STUDIES ON HEPATOTOXINS IN RELATION TO MITOCHONDRIAL FUNCTION

A

Thesis

Submitted

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by

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DECLARATION

I, P. PADMA, declare that the work presented in my thesis has been carried out by me under the supervision of Dr. O.H. Setry, and has not been submitted for any degree or diploma of any other University.

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CERTIFICATE

This is to certify that P. PADMA, has carried out the research work embodied in the present thesis entitled " **STUDIES ON HEPATOTOXINS IN RELATION TO MITOCHONDRIAL FUNCTION** " under my **supervision** and guidance for the full period prescribed under the PhD ordinance of this University. I recommend her thesis for the submission for the degree of Doctor of Philosophy of this University.

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ABBREVIATIONS

a-KG	alpha ketoglutarate
AAT	Aspartate aminotransferase
ADP	Adenosine 5'-Di phosphate
A1AT	Alanine aminotransferase
AMP	Adenosine 5'-Mono phosphate
ANS	1-anilino-8-napthalenesulfonate
ANSA	l-amino-2-napthol-4-sulfonic acid
ATP	Adenosine 5'-Tri phosphate
BSA	Bovine serum albumin
CCCP	Carbonyl cyanide p-triflouro methoxy phenyl hydrazone
CCl_4	Carbon tetrachloride
CL	Cardiolipin
DCPIP	2.6-Dichlorophenol indophenol
DEAE	Diethyl aminoethyl
2.4-DNP	2,4-Dinitrophenol
DTT	Dithioreitol
EDTA	Ethylene diamine tetra acetic acid
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
$FMNH_2$	Flavin mononucleotide (reduced)
GTP	Guanosine 5'-triphosphate
G6PDH	Glucose-6-phosphate dehydrogenase
HPLC	High perfonnance liquid chromatography
HEPES	N-2-Hydroxy piperazine-N-2-ethane sulphonic acid
i.p.	Intraperitoneally
KC1	Potassium chloride
KCN	Potassium cyanide
LDH	Lactate dehydrogenase
LPO	Lipid peroxidation
MDA	Malondialdehyde
MDH	Malate dehydrogenase
NAD(P)	Nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	Nicotinamide adenine dinucleotide (reduced)
P/O	Phosphate to Oxygen ratio
PMS	Phenazine methosulphate
PUFA	Polyunsaturated fatty acid
PC	Phosphatidyl choline
PE	Phosphatidyl ethanol amine
RCR	Respiratory control ratio
SDH	Succinate dehydrogenase

SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
SMP	Submitochondrial particles
TAA	Thioacetamide
TBARS	Thiobarbituric acid reactive substance
TCA	Trichloro acetic acid
TLC	Thin layer chromatography
TMPD	N,N,N',N' Tetramethyl ethylenediamine
TRIS	Tris (hydroxy methyl) aminomethane 2-Amino- 2-(hydroxy methyl-Arnino-2-(hydroxymethyl) propane-1,3-diol
UDP	Uridine diphosphate
UTP	Uridine tri phosphate
VLDL	Very low density lipoprotein
WHV	Wood chuck hepatitis virus

CONTENTS

INTRODUCTION

Mitochondria are regarded as the power houses of the cell. They carry the most important function, i.e.,oxidative phosphorylation. Some cytotoxic compounds interact with mitochondria, disrupt their function and lead to pathological signs.

Mitochondria in different cell types vary widely in size, shape and number. Each mitochondrion has an outer membrane that is freely permeable to large molecules and an inner membrane that is relatively impermeable and contains the respiratory chain. The inner compartment of the mitochondria, enclosed by the inner membrane, is the matrix in which Krebs cycle takes place. NADH and FADH2 (that are generated from the Krebs cycle) act as electron donors to the electron transport chain. Proton extrusion across the inner mitochondrial membrane generates an electrochemical proton gradient, which store potential energy. Oxidative phosphorylation is the process by which the energy of oxidation is coupled to the synthesis of ATP.

The respiratory chain comprises of four enzyme complexes located on the inner mitochondrial membrane (Wallace, D.C.,1992).

Complex I [NADH : Ubiquinone oxidoreductase] : It is the largest and contains 26 polypeptides, seven of which are encoded by mitochondrial DNA. NAD linked substrates feed reducing equivalents into the chain via Complex I which passes electrons down the chain to ubiquinone.

Complex II [Succinate : Ubiquinone oxidoreductase] : It consists of 5 polypeptides that are encoded by nuclear DNA. It accepts reducing equivalents from succinate and transfers to ubiquinone.

Complex III (Ubiquinol : cytochrome C reductase): It consists of 11 subunits with one subunit (cyt.b) encoded by mitochondrial DNA.

Complex IV (Cytochrome C oxidase): It is composed of 13 polypeptides 3 of which are encoded by mitochondrial DNA.

ATP Synthase : It is composed of 12 subunits , two of which are encoded by mitochondrial DNA.

Mitochondria play a role in programmed cell death (apoptosis). bcl-2, a nuclear gene product, is a mitochondrial inner membrane protein and overexpression of which (bcl-2 protein) results in inhibition of apoptosis (Hockenbury *et al,* 1990).

Chemically induced cell injury is associated with a perturbation of calcium homeostasis. This perturbation may be due to impairment of the plasma membrane calcium translocating systems, stimulation of calcium channels or inhibition of calcium sequestration by either endoplasmic reticulum or mitochondria. As a result, the cell becomes unable to maintain its cytosolic free calcium concentration within the physiological range (~ 0.1 μM). Heavy metals like mercury, lead and hepatotoxins acetaminophen, carbon tetrachloride cause cell death by elevating intracellular calcium levels (Kass *et al,* 1991 ; Nicotera *et al,* 1992).

The gastrointestinal tract of higher animals and human species is the port of entry for a variety of naturally occurring organic plant and animal poisons and a wide variety of chemicals in the form of drugs, pollutants and poisons (Mason *et al*. 1965). Before being distributed to the body through blood they are first directed into the liver, which plays a key role in the metabolism and elimination of several drugs. Liver cells are equipped with an active detoxification system called the mixed function oxidase which metabolically alters a variety of xenobiotics and guards the organism against potentially harmful drugs. In chronic liver disease particularly in cirrhosis, hepatic drug metabolism may be altered by changes in hepatic blood flow or in the activity of drug metabolising enzymes resulting in modification of the intensity of therapeutic and toxic effects (Wilkinson and Shand, 1975).

General aspects of Hepatotoxicity :

Drugs can induce a variety of liver lesions, the most frequent being hepatitis (Benhamou, 1988), which is defined as necrosis or dysfunction of hepatocytes resulting in liver failure and/or cholestasis.

Liver lesions can result from many different mechanisms. Some hepatotoxins require activation to toxic intermediates, others interfere with metabolic pathways or alter the integrity of cell membranes. Single or short term administration may induce necrosis, cholestasis, steatosis or inflammation while repeated administration may result in fibrosis and cirrhosis which lead to impairment of liver functions. Fatty liver may be caused by inhibition of VLDL secretion as it involves many steps and all of which must function normally if the process is to proceed smoothly. This steatogenic mechanism was first described in the case of CCI4 (Recknagel *et al* , 1960) and has since been shown to be involved in the hepatotoxicity of many other drugs (ethionine, phosphorous and orotic acid) and in choline deficient animals (Lombardi, 1966 ; Hoyumpa *et al*, 1975).

Galactosamine has been shown to reduce the synthesis of mRNA and of plasma protein as a result of sequestration of UTP (a precursor of RNA) (Decker and Keppler, 1974).

Orotic acid induced fatty liver is associated with an inhibition in glycosylation of apolipoprotein (Pottenger *et al* , 1973). This was confirmed by Martin *et al* (1982). The steatogenic action of ethionine is related to ATP depletion as ATP is required for activation and incorporation of amino acids into proteins (Farber, 1971). CCI4 inhibits protein synthesis (Smuckler *et al*, 1961).

The mitochondrial respiratory chain and free radicals :

The main site of oxygen utilisation in the cell is the mitochondrial respiratory chain which uses oxygen as the terminal electron acceptor and is essential for the generation of ATP. Many of the respiratory chain redox centres are one-electron carriers (iron sulphur centres, ubisemiquinone and cytochromes) and potential donors of one electron to oxygen, generating the reactive superoxide radical, which is dismutated to H_2O_2 by superoxide dismutase (SOD). In the presence of iron, hydrogen peroxide can generate the highly reactive hydroxyl radical which can attack lipids, proteins or nucleic acids. The respiratory chain is one of the main source to generate of cellular free radicals (Boveris *et al,* 1972) and is susceptible to damage. Free radicals induce denaturation of proteins, peroxidation of membrane lipids and generation of chemotactic factors, eventually destroying cellular integrity.

Lipid peroxidation is known to occur *in vivo* in a variety of pathological conditions such as cancer, autoimmune diseases (Halliwell and Gutteridge, 1985), hypoxia, ischemia (Kogure *et al,* 1982 ; Imaizumi *et al,* 1984 ;Mishra and Delivoria-Papadopoulous, 1989), aging (Harman, 1983) and a number of conditions induced by metal ions (Halliwell and Gutteridge, 1985) During LPO there is increase in the formation of free radicals and mitochondria are vulnerable targets of free radical-mediated damage. LPO occurs by a free radical chain reaction that has been identified with initiation, propagation and termination phases (Recknagel *et al,* 1991). Biological membranes rich in polyunsaturated fatty acids (PUFA) are highly susceptible to free-radical catalysed oxidation reactions. LPO has been demonstrated to occur in isolated mitochondria, lysosomes and microsomes (Tappel, 1972). In mitochondria about 90% of the fatty acids in the phospholipid are PUFAs, contained mainly in PC and PE which account for approximately 80% (Daum, 1985) of the phospholipids and are susceptible to LPO.

Phospholipids are required for normal functioning of a variety of microsomal enzymes, including G6Pase, ATPase, UDP glucuronyl transferase. and the drug metabolising mixed function oxidase (Recknagel and Glende, 1973). During LPO there is a loss in the enzymatic activity due to a critical alteration of necessary membrane phospholipids. The appearance of polar peroxidised lipids in a biological membrane increases its permeability leading to mitochondrial swelling, disintegration and hemolysis in RBC and rupture of endoplasmic reticulum etc.

Cells have evolved systems that either prevent or control LPO. These defense systems may be classified as enzymatic, nonenzymatic or combinations of the two. The first category includes enzymes that control the formation of endogenous initiators of LPO. The second category includes chain-breaking antioxidants, or radical scavenging compounds that sequester or quench radical species that are predominantly involved in propagating reactions of LPO (Scholz *et al,* 1990).

The enzymes identified in controlling LPO are superoxide dismutase, catalase and glutathione peroxidase. They play an important role in the control of LPO by catalysing reactions affecting the concentrations of superoxide anion, H_2O_2 and lipid hydroperoxides (Flohe, 1982).

Antioxidants contribute to nonenzymatic cellular defense against LPO by donating hydrogen atoms to free radicals resulting in their inactivation. Water soluble antioxidants like ascorbic acid, uric acid, cysteine and glutathione exist in the cell cytosol and prevent LPO by scavenging radicals in the aqueous phase (Chow and Khan, 1983).

Vitamin E (α-tocopherol) is known to be the major lipid soluble antioxidant of membranes (Burton *et al,* 1986). a- tocopherol is present both in the inner and outer mitochondrial membrane and is significantly higher in the outer membrane compared to inner membrane (Thomas *et al,* 1989). The normal range of vitamin E concentration in mitochondria is 0.2-0.3 n mol / mg protein (Thomas *et al* ,1989; Lang and Packer, 1987) and is 5-7 times lower than the ubiquinone concentration.

Dietary supplementation or depletion of vitamin E for several weeks produces animals with markedly different levels of endogenous vitamin E in the membranes of fast turnover tissues such as liver. Vitamin E is superior to ubiquinols with respect to its antioxidant activity via scavenging peroxyl radicals in membranes. Oxidative stress destroys vitamin E and causes its depletion rapidly within minutes and as a nutrient, vitamin E cannot replenish the membranes to meet the urgent need during an acute oxidative insult (McCay, 1985). Replenishment of vitamin E through dietary supplementation may take a long time.

Vitamin E shows a marked capacity to prevent LPO *in vitro* and *in vivo* and its capacity to do so is directly related to the vitamin E status (Chen *et al,* 1980). McCay *et al* (1971) demonstrated that by increasing the concentration of membrane (peroxidisable) PUFA, the amount of a-tocopherol required to prevent LPO was also increased. Deleterious consequences of LPO may occur when the cellular protective mechanisms are overwhelmed.

Oxygen - derived free radicals may result from various reactions, both intra- and extracellularly, but generation of oxygen free radicals from electrons escaping the mitochondrial electron transport chain in mitochondria is by far the predominant process during the life time of a " normal" healthy cell. Mitochondria are important targets of oxygen derived free radicals, and the resulting mitochondrial dysfunction has been suggested as the intracellular basis of aging (von Zglinicki and Brunk, 1993).

Spermine, the hydrophilic polycation reduces the negative charge on the surface of mitochondrial membrane, by interacting with negatively charged polar heads of phospholipids and the neutralisation of the surface charge renders the mitochondrial membrane more susceptible to induction of LPO by ADP/ Fe^{2+} (Kogure *et al,* 1993).

Dietary copper overload in the rat is associated with morphological abnormalities and LPO of hepatic mitochondria. Decreased levels of hepatic glutathione and α-tocopherol, accompanied by increased levels of mitochondrial thiobarbituric acid reactive substances (TBARS) was seen in copper overloaded rats (Sokol *et al,* 1993).

Hepatic stimulator substance protects the liver against acute liver failure induced by CCl_4 poisoning, probably by an antioxidative effect on LPO, which was increased by free radicals produced from CCl_4 . Hepatic stimulator substance also stimulates hepatocyte proliferation (Mei *et al,* 1993).

Isolated rat liver mitochondria exposed to Fe^{2+} /citrate undergo LPO and alterations in membrane proteins. There is an irreversible decrease in membrane potential and mitochondrial swelling associated with an increase in production of TBARS and these products react with membrane proteins (Castilhio *et al,* 1994).

Vitamin E deficiency in rats gives rise to a neuromuscular syndrome that includes peripheral neuropathy as well as generalised muscle wasting and weakness. Polarographic analysis of isolated muscle mitochondria revealed significant decreases in the rate of oxygen utilisation with both NADH and FADH2 linked substrates. Enzymatic analyses revealed decreases in activities of complexes I, II/ HI and IV. Measurements of membrane fluidity showed that it is reduced in mitochondria from vitamin E deficient rats, indicating reduced stability of their membranes (Thomas *et al,* 1993).

Mice treated with KCN (7mg/kg subcutaneously) showed an elevation in the conjugated diene levels (index of LPO) in the brain and kidney but liver and heart showed no such increase. These studies showed that neurotoxic damage produced by cyanide, involves hydroperoxide generation with peroxidation of lipids which leads to changes in structure and function of certain membranes (Ardelt *et al,* 1994).

Studies by Kukielka *et al* (1994) have shown that intact mitochondria isolated from chronic ethanol treated rats showed an increase in the production of reactive oxygen species. Oxidation of ethanol by alcohol dehydrogenase generates NADH and NADH-dependent production of reactive oxygen species which increases after chronic ethanol treatment.

In rat liver mitochondria during Fe^{2+} /ascorbate and $NADPH/ADP/Fe^{2+}$ induced LPO, a-tocopherol was decreased to 80% of initial level and reduced coenzyme Q declined continuously while total Q9 pool was unchanged. The oxidation of the Q9 pool constitutes a prerequisite for the onset of massive LPO in mitochondria and for the subsequent depletion of a-tocopherol (Noack *et al,* 1994).

Studies by Paradies *et al* (1994) have shown that in hypothyroid rats the activity of cytochrome oxidase is decreased significantly (30%) and the content of cytochrome aa_3 is decreased by 15%. The depression in cytochrome oxidase activity was related to a decrease in the cardiolipin.

Various hepatotoxins which induce LPO can be used as animal models of hepatic failure . These include carbon tetrachloride, nitrosamines, galactosamine, acetaminophen, ethanol, thioacetamide but each has its own limitations.

Carbon tetrachloride produces liver injury but when used alone is known for its unreliability (Bhathal *et al,* 1983). Pretreatment of animals with phenobarbital will increase both the severity and reliability of CCl_4 injury (Proctor and Chatamara, 1984), but in studies relating to receptors of neurotransmitters and their relationship to acute coma (Schafer *et al,* 1983), addition of phenobarbital would interfere.

Nitrosamines are potent hepatotoxins but the major objection to the routine use is their known carcinogenicity (Lijinsky and Epstein, 1970).

Galactosamine is a selective hepatotoxin in rats, mice and guinea pigs. Galactosamine induced liver failure in the rabbit for use as model of human fulminant hepatic failure is well documented (Blitzer *et al,* 1978). Saunders (1972) has described this model as "the best animal model of hepatic failure produced todate". However, there are limitations which prevent this substance from being the ideal hepatotoxin. Firstly, galactosamine is expensive for use in animals larger than rats, secondly precaution should be taken to insure reproducibility (Blitzer *et al,* 1978), thirdly the exact mechanism of action of galactosamine is not understood (Liehr *et al,* 1978).

Acetaminophen, a potent hepatotoxin in humans (Mitchell *et al,* 1973) has an unreliable dose-response curve in animals (Miller *et al,* 1976).

Thioacetamide when sprayed on fruit, it retards spoilage. TAA causes a dose-dependent hepatotoxicity characterized by acute necrosis at high doses and nodular cirrhosis at small doses in rats chronically exposed to the drug (Fitzhugh and Nelson, 1948). The simplicity of administration and relatively modest cost of TAA makes this an attractive hepatotoxin.

CARBON TETRACHLORIDE :

CCl_4 has been used as an industrial solvent in the manufacture of plastics and in dry-cleaning industry. CCl_4 is very effective against adult liver fluke, *Fasicola hepatica.* Since it is very inexpensive, Australian sheep herders were using it, but it was hazardous because of liver toxicity. For the same reason its use as anti-helminthic or anaesthtic in humans is discouraged. The toxic effects of the solvent are well studied in liver. Liver injury induced by CCl_4 involves the metabolism of CCl_4 to trichloromethyl free radical by the mixed function oxidase system of the endoplasmic reticulum (Brattin *et al,* 1985). A single dose of CCl_4 to a rat produces centrilobular necrosis and fatty degeneration of the liver (Slater *et al,* 1985). Administration of CCl_4 leads to various impairments in the liver including necrosis, fatty infiltration and decreased activity of microsomal enzymes that catalyse the oxidation of drugs (Chaplin and Mannering, 1970).

Biomembranes and subcellular organelles are the major sites of damage by LPO. Mitochondrial and microsomal membranes contain relatively large amounts of PUFAs in their phospholipids (Fleischer and Rouser, 1965). Studies of Hunter *et al* (1964) and McKnight *et al* (1965) have shown that LPO in mitochondria can be initiated by redox agents including ferrous iron, ascorbic acid and glutathione. It is well correlated with swelling and finally lysis and disintegration of the mitochondria. Previous studies (Tappel, 1965) showed that this deterioration of isolated mitochondria can be measured by oxygen consumption or by the TBA reaction and the reaction was completely inhibited by the addition of vitamin E or ubiquinol, both of which have lipid antioxidant activity. In the heart and liver mitochondria, NADH-cytochrome C reductase and the succinoxidase system were good indices of LPO damage (Tappel,1973). Studies of LPO in isolated electron transport particles showed random damage to cytochromes aa_3, b, c_1 and c . Mitochondria from liver of vitamin E deficient rabbits showed increased rate of peroxidation compared to those that got supplemented with vitamin E. Measurement of MDA continues to be one of the most useful methods for determination of LPO product in studies of LPO damage (Sawicki *et al,* 1963 ; Placer *et al,* 1966).

In vitamin E -deficient rabbits the ratio of phosphate esterified to oxygen consumed with succinate as substrate was decreased from 1.7 to 1.1 (Tappel, 1965). Studies of ethanol induced hepatic injury (DiLuzio and Hartman, 1969) have demonstrated damage to mitochondria concurrent with LPO. The damage could be prevented by vitamin E and other lipid antioxidants (Hartroft and Porta, 1967).

