BIOACCUMULATION, BIOTOXICITY AND BIODEGRADATION OF FENVALERATE IN RATS

A THESIS

submitted in fulfilment of the
requirements for the award of the degree
of
DOCTOR OF PHILOSOPHY
in
BIOSCIENCES AND BIOTECHNOLOGY

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JULY, 1995

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in this thesis entitled, **BIOACCUMULATION, BIOTOXICITY AND BIODEGRADATION OF FENVALERATE IN RATS**, in fulfilment of the requirement for the award of the degree of **DOCTOR OF PHILOSOPHY** submitted in the **DEPARTMENT OF BIOSCIENCES AND BIOTECHNOLOGY** of the University is an authentic record of my own work carried out during a period from **JANUARY, 1993 to JULY, 1995** under the supervision of **Prof. (Dr.) C. B. SHARMA.**

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other University.

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Signature of the Candidate

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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Abstract

Fenvalerate, [1, cyano (3-phenoxyphenyl) methyl 4-chloro-α-(1-methylethyl) benzeneacetate, Pydrin insecticide], a synthetic pyrethroid is widely used now a days to control a range of pests. In the present study bioaccumulation, biodegradation and biotoxicity of fenvalerate was investigated in liver, kidney and brain, at both cellular and subcellular levels, in rats exposed to acute and chronic treatments. In acute treatment after 6, 12, 24, 36 and 48 h of oral administration of a single dose (100 mg/Kg body weight), the organs were removed and analysed by HPLC for residual pesticide and its metabolites. Pattern of biodegradation of pesticide as judged by HPLC data was found to be nearly identical in liver, kidney and brain, except the rapid degradation of the pesticide (about 98% in 48 h) in liver compared to that in kidney and brain where the degradation was 78% and 68% respectively. Two metabolites designated as PI and PII were formed. These were isolated in pure form by repeated HPLC and identified by IR spectroscopy as [4-chloro-α-(1-methylethyl) benzeneacetic acid] and 3-phenoxybenzoic acid respectively.

The bioaccumulation pattern of fenvalerate and its metabolites was also studied under chronic toxicity after administering repeated oral doses of 5 mg/Kg and 15 mg/Kg body weight of rats on alternate days for a period of 7, 15 and 30 days. The accumulation of residual pesticide was maximum in brain, followed by kidney and liver and the pattern of accumulation was dose-dependant.

In vitro metabolism of fenvalerate was carried out to characterise the enzyme

involved in fenvalerate metabolism in rats. The enzyme was found to be localised mainly in outer mitochondrial membrane and microsomes in liver. This enzyme hydrolysed about 50% of fenvalerate with the formation of two degradation products which were found to be the same as were formed *in vivo*. The activity of the outer mitochondrial membrane localised enzyme was linear upto 30 min and to about 1.0 mg protein concentration, the pH optimum was 7.5 and K_m and V_{max} values were 26.32 mM and 0.50 mM min^{-1} mg^{-1} protein, respectively. The enzyme was stimulated at low EDTA concentrations (<0.5 mM), while higher concentrations of EDTA (> 0.5 mM) were found to be inhibitory causing 50% inhibition at 2 mM EDTA concentration. The enzyme required Mg^{2+} ions, while Mn^{2+} and Ca^{2+} were inhibitory. Presence of nonionic detergents (Triton X-100 and Nonidet P-40) in the medium stimulated the activity of the enzyme which also catalysed further degradation of PI into a third metabolite, PIII.

Fenvalerate affected various biochemical parameters in rat. There was a marked decrease in total proteins, glycogen, nucleic acids and serum cholesterol, while concentration of major components of neutral lipids (TAG, MAG) and phospholipids (PE, PC) was increased in liver, kidney and brain. Haematological parameters showed a decline in Hb and RBC counts and an elevation in SGPT, SGOT, alkaline and acid phosphatases by 3.0-, 4.0- 1.5- and 2.0- fold, respectively.

The ultrastructural changes induced by fenvalerate exposure in cells of liver and kidney of rat were investigated by transmission electron microscopy. It was found that chronic exposure to fenvalerate induced clearly noticeable changes in the ultrastructure of the rat liver cells. For example, while in unexposed rats, liver cells showed a distinct nucleus and nucleolus and a well defined endoplasmic reticulum (ER) network, the fenvalerate exposed rat liver cells showed dispersion of chromatin material, disorganisation of nucleolus, presence of crystalline rods and deposition of lipid droplets in 30 days of chronic treat-

ment with a low dose of fenvalerate (15 mg/Kg body weight). Identical ultrastructural changes were also induced in kidney cells by the fenvalerate exposure. The effect on the brain cells is likely to be identical to that of liver and kidney, although it was not investigated due to some technical difficulties.

Fenvalerate also affects the subcellular membrane structure and functions. The chronic exposure of fenvalerate to rats led to an increase in the total carbohydrates and total phospholipid contents in various subcellular fractions namely, nuclear membrane, outer and inner mitochondrial membranes and plasma membrane of rat liver. There was a decrease in the protein content of treated rat liver membranes and the SDS-PAGE protein profiles showed marked changes in the protein pattern and intensities of various protein bands in control and treated rats. The most prominent differences were: the 79 kDa protein was absent, the intensity of 225, 129, 127, 82, 72, 46, 44 and 15 kDa proteins was depressed, while the intensity of 220, 32, 24 and 17 kDa proteins was enhanced in nuclear membranes of exposed rat livers. Similarly, in outer mitochondrial membranes of the exposed rat liver, the 210, 180, 35 and 17 kDa proteins were absent, the relative intensity of 150, 82, 55, 48, 46, 41, 38, 31 and 27 kDa protein bands was greatly reduced, and the intensity of 162 and 18 kDa proteins was enhanced compared to control. The plasma membrane of treated rat livers showed maximum decrease in the intensity of 42 and 17 kDa bands, while 158 and 32 kDa protein bands were missing and 180, 90 and 75 kDa proteins showed increased intensity compared to the control. The most noticeable effect was the presence of a new 35 kDa protein in the PM of the pesticide exposed rat liver cells. This 35 kDa protein may be a "stress protein" induced to counteract the effect of the pesticide. At the moment it is not clear if the 35 kDa protein is a monomeric subunit of the well known heat shock proteins, hsp 70, family.

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In the end, I would like to say that any achievement, be it in science or in any other field, is not of any single individual. So many good and kind souls have contributed in successful completion of this thesis that I cannot even count them. It is their collective blessings and good wishes that I shall always remain indebted to.

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SWATI MISRA

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Abbreviations Used

ACh	Acetylcholine
BCF	Bioconcentration factor
°C	Degree centigrade
CNS	Central nervous system
CPIA	4-chloro - α -(1-methylethyl) benzeneacetic acid
cf	Cited from
d	Days
DA	Dopamine
DAG	Diacylglycerol
DOPAC	Dihydroxyphenylacetic acid
EC	Esterified cholesterol
EDTA	Ethylene diamine tetra acetic acid
ER	Endoplasmic reticulum
FFA	Free fatty acid
Fig.	Figure
g	Gram
GA	Golgi apparatus
GABA	γ-aminobutyric acid
GLC	Gas liquid chromatrography
h	Hours
HPLC	High performance liquid chromatography
HVA	Homovanillic acid
Hb	Haemoglobin
kDa	Kilo dalton

K_m	Michaelis - Menten constant
kdr	Knockdown rate
LC_{50}	Median lethal concentration
LD_{50}	Median lethal dose
LDH	Lactate dehydrogenase
mA	Milli ampere
MAG	Monoacylglycerol
MDH	Malate dehydrogenase
MFO	Mixed-function oxidase
mM	Millmolar
µg	Micro gram
mg	Milli gram
min	Minute
µl	Microlitre
ml	Milli litre
mol	Molar
NA	Noradrenaline
nm	Nanometer
PBO	Piperonyl butoxide
PBZ	Peripheral benzodiazepine
PI	Metabolic product I
PII	Metabolic product II
PIII	Metabolic product III
PM	Plasma membrane
ppm	Parts per million
R	Pyrethroid-resistant
R_f	Relative front

S	Pyrethroid-susceptible
SDH	Succinate dehydrogenase
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
SOD	Superoxide dismutase
TAG	Triacylgycerol
TEM	Transmission electron microscope
TEMED	N,N,N',N',-Tetramethyl ethylene diamine
TLC	Thin-layer chromatography
UV	Ultra voilet
v/v	Volume/volume
V_{MAX}	Maximum velocity
VNC	Ventral nerve cord
w/v	Weight/volume
wt.	Weight

Chapter 1

INTRODUCTION

In recent times, growing concern with methods of insect and pest control have prompted a search for eco-friendly and safe alternatives. The manufacture and use of organochlorine and organophosphate pesticides have decreased in the last decade, in part due to their adverse effects on aquatic and wildlife and tendency of these pesticides to bioconcentrate in animal and plant tissues. Replacement of these chemicals in agriculture is, lately, being done by synthetic pyrethroids. Fenvalerate combines high insecticidal activity against a broad spectrum of insect pests with moderate to low mammalian toxicity and adequate field stability. It has been used in place of banned pesticide, Toxaphene in many countries. However, caution is required since it is considered to be a potential neurotoxicant and reportedly affects the cardiovascular system, liver and kidney in mammals. Since continuous application of the commonly used synthetic pyrethroids pesticides in agriculture may result in their accumulation in various tissues of animals through food chain, bioaccumulation has, therefore, become one of the major concerns in the safe use of pesticides. The current status of knowledge regarding the toxicity and biodegradability of fenvalerate, a new generation of synthetic pyrethroid, is fairly inadequate. It was, therefore, considered essential to carry out detailed study on the metabolism of fenvalerate with regard to its detoxification and toxicity of the metabolites formed in animal tissues, in both acute and chronic exposure.

The scope of this research encompasses a deeper insight into the mechanism of pesticide action, toxicity and metabolism. Results of the present study are expected to be useful in making recommendations regarding safer use of the pesticide. In addition, this will

add to the knowledge needed in designing appropriate environmental technologies for pollution control. In view of the above, the present study was undertaken in rats with following objectives:

(1) **To study the bioaccumulation of fenvalerate in liver, kidney and brain of rats.**

(2) **To investigate the metabolic fate and isolate and characterise the degradation products formed during the biodegradation of fenvalerate in various organs.**

(3) **To study the biochemical changes and toxic effects induced by administration of fenvalerate in rats.**

(4) **To study the properties of the enzyme involved in the biodegradation of fenvalerate.**

(5) **Finally, to investigate the structural and functional damage caused by fenvalerate in tissues at cellular and subcellular levels.**

Chapter 2

LITERATURE REVIEW

2.1 PYRETHROIDS

Pyrethrum, an extract of the pyrethrum flower *Chrysanthemum cinarariaefolium*, contains four esters of complex alcohols and acids called Pyrethrins I and II and Cinerin I and II (Shepard, 1939). As an insecticide, owing to its favourable properties, it was widely used in agriculture and forestry and the home and laboratory animal quarters. Very early reports also suggest low mammalian toxicity (Negherbon, 1959).

Need of safer methods of insect control have prompted a search for alternatives. The manufacture and use of organochlorine pesticides in the U.S. have decreased in the last decade, in part due to their adverse effects on wildlife and the tendency of these chemicals to bioconcentrate. Replacement of these pesticides in the agricultural industry initially fell on the organophosphates, but more recently, on the synthetic pyrethroids (Schimmel et al., 1983). Natural pyrethrins and their synthetic analogs have found a valuable place in the control of insects. Pyrethrins I and II, first isolated from *Chrysanthemum cinarariaefolium* in 1924, were found to exhibit exceptional potency (Elliott, 1974). These compounds rapidly penetrate the insect cuticle and immobilise the pest. This property along with efficient toxicity makes these natural products desirable models for a new breed of pesticides. Pyrethrins act *per se* and no metabolic conversion to more toxic molecules is required for their action. So the

relatively short lived effects in survivors suggest that rapid detoxification of pyrethrins takes place with repair of damage to the membranes (Verschoyle and Barnes, 1972). Several synthetic derivatives have been constructed that introduce requisite commercial features such as low volatility, photostability, and even greater toxicity. The natural pyrethrins, apart from some minor irritant effects on upper respiratory tract, were not associated with any serious toxic effects on humans exposed to them and were thus considered safe to use. But later it was reported that they affect the cardiovascular system, liver and kidney (El-Sewedy et al., 1982; Vijverberg and Van den Bercken, 1982). Synthetic pyrethroids, in general, induced repetitive discharges in the nervous system of insects (Scott and Matsumura, 1983) and also vertebrates (Van den Bercken et al., 1973). In some cases, pyrethroids induced convulsions and paralysis at higher doses (Vijverberg and Van den Bercken, 1982). Synthetic pyrethroids are grouped into two classes based on structure - Type I pyrethroids and Type II pyrethroids. Type I pyrethroids do not have the α - cyano constituent and produce tremors and convulsions, while Type II pyrethroids generally contain α - cyano -3- phenoxybenzyl alcohol and produce choreoathetosis, convulsions and salivation. Earlier, pyrethroids were assumed to be non-inducing insecticides and were recommended for use in quarters housing animals undergoing pharmacologic treatment (Fouts, 1963; Conney, 1967), but later at high doses they were found to cause induction of microsomal enzymes (Springfield, 1973).

2.2 FENVALERATE

Fenvalerate [Sumicidin,(RS)- α -cyano-3-phenoxybenzyl - (RS)-2-4-(4-chlorophenyl) isovalerate] is one of the most potent pyrethroid insecticides and is used to control a range of pest insects of cotton and vegetables. It also effectively controls fowl mites (Hall et al., 1978; Loomis et al., 1979) and reduces the population of face fly, horn

fly and ticks in cattle (Davey et al., 1980; Knapp and Herald, 1981). This insecticide has four optical isomers due to the presence of two assymetric carbons in the molecule. The most potent isomer, the (2S, α S) - isomer has the absolute configuration [S] both at the C-2 of the acid moiety and at the benzylic carbon of the alcohol moiety (Kaneko et al., 1981).

Fenvalerate combines very high insecticidal activity against a broad spectrum of insect pests with moderate to low mammalian toxicity and adequate stability in fields (Ohno, 1977). This compound has undergone rapid development as an agricultural insecticide and several workers reported extensive toxicological evaluation (Ohkawa et al., 1978; Lee et al., 1985). Fenvalerate, a first generation synthetic analog, had been proposed for use in South Carolina as a substitute for the banned pesticide toxaphene (Hale et al., 1989).

2.3 FENVALERATE METABOLISM

2.3.1 Metabolism in Plants

Plant metabolism studies have been conducted with kidney bean (Ohkawa et al., 1980) and cabbage (Mikami et al., 1985). The increased use pattern of fenvalerate and its extended field residual activity require further knowledge of its metabolic fate in other crop species, especially in the grain cereals. Lee et al. (1988) reported the metabolic fate of [^{14}C]-fenvalerate on wheat plants after foliar treatment, systemic movement of [^{14}C] - fenvalerate and its metabolites was not observed. Undegraded [^{14} C] - fenvalerate was the major product recovered in the foliage or straw. The major degradation pathways included decarboxylation (photolytic decomposition) and ester cleavage. The overall metabolic fate of fenvalerate is consistent with other pyrethroids i.e., fluvalinate (Quistad et al., 1982), and decamethrin (Ruzo and Casida, 1978).

Lee et al. (1988) have also reported that in case of plants, species variability occurs. The metabolism of several pyrethroids has been examined in intact cotton plants under green house and field conditions and the excised leaf disks (Ruzo and Casida, 1979). It was observed that under field conditions, about 30% of ^{14}C - labeled permethrin was lost within one week (Gaughan and Casida, 1978). Permethrin, deltamethrin and cypermethrin are degraded on or in cotton plants primarily by photoisomerization, ester cleavage and conjugations (Roberts, 1981; Ruzo and Casida, 1979). Plant tissue culture more efficiently utilises the ^{14}C-labeled compounds and tends to optimise uptake and metabolism in pesticide studies compared to similar experiments under green house or field conditions. Ohkawa et al. (1980) found that in green house grown bean plants, fenvalerate metabolism included ester cleavage, hydrolysis of CN- group to $-CONH_2$ and -COOH groups, hydroxylation at the 2 and 4 - phenoxy positions, oxidation of 3-phenoxybenzyl alcohol derived by hydrolysis to 3-phenoxybenzoic acid and conjugation of resulting decarboxylic acids and alcohols with sugars. They also found that fenvalerate had a half-life of approximately 2 weeks under greenhouse conditions, while under field conditions, it had a half-life of 2 days in cotton (Holmstead et al.,1978) and approximately 3 weeks in wheat plant (Lee et al., 1988).

2.3.2 Metabolism in Soil

The fate of fenvalerate (Pydrin) in the soil environment was examined by Lee (1985). He found that the dissipation rates ($t_{1/2}$) of [^{14}C]-fenvalerate in the sandy loam and the silty clayloam soil under laboratory conditions were approximately 75-80 days which were somewhat slower than other reported studies ($t_{1/2}$ from 15 days to 3 months, Ohkawa et al., 1978). Earlier findings have also shown that variations in the test soil and environment produced variations in pyrethroid stability (Williams and Brown, 1979; Miyamoto and Mikami, 1983). Further study with sterilised sandy loam

soil showed the lack of biodegradation of the applied [^{14}C]- fenvalerate or formation of unextractable soil bound residues (Lee et al., 1982) even after 30 days of aerobic incubation. This indicated that soil degradation of fenvalerate was predominantly by microbial activity. They also monitored the [^{14}C]- fenvalerate and its degradation products indicating low soil mobility potential since less than 0.1 ppm of ^{14}C- residues were detected in 3-6 inch level even 2 years after application. This apparent lack of soil mobility was verified by soil column leaching and soil thin layer chromatography (Reed et al., 1983).

Rotational crop residue studies were also done by Lee et al. (1982). Lettuce, beets and wheat planted 30, 120 and 365 days after soil application were harvested and approximately 25% of total ^{14}C- residues in these crop samples were identified as the glucose conjugates of CPIA [4-chloro- α -(1-methylethyl) benzeneacetic acid] whereas majority of ^{14}C - residue was associated with the unextractable cellulose fraction. The rotational crop studies showed that very low residues of fenvalerate and its metabolites occur in plant grown in fenvalerate treated soil. Thus the rate of dissipation was significantly affected by the physicochemical properties and microbial nature of the test soils.

2.3.3 Metabolic Fate of Fenvalerate in Rats

Metabolism of fenvalerate and its various isomer compositions has been extensively studied in laboratory animals. Different fenvalerate preparations including the racemic, 2 S, RS and the resolved SS isomer, labeled with [^{14}C] at various positions (Carbonyl, -carbon and -CN moiety) have been examined by Sumitomo Chemical Company (Okhawa et al., 1979; Kaneko et al., 1981). Later, Lee et al. (1985) studied the rat metabolism of fenvalerate (Pydrin) and found that more than 90% of the

administered radioactivity from the acid moiety (chlorophenyl-^{14}C) and the alcohol moiety (phenoxyphenyl -^{14}C) was eliminated within the initial 24 h. Complete elimination of the toxin from the body occured within 5 days after dosing. They also observed that the complete metabolic reactions were independent of the isomeric composition of the test material. Moreover, fecal route was the major route of elimination, accounting for approximately 60% of the administered dose. Tissue distribution profile showed highest ^{14}C-residue levels in liver and fat tissues.

Fenvalerate follows an overall metabolic profile which is very similar to other cyano pyrethroids such as cypermethrin (Cole et al., 1982; Crawford et al., 1981), decamethrin (Ruzo et al., 1978) and deltamethrin (Cole et al., 1982). Lee et al. (1985) observed a rather complex metabolic degradation pattern of fenvalerate in rats. This included: hydroxylation of the intact molecule, ester cleavage, hydroxylation and oxidation of the acid portion of the fenvalerate molecule, hydroxylation and oxidation of the alcohol portion of the fenvalerate molecule,oxidation of the cyano moiety and conjugation.

Kaneko et al. (1981) studied the comparative metabolism of fenvalerate and the (2 S, α S) isomer in both rats and mice. Although no significant differences in the nature and amount of metabolites were seen between the sexes, dose levels or isomers, some species differences were found. The tissue residue in the mice and rats given the acid- and the alcohol - labeled compounds were very low except for fat, while the -CN labeling showed somewhat slower excretion of radiocarbon, longer half life and higher tissue residues. Earlier, Ohkawa et al. (1979) had found that concentration of fenvalerate in blood and liver reached maximum levels within 3 h after a single oral dose of ^{14}CN-S-fenvalerate and rapidly decreased, so that at 144 h, the concentration was below 0.01 ppm in both tissues. Cleavage of the ester linkage led

to the release of cyanide which was rapidly converted to thiocyanate and some extent to CO_2.

The half lives of excretion following treatment of rats at 7.0 or 8.4 mg/Kg and mice at 30 mg/Kg were 12-14 h as reported by Kaneko et al.(1981). Other systems studied with respect to fenvalerate metabolism included dogs (Kaneko et al., 1984), ruminants (Wzolek et al., 1981), chickens (Akhtar, 1983) and bobwhite quail (Bradbury and Coats, 1982). Although, most of the metabolites formed by metabolism of fenvalerate in Japanese quails (*Coturnix coturnix* japonica) were also found in earlier studies with rats, mice and dogs, quantitative differences could well be observed between the avian and mammalian species when equitoxic doses were administered (Mumtaz and Menzer, 1986).

To understand the mechanisms involved in the metabolism of fenvalerate, Mumtaz and Menzer (1986) conducted detailed *in vitro* experiments employing rat and quail liver microsomes. The major metabolites found in both rat and quail microsomal extracts were similar except 4′ OH fenvalerate which could be detected only in quail microsomal extracts. They found about 34% fenvalerate to be metabolised by Japanese quail microsomes, while earlier Akhtar (1983) had reported less than 10% metabolism of fenvalerate by chicken liver microsomes. Soderlund and Casida (1977) revealed that in *in vitro* metabolism, the esters derived from α-cyano-3-phenoxybenzyl alcohol were hydrolysed much more slowly than their primary alcohol analogues by mouse liver esterases. These esters also appeared to be more resistant to oxidation by mouse liver oxidases. However, it is to be noted that fenvalerate is rapidly metabolised in living rats mainly by ester cleavage and oxidation at 4′ of alcohol moiety. Several studies *in vivo* and *in vitro* have finally shown that by decreasing the rate of metabolism, pyrethroid toxicity can be increased.

2.3.4 Metabolic Fate of Fenvalerate in Poultry/Livestock

Very little information is available on the fate of fenvalerate in livestock and poultry. Wszolek et al. (1981) reported the detection of insecticide and one of its major metabolite in the milk and feaces, but not in urine, of the dairy cows fed with fenvalerate - fortified grain. The bovine rumen fluid did not appear to readily degrade fenvalerate *in vitro* (Wszolek et al., 1981). Lambs were also fed fenvalerate - fortified grain to study the accumulation of fenvalerate in tissues of agricultural animals. The study indicated that the insecticide accumulates in the animal's fat with much smaller residues present in other tissues. Since the expected agricultural use pattern of the insecticide may contaminate feed stuff, Akhtar et al. (1989) studied the distribution of excretion to identify the nature of residues in eggs and meat of laying hens that were fed with fenvalerate. Majumdar et al. (1994) studied the subacute toxicity of fenvalerate in broiler chicks.

2.4 FENVALERATE TOXICITY TO AQUATIC ENVIRONMENT

Meiobenthos are organisms well suited for contaminant studies since they are intimately associated with sediments. Recently, life history studies with sediment-associated contaminants have been conducted (Chandler, 1990; Chandler and Scott, 1991). Since the advent of manufactured pesticides, non-point source runoff is a major environmental problem. Much of the pesticides released into the environment never find their target organisms and are instead washed into aquatic systems (Pimental and Edwards, 1982). Schimmel et al. (1983) found that fenvalerate was one of the most toxic pesticides, with very low LC_{50} values for fish and shrimp and extremely high bioaccumulation in the oyster, *Crassostrea virginica* (bioconcentration factor = 4700). They also reported that fenvalerate, if detected, was most likely to

be associated with adverse effects on biotic composition of that system.

Fenvalerate was found to be relatively persistent when associated with the sedimentary environment. The half-life ranges from 27 to 34 days (Schimmel et al., 1983) or 12 to 41 days (Cotham and Bildeman, 1989). Since fenvalerate is very hydrophobic, it adheres to organic matter on the bottom quickly or is suspended in the water column and accumulates to >100 mg/Kg in estuarine sediments (Scott et al., 1989). The sublethal effects of sediment- associated fenvalerate on reproductive output of life history stages of the benthic copepod, *Amphiascus tenuirenis* have been studied by Strawbridge et al. (1992), but found no adverse effect of fenvalerate on their population sizes, may be due to tight adherence of fenvalerate to organic matter. Fenvalerate is reported to be super toxic to fish and macro-invertebrates (Tagatz and Ivey, 1981). Linden et al. (1979) previously reported that aqueous fenvalerate was highly toxic (LC_{50} = 1.9 g/l) to another copepod, *Nitocra spinipes*.

It has been well established that organic matter attracts hydrophobic compounds such as pesticides including fenvalerate (Caplan et al., 1984; McCarthy and Jimenez, 1985; Chiou et al., 1987; Servos and Miur, 1989). About 100% of the aqueous pesticide toxicity studies with meiobenthos have shown high mortality and inhibited life-history progressions with increasing concentration (Coull and Chandler, 1992). Schimmel et al. (1983) evaluated some pyrethroid insecticides in order to determine their acute toxicity, solubility, persistence in sea water and their respective bioconcentration factors (BCF).

The pyrethroid insecticides are extremely toxic to fish (Khan, 1983). The acute effects of fenvalerate on fish in static tests have been well documented by Coats and O'-Donnell-Jeffrey (1979). To study the pyrethroid mode of action in fish, the physi-

ological responses of rainbow trout (*Salmo gairdneri*) to fenvalerate intoxication during aqueous exposure were examined by Bradbury et al. (1987). Visible signs of intoxication included elevated cough rate, tremors and siezures. Damage to gill tissue was consistant with irritation. Bradbury et al. (1985) and Holcombe et al. (1982) reported fenvalerate flow through 96-h LC_{50} values of 5.4 to 0.69 mg/l for rainbow trout (*S. gairdneri*) and fathead minnows (*Pimephales promelas*).

The extreme toxicity of these insecticides to fish may be due to (i) efficient gill uptake (ii) inefficient detoxification and elimination, and (iii) sensitivity at the site(s) of action. Thus, low rates of fenvalerate elimination and metabolism do seem to play an important role in the piscicidal activity of this insecticide (Bradbury et al., 1986).

2.5 INSECTICIDAL ACTIVITY OF FENVALERATE

The insecticidal activity of pyrethroids involves among other factors, esterase detoxification. Also oxidase detoxification is even more significant among some species. Pyrethroid hydrolysing esterases are much less active in insects than in mouse liver preparations (Jao and Casida,1974). Populations of insects subjected to selection pressure by insecticides develop resistance to the selective agent and often other chemically related insecticides. Davies et al. (1958) found that repeated field use of piperonyl butoxide (PBO) synergised pyrethrins in Sweden in the 1950 resulted in house fly populations (*Musca domestica*) that were highly resistant to PBO-synergised pyrethrins. The mechanism of resistance associated with these flies was a general resistance mechanism that delayed knockdown (kdr) (Farnham,1971).

Some reports have demonstrated that enhanced metabolism is a major factor in permethrin resistance in both field and laboratory- selected house flies (Motoyama,

1984; MacDonald et al. 1985; Scott and Georghiou, 1986). The metabolism of fenvalerate by resistant Colorado potato beetles was studied by Soderlund et al. (1987). The distribution and metabolism of radiolabeled fenvalerate were compared in pyrethroid resistant (R) and susceptible (S) house flies (Golenda and Forgash, 1989). It was observed that excretion of fenvalerate metabolites began 24 h post treatment in S-flies while after 4 h in R-flies. No qualitative differences were observed in identified metabolites between strains, but quantitative differences were there. The effect of PBO on metabolism implicates a role for the mixed-function oxidase (MFO) system in the resistance mechanism of this strain of house fly. Golenda and Forgash (1989) found that cuticular penetration was faster and excretion of absorbed radioactive material slower in S-insects and that metabolic detoxification of the pyrethroid via oxidative and hydrolytic routes was substantially enhanced in R-insects. But Bull and Pryor (1990) found that in case of tropical application of [^{14}C]-fenvalerate in R-insects, the coadministration with synergists (PBO and DEF) slowed down the rate at which the toxicant penetrated the cuticle. The synthetic pyrethroid (permethrin) resistance was attributed to two major factors (i) a kdr like form of nerve insensitivity; and (ii) an enhanced capacity for metabolic detoxification. Based on the toxicity tests with synergists (which on coadministration with insecticides, inhibit the important detoxifying enzyme systems) and *in vivo* metabolism studies, the enhanced metabolism appeared to involve a major contribution by mixed function microsomal oxidases (MFO). Comparative studies of the *in vitro* metabolism of [^{14}C]-fenvalerate by homogenate preparations of S - and R- houseflies were carried out by Bull and Pryor (1990). They concluded that enhanced metabolic degradation is a major factor in the cross-resistance of their R-strain to fenvalerate. The existance of kdr-like resistance to pyrethroids in houseflies is well documented through brain membrane receptor binding studies with [^{3}H] Saxitoxin (Rossignol, 1988) and [^{14}C] cis-permethrin and DDT (Chang and Plapp, 1983) and electrophysiological studies for the effect of pyrethroids on the central

nervous system (Scott and Georghiou, 1986; Miller et al. 1979; Devries and Georghiou, 1981). Insecticidal activity of pyrethroids is highly dependant on the stereochemistry of both acid and alcohol moieties, so that diastereomeric and enantiomeric esters with similar physical properties may greatly differ in potency suggested by Elliott et al. (1974) and Elliott (1974). Although pyrethroids are known to alter impulse conduction in insects and arthropods nerve axons *in vivo* (Gammon, 1978) and *in vitro* (Narahashi, 1962; Burt and Goodchild, 1977; Nishimura and Narahashi, 1978), studies using a variety of insecticidally active pyrethroids have failed to show a clear correlation between the structure-activity relationships found in neurophysiological preparations and those observed for insecticidal potency (Clements and May, 1977; Narahashi et al., 1977). Gerolt (1969) had earlier suggested that lipophilic neuroactive insecticides may reach the insect nervous system by external migration in the cuticular waxes and the integument of tracheal system rather than by transport in the haemolymph. However, Soderlund (1979) suggested that more rapid uptake of a potent pyrethroid insecticide NRDC 157 in haemolymph than fat body of *P. americana* is consistent with hypothesis that the internal distribution of this compound to site action is mediated by transport in haemolymph, despite low aqueous solubility of pyrethroids. They also suggest that receptor chirality is the source of consistent differences in potency seen between pyrethroid enantiomers. Thus, the use of enantiomer pairs in neurophysiological or biochemical studies of pyrethroid action at the molecular level is a potentially powerful tool for identifying toxicologically significant responses to pyrethroids *in vitro* systems. Clements and May (1977) showed that insecticidally inactive isomers of fenvalerate were atleast 100-fold less effective than active isomers on peripheral nerve and nerve-muscle preparations from *Schistocerca gregaria*. Yellama and Reddy (1987) studied the effect of fenvalerate on behavioral aspects and spontaneous activity of ventral nerve cord (VNC) under *in vitro* and topical modes of administration and found that fenvalerate - induced toxic effects were antagonised by *in vitro* application

of acetylcholine due to opening of Na^+ channels by ACh or increase in the sensitivity of ACh receptors on the post synaptic membrane. Suppression of the spontaneous activity was seen as an intervention of fenvalerate with Na^+ and K^+ channels or due to inhibition of Ca^{2+}-ATPase and Mg^{2+}-ATPase systems (Lund and Narahashi, 1982).

During the course of insect development the activity of many enzymes, including those responsible for insecticide detoxification, may undergo dramatic changes (Krieger and Wilkinson, 1969; Yu and Terriere, 1974; Yu, 1983). Since an insect's ability to deal with xenobiotics, such as insecticides, may change during the course of development, a study was undertaken by Dowd and Sparks (1987) to understand the presence and involvement of hydrolysis in pyrethroid detoxification in *P. includens* and *H. virescens*. They suggested that hydrolysis would be a major metabolic influence on the toxicity of pyrethroid insecticides to these two insect species. Hence, reducing hydrolysis through the use of synergists that inhibit hydrolytic enzymes, as suggested for other insect species (Schnitzerling et al., 1983) may increase pyrethroid toxicity to all stages of *P. includens* and *H. virescens*. Dowd et al. (1987) suggested that organophosphate - pyrethroid combinations may provide atleast a short-term improvement in the control of pyrethroid-resistant *Heliothis virescens*.

2.6 NEUROTOXICOLOGICAL AND BIOCHEMICAL EFFECTS OF FENVALERATE

The pyrethroid insecticides are generally accepted to be potential neurotoxicants (Casida et al., 1983). Specific hypotheses regarding the effect of pyrethroids in the nervous system include interactions with (i) sodium channels (Eells et al., 1993 Narahashi et al., 1992 Narahashi,1983), (ii) the γ-aminobutyric acid-receptor-ionophore complex (Cole et al., 1984; Lawerence and Casida, 1983), and (iii) ATPase - utilising systems (Clark and Matsumura, 1982). Numerous studies have been published

indicating that pyrethroid insecticides are capable of inhibiting a variety of ATPase - utilising systems including Ca^{2+} and Ca^{2+} + Mg^{2+}-ATPases (Clark and Matsumura, 1982), mitochondrial Mg^{2+}-ATPase (El-Sebae et al., 1981; Desaiah et al., 1975) and Na^{+}-K^{+}-ATPase (Desaiah et al., 1975) which could be responsible for increase in ion loss from intoxicated rainbow trout (Bradbury et al., 1987). The peripheral benzodiazepine (PBZ) receptor are found in high concentration in adrenal cortex and skin which is correlated with high mitochondrial concentration and cytochrome oxidase activity. Thus, PBZ receptor may be regulator of mitochondrial functions and affect all growth and differentiation. Pyrethroids are found to have convulsant and proconvulsant activities in experimental animals asymptomatic with intoxication (Gilbert et al., 1990; Devand, 1986) which suggest that PBZ receptor may not constitute primary lesion in pyrethroid neurotoxicity. The interactions of 14 pyrethroids with PBZ receptors of rat brain were studied by Ramadan et al. (1988). Since PBZ receptors are also abundant in salivary glands and lungs, it is speculated that excessive salivation and persistent edema in lungs of mice intoxicated with type II pyrethroids (Grey,1985) might be due to action of pyrethroids on PBZ receptor. In addition, since activation of PBZ receptor increases neural excitability (Massati and Lucautoni, 1986) and inhibition of GABA receptor also results in increased excitation, the action of pyrethroids at these two targets contribute to the massive neurotoxicity originating from action of pyrethroids. The presence of cyano moiety in fenvalerate and other type II pyrethroids has been suggested to greatly enhance neurotoxicity in mammals (Casida et al.,1983; Grey and Soderlund,1985).The effect of systemic treatment of fenvalerate was studied by Husain et al. (1991) in adult female rats at different doses on regional brain levels of noradrenaline (NA), dopamine (DA) and their acid metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) by HPLC-EC system. Their neurochemical data suggested that repeated exposure of fenvalerate to rats resulted in changes in the levels of NA,DA, HVA and DOPAC in discrete brain

areas. The regions affected most were corpus striatum, pons medulla and hypothalamus which are related to motor function and aggression (Singhal and Merali, 1979). It has thus been observed that interference of xenobiotics with sites involved in the complex process of synaptic transmission may lead to improper information transfer with CNS and cause subtle behavioral changes. So the observed symptoms of headache, dizziness, nausea and ataxia found in fenvalerate exposed workers (He et al., 1988) may be due to alterations in metabolic disposition of catecholamines and sensitization of catecholamine receptors (Husain et al., 1991).

In large scale application of pyrethroids by crop dusting, orchard/forest spraying some of it enters the aquatic ecosystem and adversely affects fresh water fishes (Johnson, 1973). Several workers have reported that activity of aldolase has been altered by pesticides in fishes (Rao, 1984; Bhatia et al., 1973). Radaiah et al. (1989) studied the toxic effect of fenvalerate on aldolase activity in liver, kidney, gill and brain tissues of fish *Tilapia mossambica* and observed that activity of aldolase increased with duration of exposure in all tissues with maximum increase in liver; thus, indicating rapid cleavage of C-C bonds of hexoses leading to formation of trioses which were made available for further oxidation (Harper et al., 1978). The study also suggested that the pesticides shifted the aerobic metabolism with simultaneous increase in glycolytic pathway (Losota, 1973) and that fenvalerate intoxication altered the glycolytic pathway in fish.Since superoxide dismutase (SOD) and catalase play an important role as cellular defence system in scavenging the reactive oxygen intermediates, the toxic impact of fenvalerate on these enzymes in *Tilapia mossambica* was observed by Radaiah and Reddy (1989). Reddy and Yellama (1991) monitored the toxicity of fenvalerate in *Periplaneta americana* and observed a decrease in total and soluble proteins attributed to increased proteolysis. Further, the levels of glycogen, pyruvate and SDH and MDH activity dropped significantly and lactate and LDH activity showed an increase.

Fenvalerate induced biochemical changes were also observed in fresh water fish by Reddy et al. (1991). Other reports have shown that Os-Mg^{2+} and Na^{+}-K^{+}-ATPases were decreased in rat exposed to pyrethroids (El-Toukhy and Girgis, 1993) and ATP levels reduced in *Daphnia magna* exposed to fenvalerate (McKnee and Knowles, 1986). Subacute toxicity studies of fenvalerate was carried out in broiler chicks after prolonged treatment by Majumdar et al.(1994). Fenvalerate is found to increase the cholesterol level in brain, GPT activity in liver and heart and GOT activity and alkaline phosphatase in heart tissues in broiler chicks. Biochemical and toxicological effect of fenvalerate on rat lungs was studied by Tian (1993).

Sarkar et al. (1993) studied the effect of fenvalerate on membrane fluidity. It has been observed that fenvalerate interacts with the membrane by localising itself near the hydrophobic tail region of the lipid acyl chain and perturbs its ordered structure to make the membrane more fluid. The effect of fenvalerate on membrane ionic current specifically sodium current (Lund and Narahashi, 1983) might partly be exerted by this fluidisation effect because changes in membrane fluidity by cholesterol has already been shown to change ionic channel characteristics (Balotina et al., 1989).

We have studied the various aspects of fenvalerate in rats. As far as we are aware, the effect of fenvalerate on biochemical parameters such as lipids, nucleic acids etc. have not been studied up to now. Also we have not come across the effect of fenvalerate on membrane proteins at cellular and subcellular levels in rats.

