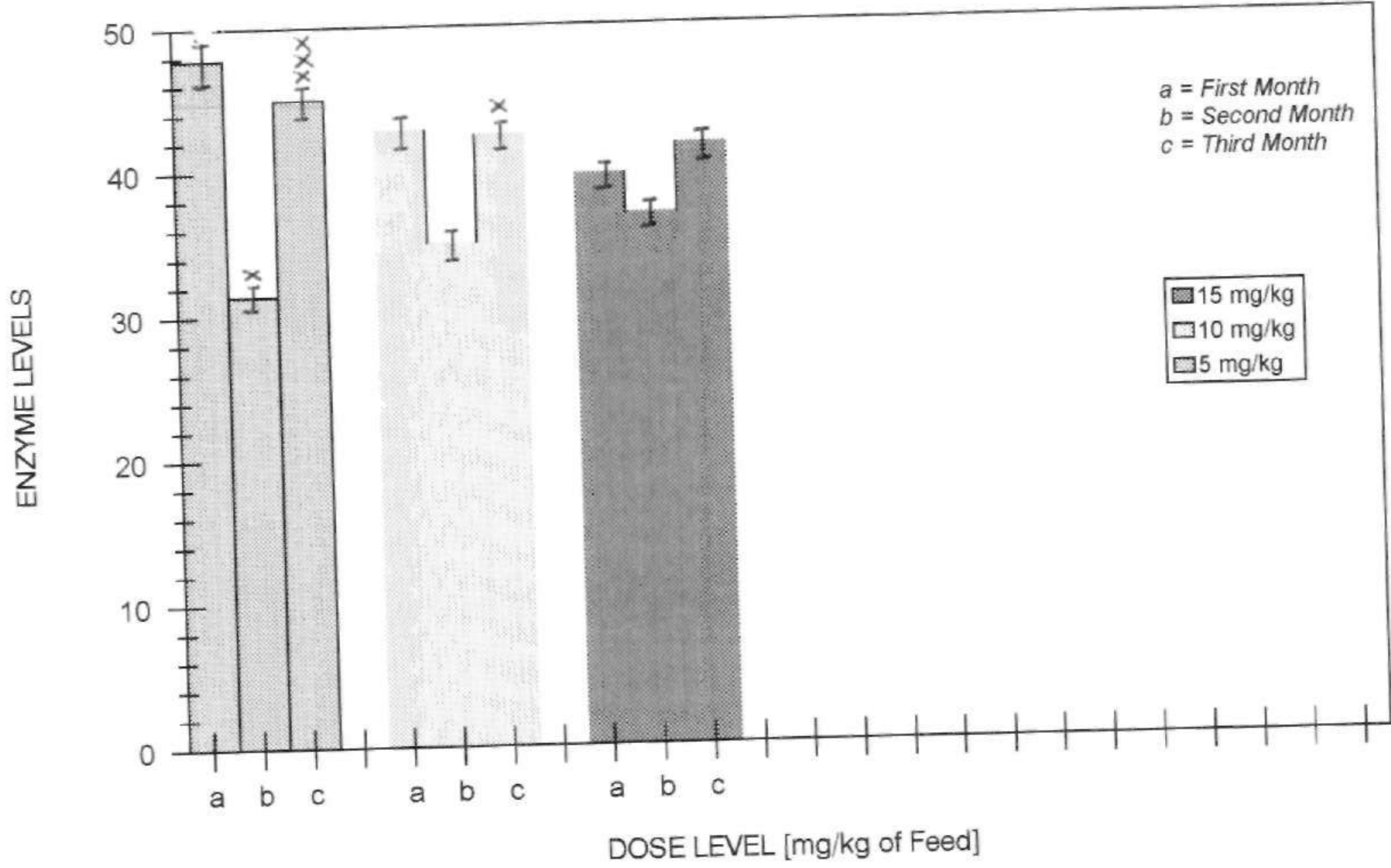

FIG. 4.9 : SA ACTIVITY PROFILE (Between Successive Months of Study)