Cirrhosis is generally regarded as a nonreversible chronic affliction of the liver that often ends fatally due to the onset of hepatic failure or other complications (Jikko *et al,* 1984). Mitochondrial function has been shown to be disturbed in patients with cirrhosis of the liver (Diaz Gil *et al,* 1977) and in rats with TAA (Moller and Dargel, 1984) and CCl_4 induced liver cirrhosis (Jikko *et al,* 1984). Similar changes in mitochondrial function have been described after chronic ethanol feeding (Cederbaum *et al* 1974 ; Schilling and Reitz 1980 ; Bottenus *et al,* 1982). Though the relationship between cirrhosis and impaired liver mitochondrial function has not been clearly established, there is growing evidence suggesting a functional and morphological alteration of mitochondria in liver disease (Hernandez-Munoz *et al,* 1992). Several research groups have characterised the effects of chronic ethanol consumption on the structure and function of liver mitochondria in both animals (Iseri *et al,* 1966 ; Koch *et al,* 1978; Bernstein and Penniall, 1978 ; French *et al,* 1983 ;Thayer, 1987) and humans (Lieber and Rubin 1968 ; Bruguera *et al,* 1977 ; Diaz Gil *et al,* 1977 ; Jenkins and Peters, 1978). Studies using flourescent membrane probes and electron paramagnetic resonance spectroscopy revealed that chronic ethanol consumption induces modification in the fluidity of mitochondrial membrane which may result

in alterations of metabolite transport across the mitochondrial membrane (Waring *et al,* 1982 ; Harris *et al,* 1987).

Regarding CCl_4 -induced cirrhosis, it has been found that alterations of oxidative phosphorylation are accompanied by a decrease of adenylate energy charge, which could be responsible for the lower albumin synthesis found in cirrhotic rats (Jikko *et al,* 1984). CCl_4 induced cirrhotic rats had exaggerated blood acetaldehyde levels after ethanol ingestion, this seems to be related to a reduced mitochondrial capacity to oxidise acetaldehyde and other substrates (Ma *et al,* 1989). It is reported that in chronic CCl_4 treated rats, liver mitochondria are morphologically and functionally intact and that the mitochondrial deficiency is a consequence of hepatocyte mass loss in the liver (Krahlenbuhl *et al,* 1989 ; Krahlenbuhl *et al,* 1990).

Chronic CCl_4 administration decreased mitochondrial respiration and P/O ratios mainly for substrates of site I, decreased membrane potential accompanied by abnormal distribution of phospholipids and cholesterol in mitochondrial membranes. Adenosine treatment prevented alterations in mitochondrial membrane composition and impairment of mitochondrial function induced by CCl_4 (Hernandez-Munoz *et al,* 1992).

Pulmonary toxicity could occur by inhalation of CCl_4 (Ma *et al,* 1989). *In vitro* studies in rat alveolar type II cells exposed to CCl_4 showed a decrease in ATP levels, which is required as a cofactor in phosphatidylcholine synthesis (Ma *et al,* 1989). The reduction in cellular ATP may be the result of a decline in glucose uptake, an inhibition of glucose metabolism and / or damage to mitochondria.

Promethazine is known to have a protective effect against the CCl_4 -induced liver necrosis. Studies were carried out using isolated hepatocytes and microsomal suspensions. *In vitro* CCl_4 was activated in both systems to free radical metabolites which initiate LPO. Promethazine acts by scavenging the trichloromethylperoxyl radical and lipid peroxyl radicals suggesting that LPO is of more importance than covalent binding in the pathogenesis of CCl_4 -induced liver necrosis (Poli *et al,* 1989).

Mitochondrial function evaluated in cirrhosis (induced by long term exposure to phenobarbital/ CCl_4 in rats) showed a decrease in O_2 consumption and ATP production. During cirrhosis of the liver there was a loss of hepatocytes leading to reduced O_2 uptake and reduced mitochondrial enzymes activities (Krahlenbuhl *et al,* 1989).

Cytochrome aa_3 concentrations of the liver increased in cirrhosis (induced by phenobarbitone and CCl_4) and were negatively correlated with the ATP synthesizing ability per unit of cytochrome aa_3. There was a decrease in hepatic energy charge accompanied by a decrease in serum albumin level (Jikko *et al* 1984).

It has been reported that in patients with liver cancer (Ozawa and Honjo, 1975) or jaundice (Ozawa and Honjo, 1977) an adaptive enhancement of mitochondrial function occurs to provide sufficient energy in response to an increased metabolic load. In the jaundiced patient with a marked increase of cytochrome aa_3 the phosphorylative activity per unit of cytochrome aa_3 decreased approximately by 50% to that of controls, indicating an inhibition in the phosphorylation by respiratory chain (Ozawa and Honjo, 1977).

Long term (chronic) administration of CCl_4 to rats resulted in a decrease of hepatic energy charge from its normal value of 0.85 to 0.78 (Jikko *et al,* 1984). The specific activity of cytochrome oxidase and the concentration of cyt.aa3 increased (Tanaka *et al,* 1987). The cyt.aa3 concentration increased with severity of cirrhosis and was reciprocally correlated with a decrease in the hepatic energy charge level.

LPO leading to cell membrane damage is known to occur in CCl_4 induced hepatotoxicity (Koster *et al,* 1977 ; Wolf *et al,* 1980 ; Curtis *et al,* 1979) . Membrane LPO is an important pathophysiological event in a variety of diseases and stress conditions. It is observed as a consequence of an intense iron dependent production of oxygen radicals or after administration of toxic drugs such as CCl_4 or acetaminophen both *in vivo* and *in vitro.* LPO results in a cascade of degenerative processes from membrane denaturation to tissue damage. The cell has built up an impressive set of system for protecting the integrity of membrane. It includes vitamin E, (a- tocopherol) which is the major natural lipophilic antioxidant. Under pathological conditions, these systems can be overwhelmed and non-toxic drugs would be required for preventing deleterious effects of LPO. This chain reaction is generally catalysed by metals and involves various intermediate free radicals, hydroxyl, peroxyl and alkoxyl radicals, therefore most inhibitors of LPO are metal chelators or antioxidants and radical scavengers.

Colchicine is a drug used in the reversion of experimental and clinical cirrhosis (Mourelle *et al,* 1981). Its hepatoprotective effect was attributed merely to an antifibrogenic action (Rojkind and Kershenobich, 1975).

Mourelle *et al* (1987) have shown that pretreatment of rats with colchicine (10 µg /day/ rat) for seven days protected them against CCl_4 induced damage. These studies have been limited to the plasma membrane. Colchicine exerts an effect at the level of the plasma membrane of the hepatocyte (Yahuaca *et al,* 1985) and therefore is effective not only in reversion of liver cirrhosis but also in the prevention of early changes in liver induced by acute CCl_4 administration in rats (Mourelle *et al,* 1987). It also inhibits leukotriene synthesis by macrophages (Simmons *et al,* 1983) and increases the formation of prostaglandins (Gemsa *et al,* 1980).

D-GALACTOSAMINE

D-Galactosamine is an amino sugar with unique hepatotoxic properties in animals. D-galactosamine is a hepatotoxin which induces liver necrosis via depletion of UTP and other uridine nucleotides. Galactosamine hepatitis is a potential model for generalised inflammatory liver injury and a tool for the screening of agents that protect the liver. It provides a method to inhibit protein synthesis, especially glycoprotein synthesis. It is more suitable than other agents as it affects specifically only liver. Keppler *et al* (1968) reported that administration of D-galactosamine hydrochloride to rats induced histological modifications similar to hepatitis. Prominent features of the syndrome are complete depletion of liver glycogen, absence of fatty infiltration, elevation of serum transaminases and decrease of serum proteins. The drug induced hepatitis is reversible, normal histology being found four weeks later. In animals treated with galactosamine the CO_2 production was found to be reduced by 50-70% suggesting an impaired citric acid cycle activity with possible mitochondrial damage. Electron micrograph studies revealed that the outer membrane of the mitochondria is intact while the cristae are hardly visible (Monier and Wagle, 1971). Galactosamine induced hepatic damage is accompanied by an overall inhibition of gluconeogenesis, the CO_2 fixation step being the most affected.

D-galactosamine can be administered by different routes (i.p., i.v.,or even s.c.) to provoke hepatotoxicity. Older rats need less galactosamine than younger ones for the same degree of liver injury. It influences neither the cytoplasmic redox state nor the energy state (ATP/ ADP) (Keppler *et al,* 1968 ; Keppler *et al,* 1969). This is one aspect in which hepatitis due to D-galactosamine differs from liver injuries induced by ethionine or CCl_4 (Kroner and Staib, 1967 ; Farber, 1971). A reduction of total adenine nucleotide level occurs in livers severely damaged by galactosamine, this reflects the marked loss of viable hepatocytes.

A dose of 2.5 g of D-galactosamine hydrochloride / kg bw is lethal for albino rats with a weight of 180-200 gm. Preliminary experiments have shown that most of the animals die 40-56 hours after the injection. From the 36th hour the animals become increasingly inactive. Histological changes are more severe 48 hours after the initial injection and resemble the observations in acute liver necrosis (Groflin and Tholen, 1978).

Galactosamine in contrast to other hepatotoxins (eg.,CCl4) has essential advantages : a) A selective liver toxicity (Keppler *et al,* 1968 ; Decker and Keppler, 1972 ; Chrito *et al,* 1977), b) all pathological changes are easily reproducible and c) a great similarity to fulminant hepatitis in men (Chrito *et al,* 1977).

Liver rapidly removes galactosamine from serum. Administration of more than 200 mg galactosamine/ kg bw leads to an accumulation of galactosamine-1-phosphate with subsequent inhibition of galactosamine metabolism in liver (Keppler and Decker, 1969).

Keppler and coworkers (1968) stressed the similarity between D-galactosamine hepatitis and the histological changes in human viral hepatitis. Light microscopic examination of the liver by Koff and coworkers (1971) indicated that the histological similarity was more apparent than real, since vacuolisation of the cytoplasm suggesting lipid accumulation was evident as early as 15 hours after injection. The presence of increased lipid within hepatocytes was confirmed both by electron microscopy and chemical determinations of hepatic triglyceride content. The combination of hepatic triglyceride accumulation in association with widespread hepatocellular necrosis and inflammation indicates that the D-galactosamine hepatitis closely resembles that induced by chemical toxins such as CCl_4 (Gamer and McLean, 1969) than viral hepatitis.

The hexosamines, D-galactosamine and D-glucosamine are found as integral units of glycoproteins, gangliosides and muco polysaccharides (Ledeen ,1979 ; Irwin, 1974 ; Margolis and Margolis, 1979). At high concentrations exogenous hexosamines exert cytotoxic effect on hepatocytes (experimentally used to develop a hepatitis model) and various tumour cell lines by affecting several metabolic systems which require uracil nucleotides to ensure their normal liver function.

Phenylmethylsulfonylflouride (PMSF) administration to rats was effective in partially preventing liver necrosis induced by thioacetamide, diethylnitrosamine or galactosamine suggesting participation of protein degradation in liver injury induced by these chemicals (de-Ferreyra *et al.* 1983).

D-Galactosamine injection induces an inhibition of carnitine palmitoyl transferase I activity with a decrease in mitochondrial phospholipids which were prevented by clofibrate suggesting that it counteracts by maintaining the mitochondrial membrane integrity (Sire *et al,* 1986).

The mechanism of liver injury by galactosamine remains controversial, a role for bacterial endotoxin has been suggested (Camara *et al,* 1983). Vitamin E improves the early fat and collagen accumulation in liver, decreases SGPT level and improves the survival rate in the D-galactosamine experimental model of acute liver injury in both conventional and germ-free rats showing that toxicity is not mediated through intestinal bacteria and / or endotoxins (Scalafani *et al,* 1986).

Galactosamine administration produces hepatitis-like liver injury in animals. Hepatotoxicity of galactosamine is attenuated by activation of reticuloendothelial (RES) system. Fructose-1,6, diphosphate increases the phagocytic activity of RES and offers protection against galactosamine toxicity (Markov *et al,* 1991).

Inhibition of mRNA synthesis and posttranslational glycosylations of proteins of the hepatocytes is instrumental in D-galactosamine induced hepatocellular necrosis (Decker, 1993).

Zinc has a protective effect on galactosamine induced liver damage. Its effect may be due to inhibition on LPO and increase in protein synthesis (Hu *et al,* 1992).

It was demonstrated that in the presence of hepatocellular regeneration, galactosamine toxicity is minimal, while in the absence it is maximum (Abdul-Hussain and Mehendale, 1992).

THIOACETAMIDE

Thioacetamide is a weak hepatocarcinogen that induces liver damage in rats, such as necrosis of hepatocytes, (Gupta, 1956 ; Cerdan *et al,* 1978 ; Castro *et al,* 1974 ; Cascales *et al,* 1982) cirrhosis and tumors (Becker, 1983, Praet and Roels, 1984) depending on the dosage and duration of the treatment. Repeated administration of thioacetamide (TAA) leads to enlarged nucleoli and elevated nucleolar RNA polymerase activity (Adams and Busch, 1963 ; Anderson *et al,* 1977).

Although the exact mechanism of action has not been elucidated, studies on acute toxicity of TAA continue to be reported (Trennery and Waring, 1983 ; Satyabhama and Padmanabhan, 1984). The simplicity of administration and relatively modest cost of TAA makes this an attractive hepatotoxin.

Hepatic fibrosis is a common and important condition in which major amounts of liver parenchyma are replaced by fibrous connective tissue. Experimental hepatic fibrosis has been induced in rats and baboons with chronic administration of hepatotoxins such as CCl_4 , ethanol or TAA (Feinman and Lieber, 1972 ; Hatahara and Seyer, 1982 ; Seyer, 1982). These animal models mimic to various degrees the pathological processes observed in human hepatic fibrosis. It has also been shown that hepatic fibrosis is accompanied by increased content of collagen (Chen and Leevy, 1971; Chen and Leevey, 1975; Rojkind and Martinez-Palomo, 1976 ; Seyer *et al,* 1977). A similar increase in collagen content has been observed in experimentally induced hepatic fibrosis in animal models (Feinman and Lieber, 1972 ; Henley *et al,* 1977; Mezey *et al,* 1977; Seyer, 1980 ;Hatahara and Seyer, 1982).

Chronic TAA intoxication of rats produced macro (TAA application for six months) or micro (application for three months) nodular liver cirrhosis depending on dosage and length of administration. Studies by Moller and Dargel (1984) showed that the structure and function of liver mitochondria are altered. Respiratory control ratio was decreased due to increased state 4 and decreased state 3 respiration in macronodular cirrhotic rats. The mitochondrial content of cytochrome aa3 and b were also lowered. Analysis of mitochondrial phospholipid fatty acids revealed marked alterations in phosphatidylcholine and cardiolipin leading to a decreased 20 :4 /18:2 ratio (Moller and Dargel, 1984).

Thioacetamide administered (100 mg/kg body weight) female rats, showed functional disturbances in liver mitochondria 24h after its administration. RCR was significantly reduced due to an increase in state 4 and a decrease in state 3 respiration (Moller and Dargel, 1985).

Liver injury was induced by a single subcutaneous administration of TAA (200 mg/ kg body weight). Levels of aspartate aminotransferase and alanine aminotransferase increased after 24 hours and 48 hours. Plasma zinc levels decreased after 24 hours and returned to normal levels after 48 hours. The study showed that a single dose of TAA resulted in profound liver damage and supplementation of zinc prior to and simultaneously with TAA maintained the zinc levels in plasma, but did not have any effect on the histological changes (Dashti *et al,* 1987).

Hepatic encephalopathy by TAA induced liver failure affected the activity of two malate- aspartate shuttle enzymes in synaptic and nonsynaptic mitochondria isolated from rat brain. Aspartate aminotransferase was decreased by 26% and malate dehydrogenase by 50% in synaptic mitochondria whereas nonsynaptic mitochondria were not affected (Faff-Michalak and Albrecht, 1991).

Studies with mitochondria isolated from rats treated with TAA for 12 weeks showed a decrease on the respiratory control ratio using either succinate or glutamate plus malate as substrates. The rate of uncoupled respiration was not altered showing that the capacity of the mitochondrial respiratory chain was not affected due to TAA treatment. Oxygen consumption with palmitoyl- coenzyme A and palmitoyl-L-carnitine by isolated mitochondria was increased in TAA treated rats on carnitine palmitoyl transferase-I activity, tissue levels of ketone bodies, carnitine and carnitine esters, β-hydroxybutyrate / acetoacetate ratio were higher in livers of TAA treated rats than in controls indicating an adaptive mechanism for maintaining energy homeostasis under conditions of impaired glucose tolerance (Nozu *et al,* 1992).

Repeated administration of TAA to CD1 mice produced hepatic failure and biochemical and behavioral effects characteristic of hepatogenic encephalopathy. Administration of 5-flouromethylornithine, a selective inactivator of ornithine aminotransferase, significantly reduced mortality and it ameliorated most of the TAA induced pathologic symptoms such as hypothermia, pathologic liver function and amino acid patterns (Sarhan *et al,* 1993).

ETHIONINE

Ethionine, a methionine analogue, induces fatty liver in rats. The mechanism by which ethionine induces fatty liver is thought to be due to the inhibition of synthesis of very low density lipoprotein (Katoh *et al,* 1991).

Chronic administration of ethionine, an alkylating hepatocarcinogen, to rats resulted in a decrease of hepatic drug metabolising enzyme activities and

cytochrome P-450 content (Matsuura *et al,* 1984). Hepatic heme oxygenase, the first enzyme of heme degradative pathway was increased following ethionine administration. The induction of hemeoxygenase due to ethionine leads to the decrease of cytochrome P-450 and drug metabolising activities in the liver.

Cultures of *Saccharomyces cerevisae* grown in the presence of ethionine showed a drastic reduction in oxygen consumption. Ethionine also impaired the incorporation of methionine and leucine into mitochondrial translation products and mitochondria utilised ethionine as a precursor amino acid for protein synthesis. Mitochondrial translational products synthesized in the presence of ethionine rapidly degraded inside the organelle compared to normal proteins synthesized under identical conditions in the mitochondria (Tellez *et al,* 1985).

Studies by Wilson *et al* (1986) revealed that ethionine administration produced partial or total uncoupling of pancreatic mitochondrial respiration. ATP levels decreased by approximately 30-40 % three to four hours after administration of L-ethionine (1 mg / gm body weight) to rats by gastric gavage (Smith *et al,* 1987). ATP levels returned to control values at the end of 8 hours. Hepatic inorganic phosphate levels rose concomitantly with the ATP fall.

The influence of D,L-ethionine (5mM) was tested on hepatocytes isolated from fed or fasted rats. Ethionine induces a decrease in intracellular ATP content both in the cytosol and mitochondria of fed and fasted rats . In fed rats there was increase in glycolysis, decrease in mitochondrial ATP/ADPXPO4 and mitochondrial NAD^+/NADH ratios. In fasted rats there was a decrease in neoglucogenesis from lactate + pyruvate or alanine, decrease in cytosolic ATP/ADPxPO4 and cytosolic NAD^+/NADH ratios (Lavoinne *et al,* 1983).

Studies by Rawson *et al* (1994) have shown that rats given L-ethionine, reduces ATP in the liver by trapping adenosine as S-adenosyl-L-ethionine which stimulates food intake 4 to 8 hrs after administration of ethionine. Liver lactate and pynivate levels were not affected by ethionine treatment while liver glycogen was decreased by 15% in ethionine treated rats.

Medicinal plant : *Phyllanthus fraternus*

P.fraternus is a perennial herb, upto 60 cms in height, occurring as a winter weed throughout the hotter parts of India. Plant, fresh leaves and roots are used for various medicinal purposes.

Properties and uses ascribed for *P.fraternus* in traditional medicine:

The plant is bitter in taste, astringent, stomachic, diuretic and antiseptic. It is used in gastric complaints including dyspepsia, colic, diarrohea and dysentery ; also employed in dropsy and diseases of urinogenital system. The plant is also said to be useful in diabetes. A decoction of the leaves is used as a refrigerant for

scalp; leaves and roots are made into poultice with rice water for application on oedamatous swellings and ulcers. The latex is also applied to offensive sores and ulcers, mixed with oil, it is used in opthalmia. The fresh leaves are also considered as a remedy for jaundice.

Chemical studies of the plant have reported that the leaves of *P.fraternus* contain a number of lignans and alkaloids. Phyllanthin (a bitter constituent) and hypophyllanthin (a non-bitter compound) were isolated from the leaves of *P.fraternus* and identified as lignans (Row *et al*, 1964). Phyllanthin was found to be (+) 3,4,3', 4, 9,9' - hexamethoxy-8,8' -butyrolignan with absolute (8s,8' s) configuration (Row *et al,* 1967). The hexane extract of the leaves gave three additional lignans viz., niranthin, nirtetralin and phyltetralin (Anjaneyulu *et al,* 1973). The distribution of the lignans in the leaves varied considerably with geographic location of the plant (Anjaneyulu *et al,* 1973). The aerial parts of *P.fraternus* yielded four alkaloids, 4-methoxy securinine (phyllanthine) and 4-methoxy-norsecurinine. The ethyl acetate portion of the water soluble fraction of the ethanolic extract of the *P.fraternus* roots yielded two new glycoflavones, which are characterised as 3,5,7-trihydroxy flavonal-4'o-α-L-rhamnopyranoside (Chauhan *et al,* 1977). Another new compound viz., lintetralin was also isolated from *P.fraternus* (Ward *et al,* 1979).