Chapter 3

MATERIALS AND METHODS

3.1 MATERIALS

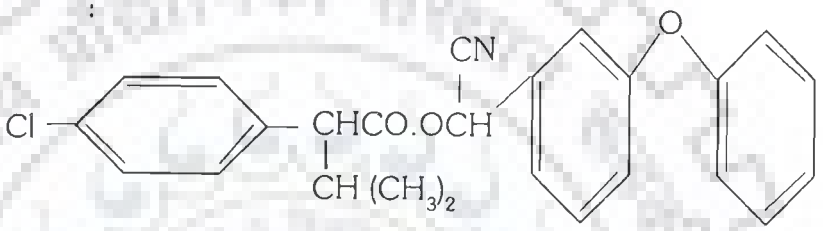
3.1.1 Chemicals

Fenvalerate (Technical grade) was a gift from Gujrat Ankleshwar Ltd., (India). Bovine serum albumin, phospholipid and neutral lipid standards, Triton X-100, Tris, sodium dodecyl sulphate(SDS), Nonidet P-40 were purchased from Sigma Chemical Company (St. Louis,USA). Acrylamide, N,N- methylene-bis-acrylamide and TEMED were obtained from Serva (Germany). Digitonin, β-mercaptoethanol, ammonium persulphate, p-nitrophenyl phosphate and HEPES buffer were procured from E.Merck. The molecular weight standards were obtained from Biorad (USA). The organic solvents used were HPLC grade obtained from Spectrochem, (Bombay, India). All other chemicals were reagent grade from standard firms. Male albino rats (*Rattus rattus* albino) weighing 100 ±10 g were obtained from Indian Drug and Pharmaceutical Ltd. (Rishikesh India).

3.1.2 Animals

Male albino rats (*Rattus rattus* albino), weighing 100± 10 g, were housed in cages with galvanised iron wire bar tops in a room maintained at 25 ± 2°C and an alternate 12 h light - dark photoperiod. The animals were on standard pellet diet (Lipton, India) and water *ad libitum* throughout the study period.

FIG. 3.1 CHEMICAL IDENTITY AND PHYSICO-CHEMICAL PROPERTIES OF FENVALERATE

CHEMICAL NAME (IUPAC)	:	(RS)-α-cyano-3-phenoxybenzyl (RS)-2 (4-chlorophenyl)-3-methylbutyrate
FORMULATIONS	:	Sumicidin, Pydrin, Belmark.
MOLECULAR FORMULA	:	$C_{25}H_{22}Cl\,NO_3$
MOLECULAR WEIGHT	:	419.9
STRUCTURE	:	

CHARACTERSTIC PROPERTIES :		
PHYSICAL STATE	:	Viscous liquid, partly crystalline at room temperature
ODOUR	:	Slightly pungent
COLOUR	:	Yellowish - Brown
VAPOUR PRESSURE (25°C)	:	0.037 mPa.
SOLUBILITY IN WATER (20°C)	:	< 1 mg/l
ORGANIC SOLVENTS (23°C)	:	> 1 Kg/Kg acetone, chloroform cyclohexanone, ethanol, xylene; 155 g/Kg hexane.
STABILITY	:	Stable to heat and sunlight. More stable in acid than in alkaline media with optimum stability at pH 4.0.

TOXICOLOGY

ACUTE ORAL LD_{50} (rats)	:	451 mg/Kg
ACUTE PERCUTANEOUS LD_{50} (rats)	:	> 5000 mg/Kg
ACUTE ORAL LD_{50} (domestic fowls)	:	> 1600 mg/Kg

3.2 METHODS

3.2.1 Fenvalerate Treatment of Rats

For oral toxicity studies, doses were prepared by dissolving fenvalerate in peanut oil. Ten rats in two groups each were weighed prior to dosing and treated with fenvalerate through oral gavage . Long term studies (chronic treatment) were carried out by administering oral doses of 5 mg/Kg ($1/90^{th}$ of LD_{50}) and 15 mg/Kg ($1/30^{th}$ of LD_{50}). In chronic treatment, rats were given dose for 7, 15 and 30 days on alternate days. In acute treatment rats were administered a single oral dose of 100 mg/Kg body weight and the animals were sacrificed 0, 6, 12, 24, 36, and 48 h postdosing. Control rats were administered an equal volume of peanut oil (0.2 ml/100 g body weight). Before terminating the experiments at designated intervals of time, the animals were weighed.

3.2.2 Extraction of Fenvalerate Residues and its Metabolic Products from Rat Tissues

For the extraction of fenvalerate residues and its metabolic products from rat tissues, the animals were sacrificed at indicated time intervals after the administration of pesticide. Liver, kidney, and brain tissues were removed and used immediately for the extraction of residual fenvalerate and its metabolites.

Tissue (1 gm fresh weight) was washed twice in chilled saline (0.9% NaCl) and homogenised in 2 ml of 50 mM Tris-HCl buffer, pH 7.4. The homogenate was extracted twice with 5 ml of chloroform. The phases were separated by centrifugation at 1000 x g for 10 min. The lower organic layer was washed with 5 ml of 0.9% NaCl solution, acidified to pH 2.0, by vigorous shaking. The lower organic layer

($CHCl_3$ layer) was collected and dried under a stream of nitrogen gas. Final sample was redissolved in 0.5 ml of $CHCl_3$ for further analysis.

3.2.2.1 Analysis of Fenvalerate Residues and Metabolic Products by High Performance Liquid Chromatography (HPLC)

Fenvalerate and its metabolic products were analysed by HPLC using a Shimadzu (Japan) Liquid Chromatograph (LC-4A) equipped with a UV-detector and a reverse phase Zorbax ODS- C_{18} column and eluted with a mobile phase consisting of acetonitrile: water (80:20, v/v) at a flow rate of 1.5 ml/min. The elution was monitored by UV absorption at 280 nm and quantitation was based on a direct comparision of peak areas with that of pure fenvalerate standard used for caliberating the instrument. Multiple injections were given on the same column to collect sufficient amount of various metabolites. Fractions were then repeatedly chromatographed on the reverse phase Zorbax ODS- C_{18} column until purified to homogeneity as judged by the single sharp peak and also by single spot on TLC.

3.2.2.2 Analysis of Fenvalerate and its Metabolic Products by Thin-Layer Chromatography (TLC)

Thin-layer chromatography (TLC) of fenvalerate was performed according to the modified method of Shaikh et al. (1990). Fenvalerate and its metabolic products were separated on TLC plates (coated with Silica Gel G, E.Merck of 0.50 mm thickness).

The solvent system used for chromatogram development was

Hexane : Acetone : Ethylacetate

12 : 5 : 3

The spots were located by exposure to iodine vapours. The spots were identified by comparing their R_f values with authentic reference standards.

3.2.3 Infra Red Spectroscopy

The purified metabolites were characterised by Infrared spectroscopy using a Perkins Elmer (model 883) double beam spectrophotometer (4000-200).

3.2.4 Transmission Electron Microscopy (TEM)

The ultrastructural changes in rat liver and kidney were viewed under transmission electron microscope, using CM10 (Phillips) TEM. Samples of liver and kidney obtained from rats after different intervals of time were primarily fixed in 2.5% gluteraldehyde overnight at 4°C. Samples were then washed with 0.1 M phosphate buffer three times, changing after every 30 min at 4°C. Post fixation was done in 1% osmium tetroxide for 2 h at 4°C. Again washing was done with 0.1 M phosphate buffer. Dehydration through a graded series of ethanols was carried out and sections were cleared in toluene for 2 h at room temperature. Infiltration was done using 3:1, 2:2, 1:3 and 0:4 part of toluene :araldite for 2 h each at room temperature, which was followed by embedding in beam capsules. Sections were then kept at 60°C for 72 h. Sections (60-90 nm thick) were stained with uranyl acetate and lead citrate for 15 min each.

3.2.5 *In Vitro* Metabolism

3.2.5.1 Enzyme Preparation

Excised rat livers (~10 gm) were homogenised in a glass-teflon homogeniser in 4 volumes of 0.01 M phosphate buffer, pH 7.4. The homogenate was centrifuged at

1000 x g for 10 min to remove nuclei and other cell debris. The supernatant was again spun at 12,000 x g for 20 min to pellet down mitochondria. The inner and outer mitochondrial membranes were separated as described in Fig. 3.4 and used as enzyme source. The post mitochondrial fraction was centrifuged at 100,000 x g for 60 min. The microsomal pellet obtained was resuspended in 0.01 M phosphate buffer, pH 7.4.

3.2.5.2 Enzyme Assay

In order to assess the enzyme activity, the conversion of fenvalerate to its hydrolytic products was measured. The incubation medium contained outer mitochondrial membrane (1 ml, 1 mg/ml protein), 8 µmol $MgCl_2$, 1.8 µmol NADPH and 150 µmol KCl and volume made upto 2.5 ml with 0.1 M phosphate buffer (pH 7.5). The incubation mixture equilibrated for 5 min before incubation was initiated with the addition of 0.50 mg fenvalerate dissolved in 100 µl of acetone. The incubations with membranes was carried out at 37°C and terminated after 30 min, unless stated otherwise, by adding 5 ml of chloroform. In control incubations an equal volume of phosphate buffer replaced either membrane fraction or substrate. After termination of the *in vitro* incubation, the incubation medium was extracted with 5 ml of chloroform twice under acidic pH conditions. The two phases were then separated by centrifugation at 3000 x g for 10 min. The pooled extracts were dried under a stream of nitrogen gas, redissolved in 0.5 ml of $CHCl_3$ and finally analysed for residual fenvalerate and its metabolites on HPLC as described earlier. The flow chart is shown in Fig. 3.2.

3.2.6 Subcellular Membrane Fractions from Rat Liver

3.2.6.1 Preparation of Nuclear Membrane

Nuclear membrane was prepared by the modified method of Franke et al.

FIG. 3.2 : FLOW CHART FOR *IN VITRO* ENZYME ASSAY

The incubation mixture consist of :

Membrane fraction	:	1.0 ml (~ 1.0 - 1.5 mg protein)
$MgCl_2$ (8 μM)	:	0.2 ml
KCl (150 μM)	:	90 μl
0.1 M PO_4^{2-} Buffer (pH 7.5)	:	1.0 ml
NADPH (1 μM)	:	10 μl
Fenvalerate (500 μg)	:	100 μl
		2.4 ml

↓

Incubation at 37°C for 30 min with shaking

↓

Reaction terminated by 5 ml of $CHCl_3$.

↓

Extraction of pesticide and its products by 5 vols. of $CHCl_3$ twice.

↓

$CHCl_3$ extract washed with acidified 0.9% NaCl (aq.).

↓

Separation of $CHCl_3$ layer and dry under N_2 gas.

↓

Redissolved in 0.5 ml $CHCl_3$ for **HPLC/TLC** analysis.

(1973). Rat livers were minced in cold homogenising medium (0.45 M sucrose, 0.07 M KCl, 0.01 M Tris - HCl, pH 7.2). The homogenate was filtered through 2 layers of cheese cloth and filtrate centrifuged at 1800 x g for 20 min. The pellet suspended in the same medium was purified by mixing with dense sucrose medium (1.8 M), layered over pure dense sucrose medium (2.0 M sucrose, 0.07 M KCl, 0.01 M Tris, pH 7.2) and centrifuged at 60,000 x g for 90 min. The nuclear membrane pellet so obtained was sonicated in high salt extraction medium (0.3 M sucrose, 1.5 M KCl, 0.01 M Tris - HCl, pH 7.2). The suspension was stirred for 3-4 h at 4°C and later centrifuged at 800 x g for 20 min, wherein larger nuclear fragments were removed. The supernatant was spun at 110,000 x g for 2 h and the pellet so obtained was resuspended in 0.1 Tris - HCl, pH 7.2. This pellet contained nuclear membrane. The Scheme for nuclear membrane preparation is shown in Fig. 3.3.

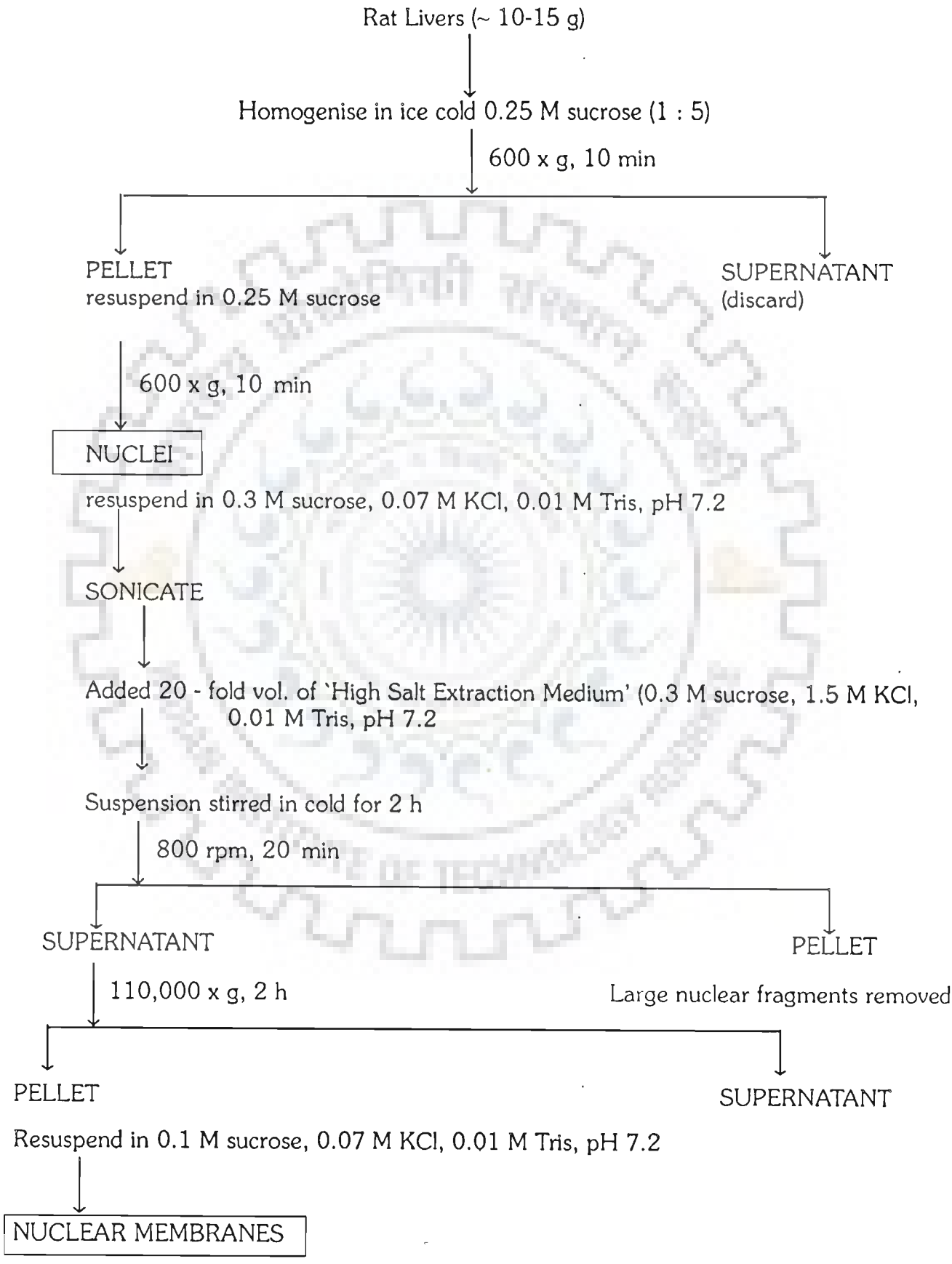
3.2.6.2 Preparation of Mitochondrial Membrane

Mitochondrial membranes were prepared by the method described by Morimoto et al. (1976). Briefly, rat livers were homogenised in cold medium H (220 mM D-Mannitol, 70 mM sucrose, 2 mM HEPES, pH 7.4, 1 mM EDTA) and centrifuged at 660 x g for 10 min. Pellet was discarded and supernatant was spun at 4400 x g for 10 min. The mitochondrial pellet was washed with medium H and final mitochondrial pellet was obtained.

3.2.6.3 Separation of Outer and Inner Mitochondrial Membranes

Freshly isolated mitochondria was treated with digitonin (1 mg digitonin/10 mg mitochondrial protein) for 15 min. Suspension was diluted 10-fold with cold medium H and centrifuged at 12,000 x g for 5 min. The pellet i.e., mitoplast (inner

FIG. 3.3 : FLOW CHART FOR NUCLEAR MEMBRANE PREPARATION FROM RAT LIVER



mitochondrial membrane) was washed with medium H at 12000 x g for 15 min twice and final mitoplast fraction was obtained, while the supernatant was centrifuged at 144,000 x g for 30 min to sediment the outer membrane vescicles. The complete scheme for mitochondrial membrane preparation is given in Fig. 3.4.

3.2.6.4 Preparation of Plasma Membrane

The method of Fleischer and Kervina (1974) was used for the plasma membrane preparation from rat livers. 15 gm rat livers were homogenised at 4°C in 0.25 M sucrose, 0.01 M HEPES, pH 7.5, filtered through 4 layers of cheese cloth and centrifuged at 1000 x g for 10 min. The pellet containing nucleus, mitochondria and plasma membrane was resuspended in 0.01 M HEPES containing 0.25 M sucrose and 1 mM $MgCl_2$. This was layered on a 2- step gradient (0.25 M/1.6 M sucrose) and spun at 80,000 x g for 70 min. The interface band rich in mitochondria and plasma membrane was purified by layering on 0.25 M sucrose and spun at 1200 x g for 10 min. The band was layered on 0.25 M/1.45 M sucrose and centrifuged at 68,000 x g for 60 min. The interphase band now containing plasma membrane fraction was resuspended in 0.01 M HEPES containing 0.25 M sucrose, 1 mM EDTA and pelleted down. The pellet was layered on 0.25 M/1.35 M sucrose and centrifuged at 68,000 x g for 60 min. About 5 mg of whitish pellet of purified plasma membrane was obtained. The total scheme for the isolation of plasma membrane fraction is shown in Fig. 3.5.

FIG. 3.4 : FLOW CHART FOR MITOCHONDRIAL MEMBRANE PREPARATION FROM RAT LIVER

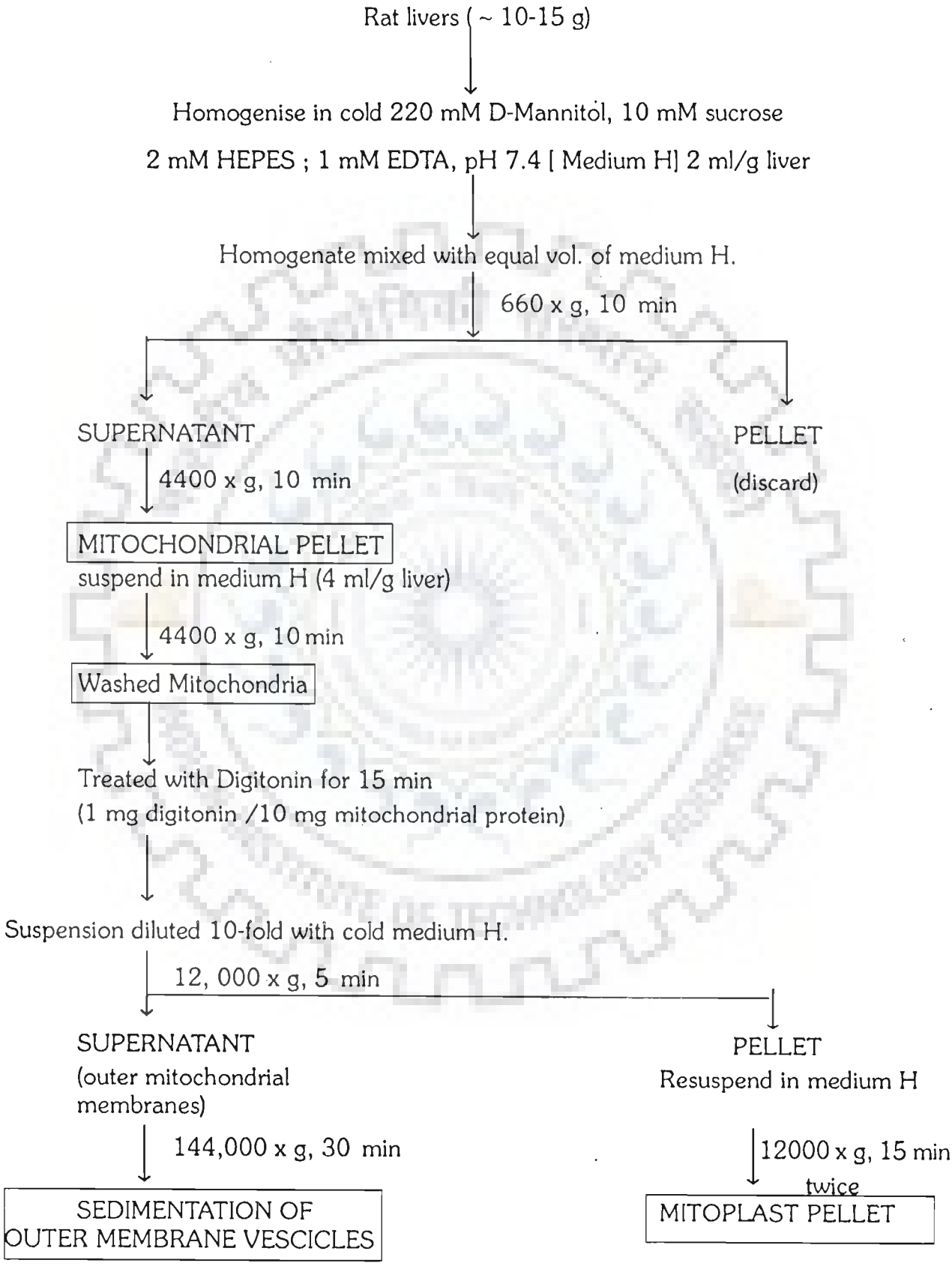
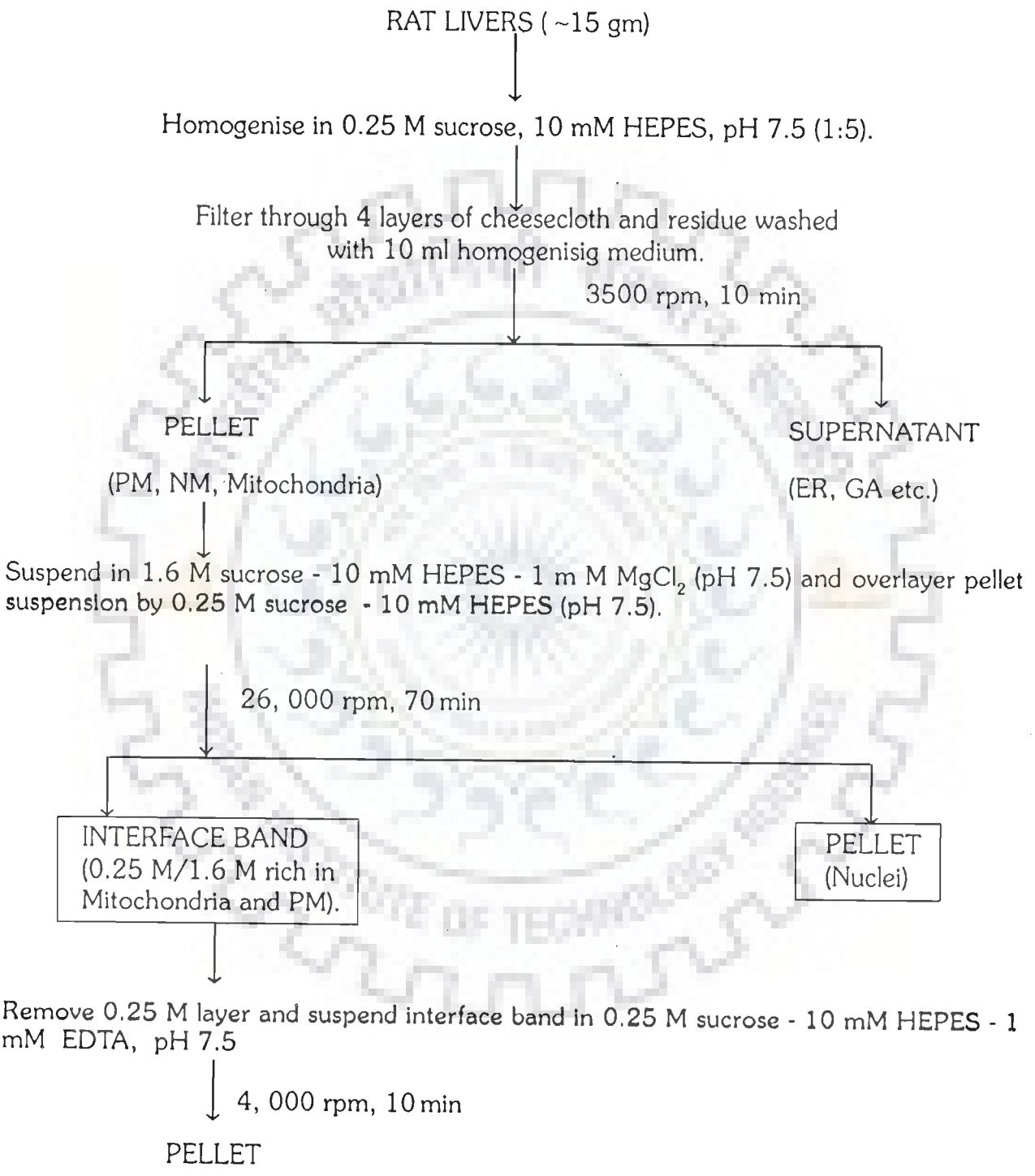
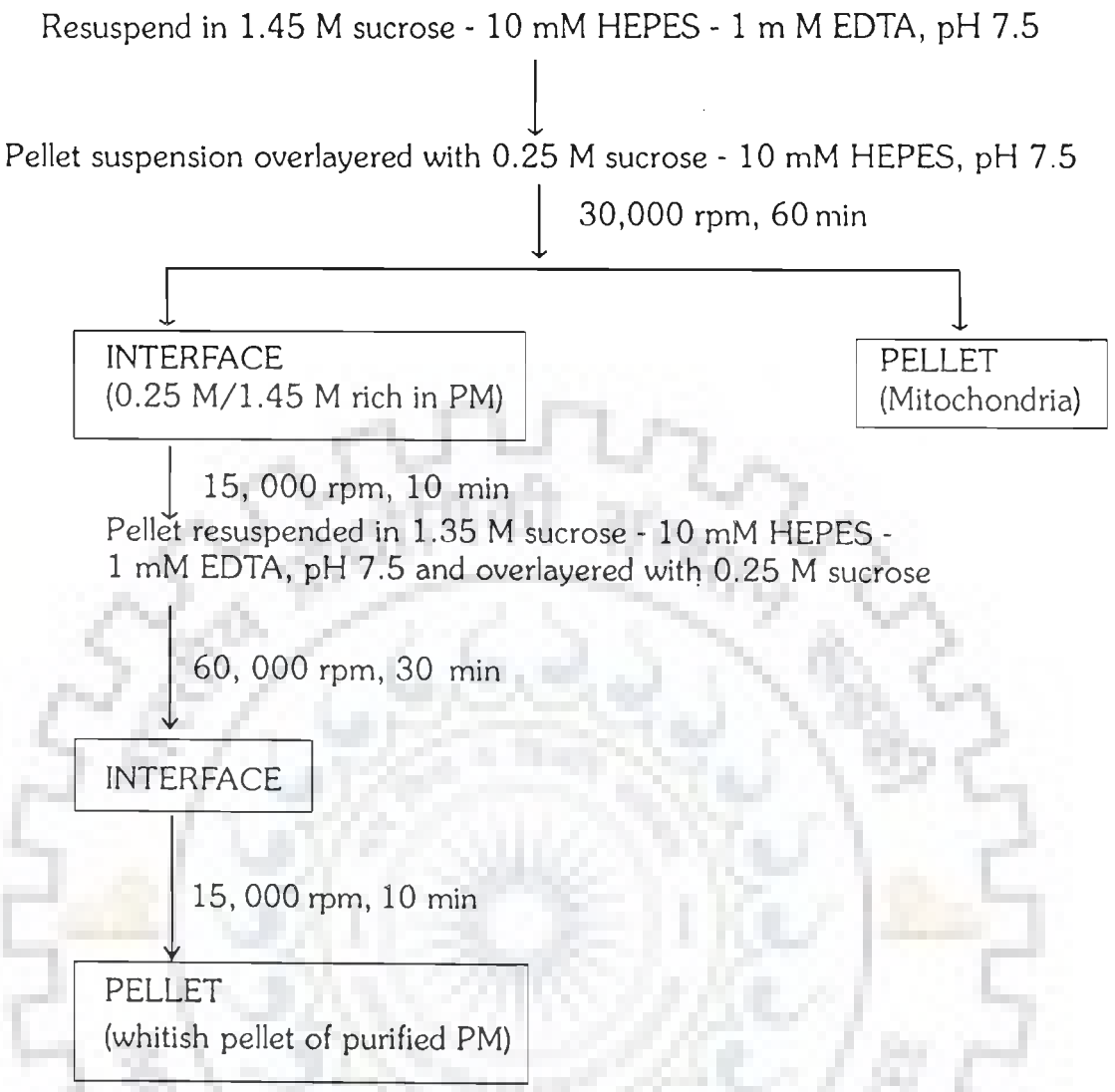


FIG. 3.5 : FLOW CHART FOR PREPARATION OF PLASMA MEMBRANE FROM RAT LIVER




Resuspend in 1.45 M sucrose - 10 mM HEPES - 1 m M EDTA, pH 7.5
Pellet suspension overlayered with 0.25 M sucrose - 10 mM HEPES, pH 7.5
30,000 rpm, 60 min
INTERFACE
(0.25 M/1.45 M rich in PM)
PELLET
(Mitochondria)
15, 000 rpm, 10 min
Pellet resuspended in 1.35 M sucrose - 10 mM HEPES -
1 mM EDTA, pH 7.5 and overlayered with 0.25 M sucrose
60, 000 rpm, 30 min
INTERFACE
15, 000 rpm, 10 min
PELLET
(whitish pellet of purified PM)

3.2.7 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out by the procedure of Laemmli (1970). The solutions used for 10% gel electrophoresis were as following :

Stock Solutions :

Solution A : 30% acrylamide mix (w/v)

Solution B : 1.5 M Tris-HCl buffer, pH 8.8

Solution C : 0.5 M Tris-HCl buffer, pH 6.8

Solution D : 10% ammonium persulphate

SDS stock solution : 10% (w/v)

Electrophoresis buffer : Tris-Glycine, pH 8.3 and 0.1% SDS.

Sample buffer : 0.0625 M Tris-HCl buffer (pH 6.8), 2% SDS, 10% glycerol and 5% 2-mercaptoethanol.

Staining solution :

0.1% Coomassie Brilliant Blue (CBB) R-250 in 25% methanol and 10% acetic acid and volume made upto 100 ml with water. The staining solution was filtered before use.

Working solutions :

10% Separating gel:

Water	15.8 ml
Solution A	13.3 ml
Solution B	10.0 ml
10% SDS	0.4 ml
Solution D	0.4 ml
TEMED	0.016 ml

5% Stacking gel

Water	6.8 ml
Solution A	1.7 ml
Solution C	1.25 ml
10% SDS	0.1 ml
Solution D	0.1 ml
TEMED	0.01 ml

The polymerised slab gel was fitted to electrophoretic apparatus. The electrophoresis buffer was filled in both lower and upper chambers. Protein samples were concentrated, mixed with sample buffer by heating them in boiling water bath for 2 min to effect dissociation. High molecular weight protein standards were treated in same manner. Tracking dye was added to each protein sample and samples were layered in the wells. Electrophoresis was carried out with a current of 30 mA for stacking gel and 40 mA for separating gel at 25°C until the bromophenol blue tracking dye reached the bottom of the gel (about 2-3 h). The gels were stained for 4-6 h by CBB. Destaining was done in methanol-acetic acid-water (25:7:68, v/v/v)

3.2.7.1 Silver Staining

Silver staining of gels was carried out by the method of Davis et al. (1986). Briefly, the gels were fixed in 50% methanol and placed in the orbital shaker for 2 h. The silver staining solution was prepared just before use by mixing 21 ml of 0.36% NaOH with 1.4 ml of 14.8 M NH_4OH and then adding 4 ml of silver nitrate solution (0.8 g $AgNO_3$) dropwise with vigorous vortexing. The solution was made upto 100 ml with double distilled water. This staining solution was added to the gel followed by gentle shaking in orbital shaker for 15 min. After this the gel was washed for 30 min in 3-4 changes of water by shaking and treated with freshly prepared developing solution (25 mg citric acid and 0.25 ml of 38% formaldehyde in a total volume of

500 ml). When the bands reached the desired intensity, the gel was immediately rinsed with water and the reaction was stopped by agitating the gel in methanol-acetic acid-water (45 :5 :50, v/v/v). The stained gels were stored in this solution in dark.

3.2.7.2 Molecular Weight Determination of Membrane Proteins.

Mobilities of each band were determined relative to the migration of bromophenol blue as the tracking dye. Molecular weight standard proteins were : Myosin (205 kDa);β-Galactosidase (116 kDa); Phosphorylase B (97.4 kDa); Bovine albumin (66.2 kDa); Ovalbumin (45 kDa); Carbonic anhydrase (29 kDa). The molecular weight of unknown proteins is computed from the caliberation plot of $\log_{10}$ molecular weight vs. relative mobility of standards proteins.

3.2.8 Lipid Analysis

3.2.8.1 Extraction of Total Lipids from Rat Tissues

Total lipids were extracted from rat tissues i.e., liver, kidney and brain by the method of Folch et al. (1957). Briefly, 1 gm of tissue was homogenised in 5 ml of chloroform: methanol (2:1) containing 0.01% BHT to prevent oxidation of lipids. The homogenate was vortexed for 15 min and the lower organic layer collected. This step was repeated thrice with the same volume of chloroform: methanol (2:1). The organic layers were pooled and dried under nitrogen gas. The residue was redissolved in 10 ml of C:M (2:1) and washed with 0.9% NaCl by vigorous shaking followed by keeping overnight at 4°C. The organic layer was again concentrated under a stream of N_2 gas and finally redissolved in 1ml of C:M (2:1). This was further analysed for

phospholipids, neutral lipids and total triglycerides.

3.2.8.2 Analysis of Phospholipid Components by HPLC

Total lipids extracted from tissues were used for the analysis of phospholipid components by HPLC according to Curstedt et al. (1983) on a Shimadzu LC-4A equipped with a SPD-2AS variable wavelength UV- spectrophotometric detector and a chromatopac C-RZA integrator. The separation of phospholipids was achieved on a nucleosil-5 C_{18} RP column (25 cm x 4.6 mm i.d.) using methanol-water-acetonitrile (70:20:10, v/v/v) at a flow rate of 1.5 ml/min isocratically. The phospholipid peaks were detected at 214 nm. The column was caliberated using authentic phospholipid standards.

3.2.8.3 Analysis of Neutral Lipids by Thin-Layer Chromatography

TLC of neutral lipids was performed according to the method of Mangold (1965). Silica gel G coated (0.25 mm) glass plates (20 x 20 cm) were routinely used. Prior to use, plates were activated by heating in an oven at 110°C for 60 min. The plates were cooled to room temperature and lipid samples were applied on plates with the help of micropipettes.

The chromatoplates were developed in the following solvent systems :

solvent I : Hexane : Diethylether : Glacial acetic acid

60 : 40 : 1

solvent II :Hexane : Diethylether : Glacial acetic acid

90 : 10 : 1

The plates were developed in solvent I upto a height of 7 cm. The plates

were dried in air and further developed in solvent II upto a height of 15 cm. The plates were air dried once again and placed in an iodine chamber to locate spots of various neutral lipids (monoacylglycerol-MAG; diacylglycerol-DAG; triacylglycerol-TAG; free fatty acids-FFA; and esterified cholesterol-EC)

3.2.8.4 Quantitative Estimation of Triacylglycerols by Gas Liquid Chroma tography (GLC)

The amount of triacylglycerol in the neutral lipid fraction was quantitatively estimated by GLC according to Mares and Husek (1985). Analysis was carried out on the Hewlett-Packard 1 (HP 1) methyl silicon gum gas chromatograph equipped with flame ionisation detector and linear temperature programme. Capillary columns (5 m x 0.55 mm x 2.65 mm) were used . Prior to use, columns were stabilised for 2 h. Operational conditions were :

column temperature	150 °C
injection temperautre	200 °C
FID temperature	220 °C
injection volume	1 µl
flow rate of carrier gas	2.5 ml/min

1,3-diolein dissolved in chloroform:methanol (2:1) was used as standard to calibrate the instrument.

3.2.9 Analysis of Biochemical Parameters

3.2.9.1 Haematological Parameters

For haematological analysis, blood samples from rats of each group were collected

directly by cardiac puncture with the help of a 2 ml presterilised plastic disposable syringe in two vials, one containing EDTA (1.5 mg/ml blood) to prevent blood clotting and other without EDTA. No haemolysis was observed during collection of blood. The blood sample in former vial (with EDTA) was analysed for total RBC, WBC and haemoglobin counts. RBC and WBC were counted by Neubauer double haemocytometer. The diluting fluid for RBC contained 0.69 g NaCl, 1 g sodium citrate and 1 g formalin in 100 ml double distilled water. For WBC, the composition of the diluting fluid was glacial acetic acid (1.5 ml), gentian voilet 1 mg and 98.5 ml distilled water. The blood sample collected without EDTA was first allowed to clot at room temperature for 60 min followed by 6 h incubation at 0-4°C. The serum was separated by centrifugation at 5000 x g for 5 min . The clear serum was analysed for various parameters using standard analytical procedures. Blood parameters studied were serum alkaline and acid phosphatases by the method of Morton (1955). Serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) were estimated by the procedure of Reitman and Frankel (1957). Haemoglobin content in blood was measured by Cyanmethamyolobin method while total RBC and WBC were estimated by method of Dacie and Lewis (1977).

3.2.9.2 Nucleic Acid Estimation

Nucleic acid content was estimated by the method of Munro and Fleck (1966). Briefly, rat liver (0.5 gm) was homogenised in 50 mM maleate buffer, pH 6.8 and 0.1 ml sample was taken from this homogenate. 2.5 ml of 0.22 N cold perchloric acid was added to homogenate, kept in ice for 10 min and centrifuged at 3,000 rpm at 0-4°C for 15 min. 3.2 ml of 0.3 N KOH was then added to the pellet and incubated for 1 h at 37°C. Then 0.8 ml of 2.2 N cold perchloric acid was added and centrifuged at 1000 x g for 5 min. The supernatant was diluted 1:1 with distilled

water and absorbance measured at 260 nm for RNA. While 2.0 ml of 1 N $HClO_4$ was added to the precipitate and incubated at 80°C for 1 h, followed by centrifugation at 1000 x g and absorbance read at 260 nm for DNA content.