TEST OF SIGNIFICANCE [Between Successive Months]
- * Represents $P < 0.05$
- ** Represents $P < 0.02$ OR $P < 0.01$
- *** Represents $P < 0.001$

month compared with the first month ($P<0.001$) and significantly elevated in the third month compared with the second month. The ALP activity was significantly reduced at second month when compared with first month and slightly elevated in third month when compared with second month (Fig. 4.7). No significant difference was observed in serum total protein and albumin at this dose level over the three month of study (Fig. 4.8 & 4.9)

Table 4.8 Average Weight Per Experimental Group Over the Twelve Weeks Study Period

Week of Study	Control Group	5 mg/kg Dose Group	10 mg/kg Dose Group	15 mg/kg Dose Group
0	133	160	178	140
1	134	164	183	143
2	138	169	187	145
3	140	173	188	147
4	145	174	193	148
5	145	179	195	151
6	149	162*	195	156
7	155	159*	192	156
8	156	160	189	152
9	161	163	190	151
10	165	170	191	150
11	172	172	194	152
12	178	177	201	152

*Loss of weight

In the twelve weeks of study, in the control there was gain in weight throughout the study period. In the 5 mg/kg dose group there was gain in weight from the onset of experiment until the sixth and seventh week when decrease in weight was observed. The animal started increasing in weight again at the eighth week of study. In 10 mg/kg dose group decrease in weight was observed at eighth week while decrease in weight was also observed at eighth week in 15 mg/kg dose group. In the two groups(10 mg/kg and 15 mg/kg dose group) there was increase in weight gain in subsequent month as observed in Table 4.8.

4.3 HISTOPATHOLOGICAL EXAMINATION

At the first month of study congestion was observed in the 5 mg per kg body weight group, periportal round cells inflammation and prominent sinusoidal cells were observed in the 10 mg and 15 mg per kg body weight group.

At the second month of study congestion was observed only in the 15 mg lindane per kg body weight group while periportal round cell inflammation, sinusoidal ectasia and plump sinusoidal cells were observed in all the drugs treated group with relative increase in round cell inflammation in the 15 mg lindane/kg body weight group.

At the third month of study periportal round cell inflammation, plump sinusoidal cells and sinusoid ectasia (all mild changes) was noted in all the drug treated groups. However, mild fatty change was observed in the 15 mg lindane per kg body weight group.

Plates 4.1 to 4.4 show control, congestion and inflammation, plump sinusoid, sinusoid ectasia and fatty changes at high magnification : Mag x 100 of the liver tissue.