Pharmacological studies on *P.fraternus :*

The aqueous extract of *P.fraternus* leaves was reported to produce hypoglycaemic action in normal as well as alloxan -diabetic rabbits. The extract lowered the blood sugar level even when it was administered one hour after glucose administration. The hypoglycaemic activity of the leaf extract appeared to be higher than that of tolbutamide (Ramakrishnan *et al,* 1982). Petrol extract of *P.fraternus* (whole plant) showed antifungal activity against *Helminthosporium sativum* (Bhatnagar *et al,* 1961). An aqueous extract of the plant inhibits DNA polymerase of wood chuck hepatitis virus (WHV) and binds to the surface antigen of WHV *in vitro* (Venkateswaran *et al,* 1987). *P.fraternus* has been shown to be effective as an adjunct with other siddha drugs in the treatment of jaundice due to infective hepatitis (Ramanan and Sainani, 1961)

OBJECTIVES

OBJECTIVES AND SCOPE OF THE PRESENT STUDY

Mitochondria are important organdies involved in TCA cycle and oxidative phosphorylation. Membrane integrity is essential for the synthesis of ATP by oxidative phosphorylation. Various agents and pathological conditions produce mitochondrial dysfunction which can cause loss of cell function and cell death. Liver is a prominent site for drug metabolism and mitochondria are the initial target organelles within the hepatocyte. In this study, the effect of four hepatotoxins (ie.,CCl4, TAA, ethionine and galactosamine) on mitochondrial function was studied using rat as an experimental model.

CCl_4, TAA, ethionine and galactosamine differ in the mechanism by which they bring about hepatic failure. CCl_4 and TAA reqiure to be converted to toxic metabolites within the liver while galactosamine and ethionine cause toxicity by inducing metabolic deficit.

CCl_4, a notorious hepatotoxin is very effective against adult liver fluke, *Fasicola hepatica* but because of its toxicity its use as an antihelminthic or anaesthtic is discouraged. It is also a widely used solvent in plastic and dry cleaning industry and is a classic model of hepatotoxicity. Thioacetamide is known to retard fruit spoilage and is also widely used to induce cirrhosis and necrosis of the liver. Galactosamine induced liver injury is known to resemnle human viral hepatitis in its morphological and functional features (Keppler *et al*, 1968). Ethionine, an ethyl analog of methionine is a known alkylating hepatocarcinogen and produces a variety of physiological and pathological effects in liver and pancreas of animals.

In this study it was observed that all these hepatotoxins induce toxicity by the initiation of lipid peroxidation. Malondialdehyde (MDA), a secondary product of lipid peroxidation was measured by TBARS, which is an index of membrane damage. As vitamin E (biological antioxidant) was shown to prevent lipid peroxidation both *in vitro* and *in vivo*, it was employed to prevent the toxicity induced by these hepatotoxins. Colchicine which was shown earlier to reverse the experimental and clinical cirrhosis, was used to prevent the CCl_4 induced liver damage. Recent studies in our laboratory have shown that the administration of the aqueous extract of *P.fraternus* along with alcohol prevented most of the alcohol induced liver damages. In this study the ability of £ *fraternus* in protecting against CCl_4 induced liver toxicity was also examined.

Objectives of the study :

1) To study the effect of hepatotoxins on the rate of transfer of electrons through different segments of the electron transport chain. Lipid peroxidation and

phospholipid composition, were also studied to relate the effect of hepatotoxins to the membrane integrity and finally the ability of the system to make ATP.

2) Cytochrome oxidase from control and CCl_4 administered rats was purified to study the kinetic properties and the subunit composition of the enzyme compared to controls.

3) The most important is to find a mechanism to prevent the toxicity that is induced by these hepatotoxins, which has an applied value. For this study, vitamin E, colchicine or an aqueous extract of *P.fraternus* was administered independently along with these hepatotoxins.

MATERIALS AND METHODS

Animals :

All the experiments were performed in colony bred rats, derived from Wistar strain, raised in animal house facility of University of Hyderabad. Rats weighing about 100 + 120 gms were used for the present study. They were given food (balanced pellet food supplied by Hindustan Lever Ltd., India) and water *ad Libitum.* They were maintained in an air conditioned room at 25 + 2° C under natural day and night cycles. The weight of the rats was monitored for atleast 2-4 days before starting the experiment.

Chemicals:

Adenosine di phosphate (ADP), Cytochrome C, L- Glutamic acid, L- Malic acid, Succinic acid, 1-anilino-8-naphthalene sulfonate (ANS), Lactate dehydrogenase (LDH), Malate dehydrogenase (MDH), Nicotinamide adenine dinucleotide (reduced) (NADH), Pyruvate kinase, Phosphoenol pyruvate, Triton X-114, Carbonyl cyanide p-triflouro methoxy phenyl hydrazone (CCCP), D-Galactosamine hydrochloride, L-Ethionine, 2,4- Dinitrophenol (2,4-DNP), 1,1,3,3-Tetra methoxypropane, were obtained from Sigma chemical company, St. Louis,MO, USA. DEAE - Sephacel was purchased from Pharmacia,Sweden. [^{35}S] Methionine (800 Ci/ m mol) was obtained from Amersham, U.K. All other chemicals were obtained from commercial sources in India and were of analytical grade.

Glassware **and** solutions: All the glassware used for the experiments were routinely cleaned by immersion in a hot chromic acid bath. They were thoroughly washed with tap water, rinsed with single and then with double distilled water. Triple distilled water was used for the isolation of mitochondria. The water was boiled and cooled before preparing the isolation medium A and B. Buffers and isolation medium were prepared fresh daily and adjusted to the desired pH.

Preparation of *Phyllanthu.fraternus* extract : The whole plant of *P.fraternus* including roots were homogenised in water (5 gm per 12.5 ml) using a motor and pestle. The homogenate was then filtered through a cheese cloth. An aqueous extract equivalent to 100 mg dry powder of the plant per kg body weight was administered daily to each rat. The dry weight content of the plant was determined after drying the plant in an oven and was found as 25% of the wet tissue.

Preparation and administration of Vitamin E: Vitamin E, commercially available as Dl-α-Tocopherol acetate was diluted (1——>5) with mineral oil. 20 IU/day/rat was given orally with the help of gastric tube for a period of twenty

days. Circulating vitamin E levels were raised by 50% (measured by HPLC) by this treatment.

TREATMENT OF RATS :

Experiments using Carbon tetrachloride (CCl_4) as hepatotoxin :

Controls : received 0.5 ml of mineral oil.

CCl_4 : received CCl_4 (4 g/kg bw) dissolved in 0.5 ml of mineral oil through an intragastric tube and sacrificed 24h after the administration of CCl_4

Vitamin E : received vitamin E (20 IU /day/rat) for 20 days orally, 24h after the last dose of vitamin E, 0.5 ml of mineral oil was administered and sacrificed 24h later.

Vitamin E + CCl_4 : received vitamin E (20 IU /day/rat) for 20 days orally, then CCl_4 (4g/kg bw) dissolved in 0.5 ml of mineral was administered and sacrificed 24h later.

P.fraternus : received an aqueous extract equivalent to 100 mg dry powder of the plant in 0.5 ml of water per kg body weight orally for a period of five days and 24h after the last dose of *P.fraternus,* 0.5 ml of mineral oil was given and sacrificed 24 h later.

P.fraternus + CCl_4 : received an aqueous extract of *P.fraternus* (100 mg powder in 0.5 ml of water /kg bw) orally for five days and then CCl_4 in 0.5 ml of mineral oil (4 g/ kg bw) was given and sacrificed 24h later.

Colchicine : received colchicine (10 µg in 0.2 ml of saline/day/rat) for seven days through an intragastric tube. 24h after the last dose of colchicine, 0.5 ml of mineral oil was given and sacrificed 24 later.

Colchicine + CCl_4 : received colchicine (10 µg in 0.2 ml of saline/day /rat) orally for seven days and after the last dose of colchicine, CCl_4 in 0.5 ml of mineral oil (4 g/kg bw) was given and sacrificed 24h later.

Experiments using Thioacetamide (TAA) as hepatotoxin :

Controls : received 0.2 ml of saline intraperitoneally.

Thioacetamide : TAA (25mg in 0.2ml of saline/100g bw) was administered i.p. for four days and sacrificed 24h later.

Vitamin E : received vitamin E (20 IU /day/rat) orally for 20 days, 24h after the last dose of vitamin E, 0.2 ml of saline was administered i.p. and sacrificed 24h later.

Vitamin E + TAA : received vitamin E for 20 days orally (20 IU /day /rat) then, TAA (25 mg in 0.2 ml saline /100 g bw) was administered i.p. for four days and sacrificed 24h later.

P.fraternus : received an aqueous extract equivalent to 100 mg dry powder of the plant in 0.5 ml of water per kg body weight orally for a period of five days and 24h after the last dose of *P.fraternus,* 0.2 ml of saline was given i.p. and sacrificed 24 h later.
P.fraternus + TAA : received the extract of *P.fraternus* (100 mg in 0.5 ml of water/kg bw) orally for five days and then given TAA (25 mg in 0.2 ml of saline /100 g bw) i.p. for four days and sacrificed 24h later.

Experiments using Galactosamine as hepatotoxin :

Controls : received 0.5 ml of saline intraperitoneally.
Galactosamine : was injected i.p., at a dose of 2.5g in 0.5 ml of saline per kg bw. An aqueous solution of the drug was used after the pH was adjusted to 6.7-7.0 with NaOH. Animals were killed at 12, 24 and 36 hrs after drug administration.

Experiments using Ethionine as hepatotoxin :

Controls : received 0.5 ml saline i.p.
Ethionine : was administered i.p.,(100 mg in 0.5 ml saline /100 g bw) for four days and sacrificed 24h after the last dose.
Vitamin E : received vitamin E (20 IU /day/rat) orally for 20 days, 24h after the last dose of vitamin E , 0.5 ml of saline was administered i.p. and sacrificed 24h later.
Vitamin E + Ethionine : received ethionine (100 mg in 0.5 ml of saline/100 g bw) for four days after the pretreatment with vitamin E (20IU/day/rat) for 20 days and sacrificed 24 h later.

Preparation of liver mitochondria :

Mitochondria were isolated from liver according to the method of Lawrence and Davies (1986). The isolation medium A consists of 70 mM sucrose, 220 mM mannitol, 2 mM HEPES, 0.2 mM EDTA, and 36 mg BSA per 100 ml. The pH was adjusted to 7.4. Isolation medium B consists of 0.25 M sucrose. The experimental rats were killed by stunning and livers were excised and transferred immediately to ice-cold homogenising medium A. All the subsequent operations were carried at 4^0 C. Livers were blotted with filter paper, washed twice with medium A to remove traces of blood. They were then weighed and minced finely. A 10% (w/v) homogenate was prepared in medium A using a Potter-Elvehjem homogeniser with a teflon pestle. The pestle was driven by an electric motor at 3500 rpm and a maximum of 4 up and down strokes were given for complete homogenisation. The 10% homogenate was centrifuged at 700 x g for 10 min in Beckman J2-21 M/E refrigerated centrifuge using JA-20 rotor. The pellet was

discarded and the supernatant was centrifuged at 7000 x g for 10 min. The supernatant thus obtained contains a white fat layer which was removed with a cotton swab. The pellet containing mitochondria was suspended in 0.25 M sucrose and centrifuged at 7000 x g for 10 min. This step was repeated thrice to wash the mitochondria. The final pellet was suspended in medium B to a protein concentration of 15-20 mg/ml and a portion was used immediately for polarographic studies and the other portion was stored in liquid nitrogen for future studies.

Preparation of kidney mitochondria :

Killing of the animal and removal of the tissue was same as described for liver. The kidney capsule was removed gently by squeezing the kidney by the thumb and fore finger. The kidney was cut and the medullary portion was removed and discarded. Mitochondria was then prepared from the cortex portion by the method described for liver.

Protein estimation:

The protein was determined by Biuret method (Gornall *et al*, 1949) with bovine serum albumin as the standard. Deoxycholate was used for solubilisation. 100 µl of mitochondrial suspension (containing 1-2 mg protein) was pipetted out and to this 0.2 ml of 1% deoxycholate was added . The volume was then made upto 1 ml with double distilled water. 4 ml of Biuret reagent was added to this and mixed well. Simultaneously a blank was also run. A standard protein solution was also run which consisted of 100 µl of BSA (10 mg/ml), 0.2 ml deoxycholate, 0.7 ml water and 4 ml of reagent. The tubes were left at room temperature for 30 min and optical density was measured at 540 nm.

Preparation of submitochondrial particles :

Submitochondrial particles were obtained from freshly prepared liver mitochondria according to the procedure of Hackenbrock and Hammon (1975). Mitochondria suspended in 0.25 M sucrose (50 mg protien/ml) were mixed with digitonin (0.12 mg /mg protein) and stirred at 4^0 C for 15 min. The suspension was centrifuged at 12,000 x g for 15 min and the pellet was washed with 1 ml of 0.25 M sucrose. The mitoplasts thus obtained were suspended in ice-cold water (50 mg protein in 25 ml). The suspension was centrifuged at 10,000 x g for 10 min and the pellet was suspended in a small volume (5-7 ml) of ice-cold water and sonicated with MSE ultrasonicator using a microprobe. Three pulses each of 30 seconds duration were given and then centrifuged at 12,000 x g for 10 min. From the supernatant fraction SMP were sedimented by centrifugation at 105,000 x g for

one hour in a Beckman Ultracentrifuge. The pellet containing SMP was suspended in 0.25 M sucrose to a protein concentration of 15-20 mg/ml and used immediately or stored in liquid nitrogen depending upon the parameter that is to be assayed. Protein estimation was done by Lowry method (1951). Recovery of SMP protein was 30% of mitochondrial protein.

Assay of Aminotransferases :

Alanine amino transferase and aspartate amino transferase were assayed in the plasma and liver samples of control and experimental animals. Following decapitation, blood was collected from the jugular vein into a tube containing an anticoagulant, heparin. The blood was centifuged at 3000 rpm for 10 min at room temperature and the supernatant was taken as plasma. A 5% liver homogenate was prepared in ice-cold 0.32 M sucrose using a motor driven Potter - Elvehjem homogeniser with a teflon pestle. Assays of these enzymes were performed in a Shimadzu-160A spectrophotometer. Time periods and enzyme concentrations were adjusted in such a way that a linear curve was obtained for atleast 3-5 minutes.

Aspartate aminotransferase (AAT **E.C.2.6.1.15):**

The method of Bergmeyer and Bernt (1974) was adopted for the assay. The oxaloacetate formed in the reaction was converted to malate using purified malate dehydrogenase (MDH) and the NADH consumed in the course of the reaction, which will be proportional to AAT activity, was measured.

$$\text{L-aspartate} + \alpha\text{-ketoglutarate} \xrightarrow{\text{liver/plasma AAT}} \text{glutamate} + \text{oxaloacetate}$$

The oxaloacetate formed in the reaction was converted to malate using purified malate dehydrogenase (MDH) and the NADH consumed in the course of the reaction, which will be proportional to AAT activity, was measured.

$$\text{oxaloacetate} + \text{NADH} \xrightarrow{\text{commercial MDH}} \text{malate} + \text{NAD}^+$$

Assay mixture : 160 μ moles potassium phosphate, 20 μ moles aspartic acid (pH 7.4), 18 μ moles of α-ketoglutaric acid (pH 7.4), 0.4 μ moles NADH, 1% Triton X-100, 5 μl of MDH (0.5 mg protein/ ml) and 25 μl plasma or 40 μg of liver homogenate in a total volume of 1.0 ml.

The reaction mixture was preincubated for 10 min in the absence of α-ketoglutarate and the reaction was initiated by the addition of a-KG and the decrease in absorbancy due to NADH oxidation was followed at 340 nm for 10

min. Enzyme activity was calculated using a mM extinction coefficient of 6.22 for NADH and is expressed as μ moles NADH oxidised per mg protein per hr.

Alanine aminotransferase (A1AT E.C.1.1.1.42) :

A similar method was adopted for the assay of A1AT (Bergmeyer and Bernt, 1974).

liver/plasma A1AT

alanine + α-ketoglutarate ——————> pyruvate + glutamate

The pyruvate so formed, was converted to lactate in the presence of NADH and purified lactate dehydrogenase (LDH).

commercial LDH

pyruvate + NADH <——————> lactate + NAD^+

Assay mixture : 160 μ moles potassium phosphate pH 7.4, 40 μ moles alanine pH 7.4, 18 μ moles a-ketoglutarate pH 7.4, 0.42 μ moles NADH, 1% Triton X-100, 5 μl of MDH (0.5 mg protein/ml) and 25 μl plasma or 40 μg liver tissue in a total volume of 1.0 ml.

Change in absorbancy due to NADH oxidation was measured at 340 nm for 10 min. Activity was calculated and expressed as mentioned above for AAT activity.

Assay of oxidative phosphorylation :

Polarographic determination of oxidative phosphorylation was carried out according to Estabrook (1967) using a Gilson 5/6 oxygraph fitted with a Clark type electrode. After obtaining a steady base line 2.5-3 mg protein was added to the reaction chamber containing 1.7 ml of buffer (50 mM Tris- Cl, 20 mM potassium phosphate, 2 mM EDTA, 7 mM $MgCl_2$, 50 mM sucrose, pH 7.4). Respiration was initiated by addition of substrate (either succinate or glutamate + malate). State 3 respiration was obtained by addition of ADP and the ADP exhausted respiration was state 4. Respiratory control ratio was calculated from the ratio of state 3 to state 4 respiration. P/O ratio was calculated from the amount of ADP added and the amount of oxygen utilised during the rapid state 3 period. To measure the rate of respiration in the presence of uncoupler, 0.1 μM of CCCP was added.

Succinate oxidase: was measured polarographically using a Clark type oxygen electrode, in a reaction medium containing 50 mM Tris-Cl, 20 mM potassium phosphate, 2 mM EDTA, 7 mM $MgCl_2$, 50 mM sucrose (pH 7.4) and 9 mM succinate. State 3 respiration was measured by adding 150 n moles ADP in a total volume of 1.7 ml containing 2.5 mg mitochondrial protein.

NADH oxidase : NADH cannot be transported across the inner mitochondrial membrane if the mitochondria is tightly coupled. Since externally added NADH cannot be oxidised, glutamate and malate were used to reduce NAD^+ in the matrix to NADH, which then gets oxidised through electron transport chain. NADH oxidase was measured polarographically using glutamate and malate as substrates. The reaction medium was the same as used for succinate oxidase except that 2 mM glutamate and 4 mM malate were used instead of 9 mM succinate as substrates. State 3 respiration was measured by adding 200 n moles ADP.

Cytochrome C oxidase: The enzyme was assayed by following the decrease in absorbance of ferrocytochrome C at 550 nm as described earlier (Cooperstein and Liazarow, 1951). The reaction was initiated by the addition of 1.0 µg mitochondrial protein. (E_{mM} =19.1).

Preparation of reduced cytochrome C : 17 mg of cytochrome C was dissolved in 20 ml of 30 mM potassium phosphate buffer, pH 7.4. It was then reduced by addition of small amounts of sodium dithionite (Yonetani, 1967). Excess sodium dithionite was removed by dialysis against 30 mM phosphate buffer ,pH 7.4 for 10-20 hours with four changes of buffer.

NADH dehydrogenase : [NADH : (acceptor) oxidoreductase,E.C.1.6.99.3]

NADH dehydrogenase was measured using potassium ferricyanide as electron acceptor (King and Robert, 1967). The reaction system contained 250 mM sucrose, 30 mM Tris - Cl, 10 mM potassium phosphate, 5 mM $MgCl_2$, 1 mM KCN, 1 mM $K_3(Fe)CN_6$, pH 7.4 and 20 µg of mitochondrial protein in a reaction volume of 1 ml. The reaction was started by the addition of NADH (1.5 mM) and the rate of reduction of ferricyanide was followed at 420 nm (E_{mM} = 1.0).

Succinate dehydrogenase [Succinate : (acceptor) oxidoreductase, **E.C.1.3.99.1)**

SDH was assayed using DCPIP as electron acceptor (King, 1967). The reaction system was the same as used for NADH dehydrogenase except that potassium ferricyanide was substituted with 1 mM PMS and 70 µM dichlorophenol indophenol. The rate of the reduction of DCPIP was followed at 600 nm . 10 µg of mitochondrial protein was incubated with 10 µl of 0.5 M sodium succinate (pH 7.4) at room temperature for 10 min before assaying SDH activity (E_{mM} = 16.9).