3.2.9.3 Serum Cholesterol

Serum cholesterol was measured according to the method of Bowman and Wolf (1962). The colour development was complete after 30 min and the absorbance was read at 550 nm. 0.05 ml of serum sample was added to 2.5 ml of ethanol to produce a protein precipitate. The sample was centrifuged at 2000 rpm for 5 min. To 2.0 ml of ethanolic supernatant about 2.0 ml of color reagent (8ml of 2.5% $FeCl_3$ diluted to 100ml with conc. H_2SO_4) was added and mixed for 10-15 s and tubes cooled. Cholesterol standard (stock 1 mg/ml) was also run concurrently.

3.2.9.4 Estimation of Total Phospholipid Phosphorous Content

The total phospholipid phosphorous content was estimated colorimetrically by the method of Marinetti (1962). The method is based on the principle that orthophosphoric acid forms a complex with molybdate and on reduction with reducing agent produces a blue colour. Appropriate aliquotes of lipid samples were evaporated to dryness. To these samples 1 ml of $HClO_4$ was added and contents of the tubes digested at 100-150°C for 15-20 min till fumes subsided. After cooling at room temperature, 7 ml of distilled water was added followed by addition of 0.5 ml ammonium molybdate and 0.2 ml of reducing reagent (30 g sodium bisulfite, 6.0 g sodium metabisulfite and 0.5 g ANSA was mixed thoroughly and dissolved in 250 ml of distilled water. This was made to stand for 3 h in dark). Contents of the tube were mixed and placed in boiling bath for 7 min. Cooling was done for 20 min and colour

intensity measured at 830 nm against blank. Standard KH_2PO_4 was also run concurrently and phosphorous values were multiplied by 25 to get phospholipid values.

3.2.9.5 Estimation of Total Carbohydrate Content

Total carbohydrate content was determined by the Phenol/ Sulphuric acid method of Dubois et al. (1956). An appropriate volume of the enzyme (20-50 mg carbohydrate) was diluted with distilled water to a final volume of 2 ml. Then 5 ml of 90% phenol solution and 5 ml of concentrated sulphuric acid were added followed by thorough mixing on a Vortex mixer. The mixture was allowed to stand at room temperature for 30 min and the absorbance was measured at 490 nm against a blank to which no enzyme was added. The amount of carbohydrate was computed from the standard curve of glucose prepared under identical conditions.

3.2.9.6 Enzyme Assays

3.2.9.6.1 Acid Phosphatase

Acid phosphatase activity was measured by a slightly modified method of Odds and Hierholzer (1987) using p-nitrophenyl phosphate as substrate. The incubation mixture (1.0 ml) contained 10-200 µg protein, 1 mM p-nitrophenyl phosphate and 50 mM acetate buffer, pH 5.0. The reaction was terminated after 15 min incubation at 30°C by the addition of 1.5 ml of 4% Na_2CO_3. The absorbance of yellow colour of p-nitrophenol released was measured at 420 nm against blank.

3.2.9.6.2 Alkaline Phosphatase

Alkaline phosphatase activity in rat liver was assayed by method of Morton

(1955) using p-nitrophenyl phosphate as substrate. The reaction mixture contained the following in a volume of 3 ml. 0.8 ml of 0.1 M sodium carbonate-bicarbonate buffer, pH 9.3 was added to 2 ml of 5 mM p-nitrophenyl phosphate and mixed thoroughly Incubation was initiated by addition of 0.2 ml of enzyme preparation and change in absorbance was read at 405 nm.

3.2.9.7 Other Methods

Protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Liver glycogen was measured by the method of Hassid and Abraham (1957).

Chapter 4

RESULTS

4.1 ACUTE TREATMENT

4.1.1 Biodegradation of Fenvalerate in Rat Tissues

Accumulation of fenvalerate in liver , kidney and brain was studied after administering a single sublethal dose of 100 mg fenvalerate/ Kg of body weight, (LD_{50} : 451 mg/ Kg) . After treatment, the animals were sacrificed at intervals varying from 0 - 48 h and liver, kidney and brain were quickly removed and analysed on a HPLC C_{18} column for the concentration of the pesticide. The HPLC profiles showing time-dependant accumulation and biodegradation of the parent pesticide in liver, kidney and brain are given in Fig.4.1 , 4.2 and 4.3, respectively. These results show that fenvalerate underwent rapid metabolism and two metabolites designated as PI and PII were detected in all tissues by HPLC. The kinetics of biodegradation in liver was very rapid and it was found that the highest accumulation of pesticide in liver occured within 6 h following oral administration of the pesticide (Fig. 4.4). Following this initial period of accumulation, the pesticide was rapidly biodegraded and during the next 48 h it was almost removed from the liver. For example, the % molar concentration of fenvalerate in liver after 6 h of oral administration was about 70% which was reduced to less than 5% within 48 h with concomitant accumulation of metabolites (Table 4.1). As can be seen in liver, there was a preferential accumulation of metabolite PI while metabolite PII was not significantly accumulated.

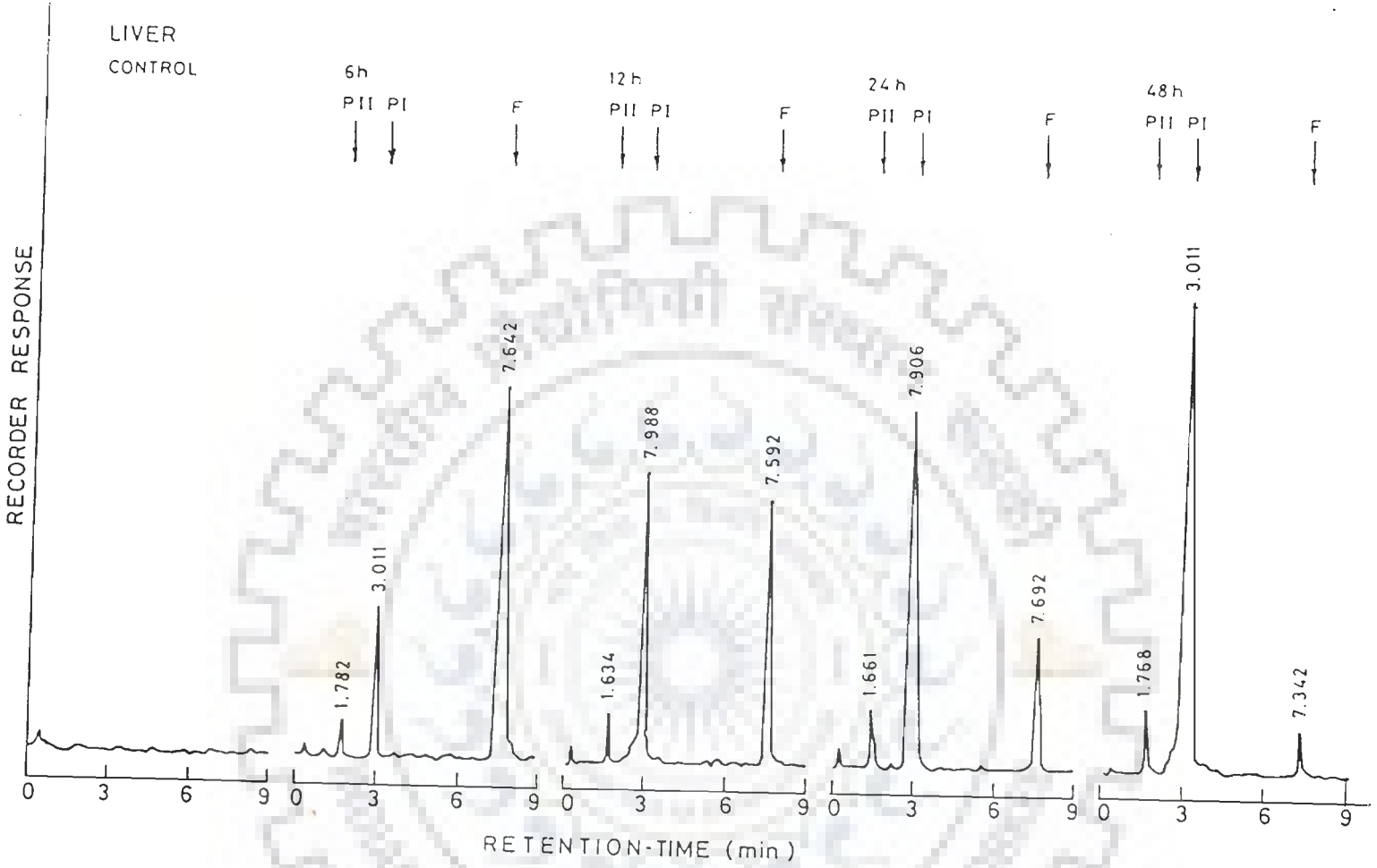


Fig. 4.1 HPLC Spectra Showing Biodegradation of Fenvalerate (F) into its Metabolic Products (PI and PII) in Liver Under Acute Treatment (100 mg/Kg) as a Function of Time.

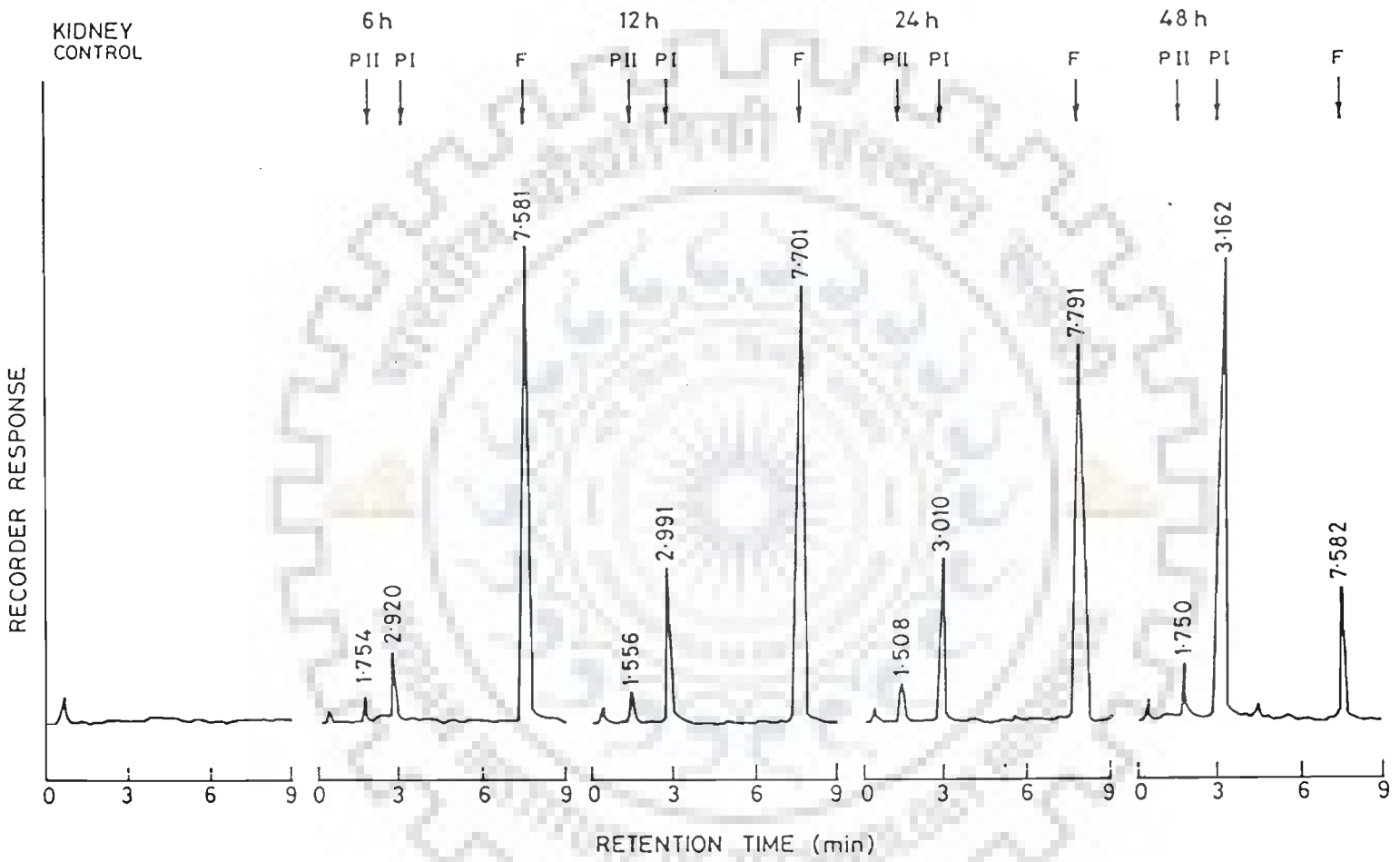


Fig. 4.2 HPLC Spectra Showing Biodegradation of Fenvalerate (F) into its Metabolic Products (PI and PII) in Kidney Under Acute Treatment (100 mg/Kg) as a Function of Time.

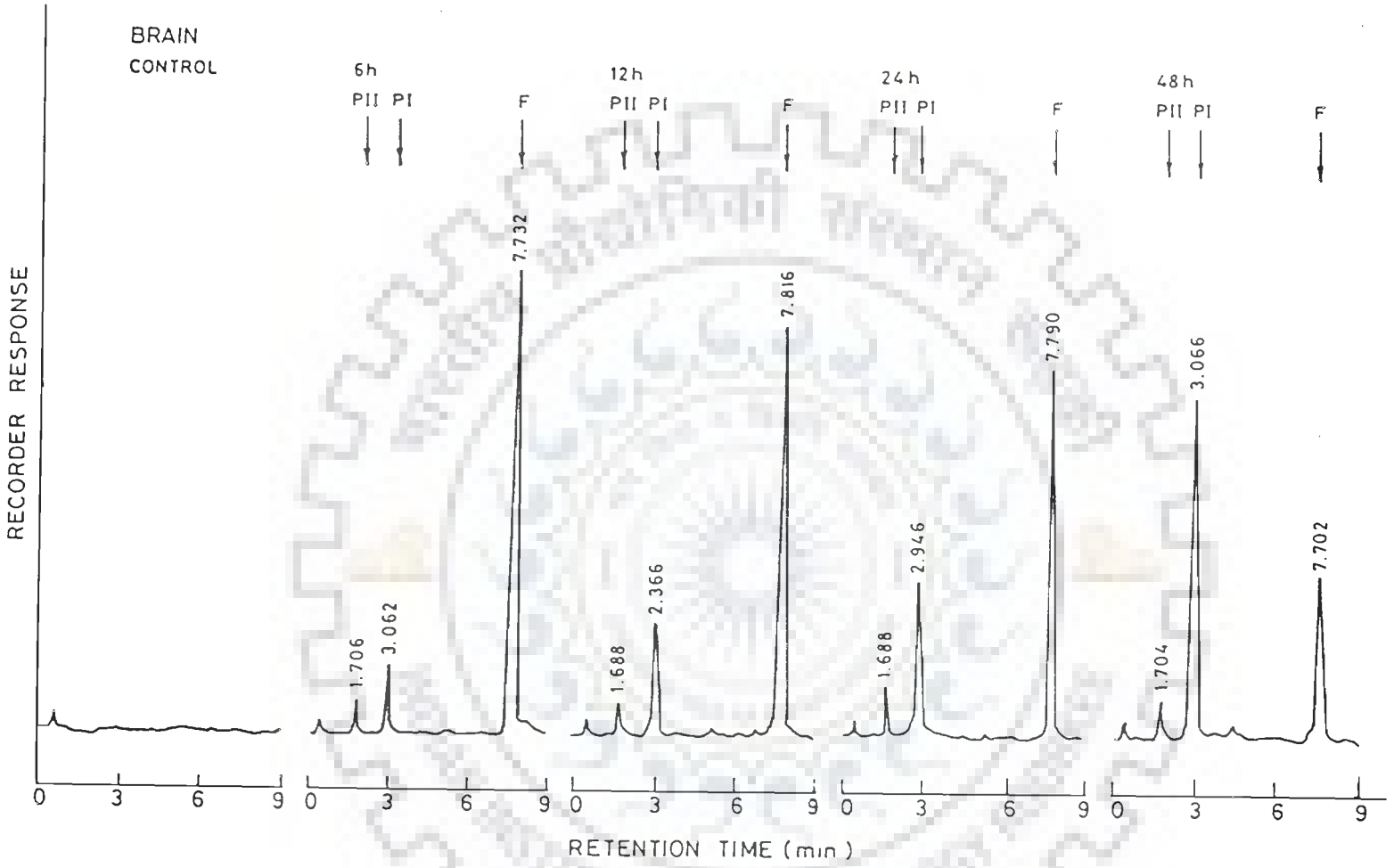


Fig. 4.3 HPLC Spectra Showing Biodegradation of Fenvalerate (F) into its Metabolic Products (PI and PII) in Brain Under Acute Treatment (100 mg/Kg) as a Function of Time.

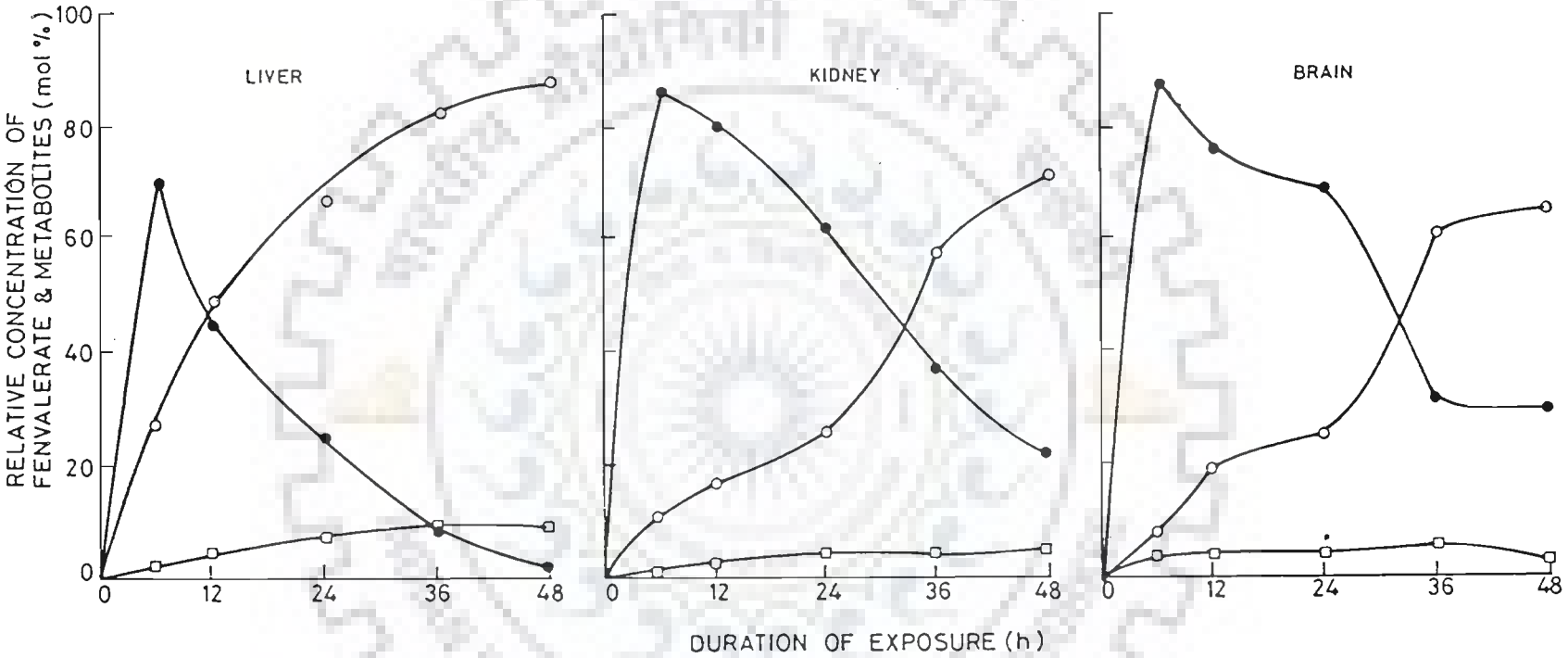


Fig. 4.4 Relative Distribution of Fenvalerate and its Metabolites (PI and PII) in Rat Liver, Kidney and Brain as a Function of Time. Treatment Dose : 100 mg/Kg. Fenvalerate (●-●), PI (0-0), PII (□-□).

Table 4.1 : BIODEGRADATION OF FENVALERATE INTO ITS METABOLIC PRODUCTS IN RAT LIVER AS A FUNCTION OF TIME

Fenvalerate (100 mg/Kg body weight) dissolved in groundnut oil was admin istered orally to a group of 10 rats. At indicated intervals, various organs were obtained and the amounts of residual pesticide and the metablite(s) formed were determined by HPLC as described in Materials and Methods.

Duration of Exposure (h)	Dose (mg/Kg body wt.)	Relative Concentration (mol %)		
		Liver		
		Residual Fenvalerate	PI	PII
Control	0.00	0.00	00.00	0.00
6	100.00	70.34	27.55	2.10
12		45.74	49.58	4.68
24		25.80	67.19	7.01
36		8.23	82.78	8.99
48		2.20	89.70	8.10

PI and PII are the metabolic products formed.

Identical pattern was observed in kidney and brain , except that the degradation was significantly slower in both kidney and brain than that in liver. For instance, the relative residual concentration of the pesticide in kidney and brain was about 70% compared to only about 25% in liver. There was corresponding accumulation of biodegradation product PI which increased with time. The product PII however was low in concentration. In other words , the pesticide was completely degraded in liver, while in kidney and brain there was substantial amount remaining unmetabolised even after 48 h . This confirms the earlier findings that fenvalerate, a synthetic pyrethroid is more toxic and damaging to the brain and nervous system, i.e. neurotoxic.(Saleh et al., 1993). The characterisation of metabolites formed *in vivo* in liver, kidney and brain will be presented later in the thesis .

4.1.2 Effect of Fenvalarate on Nucleic Acid Content in Rat Liver

Table 4.2 shows the effect of fenvalerate on nucleic acid content in rat liver. The results indicate that both DNA and RNA content of liver were significantly affected by the pesticide exposure. It was found that within 6 h after treatment, there was almost 50% decrease in both DNA and RNA contents in rat liver. However, following the initial period of 6 h, the RNA content started to increase and its level (1.1488 mg g^{-1} tissue) was comparable to that of control (1.576 mg g^{-1} tissue). In case of DNA , the decrease in content continued upto 12 h. Thereafter the DNA content of liver increased and reached the normal value in 48 h. These results indicate that the *in vivo* effect of fenvalerate on nucleic acid content in liver was time-dependant and was related to the accumulation of pesticide in liver, as the accumulation of pesticide in liver reached maximum level in 6 h after oral administration of pesticide. In other words, when the accumulation of pesticide in liver was maximum during first 6 h, the synthesis of DNA was also inhibited. Since during the later period (12-48 h)

the concentration of pesticide in liver was reduced due to its degradation , the effect on nucleic acid synthesis was also decreased. Furthermore, the metabolites (PI and PII) which accumulated in the liver at the cost of the parent pesticide did not seem to inhibit the DNA and RNA synthesis indicating that the metabolites were not toxic and the damage was entirely due to the pesticide. Once the pesticide was completely degraded in 48 h, the toxic effect of the pesticide on the nucleic acid synthesis was also reversed.

Table 4.2 : EFFECT OF FENVALERATE ON NUCLEIC ACID CONTENT IN RAT LIVER

Duration of Exposure (h)	Dose (mg/Kg body wt.)	Nucleic Acid Content (mg g^{-1} liver)[a]	
		RNA	DNA
Control	0.00	1.1576 ± 0.01	0.0754 ± 0.004
6	100.00	0.7628 ± 0.02	0.0458 ± 0.001
12		0.9751 ± 0.02	0.0412 ± 0.001
24		1.1570 ± 0.04	0.0658 ± 0.002
48		1.1488 ± 0.01	0.0741 ± 0.001

[a]Each value is an average ± standard error of 6 determinations from 4 animals of same age group.

4.1.3 Effect of Fenvalerate on Total Triglyceride Content in Rat Tissues

The total triglycerides in rat tissues i.e., liver, kidney and brain were analysed by gas liquid chromatography. Fig. 4.5 shows that on exposure of fenvalerate , the triglyceride content markedly increased upto 24 h in liver, kidney and brain. For instance , the increase in total triacylglycerols (TAG) in liver, kidney and brain due to the pesticide treatment of rats was about 3.5 -, 7.0 - and 10.0-fold respectively. This increase in TAG content in these organs may be due to the blocking of their secretion (Hoyumba et al., 1978). After 24 h of

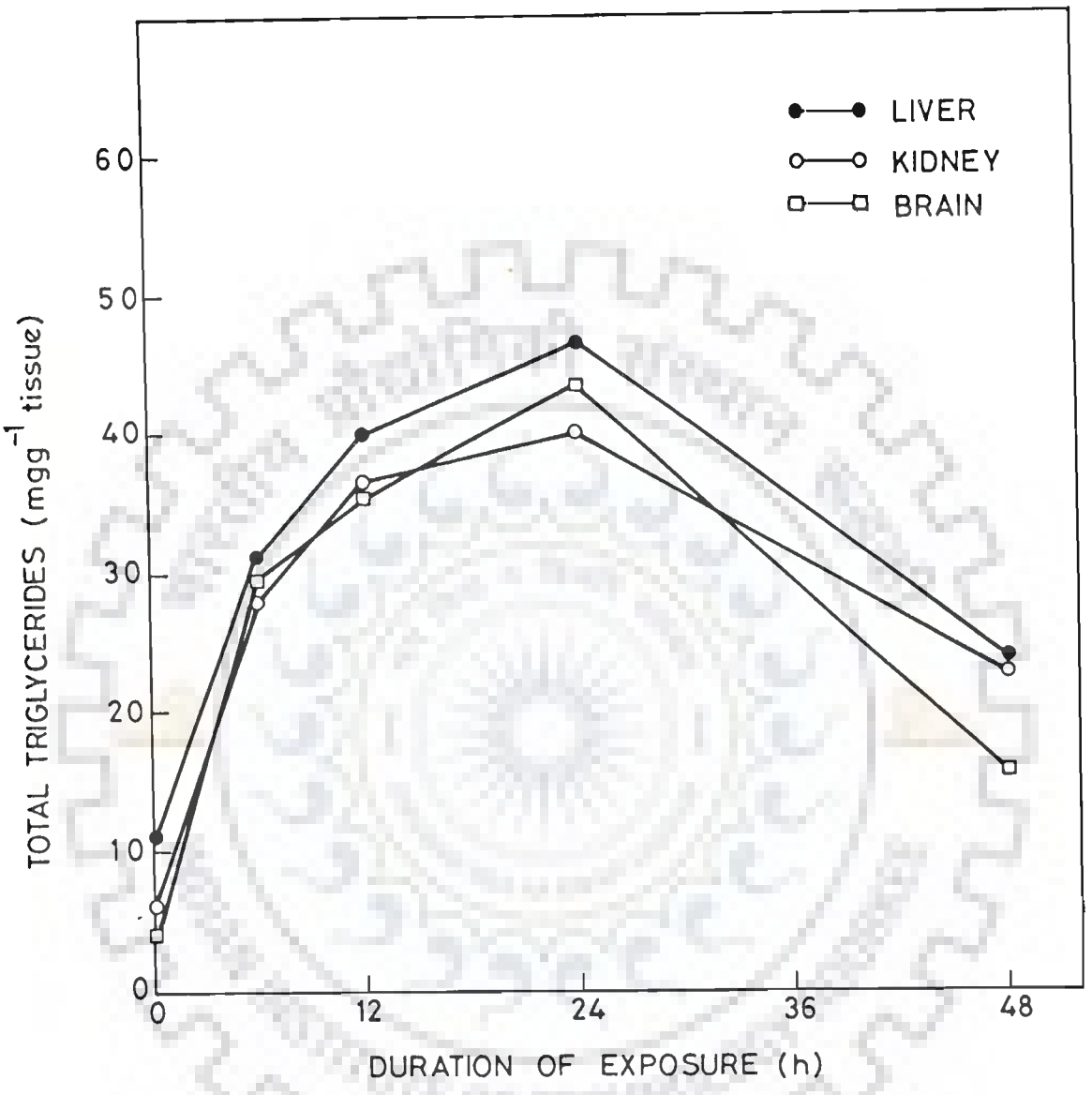


Fig. 4.5 Effect of Fenvalerate (100 mg/Kg) on Total Triglycerides in Rat Organs as a Function of Time.

pesticide treatment, the triglyceride content of liver, kidney and brain decreased rapidly. These results clearly suggest that the effect of pesticide after 24 h was greatly diluted perhaps due to the biodegradation of pesticide. As pointed out earlier, although the metabolites PI and PII accumulated in these tissues, they do not seem to affect as far as triglyceride metabolism is concerned. In this regard, the effect of pesticide on triglyceride accumulation parallels the effect on nucleic acids.

4.1.4 Effect of Fenvalerate on Total Protein and Glycogen Contents in Rat Liver

Fig. 4.6 shows the effect of fenvalerate on total proteins and glycogen in rat liver as a function of time. It can be seen that the concentration of total proteins in liver was decreased from about 110 to 65 mg g^{-1} liver within 12 h of the oral administration of pesticide to rats. After 12 h, the animal showed recovery and in 48 h the protein concentration was comparable to control. The results further support the earlier conclusion, that in the initial period the accumulation of pesticide in rat liver was sufficiently high which affected the rate of metabolism. Once the concentration of the parent compound in liver was reduced due to biodegradation, its inhibitory effect was also removed. The effect on liver glycogen was not as pronounced as on proteins, but it followed the same pattern as was observed in case of proteins and nucleic acids.

The decrease in total proteins may be attributed to increased proteolysis as reported by Reddy and Yellama (1991). The decrease in protein content may also be due to disintegration and damage to cellular functions with consequent impairment of the protein synthesis machinery (Reddy et al., 1991). The lowering of liver glycogen might be due to the increased utilisation into the anaerobic glycolysis to meet the

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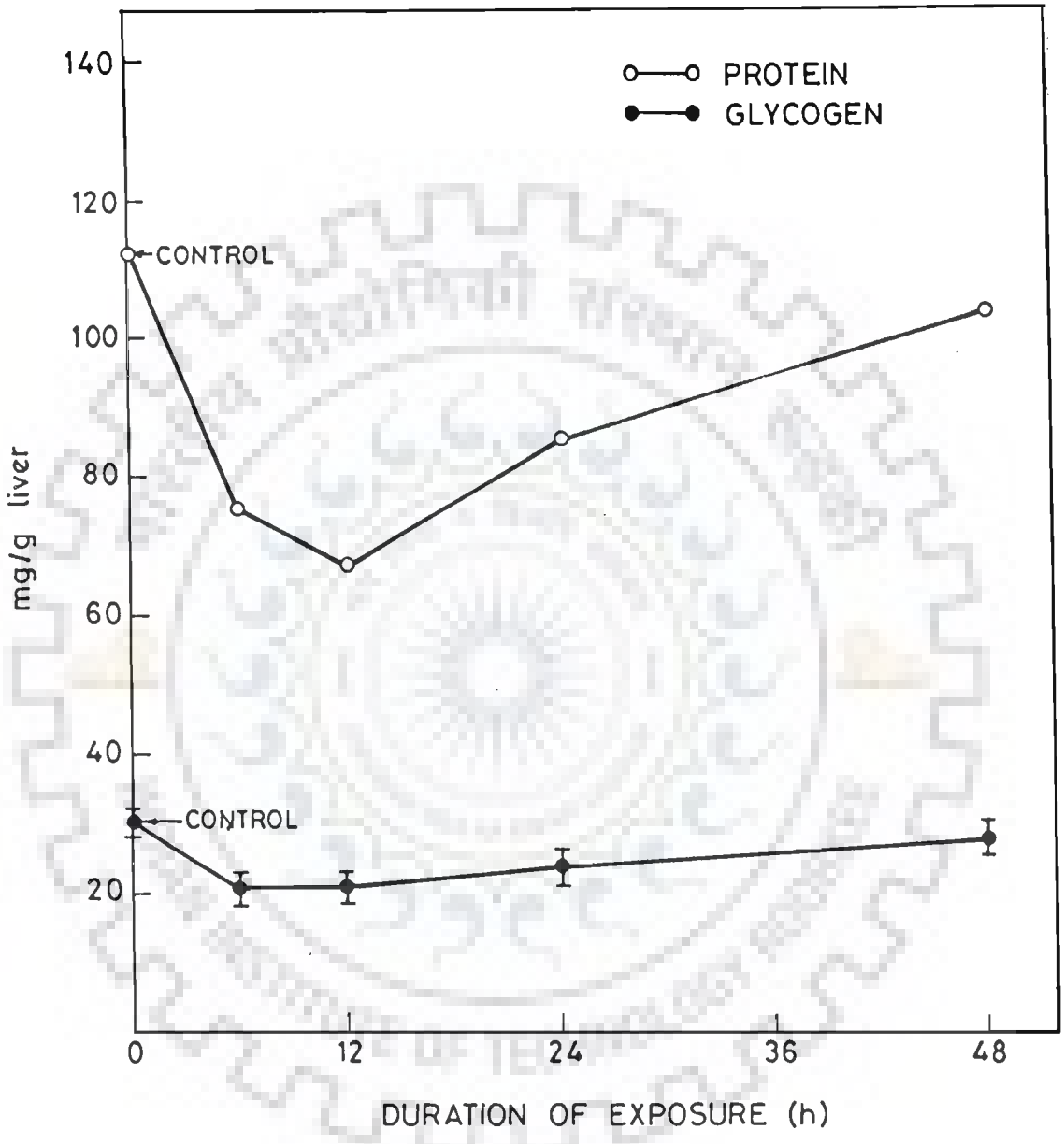


Fig. 4.6 Effect of Fenvalerate (100 mg/Kg) on the Protein and Glycogen Contents in Rat Liver as a Function of Time.

energy requirements to counteract the toxic stress induced by fenvalerate (Reddy and Yellama, 1991).

4.1.5 Effect of Fenvalerate on Serum Cholesterol

The acute treatment of rats with fenvalerate lowered the total cholesterol in serum of rats compared to the control (Fig.4.7). It can also be seen that like nucleic acids, total proteins and glycogen, the concentration of total cholesterol in serum rapidly decreased following the administration of pesticide to rats. It was found that the concentration of cholesterol in serum decreased from approximately 160 to 80 mg/100 ml serum within 6 h. Once again, like other parameters, the serum cholesterol level started to increase and reached the normal level in 48 h. From these results, it is evident that fenvalerate inhibits the synthesis of cholesterol *in vivo*. The decrease of cholesterol level may be an indication of hepatic dysfunction.

4.1.6 Effect of Fenvalerate on Haematological Parameters in Rat

The effect of fenvalerate on the rat blood parameters (haemoglobin, white blood cells and red blood cells) was investigated as a function of time . The results are summarised in Table 4.3. The Hb content decreased from 15.5 to 10.5 gm/dl within 12 h, but it increased upto 13.4 gm/dl in the next 48 h. WBC counts increased from 4.62 to 7.60 x 10^3/cmm in first 12 h after administration of pesticide, but following this period, the animal recovered and reached almost normal level in the next 36 h. RBC decreased rapidly from 5.5 to 2.16 x 10^6/cmm during first 12 h after which the RBC level was almost normal. These results also indicate that the effect on blood parameters is dependant on the concentration of pesticide accumulated in tissues.

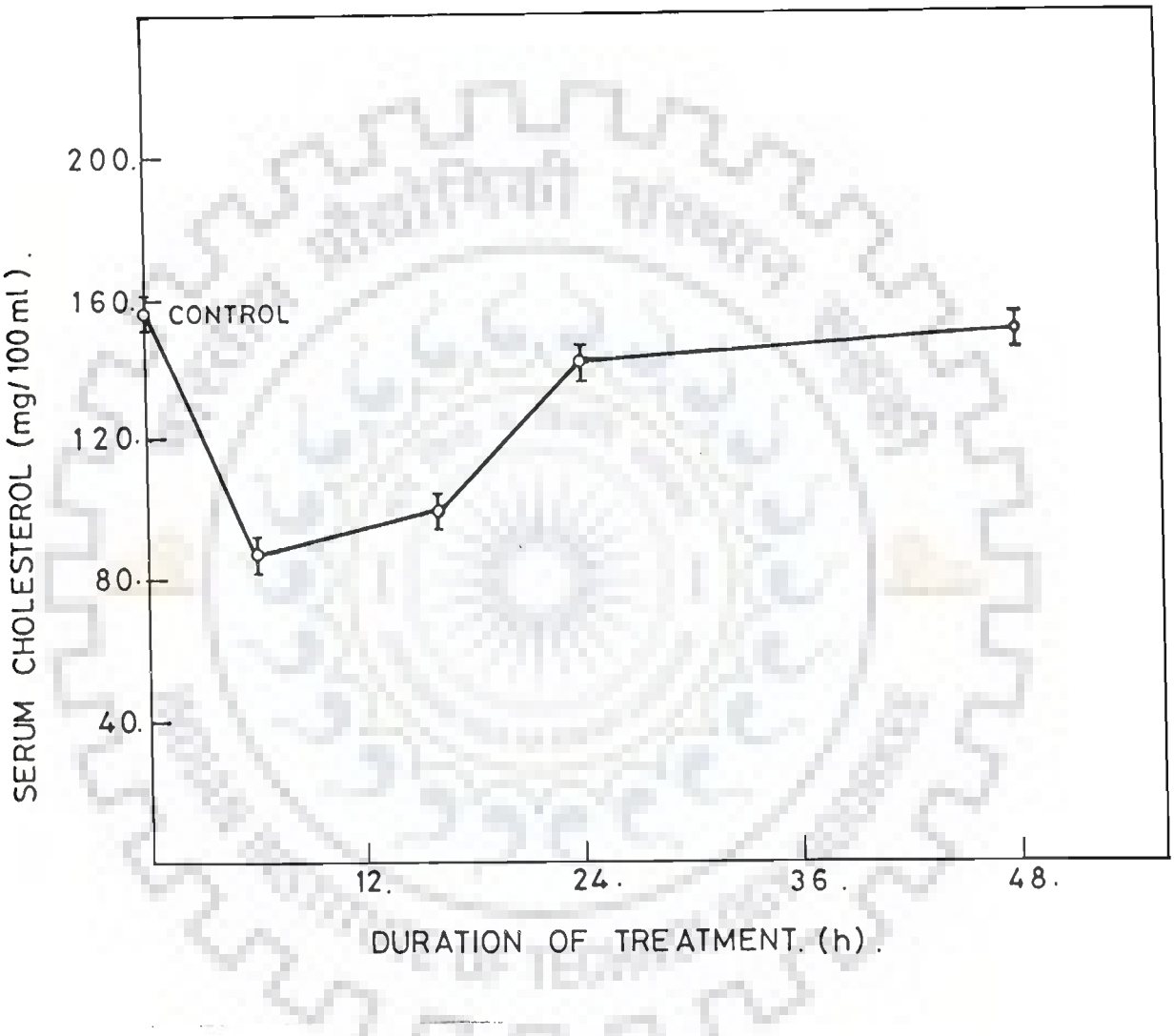


Fig. 4.7 Concentration of Serum Cholesterol in Control and Fenvalerate Treated (100 mg/Kg) Rats.