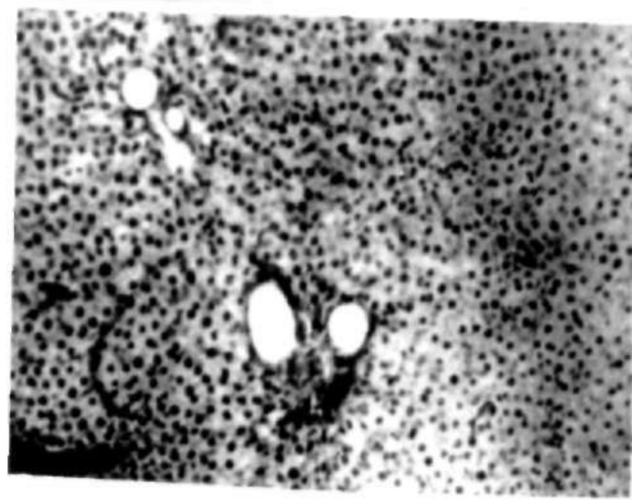

PLATE 4.1 CONTROL

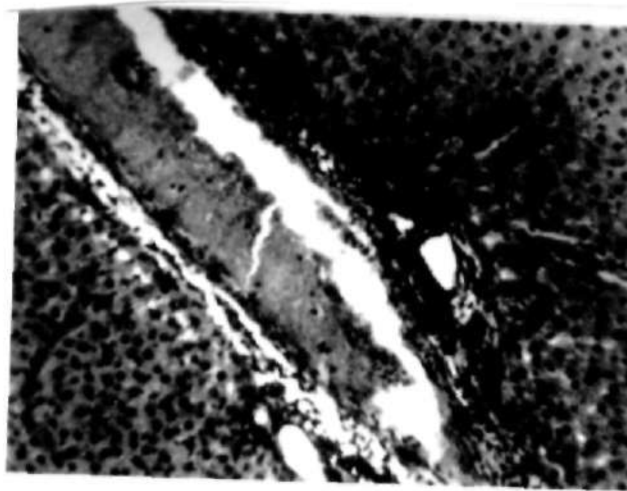

PLATE 4.2 CONGESTION AND INFLAMMATION OF THE LIVER TISSUE AT HIGH MAGNIFICATION

PLATE 4.3 PLUMPED SINUSOID AND FATTY CHANGE OF LIVER TISSUE OF LINDANE FED RAT.

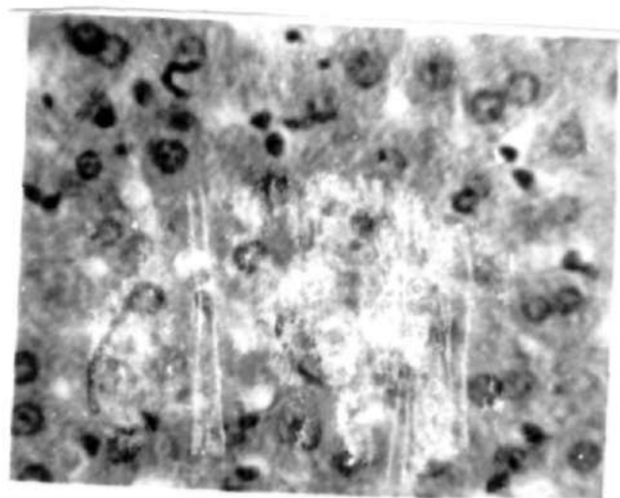

PLATE 4.4 SINUSOID ECTASIA AND FATTY CHANGE OF LINDANE FED RAT.

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

5.1 ACUTE TOXICITY STUDY

Acute toxicity study in experimental animals is aimed at detecting the short term effects of high doses of a compound, and this provides fundamental data for the design of more complex studies in animal in order to estimate the safety level for humans. The simplest acute toxicity study usually employed is determination of the median lethal doses (LD_{50}) 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.

The LD_{50} of lindane intraperitoneally was 25.4 mg per kg body weight. Matsumura (1975) had classified chemicals according to their oral LD_{50} values using the following criteria:

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

This classification gives a simple expression of the degree of toxicity of any chemical and, though convenient, is only a rough standard This classification therefore shows that lindane is highly toxic when administered intraperitoneally.

Route of administration of chemicals however strongly influences the LD_{50} values According to Matsumura, oral LD_{50} of lindane was 91mg/kg, which makes lindane a moderately toxic pesticide.

5.2 CHRONIC TOXICITY STUDY

Chronic toxicity studies were usually designed to provide data on the toxic effect of a chemical and to determine the dose level and time course required for these effects to manifest. In addition to determining clinical effects, this study also involves a variety of biochemical and pathologic evaluation.

The most widely used criteria for evaluating the toxic action of a substance in animal as reported by Cutler (1974) are: the reduction in the rate of body weight gain, detection of gross and histopathologic abnormalities in the organ, changes in organ weight and the increase in mortality rate. The mortality rate is not relevant here as no death was recorded throughout the study period in all the experimental groups.

The values of the liver function tests depends on their specificity for damage as well as on their sensitivity (Cutler, 1974). Aminotransferases are among a group of ubiquitous enzymes whose serum activity is elevated in many different disease states including myocardial infarction, viral and toxic hepatitis, muscular dystrophy and other muscular diseases and a small percentage of cancer patients, probably those with liver involvement (Schwartz, 1971). It was further observed by Schwartz (1971) that the extent of serum elevation is related to the particular disease and very often, to the stage of the disease.