Reverse electron transport :

Energy linked (ATP driven) succinate mediated reduction of NAD^+ by reverse electron transport in submitochondrial particles was assayed essentially as described by Ernster and Lee (1967). The reaction system contained 250 mM sucrose, 50 mM Tris, 5 mM $MgCl_2$, 1 mM KCN, 1 mM NAD^+, 6 mM succinate and 75 µg of submitochondrial particle protein in a total reaction volume of 1 ml. The reaction was started by the addition of 1.25 mM ATP and the rate of reduction of NAD^+ was followed at 340 nm. The increase in absorbance was due to the reduction of NAD^+ to NADH (E_{mM} = 6.22).

Succinate cytochrome C reductase : (Succinate : Ubiquinone oxidoreductase EC.1.9.3.1 and Ubiquinol : ferricytochrome C oxidoreductase EC 1.10.2.2)

The activity of cytochrome C reductase was determined by the modified method of Tsidale (1967). The reaction was carried out in a total volume of 1 ml and consisted of buffer (10 mM KH_2PO_4, 0.8 mM EDTA, 0.25 M sucrose pH 7.4), 5 mM succinate, 1 mM KCN, 2 n moles rotenone and 130 n mole of ferrocytochrome C. The reaction was initiated by adding 20 µg of mitochondrial protein. The reaction was followed for 1 min by recording the increase in absorbance of cytochrome C at 550nm (E_{mM} = 19.1).

NADH oxidase: The activity in SMP from liver was determined by spectrophotometric method (Estabrook,1967). The reaction was carried out in 1 ml buffer (containing 50 mM Tris, 20 mM potassium phosphate, 2 mM EDTA, 7 mM $MgCl_2$ and 50 mM sucrose) adjusted to pH 7.4. 0.5 mM NADH was added to it and mixed well. The reaction was started by addition of 60 µg of submitochondrial protein (E_{mM} = 6.22).

Estimation **of cytochromes** :

The concentrations of cytochromes : b, c, and aa_3 were estimated from difference spectra as described by Williams (1964). For quantitation of the individual cytochromes, the α-band regions of reduced minus oxidised difference spectra were analysed by measuring the peak height at the maximum absorbance relative to a baseline formed by connecting the isosbestic points at 540, 575 and 630 nm for cytochrome c, b and aa_3 respectively.

The reaction system contained 200 mM potassium phosphate pH 7.4, 15 µ M potassium ferricyanide, 4 mg mitochondrial protein and deoxycholate (0.5 mg/mg protein). It was mixed thoroughly and distributed equally into both reference and sample cuvettes. After obtaining a steady base line, the contents of the sample cuvette were reduced with a few grains of sodium dithionite and the

spectrum recorded from 500- 650 run using a Hitachi dual wave length spectrophotometer. The spectrum gave the peaks of cytochrome c and cytochrome aa_3 . The peak due to cytochrome b is not distinct as it is masked by cytochrome c peak.

To obtain the spectrum of cytochrome b, the contents of the reference cuvette were reduced by adding 3 mM sodium ascorbate and 200 μM TMPD. The difference spectrum (dithionite reduced minus ascorbate + TMPD reduced) was recorded.

Wavelength pairs and the mM coefficients used forthe calculation of the quantities of individual cytochromes was as follows :

cytochrome b	E560-575	$= 20.0\ mM^{-1}\ cm^{-1}$;
cytochrome c	E540-550	$= 19.1\ mM^{-1}\ cm^{-1}$;
cytochrome aa_3	E605-630	$= 16.0\ mM^{-1}$ cm"' (von Jagow, 1973).

Assay of Lipid peroxides by Thiobarbituric acid reaction :

Lipid peroxide level was determined in the liver homogenates and mitochondria of control and experimental animals according to the procedure of Ohkawa *et al* (1979). A 10% liver homogenate was prepared in 1.15% KCl by using a Potter-Elvehjem homogeniser. Mitochondria were washed with 1.15% KCl and suspended in the same medium. Protein estimation was done by Biuret method. To 5 mg protein, 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid (adjusted to pH 3.5 with NaOH) and 1.5 ml of 0.67% (w/v) aqueous solution of thiobarbituric acid were added. The total volume was made upto 4.0 ml with distilled water and the tubes heated in a water bath at 95^0 C for 60 min using a marble as condenser. A blank was also run simultaneously and tetra methoxypropane was used as an external standard. After cooling, 1.0 ml of distilled water and 5 ml of n- butanol were added and the tubes were vortexed and then centrifuged at 4000 rpm for 10 min at room temperature. The absorbance of the organic layer was measured at 535 nm. The extent of lipid peroxides was expressed as n mol of MDA formed per 100 mg protein.

ATPase assay :(ATP phosphohydrolase **E.C.3.6.1.3.)**

This assay was carried out according to the method of Veldesma-Currie and Slater (1968). 1 ml of the buffer (consisting of 20 mM Tris, 100 mM KC1, 5 mM $MgCl_2$, 1 mM EDTA) pH 7.8 and about 200 μg of mitochondrial protein was incubated for 5 min at 37^0 C. The reaction was started by the addition of 2.5 mM of ATP (neutralised solution) and was terminated by adding 10% TCA at different time intervals, i.e.,0, 2 and 4 min. For uncoupler stimulated ATPase activity the mitochondria were incubated in a reaction mixture containing 0.1 mM of 2,4 - dinitrophenol. The reaction was terminated at zero time and used as blank. The

reaction mixture was precipitated with TCA and centrifuged at 3000 rpm for 10 min. The inorganic phosphate present in the supernatant was estimated by Fiske-Subbarow (1925) method.

To 1 ml of the supernatant, 4 ml water, 0.4 ml of ammonium molybdate (2.5% dissolved in 3N H_2SO_4) and 0.2 ml ANSA (0.2%) were added and vortexed. After 10 min the color was measured at 640 nm. The Pi released was calculated using a standard.

Mitochondrial Swelling :

About 20-40 µg of mitochondrial protein was suspended in a buffer containing 10 m mol Tris, 50 m mol sucrose, 5 m mol $MgCl_2$,10 m mol KCl and 0.25 m mol ADP. The reaction was started by the addition of succinate (5 m mol/lit). Swelling was followed by change in absorbance at 520 nm in a Shimadzu-160A spectrophotometer (Packer, 1967).

Preparation of tissue extracts for metabolite (ATP, ADP and AMP) assays :

Rats were killed by stunning, liver was excised and transferred immediately to liquid nitrogen and frozen at this temperature for 10 min. Liver was powdered using a stainless steel pestle and mortar (both mortar and pestle were precooled with liquid nitrogen) Tissue powder was rapidly transferred into preweighed tubes containing 3 ml of ice-cold 10% (w/v) perchloric acid and the tubes were weighed again. The powder was dispersed well and homogenised using Potter-Elvehjem homogeniser. Samples were allowed to stand on ice for 15 min and centrifuged at 5000 rpm for 10 min at 4^0 C. The supernatant was neutralised with solid potassium carbonate till pH was 7 to 7.2 and was centrifuged at 10,000 rpm for 10 min at 4^0 C. The supernatant was used for assay of metabolites.

Assay of metabolites : Metabolites present in the neutralised perchloric acid extract were assayed by spectrophotometric method (using commercial enzymes) using a Shimadzu spectrophotometer (Model No UV-160A). Metabolite assays were coupled to an appropriate NAD(P) or NAD(P)H dependent dehydrogenases (in a final volume of 750 µl) and change in absorbancy was recorded at 340 nm till three successive measurements of absorbancy were constant at 25 + 2^0 C. Authentic standards (different concentrations) were used for preparing a standard curve and for the calculation of metabolite concentrations. The method of Lowry and Passonneau (1972) was followed for all metabolite assays.

ATP : Reaction mixture consists of 150 mM Tris-HCl (pH 8.1), 0.1 mM $MgCl_2$, 0.5 mM DTT, 500 µM NADP, 1 mM glucose, 2 µg or 0.28 U/ ml Hexokinase , 0.15 µg or 0.07 U /ml of glucose-6-phosphate dehydrogenase (G6PDH). Reaction

was started with the addition of 25 µl of sample and change in absorbance was followed.

$$\text{ATP} + \text{glucose} \xrightarrow{\text{Hexokinase}} \text{glucose-6-phosphate} + \text{ADP}$$

$$\text{glucose 6-phosphate} + \text{NADP} \xrightarrow{\text{G6PDH}} \text{6-phospho gluconolactone} + \text{NADPH} + \text{H}^+$$

ADP : Reaction mixture consists of 30 mM imidazole -HC1 buffer, pH 7.0, 2 mM $MgCl_2$, 75 mM KC1, 50 µM NADH, 300 µM phosphoenol pyruvate, pyruvate kinase 2 µg or 0.3U/ ml, lactate dehydrogenase 2 µg or 0.4 U/ml. Reaction was started with addition of 25 µl of sample and change in absorbancy was followed at 340 run.

$$\text{ADP} + \text{phosphoenol pyruvate} \xrightarrow{\text{Pyruvate kinase}} \text{ATP} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+$$

AMP : Reaction mixture consists of 30 mM imidazole -HC1 buffer, pH 7.0, 2 mM $MgCl_2$, 75 mM KC1, 100 µM ATP, 50 µM NADH, 300 µM phosphoenol pyruvate, myokinase 1 µg or 0.36 U/ml, pyruvate kinase 2 µg or 0.3U / ml, lactate dehydrogenase 2 µg or 0.4 U/ml. Reaction was started with addition of 25 µl of sample and change in absorbancy was followed at 340 nm.

$$\text{AMP} + \text{ATP} \xrightarrow{\text{Myokinase}} 2\ \text{ADP}$$

$$\text{ADP} + \text{phosphoenol pyruvate} \xrightarrow{\text{Pyruvate kinase}} \text{ATP} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+$$

Measurement of membrane potential $(\Delta\Psi)$ in submitochondrial particles:

The membrane potential $(\Delta\Psi)$ was determined by the distribution of the flourescent probe, 1- anilino-8-napthalenesulfonate (ANS) across the submitochondrial membrane (Azzi, 1971). A flourescence increase of the added flourochromes was observed when energy was supplied to the membranes of the submitochondrial particles, while a decrease in flourescence was observed with intact mitochondria

Submitochondrial particles (SMP) prepared by sonic disruption of rat liver mitochondria were used for this study. The reaction system contained submitochondrial particles (380 µg protein) preincubated for 5 min in a buffered medium (containing 250 mM sucrose, 10 mM Tris- HC1, 5 mM $MgCl_2$, pH 7.5) and 10 µM ANS in a total volume of 1.0 ml. The intensity of flourescence was

measured in a Hitachi F3010 Flourescence spectrophotometer. The excitation and emission wavelengths used were 350 nm and 480 nm respectively. 1 mM succinate was added to the reaction system to energise the membrane and the flourescence change was recorded The addition of succinate induced an increase in ANS flourescence. After the flourescence change reached to a steady state, CCCP (a potent uncoupler) was added (0.3μM) to shift the membrane to de-energised state.

The changes of ANS flourescence in submitochondrial particles in the energised and de-energised state was used for the calculation of membrane potential ($\Delta\Psi$). The internal volume of the submitochondrial particles used for calculation was 2 μl per mg protein (from earlier studies). Membrane potential was calculated using Nernst equation as follows :

at room temperature,

$$\Delta\Psi = -59 \log \left[\frac{C_1}{C_2} + \frac{(C_1 - C_2)\ V}{C_2\ v} \right]$$

C_1 = concentration of ANS inside SMP,
C_2 = concentration of ANS outside SMP,
v = internal volume of submitochondrial particles and
V = external volume outside SMP.

Analysis of lipids in mitochondrial membranes :

Thin layer chromatography (TLC) was used for separation and identification of phospholipids

Preparation of TLC plates :

TLC plates were prepared with Silica Gel G (Merck). 50 gms of silica gel was used for 100 ml of distilled water and the slurry was spread on glass plates with an applicator. The plates were dried at room temperature and activated at 110^0 C for one hour before use. Mitochondrial lipids were extracted by the procedure of Bligh and Dyer (1959). Approximately 6-8 mg of mitochondrial protein was taken in a stoppered test tube to which 4 ml of methanol and 2 ml of chloroform was added and left at room temperature for one hour. To this, 2 ml of chloroform and 2 ml of water were added, mixed and centrifuged at 1000 rpm for 10 min. The bottom chloroform layer was taken into test tubes and applied (about 200 μl) as a streak on TLC plates. The phospholipids were separated by using a solvent system containing chloroform : methanol : water (65:25:4). When the solvent reaches the top, the plates were taken out, dried at room temperature and exposed to iodine vapour to detect various subclasses of phospholipids using

authentic standards. For the quantitation of individual phospholipids corresponding area on the TLC plate was scraped out into test tubes and digested with 1 ml of 60% perchloric acid at 170-180° C (until the sample became colorless) and then the Pi was estimated.

Phosphorous determination :

After digestion, the tubes were cooled to room temperature and 7.5 ml of double distilled water, 0.4 ml of ammonium molybdate (2.5% dissolved in 3N H_2SO_4), 0.2 ml of ANSA (0.2%) were added and mixed well. The tubes were heated in boiling water bath for 10 min. The total volume was made to 10 ml with distilled water. The tubes were centrifuged to sediment silica gel and the color in the supernatant was measured at 640 run along with a suitable standard. Phospholipid phosphorous per mg protein was calculated.

Purification of Cytochrome oxidase :

Cytochrome C oxidase was purified from liver mitochondria according to the method of Kadenbach *et al* (1986).

Step **I : Isolation** of mitochondria :

Mitochondria were isolated according to Lawrence & Davies (1986). Freshly prepared liver mitochondria from control and CCl_4 treated rats were washed once with 200 mM phosphate buffer, pH 7.2 and suspended in the same buffer to a protein concentration of 25 mg/ml.

Step II : Extraction of mitochondria with Triton **X-114** :

Mitochondria were solubilised by the addition of 20% Triton X-114 (v/v). About 5 µl of 20% Triton X-114 per mg of mitochondrial protein was slowly added by stirring at 4^0 C. The mixture was stirred for 2 hrs at 4^0 C and centrifuged at 105,000 x g for 60 min in a Beckman Ultracentrifuge using Ti80 rotor. The supernatant was discarded, the pellet was washed with 200 mM phosphate buffer pH 7.2 and resuspended in a small volume (5-7 ml) of 150 mM phosphate buffer pH 7.2 containing Triton X-100 (5% v/v). The mixture was stirred for 2 hrs at 4 ^{0}C and centrifuged at 105,000 x g for 30 min.

Step HI : Chromatography on DEAE -Sephacel

The DEAE-Sephacel (Pharmacia) column was equilibriated with 50 mM potassium phosphate buffer pH 7.2, containing 0.05% Triton X-100 and the

supernatant obtained in the above step was diluted with three volumes of double distilled water and was layered on the column (1.5 x 15 cm) and washed with two column volumes of the same buffer. The bound protein, cytochrome C oxidase was eluted with 200 mM potassium phosphate buffer, pH 7.2, containing 0.05% Triton X-100.

Step IV : Fractionation **with ammonium** sulphate

The fractions containing the protein (by checking OD_{280}) were pooled and solid sodium cholate was added (1% w/v). A neutralised saturated (0°) ammonium sulphate was slowly added under stirring until a persistent turbidity was formed (25 -27% saturation) and the solution was kept overnight at 0° C. The solution was centrifuged for 15 min at 12,000g and the supernatant obtained was subjected to ammonium sulphate fractionation (38%). After 30 min the solution was centrifuged for 15 min at 12,000 x g and the precipitate containing cytochrome oxidase was suspended in a small volume of buffer (containing 0.25 M sucrose, 10 mM Tris-HCl, 2 mM EDTA adjusted to pH 7.4). The ammonium sulphate was removed by extensive dialysis and protein was estimated by Lowry method (1951).

SDS-PAGE was performed according to the method of Laemmli (1970). The subunits were separated on a 12% gel using 0.025 M Tris, 0.192 M glycine and 0.1% SDS (pH 8.3) as electrode buffer. The protein solution was mixed with an equal volume of 2X sample buffer (containing 0.125 M Tris- HC1 (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol and 0.002 % bromophenol blue). The sample was denatured at 100^0 C for five min. Electrophoresis was carried out at a constant voltage of 120 volts until the tracking dye reached 1 cm above the base of the resolving gel.

Native gel electrophoresis was carried out on a 5% gel under the same conditions except that SDS was not included in the sample and electrode buffer.

Silver staining of the gel was done according to the method of Bloom *et al* (1987). The gel was fixed in a fixative solution (50% methanol, 12 % acetic acid and 50 µl of 37% formaldehyde / 100 ml) for one hour. The gel was then rinsed with double distilled water and placed in 50% ethanol for 20 min and treated with sodium thiosulphate (0.02 %) for one min and then washed extensively with double distilled water. The gel was then impregnated with 0.2% silver nitrate (containing 75 µl of formaldehyde per 100 ml) for 20 min and then rinsed with double distilled water. The gel was developed using sodium carbonate (6% Na_2CO_3, 50 µl of formaldehyde per 100 ml) for 10 min and was washed with double distilled water. The reaction was stopped by placing the gel in 12% acetic acid.

Protein synthesis in isolated mitochondria :

Mitochondria were isolated according to Lawrence & Davies (1986). The isolated mitochondria were suspended in 0.25 M sucrose **to** a protein concentration of 20-25 mg /ml. Protein estimation was done by Biuret method (Gornall *et al,* 1949).

Isolated rat liver mitochondria were radiolabeled with [^{35}S] methionine essentially as described by Hofmann and Hosein (1978) with modifications suggested by Mills *et al* (1983). Mitochondria were incubated with 20 μ Ci [^{35} S] methionine at 30° C in a mixture containing 50 mM Tris-HCl pH 7.4, 90 mM KCl, 2 mM EDTA, 5 mM potassium phosphate, 10 mM ATP, 10 mM $MgCl_2$, 5 mM phosphoenol pyruvate, 50 μg/ml pyruvate kinase, 20 mM of each amino acid (except methionine), 500 μg cycloheximide and 100 mg mitochondrial protein in a total volume of 50 pi The pH of the reaction mixture was 7.5.

Analysis of [^{35}S] methionine incorporation : About 5 μl aliquot of the ***in*** *vitro* reaction mixture was spotted on Whatman No.540 filters (24 mm diameter and numbered with soft carbide pencil) at different time intervals i.e., 0,5,10,15,20,25 min. These filters were air-dried for five min and then placed in 10% (w/v) TCA for 30 min. These filters were then transferred into a beaker containing 5% TCA and heated at 90^0 C for 5 min and then rinsed in 5% TCA kept at room temperature, washed in absolute ethanol followed by acetone wash. The filters were air-dried and the radioactivity incorporated was counted in a liquid scintillation counter.

Polyacrylamide gel electrophoresis **and autoradiography** :

Mitochondrial proteins were separated according to molecular weight on a 10% gel by using the Tris-glycine, SDS-polyacrylamide gel system of Laemmli (1970). Incorporation of [^{35}S] methionine was followed by autoradiography by exposing the gel to X-ray film for three months.

Statistical Analysis of Data :

Computer programmes were used for calculating P values and to assess the statistical significance of the changes obtained in the experiments by Student's t' test. Probability values (P) less than 0.05 were considered statistically significant. All the data were expressed as means + SD.

RESULTS

The results of some parameters in this study are calculated and expressed in more than one way. This is explained here taking the example of cytochrome oxidase (Table 2). The activity of the enzyme is given as n moles of cytochrome oxidised per min per mg protein. This value for control group, CCl_4 group and *P.fraternus*+ CCl_4 group is 1702, 836 and 1303 respectively. Parameters which were affected by the hepatotoxin to a significant level and which are important for discussion are expressed relative to control, which is taken as 100 (i.e., percent control). So the same data is expressed as 100, 49 and 77 (Table 37) for the three groups (mentioned above) respectively. The activity of cytochrome oxidase in CCl_4 group is only 49% of the control group i.e., it was inhibited by (100-49) 51% by CCl_4 treatment. Similarly the treatment of *P.fratemus* prior to CCl_4 administration has shown a protective effect. The inhibition on cytochrome oxidase was only 33% (100-77) compared to 51% with CCl_4 alone. So part of the CCl_4 induced inhibition was relieved by *P.fratemus.* When the inhibition totally disappears, then we call it as 100% relief or recovery or protective effect. If the inhibition was partly relieved as seen above, then the percent relief was calculated as follows. Amount of inhibition that was to be relieved to give 100 percent relief was 51%. The amount of inhibition that was observed in *P.fraternus* + CCl_4 group was 23 and the relief was 28. The percent relief was calculated as 100/51 x 28 which is equal to 55 (Table 37). This means, *P.fratemus* can relieve 55% of the CCl_4 induced inhibition on cytochrome oxidase. The percent relief was calculated to compare the ability of different protective agents in relieving the inhibition on the same parameter.