Table 4.3: ALTERATIONS IN BLOOD PARAMETERS OF RAT EXPOSED TO FENVALERATE

Duration of Exposure (h)	Dose (mg/Kg body wt.)	Hb (g/dl)	WBC (10^3/cmm)	RBC (10^6/cmm)
Control	0.00	15.50 ± 0.65	4.62 ± 0.12	5.50 ± 0.28
6	100.00	12.20 ± 0.64	4.64 ± 0.10	3.22 ± 0.10
12		10.50 ± 0.20	7.60 ± 0.06	2.16 ± 0.20
24		12.80 ± 0.20	7.00 ± 0.10	3.40 ± 0.11
48		13.40 ± 0.11	5.28 ± 0.24	5.00 ± 0.20

All the values are mean ± S.E. (4 estimation)

On the basis of the biotoxicity data presented here, it can be concluded that fenvalerate rapidly accumulates in tissues and inflicts its toxic effect within few hours of pesticide administration. Once the level of pesticide is lowered due to its degradation, the toxic effect on biochemical parameters is also removed. Further, the metabolites formed by degradation of fenvalerate do not seem to have any major toxic effects.

4.2 *IN VITRO* METABOLISM

4.2.1 *In Vitro* Biodegradation of Fenvalerate by Various Subfractions of Rat Liver

In order to study *in vitro* biodegradation of fenvalerate, various subcellular fractions from rat liver were prepared using standard procedure (Fleischer and Kervina, 1974) as described in Materials and Methods. Fenvalerate (11.90 mM) was incubated with different subcellular fractions containing comparable protein content (about 1 mg) as determined by Lowry et al. (1957).

The incubations were carried out for 30 min at 37^0 C and the residual fenvalerate and metabolites were analysed by HPLC as described in Methods (Fig. 3.2). The results

of the relative concentrations of fenvalerate and metabolites in mol % are summarised in Table 4.4. It was found that the total tissue homogenate, under the said experimental conditions, degraded the pesticide and nearly 60% of the pesticide used was converted into two metabolites with relative molar concentrations of approximately 57% and 2% respectively. From these results it is clear that the enzyme responsible for the degradation of fenvalerate is present in the tissue homogenate. Further analysis of the *in vitro* degradation by subcellular fractions, namely, nucleus, whole mitochondria, outer mitochondrial membrane, inner mitochondrial membrane and crude microsomes was carried out. A comparision of the data showed that very little degradation occured with nuclear fraction and inner mitochondrial membrane, whereas mitochondria as a whole was able to degrade about 40% of the pesticide used, with concomitant formation of the metabolites PI and PII accounting for about 35 and 4 mol % respectively. It is interesting to note that most of the degradative activity was carried out by the outer mitochondrial membrane. Under identical conditions, the outer mitochondrial membrane degraded approximately 49% fenvalerate, resulting in the formation of metabolites PI and PII. Relative molar concentrations of PI and PII were 42% and 7% respectively. Since no significant degradation of the pesticide occured in the inner membrane of mitochondria, it was tempting to assume that the degradation enzymes were localised in the outer membrane of mitochondria, and whatever little degradative activity was seen in the inner mitochondrial membrane may be due to contamination of the outer membranes. As expected, the crude microsomes i.e., post mitochondrial membrane pellet also showed very high degradative activity towards fenvalerate. For instance, under identical incubation conditions, approximately 65% of the pesticide added to the incubation medium was converted to its metabolites, PI and PII accounting for about 58 and 6 mol % respectively. It was not unexpected that microsomes showed very high degradation activity because smaller mitochondria are difficult to separate from mi-

Table 4.4: *IN VITRO* BIODEGRADATION OF FENVALERATE IN VARIOUS SUBCELLULAR FRACTIONS OF RAT LIVER

Subcellular Fraction	Relative Concentration (mol %)		
	Residual Fenvalerate	PI	PII
Total Tissue homogenate	41.14	56.86	2.00
Nuclei	92.62	7.38	N.D.
Whole Mitochondria	62.40	33.30	4.29
Outer Mitochondrial membrane	51.09	42.01	6.90
Inner Mitochondrial membrane	85.99	12.70	1.31
Crude Microsomes	35.31	58.27	6.42

PI and PII are metabolites formed.

All incubations were carried out at 37°C for 30 min with equivalent amount of protein.

crosomes. At the moment, it is not certain however, whether the enzymes responsible for degradation of fenvalerate *in vitro* in mitochondria and microsomes are same or two different enzyme systems.

4.2.2 Subcellular Distribution of Fenvalerate Hydrolysing Enzymes in Rat Liver

The results in the preceeding paragraph showed that fenvalerate metabolising enzyme activity in rat liver can be assayed quite accurately by formation of metabolite PI which accounts for nearly 85% of the total degradation products formed. Thus for all practical purposes, the initial concentration of the metabolite PI may be taken as a measure of the enzyme activity. The amounts of PI (mol %) formed by various subcellular fractions are shown in Fig. 4.8. The specific activity of the enzyme calculated using rate of formation of PI *in vitro* by subcellular fraction are shown in Table 4.5. Besides crude homogenate and crude microsomal fraction, the maximum enzyme activity was found to be localised in the outer membrane of mitocondria. We have therefore characterised the enzyme activity in the outer mitochondrial membrane.

4.2.3 Characterisation of Fenvalerate Hydrolysing Enzyme in Outer Mitochondrial Membrane

4.2.3.1 Time Course

Fig. 4.9 shows the time course of the fenvalerate metabolising enzyme in outer mitochondrial membrane fraction. It can be seen that the reaction was nearly linear upto 30 min and thereafter the product formation levels off which is a maximum value and the enzyme was found to follow a Michaelis-Menten kinetics. In all experi-

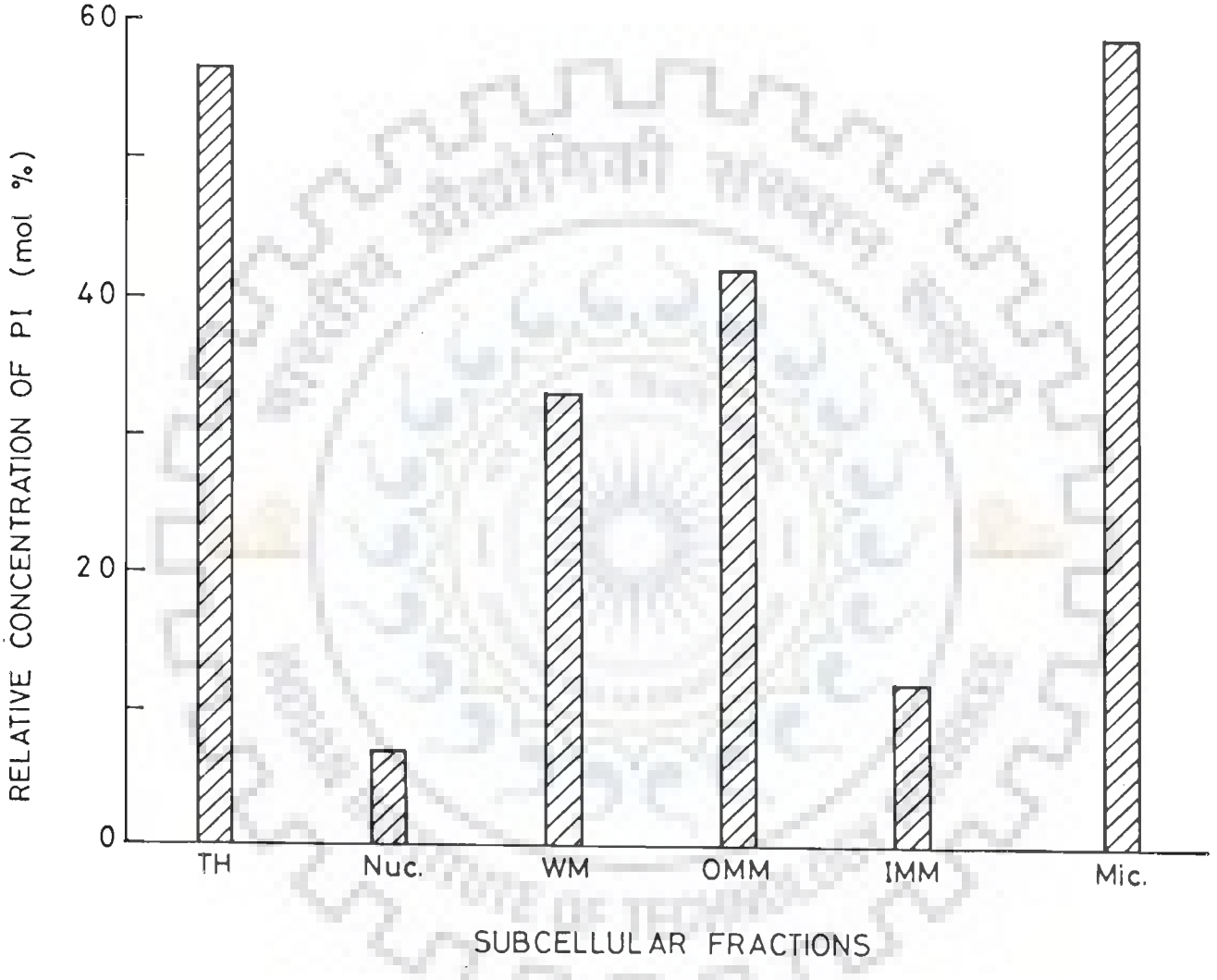


Fig. 4.8 Subcellular Localisation of Fenvalerate Hydrolysing Enzyme in Rat Liver. Incubation Mixture and Conditions as Described in Materials and Methods. TH : Tissue Homogenate ; Nuc : Nuclei ; WM : Whole Mitochondria ; OMM : Outer Mitochondrial Membrane ; IMM : Inner Mitochondrial Membrane ; Mic. : Microsomes.

Table 4.5 : SUBCELLULAR DISTRIBUTION OF THE FENVALERATE METABOLISING ENZYME IN RAT LIVER

Subcellular Fraction	Enzyme Activity
	mM PI formed/min/mg protein
Total Tissue homogenate	0.225
Nuclei	0.030
Whole Mitochondria	0.132
Outer Mitochondrial membrane	0.166
Inner Mitochondrial membrane	0.050
Crude Microsomes	0.244

Incubations were carried out at 37°C for 30 min.

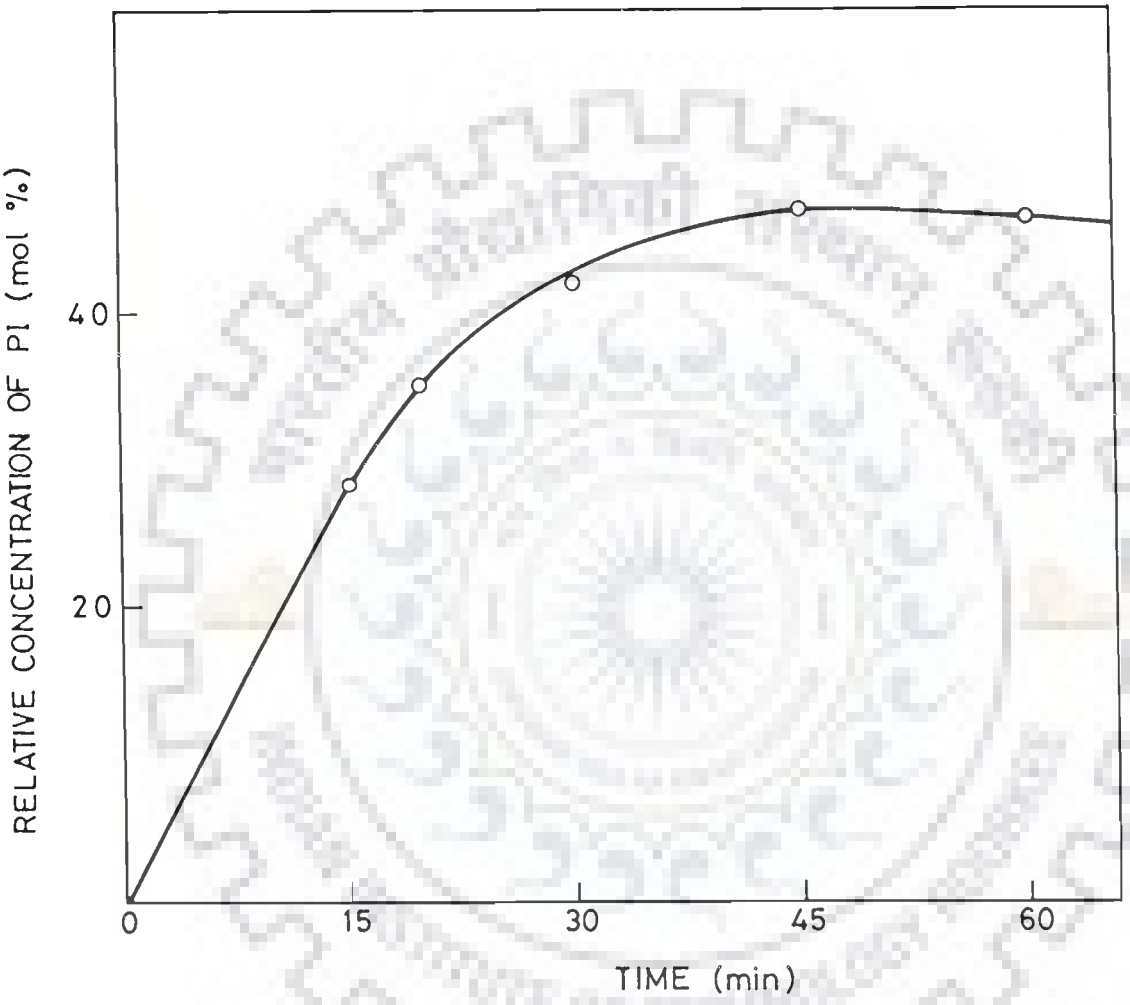


Fig.4.9 Rate of Degradation of Fenvalerate by Rat Liver Outer Mitochondrial Membrane with Varying Incubation Time from 0 to 60 min.

ments to follow, the incubation period was around 30 min.

4.2.3.2 Effect of Protein Concentration

Table 4.6 shows the relative concentration (mol %) of residual fenvalerate and its metabolic products formed, while Fig.4.10 shows the enzyme activity as a function of protein concentration. The reaction under standard assay conditions was approximately linear upto 1.0 mg per assay. Further increase in the enzyme concentration did not result in increase in rate of degradation indicating that substrate concentration becomes the limiting factor.

4.2.3.3 Optimum pH

Table 4.7 shows the relative mol % of residual fenvalerate and metabolic products (PI and PII), while the pH profile of the enzyme activity is shown in Fig.4.11. It can be seen that the optimum pH for the enzyme is 7.5 and the activity below or above this pH declines sharply. For instance, the enzyme activity at pH 7.0 and 8.0 was only 59% and 80% of the maximum.

4.2.3.4 Kinetic Parameters

The kinetic parameters (K_m and V_{max}) were determined by Lineweaver-Burk plot at saturating substrate concentration (Fig. 4.12.) The K_m and V_{max} values were found to be 26.32 mM and 0.50 mM min^{-1} mg^{-1} protein, respectively. From the Lineweaver-Burk plot at higher substrate concentration, the curve shows an upward trend indicating substrate inhibition.

Table 4.6 : EFFECT OF ENZYME CONCENTRATION ON FENVALERATE HYDROLYSIS

Protein Concentration (mg)	Relative Concentration (mol %)		
	Residual Fenvalerate	PI	PII
0.50	62.81	31.58	5.61
1.00	50.76	41.56	7.68
1.50	53.88	43.07	3.05
2.00	52.89	45.35	1.76
2.50	51.01	45.50	3.49

PI and PII are metabolites formed

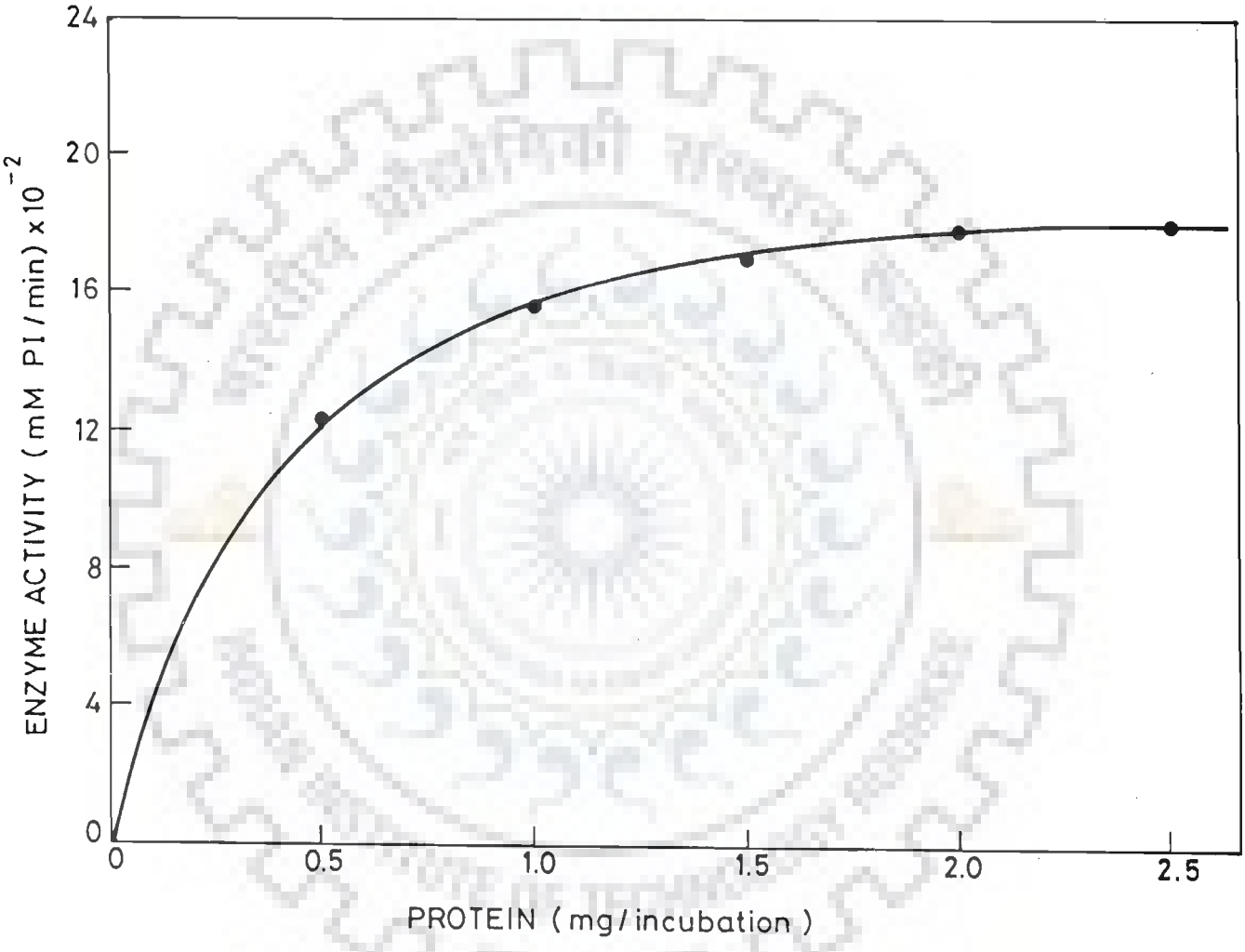


Fig. 4.10 Effect of Outer Mitochondrial Protein Concentration on Fenvalerate Hydrolysis. Incubation Mixture and Conditions are as Described.

Table 4.7 : EFFECT OF pH ON FENVALERATE HYDROLYSIS

pH	Relative Concentration (mol %)		
	Residual Fenvalerate	PI	PII
3.5	87.36	8.04 (19)	3.67
4.0	86.26	10.39 (25)	3.34
5.0	78.51	18.15 (43)	1.46
6.0	79.30	18.19 (44)	2.50
6.5	78.11	19.53 (47)	2.36
7.0	73.51	24.46 (59)	2.03
7.5	50.20	41.79 (100)	8.01
8.0	60.89	33.60 (80)	5.51

PI and PII are metabolites formed

Values in Parentheses are expressed as % of maximum activity

pH 3.5-8.0 : 0.1 M Sodium acetate buffer

pH 6.0-8.0 : 0.1 M Phosphate buffer

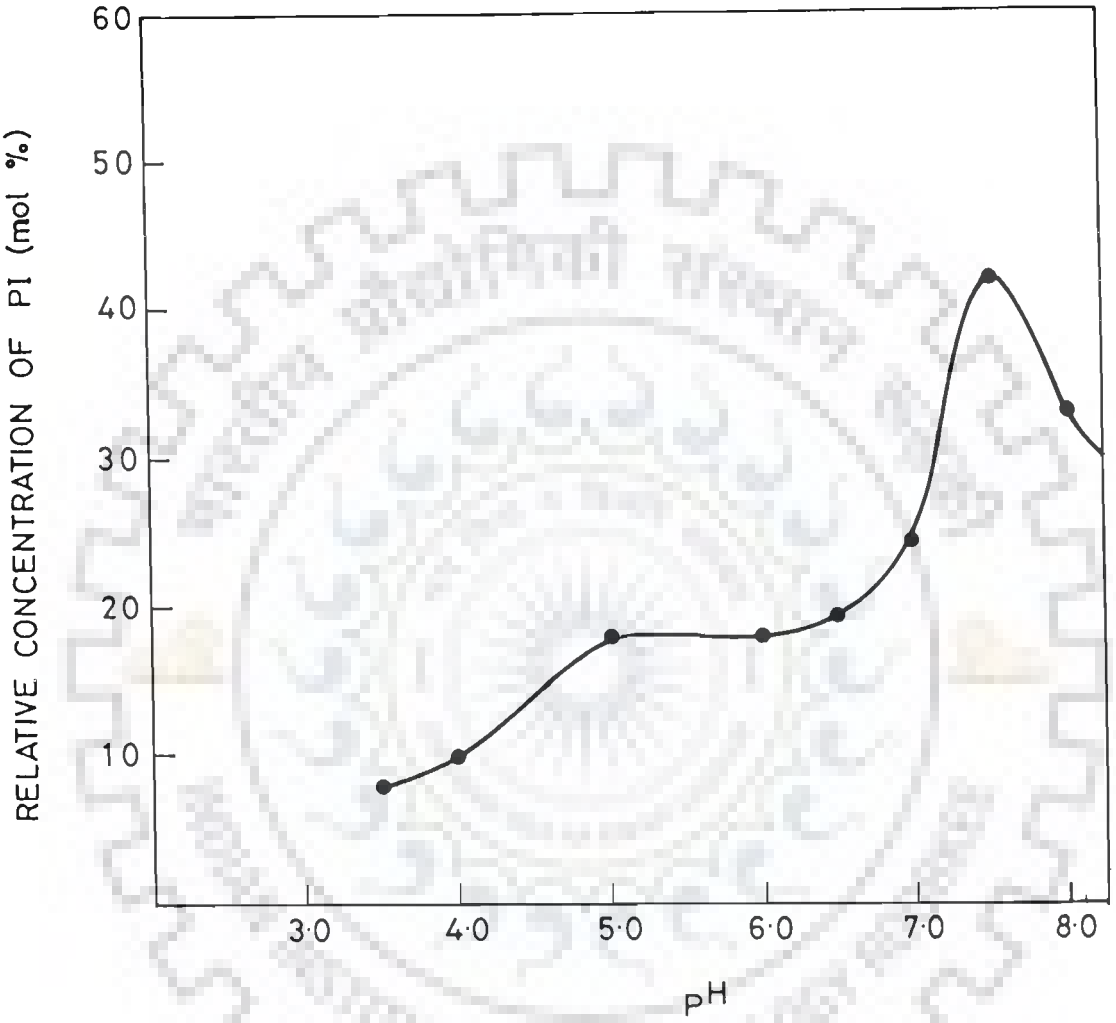


Fig. 4.11 pH- Activity Curve for Fenvalerate Hydrolysing Enzyme from Rat Liver Outer Mitochondrial Membrane. Incubation Mixture Consisted of 0.1 M Sodium Acetate Buffer (pH : 3.5 - 5.0) and 0.1 M Phosphate Buffer (pH : 6.0 - 8.0).

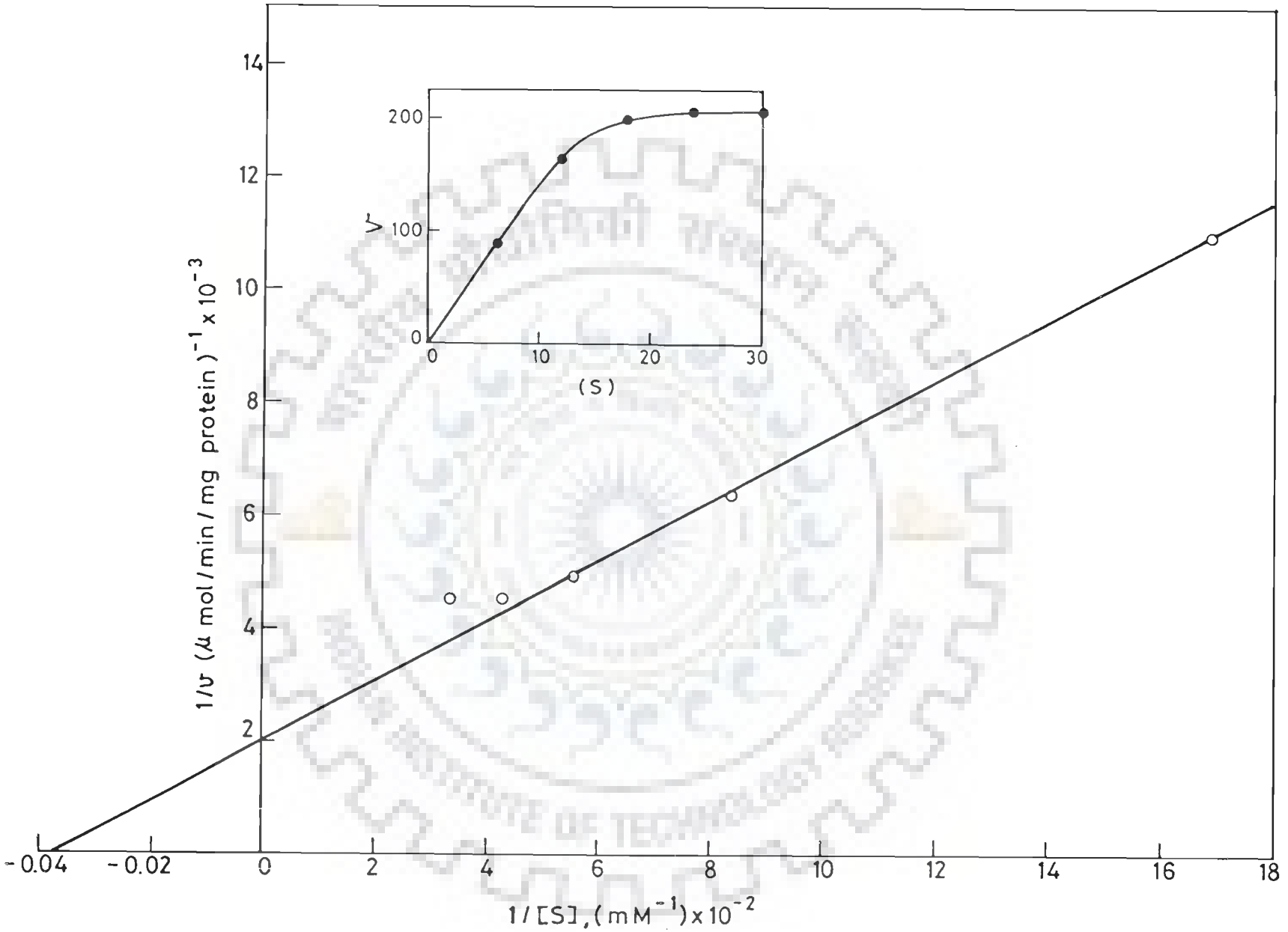


Fig. 4.12 Lineweaver - Burk Plot Showing K_m and V_{max} for Fenvalerate Hydrolysis by Rat Liver Outer Mitochondrial Membrane.

4.2.3.5 Effect of EDTA and Metal Ions on the Fenvalerate Metabolising Enzyme

Fig. 4.13 shows the enzyme activity as a function of EDTA concentration. It can be seen that in presence of low EDTA concentration (below 0.5 mM), the enzyme activity was significantly stimulated. But at higher concentration (above 0.5 mM), the enzyme activity was strongly inhibited. For example, at 2 mM concentration of EDTA, the activity was only about one third of the maximum activity observed in presence of 0.5 mM concentration of EDTA or about half of the activity observed in absence of EDTA. These results show that whereas low concentration of EDTA was probably helping in making active the enzyme-substrate complex, the higher concentration of EDTA induced unfavourable changes in the conformation in the enzyme or chelating metal ions required by the enzyme for its activity .

Table 4.8 shows the effect of some metal ions on the enzyme activity *in vitro*. It was found that addition of 2 mM EDTA resulted in reduction of the enzyme activity to from about 0.166 mM min^{-1} to 0.10 mM min^{-1}. The addition of Mg^{2+} (10 mM) sufficient to neutralise EDTA, restored full activity of the enzyme which was comparable to that of enzyme without EDTA. There was a slight inhibition with EDTA + Mg^{2+}. Zn^{2+} did not affect the activity of the enzyme.Mn^{2+} and Ca^{2+} were found to be potential inhibitors of the enzyme activity.

4.2.3.6 Effect of Detergents

With a view to find a suitable detergent for solubilising membrane bound enzyme, the effect of detergent on the enzyme activity was determined. The results are shown in Table 4.9. In absence of detergents, nearly 50% of the pesticide remained

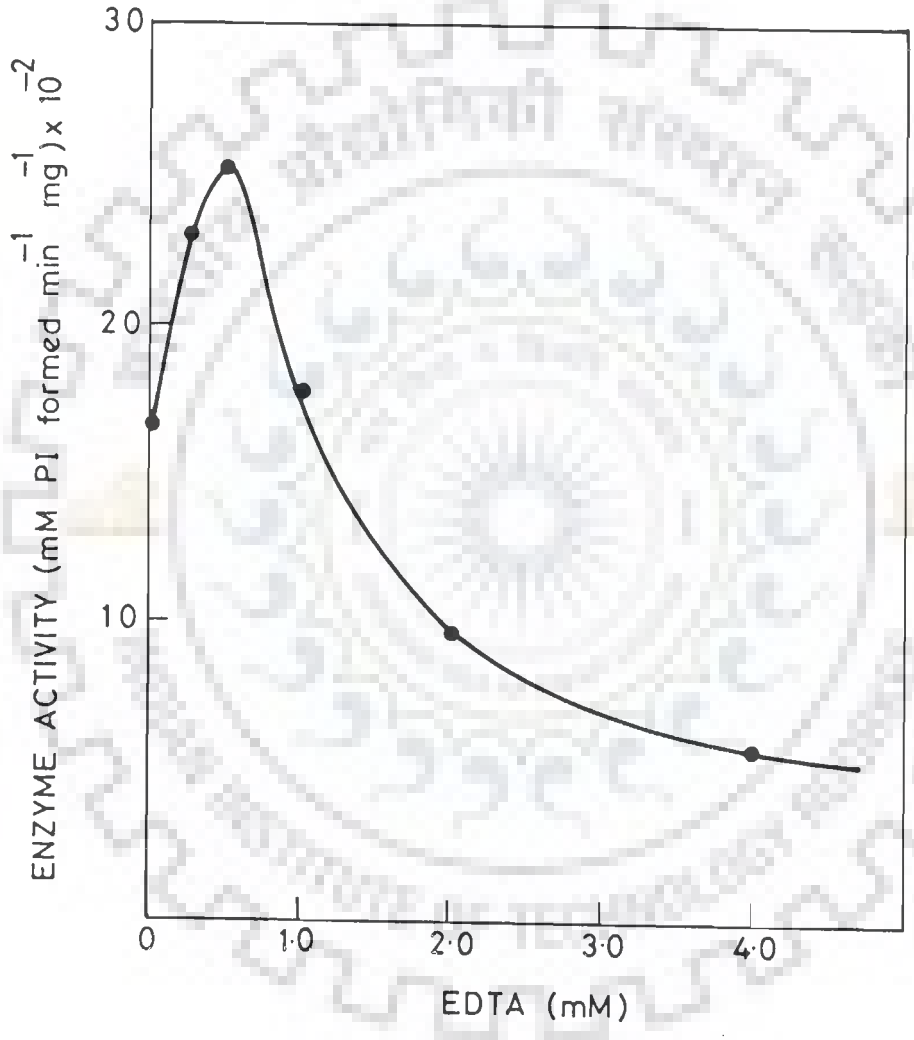


Fig. 4.13 EDTA - Activity Profile for the Fenvalerate Hydrolysing Enzyme in Outer Mitochondrial Membrane of Rat Liver.

Table 4.8 : EFFECT OF METALS IONS AND EDTA ON FENVALERATE HYDROLYSIS

Conditions[a]	Relative Concentration (mol %)		
	Residual Fenvalerate	PI	PII
Control	50.24	42.58 (100)	7.18
0.0 mM metal[b]	76.70	22.33 (53)	1.00
Mg^{2+}	45.95	50.03 (117)	4.02
Mg^{2+} + EDTA	48.98	46.45 (109)	4.57
Zn^{2+}	51.21	46.26 (108)	2.53
Zn^{2+} + EDTA	53.27	43.40 (102)	3.33
Ca^{2+}	60.02	37.96 (89)	2.02
Ca^{2+} + EDTA	64.34	33.41 (79)	2.25
Mn^{2+}	62.21	36.41 (86)	1.38
Mn^{2+} + EDTA	58.36	38.82 (91)	2.82

PI and PII are metabolites formed

Values in Parentheses indicate % of control (complete incubation medium).

[a]All cations with exception of Zn^{2+} which was added as the sulfate form, were in the chloride form, and added to a final concentration of 1×10^{-2} M. Final EDTA concentration was 2 mM.

[b]Complete incubation medium with 2 mM EDTA.

undegraded with the formation of 41% major product PI. In presence of detergents the *in vitro* degradation of fenvalerate was significantly enhanced. For example, the amount of undegraded pesticide in presence of 0.5% Triton X-100 was only approximately 35% compared to 50% without detergent. Similarly, in presence of Nonidet P-40 and Digitonin, the concentration of residual fenvalerate was 40% and 35% respectively. Unexpectedly, Triton X-100 and Nonidet P-40 seem to catalyse further the degradation of metabolite PI into a third metabolite (PIII) as indicated by concomitant increase in the concentration of PIII and decrease in the concentration of PI . In contrast, effect of Digitonin was comparable with the control i.e., concentration of metabolite PI paralleled the degradation of the parent pesticide, fenvalerate with no accumulation of metabolite PIII (Fig. 4.14).

Table 4.9 : EFFECT OF DETERGENTS ON FENVALERATE HYDROLYSIS

Detergent	Relative Concentration (mol %)			
	Residual Fenvalerate	PI	PII	PIII
Control[a]	50.09	42.01	7.90	N.D.
Triton X - 100	34.54	13.66	4.11	47.69
Nonidet P - 40	41.42	7.93	4.28	46.37
Digitonin	35.58	59.4	5.02	N.D.

Detergent was added to the membrane before using it as enzyme source in the *in vitro* assay.
[a]Complete incubation medium with no detergent .

The mechanism of the action of detergent on metabolite formation is not known at the moment. However, it appears that Triton X-100 and Nonidet P-40

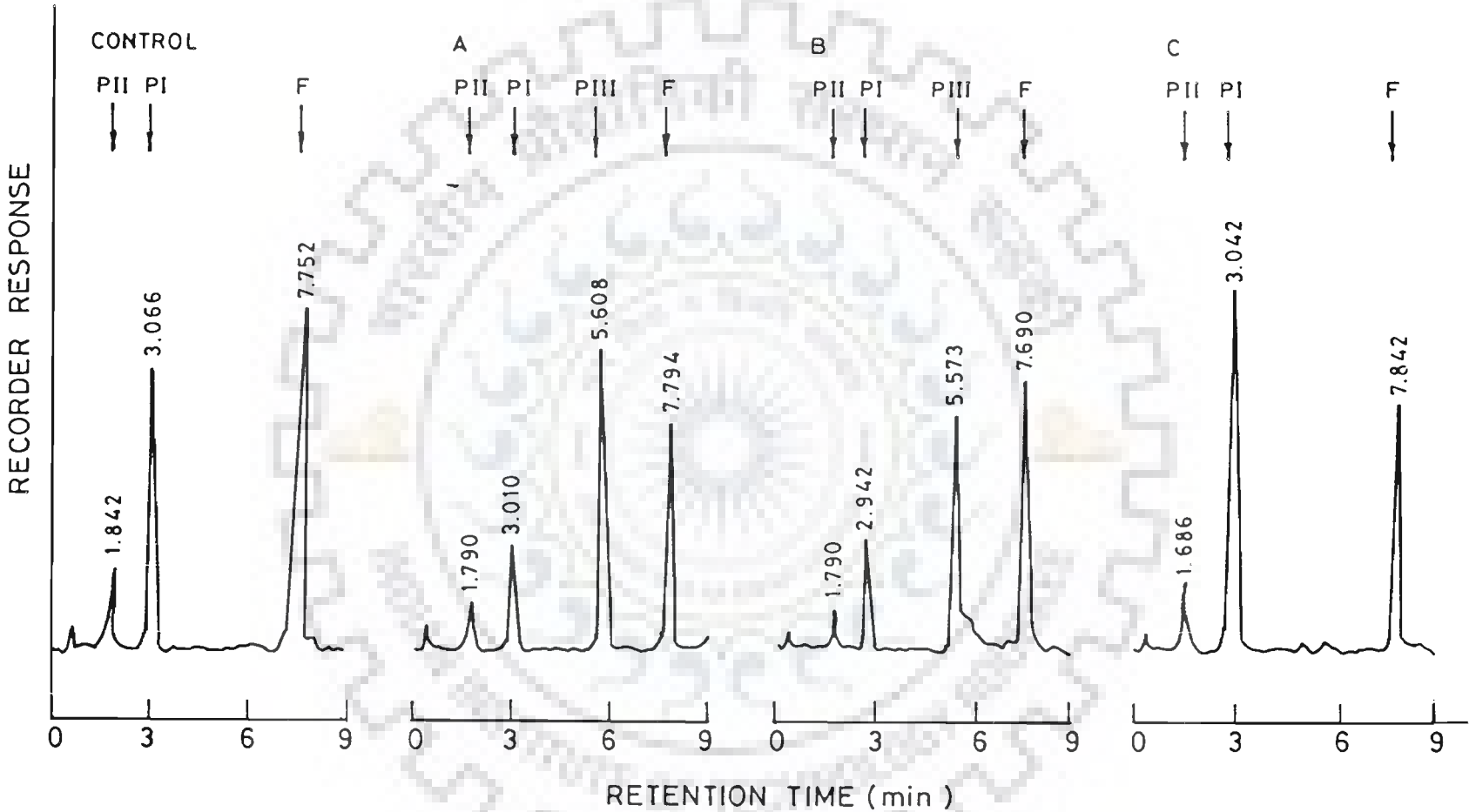


Fig. 4.14 HPLC Spectra of *In Vitro* Metabolism of Fenvalerate (F) into its Products (PI, PII and PIII) in Presence of Detergents. (A: Triton X-100; B: Nonidet P-40; C: Digitonin)

release the enzyme from the membrane which is responsible for the degradation of PI into PIII. Further work would be necessary to understand the effect of detergent on the metabolic pathway of the degradation of fenvalerate.