The values obtained for the ALT activity from the prolonged exposure of the animals to lindane were not within the normal range. ALT is a cytoplasmic enzyme and is mostly found in the liver (Wroblewski, 1955). Mean serum concentration of ALT showed

the most significant increase throughout period of study, except in the second month for rats treated with 15mg lindane per kg Feed which showed a significant decrease. The initial increase in serum ALT value could be due to the release of the enzyme into the extrahepatic fluid as a result of acute damage to the liver. Wolf et al (1972), reported that the extent of rise of the transaminase usually reflects the severity of the hepatic change, and could be attributed to the liberation of enzymes from the damaged tissue into the general circulation. The significant decrease in ALT activity observed could be explained in line with a report by De Bruin (1976), that a decrease in serum enzyme activity may occasionally arise in case of specific enzyme deficiency as a result of blockage of de-novo enzyme synthesis due to liver damage from prolonged exposure to toxicant.

Significant increase in the activities of the transaminases in serum were also observed in acute hepatitis and liver cell injury, with activity of ALT being greater than that of AST, particularly in infective hepatitis (Kachmar and Moss, 1976). Gowenlock (1988) also observed that with liver cell necrosis or abnormal membrane permeability, the transaminase are released from the cell and their serum level increase. Level of transaminase in serum increases in acute hepatocellular damage and are also increased in chronic hepatocellular and cholestatic disease. The picture observed in toxic hepatitis is similar to that found in infectious hepatitis with very high ALT and AST activities in severe cases. In non liver disease for example heart disease, AST may be increased whereas non-hepatic ALT elevation is unusual (Wilkinson, 1976). In myocardial infarction, blood serum AST is seen to increase significantly with a significant rise in the AST/ALT activity quotient.

AST activity increased significantly, $P<0.001$ at the first month of study in all the lindane treated rats but in subsequent months there was no significant increase. Previous reports showed that AST is highly concentrated in the several tissues including heart

muscle, liver, skeletal muscle and kidney (Wilkinson, 1976). ALT is the more liver-specific enzyme and is therefore generally more sensitive to hepatic damage than AST. Thus, measurement of ALT activity in serum is of greater diagnostic specificity in confirming liver damage. It could further be explained that since the liver is the major source of ALT, any damage to the hepatocyte could therefore specifically affect its synthesis while the activity of AST may not be affected. AST and ALT are both involved in transamination reaction and due to protein turnover, the ALT already available in circulation could become depleted without being replenished thereby significantly reducing the level in the serum as observed in the second month of study in animal treated with 15mg lindane per kg feed. The above observations could therefore explain the more significant alterations observed in the ALT activities in the current study.

The reference range (Mean ± 2SD) of normal values for the current study was also calculated using the mean values for the control groups. The values obtained further showed that ALT activity may be a sensitive index of degree of liver damage, since the value of ALT obtained with prolonged exposure to the toxicant were not within the normal range.

Wilkinson (1976) and Gowenlock (1988) have independently reported that the highest serum ALP activity occurs in post hepatic obstruction and this is said to be related in part to increased synthesis of this enzyme. This could explain the slightly significant increase in ALP activity observed in the 10mg and 15mg lindane per kg feed group at first and second month of study.

The other biochemical parameter investigated i.e total protein and albumin were less sensitive in detecting an impairment of liver function in the rats of the treated groups as compared with the controls, Total protein gave values which showed slightly significant

differences between the treated and control in two treated groups, while serum albumin test failed to detect hepatic insufficiency between the treated and the control. Significant differences were however observed in total protein level at first and third month in 10mg per kg feed lindane group (Table 4.4). Significant increase in total protein concentration in disease arise from an increase in total globulins, usually of gamma globulins (Gowenlock, 1988). De Bruin (1976) also reported that the characteristic changes in chronic liver disease is a rise in globulin levels and a fall in serum albumin. This observation, could therefore explain the significant increase in the mean serum total protein level for the 10mg/kg feed group at first and third month.