CARBON TETRACHLORIDE :

In the present study the effect of carbon tetrachloride (4g/kg bw) on mitochondrial function, the protective effect of vitamin E, colchicine and an aqueous extract of *Phyllanthus fraternus* against its toxicity was studied. Activities of various enzymes of mitochondrial electron transport chain namely NADH oxidase. succinate oxidase, cytochrome oxidase, NADH dehydrogenase and succinate dehydrogenase have been determined. The effect was also studied on cytochrome and phospholipid content of mitochondria and membrane potential (AT) in SMP.

Studies on oxidative phosphorylation :

NADH oxidase (through **glutamate** and **malate)**

Fig. 1 shows the effect of administration of CCl_4 and the protective effect of vitamin E on state 3 respiration, respiratory control ratio (RCR) and P/O ratio.

Externally added NADH cannot penetrate the tightly coupled mitochondria. So, glutamate and malate were used to reduce the internal NAD^+ pool and generate NADH in the matrix which gives electrons to the respiratory chain. Studies on this enzyme complex gives the information on the ability of transfer of electrons through all the three sites. The P/O ratio is the index of the efficiency of the system to conserve energy in the form of ATP, while RCR gives an index of the integrity of the mitochondrial membrane.

In CCl_4 treated group state 3 respiration was decreased by 93% when compared to controls. RCR decreased by 81% while P/O ratio was completely abolished in the CCl_4 treated group (Fig. 1). Addition of CCCP (uncoupler) stimulated respiration and gave an RCR of 7.0 + 0.2 in controls. In CCl_4 treated rats such stimulation was not observed on addition of CCCP showing that the mitochondria are uncoupled.

Administration of vitamin E prior to CCl_4 treatment relieved the inhibition (induced by CCl_4) on rate of respiration, RCR and P/O ratio by 84%, 57% and 94% respectively. Vitamin E alone did not have any significant effect on these parameters.

Fig. 2 shows the effect of administration of CCl_4 along with an aqueous extract of *P.fraternus*. *P.fraternus* by itself has no significant effect either on rate of respiration, RCR and P/O ratio. Pretreatment of rats with an aqueous extract of *P.fraternus* before the administration of CCl_4 improved the mitochondrial function to a large extent. The amount of inhibition (induced by CCl_4) relieved was 87% on rate of respiration, 48% on RCR and 94% on P/O ratio.

Fig. 3 shows the protective effect of colchicine on CCl_4 treatment. Prior administration of colchicine (10 μg/day rat) for seven days showed a protective effect on mitochondrial functions to a large extent. From the Fig. 3 it can be seen that colchicine by itself had a small effect on rate of respiration (10%), RCR (11%) and P/O (4%) ratio. Pretreatment of rats with colchicine relieved the inhibition (induced by CCl_4) by 81% on rate of respiration ,74% on RCR and 94% on P/O ratio.

Studies on Succinate oxidase :

Fig. 4 shows the effect of CCl_4 treatment on the rate of respiration, P/O ratio and RCR using succinate as the substrate.

The study on this enzyme complex gives information on the effect of CCl_4 treatment on the transfer of electrons from succinate to molecular oxygen through site II and site III. The results given in Fig. 4 shows that there is a significant decrease (65%) in the rate of respiration in rats 24 hours after CCl_4 treatment compared to controls. RCR decreased by 78% while P/O ratio was completely abolished by CCl_4 treatment. In the presence of CCCP, control rats showed a

RCR of 4.4+0.2 while in CCl_4 treated rats such stimulation was not seen showing that the mitochondria were uncoupled.

Fig. 4 also shows the protective effect of vitamin E on CCl_4 induced changes. Vitamin E alone did not show any effect on rate of respiration, RCR and P/O ratio. In the group in which vitamin E was given prior to CCl_4 treatment, it was observed that the inhibition induced by CCl_4 was relieved by 74% on state 3 respiration, 67% on RCR and 84% on P/O ratio.

Fig. 5 shows the effect of CCl_4 with or without the prior administration of the aqueous extract of *P.fraternus*. *P.fraternus* alone had showed small effect on rate of respiration (8%), RCR (10%) and P/O ratio (10%). The inhibition was very small that one can expect a variation of this magnitude within the group. In the group in which *P.fraternus* was given prior to CCl_4, it was seen that inhibition induced by CCl_4 was relieved to a large extent. The amount of inhibition relieved was 68% on state 3, 58% on RCR and 78% on P/O ratio.

Fig.6 shows the protective effect of colchicine on CCl_4 induced changes on mitochondrial function. Colchicine by itself had a small effect on rate of respiration, RCR and P/O ratio. In rats pretreated with colchicine before the administration of CCl_4, most of the inhibition induced by CCl_4 was relieved. The amount of inhibition relieved was 75% on rate of respiration, 61% on RCR, and 88% on P/O ratio.

Studies on **NADH** dehydrogenase, succinate dehydrogenase, cytochrome oxidase and succinate cytochrome C reductase :

Studies on NADH dehydrogenase gives information on the transfer of electrons from NADH to the prosthetic group of flavoprotein (FP 1) [E-FMN to E-FMNH2]. Study of succinate dehydrogenase gives information on the transfer of electrons from succinate to the prosthetic group of the flavoprotein (FP 2) [E-FAD to E-FADH2]. The study on cytochrome oxidase, the third segment of the electron transport chain will give information about the electron carriers from ferrocytochrome C to molecular oxygen. Studies on succinate cytochrome C reductase gives information about site 2 of the electron transport chain.

Table 1 shows the effect of CCl_4 administration with or without a pretreatment with either vitamin E or *P.fraternus* or colchicine on NADH dehydrogenase and succinate dehydrogenase. From this table it can be seen that the activity of NADH dehydrogenase and succinate dehydrogenase in CCl_4 treated rats was decreased by 35% and 76% respectively. Administration of vitamin E or an aqueous extract of *P.fraternus* or colchicine did not show any significant effect on these enzymes. Prior treatment with vitamin E or *P.fraternus* or colchicine relieved the inhibition (induced by CCl_4) to a large extent. The amount of inhibition relieved was 60%, 71% and 86% by vitamin E, *P.fraternus* and colchicine respectively on NADH dehydrogenase. CCl_4 induced inhibition

on succinate dehydrogenase was relieved completely by vitamin E (121%), while 99% and 87% recovery was observed with *P.fraternus* and colchicine.

Table 2 shows the effect of CCl_4 with or without pretreatment with either vitamin E or *P.fraternus* or colchicine on succinate cytochrome C reductase and cytochrome oxidase. Administration of vitamin E or *P.fraternus* or colchicine did not show any significant effect on the above parameters. In CCl_4 treated rats the succinate cytochrome C reductase activity was decreased by 68%. Administration of vitamin E or *P.fraternus* or colchicine prior to CCl_4 treatment relieved the inhibition (induced by CCl_4) by 71%, 63%, and 74% respectively on succinate cytochrome C reductase. Cytochrome oxidase activity was decreased by 51% in CCl_4 treated rats. Inhibition on cytochrome oxidase was relieved completely with vitamin E pretreatment while 55% and 76% relief was observed for the pretreatment with *P.fraternus* or colchicine respectively.

Recovery Studies : The recovery studies were carried out to examine how the mitochondria that was damaged by CCl_4 recover on their own over a period of time. For this study rats were allowed to recover on their own for 48h and 96h after the administration of a single dose of CCl_4 . A progressive recovery was observed. NADH oxidase was recovered by 48% and 78% (at the end of 48h and 96h) and succinate oxidase by 38% and 60% at the end of 48h and 96h respectively. At the end of 48h succinate dehydrogenase was recovered by 52% and cytochrome oxidase by 10% while no recovery was observed on NADH dehydrogenase. Succinate dehydrogenase and cytochrome oxidase were recovered completely , and NADH dehydrogenase showed only a 7% recovery even at the end of 96h (data not shown).

Studies on submitochondrial particles : Table 3 gives the effect of CCl_4 treatment on different enzymes in submitochondrial particles. NADH oxidase, NADH dehydrogenase, succinate dehydrogenase and cytochrome oxidase were decreased significantly (50%, 17%, 53% and 45% respectively). In order to see the effect of CCl_4 administration on electron carriers present at site I of the respiratory chain, its effect on energy linked succinate mediated reduction of NAD^+ in sonic submitochondrial particles was assayed. Succinate was the electron donor and NAD^+ was the electron acceptor, ATP was added to the reaction system to provide energy necessary for the reverse electron transport. The activity of this enzyme decreased significantly (by 64%) in CCl_4 treated rats compared to controls. Membrane potential (AT) was also decreased significantly (by 31%) in CCl_4 treated rats.

Studies on ATPase activity :

In tightly coupled mitochondria the ATPase activity is very low and when an uncoupler is added there is a stimulation in the enzyme activity. The activity of the enzyme was expressed as μg Pi liberated per min per mg protein and was measured in the presence and absence of an uncoupler, 2 4-dinitrophenol (2 4-DNP).

Table 4 gives the percent stimulation with 2,4-DNP in controls, CCl_4 treated with or without a pretreatment of vitamin E. Addition of 2,4-DNP to control rats showed a 192% stimulation in ATPase while the CCl_4 treated rats showed only a 20% stimulation. Pretreatment of rats with vitamin E alone showed stimulation similar to controls (165%). In rats pretreated with vitamin E prior to CCl_4 administration, a 173% stimulation in ATPase activity was observed (almost close to control levels). The stimulation on ATPase which was lost by CCl_4 treatment was recovered by pretreatment with vitamin E.

Lipid peroxidation level : Table 5 shows the effect of CCl_4 alone or in the presence of high levels of vitamin E or pretreatment with the extract of *P.fraternus* on lipid peroxide level in liver homogenate and mitochondria. CCl_4 administration increased the lipid peroxide level by 139% and 131% in homogenate and mitochondria respectively. Administration of vitamin E or *P.fraternus* did not show any significant effect on lipid peroxide level, but when CCl_4 was administered after the pretreatment of vitamin E or *P.fraternus*, they largely suppressed the CCl_4 induced lipid peroxide level compared to controls. Presence of vitamin E along with CCl_4 brings down the stimulation in peroxide level (induced by CCl_4) from 139% and 131% to 49% and 45% in homogenate and mitochondria respectively. Pretreatment with *P.fraternus* extract prior to CCl_4 administration reduces the CCl_4 induced stimulation in peroxide level from 139% and 131% to 16% and 18% in homogenate and mitochondria respectively.

Cytochrome Content : The cytochromes : aa_3 , b and c were measured by difference spectroscopy using a double beam spectrophotometer.

Table 6 gives the effect of administration of CCl_4 with or without a pretreatment with either vitamin E or *P.fraternus* or colchicine on the cytochrome content of liver mitochondria. The concentration of cytochrome aa_3 was decreased significantly (34%) in CCl_4 treated rats when compared to controls. The cytochrome b and c concentrations were not significantly affected with CCl_4 treatment. Administration of vitamin E or *P.fraternus* or colchicine alone did not show any significant change on the concentration of cyt aa_3 , b and c. CCl_4 induced inhibition on cyt aa_3 was relieved completely by pretreatment with vitamin E. In rats pretreated with *P.fraternus* or colchicine the inhibition (induced by CCl_4) on cyt.aa3 was relieved by 62% and 50% respectively.

Phospholipid composition : Table 7 shows the effect of CCl_4 with or without a pretreatment with either vitamin E or *P.fraternus* on phospholipid composition of mitochondria. In CCl_4 treated rats phosphatidylcholine(PC), phosphatidyl ethanolamine (PE) and cardiolipin (CL) decreased significantly (38%, 47% and 58% respectively). In the group in which vitamin E alone was given the decrease in PC, PE and CL (8%, 4% and 18% respectively) was not significant. In rats pretreated with vitamin E prior to CCl_4 , the amount of inhibition (induced by CCl_4) was relieved by 26%, 47% and 60% on PC, PE and CL respectively. In the group in which *P.fraternus* alone was administered PC decreased by 8% while PE and CL were increased by 5% and 12% respectively but it was statistically insignificant. In rats pretreated with aqueous extract of *P.fraternus* followed by CCl_4 the inhibition (induced by CCl_4) was relieved by 37% for PC, 64% for PE and 33% for CL.

Adenine nucleotides : Table 8 shows the effect of CCl_4 treatment on the levels of ATP, ADP, AMP and energy charge of the cell. In CCl_4 treated rats the ATP content decreased significantly (42%) accompanied by a significant increase in AMP levels (183%). The ADP levels remained unchanged. Energy charge of the cell was decreased significantly (27%) in CCl_4 treated rats.

Transaminases : Table 9 gives the activity of alanine and aspartate amino transferases in control and CCl_4 treated rats in both liver and plasma. The activity of alanine aminotransferase decreased by 78% in the liver whereas three fold (344%) increase was observed in the plasma . Aspartate aminotransferase decreased by 63% in the liver accompanied by a nine fold increase in the plasma (860%).

Studies on kidney mitochondria : Table 10 gives information about the effect of CCl_4 on state 3, state 4, RCR and P/O ratio. NADH oxidase was estimated using glutamate and malate as substrates while succinate was used as substrate for succinate oxidase.

NADH oxidase: State 3 and state 4 respiration was decreased significantly (about 70%) by CCl_4 treatment. RCR and P/O ratio decreased by 30% and 73% respectively in CCl_4 treated rats when compared to controls.

Succinate oxidase: State 3 respiration decreased by 84% and state 4 by 50% in CCl_4 treated rats while RCR and P/O ratio were inhibited by 66% and 100% respectively.

Table 11 shows the activities of NADH dehydrogenase, succinate dehydrogenase and cytochrome oxidase in control and CCl_4 treated rats. The activities of NADH

dehydrogenase, succinate dehydrogenase and cytochrome oxidase decreased by 47%, 43% and 31% respectively in CCl_4 treated rats.

Studies on mitochondrial swelling : Swelling was monitored by decrease in absorbance as a function of time. Fig.7 gives the effect of CCl_4 , thioacetamide and ethionine on mitochondrial swelling. Mitochondria from animals treated with CCl_4 , TAA and ethionine showed a significant swelling when compared to controls.

Studies on *in Vitro* protein synthesis : Fig.8 shows effect of administration of CCl_4 on the incorporation of $[^{35}S]$ methionine into mitochondrial protein products *in vitro.* In CCl_4 treated rats there was a significant decrease (about 50%) in the incorporation of radiolabeled methionine into mitochondrial protein products (at different time intervals) when compared to controls.

In order to see if there is any difference in the synthesis of mitochondrial proteins in control and CCl_4 treated rats the $[^{35}S]$ methionine labeled mitochondrial protein was separated by SDS-PAGE and subjected to autoradiography. The results in Fig.9 show that in CCl_4 treated rats there was a decrease in the synthesis of proteins with molecular weight of approximately 62 kD.

Purification of cytochrome oxidase : The enzyme was purified on a DEAE-Sephacel column from control and CCl_4 treated rats. The purity of the preparation was checked by measuring the heme a content and enzyme activity. The content of heme a in mitochondria and purified cytochrome oxidase was 0.21 and 4.1 n mol per mg protein. A purification of 19 fold was achieved by this method (Table 12).

Kinetic properties of the enzyme was studied in control and CCl_4 treated rats using reduced cytochrome C as substrate. At lower temperature i.e., 15° C and 25° C there was no significant difference in K_m and V_{max} of control and CCl_4 treated rats. At 37° C the K_m increased by 60% (from 2.5 μM to 4.0 μM) and V_{max} decreased by 44% (from 22000 to 12,320 n moles per min per mg protein) in CCl_4 treated rats (Fig. 10).

The purity of the isolated protein was confirmed from native gel electrophoresis in which a single band was obtained (Fig. 11). The polypeptide composition on SDS-PAGE was performed using the enzyme protein from control and CCl_4 treated rats. Fig. 12 gives the polypeptide composition of cytochrome oxidase in control and CCl_4 treated rats. Quantitative measurement by scanning densitometry showed that in CCl_4 treated rats there was a significant decrease (about 60%) in polypeptides 1, 2, 3 and 6 while polypeptides 4 and 5 were significantly increased (64% and 196% respectively).

Experimental details :

Control group received mineral oil.

CCl_4 group received one dose of CCl_4 (4g/kg bw).

Vitamin E group received vitamin E (20IU/day/rat) for 20 days.

Vit.E + CCl_4 group received CCl_4 after a pretreatment with vitamin E.

Results are expressed as percent control. About 2.5 mg protein was used for each assay. Glutamate and malate were used to reduce matrix NAD^+ to NADH. State 3 (ADP added) respiration was calculated as ng atom of oxygen per min mg protein.

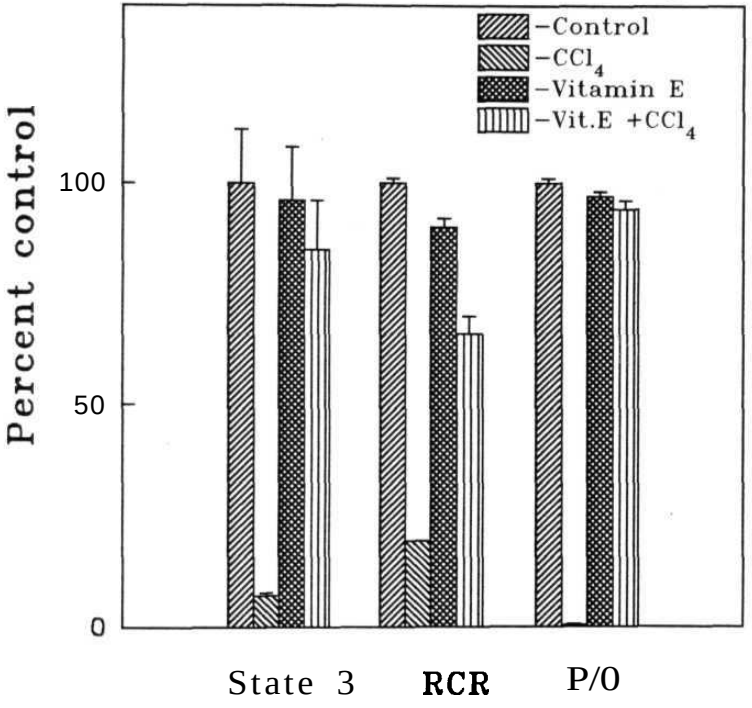
Respiratory control ratio (RCR) is the ratio of ADP stimulated to ADP exhausted respiration.

P/O = ADP/O ratio.

All the values are expressed relative to control which was taken as 100. The control value for state 3_{ADP} respiration, RCR and P/O ratio was 73 + 9, 5.2 + 0.24 and 3.03 + 0.02 respectively.

Values are Mean + SD of atleast 12 animals.

Fig.1 Effect of administration of CCl_4 with or without pretreatment with vitamin E on NADH oxidase.



Experimental details :

Control group received mineral **oil.**

CCl_4 group received one dose of CCl_4 (4g/kg bw)

P.fraternus group received the extract of *P.frat ernus* (100mg/dry powder / kg bw) for five days.P.frat + CCl_4 group received CCl_4 after a **pretreatment** with *P.fraternus.*

Results are expressed as percent control. About 2.5 **mg** protein was used for each assay. **Glutamate** and **malate** were used to reduce matrix NAD^+ to NADH. State 3 (ADP added) respiration was calculated as ng atom of oxygen per **min** mg protein.

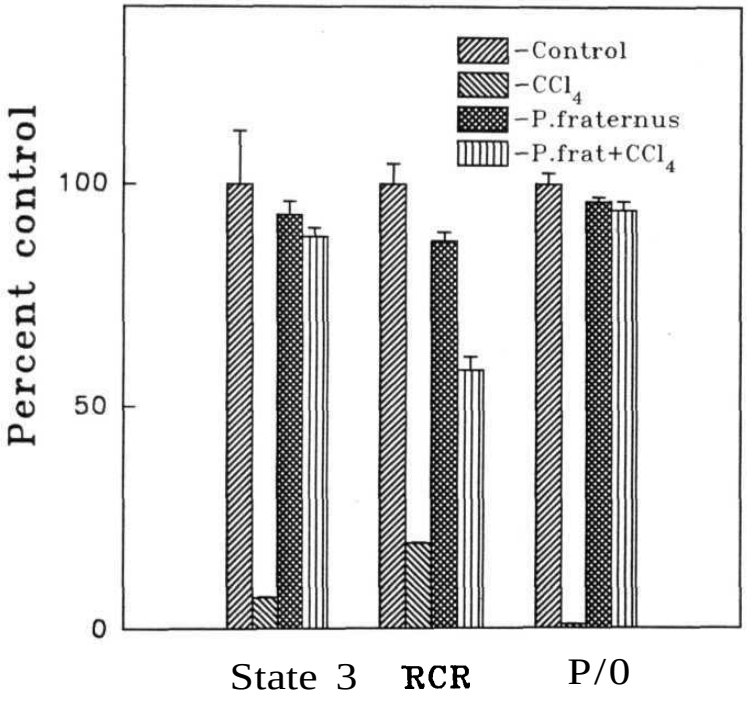
Respiratory control ratio (RCR) is the ratio of ADP stimulated to ADP exhausted respiration.