4.2.4 Identification of Fenvalerate and Metabolites by TLC

The TLC of *in vitro* assay of fenvalerate metabolism in rat liver subcellular fractions was also carried out. Two new metabolites PI and PII were formed which substantiate the earlier results obtained by HPLC analyses. The R_f values were :

Fenvalerate : 0.94
PI : 0.88
PII : 0.49

4.3 CHARACTERISATION OF FENVALERATE AND ITS METABOLITES BY INFRARED SPECTROSCOPY

For characterisation of fenvalerate and its metabolites formed, they were purified in sufficient quantities by HPLC as described in Materials and Methods. The IR spectra of fenvalerate and the metabolites PI and PII are shown in Fig.4.15.

The IR spectrum of fenvalerate compound shows the characterstic peak of ester group linkage in the region of 1750 cm^{-1}. The parent compound also shows peaks in the region of 762 cm^{-1} representing aromatic substitution with halogen, in this case -C-Cl bond. The -C ≡ N vibration is characterised by the peak at 2290 cm^{-1}, while the peaks at 1384 cm^{-1}, 1450 cm^{-1} and 1148 cm^{-1} suggest presence of gem-dimethyl group i.e., $(-CH<^{CH_3}_{CH_3})$. The aromatic rings present in the compound are shown by the characterstic peaks at 1594 cm^{-1}.

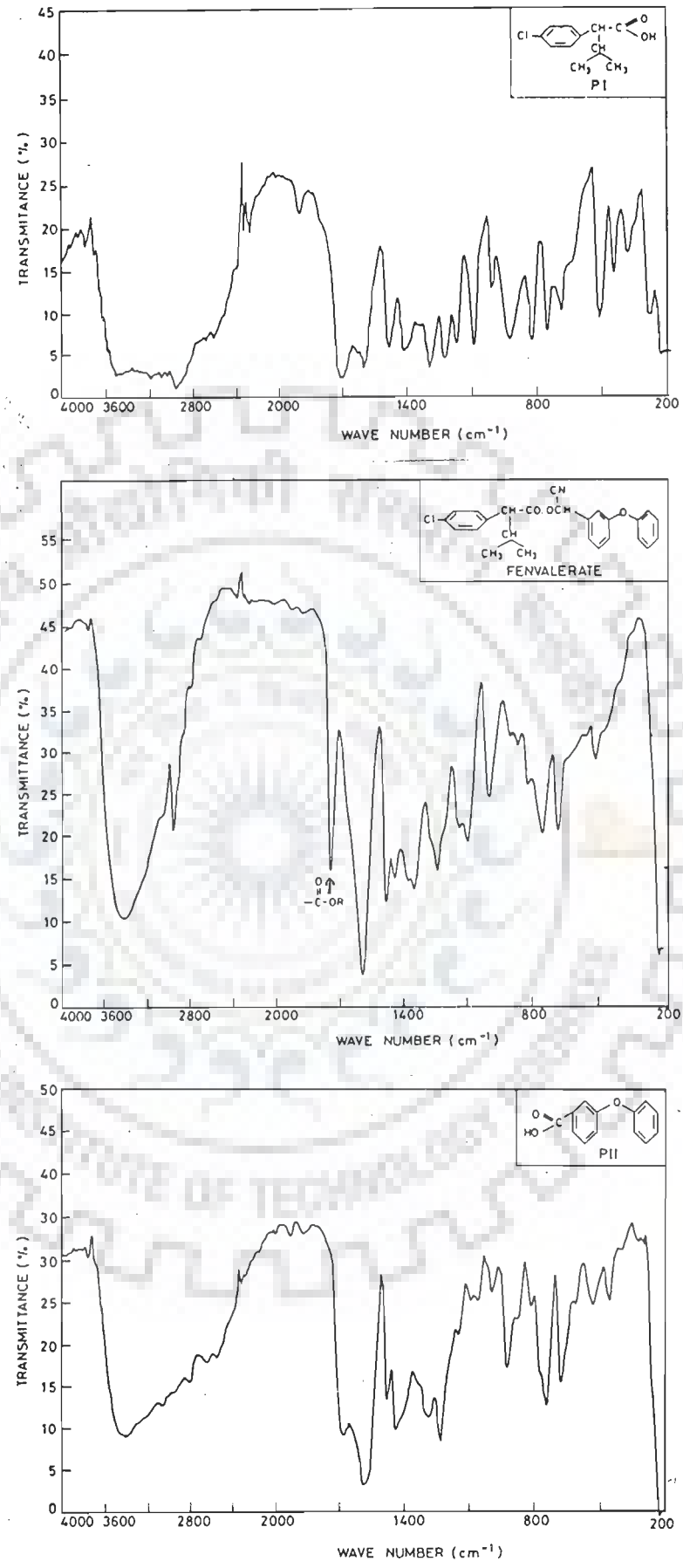


Fig. 4.15 Infrared Spectra of Fenvalerate and its metabolites (PI and PII). Arrow Denotes the Position of Ester Peak in Fenvalerate which is Missing in PI and PII.

In case of metabolite PI, the 1752 cm^{-1} peak (ester group) was completely missing indicating the cleavage of the ester bond. A group of 5-6 new peaks in the region of 2682 cm^{-1} shows the presence of a carboxylic group. Further the -OH of carboxylic group is characterised by a peak at 2962 cm^{-1}. These results indicate that fenvalerate has undergone hydrolytic ester cleavage *in vivo* as the first step of biodegradation. Formation of this metabolite PI has also been observed in rats, both *in vivo* and *in vitro*, as a major product (Kaneko et al., 1981; Mumtaz and Menzer, 1986). The presence of a gem-dimethyl group in the region of the 1174 cm^{-1} and -C-Cl band at 756 cm^{-1} in the metabolite PI is also seen. The characterstic peak of aromatic ring at 1600 cm^{-1} is present in the metabolite.

The IR spectrum of metabolite PII, which was found in liver, kidney and brain after 6 h exposure of pesticide to rats did not give the characterstic peaks at 1148 cm^{-1} and 1752 cm^{-1} indicating absence of the gem-dimethyl and ester groups respectively. The characterstic peak present in this compound was in the region of 1592 cm^{-1} representing presence of substitution in aromatic ring. The peaks for benzoic acid at 1302 cm^{-1} and 1920 cm^{-1} are also present in this compound. The peak at 1240 cm^{-1} showing the presence of =C-O-C group was present in both, metabolite PII as well as the parent compound,indicating *in vivo* degradation of fenvalerate into PII containing this group intact. Moreover, the peak at 2290 cm^{-1} which is characterstic for -C≡N group and present only in fenvalerate is missing in both the metabolites PI and PII, indicating loss of the cyano group. As reported in literature, the cyano-group is converted into either thiocyanate in blood or lost as CO_2.(Ohkawa et al.,1979).

Thus the hydrolytic cleavage of the ester bond gave rise to two new products i.e., PI and PII, which are charaterised as 4-chloro-α-(1-methylethyl) benzeneacetic acid (CPIA) and 3-phenoxybenzoic acid (PBacid) respectively, as a primary step of the *in*

vivo biodegradation in rat tissues. However, *in vitro* studies have revealed the presence of a third metabolite PIII which is presumed to be formed by further degradation of the major product PI. On the basis of the kinetic data on bioaccumulation of metabolites PI and PII and the IR spectra, the proposed metabolic pathway of fenvalerate in rat is shown in Fig. 4.16.

4.4 ELECTRON MICROSCOPIC STUDIES

4.4.1 Effect of Fenvalerate on the Ultrastructure of Liver Cells

Electron microscopic studies were carried out to investigate further the effect of pesticides on the ultrastructure of liver cell, a hepatocyte. Since numerous metabolic functions occur in liver, hepatocytes have been thoroughly studied for many years and the cellular organisation of a eukaryotic cell of animal origin is well illustrated by hepatocytes. Fig. 4.17 a,b show the electron micrographs of liver cells of the control (untreated) rat. It can be seen that the nucleus of liver cells of control rat shows intact nucleus, a well defined nuclear membrane and a distinct nucleolus. Also the presence of rough endoplasmic reticulum (RER) and mitochondria is evident in the electron micrographs. When the fenvalerate exposed liver cells were viewed under transmission electron microscope (TEM), it was observed that the nuclear membrane was more or less intact, but contained dispersed chromatin material and after 7 days treatment, the nucleolus becomes almost invisible (Fig. 4.17c). However, there was an increase in the chromatin material (CH) with formation of two nucleolus after 30 days treatment (Fig. 4.17d). It was also found that when dose rate was increased from 5 to 15 mg/ Kg body weight, the number of nucleolus appeared to increase and also there was a tendency of margination of heterochromatin along the nuclear membrane (Fig. 4.17e,f). A closer look at the electron micrographs also showed presence of crystal rods (CR) around the nuclear membrane and deposition of lipids in the

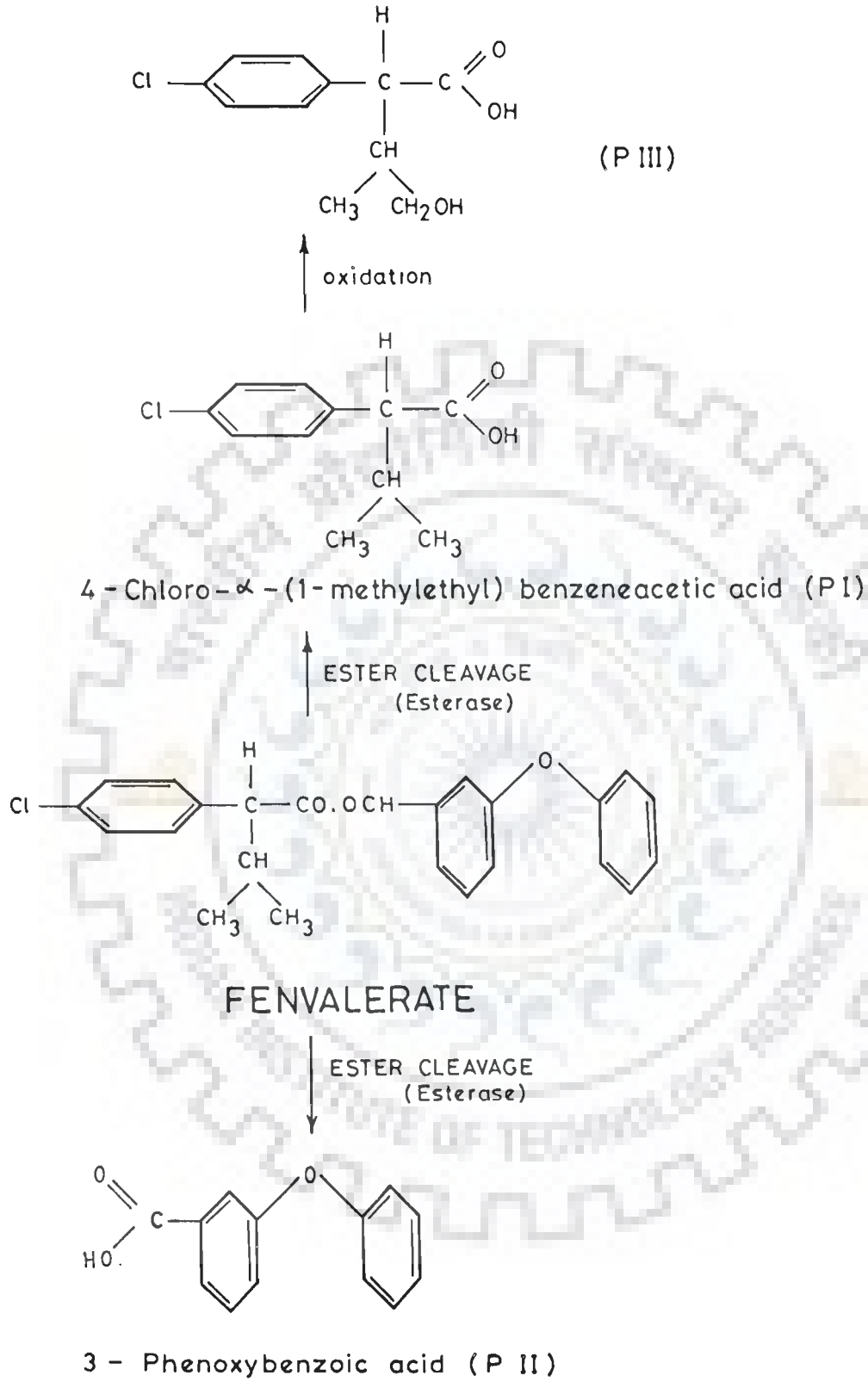


Fig. 4.16 Proposed Metabolic Pathway for Fenvalerate in Rats.

Fig. 4.17 (a) Electron Micrograph of Liver Cell of Control Rat. (× 4600)
Nucleus (N) ; Nucleolus (Nu) ; Nuclear membrane (NM);
Lipid (Li)

Fig. 4.17(b) Electron Micrograph of Liver Cell of Control Rat (× 6700)
Rough Endoplasmic Reticulum (RER); Mitochondria (M).

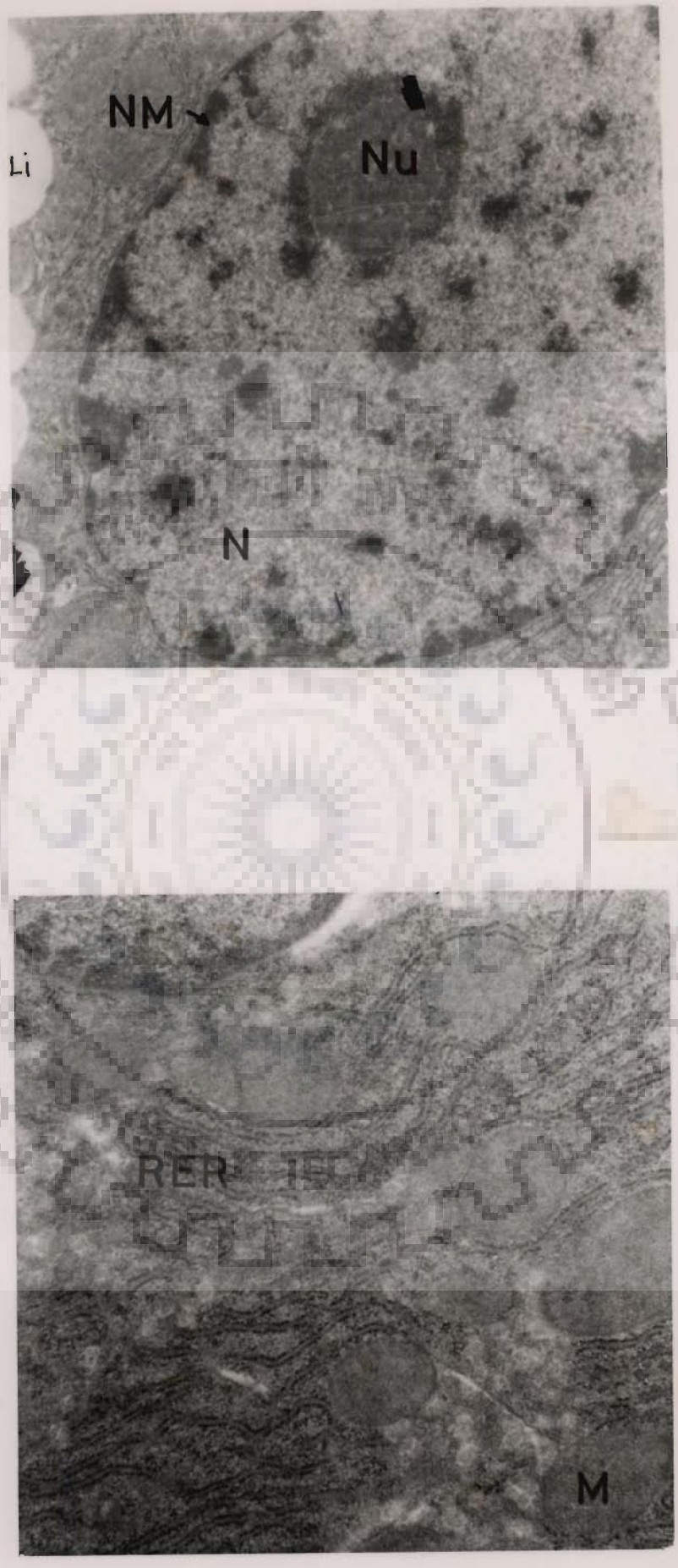
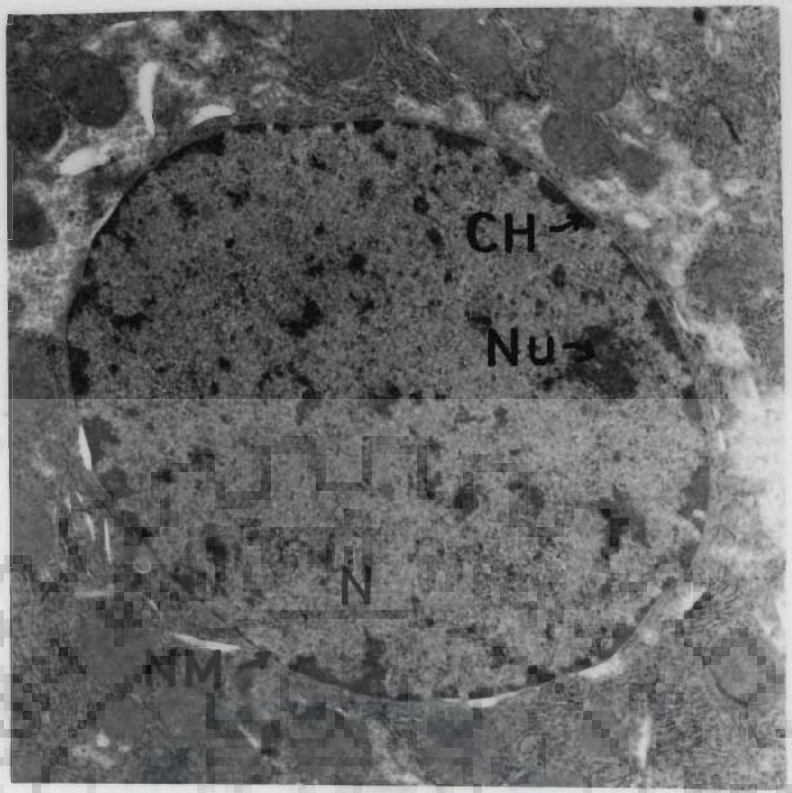

NM
Nu
Li
N
RER
M

Fig. 4.17(c) Electron Micrograph of Liver Cell of Fenvalerate Treated (7 days, 5 mg/Kg) Rat (× 4600)

Nucleus (N) ; Nucleolus (Nu); Nuclear Membrane (NM); Chromatin (CH)

Fig. 4.17(d) Electron Micrograph of Liver Cell of Fenvalerate Treated (30 days, 5 mg/Kg) Rat (× 4600)

Nucleus (N); Nucleolus (Nu); Chromatin (CH)


CH
Nu
N
NM

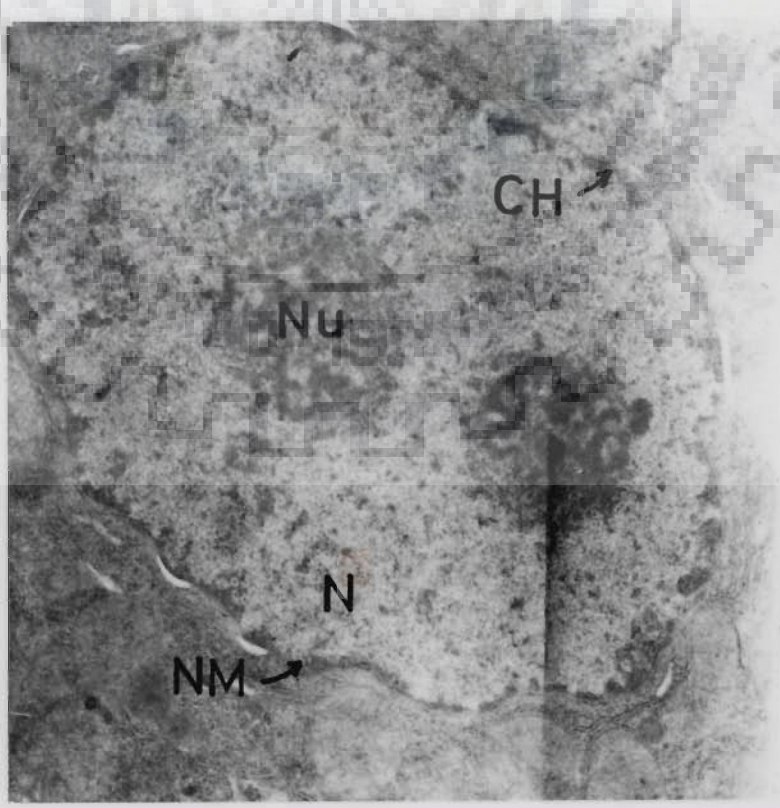

CH
Nu
N
NM

Fig. 4.17(e) Electron Micrograph of Liver Cell of Fenvalerate Treated (7 days, 15 mg/Kg) Rat (× 4600)

Nucleus (N); Chromatin (CH); Endoplasmic Reticulum (ER)

Fig. 4.17(f) Electron Micrograph of Liver Cell of Fenvalerate Treated (30 days, 15 mg/Kg) Rat (× 4600)

Nucleus (N); Nucleolus (Nu); Mitochondria (M); Microbodies (MB); Chromatin (CH).


ER
N
CH
B
M
N
CH₂

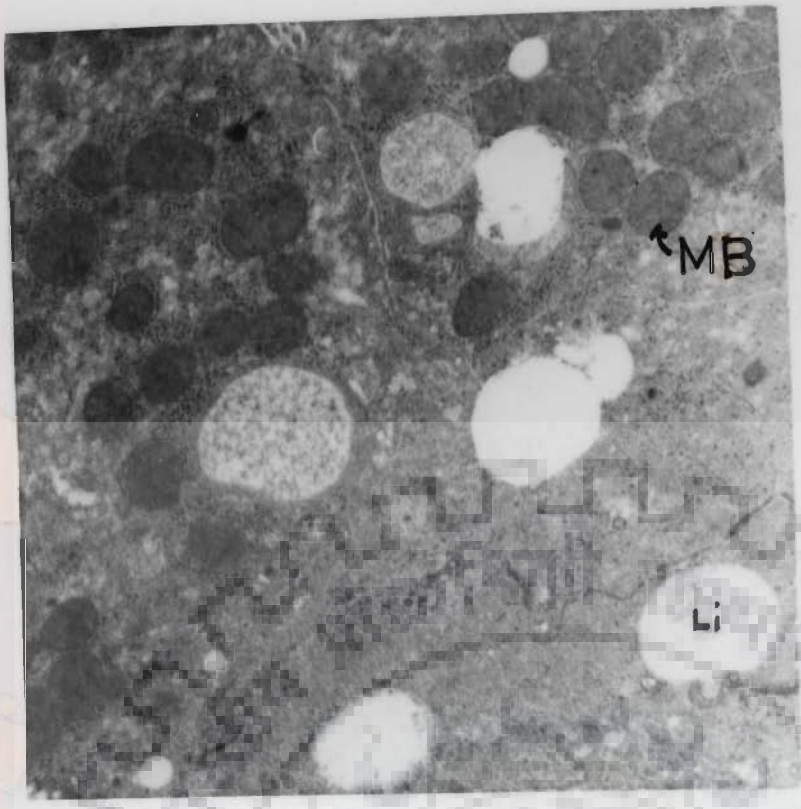
pesticide treated cells. A large number of microbodies (MB) were also visible around the nucleus in the liver cells of treated animals. Deposition of fat droplets and MB were clearly visible in the electron micrographs (Fig. 4.17g). The electron micrograph (Fig. 4.17h) also showed deposition of lipids crystal rods which may be of cholesterol esters (Okuno et al.,1986). This accumulation of unsaturated lipid (USL) droplets in 7 and 30 days treated rats could be correlated to the increase in total lipids due to pesticide exposure. Even low dose (5 mg/Kg) led to dilation of bile canaliculi (BC) with indication of some broken microvilli (Fig.4.17i). The deposition of dense microbodies on the surface of endoplasmic reticulum (ER) was quite evident in the liver cells of fenvalerate treated rats (Fig. 4.17j). It appears that the fenvalerate treatment had blocked the intracellular translocation of newly synthesized proteins from the surface of endoplasmic reticulum.

4.4.2 Effect of Fenvalerate on the Ultrastructure of Kidney Cells

The electron micrographs of cross section of kidney cells of control and fenvalerate treated rats are shown in Fig 4.18. The control kidney showed normal nucleus with a distinct nucleolus (Fig. 4.18a). After 7 days treatment of rats with fenvalerate, the nucleolus started to disorganise (Fig. 4.18b) and 30 days exposure to pesticide resulted in marked disorganisation of the chromatin material in nucleus which was seen dispersed all along the nuclear membrane. Also in the cytoplasm, deposition of fat droplets could be seen. In addition, dense microbodies (MB) and deformed mitochondria were present in the cytoplasm of the kidney cells from the rat exposed for 30 days to the pesticide (Fig.4.18c). From these results, it therefore appears that fenvalerate has significant effect on the ultrastructure of nucleus especially with regard to nucleolus, distribution of chromatin, and deposition of crystalline rods and lipid droplets.

Fig. 4.17(g) Electron Micrograph of Liver Cell of Fenvalerate Treated (30 days, 15 mg/Kg) Rat (× 4600)
Microbodies (MB); Lipid Droplets (Li).

Fig. 4.17(h) Electron Micrograph of Liver Cell of Fenvalerate Treated (7 days, 5 mg/Kg) Rat (× 4600)
Unsaturated Lipid Droplets (USL); Crystal Rods (CR); Chylomicrons (C)


MB
Li


USL
C
CR

Fig. 4.17(i) Electron Micrograph of Liver Cell of Fenvalerate Treated (30 days, 5 mg/Kg) Rat (× 4600)
Bile Canaliculi (BC)

Fig. 4.17(j) Electron Micrograph of Liver Cell of Fenvalerate Treated (7 days, 15 mg/Kg) Rat (× 4600)
Microbodies (MB), Endoplasmic Reticulum (ER)


BC

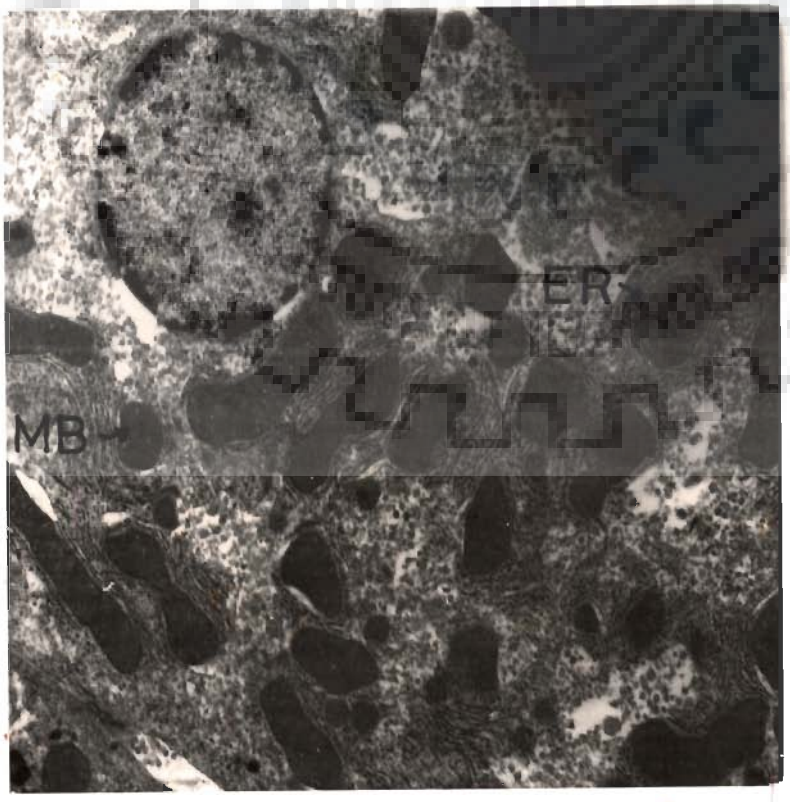

ER
MB

Fig. 4.18(a) Electron Micrograph of Kidney Cell of Control Rat (× 4400) Nucleus (N); Nucleolus (Nu).

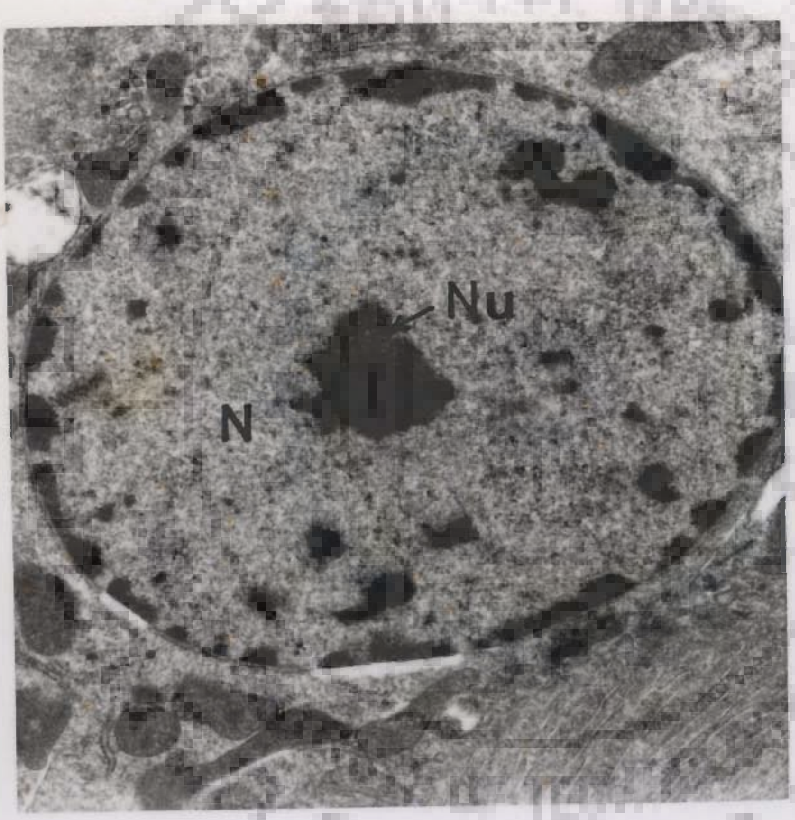
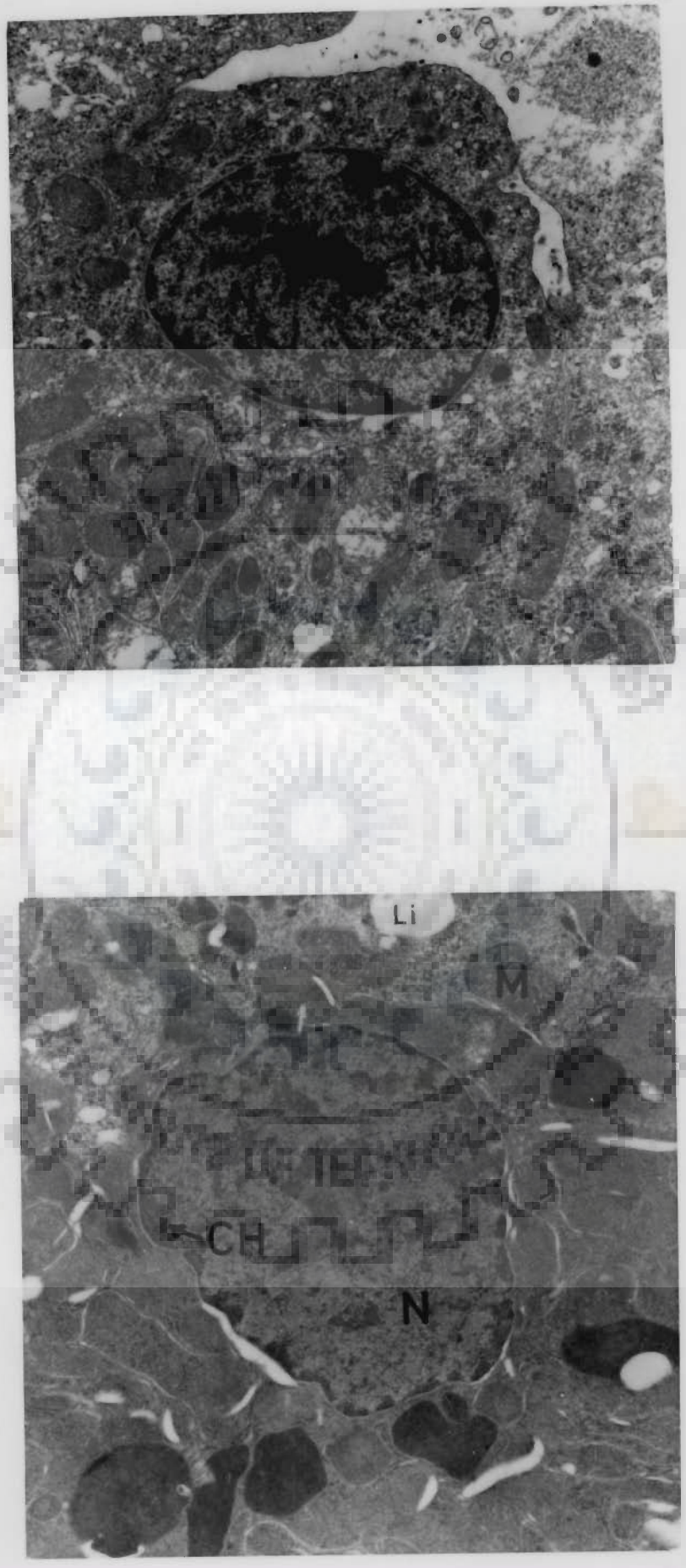

Nu
N

Fig. 4.18(b) Electron Micrograph of Kidney Cell of Fenvalerate Treated (7 days, 5 mg/Kg) Rat. (× 4200)
Nucleus (N); Nucleolus (Nu).

Fig. 4.18(c) Electron Micrograph of Kidney Cell of Fenvalerate Treated (30 days, 5 mg/Kg) Rat. (× 4200)
Nucleus (N); Chromatin (CH); Mitochondria (M); Lipid (Li).


Li
M
CH
N

4.5 CHRONIC STUDIES

4.5.1 General Observations

As the chronic toxicity studies involved repeated exposure of rats to fenvalerate for a prolonged period of time, the physical signs of intoxication included hyperactive and aggressive behaviour of rats during 8-10 h postdosing period. The partial loss of body hair was also observed after 24 h of pesticide exposure (Fig. 4.19a, b). The animals, however, returned to normal behaviour after 2 days following exposure to the pesticide. Thus, the animals took about 2 days to get adjusted to the initial irritational reactions of the pesticide. Moreover, the animals did not show any significant loss in body weight.

4.5.2 Bioaccumulation and Distribution of Fenvalerate and Metabolites in Rat Organs

For bioaccumulation of fenvalerate and its degradation products in different rat organs, chronic treatment was given to rats over a period of 30 days on alternate days. At indicated intervals, the animals were sacrificed, organs removed and analysed by HPLC for the accumulation of the parent pesticide and its metabolites. The experiments were conducted using two different doses of fenvalerate i.e., 5 and 15 mg/Kg body weight. The representative HPLC profiles of the accumulation of pesticide and its metabolites in liver, kidney and brain after 7, 15 and 30 days exposure are shown in Figs. 4.20, 4.21 and 4.22. It was found that the pesticide was rapidly metabolised and two slow moving products PI and PII with retention times (RT value) of 1.725 min and 3.09 min were formed after 7 days of treatment. At this stage of treatment,substantial concentration of residual fenvalerate (RT : 7.78 min) was also detected. Further treatment of animals i.e., after 15 days and 30 days, gave

Fig. 4.19(a) Rat Before Treatment.

Fig.4.19(b) Rat After Treatment.