The histopathology result suggest that congestion of the liver renders the hepatic cells more susceptible to the action of toxic substances, absorbed by the hepatic cells, transported to the liver. This could lead to inflammation. Inflammation can be provoked by irritants. An example of causes of inflammation is chemical poisons. The inflammations observed throughout the study period can be said to be due to lindane. When irritants persists for a long period of time it results to chronic inflammation and the body responds by producing excessive amounts of connective tissue and epithelium in the area. This could explain the plump sinusoidal lining cells and sinusoid ectasia observed. When the mitochondria are damaged due to irritants, the enzymes concerned in the metabolism of fats are disrupted and so the fat that should be in a colloidal state in the protoplasm, accumulates and become visible a condition known as fatty degeneration, sometimes referred to as fatty change. It is a manifestation of cell sickness and is found in the parenchyma cells of the liver caused by hypoxia and poisons. Poisons which are hepatotoxic agents damage or denature enzymes, thus causing fatty degeneration, The mild fatty change

observed at the third month of study in the 15mg/kg dose group may be as a consequence of a disruption of fat metabolism.

Ringler and Dabich (1980) reported that increase in serum concentration of a particular enzyme, indicates damage to the cells that releases the enzyme and that serum enzyme responses usually, vary with the rate and magnitude of absorption of the toxicant and hence may serve as an indication of severity of exposure. It was therefore concluded by Ringler and Dabich (1980), that a correlation exists between rises in serum enzyme activity and severity of toxic damage to a particular tissue.

The histopathology report indicate liver cell damage, which suggests that the alterations in the transaminase activity observed in the current study could be due to the distruption of the liver cell integrity.

5.3 CONCLUSION

The toxic effect of lindane, a synthetic organochlorine insecticide, on the mammalian (rat) has been investigated using serum biochemistry and histopathology as indices of toxicity. The median lethal dose of lindane (LD_{50}), was calculated to be 25.4 mg per kg body weight using the IP route. Prolonged period of exposure led to an increase level of hepatic enzymes. ALT appears to be most sensitive enzyme released during liver damage than AST. ALP, total protein and albumin showed little or no significant alteration through out the study period.

The histopathology result showed mild degeneration of the liver. Inflammation of the liver was observed at lower doses and fatty degeneration at higher doses after prolonged exposure to lindane.

5.4 RECOMMENDATIONS

Lindane is an organochlorine with high lipid/water partition coefficient thus, it tends to accumulate in biological tissue ; persistent in nature and resistant to biological degradation. At low doses it is not very toxic and in as much as the dose requirement as an insecticide is considerably below the reported toxic dose, it perhaps may be a preferred insecticide for indoor use to the cholinesterase inhibitors which are known to be of high toxicity at very low doses.

However, the development of resistances by insect to the compound may be a major constraint thus, dose requirement for pest elimination may go up. Nevertheless, there is still a strong case for lindane use.

Moreover for indoor uses lindane may be preferred to organophosphate because of the high residual level which may allow longer period between sprays. The persistence in the environment and the long term consequence of this as a result of bioaccumulation may strengthen the case for caution and search for alternative pesticides.

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APPENDICES

APPENDIX I

PREPARATION OF REAGENTS FOR TRANSAMINASES ASSAY

1. PHOSPHATE BUFFER (pH 7.4)

11.3g of dry anhydrous disodium hydrogen phosphate and 2.7g of dry anhydrous potassium dihydrogen phosphate were dissolved in 1 litre of water, pH checked using the pH meter. The buffer was stored at 4°C.

2. SUBSTRATES (200 mmol)

For AST; exactly 6.6g l- Aspartic acid was dissolved in about 90ml of 1N NaOH. 0.146g of alpha-ketoglutarate was added and dissolved by adding a little more NaOH. The pH was adjusted to 7.4 and the solution made up to 500 ml with phosphate buffer.

For ALT; exactly 4.5g L-alanine was dissolved in 90 ml of water and 2.5 ml of 1N NaOH was added to adjust the pH to 7.4, 0.146g of alpha - keto glutarate was added and dissolved by adding a little more NaOH. The pH was adjusted to 7.4 and the solution made up to 500 ml with phosphate buffer.