P/O = ADP/O ratio.

All the values are expressed relative to control which was taken as 100. The control value for state 3_{ADP} respiration, RCR and P/O ratio was 73 + 9, 5.2 + 0.24 and 3.03 + 0.02 respectively.

Values are mean + SD of atleast 8 animals.

Fig 2 Effect of administration of CCl_4 with or without pretreatment with P.fraternus on NADH oxidase.



Experimental details :

Control group received mineral oil.

CCl_4 group received one dose of CCl_4 (4g/kg bw).

Colchicine group received colchicine (10 μg/day/rat) for seven days.

Colc.+ CCl_4 group received CCl_4 after a pretreatment with colchicine.

Results are expressed as percent control. About 2.5 mg protein was used for each assay. Glutamate and malate were used to reduce matrix NAD^+ to NADH. State 3 (ADP added) respiration was calculated as ng atom of oxygen per min mg protein.

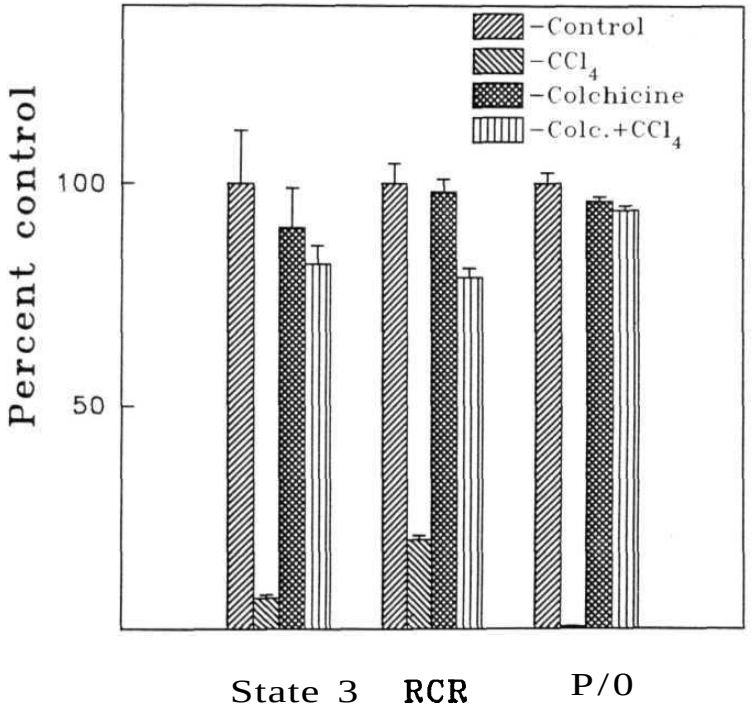
Respiratory control ratio (RCR) is the ratio of ADP stimulated to ADP exhausted respiration.

P/O = ADP/O ratio.

All the values are expressed relative to control which was taken as 100. The control value for state 3_{ADP} respiration, RCR and P/O ratio was 73 + 9, 5.2 + 0.24 and 3.03 + 0.02 respectively.

Values are Mean + SD of atleast 8 animals.

Fig.3 Effect of administration of CCl_4 with or without pretreatment with Colchicine on NADH oxidase.



Experimental details :

Control group received mineral oil.

CCl_4 group received one dose of CCl_4 (4g/kg bw).

Vitamin E group received vitamin E (20IU/day/rat) for 20 days.

Vit.E + CCl_4 group received CCl_4 after a pretreatment with vitamin E.

Results are expressed as percent control. About 2.5 mg protein was used for each assay. Succinate was used as the substrate. State 3 (ADP added) respiration was calculated as ng atom of oxygen per min mg protein.

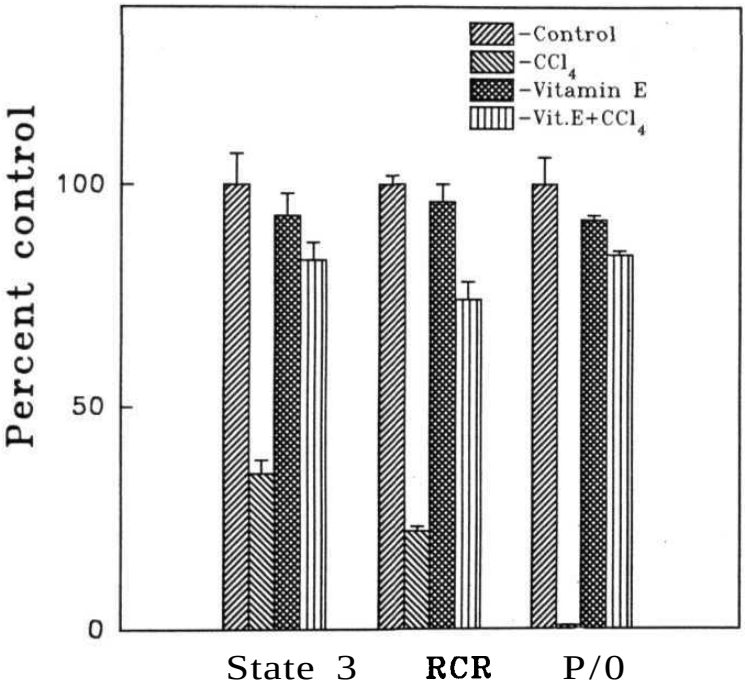
Respiratory control ratio (RCR) is the ratio of ADP stimulated to ADP exhausted respiration.

P/O = ADP/O ratio.

All the values are expressed relative to control which was taken as 100. The control value for state 3_{ADP} respiration, RCR and P/O ratio was 82 + 6, 4.6 + 0.1 and 2.1 + 0.02 respectively.

Values are Mean + SD of atleast 12 animals.

Fig.4 Effect of administration of CCl_4 with or without pretreatment with vitamin **E on Succinate** oxidase.



Experimental details :

Control group received mineral oil.

CCl_4 group received one dose of CCl_4 (4g/kg bw).

P.fraternus group received the extract of *P.fraternus* (100mg/dry powder /kg bw) for five days.

P.frat + CCl_4 group received CCl_4 after a pretreatment with *P.fraternus.*

Results are expressed as percent control. About 2.5 mg protein was used for each assay. Succinate was used as the substrate.State 3 (ADP added) respiration was calculated as ng atom of oxygen per min mg protein.

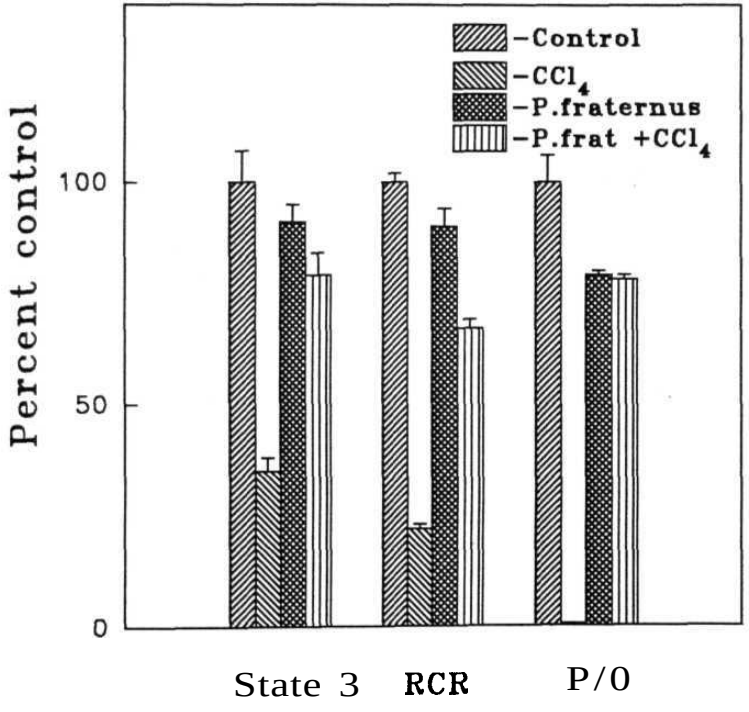
Respiratory control ratio (RCR) is the ratio of ADP stimulated to ADP exhausted respiration.

P/O = ADP/O ratio.

All the values are expressed relative to control which was taken as 100. The control value for state 3_{ADP} respiration, RCR, ADP and P/O ratio was 82 + 6, 4.6 + 0.1 and 2.1 + 0.02 respectively.

Values are Mean + SD of atleast 8 animals.

Fig.5 Effect of administration of CCl_4 with or without pretreatment with P.fraternus on Succinate oxidase.



Experimental details :

Control group received mineral oil.

CCl_4 group received one dose of CCl_4 (4g/kg bw).

Colchicine group received colchicine (10μg/day/rat) for seven days.

Colc.+ CCl_4 group received CCl_4 after a pretreatment with colchicine.

Results are expressed as percent control. About 2.5 mg protein was used for each assay. Succinate was used as the substrate. State 3 (ADP added) respiration was calculated as ng atom of oxygen per min mg protein.

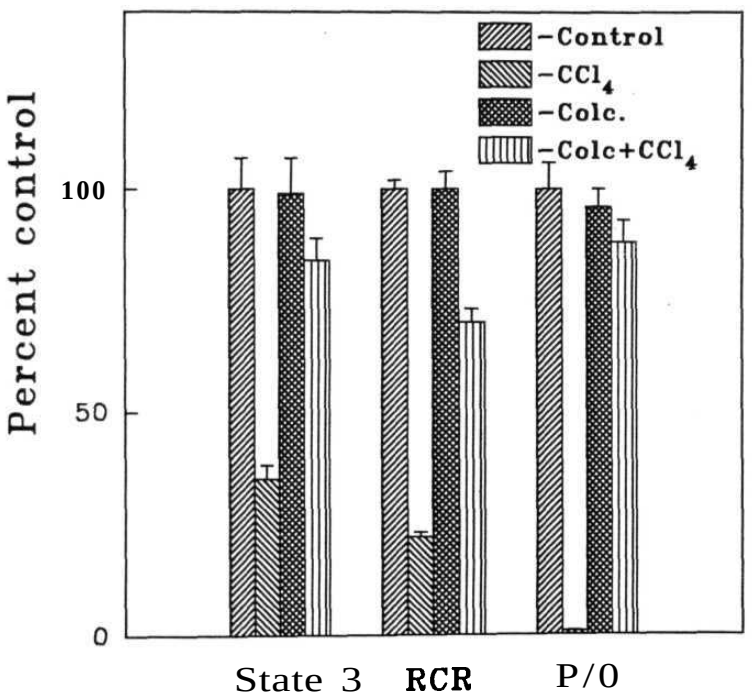
Respiratory control ratio (RCR) is the ratio of ADP stimulated to ADP exhausted respiration.

P/O = ADP/O ratio.

All the values are expressed relative to control which was taken as 100. The control value for state 3_{ADP} respiration, RCR and P/O ratio was 82 + 6, 4.6 + 0.1 and 2.1 + 0.02 respectively.

Values are Mean + SD of atleast 8 animals.

Fig.6 Effect of administration of CCl_4 with or without pretreatment with Colchicine on Succinate oxidase.



Experimental details :

Controls received saline/ oil.

CCl_4 group received one dose of CCl_4 (4 g/kg bw).

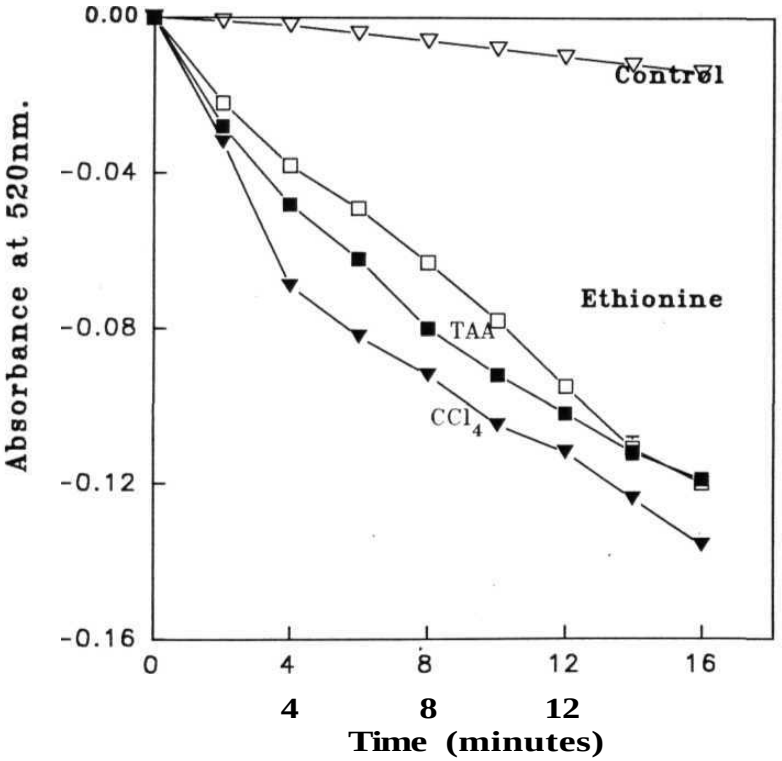
TAA group received thioacetamide (25mg/ 100 g bw) for four days.

Ethionine group received ethionine (100 mg/100 g bw) for four days.

About 40 µg per ml of mitochondria was suspended in a buffered medium. Respiration was initiated by addition of 50 m mol succinate and the decrease in OD was followed at 520 nm.

Values are Mean + SD of atleast four animals.

Fig.7 Effect of CCl_4, Thioacetamide and Ethionine on the swelling of mitochondria.



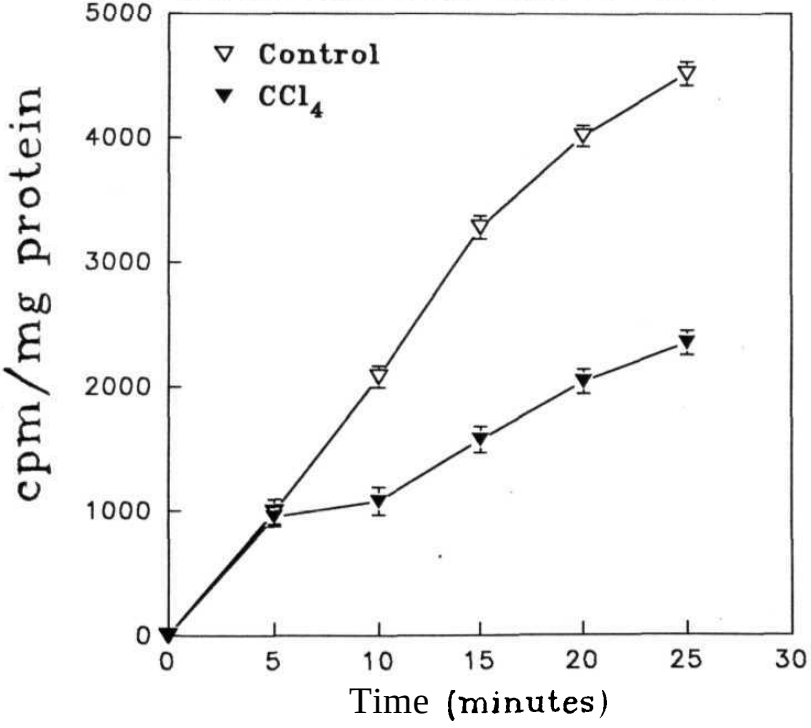
Experimental details :

Control group received oil.

CCl_4 group received one dose of CCl_4 (4 g/kg bw).

100 mg protein was incubated with ^{35}S methionine and its incorporation determined. Each point represents the average radioactivity incorporated per mg mitochondrial protein from four animals.

Fig.8 Effect of administration of CCl_4 on the incorporation of [^{35}S] methionine into mitochondrial protein products in vitro.



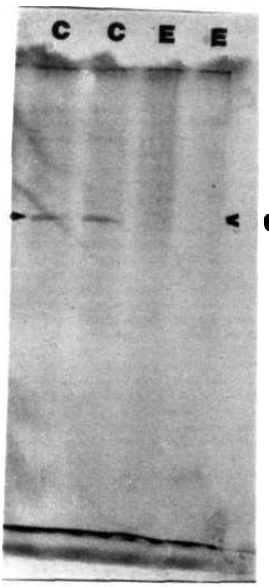
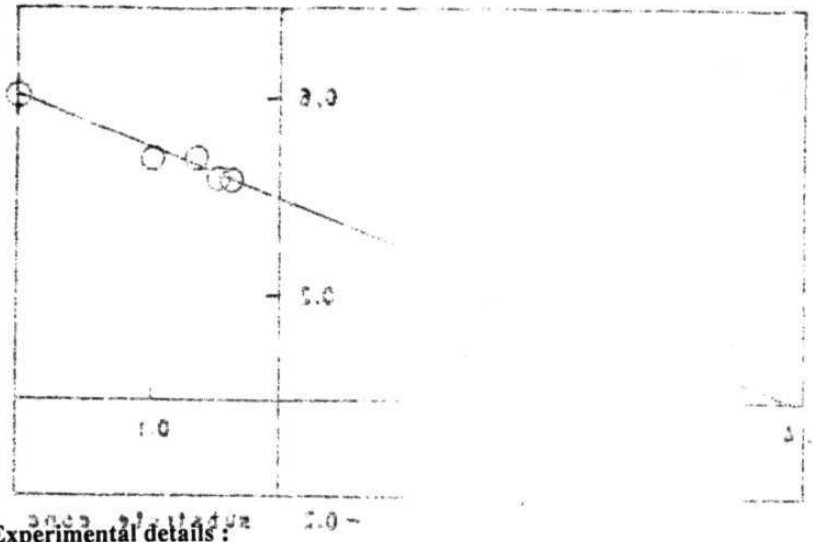


Fig.9 : Autoradiogram of ^{35}S methionine labeled mitochondrial protein products from control and CCl_4 treated rats.

Lane C : Control group which received aineral oil.

Lane E : CCl_4 group which received one dose of CCl_4(4g/kg bw).

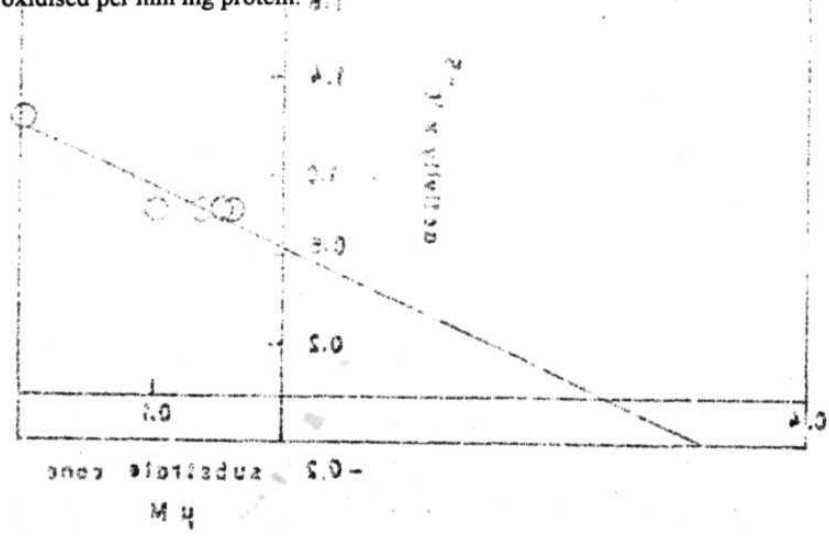


Experimental details :

Control group received oil.