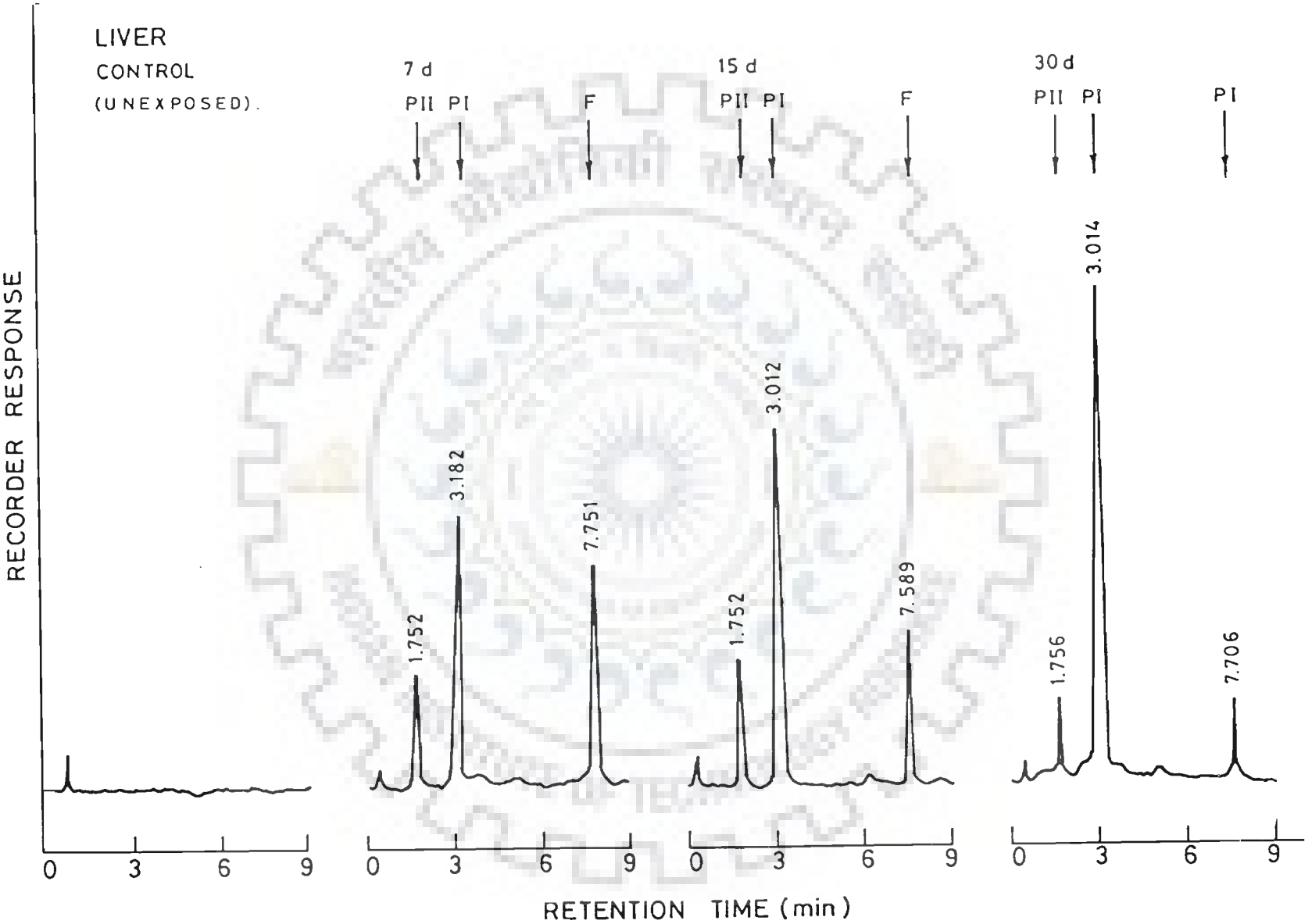


Fig. 4.20 HPLC Profiles of Bioaccumulation of Fenvalerate (F) and its Metabolic Products (PI and PII) in Liver under Chronic Treatment (15 mg/Kg) as a Function of Time

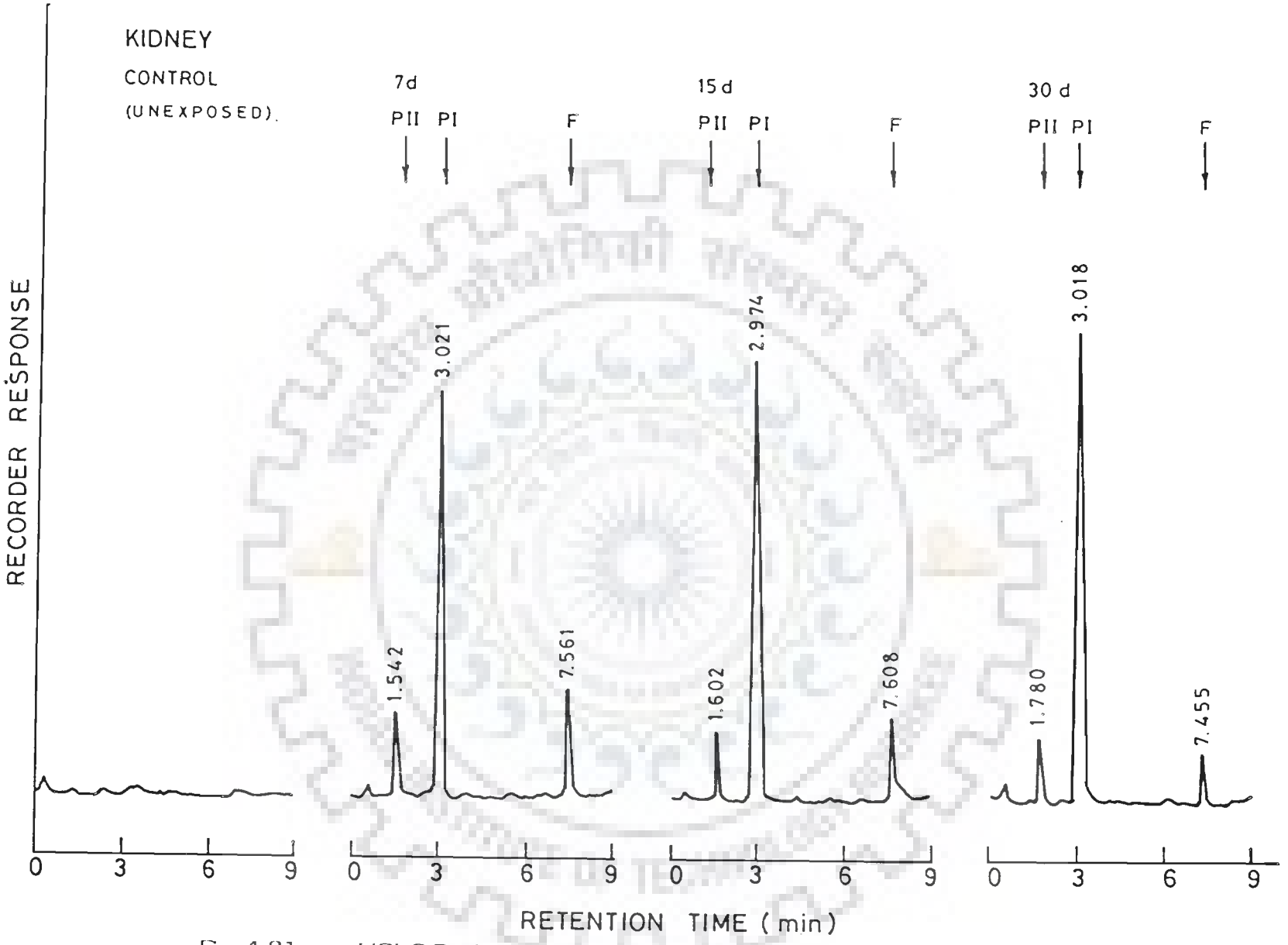


Fig. 4.21 HPLC Profiles of Bioaccumulation of Fenvalerate (F) and its Metabolic Products (P1 and PII) in Kidney under Chronic Treatment (15 mg/Kg) as a Function of Time.

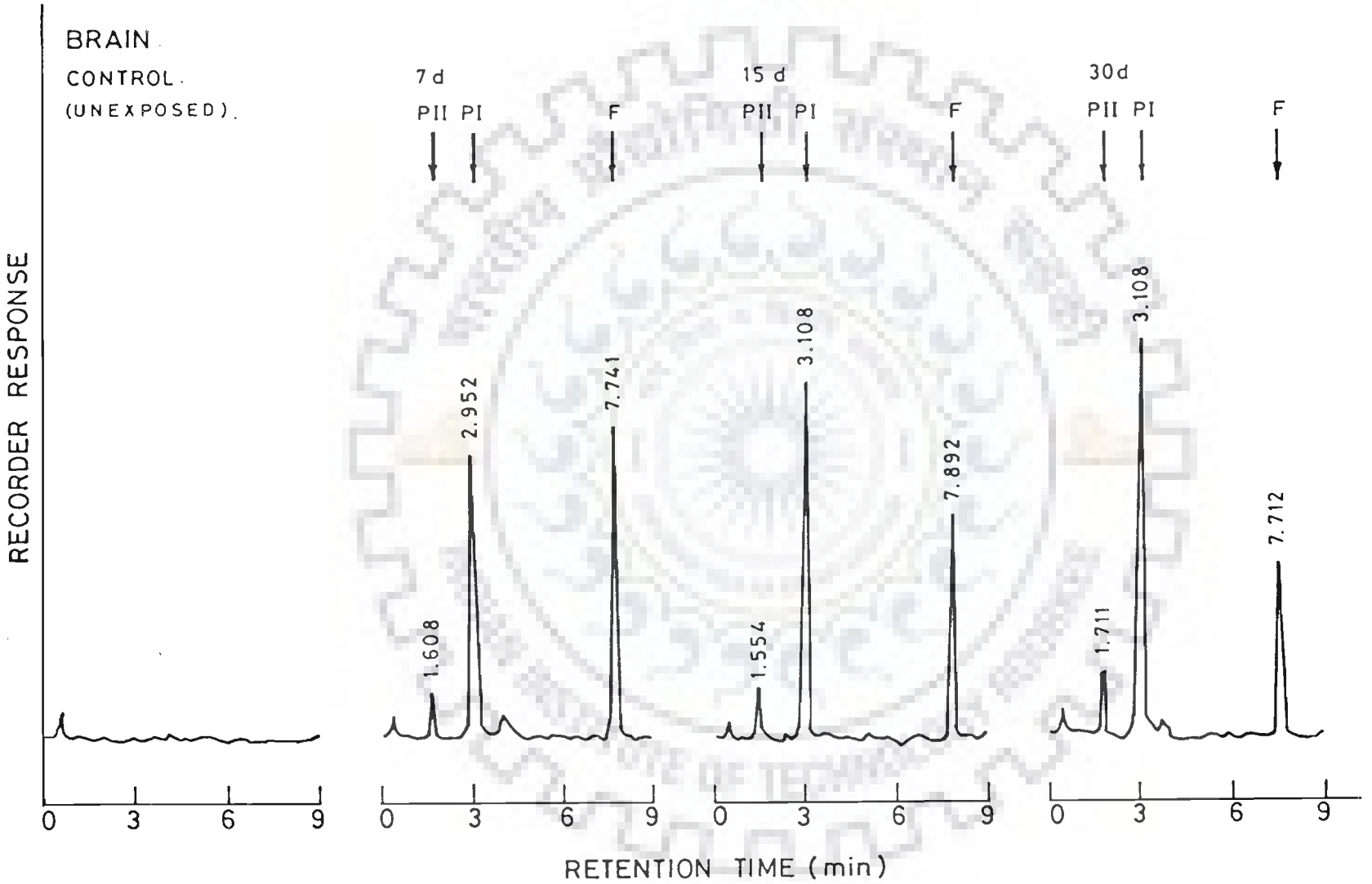


Fig. 4.22 HPLC Profiles of Bioaccumulation of Fenvalerate (F) and its Metabolic Products (PI and PII) in Brain Under Chronic Treatment (15 mg/Kg) as a Function of Time.

similar profiles except that the concentration of the parent pesticide decreased with concomitant increase in the relative concentration of the main product (PI) with time. The quantitative data of the relative concentration (mol %) of the residual fenvalerate and its degradation products PI and PII in liver are shown in Table 4.10. These results also indicate that unlike PI, the accumulation of PII at higher dose exposure i.e., 15 mg/Kg did not increase with time. In fact, after a slight increase between 7 and 15 day period, there was a rapid decrease in its relative concentration in liver. For example, after 15 days treatment the relative molar concentration of residual fenvalerate, PI and PII were approximately 20%, 60% and 20 % respectively. After 30 days of treatment, the residual molar concentrations were 8%, 82% and 9% respectively. Thus, whereas the concentration of PI increased at the cost of fenvalerate, the concentration of PII decreased between treatment period of 15 days and 30 days. This deviation may be due to rapid excretion or rapid metabolism of this compound resulting in lower accumulation in liver.

Bioaccumulation of fenvalerate and its metabolites in kidney and brain of rats as a function of time at 5 and 15 mg/Kg body weight doses is shown in Fig. 4.23. For comparison, profile of the relative accumulation of the pesticide and its products in liver is also included in the histogram. From these results, it is quite apparent that the degradation pattern in kidney and brain is identical to that of liver, except that at high dose level (15 mg/Kg) the accumulation of PII in brain does not change much after 15 days treatment. But at low dose level (5 mg/Kg), there is a gradual increase in the relative concentration of PII with time.

4.5.3 Effect of Fenvalerate on Nucleic Acids in Liver

The chronic exposure of rats to low (5 mg/Kg) and high (15 mg/Kg) dose levels

Table 4.10 : BIOACCUMULATION OF FENVALERATE AND ITS METABOLIC PRODUCTS IN RAT LIVER AS A FUNCTION OF TIME

Fenvalerate (5 and 15 mg/Kg of body weight) dissloved in groundnut oil was adminsitered orally to different groups of rats. At indicated intervals, postdosing, various organs were removed and amounts of residual fenvalerate and the metabolites (PI and PII) formed were determined by HPLC as described in Methods.

Duration of Exposure (Days)	Dose (mg/Kg body wt.)	Relative Concentration (mol %)		
		Residual Fenvalerate	PI	PII
Control	0.00	00.00	00.00	0.00
7	5.00	21.06	67.95	10.99
15		10.25	75.87	13.87
30		6.14	80.76	13.10
7	15.00	38.31	45.16	16.52
15		20.26	60.44	19.34
30		8.35	82.20	9.52

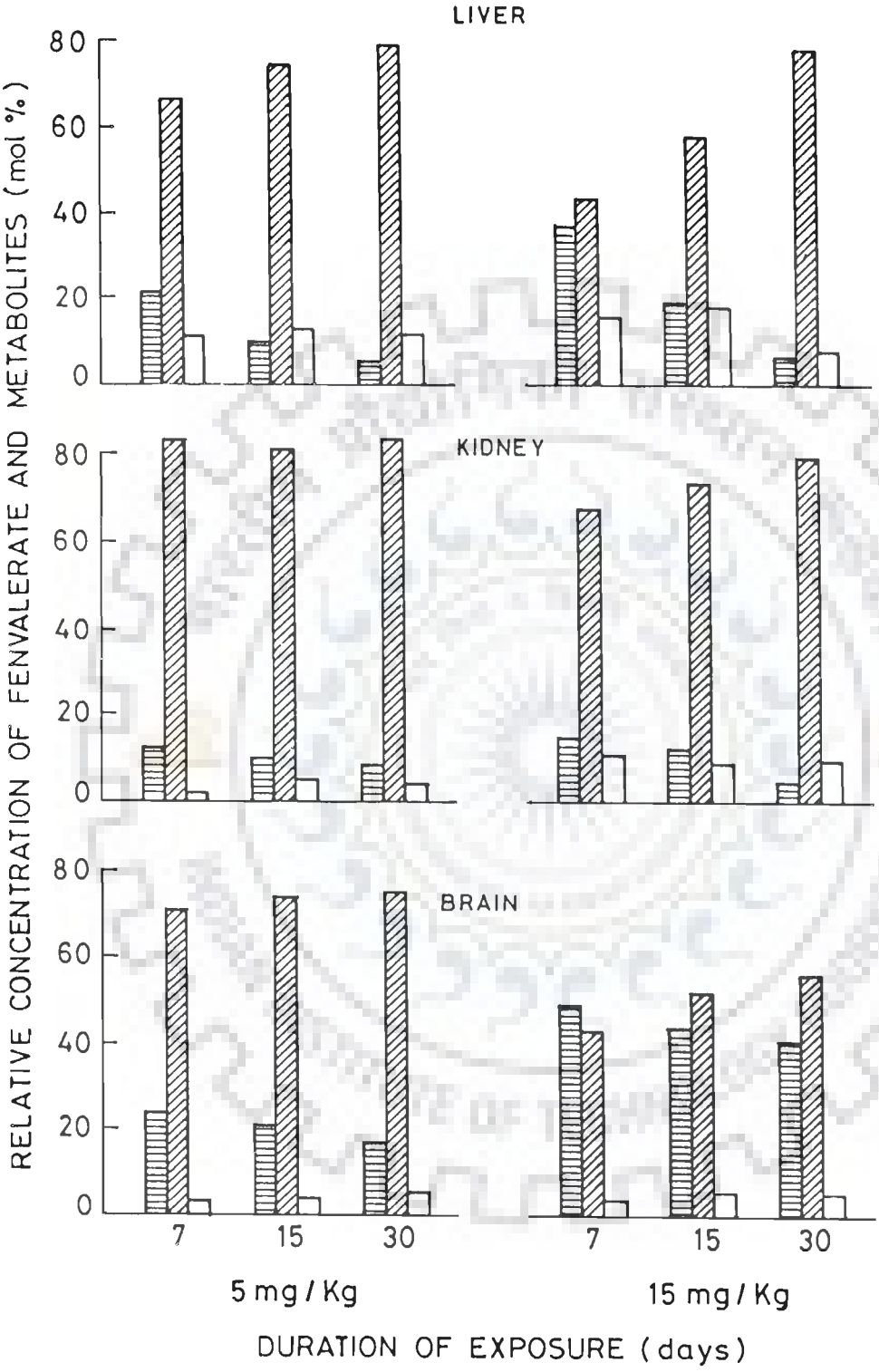


Fig. 4.23 Bioaccumulation of Fenvalerate and Metabolic Products (PI and PII) in Rat Organs at Different Doses of Fenvalerate as a Function of Time. Fenvalerate (▤), PI (▨), PII (□).

induced a marked reduction in the nucleic acids (RNA and DNA) content in rat liver compared to control (Table 4.11). At both dose, the maximum decrease was observed after 30 days exposure to pesticide. Thus the effect was time dependent and there was no sign of recovery. In this respect the results of chronic exposure were different from acute treatment reported earlier in the Thesis. In the latter case, after initial decrease the animal tends to neutralise the damaging effect caused by the pesticide. As expected, the effect of fenvalerate on nucleic acids in liver was more pronounced with higher dose, but the pattern remained the same as observed with lower dose.

4.5.4 Effect of Fenvalerate on Alkaline and Acid Phosphatases

Fenvalerate influenced the specific activities of alkaline and acid phosphatases in rat liver (Figs. 4.24 and 4.25). It can be seen that at both the doses of pesticide (5 and 15 mg/Kg), the specific activity of alkaline phosphatase increased significantly. At lower dose, the alkaline phosphatase specific activity increased approximately 2.5-fold. The activity at high dose was comparable to low dose as the increase in dose level did not significantly enhance the effect, although the effect was obvious. In contrast, the specific activity of acid phosphatase decreased significantly with time and after 30 days exposure with low dose, the activity of the enzyme was reduced to half. At higher dose, once again, the results were comparable to that of lower dose.

4.5.5 Effect of Fenvalerate on Haematological Parameters

Fenvalerate toxicity influenced mostly all the blood and serum parameters in rat listed in Table 4.12. The results show that chronic exposure of rats to the pesticide resulted in several fold increase in the activity of serum enzymes i.e., serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic

Table 4.11: EFFECT OF FENVALERATE ON NUCLEIC ACID CONTENT OF RAT LIVER

Duration of Exposure (Days)	Dose (mg/Kg body wt.)	Nucleic Acid Content (mg g^{-1} liver)[a]	
		RNA	DNA
Control	0.00	1.157 ± 0.03	0.075 ± 0.004
7	5.00	1.110 ± 0.05	0.045 ± 0.001
15		1.023 ± 0.02	0.043 ± 0.002
30		0.874 ± 0.02	0.038 ± 0.002
7	15.00	1.088 ± 0.01	0.054 ± 0.001
15		0.963 ± 0.04	0.42 ± 0.004
30		0.724 ± 0.01	0.028 ± 0.006

[a]Each value is an average ± S.E. of 4 determinations from 4 animals of same age group.

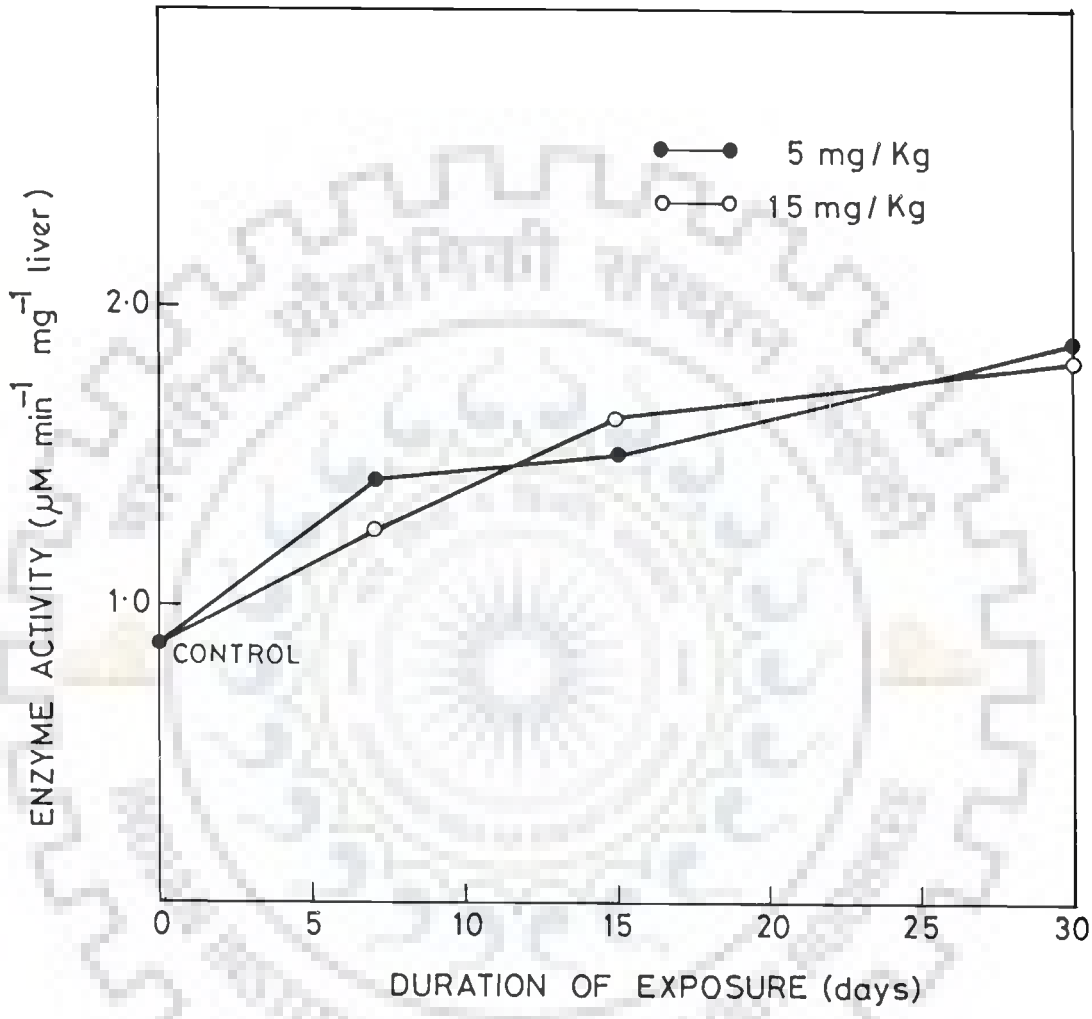


Fig. 4.24 Effect of Graded Doses of Fenvalerate on Alkaline Phosphatase Activity in Rat Liver.

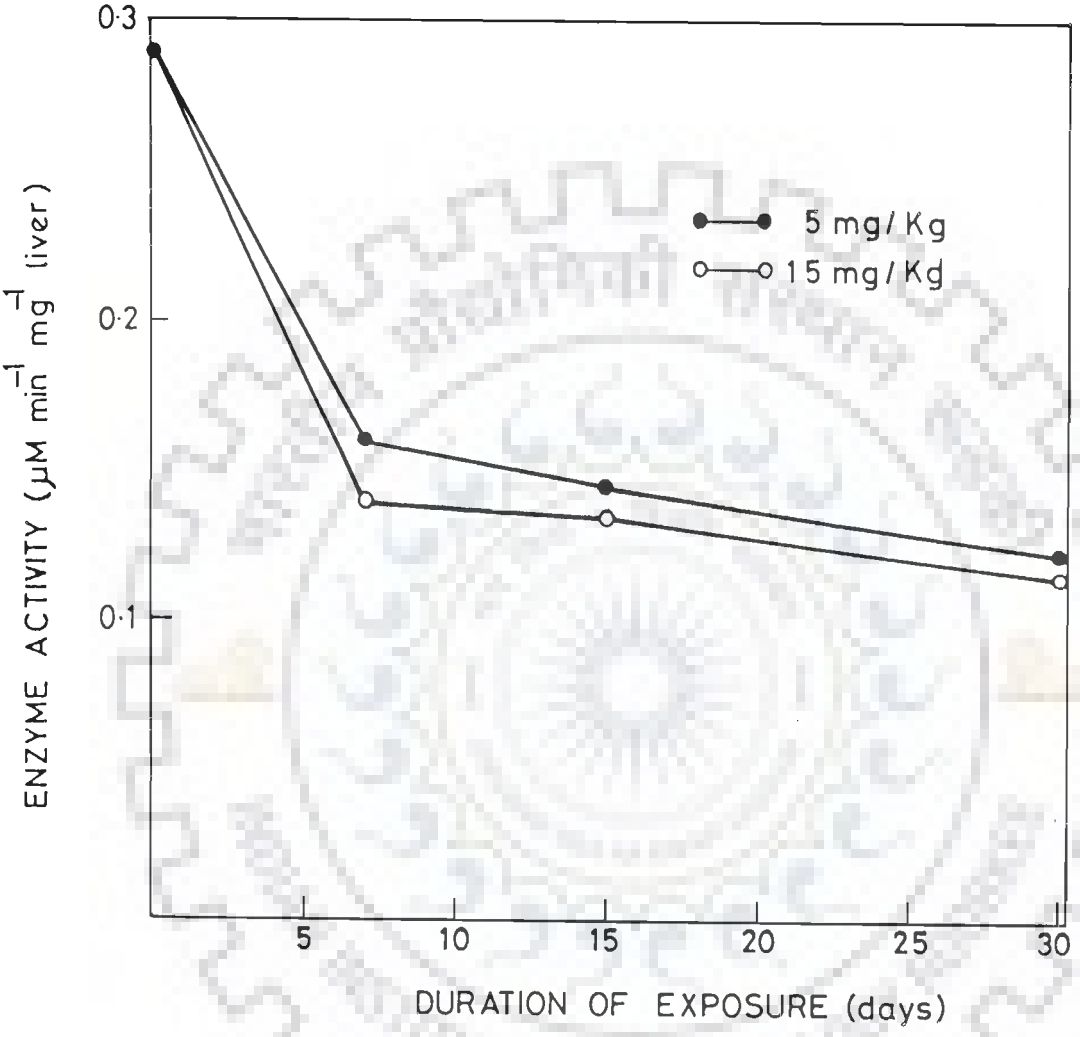


Fig. 4.25 Effect of Chronic Exposure of Graded Doses of Fenvalerate on Acid Phosphatase Activity in Rat Liver.

Table 4.12 : ALTERATIONS IN HAEMATOLOGICAL PARAMETERS IN RATS INDUCED BY CHRONIC EXPOSURE OF FENVALERATE

Duration of Exposure (days)	Dose (mg/kg)	Blood Parameters[a]						
		SGOT (U/ml)	SGPT (U/ml)	ALP (KA/ml)	AP (KA/ml)	Hb (g/dl)	WBC (10^3/cmm)	RBC (10^6/cmm)
Control		50 ± 1.0	20 ± 1.0	41 ± 0.6	22.0 ± 0.50	15.5 ± 1.10	4.0 ± 1.0	5.4 ± 0.81
7	15.0	105 ± 2.10	33 ± 1.30	78 ± 2.0	3.9 ± 0.40	9.6 ± 0.40	6.0 ± 0.66	4.0 ± 0.06
15		116 ± 1.60	41 ± 1.20	66 ± 1.8	4.0 ± 0.32	8.0 ± 0.32	6.4 ± 0.82	3.8 ± 0.2
30		145 ± 4.20	80 ± 1.88	58 ± 2.26	4.2 ± 0.20	7.5 ± 0.10	7.2 ± 0.6	3.2 ± 0.2

All the values are mean ± S.E. (3 estimations)
[a]Represents average of values for 10 rats of same age group
SGOT - Serum glutamic oxaloacetic transaminase; SGPT - Serum glutamic pyruvic transaminase;
ALP - Alkaline Phosphatase ; AP - Acid phosphatase ; Hb - Haemoglobin ;
WBC - White blood corpuscles ; RBC - Red blood corpuscles

transaminase(SGPT), alkaline phosphatase and acid phosphatase. Toxic stress caused by fenvalerate increased the SGOT and SGPT activities by approximately 3.0-fold and 4.0-fold respectively, after 30 days treatment. Unlike liver, the acid phosphatase level increase in serum was 1.5-fold, while alkaline phosphatase activity was elevated by about 2.0-fold after 7 days of treatment, but during the later period, a slight decrease in activity was observed. Simultaneously, the blood parameters i.e., haemoglobin (Hb), WBC and RBC were also analysed in fenvalerate treated rats which showed a slight change upto 30 days treatment. The Hb content showed a 50% decline and RBC counts also decreased upto 30 days exposure of fenvalerate to rats. Meanwhile, the WBC counts registered a gradual increase upto 30 days (10.2×10^3/cmm) as compared to control (7.0×10^3/cmm). The decrease in Hb and RBC contents due to fenvalerate exposure indicates the anaemic condition of rats.

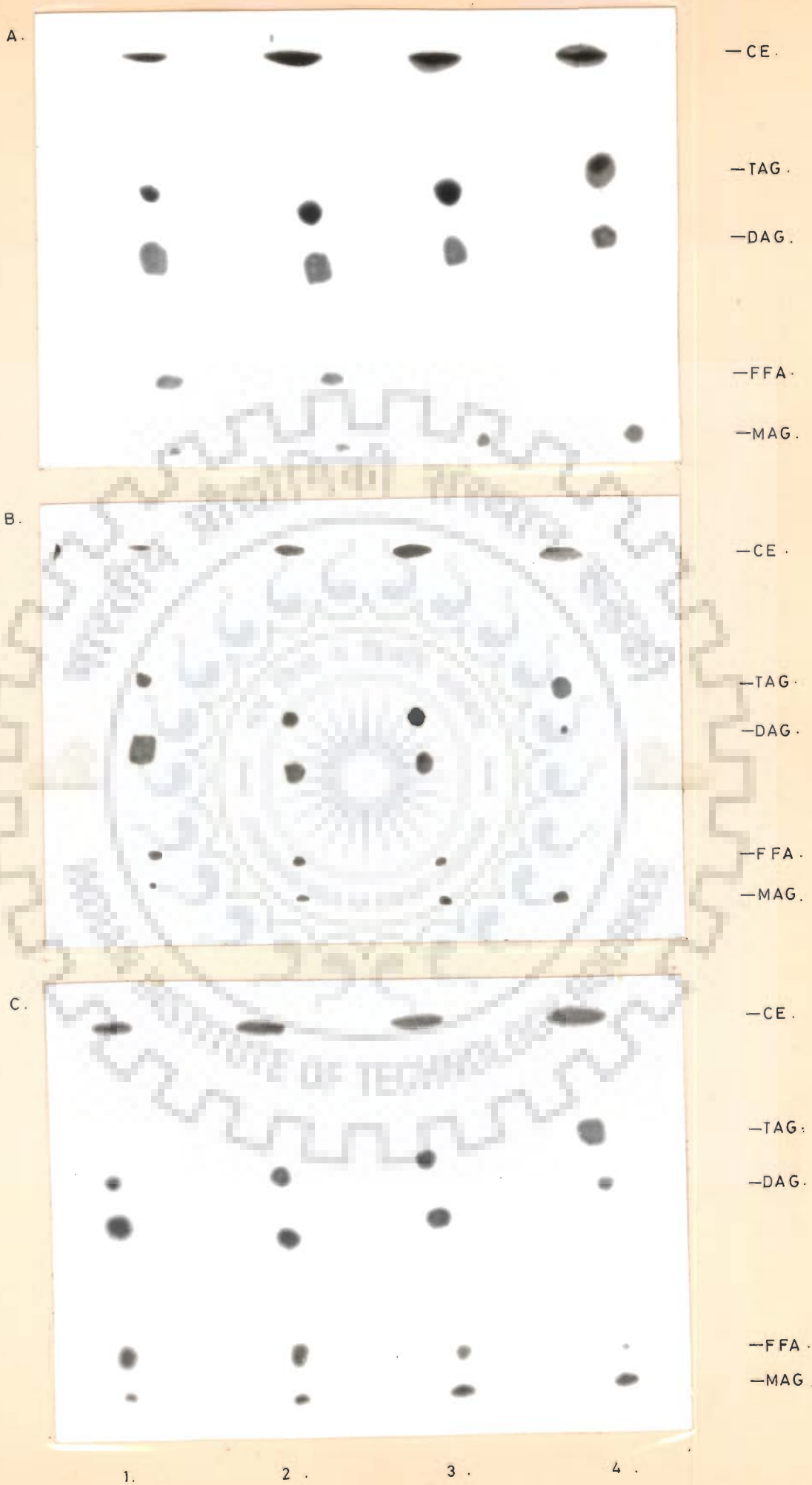
4.5.6 Effect of Fenvalerate on Neutral Lipids

The effect of fenvalerate on neutral lipids in liver, kidney and brain was analysed by thin layer chromatography (TLC). The results are shown in Fig. 4.26 a, b and c. A qualitative analysis of the data shows that fenvalerate significantly affects the composition of the neutral lipids in liver, kidney and brain. For instance, in liver, concentration of triacylglycerol (TAG) seem to increase with the duration of treatment with concomitant decrease in concentration of diacylglycerol (DAG) and free fatty acid (FFA). Meanwhile FFA was not detected after 15 and 30 days exposure to the pesticide. On the basis of these results it seems that the accumulation of TAG may be due to either enhanced synthesis of TAG or reduction in hydrolysis of triacylglycerols into lower glycerides. In other words, the activity of lipase seems to be affected. Interestingly however, there seems to be a gradual increase in monoacylglycerol (MAG) concentration. Similar trend was observed in case of kidney and brain.

Fig. 4.26 (a) Thin - Layer Chromatogram of Neutral Lipids of Liver from Rats of (1) Control, (2) 7 day (3) 15 day, (4) 30 day Exposure of Fenvalerate (15 mg/Kg). Spots of Cholesterol Ester (CE) ; Triacylglycerol (TAG) ; Diacylglycerol (DAG) ; Free Fatty Acids (FFA) and, Monoacylglycerol (MAG) are Seen.

Fig. 4.26(b) Thin - layer Chromatogram of Neutral lipids of Kidney from Rats of (1) Control, (2) 7 day, (3) 15 day, (4) 30 day Exposure of Fenvalerate (15 mg/Kg). Spots of Cholesterol Ester (CE) ; Triacylglycerol (TAG); Diacylglycerol (DAG) ; Free Fatty Acids (FFA) and Monoacylglycerol (MAG) are seen.

Fig. 4.26(c) Thin-layer Chromatogram of Neutral Lipids of Brain from Rats of (1) Control, (2) 7 day, (3) 15 day, (4) 30 day Exposure of Fenvalerate (15 mg/Kg). Spots of cholesterol Ester (CE); Triacylglycerol (TAG); Diacylglycerol (DAG); Free Fatty Acids (FFA); and Monoacylglycerol (MAG) are Seen.


A.
—CE.
—TAG.
—DAG.
—FFA.
—MAG.
B.
—CE.
—TAG.
—DAG.
—FFA.
—MAG.
C.
—CE.
—TAG.
—DAG.
—FFA.
—MAG.
1.
2.
3.
4.

The effect of fenvalerate on total triglycerides in rat tissues i.e., liver, kidney and brain was quantitatively determined by Gas Liquid Chromatography (GLC). Results are summarised in Fig. 4.27. Experiments were carried out at two different doses of pesticide i.e., 5 and 15 mg/Kg body weight. From these results it can be seen that in liver the triacylglycerol concentration increased from about 10 to 50 mg/g liver. Thus there was a five-fold increase in triglycerides when 15 mg/Kg fenvalerate dose was used in treatment over a period of 30 days. Nearly identical pattern with regard to the accumulation of triglycerides in kidney and brain was also observed. It can be seen that the effect of fenvalerate was dose-dependant and became more dominant with increasing period of exposure.

4.5.7 Effect of Fenvalerate on Phospholipids in Rat Organs

The effect of fenvalerate, on component phospholipids of rat liver, kidney and brain was analysed by HPLC. The results (Table 4.13) show relative concentration (mol %) of the component phospholipids in liver. There was a significant increase in the relative concentration of phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE), the major phospholipids present in the rat liver as compared to control. The increase in PC was time dependant. Slight increase in the mol % of sphingomyelin (SM) in liver was also observed. In contrast, however the minor phospholipids, namely, phosphatidyl inositol (PI), phosphatidyl serine (PS) and phosphatidyl glycerol (PG) continuously decreased with increasing exposure period (Fig. 4.28).