3. STOCK PYRUVATE STANDARD (200 mmol/l)

220 mg of sodium pyruvate was dissolve in 100ml of phosphate buffer solution, and was stored at -15°C

4. working pyruvate standard (4 mmol/l)

Stock standard was diluted one in five with phosphate buffer and stored at -15°C.

5. 2.4 DINITROPHENYL HYDRAZINE (DNPH 1mm).

Exactly 19.8 mg of DNPH was dissolved in 10 ml of concentrated Hydrochloric acid and made up to 100 ml with water, solution was stored in brown bottle at room temperature.

6. Sodium hydroxide (NaOH 0.4N)

Exactly 16g of NaOH was dissolved in 1 litre of water to gi ve a 0.4N solution.

APPENDIX II

TABLE 3.1 Procedure for Assay of AST using Reitman and Frankel Method

S/No	PROCEDURE	TEST (ml)	TEST-BLANK (ml)	STD (ml)	STD BLANK (ml)
1.	Buffer Substrate Solution: Incubate in H_2O bath (37^0C) for 5 minutes.	0.5	0.5		0.5
2.	Serum(Fresh Unhaemolysed)	0.1	-		-
3.	Pyruvate Standard	-	-	0.1	-
4.	Distilled Water	-	-	-	-
5.	Mixed, incubate at 37^0C for 60 minutes. Remove and add DNPH.	0.5	0.5	0.5	0.5
6.	Mix, stand at room temperature for 20 minutes, add 0.4N NaOH.	5.0	5.0	5.0	5.0
7.	Stand the resultant solution for further 5 minutes and finally read at 510 nm after zeroing the instrument with distilled water.				

APPENDIX III

PREPARATION OF REAGENTS FOR ASSAY OF ALP

1. BUFFER - pH 10.0

    6.3g of anhydrous sodium carbonate (Na_2CO_3) and 13.36g of sodium bicarbonate ($NaHCO_3$) were dissolved in 1 litre of distilled water. This solution was stored at 4^0C

2. SUBSTRATE - 0.01M disodium phenol phosphate.

    2.18g of sodium phenol phosphate was dissolve in 1 litre of water. The solution was quickly boiled to kill any organisms and cooled immediately. The solution was then preserved with a little chloroform (4m/1) and store at 4^0C . Substrate solution is stable for 3-4 weeks.

3. STOCK PHENOL STANDARD of 1mg/ml

    1g pure crystalline phenol was dissolve per 1 litre in 0.1N hydrochloric acid. The standard stock solution was stored at 4^0C in a brown bottle.

4. WORKING PHENOL STANDARD (1mg/100ml)

    1ml of stock standard was diluted to 100ml with distilled water. Solution was preserved with a few drops of chloroform and stored at 4^0C in a brown bottle.

5. SODIUM HYDROXIDE (0.5N)

    20g of sodium hydroxide (NaOH) was dissolved in 1 litre of distilled water to give 0.5N solution.

6. SODIUM BICARBONATE (0.5N)

    42g of sodium bicarbonate ($NaHCO_3$) was dissolved in 1 litre of distilled water to give 10.5N solution

7. 4 - AMINOANTIPYRENE.

    6g of 4-aminoantipyrene was dissolved in 1 litre of distilled water,

8. POTASSIUM FERRICYANIDE (K_3 Fe $(CN)_6$)

    24g of potassium ferricyanide was dissolved in 1 litre of distilled water and stored in a brown bottle.

APPENDIX IV

TABLE 3.2 PROCEDURE FOR ASSAY OF ALKALINE PHOSPHATASE USING KING-ARMSTRONG METHOD

S/No	PROCEDURE	TEST (ml)	TEST-BLANK (ml)	STD (ml)	STD BLANK (ml)
1.	Buffer Solution:	1	0.5		0.5
2.	Phenylphosphate substrate. Place at (37^{0}C) for 3 minutes.	1			
3.	Serum(Fresh Unhaemolysed)	0.1	-		-
4.	Phenol Standard	-	-	0.1	-
5.	Distilled Water	-	-	-	-
6.	Mixed, incubate at 37^{0}C for 5 minutes. Remove and add 0.5N NaOH.	0.8	0.5	0.5	0.5
7.	0.5N Na_2CO_3.	1.2	5.0	5.0	5.0
8.	4-Amino antipyrene.	1.0			
9.	Potassium Ferricyanide.	1.0			
10.	Solutions were finally read zeroing the instrument with the blank.				