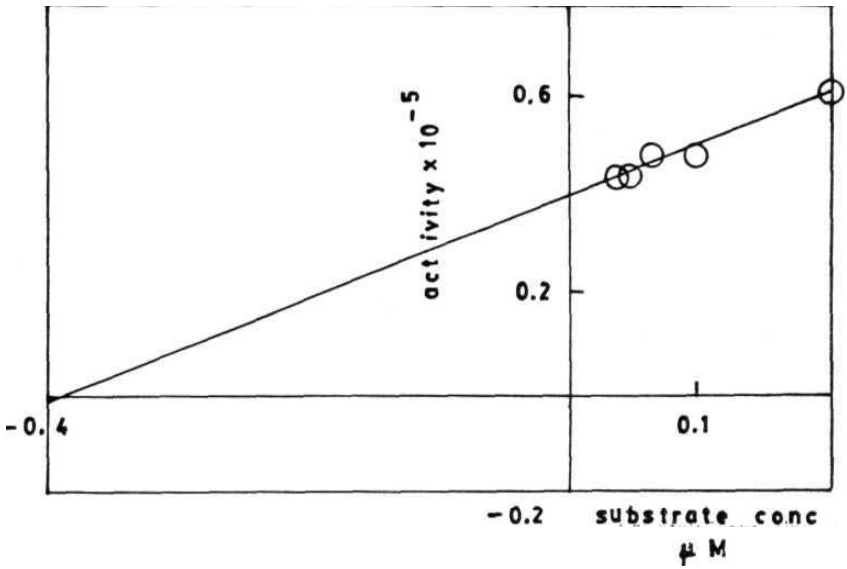
CCl_4 group received one dose ofCCl_4 (4g/kg bw)

25 ng protein was used for each assay. Reduced cytochrome C was used as substrate. Cytochrome C oxidase activity is expressed as n moles of cytochrome C oxidised per min mg protein.



Line -Weaver Burke plot of purified cytochrome oxidase
a) Control b) CCl_4 treated

10 (0)



10 (b)

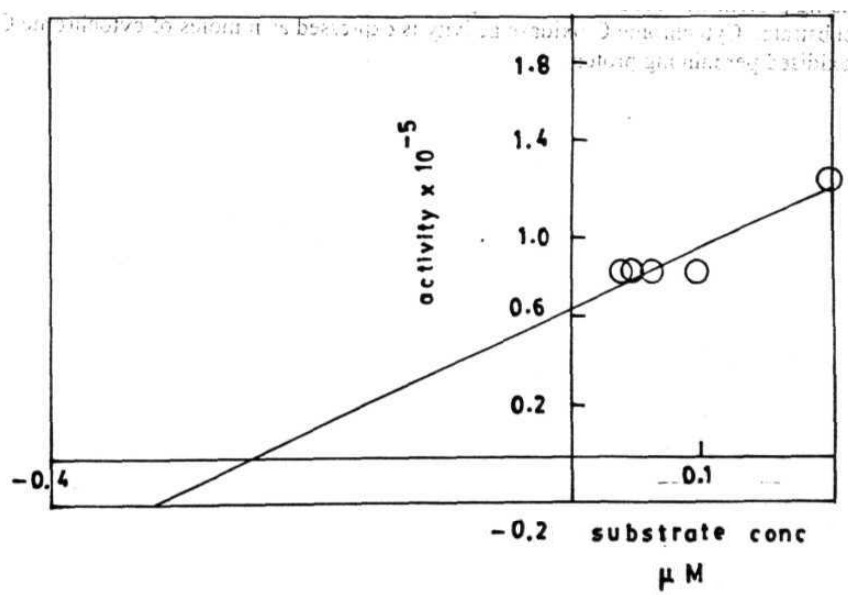


Fig.11 : 5% native gel of the purified of cytochrome oxidase.

The gel was silver stained.

Lane C : Control group which received mineral oil.

Lane E : CCl_4 group which received one dose of CC1 (4g/kg bw).

Fig. 11 : Native gel of the purified cytochrome oxidase.

Fig. 12 : 12% SDS-polyacrylamidegel electrophoresis of the purified cytochrome oxidase.

Lane C : Control group which received mineral oil.

Lane E : CCl_4 group which received one dose of CCl_4 (4g/kg bw).

The gel was silver stained. Band 1 corresponds to 57kD, 2 to 29 kD, 3 to 17kD, 4 to 12kD, 5 to 10kD, 6 to 6kD and 7 to 5kD.

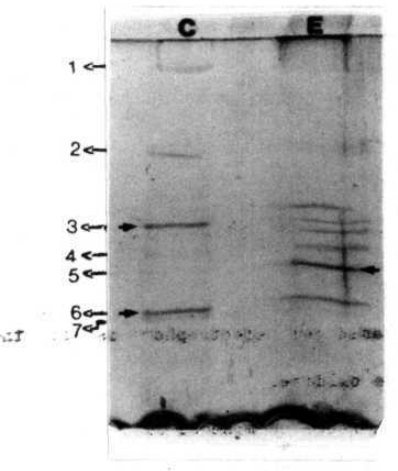


Fig. 12 : SDS-PAGE of the purified cytochrome oxidase from control and CCl_4 treated rats.
Lane C : control group which received oil.
Lane E : CCl_4 group which received one dose of CCl_4

Table 1: Effect of administration of Carbon tetrachloride with or without pretreatment with Vitamin E or *P.fraternus* or Colchicine on a) NADH deydrogenase and b) Succinate dehydrogenase of liver mitochondria.

GROUP	NADH dehydrogenase (a)	Succinate dehydrogenase (b)
Control	2002 ± 264	139 + 20
CCl_4	1292 ± 173@	33 ± 11*
Vitamin E	2120 ± 75	165 + 8
Vit.E + CCl	1725 + 183	160 ± 13
P.fraternus	2150 ± 86	168 ± 5
P.frat.+ CCl	1800 ± 172	137 ± 17
Colchicine	1950 ± 134	134 + 15
Colc. + CCl	1893 ± 124	125 ± 12

Experimental details : Control group received mineral oil, CCl group received one dose of CCl (4g/kg bw), Vitamin E group received vitamin E (20IU/day/rat) for 20 days, Vit.E + CCl group received CCl after a pretreatment with vitamin E, *P.fraternus* group received the extract of *P.fraternus* (100mg dry powder/kg bw) for five days, P.frat.+ CCl_4 group received CCl_4 after a pretreatment with *P.fraternus*, Colchicine group received colchicine (10µg/day/rat) for seven days, Colc.+ CCl group received CCl_4 after a pretreatment with colchicine.

a) 20 µg protein was used for each assay. NADH was used as substrate. NADH dehydrogenase activity is expressed as NADH units per min per mg protein. (One NADH unit • one n mole of potassium ferricyanide reduced per min per mg protein).

b) 10 fig protein was used for each assay. Succinate was the substrate. SDH activity is expressed as SDH units per min per mg protein. (One SDH unit = one n mole of DCPIP reduced per min per mg protein).

Values are Mean ± SD of atleast twelve animals.

@ = $P < 0.02$; * = $P < 0.005$.

Table 2: Effect of administration of Carbon tetrachloride with or without pretreatment with Vitamin E or *P.fraternus* or Colchicine on a) Succinate Cyt.C reductase and b) Cytochrome oxidase of liver mitochondria.

GROUP	Succinate Cyt.C reductase (a)	Cytochrome oxidase (b)
Control	60 ± 2	1702 ± 134
CCl_4	19 ± 2**	836 ± 164*
Vitamin E	58 ± 3	1610 + 120
Vit.E + CCl_4	48 ± 2	1770 + 118
P.fraternus	54 ± 4	1761 ± 98
P.frat. + CCl_4	45 ± 4	1303 ± 240
Colchicine	58 ± 3	1576 ± 122
Colc. + CCl_4	49 + 4	1490 ± 112

Experimental details are same as described for Table 1.

a) 20 µg protein was used for each assay. Activity is expressed as n moles of cytochrome C reduced per min per mg protein.

b) 1.0 µg protein was used for each assay. Reduced cytochrome C was used as substrate. Cytochrome C oxidase activity is expressed as n moles of cytochrome C oxidised per min per mg protein.

Values are Mean ± SD of atleast twelve animals.

• = $P < 0.005$; •• = $P < 0.001$.

Table 3 : Effect of administration of CCl_4 on the activities of various enzymes in sub mitochondrial particles.

ENZYME	CONTROL	CCl_4 treated
1)NADH oxidase	342 ± 12	171 + 18
2)NADH dehydrogenase	4200 ± 312	3500 + 212
3)Succinate dehydrogenase	233 ± 28	110 + 14 *
4)Cytochrome oxidase	1552 ± 140	850 ± 181 $
5)Reverse electron transport	28 ± 2	10 ± 2 **
6)Membrane potential	235 ± 20	163 + 14 $

Experimental details are same as described for Table 1.

1) 60 μg protein was used for each assay. Activity is expressed as n moles of NADH oxidised per min per mg protein.

2) 20 μg protein was used for each assay. Activity is expressed as NADH units per min per mg protein. (One NADH unit = one n mole of potassium ferricyanide reduced per min mg protein).

3) 10 μg protein was used for each assay. Activity is expressed as SDH units per min per mg protein. (One SDH unit = one n mole of DCPIP reduced per min mg protein).

4) 1.0 μg protein was used for each assay. Activity is expressed as n moles of cytochrome C oxidised per min per mg protein.

5) 360 μg protein was used for each assay. Activity is expressed as n moles of NAD reduced per min per mg protein.

6) 380 fig protein was used for each assay. 1 mM succinate was added to energise the membrane and 0.3 μM CCCP to deenergise the membrane. Membrane potential is expressed as millivolts.

Values are Mean ± SD of atleast 6 animals.

$ = $P < 0.01$; • = $P < 0.005$ j •• • $P < 0.001$.

Table 4 : Effect of administration of CCl_4 with or without pretreatment with Vitamin E on ATPase activity of liver mitochondria in the presence and absence of DNP.

GROUP	-DNP	+DNP	% stimulation with DNP
Control	0.70 + 0.08	2.05 ± 0.50	192
CCl_4	3.81 ± 0.15**	4.61 ± 0.13*	20
Vitamin E	1.23 + 0.20	3.26 + 0.02	165
Vit.E + CCl	1.12 ± 0.12	3.06 ± 0.02	173

Experimental details are same as described for Table 1.

ATP was the substrate. Activity is expressed as μg Pi released per min per mg protein in the presence and absence of the uncoupler, 2,4-DNP.

Values are expressed as Mean + SD of atleast six animals.

• = $P < 0.005$; ** = $P < 0.001$.

Table 5 : Effect of administration of CCl_4 with or without a pretreatment either with Vitamin E or *P. fraternus* on lipid peroxide level in liver homogenate and mitochondria.

GROUP	LIPID PEROXIDE LEVEL	
	liver homogenate	mitochondria
Control	130 ±42	64 ± 14
CCl_4	311 ± 14	148 ± 38$
Vitamin E	122 ± 8	80 ± 5
Vit.E +CCl_4	194 ±20	93 ± 8
P.fraternus	123 ±14	68 ± 12
P.frat. + CCl_4	152 ±12	76 ± 8

Experimental details are same as described for Table 1.

Approximately 2-3mg protein from 10% liver homogenate and mitochondria was used for each assay. Tetra methoxy propane was used as an external standard. Lipid peroxide level is expressed as n mol MDA formed per 100mg protein.

Values are Mean + SD of atleast eight animals.

$ = $P < 0.01$; •* = $p < 0.001$.

Table 6 : Effect of administration of CCl_4 with or without a pretreatment with vitamin E or *P. fraternus* or colchicine on the cytochrome content of rat liver mitochondria.

GROUP	cytochrome concentration a + a	b	c
Control	100 ± 17	100 ± 11	100 ± 2
CCl_4	66 ± 11*	117 + 24	97 ± 11
Vitamin E	107 ± 10	111 + 11	91 ± 2
Vit.E + CCl_4	115 +5	133 + 22	103 ± 9
P. fraternus	107 ± 7	111+11	102 ± 1
P.frat.+ CCl_4	87 ± 10	116 +11	97 + 1
Colchicine	108 +9	111 + 17	89 ± 10
Colc. + CCl_4	83 + 16	105 +16	91 ± 5

Experimental details are same as described for Table 1,

Results are given as percent controls. 4 mg protein was used for each assay. mM extinction coefficients used was 16.0, 20.0 and 19.1 for cyt aa , cyt b and cyt c respectively. Concentration of each cytochrome was calculated as n moles per mg protein and expressed relative to control which was taken as 100. The control value for cyt aa , cyt b and cyt c was 0.196 i 0.033, 0.18 ± 0.021 and 0.334 ± 0.001 n moles per mg protein respectively.

Values are represented as Mean ± SD of atleast six animals.

= $P < 0.05$.

Table 7 : Effect of administration of CCl_4 with or without a pretreatment either with vitamin E or *P. fraternus* on phospholipid composition of mitochondria.

GROUP	Phosphatidyl choline	Phosphatidyl ethanolamine	Cardiolipin
Control	100 + 7	100 ± 14	100 ± 18
CCl_4	62 ± 5$	53 ± 6	4 2 + 2
Vitamin E	92 + 6	96 *t* 12	82 ± 9
Vit.E + CCl_4	72 ± 6*	75 ± 13#	77 ± 12
P.fraternus	92 + 8	105 ± 16	113 + 17
P.frat.+ CCl_4	76 + 6	83 ± 9	61 + 17

Experimental details are same as described for Table 1.

Results are given as percent controls. Phospholipids were separated by TLC using chloroform : methanol : water as solvent. Approximately 200 µl of the lipid extract was applied as a streak. The separated phospholipids were digested with perchloric acid (60%) and the concentration of inorganic phosphate was estimated according to Fiske-Subbarow method. The concentration of phospholipids are expressed as µg phospholipid phosphorous / gm tissue and expressed relative to control which was taken as 100. The control value for phosphatidyl choline, phosphatidyl ethanolamine and cardiolipin are 25.1 ± 1.8, 18.2 4 2.5 and 12.5 + 2.2 µg phospholipid phosphorous / gm tissue.

Values represented are Mean 1 SD of atleast six animals.

$ = $P < 0.01$; • = $P < 0.005$.

Table 8: Effect of administration of CCl_4 on the levels of **ATP,** ADP, **AMP** and energy charge of liver homogenate.

GROUP	ATP	ADP	AMP	ENERGY CHARGE
Control	1.91 ± 0.01	0.82 ± 0.1	0.272 ± 0.02	0.77 ± 0.04
CCl_4	1.1 ± 0.01	0.80 ± 0.1	0.77 ± 0.07	0.56 ± 0.03

Experimental details are same as described for Table 1,

Levels of ATP, ADP and AMP are expressed as μ moles per gm of liver wet weight.

$$\text{Energy charge} = \frac{[\text{ATP} + 1/2\ \text{ADP}]}{[\text{ATP} + \text{ADP} + \text{AMP}]}$$

Values are Mean ± SD of atleast six animals.

* = $P < 0.005$; ** = $P < 0.001$.

Table 9 : Effect of administration of CCl_4 on the activities of liver and plasma transaminases.

	liver transaminases		plasma transaminases	
	Al AT	AAT	**Al AT**	**AAT**
Control	18 + 3	89 ± 10	0.36 ± 0.11	0.05 ± 0.01
CCl_4	4 + 0.2	33 ± 4	1.6 ± 0.11	0.48 + 0.06

Experimental details are same as described for Table 1.

Activities of transaminases are expressed as μ moles of NADH oxidised per hour per mg protein. A1AT : alanine aminotransf erase, **AAT** : apartate aminotransferase.

Values are Mean ± SD of atleast eight animals.

• = $P < 0.005$; •* = $P < 0.001$.

Table 10 : Effect of administration of CCl_4 on NADH oxidase and Succinate oxidase of kidney mitochondria.

GROUP	STATE 3	STATE 4	RCR	P/0
NADH OXIDASE:				
CONTROL	90+5	43+2	2.06 ± 0.1	1.97+0.01
CCl_4	19 ± 3	13 ± **1.5**	1.44 + 0.02	0.54 ± 0.01
SUCCINATE OXIDASE :				
CONTROL	38+8	12+2	2.9 + 0.01	2.83 ± 0.05
CCl_4	6+1.5	6 ± 1.5	1.0 ± 0.12	0

Experimental details are same as described for Table 1.

State 3 (ADP added) and state 4 (ADP exhausted) respiration rates are expressed as ng atom oxygen per min per mg protein.

Respiratory control ratio (RCR) is the ratio of state 3/state 4.

P/0 = ADP/O.

Values are Mean 1 SD of atleast eight animals.

* = $P < 0.005$; ** = $P < 0.001$.

Table 11: Effect of administration of CCl_4 on a) NADH dehydrogenase b) Succinate dehydrogenase and c) Cytochrome oxidase of kidney mitochondria.

GROUP	NADH dehydrogenase (a)	Succinate dehydrogenase (b)	Cytochrome Oxidase (c)
Control	1150 ± 173	150 ± 22	1099 + 186
CCl_4	612 ± 12^{S}	8 6 + 7$^{\$}$	758 ± 198

Experimental details are same as described for Table 1.

a) 20 μg protein was used for each assay. NADH was used as substrate. NADH dehydrogenase activity is expressed as NADH units per min per mg protein. (One NADH unit = One n mole of potassium ferricyanide reduced per min per mg protein).

b) 10 μg protein was used for each assay. Succinate was the substrate. SDH activity is expressed as SDH units per min per mg protein. (One SDH unit = one n mole of DCPIP reduced per min per mg protein).

c) 1.0 μg protein was used for each assay. Reduced cytchrome C was used as substrate. Cytochrome C oxidase activity is expressed as n moles of cytochrome C oxidised per min per mg protein.

Values are Mean + SD of atleast eight animals.

$ = $P < 0.01$.

Table 12 : Purification of Cytochrome C oxidase from rat liver.

Fraction	Total protein (mg)	Conc.heme a (n mol/mg pr.)	'/. yield
1 Mitochondria	400	0.21	100
2)Triton X-114 extraction	80	0.72	20
3)Triton X-100 supernatant	20	1.5	5
4)DEAE-Sephacel green eluate	12	2.3	3
5)38% ammonium sulphate ppt.	1.2	4.1	0.3

Protein was determined by Lowry method. The heme a content was calculated from the difference spectra (dithionite-reduced minus air oxidised) using a $AE_{605-630nm}$ = 16mM cm .

THIOACETAMIDE :

The effect of thioacetamide (TAA) (25 mg/100g bw for 4 successive days) on structural and functional properties of liver mitochondria in rats was studied. The rats were sacrificed 24h after the last dose of TAA.

NADH oxidase : This was studied using NAD^+ linked substrates like glutamate and malate. Fig. 13 shows that there is a significant decrease in state 3, RCR and P/O ratio and the decrease was 59%, 80%, and 99% respectively when compared with controls. Effect of the administration of TAA in the presence of higher level of circulating vitamin E was studied. Higher levels of vitamin E relieved most of the TAA induced inhibition. State 3 respiration was completely relieved while RCR and P/O ratio were relieved by 76% and 94% respectively. Addition of CCCP (an uncoupler) stimulated respiration and gave a RCR of 7.2 + 0.4 in controls. Such stimulation was not seen in TAA treated rats, showing that the mitochondria are uncoupled.

Fig. 14 shows the protective effect of *P.fraternus.* The plant extract was administered along with TAA and the effect was studied on the above parameters. *P.fraternus* showed a protective effect against TAA. The TAA induced inhibition was relieved to an extent of 76% on state 3, 59% on RCR and 86% on P/O ratio respectively.

Succinate oxidase : Fig. 15 shows the effect of TAA with or without the prior administration of vitamin E on succinate oxidase activity. Respiration was initiated by succinate which gives the rate of transfer of electrons for site 2 and site 3. State 3, RCR and P/O ratio were decreased significantly and the percent inhibition was 47% on state 3, 73% on RCR and 49% on P/O ratio. Addition of CCCP stimulated respiration and gave a RCR of 4.4 + 0.2 in controls while in TAA treated rats such stimulation was not observed showing that the mitochondria are uncoupled. In the group in which vitamin E was given prior to TAA, the inhibition (induced by TAA) was relieved by 81% on state 3 , 80% on RCR and 88% on P/O ratio.

Fig. 16 shows the protective effect of *P.fraternus* on TAA induced inhibition on succinate oxidase. In the group in which *P.fraternus* was given alone no significant changes were seen in the parameters studied. Administration of *P.fraternus* extract prior to TAA treatment relieved the inhibition on state 3 by 47%, on RCR by 59% and on P/O ratio by 79%.