Nearly identical pattern of mol % increase in PC and PE was observed in rat kidney (Table 4.14 and Fig. 4.29). The PI content remained nearly the same upto 30 days when compared with control. The relative concentrations of SM, PS and PG were reduced considerably. Again PG was not detected after 30 days exposure

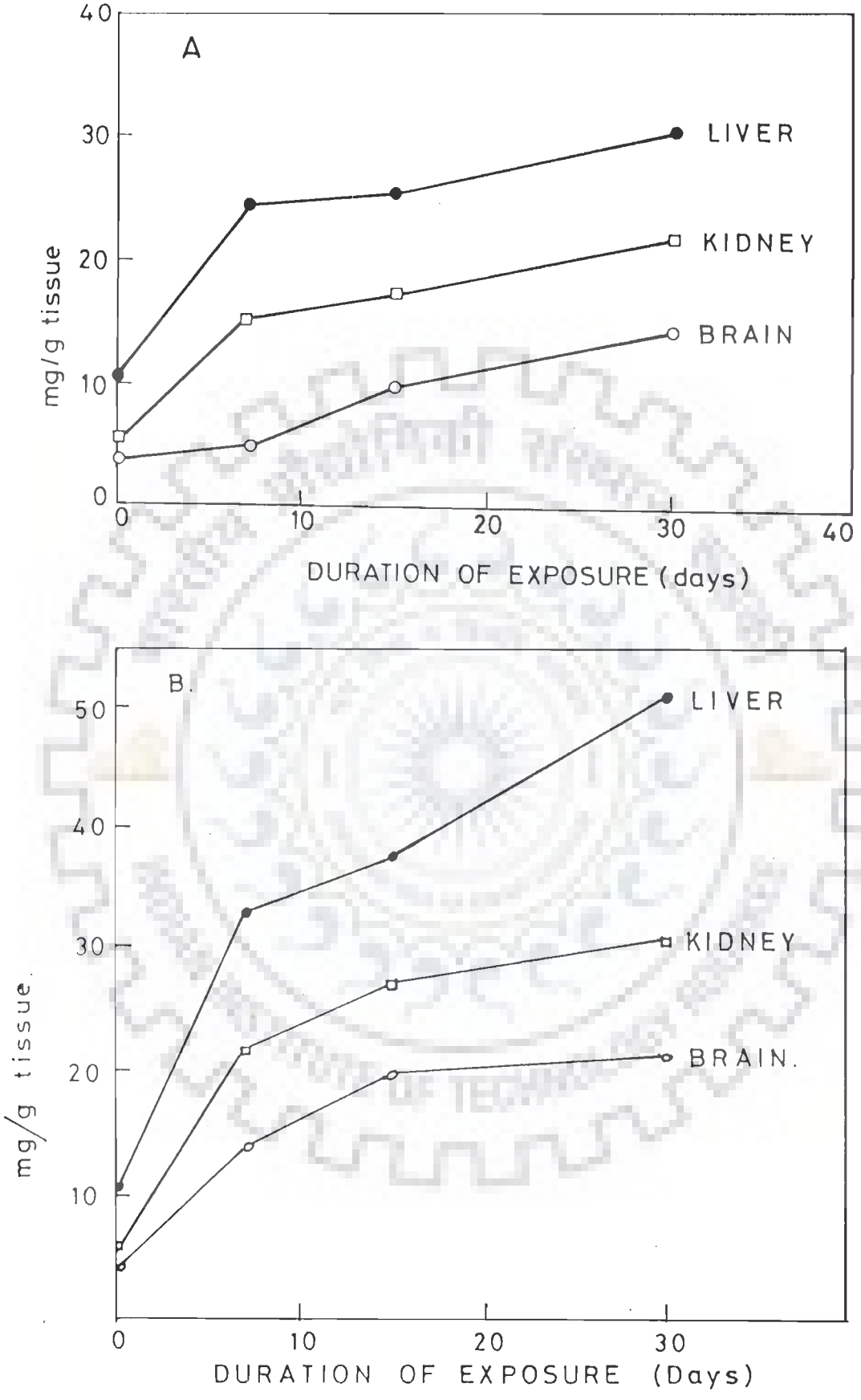


Fig. 4.27 Effect of Fenvalerate at A. 5 mg/Kg and B. 15 mg/Kg Doses on Total Triglyceride Content in Rat Liver, Kidney, Brain.

Table 4.13 : EFFECT OF FENVALERATE ON PHOSPHOLIPID COMPOSITION IN RAT LIVER

Duration of Exposure[1] (Days)	Relative Concentration of Phospholipid Components (mol %)					
	PI	PC	SM	PE	PS	PG
Control	7.83	51.95	3.41	27.94	4.40	4.45
7	4.98	54.46	3.88	29.67	4.23	2.78
15	4.94	56.54	4.59	30.49	2.96	0.48
30	2.67	59.00	4.89	32.56	0.88	N.D

[1]Rats were administered an oral dose of fenvalerate (15 mg/Kg body weight) on alternate days for a period of 7, 15 and 30 days

P1 - Phosphatidyl inositol ; PC - Phosphatidyl choline

SM - Sphingomyelin ; PE - Phosphatidyl ethanolamine

PS - Phosphatidyl serine ; PG - Phosphatidyl glycerol

N.D - Not detected.

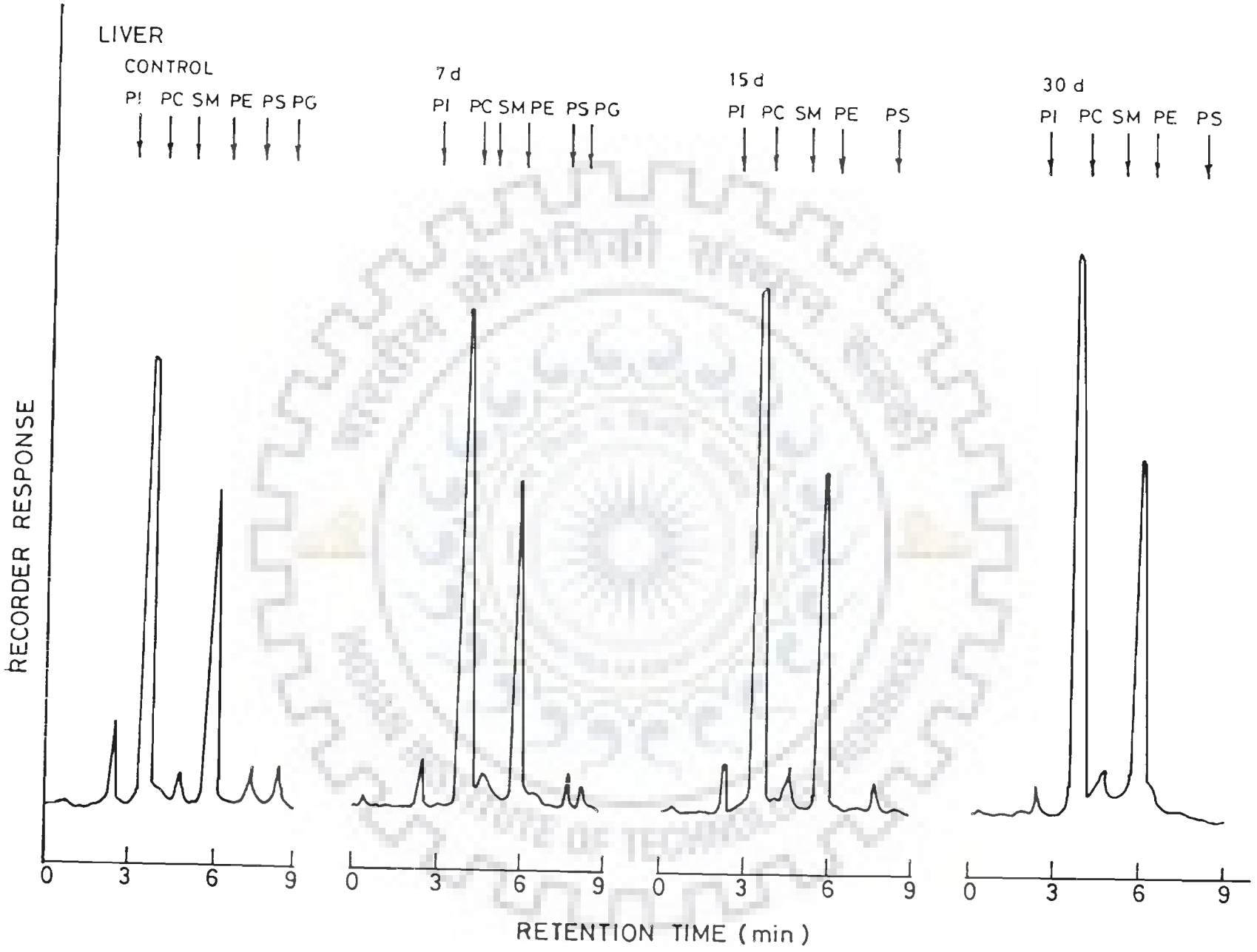


Fig. 4.28 HPLC Profiles Showing Phospholipid Components in Liver of Control and Fenvalerate Treated (15 mg/Kg) Rats.

Table 4.14 : EFFECT OF FENVALERATE ON PHOSPHOLIPID COMPOSITION IN RAT KIDNEY

Duration of Exposure[1] (Days)	Relative Concentration of Phospholipid Components (mol %)					
	PI	PC	SM	PE	PS	PG
Control	5.89	39.61	9.72	34.32	7.68	2.78
7	6.01	43.73	8.60	36.36	4.30	1.00
15	6.52	45.89	6.44	36.59	3.44	1.12
30	6.46	48.06	5.96	37.52	2.00	N.D

[1]Rats were administered an oral dose of fenvalerate (15 mg/Kg body weight) on alternate days for a period of 7, 15 and 30 days

PI - Phosphatidyl inositol ; PC - Phosphatidyl choline

SM - Sphingomyelin ; PE - Phosphatidyl ethanolamine

PS - Phosphatidyl serine ; PG - Phosphatidyl glycerol

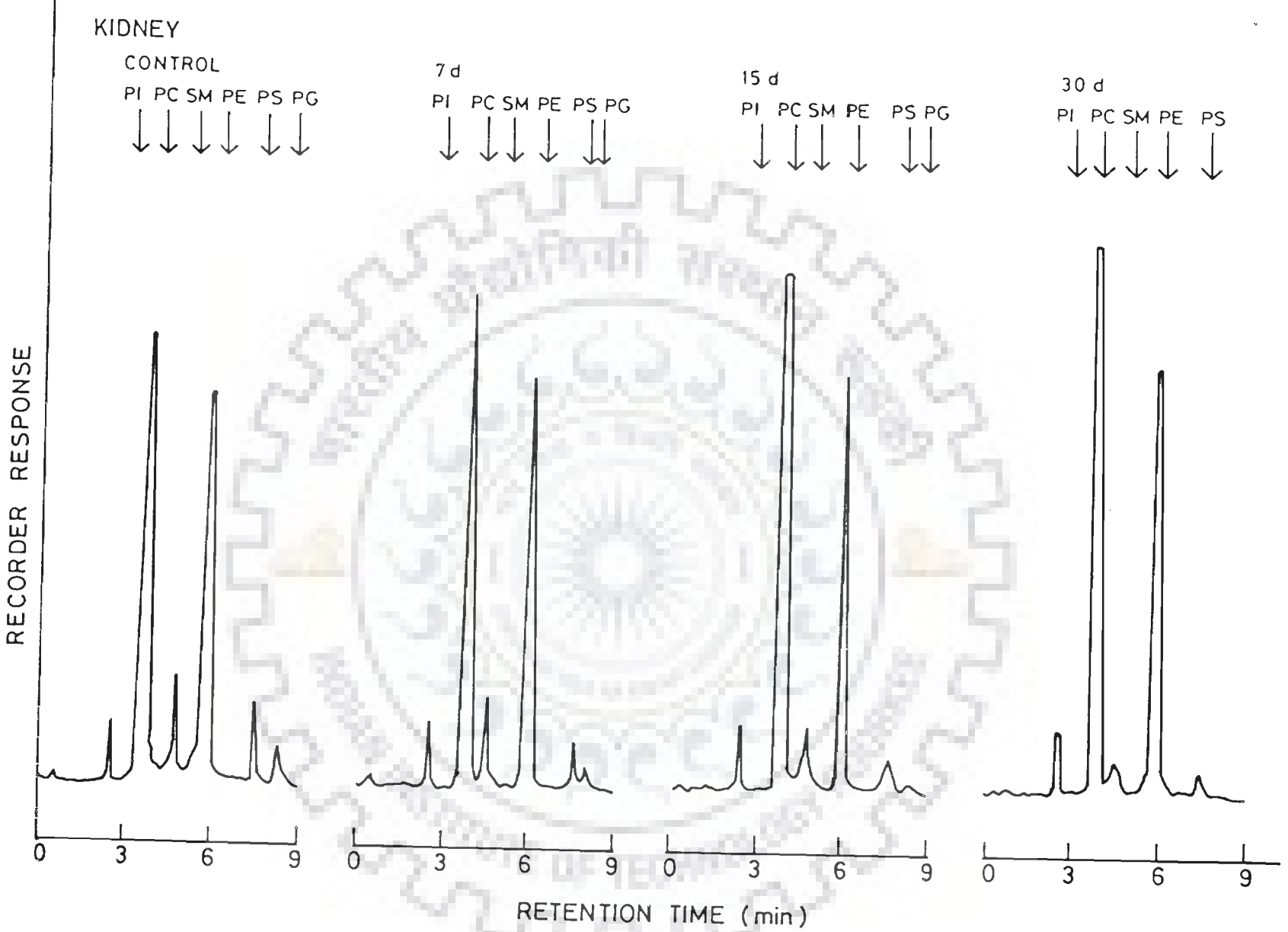


Fig. 4.29 HPLC Profiles Showing Phospholipid Components in Kidney of Control and Fenvalerate Treated (15 mg/Kg) Rats.

to the pesticide. In rat brain also , the effect of fenvalerate was comparable to that of liver (Table. 4.15) As expected, the PC content increased upto a period of 30 days on administering a dose of 15 mg/Kg fenvalerate. Similarly PE also showed a slight increase, whereas the concentration of PI increased upto 15 days after which there was a decrease compared to control. The relative concentrations of PS and PG (mol %) were reduced to the extent that the PG was not detected after 30 days pesticide exposure as in other tissues (Fig. 4.30).

Table 4.15 : EFFECT OF FENVALERATE ON PHOSPHOLIPID COMPOSITION IN RAT BRAIN

Duration of Exposure[1] (Days)	Relative Concentration of Phospholipid Components (mol %)					
	PI	PC	SM	PE	PS	PG
Control	3.65	36.86	6.87	39.27	10.58	2.77
7	3.76	42.86	6.55	38.09	7.33	1.41
15	3.82	44.65	5.13	38.26	7.02	1.12
30	2.79	48.55	4.98	40.62	3.06	N.D

[1]Rats were administered an oral dose of fenvalerate (15 mg/Kg body weight) on alternate days for a period of 7, 15 and 30 days

PI - Phosphatidyl inositol ; PC - Phosphatidyl choline

SM - Sphingomyelin ; PE - Phosphatidyl ethanolamine

PS - Phosphatidyl serine ; PG - Phosphatidyl glycerol

From these results it seems that fenvalerate changes the composition of the phospholipids in liver, kidney and brain. Since phospholipids are important components of cell

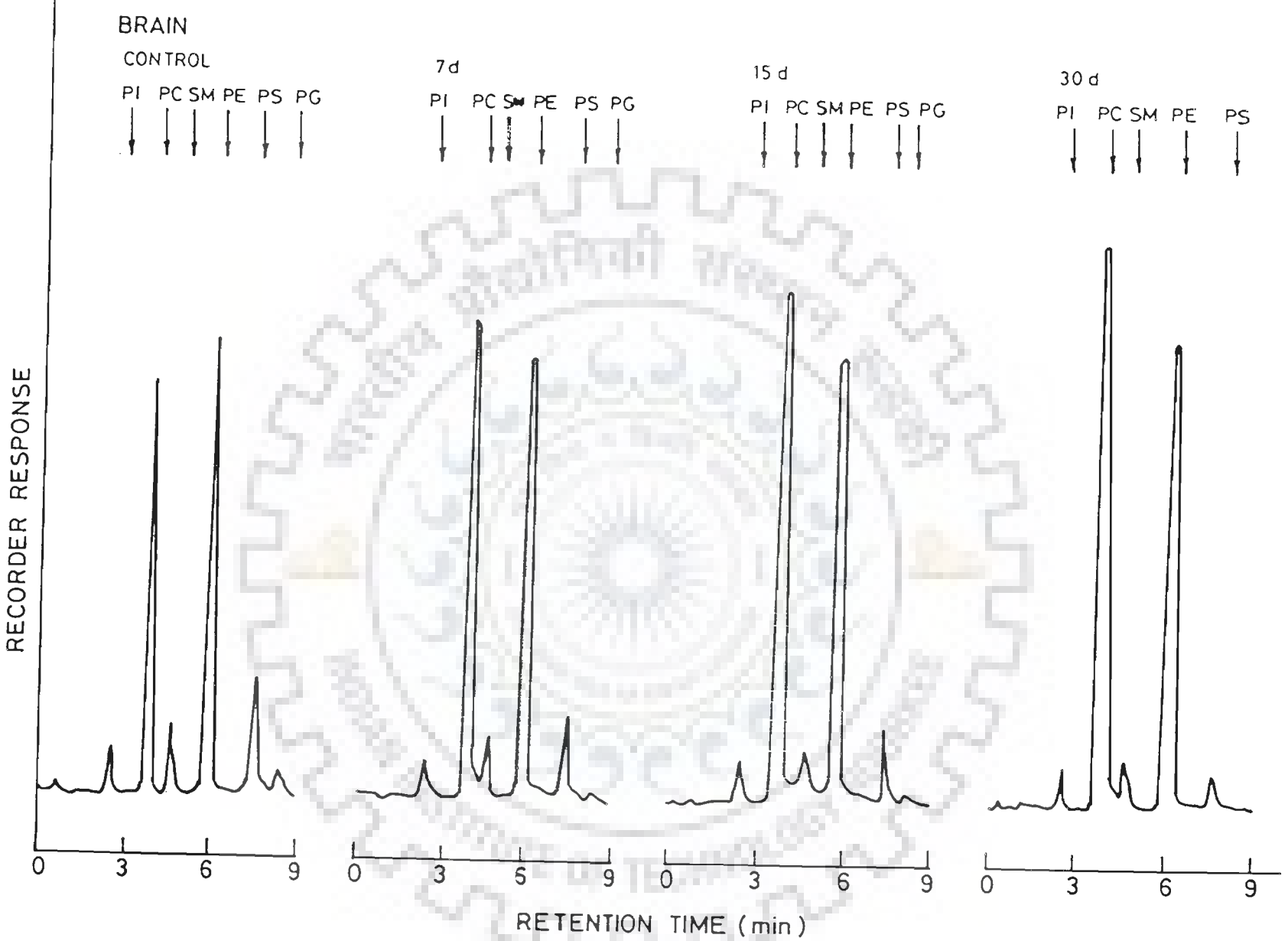


Fig. 4.30 HPLC Profiles Showing Phospholipid Components in Brain of Control and Fenvalerate Treated (15 mg/Kg) Rats.

membrane and membranes of the organelles, such as nuclei, mitochondria, endoplasmic reticulum, golgi, etc., the change in the phospholipid composition indicates change in membrane structure which may in turn affect the permeability of the membrane.

4.6 EFFECT OF FENVALERATE ON SUBCELLULAR MEMBRANES

As pointed out in the preceeding section, fenvalerate treatment appeared to damage the membrane structure of rat liver. In order to understand more about the damage caused by the chronic treatment of fenvalerate to the membranes, various subcellular fractions were prepared and biochemical parameters namely proteins, phospholipids and carbohydrates were determined.

4.6.1 Effect of Fenvalerate on Carbohydrate Content in various Subcellular Fractions of Rat Liver

The changes in the carbohydrate content of the nuclear membrane, Plasma membrane ,outer mitochondrial and inner mitochondrial membranes are shown in Fig. 4.31. It can be seen that in all cases, the carbohydrate content of the membrane were significantly increased as a result of fenvalerate treatment. For instance, the increase in carbohydrate content in nuclear membrane ,plasma membrane, outer mitochondrial and inner mitochondrial membrane fractions were about 1.5-, 2.5, 2.0 - and 1.5-fold respectively. Increase in the carbohydrate content shows enhancement of glycosylation of membrane glycoproteins and glycolipids.

4.6.2 Effect of Fenvalerate on Total Phospholipids in Subcellular Fractions of Rat Liver

The effect of fenvalerate on total phospholipids in nuclear, mitochondrial

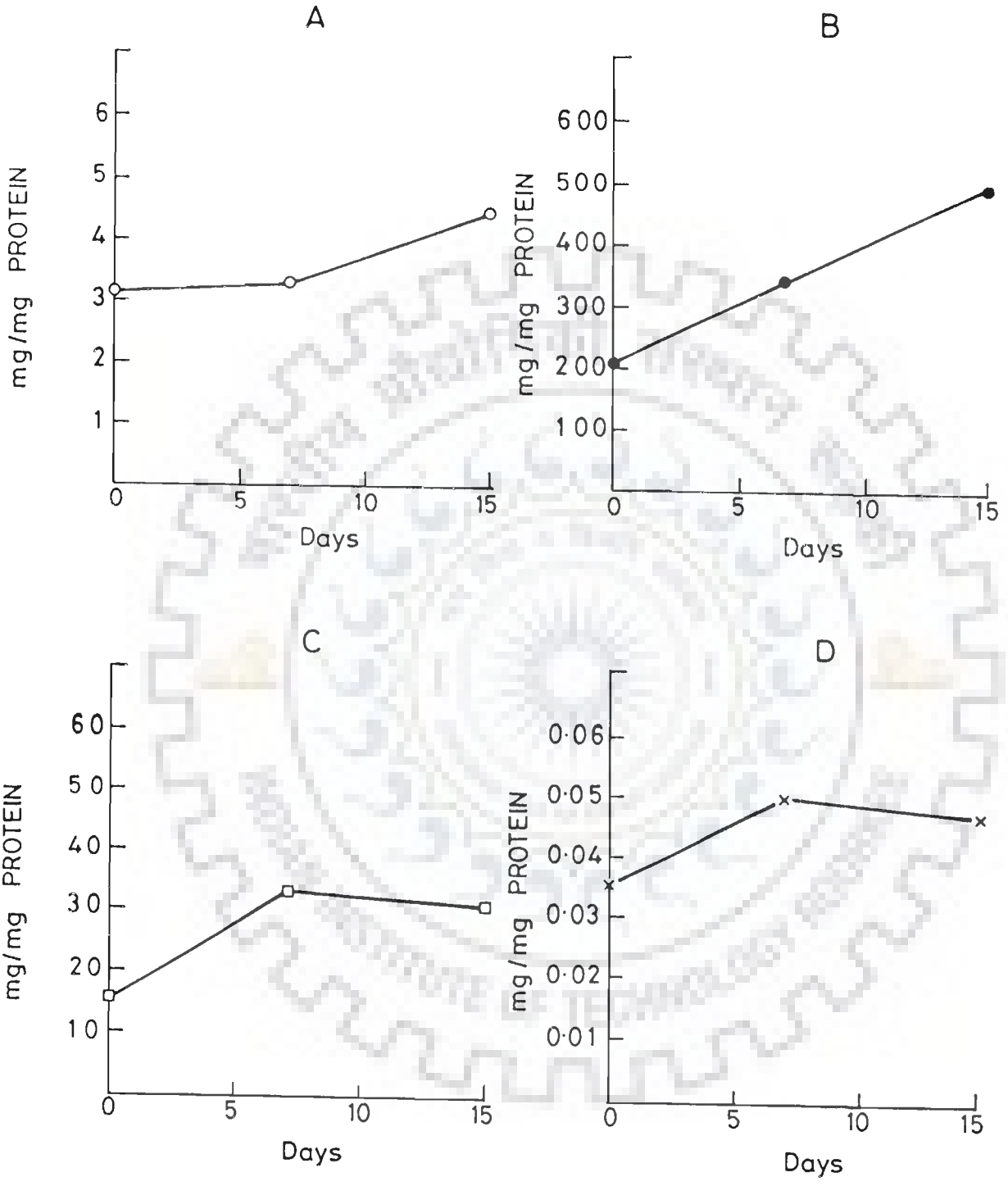


Fig. 4.31 Total Carbohydrate Content in Various Subcellular Fractions from Liver of Control and Fenvalerate Treated (15 mg/Kg) Rats. A. Nuclear Membrane; B. Plasma Membrane; C. Outer Mitochondrial Membrane and D. Inner Mitochondrial Membrane.

and plasma membrane fractions is shown in Fig.4.32. As expected, the concentration of total phospholipids markedly increased in the pesticide treated rat liver membrane fractions, the maximum increase being in the plasma membrane fraction. Once again in line with the earlier observations, these results show the ultrastructural changes in the subcellular membranes of the liver tissues.

4.6.3 Effect of Fenvalerate on Total Proteins of Subcellular Membrane Fractions from Rat Liver

Fig. 4.33 shows the effect of fenvalerate treatment on total protein content of nuclear, mitochondrial and plasma membrane subcellular fractions. The results are represented in terms of mg g^{-1} liver. It was found that protein content of all the subcellular fractions tested were significantly reduced by fenvalerate treatment. The maximum effect was on plasma membrane. Since plasma membrane (PM) proteins are synthesised in rough endoplasmic reticulum (RER) and then translocated to the plasma membrane, these results were interpreted to mean , but by no means prove, that fenvalerate impairs the translocation of proteins from ER to PM. Mitochondria can synthesise its own proteins and also proteins sythesised in endoplasmic reticulum are translocated into it. The decrease in protein content may also be interpreted as either translocation of protein to mitochondria or protein synthesis into mitochondria is affected by the pesticide treatment.

4.6.4 Analysis of Rat Liver Membrane Proteins by SDS-PAGE

To further investigate the effect of fenvalerate, the subcellular membrane proteins were analysed by SDS-PAGE. The apparent molecular weights of the individual protein bands were determined refering to the standard molecular weight proteins

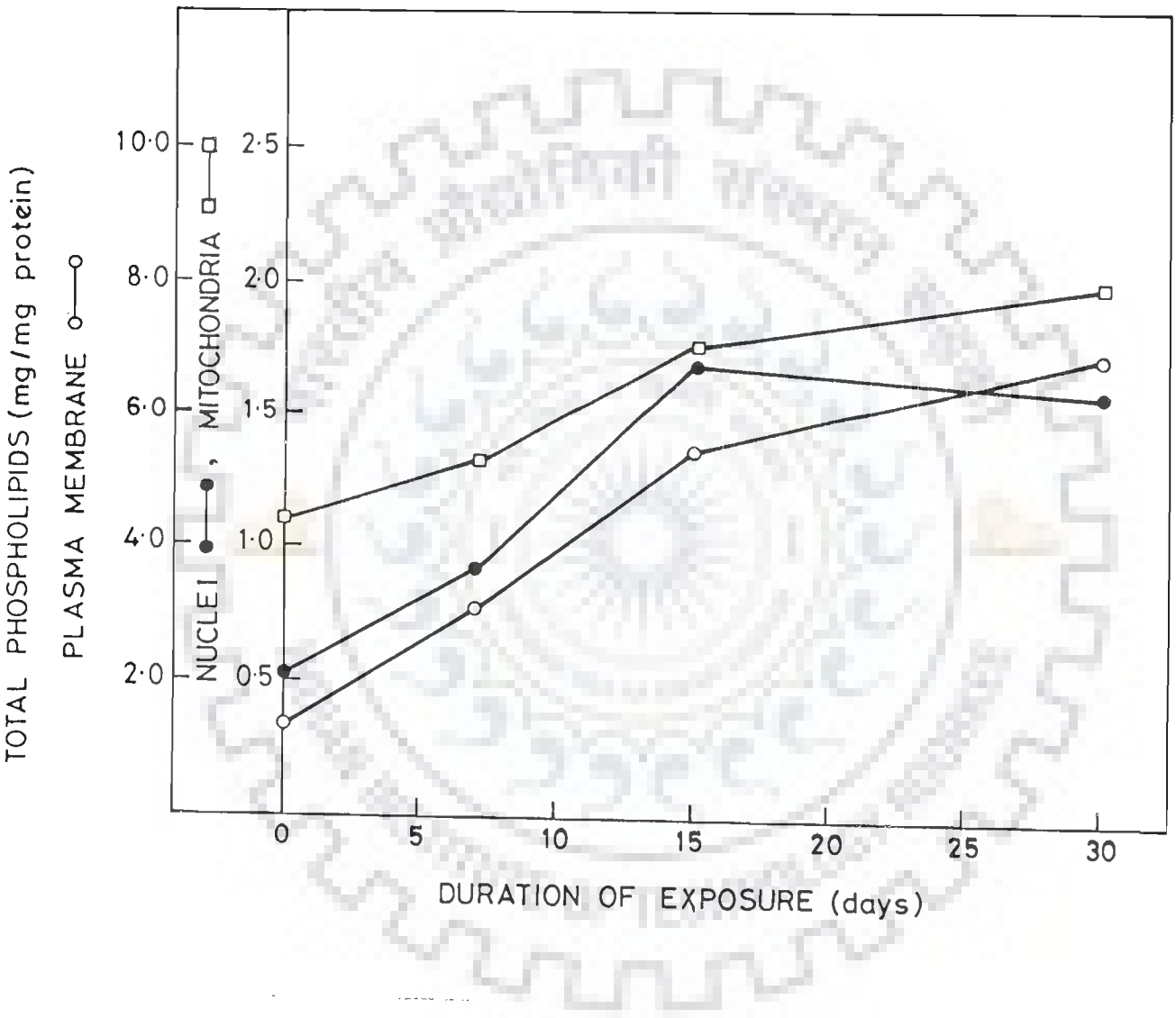


Fig. 4.32 Total Phospholipid Content in Various Subcellular Fractions from Liver of Control and Fenvalerate Exposed (15 mg/Kg) Rats.

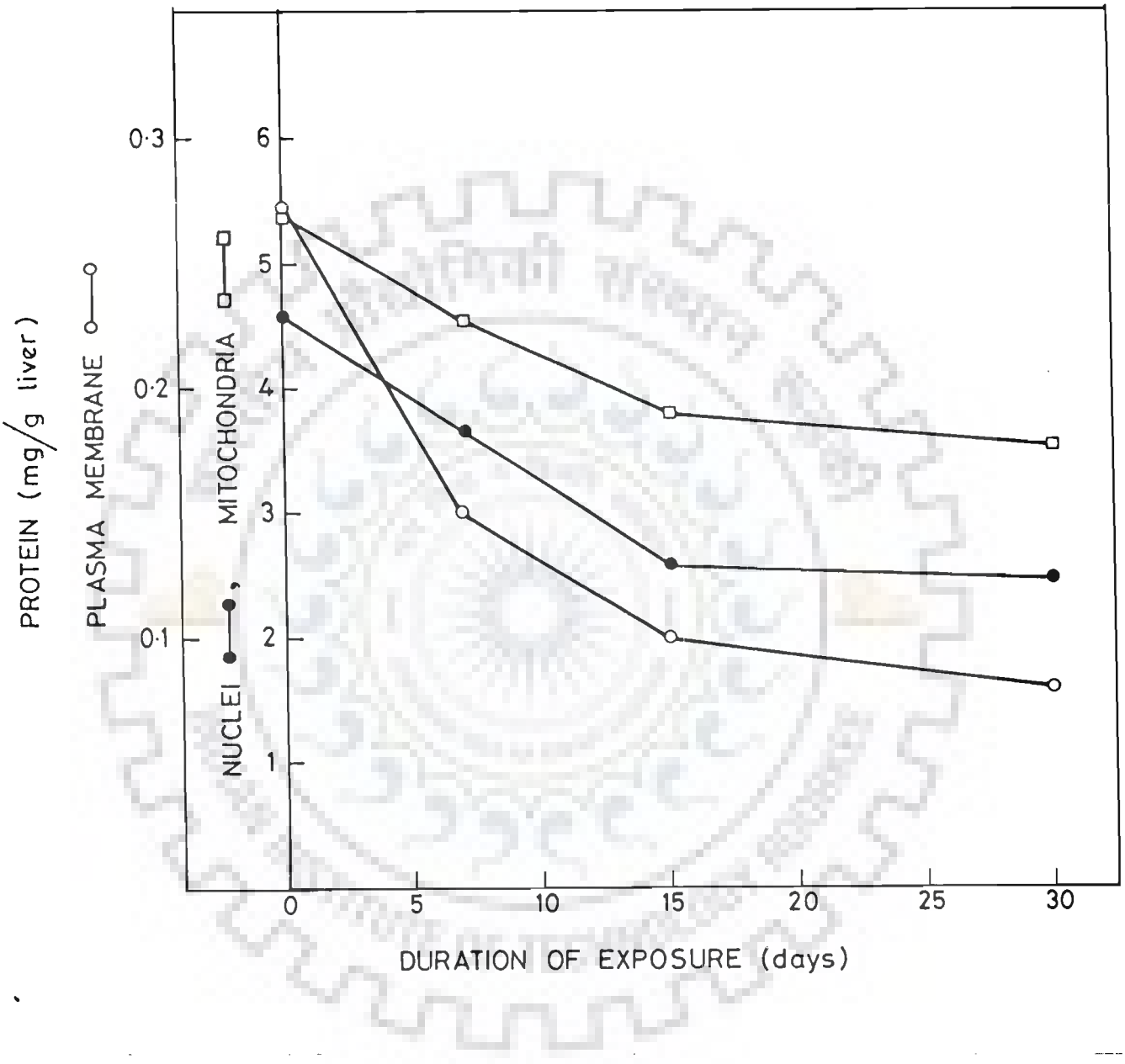


Fig. 4.33 Total Protein Content in Various Subcellular Fractions from Liver of Control and Fenvalerate Treated (15 mg/Kg) Rats.

prepared and run simultaneously along with known molecular weight proteins. The SDS-PAGE pattern of the proteins in the nuclear membranes from treated and untreated rat liver is shown in Table 4.16 and Fig.4.34. It was observed that in addition to the general effect on a large number of protein bands in nuclear membranes the 225, 129, 127, 82, 72, 46, 44 and 15 kDa proteins were markedly decreased in the nuclei. In addition, the 79 kDa band was completely missing in the nuclear membrane from the pesticide treated rat livers, while the intensity of 220, 32, 24 and 17 kDa proteins was enhanced. Thus there seems to a selective effect in nuclear membranes. To what extent these changes in protein affect the function of nuclei is not known. It may also be pointed out here that the electron microscopy (TEM) data also showed ultrastructural damage in the nucleus.

Table 4.17 and Fig. 4.35 show protein profiles of the outer mitochondrial membrane proteins of the control and pesticide treated rat liver. It can be seen that there is a significant decrease in the intensity of protein bands of 150, 82, 55, 48, 46, 41, 38 and 27 kDa, in pesticide treated rats with subsequent increase in 162 kDa and 18 kDa proteins. Furthermore, the pesticide treatment resulted in complete absence of 210, 180, 35 and 17 kDa proteins. This protein pattern of outer mitochondrial membranes is markedly altered by the fenvalerate treatment and there is some kind of protein specific inhibition as well as stimulation. It would be interesting to study in greater detail the implication of these changes in protein components in outer mitochondrial membranes.

The changes in plasma membrane SDS-PAGE profile is shown in Fig. 4.36 and the relative intensity of each protein band as compared to control is listed in Table 4.18. The maximum decrease in intensity of protein bands in plasma membrane due to fenvalerate treatment was observed in 42, and 17 kDa proteins. The 158 kDa and

Table 4.16 : INTENSITY OF NUCLEAR MEMBRANE PROTEIN BANDS IN CONTROL AND FENVALERATE EXPOSED RAT LIVER

Relative mobility[a]	MW (kDa)	Band Intensity[b]	
		Control	Treated
0.03	243	L	L
0.04	225	M	L
0.06	220	Trace	M
0.11	172	M	M
0.13	158	VL	VL
0.16	130	M	M
0.17	129	M	L
0.18	127	M	VL
0.20	119	L	L
0.23	102	VL	VL
0.24	97	VL	VL
0.28	82	S	M
0.29	79	M	Absent
0.31	72	M	VL
0.33	65	M	M
0.36	58	L	VL
0.38	53	L	L
0.41	46	VS	M
0.42	44	VS	S
0.49	32	M	VS
0.50	31	L	L
0.54	26	VL	VL
0.56	24	L	M
0.63	17	M	S
0.66	16	M	M
0.67	15	S	M

VL - Very Light; L - Light; M - Medium; S - Strong; VS - Very Strong

[a]Rm represent migration of proteins relative to the tracking dye BPB.

[b]Equal quantity of proteins (100µg) was loaded on SDS - PAGE.

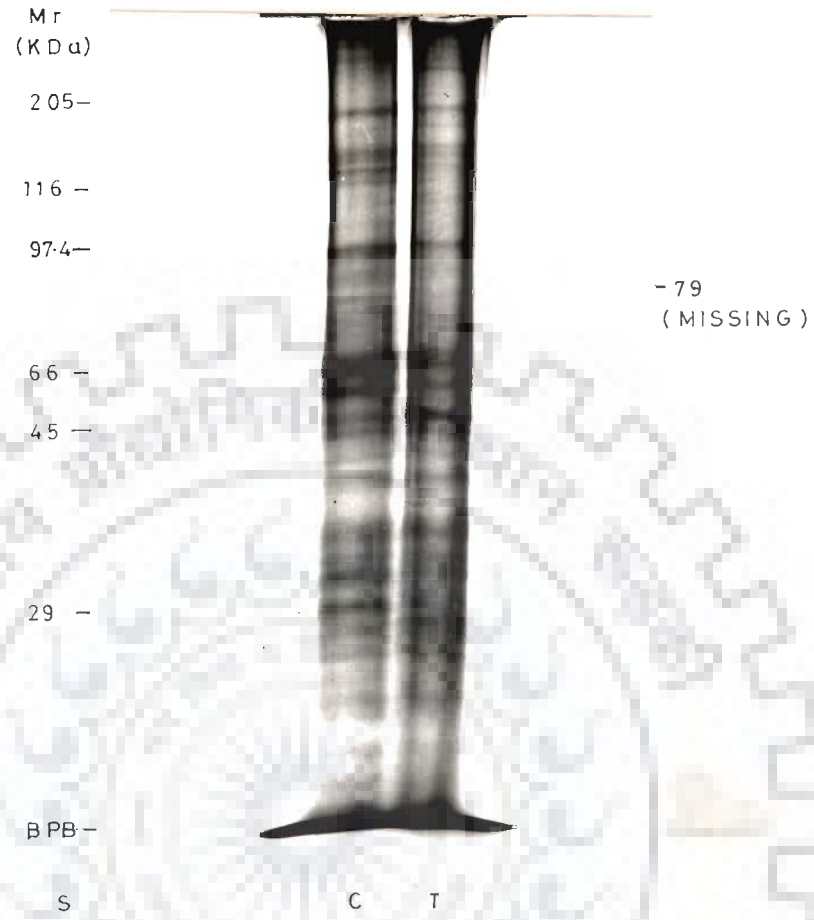


Fig. 4.34 SDS-PAGE Patterns of Hepatic Nuclear Membranes from Control (C) and Fenvalerate - Treated (T) Rats. Standard Protein Mixture (S) Contained Myosin (205 kDa) β - Galactosidase (116 kDa), Phosphorylase B (97.4 kDa), Bovine Albumin (66.2 kDa), Ovalbumin (45 kDa) and Carbonic Anhydrase (29 kDa).

Table 4.17 : INTENSITY OF OUTER MITOCHONDRIAL MEMBRANE PROTEIN BANDS IN CONTROL AND FENVALERATE EXPOSED RAT LIVER

Relative mobility[a]	MW (kDa)	Band Intensity[b]	
		Control	Treated
0.07	210	VL	Absent
0.10	180	L	Absent
0.12	162	M	VS
0.14	150	VS	L
0.16	139	VL	VL
0.19	120	L	L
0.22	104	L	L
0.25	93	L	L
0.27	85	L	VL
0.28	82	S	M
0.30	75	M	M
0.35	60	M	M
0.37	55	M	L
0.38	53	L	L
0.39	50	L	L
0.40	48	M	L
0.41	46	M	L
0.44	41	VS	VL
0.45	38	S	L
0.47	35	S	Absent
0.50	31	VS	M
0.51	30	S	S
0.53	27	S	L
0.56	24	S	L
0.62	18	M	S
0.64	17	S	Absent
0.67	15	S	S

VL - Very Light; L - Light; M - Medium; S - Strong; Vs - Very Strong
[a]Rm represents migration of proteins relative to the tracking dye BPB.
[b]Equal quantity of membrane protein (80 µg) was subjected to SDS-PAGE.