APPENDIX V

PREPARATION OF REAGENTS FOR ASSAY OF SERUM PROTEIN.

1. BIURET REAGENT (STOCK)

45 g of sodium potassium tartrate was dissolved in 500 ml of 0.2N sodium hydroxide, then 15 g of finely powdered copper sulphate ($CuSO_4.5H_2O$) was added and stirred continuously until completely dissolved. 5g of potassium iodide was then added. The solution was made up to 1 litre with 0.2N NaOH.

2. WORKING BIURET REAGENT:

200 ml of stock solution was made to 1 litre with 0.2N NAOH containing 5g potassium iodide

3. STANDARD BOVINE SERUM ALBUMIN (1.0 g/100 ml in 0.9% NaCl),

4. TARTRATE - IODIDE SOLUTION

9g of sodium potassium tartarate was dissolved in 1 litre of 0.2N NaOH containing 5g potassium iodide.

APPENDIX VI

TABLE 3.3 Assay Method for Protein Determination

S/No	SOLUTION	TEST (ml)	STANDARD (ml)
1.	Biuret Solution	5.9	5.9
2.	Serum	0.1	-
3.	Standard Bovine Serum Albumin	-	0.1

APPENDIX VII

DETERMINATION OF SERUM ALBUMIN

Modified Bartholomew and Delaney Method - 1966.

Albumin reacts with the dye - Bromocresolgreen which has specific affinity for it in acidic solutions to produce a coloured complex which is measured colorimetrically. The intensity of the colour produced is proportional to the concentration of albumin in the sample.

PREPARATION OF REAGENTS

1. SUCCINATE BUFFER - 0.1 M. pH 4

11.9 g of succinic acid was dissolved in about 800 ml of water. The pH was adjusted to 4.1 with NaOH and made up to 1 litre with water. Buffer was stored at 4°C.

2. DYE STOCK - 0.6M

419 mg of Bromocresolgreen was dissolved in 10 ml of 0.1m NaOH. The solution was then stored at 4°C.

3. WORKING DYE SOLUTION

One volume of Bromocresolgreen (BCG) solution was mixed with 3 volumes of succinate buffer. 4 ml of 30 % Brij 35 solution per litre was then added and pH adjusted to 4.2 + 0.05 by the addition of 5 mls 5N NaOH. Solution was stored at 4°C.

4. STANDARD

Bovine serum albumin (1:10) was diluted with normal saline to give a 30 g/l solution.

METHOD :

0.02 ml serum was added to 4.0 ml working dye solution. The mixture was mixed and incubated at 37°C for 10 minutes. The absorbance was read at 628 nm using Bromocresolgreen to set the instrument at zero.

APPENDIX IX

SCHEME FOR THE SUCCESSIVE DILUTION OF THE BOVINE SERUM ALBUMIN(BSA) (10g/l in 0.9% NaCl) USED FOR THE ANALYTICAL CURVE.

TUBE No.	ML Standard BSA Solution (10g/l)	ML 0.9% NaCl	Protein Conc. (Gm/l) serum	Absorbance Reading (540 nm)
1.	0.00	1.00	0.00	0.00
2.	0.10	0.90	1	0.05
3.	0.20	0.80	2	0.11
4.	0.30	0.70	3	0.15
5.	0.40	0.60	4	0.20
6.	0.50	0.50	5	0.26
7.	0.60	0.40	6	0.32
8.	0.70	0.30	7	0.32
9.	0.80	0.20	8	0.41
10.	0.90	0.10	9	0.44
11.	1.00	0.00	10	0.50