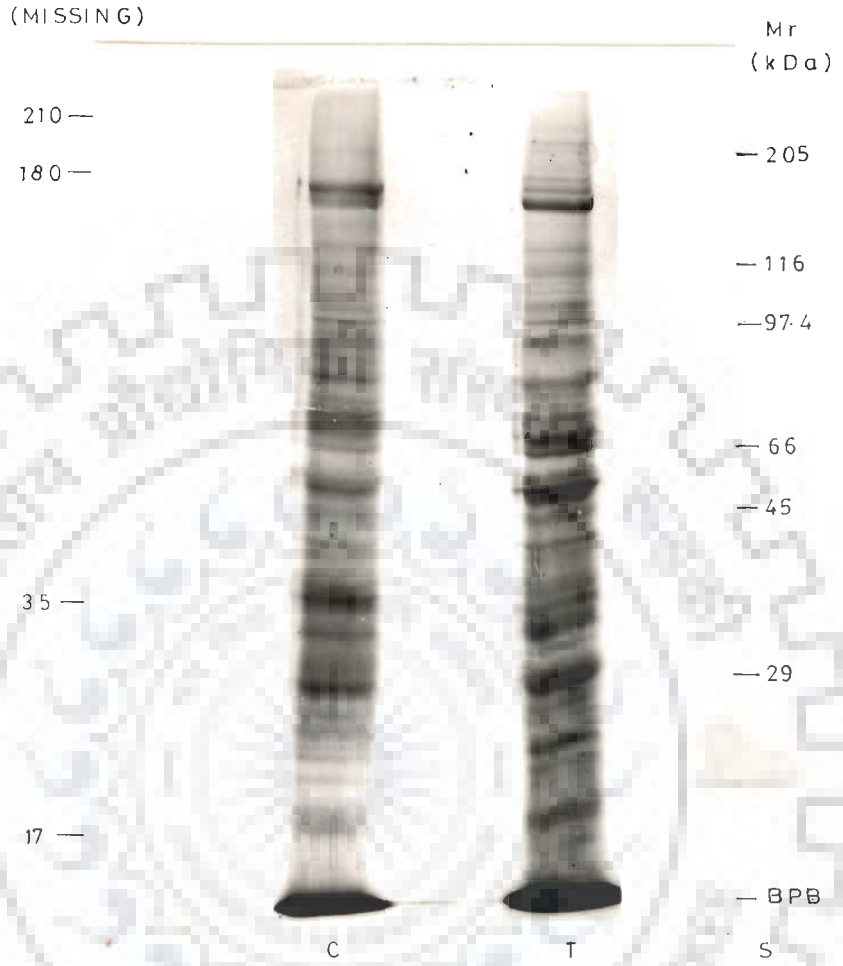


Fig. 4.35 SDS - PAGE (Silver Staining) Patterns of Hepatic Outer Mitochondrial Membrane from Control (C) and Fenvalerate - Treated (T) Rats. Standard Protein Mixture (S) contained Myosin (205 kDa), β - Galactosidase (116 kDa), Phosphorylase B (97.4 kDa), Bovine Albumin (66.2 kDa), Ovalbumin (45 kDa) and Carbonic Anhydrase (29 kDa).

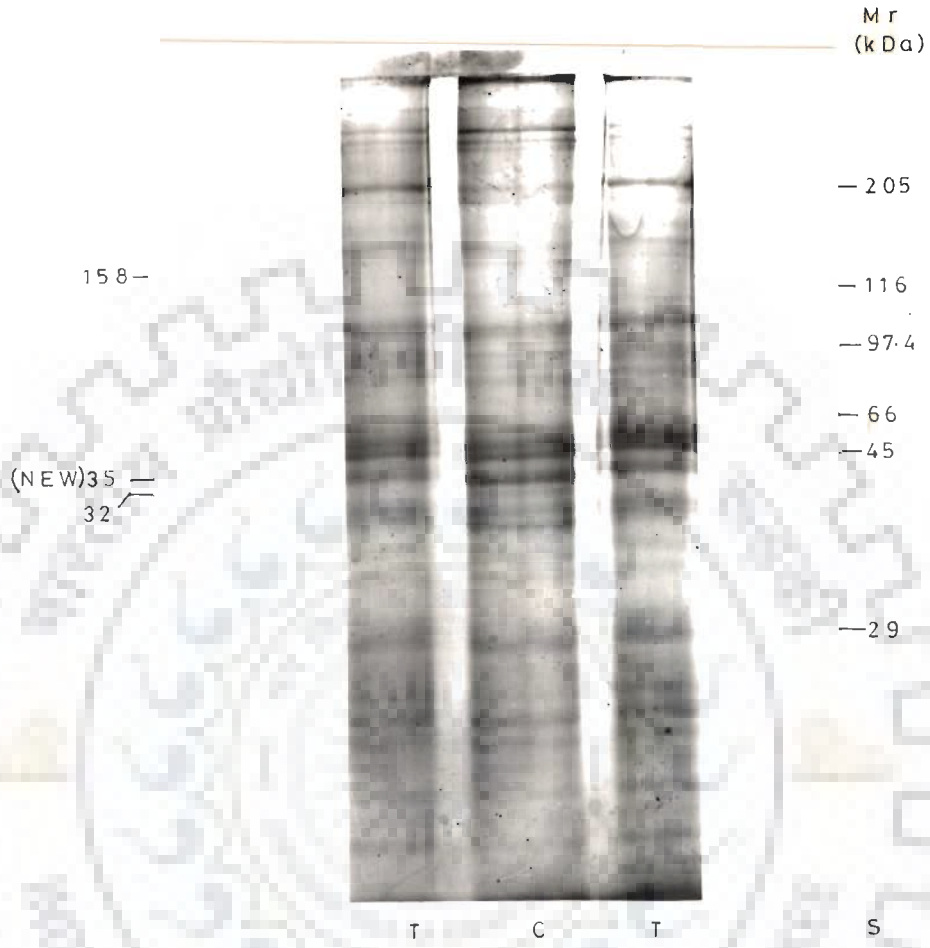


Fig. 4.36 SDS - PAGE Patterns of Hepatic Plasma Membranes from Control (C) and Fenvalerate - treated (T) Rats. Standard protein Mixture (S) Contained Myosin (205 kDa) β - Galactosidase (116 kDa), Phosphorylase B (97.4 kDa), Bovine Albumin (66.2 kDa), Ovalbumin (45 kDa) and Carbonic Anhydrase (29 kDa).

Table 4.18 : INTENSITY OF PLASMA MEMBRANE PROTEIN BANDS IN CONTROL AND FENVALERATE EXPOSED RAT LIVER

Relative mobility[a]	MW (kDa)	Band Intensity[b]	
		Control	Treated
0.05	228	M	M
0.06	220	M	M
0.08	200	L	Trace
1.10	180	L	M
0.13	158	L	Absent
0.18	127	L	L
0.20	119	VL	VL
0.23	102	M	M
0.26	90	M	S
0.30	75	L	M
0.31	72	S	S
0.32	69	L	L
0.34	63	VL	VL
0.39	50	M	S
0.40	48	S	S
0.43	42	S	M
0.44	41	S	VS
0.48	35	Absent	S
0.49	32	S	Absent
0.51	30	L	L
0.53	27	M	M
0.55	25	M	M
0.57	23	VL	VL
0.64	17	M	L
0.68	14	M	M
0.71	13	L	L

VL - Very Light; L - Light; M - Medium; S - Strong; VS - Very Strong

[a] Rm represents migration of proteins relative to the tracking dye BPB

[b] Equal quantity of membrane proteins (80 µg) was subjected to SDS - PAGE.

32 kDa protein bands were completely missing from the plasma membrane of pesticide treated rats. The intensity of these two bands in the control i.e., plasma membrane of untreated rats indicated that they are present as major proteins in PM. In addition, a number of protein bands (180, 90 and 75 kDa) showed an increase in intensity. The characterstic proteins of all plasma membranes i.e., 48 kDa which is considered a structural protein and comprise 3-10% in liver and kidney was not affected as indicated by the presence of strong intensity proteins bands in both control and fenvalerate treated rat liver membranes.

The most interesting effect seems to be the appearance of a new protein i.e., 35 kDa in plasma membrane. This seems to be a kind of " stress protein" formed as a result of fenvalerate treatment. Further studies may provide more insight into the functions of these proteins.

Thus, in general, the chronic treatment of fenvalerate for 30 days caused a massive damage in the protein constituents in the rat liver membranes which is a definite indication of the damage of native structure of membranes and its function. Increase in some selective protein concentrations and synthesis of new proteins was induced under fenvalerate toxicity.

Chapter 5

DISCUSSION

5.1 *IN VIVO* METABOLISM OF FENVALERATE

The present study describes the results of a synthetic pyrethroid, fenvalerate with reference to its biodegradation, bioaccumulation and distribution, as well as the biochemical and ultrastructural effects of fenvalerate both at cellular and subcellular levels in rat organs.

The overall metabolic profile of fenvalerate was found to be similar to other cyano pyrethroids such as cypermethrin (Cole et al., 1982; Crawford et al., 1981), decamethrin (Ruzo et al., 1978) and fluvalinate (Quistad et al., 1982) in rats. The metabolic pathway of fenvalerate has been studied earlier in rats (Ohkawa et al., 1979), which more or less, is in agreement with our results. In our study, the major reaction in the degradation pathway was the hydrolysis of the ester linkage of fenvalerate, with subsequent formation of two metabolites (Fig.4.16). These derivatives from the acid and alcohol moieties of fenvalerate undergo further metabolic reactions including oxidation and conjugation with glycine, sulfate etc..

In order to study the degradation pattern of fenvalerate in male rats, we carried out both acute and chronic toxicity studies to understand the effects of short term as well as long term fenvalerate exposure to rats.

The acute toxicity studies were carried out for a period of 48 h after administration of a single oral dose (100 mg/Kg body weight) of the pesticide. It was seen that fenvalerate underwent rapid metabolism in rat liver and within 48 h less than 5% of the parent compound was detected in liver. Moreover, after 12 h about 50% of fenvalerate was degraded in liver which agrees with the reports that the half-life of fenvalerate in rats is 12-14 h (Ohkawa, 1979). Similarly in kidney and brain, fenvalerate underwent not so rapid degradation into two slow moving products designated as PI and PII. These were identified as [4-chloro-α-(1-methylethyl) benzeneacetic acid] and [3-phenoxybenzoic acid] respectively. Such metabolites were also reported by Lee et al. (1985). It was seen that after 48 h exposure period, about 30% of residual fenvalerate was still left in brain corroborating to the earlier reports that fenvalerate is considered to be a potential neurotoxin (Saleh. et al., 1993). Also, it may be that the undegraded fenvalerate residues accumulated in brain lead to neurotoxicity. Several reports, including those of Malaviya et al. (1993) have shown that gestational exposure to fenvalerate caused disturbances in dopaminergic and cholinergic pathways and thus lead to a functional delay in brain maturation. Moreover, our results indicated that the two metabolites formed were apparently not as toxic as the parent compound since they did not produce any major toxic effects on the animal or the biochemical parameters studied so far. Cantalamessa (1993) suggested that ester hydrolysis was an important pyrethroids detoxification reaction in the adult rats.

Very little information is available to date on the effect of fenvalerate on biochemical parameters like nucleic acids, phospholipids, neutral lipids, total triacylglycerols, proteins etc., at tissue, cellular and subcellular levels. We found that under acute treatment fenvalerate showed a time-dependant effect on these biochemical parameters. For instance, the level of nucleic acids was suppressed uptil 12 h (i.e., when about 50% of fenvalerate was still left unmetabolised in liver), after which the level of

nucleic acids was elevated back to normal. This reversal of the toxic effect might be due to the rapid metabolism and excretion of the parent pesticide from the rat body. Similar pattern was also observed in case of triacylglycerol (TAG), where fenvalerate caused accumulation of this storage lipid in liver, kidney and brain of rats. This effect of pesticides on TAG content in rat liver has also been reported earlier (Elgin et al., 1990). The effect of fenvalerate on total proteins, and glycogen content in liver and serum cholesterol were also analysed and similar trend was observed. Reddy and Yellama (1991) studied the effect of fenvalerate on biochemical parameters in cockroach, *Periplaneta americana* and attributed this decrease in protein content to the increased proteolysis, while Reddy et al. (1991) studied the biochemical changes in fish, *Cyprinus carpio* and reported that fenvalerate leads to the disintegration of the cellular functions which impairs the protein synthesising machinery. The decrease in protein levels has already been reported in *Tribolium castaneum* when exposed to synthetic pyrethroids i.e., deltamethrin and permethrin (Saleem and Shakoori, 1985). However, Hassan (1990) reported that the protein content remained constant compared to control rats after administration of a single and repeated doses of fenvalerate. Recently, certain biochemical parameters were studied in broiler chicks (Majumdar et al, 1994) and they reported a change in the cholesterol content in liver, kidney and heart and the glycogen levels in liver. The decrease in liver glycogen possibly involved the adrenergic system. Fenvalerate also affects the glycogen metabolism in fishes (Radhaiah and Rao, 1990). Their findings are in agreement with our results that fenvalerate causes a decline in the level of cholesterol in serum and glycogen content in liver.

In chronic treatment the rats were administered a low (5 mg/Kg body weight of rats) and high dose (15 mg/Kg body weight) of fenvalerate on alternate days for a period of 7, 15 and 30 days. The chronic treatment of rats did not reduce the body weight upto 30 days. Mumtaz and Menzer (1986) too reported no loss in body weight

of quails on treatment with fenvalerate. But another study with mirex, an organophosphorus pesticide showed an increase in body weight when compared to control animals (Elgin et al., 1990), whereas deltamethrin caused a retardation of body growth (Abdel Khalik et al., 1993). Although we observed some signs of physical intoxication due to fenvalerate exposure in rats, such effects were not reported in birds (Mumtaz and Menzer, 1986), while mild intoxication was seen in american kestrels (Rattner and Franson, 1984) and in bobwhite quails (Bradbury and Coats, 1982).

The bioaccumulation and distribution of fenvalerate in various rat organs was studied. The HPLC analysis showed that after 7, 15 and 30 days treatment with fenvalerate, the residual parent compound and two slow moving metabolites were obtained which were similar to those seen in acute treatment. The quantitative distribution (mol %) of these compounds in liver is shown in Table 4.1. As evident, the concentration of fenvalerate decreased upto 30 days, the majority of residue either excreted or converted into its metabolites PI and PII. Similar trend was observed in kidney and brain. It was also observed from these results that when the same bioaccumulation experiment was carried out at two different doses, the accumulation of fenvalerate was dose-dependant i.e., at higher dose levels, larger quantities of fenvalerate residues were left unmetabolised. This kind of dose-dependant accumulation and metabolism of fenvalerate in rats was also observed by Kaneko et al. (1981). Moreover, the residual concentration of fenvalerate was much higher in brain compared to liver and kidney after 30 days treatment. Recently, Majumdar et al. (1994) observed very high fenvalerate residues in various organs of broiler chicks. However, they reported that intestine accumulated most of the fenvalerate residues followed by fat, brain, liver and kidney. Their basis for this observation was that since route of pesticide administration was oral, it was absorbed the most by intestines. Other workers too have reported a very high accumulation of fenvalerate in fat,skin, hair etc., in rat (Kaneko

et al.,1981). On the other hand, Gong (1990) reported greater affinity and longer half-life of fenvalerate in brain.

As in acute toxicity studies, the biochemical parameters were also affected by the chronic treatment, although the animals behaved very differently in this case. Repeated exposure of fenvalerate to rats led to no signs of recovery, partially impairing various biochemical parameters. This effect was manifested as suppression of nucleic acids and haematological parameters such as RBC and Hb counts, while there was an elevation in levels of alkaline phosphatase in liver, serum enzymes - SGOT, SGPT, acid and alkaline phosphatases and certain components of neutral and phospholipids. The increase in transaminases and alkaline phosphatase activity might be as a result of either cellular alterations or toxic stress condition induced by fenvalerate toxicity. Elevated levels of both transaminases in serum are indicative of liver damage (Martin et al., 1981), while increase in alkaline phosphatase activity occurs mainly in bone, liver and biliary tract diseases. Majumdar et al. (1994) reported an increase in GPT activity. In our studies, acid phosphatase activity decreased in liver but increased in serum. Such toxic stress conditions resulting in elevation of certain enzymes induced by fenvalerate has also been reported in cockroach with regard to SDH, MDH and LDH activity (Reddy and Yellama, 1991), and AAT and AlAT enzymes in case of fish (Reddy et al., 1991). Fenvalerate had a slight effect on brain cholinesterase activity in American kestrels as reported by Rattner and Franson (1985), whereas cypermethrin is highly toxic to ACHE activity in *Cyprinus carpio* (Reddy and philip, 1994). Fenvalerate also influenced several enzymes of tryptophan metabolism (Hassan et al., 1990) as well as inhibition of Mg^{2+}-ATPase and Ca^{2+}-ATPase in the squid (Clark and Matsumura, 1982).

Till date, no work has been reported on the effect of fenvalerate on neutral

and phospholipids in rats. We have tried to find out the effect of fenvalerate on various components of neutral lipids as well as phospholipids in liver, kidney and brain. The results obtained so far have shown that pesticide toxicity caused a marked increase in some components of neutral lipids such as TAG and MAG when analysed on TLC, while the levels of DAG and FFA were reduced in liver, kidney and brain . In case of phospholipids, the two major components i.e., PC and PE were substantially increased under fenvalerate toxicity in liver, kidney and brain, while SM was only slightly increased in liver and showed a decrease in kidney and brain. The other phospholipids determined by HPLC analysis, i.e., PI, PS and PG showed varied changes in all the organs. DAG plays a pivotal role as it can be acylated to TAG and deacylated to MAG. A decrease in DAG concentration may affect the PC concentration as it enters the PC synthesis pathway and stimulates its synthesis. DAG may also be used up in conversion to PE, thus increasing its concentration which explains the marked increase of PC and PE and a concomitant decrease in DAG levels under fenvalerate toxicity as observed by our results. Narayan et al.(1990) and Ishidate et al. (1980) have shown that DDT, endosulfan etc., caused an increase in microsomal PC by stimulating the activities of choline kinase and phosphocholine cytidyltransferase. The hepatic lipids were reportedly affected by polychlorinated biphenyls (PCBs), a group of toxic compounds and here also the phospholipids were increased (Poul, 1991). It seems that like PCBs and mirex, fenvalerate also affects some enzymes of lipid metabolism. Excessive accumulation of lipid mainly triacylglycerols is indicative of fatty liver. This may result from: (1) Increased mobilization of FFA in blood from the stored triacylglycerol of adipose tissues by action of triacylglycerol lipase. Lipogenesis from carbohydrates and hydrolysis of lipoproteins (triacylglycerol of chylomicrons) by lipoprotein lipase in extrahepatic tissues are also a source of FFA. The increased FFA are taken up by the liver and are esterified to triacylglycerol. The formation of lipoprotein is slower than formation of TAG from FFA. So TAG accumulates in liver. Fenvalerate seems to induce the process.

(2) In addition, decreased oxidation of FFA and inadequate utilization of TAG produce lipoproteins from transport to other tissues through blood. The synthesis of lipoproteins may decrease by decreased supply of phospholipids and apoproteins. The accumulation may result from the failure of the secretory mechanism. Therefore, treatment with a toxic substance interfering with lipid metabolism may have long term serious consequences (Jamdar et al., 1978; Hahn, 1982).

In other words, *in vivo* both acute and chronic treatments of rats with fenvalerate induces biochemical and haematological alterations, which in turn, affect the normal functioning of the animals.

5.2 *IN VITRO* METABOLISM OF FENVALERATE

The *in vitro* metabolism of fenvalerate by various subcellular fractions of rat liver was studied in detail. Characterisation of the fenvalerate hydrolysing enzyme was carried out under *in vitro* conditions. It was found that apart from tissue homogenate which contains all the organelles, mitochondria and microsomes contain maximum degradative enzyme activity. Also the fenvalerate metabolising enzyme in mitochondria was found to be localised mainly in the outer mitochondrial membranes. Most of the studies on *in vitro* metabolism so far have been carried out using hepatic microsomal enzymes (Krechniak and Wrzesniowska, 1991; Mumtaz and Menzer, 1986; Suzuki and Miyamoto, 1974) or whole body homogenates (Dowd and Sparks, 1987). Previous studies have indicated that some krebs cycle intermediates affected drug metabolism in liver slices and homogenates (Cinti et al., 1972). Moldeus et al. (1973) also reported that mitochondria stimulate the hepatic endoplasmic reticulum MFO of drugs and chemicals *in vitro*.

We therefore characterised the fenvalerate hydrolysing enzyme present in the outer mitochondrial membranes of rat liver. When outer mitochondrial membrane was used as enzyme source for the degradation of fenvalerate, two new products PI and PII were formed and on analyses by HPLC and TLC, were found to be similar to the metabolites formed by acute treatment of rats. But a third metabolite (PIII) was also formed *in vitro* metabolism, which is supposed to be formed by the metabolic reaction (oxidatuion) of PI. The product PIII was more prominent when nonionic detergent was added to the incubation medium. This is in agreement with reports of Mumtaz and Menzer (1986), who reported that 4'-OH CPIA, an oxidation product of the major metabolite CPIA (PI) was only found *in vitro* and minute quantities of this product were present *in vivo* metabolism. They also reported differences in other metabolites quantity on comparing *in vivo* vs *in vitro* metabolism and concluded that hydroxylases are more active in purified microsomal fractions, but in the whole animal other factors such as transport of fenvalerate from alimentary canal to liver regulate the rate of metabolism.

The time course studies showed that the reaction was linear upto 30 min with the product formation levelling off thereafter, while Mumtaz and Menzer (1986) showed maximum product formation only after 10 min incubation in quails. The protein concentration used in the medium was around 1.0 mg protein per assay. All the fractions were thus suitably diluted to obtain a final concentration of about 1.0 mg. The optimum pH for the enzyme was around 7.5. Shishido and Fukami (1972) studied the enzymatic hydrolysis of diazoxon by rat tissue homogenates and found that the optimum pH for enzyme was 8.8. We observed that in presence of low (below 0.5 mM) EDTA concentration the enzyme activity was stimulated, while higher EDTA concentration led to a strong inhibition. Our interpretation is that low concentration of EDTA i.e., upto 0.5 mM helps in making the enzyme-substrate complex active while

higher concentration i.e., about 2.0 mM, EDTA induced unfavourable conformational changes in the enzyme or chelating certain metal ions required for enzyme activity. The effect of some divalent metal ions was also studied and it was found that 10 mM Mg^{2+} activate the enzyme, while 10 mM Ca^{2+} and Mn^{2+} showed slight inhibition. This is in contrast to the report of Shishido and Fukami (1972), who reported that 0.1 mM Ca^{2+} and Mg^{2+} activated the enzyme, while 0.1 mM Zn^{2+} inhibited the enzyme activity. However, they also showed that above 1 mM Ca^{2+}concentration, there was no effect on the activity of the enzyme and 0.1 mM EDTA inhibited the mitochondrial as well as microsomal enzymes. The kinetic studies of fenvalerate hydrolysing enzyme showed that the K_m and V_{max} were 26.32 mM and 0.50 mM min^{-1} mg^{-1} protein. The Lineweaver-Burk plot showed an upward trend indicating substrate inhibition. The K_m and $V_{max}$for mouse liver esterases hydrolysing (+)-trans-Resmethrin were reported to be 125 x 10^{-7} M and 2083 pmole /mg protein /min respectively (Jao and Casida, 1974).

No reports have appeared so far regarding the effect of detergents on the fenvalerate hydrolysing enzyme. It can be seen that in presence of detergents i.e., 0.5 % digitonin only 40% residual pesticide was left compared to 50% without detergent. Surprisingly, Triton X-100 and Nonidet P-40, seem to catalyse further the degradation of PI into PIII, a new metabolite. This product PIII has not been characterised fully, but some reports show that it might be the hydroxylated derivative of CPIA (Lee, 1985; Mumtaz and Menzer, 1986). However, work would be necessary i.e., solubilise the enzyme and characterise it further.

5.3 ELECTRON MICROSCOPY

The ultrastructural studies of fenvalerate exposed liver and kidney cells viewed

under TEM have revealed distinct perturbations in the cellular structure when compared with control. The ultrastructure of fenvalerate treated cells showed disorganisation of nucleolus and chromatin material. Kumar et al. (1993) reported fenitrothion, an organophosphorous pesticide, induced damage to the nuclear membrane of rat liver cells in acute toxicity studies and found that the damage was reversible. The disorganisation of nucleolus induced by fenvalerate treatment might lead to subsequent disruption of nucleolus specific functions as synthesis of rRNA and biogenesis of ribosomes. Sporadic dispersion of lipid droplets in cytoplasm, presence of some crystalline rods and dilation of bile canaliculi in liver cells were also observed all of which were absent in the control liver cells. Previous studies have established that lipospheres staining predominantly for phospholipids and neutral fat were absent from control animals and present only when exposed to DDT (Ortega et al.,1956;1957). Some changes such as margination of heterochromatin, presence of crystalline rods and formation of microgranulomas have already been reported in rats (Okuno et al., 1986) and dogs (Parker et al., 1984). However, fenvalerate was not reported to cause microgranulomatous changes in such tissues as heart, kidney and adrenal glands in mice (Parker, 1983). Kaneko et al. (1986) concluded that the causative agent of microgranulomatous changes induced by fenvalerate was CPIA-cholesterol produced by fenvalerate βα-isomer in tissues of rat. Fenvalerate enhanced the incidence of γ-glutamyl transpeptidase-positive enzyme-altered foci in rats (Flodstrom, 1988). Fenvalerate inhibited intercellular communication *in vitro* in rat liver, suggesting that this substance could act as tumor promoter. (Hemming, 1988). It was reported that fenvalerate was not teratogenic in mice and rats (FAO/WHO, 1979). Cabral and Golenda (1990) studied the carcinogenicity of fenvalerate in mice in long-term experiments and reported no evidence of carcinogenicity in (C57B1/6) mice.

We also studied the ultrastructure of kidney cells and only slight differences in control

and fenvalerate exposed rats was observed. The nucleolus and mitochondria were deformed. Electron microscopic studies of kidney cells have not been reported so far. However, the light microscopic studies of kidney cells have shown glomerular and tubular necrosis in kidney cells exposed to fenvalerate (Majumdar et al., 1994).The ultrastructural changes in rat lungs induced by fenvalerate was repoted by Wang and Zhai (1991).

5.4 SUBCELLULAR MEMBRANE FRACTION STUDIES

The effect of fenvalerate on the biochemical parameters of subcellular membranes fractions from rat liver were also analysed in control and fenvalerate exposed rats. It was found that total carbohydrate was markedly increased in all the three membrane fractions i.e., nuclear, mitochondrial and plasma membrane.

Fenvalerate also increased the total phospholipids at cellular and subcellular levels. Since phospholipids are important functional components of cell membranes and membranes of organelles such as endoplasmic reticulum (ER) and golgi apparatus (GA), fenvalerate toxicity seems to perturb the membrane structure and functions. Sarkar et al. (1993) reported the effect of fenvalerate on membrane fluidity. They showed that fenvalerate interacts with membranes by localising itself near the hydrophobic tail region of the lipid acyl chain and perturbs its ordered structure to make the membrane more fluid. Since pyrethroids are very hydrophobic molecules, they can be expected to bind extensively to biological membranes (Jones and Lee, 1986).

The effect of fenvalerate on proteins, both qualitatively and quantitatively have been studied in detail. The total protein content of subcellular membranes showed a decrease while SDS-PAGE pattern of proteins of plasma membrane, nuclear membrane, and outer mitochondrial membrane further confirmed the results by showing

reduced intensity of protein bands in fenvalerate exposed rat liver membrane when compared to control.

The decrease in protein intensity in nuclei can be correlated to the damage caused by fenvalerate treatment to the nuclear membrane structure as observed by the ultrastructural examination of liver cells under TEM. The protein composition of control and fenvalerate treated rat liver outer mitochondrial membranes showed protein specific stimulation as well as inhibition.

A number of protein bands of plasma membrane of rat livers of fenvalerate exposed rats showed an increase in intensity in 4-5 bands, while some proteins showed a decrease. Brewster et al. (1982) studied the influence of a toxic pesticide 2,3,7,8-TCDD on the protein composition of the plasma membrane of hepatic cells from rat and found that level of many proteins were reduced in the membrane from the TCDD treated rats as shown by SDS-PAGE and was further confirmed by a reduction in binding of ^{3}H-concanavalin A to the TCDD - treated plasma membrane. Several reports have shown that *in vivo*, fenvalerate inhibits the plasma membrane bound enzymes Na^{+} - K^{+} - ATPase and Mg^{2+} - ATPase (Reddy et al., 1991; Clark and Matsumura, 1982) indicating that fenvalerate perturbs the structure and functions of plasma membrane.

A new protein band of about 35 kDa was induced under fenvalerate toxicity which was absent in control. This kind of 'stress proteins' might be formed as a result of fenvalerate treatment. Appearance of a new protein band of 35 kDa in fenvalerate treated rat liver plasma membrane, raises a question whether or not 35 kDa protein, which is absent in control PM, represented a monomeric subunit of the protein of hsp 70 family ? Also, if this protein has any structural and genetic relationship with the multi-drug-resistance membrane protein reported by Raymond and

Gros (1989). Further studies on the biosynthesis of the 35 kDa protein using radioactive labeled amino acids and antibodies are likely to reveal more about the protein. Further work will be directed in molecular characterisation of protein.

Chapter 6

SUMMARY

Fenvalerate, [1, cyano (3-phenoxyphenyl) methyl 4-chloro-α-(1-methylethyl) benzeneacetate, Pydrin insecticide], is a contact pesticide, which is used to control a wide range of agricultural, poultry, dairy and household pests. It combines very high insecticidal activity against a broad spectrum of insect pests with moderate mammalian toxicity and adequate field stability. The LD_{50} for rats is about 450 mg/Kg of body weight. In the present study, acute and chronic exposures of rats to pesticide were investigated in terms of its metabolic fate, bioaccumulation of the pesticide and its degradation products in liver, kidney and brain and finally ultrastructural and biochemical effects at both cellular and subcellular levels were studied.

6.1 METABOLIC FATE OF FENVALERATE

The metabolic fate of fenvalerate in rats was studied in detail in three organs, namely, liver, kidney and brain. After the oral administration of a single dose of 100 mg fenvalerate/Kg body weight of rats, liver, kidney and brain tissues were removed after 6, 12, 24, 36 and 48 h and analysed for residual pesticide and its metabolites by HPLC. It was found that upto 6 h after administration, fenvalerate accumulated in all tissues with very little detection of degradation products. However, following this period, the pesticide rapidly degraded and two major products, designated as PI and PII, were formed and accumulated in all tissues. The degradation in liver was most rapid with almost 75% of fenvalerate being degraded 24 h after exposure

to rats. Meanwhile, the concentration of major product PI increased concomitantly with time, while metabolite PII did not accumulate in significant proportions. Almost identical pattern was observed in kidney and brain, but the metabolism in these was much slower than that in liver. Even after 48 h exposure, only about 22 and 32 mol % of fenvalerate remained unmetabolised in kidney and brain respectively compared to less than 5% in liver. Thus it can be inferred that liver undergoes rapid detoxification, while brain tissues show a very slow metabolism. The metabolic products i.e., PI and PII were identified with help of IR spectroscopy as 4-chloro-α-(1-methylethyl)benzeneacetic acid and 3-phenoxybenzoic acid, respectively.

The bioaccumulation and distribution of fenvalerate and its metabolites in rat organs was also studied under chronic toxicity. As chronic treatment of rats involved repeated exposure to fenvalerate for a prolonged period of time i.e., 7, 15 and 30 days, the rats showed mild signs of intoxication for the first 1-2 days. The bioaccumulation of fenvalerate and its metabolites was assessed using two dose levels (5 mg and 15 mg/Kg body weight) to obtain a dose-dependant effect. The chronic treatment resulted in quite low accumulation of pesticide in liver (about 8 mol %) at 30 days, while in kidney and brain, the accumulation was comparatively much higher i.e.,about 10 and 40 mol % respectively. Furthermore, the concentration of residual fenvalerate was more at 15 mg/Kg than at 5 mg/Kg. The formation of two slow moving metabolites, PI and PII showed similar accumulation patterns as in acute treatment of rats, except that the concentration of PII was higher in all tissues.

The *in vitro* metabolism of fenvalerate was carried out to characterise the enzyme involved in the biodegradation of fenvalerate into its metabolites. Formation of the major product PI was taken as a measure of enzyme activity .The enzyme was found to be localised in the outer membrane of mitochondria, apart from microsomes.

Thus, the fenvalerate hydrolysing enzyme in outer mitochondrial membrane was studied in detail. This enzyme hydrolysed about 50 mol % of the pesticide with the formation of two products i.e., PI and PII, which were possibly the same as were formed *in vivo*. The enzyme activity was linear upto 30 min, the optimum pH was around 7.5, and the K_m and V_{max} values were found to be 26.32 mM and 0.50 mM min^{-1} mg^{-1} protein, respectively. The enzyme activity was significantly stimulated at low concentrations of EDTA (< 0.5 mM), while higher concentrations were inhibitory with 50% activity inhibited at 2 mM EDTA concentration. The enzyme showed a requirement for Mg^{2+}, while Mn^{2+} and Ca^{2+} inhibited the activity. Zn^{2+} had no effect on the enzyme. The *in vitro* degradation of fenvalerate was substantially enhanced in the presence of nonionic detergents. For instance, the amount of residual pesticide in presence of 0.5% Triton X-100 was approximately 35% compared to 50% without detergent in the incubation medium. Moreover, the presence of Triton X-100 and Nonidet P-40 in the complete medium seemed to catalyse further degradation of metabolite PI into a third metabolite PIII probably by oxidation. However, the mechanism of action of detergents on the formation of metabolites is not known at the moment.

6.2 BIOCHEMICAL AND HAEMATOLOGICAL ALTERATIONS IN RATS

The effect of fenvalerate toxicity on various biochemical parameters such as nucleic acids, total proteins, glycogen, serum cholestrol, certain enzymes and neutral and phospholipids was observed in both acute and chronic treatment of rats. There was a marked reduction in the level of nucleic acids (RNA and DNA), total proteins and glycogen in liver and serum cholestrol upto 12 h on acute exposure, after which their level returned back to normal in 48 h. These results indicate that, *in vivo*, the acute effects of fenvalerate on these parameters is time-dependant and relates to

the accumulation pattern of pesticide in liver. These acute effects were in contrast to the alterations induced by the chronic exposure of fenvalerate in rats. At both dose levels i.e.,5 mg and 15 mg/Kg, the nucleic acids content continued to decrease upto 30 days exposure. Furthermore, while alkaline phosphatase activity in liver was increased significantly compared to control, the specific activity of acid phosphatase decreased with time and was reduced by 50% after 30 days in treated rats. These effects were observed to be dose dependant. The haematological parameters also showed a marked change in their levels on fenvalerate exposure. The serum enzymes SGOT (serum glutamic oxaloacetic transaminase), SGPT (serum glutamic pyruvic transaminase), alkaline and acid phosphatases were increased by approximately 3.0-, 4.0-, 1.5- and 2.0-fold respectively. Meanwhile, haemoglobin and RBC count showed a decline and WBC counts were increased continuously upto 30 days on pesticide exposure. The neutral lipids i.e., triacylglycerol (TAG) and monoacylglycerol (MAG) concentrations were increased, while level of diacylglycerol (DAG) and free fatty acids (FFA) was reduced so much that FFA was not detected in liver and kidney after 30 days treatment otherwise the trend was similar in liver, kidney and brain. The total TAG content, when analysed on GLC, also showed accumulation of this storage lipid in all the three organs on fenvalerate treatment. Similarly, fenvalerate affected all the components of phospholipid in liver, kidney and brain of rats. Exposure to pesticide caused a marked increase in the two major phospholipid components i.e., phosphotidyl choline (PC) and phosphotidyl ethanolamine (PE).On the other hand, phosphotidyl serine (PS) and phosphotidyl glycerol (PG) decreased substantially in all the three organs. Sphingomyelin (SM) and phosphotidyl inositol (PI) showed varied response to the pesticide treatment in liver, kidney and brain, while PG was not detected after 30 days.

6.3 ULTRASTRUCTURAL STUDIES

The ultrastructural studies by transmission electron microscope (TEM) of control and fenvalerate treated rat liver cells showed the nucleus with a well defined nuclear membrane, a distinct nucleolus, normal looking mitochondria and an organised ER network in control liver cells. In contrast, the pesticide treated cells indicated marked disorganisation of nucleolus to the extent that in some cells, two nucleolus were formed after 30 days. This effect was dose dependant. Dispersion of chromatin material and flattening of mitochondria was observed. Presence of crystalline needles and lipid droplets and to some extent dilation of bile canaliculi (BC) was also observed. The electron micrographs of kidney cells of treated rats also showed similar effects.

6.4 EFFECT OF FENVALERATE ON SUBCELLULAR MEMBRANES

As pointed out earlier, chronic treatment of fenvalerate appeared to damage the structure of membranes of rat liver. Thus, various subcellular membrane fractions were prepared to investigate further the effect on proteins, total carbohydrate and total phospholipids. The carbohydrate content was substantially increased in nuclear membrane, outer and inner mitochondrial membranes and plasma membrane which was about 1.5-, 2.5-, 2.0- and 1.5-fold, respectively. Similarly, as expected, the total phospholipids in nuclear, mitochondrial and plasma membrane fractions showed an increase in pesticide treated rat liver, the maximum increase being in the plasma membrane. The membrane protein pattern was also compared in control and treated rat liver by subjecting various subcellular fractions to SDS-PAGE. The nuclear proteins of treated rats displayed an increase in intensity of 220, 32, 24 and 17 kDa, while a suppression in 225, 129, 127, 82, 72, 46, 44 and 15 kDa intensity, while a 79 kDa protein was found absent. In case of outer mitochondrial membranes, the intensity of

150, 82, 55, 48, 46, 41, 38 and 31 kDa protein bands was decreased with subsequent increase in 162 and 18 kDa proteins. The 210, 180, 35 and 17 kDa proteins were absent. The plasma membrane SDS-PAGE profile, when analysed, showed maximum enhancement in the intensity of 180, 90, 75 kDa proteins, while 42 and 17 kDa protein levels were decreased significantly. The 158 and 32 kDa bands were completely missing. Surprisingly, a new protein band of 35 kDa appeared in plasma membrane of fenvalerate exposed rat liver which was absent in control. This seemed to be a kind of 'stress protein' induced as a result of fenvalerate toxicity.

The maximum effect was thus observed in plasma membrane. Since PM proteins are synthesised in ER and then transported to plasma membrane, these results were interpreted to mean, but by no means proved, that fenvalerate impairs the translocation of proteins from endoplasmic reticulum to plasma membrane.

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