APPENDIX X

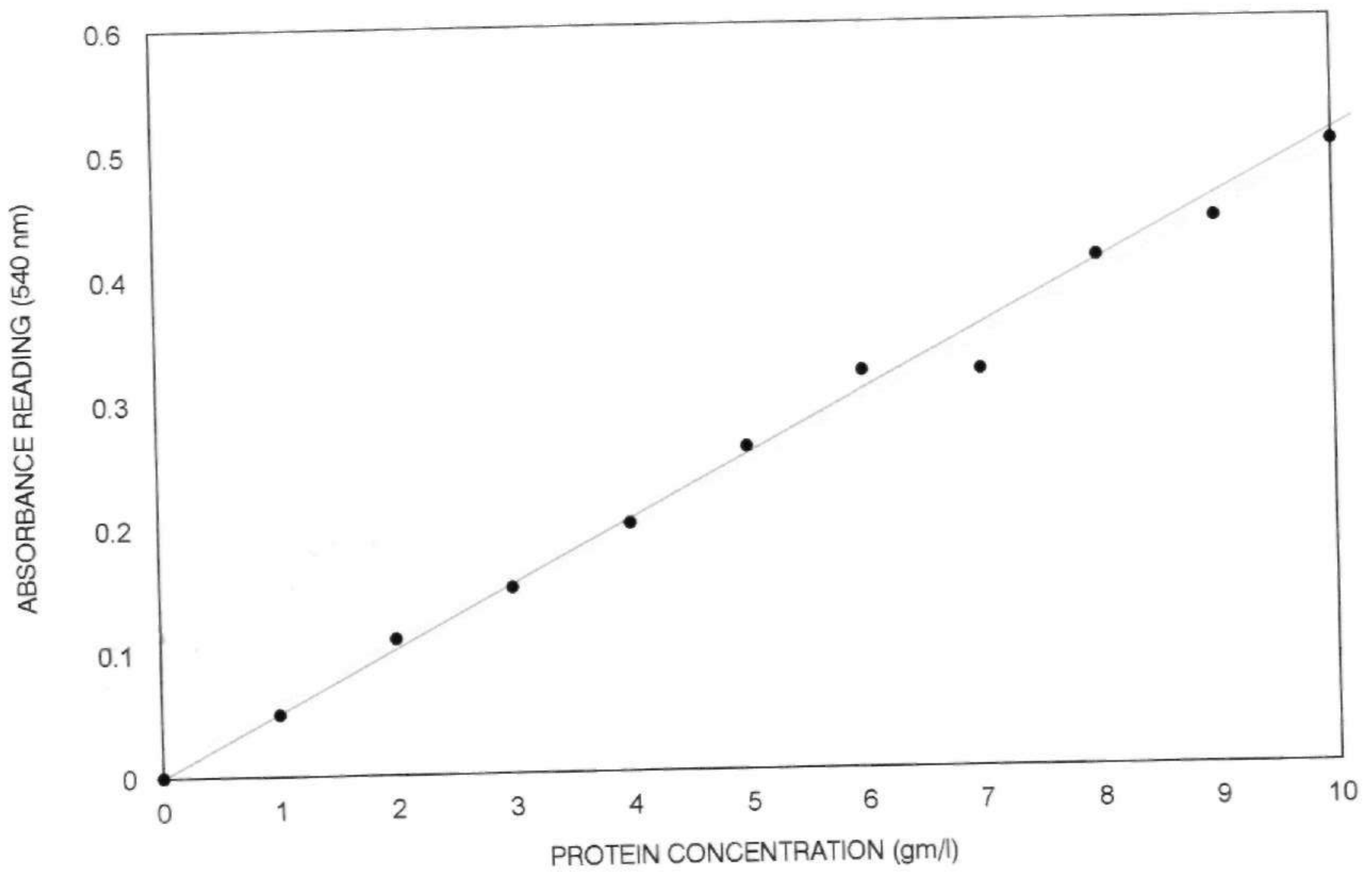

Fig 3.2 STANDARD CALIBRATION CURVE