Table 13 shows the effect of administration of thioacetamide and the protective effect of vitamin E or *P.fraternus* on NADH dehydrogenase and succinate dehydrogenase. The activity of NADH dehydrogenase was decreased significantly (61%) in TAA treated rats. Administration of vitamin E or

P.fraternus prior to TAA treatment relieved the inhibition (induced by TAA) on NADH dehydrogenase by 72% and 79% respectively. SDH did not show any significant effect either by TAA alone or TAA in the presence of high levels of vitamin E or *P.fraternus.*

Table 14 shows the effect of administration of TAA and the protective effect of vitamin E or *P.fraternus* on succinate cytochrome C reductase and cytochrome oxidase. Administration of TAA decreased the activities of succinate cytochrome C reductase and cytochrome oxidase significantly (by 54% and 37% respectively). In rats given only vitamin E or *P.fraternus,* no significant effect was observed on the above parameters. Pretreatment with vitamin E or *P.fraternus* relieved the inhibition (induced by TAA) by 56% and 46% respectively on succinate cytochrome C reductase. The inhibition on cytochrome oxidase was relieved by 51% by vitamin E and 70% by *P.fraternus.*

Studies on submitochondrial particles : Table 15 shows the effect of TAA on the activities of enzymes in SMP. A significant inhibition was observed by TAA on NADH oxidase, NADH dehydrogenase, cytochrome oxidase, reverse electron transport through site I and membrane potential. Maximum inhibition was observed for NADH dehydrogenase, cytochrome oxidase and for reverse electron transport (i.e., an inhibition of about 50% or more as compared to controls).

Studies on ATPase : Table 16 shows the effect of TAA in the presence or absence of high levels of vitamin E on ATPase activity in the presence and absence of 2,4-DNP. TAA treatment significantly increased the ATPase activity both in the presence and absence of 2,4-DNP. ATPase stimulation with 2,4-DNP was significantly decreased in TAA treated rats as compared to controls (32% in TAA compared to 139% in controls). Vitamin E restored the 2,4-DNP stimulation that was knocked out by TAA, close to control levels (94% compared to 139% in controls). Tightly coupled mitochondria show very little ATPase activity and it is elicited by the addition of an uncoupler. On the other hand uncoupled mitochondria show very high ATPase activity and cannot be stimulated further with uncoupler like 2,4-DNP.

Lipid peroxide level : Table 17 shows the effect of TAA alone or in the presence of high levels of vitamin E or pretreatment with the extract of *P.fraternus* on lipid peroxide level in homogenate and mitochondria. TAA adminstration increases the lipid peroxide level significantly both in the homogenate and mitochondria. The maximum increase was seen in homogenate (31% increase in mitochondria and 61% increase in homogenate). Administration of vitamin E or *P.fraternus* alone did not show any significant effect on lipid peroxide level, but when TAA was administered in the presence of either high level of vitamin E or *P.fraternus,* they largely suppressed the TAA induced lipid peroxide level compared to controls.

Presence of vitamin E along with TAA brings down the stimulation in peroxide level from 61% and 31% to 15% and 8% in homogenate and mitochondria respectively. Pretreatment of *P.fraternus* prior to TAA brings the TAA induced stimulation in peroxide level to 26% and 8% (in homogenate and mitochondria). Lipid peroxide level is an index of lipid peroxidation of membrane lipids which has a direct correlation to membrane damage.

Cytochrome content : Table 18 shows the effect of TAA, or vitamin E or *P.fraternus* alone or in combination with TAA on the cytochrome content of liver mitochondria. TAA induced a significant decrease on the concentration of cytochrome aa_3 and b (24% and 22% respectively). The decrease in the concentration of cytochrome c was insignificant (12%). The decrease in the level of cytochrome aa_3 induced by TAA was relieved by 46% and 67% by pretreatment with vitamin E or *P.fraternus.* Administration of vitamin E or *P.fraternus* prior to TAA treatment relieved the inhibition on cyt.b by 82% and 32% respectively.

Phospholipid composition : Table 19 shows the effect of TAA with or without the pretreatment of either vitamin E or *P.fraternus* on phosphatidylcholine, phosphatidylethanolamine and cardiolipin. There was a significant decrease in the level of PC, PE and CL by TAA treatment. The decrease is 42%, 28% and 44% respectively as compared to controls. Administration of vitamin E or *P.fraternus* alone did not affect the level of PC, PE and cardiolipin. Vitamin E or *P.fraternus* reversed the effects of TAA on the phospholipid composition and raised the levels close to control levels. In vitamin E pretreated rats the inhibition on PC, PE and CL was relieved by 62%, 50% and 115% respectively, while in *P.fraternus* pretreated rats the inhibition on PC, PE and CL was relieved by 74%, 68% and 82% respectively.

Adenine nucleotides : Table 20 shows the effect of TAA on the energy charge of the cell. It shows that there is a significant decrease in the level of ATP (57%) and a significant increase (103%) in the level of AMP in the TAA treated rats compared to controls. No change was observed on ADP levels. The energy charge of the cell was decreased significantly (24%) in TAA treated rats.

Transaminases : Table 21 shows the effect of TAA treatment on liver and plasma transaminases. Both the transaminases were significantly increased in liver. The increase in plasma was several folds in TAA treated rats as compared to controls. These transaminases are known to decrease in liver and increase in plasma during liver damage and are good index of the extent of the liver damage.

Experimental details :

Control group received saline.

Thiocetamide (TAA) group received TAA (25mg/100 g bw) for four days.

Vitamin E group received vitamin E (20IU/day/rat) for 20 days.Vit.E + TAA group received TAA after a pretreatment with vitamin E.

Results are expressed as percent control. About 2.5 mg protein was used for each assay. Glutamate and malate were used to reduce matrix NAD^+ to NADH. State 3 (ADP added) respiration was calculated as ng atom of oxygen per min mg protein.

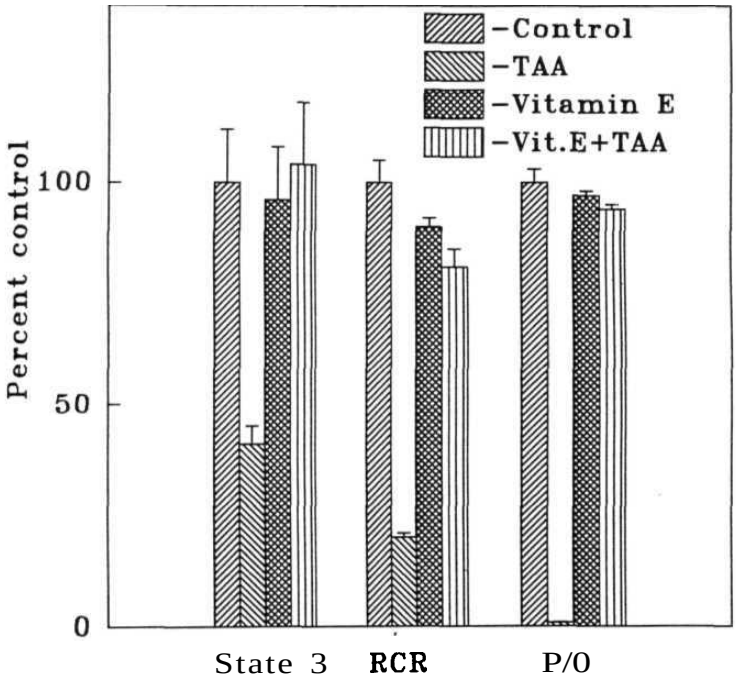
Respiratory control ratio (RCR) is the ratio of ADP stimulated to ADP exhausted respiration.

P/O = ADP/O ratio.

All the values are expressed relative to control which was taken as 100. The control value for state 3_{ADP} respiration, RCR and P/O ratio was 73 + 9, 5.2+ 0.24 and 3.03 ± 0.02 respectively.

Values are Mean + SD of atleast 12 animals.

Fig. 13 Effect of administration of **Thioacetamide** with or without pretreatment with Vitamin E on NADH oxidase.



Experimental details :

Control group received saline.

Thiocetamide (TAA) group received TAA (25mg/100 g bw) for four days.

P.fraternus group received the extract *oi P.fraternus* (100mg/dry powder / kg bw) for five days.

P.frat + TAA group received TAA after a pretreatment with *P.fraternus.*

Results are expressed as percent control. About 2.5 mg protein was used for each assay. Glutamate and malate were used to reduce matrix NAD^+ to NADH. State 3 (ADP added) respiration was calculated as ng atom of oxygen per min mg protein.

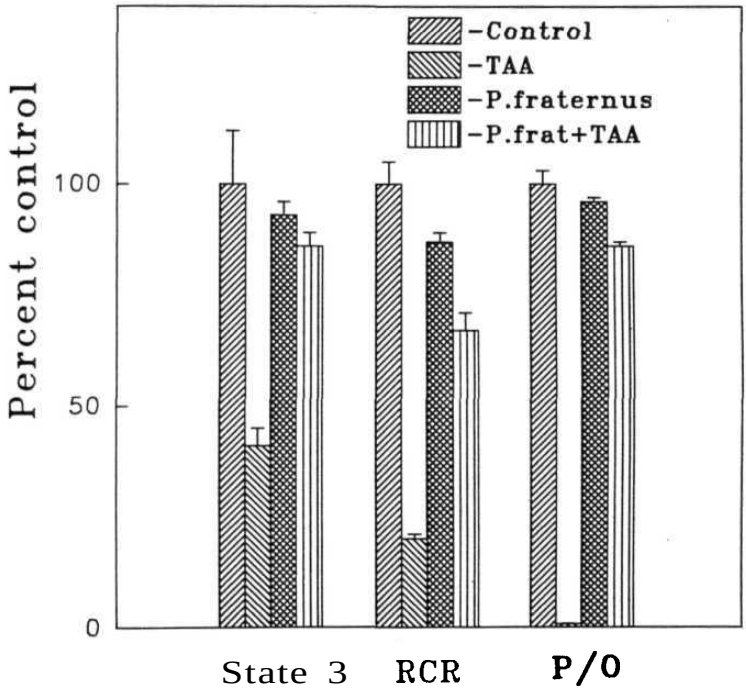
Respiratory control ratio (RCR) is the ratio of ADP stimulated to ADP exhausted respiration.

P/O = ADP/O ratio.

All the values are expressed relative to control which was taken as 100. The control value for state 3_{ADP} respiration, RCR and P/O ratio was 73 + 9, 5.2 + 0.24 and 3.03 + 0.02 respectively.

Values are Mean + SD of atleast 8 animals.

Fig.14 Effect of administration of Thioacetamide with or without pretreatment with P.fraternus on NADH oxidase.



Experimental details :

Control group received saline.

Thiocetamide (TAA) group received TAA (25mg/100 g bw) for four days.

Vitamin E group received vitamin E (20IU/day/rat) for 20 days.

Vit.E + TAA group received TAA after a pretreatment with vitamin E

Results are expressed as percent control. About 2.5 mg protein was used for each assay. Succinate was used as the substrate.State 3 (ADP added) respiration was calculated as ng atom of oxygen per min mg protein.

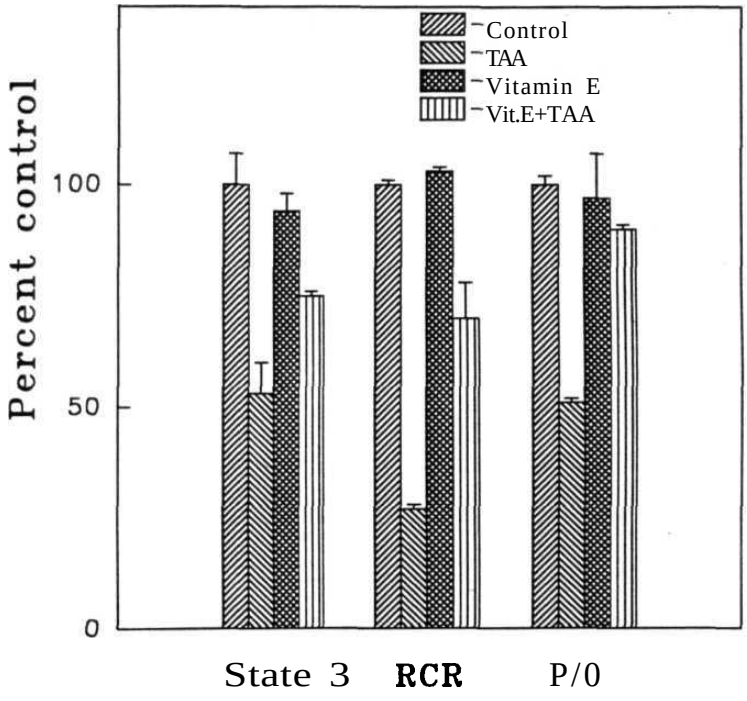
Respiratory control ratio (RCR) is the ratio of ADP stimulated to ADP exhausted respiration.

P/O =ADP/O ratio.

All the values are expressed relative to control which was taken as 100. The control value for state 3_{ADP} respiration, RCR and P/O ratio was 80 + 6, 4.06 + 0.05 and 1.96 + 0.04 respectively.

Values are Mean + SD of atleast 8 animals.

Fig. 15 Effect of administration of Thioacetamide with or without pretreatment with vitamin E on Succinate oxidase.



Experimental details :

Control group received saline.

Thiocetamide (TAA) group received TAA (25mg/100 g bw) for four days.

P.fraternus group received the extract of *P.fraternus* (100mg/dry powder / kg bw) for five days.

P.frat + TAA group received TAA after a pretreatment with *P.fraternus.*

Results are expressed as percent control. About 2.5 mg protein was used for each assay. Succinate was used as the substrate. State 3 (ADP added) respiration was calculated as ng atom of oxygen per min mg protein.

Respiratory control ratio (RCR) is the ratio of ADP stimulated to ADP exhausted respiration.

P/O = ADP/O ratio.

All the values are expressed relative to control which was taken as 100. The control value for state 3_{ADP} respiration, RCR and 80 + 6, 4.06 + 0.05 and 1.96 + 0.04 respectively.

Values are Mean + SD of atleast 8 animals.

Fig. 16 Effect of administration **of Thioacetamide with or without pretreatment with P.fraternus on Succinate oxidase.**

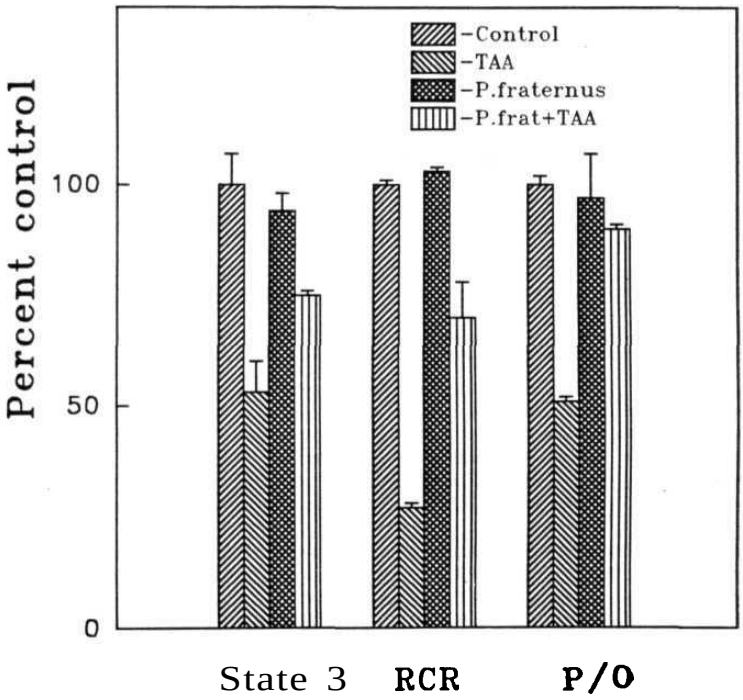


Table 13: Effect of administration of Thioacetamide with or without pretreatment with Vitamin E or *P. fraternus* on a) NADH dehydrogenase b) Succinate dehydrogenase of mitochondria.

GROUP	NADH dehydrogenase (a)	Succinate dehydrogenase (b)
Control	2250 ± 50	159 + 9
Thioacetamide	878 ± 157**	161 ± 18
Vitamin E	2120 ± 75	165 ± 8
Vit.E +TAA	1875 ± 100	156 ± 8
P. fraternus	2150 ± 112	150 i 10
P.frat.+ TAA	1950 + 104	147 ± 9

Experimental details : Control group received saline, TAA group received TAA (25mg/100 g bw) for four days, vitamin E group received vitamin E (20IU/day/rat) for 20 days, Vit.E + TAA group recieved TAA after a pretreatment with vitamin E, *P. fraternus* group received an extract of *P. fraternus* (100 mg dry powder/kg bw) for five days, P.frat.+ TAA group received TAA after a pretreatment with *P. fraternus.*

a) 20 μg protein was used for each assay. NADH was used as substrate. NADH dehydrogenase activity is expressed as NADH units per min per mg protein. (One NADH unit = one n mole of potassium ferricyanide reduced per min per mg protein).

b) 10 μg protein was used for each assay. Succinate was the substrate. SDH activity is expressed as SDH units per min per mg protein. (One SDH unit = one n mole of DCPIP reduced per min per mg protein).

Values are Mean ± SD of atleast eight animals.

•• - $P < 0.001$.

Table 14 : Effect of administration of Thioacetamide with or without pretreatment with Vitamin E or *P.fraternus* on a) Succinate Cyt.C reductase and b) Cytochrome oxidase of mitochondria.

GROUP	Succinate cytochrome C reductase (a)	Cytochrome oxidase (b)
Control	68 ± 16	1469 ± 21
Thioacetamide	31 + 4@	931 + 55**
Vitamin E	59 ± 3	1525 ± 25
Vit.E + TAA	5 2 + 4	1200 + 115
P.fraternus	6 2 + 2	1434 ± 32
P.frat. + TAA	4 8 + 4	1302 + 24

Experimental details same as described for Table 13.

a) 20 µg protein was used for each assay. Activity is expressed as n moles of cytochrome C reduced per min per mg protein.

b) 1.0 µg protein was used for each assay. Reduced cytochrome C was used as substrate. Cytochrome C oxidase activity is expressed as n moles of cytochrome C oxidised per min per mg protein.

Values are Mean ± SD of atleast six animals.

@ P = < 0.02 ; ** = P < 0.001.

Table 15 : Effect of administration of Thioacetamide on the activities of various enzymes in submitochondrial particles.

ENZYME	CONTROL	THIOACETAMIDE
1)NADH oxidase	342 ± 12	245 ± 15
2)NADH dehydrogenase	4200 + 30	2150 ± 229
3)Succinate dehydrogenase	233 ± 28	197 + 17
4)Cytochrome oxidase	1552 ± 220	610 ± 120
5)Reverse electron transport	1 5 + 2	7 + 1
6)Membrane potential	197 + 12	150 ± 4

Experimental details same as described for Table 13.

1) 60 µg protein was used for each assay. Activity is expressed as n moles of NADH oxidised per min per mg protein.

2) 20 fig protein was used for each assay. Activity is expressed as NADH units per min per mg protein. (One NADH unit = one n mole of potassium ferricyanide reduced per min mg protein).

3) 10 µg protein was used for each assay. Activity is expressed as SDH units per min per mg protein. (One SDH unit = one n mole of DCPIP reduced per min mg protein).

4) 1.0 µg protein was used for each assay. Activity is expressed as n moles of cytochrome C oxidised per min per mg protein.

5) 360 µg protein was used for each assay. Activity is expressed as n moles of NAD reduced per min per mg protein.

6) 380 µg protein was used for each assay. 1 mM succinate was added to energise the membrane and 0.3 µM CCCP to deenergise the membrane. Membrane potential is expressed as millivolts.

Values are Mean ± SD of atleast six animals.

• = $P < 0.005$; •• = $P < 0.001$.

Table 16 : Effect of administration of Thioacetamide with or without pretreatment with Vitamin E on ATPase activity of liver mitochondria in the presence and absence of DNP.

GROUP	-DNP	+DNP	% stimulation with DNP
Control	1.53 ± 0.25	3.66 ± 0.14	139
Thioacetamide	5.34 ± 0.15	7.06 ± 0.24 *	32
Vitamin E	1.6 ± 0.12	3.26 ± 0.21	103
Vit. E + TAA	1.73 ± 0.35	3.36 ± 0.20	94

Experimental details are same as described for Table 13.

ATP was the substrate. Activity is expressed as μg Pi released per min per mg protein in the presence and absence of the uncoupler, 2.4-DNP.

Values are expressed as Mean ± SD of atleast six animals.

** = $P < 0.001$.

Table 17 : Effect of administration of Thioacetamide on lipid peroxide level in homogenate and mitochondria with *or* without a pretreatment either with Vitamin E or an aqueous extract of *P. fraternus.*

	LIPID PEROXIDE LEVEL	
GROUP	liver homogenate	mitochondria
Control	130 ±10	86 + 12
Thioacetamide	210 ± 22*	113 ± 9*
Vitamin E	128 ±12	80 ± 5
Vit.E + TAA	150+12	93 + 5
P. fraternus	145 ± 9	89 + 6
P.frat.+ TAA	165 +21	93 ± 9

Experimental details are same as described for Table 13.

About 2- 3 mg protein from 10% liver homogenate and mitochondria was used for each assay. Tetra methoxy propane was used as an external standard. Lipid peroxide level is expressed as n mol MDA formed per 100 mg protein.

Values are Mean ± SD of atleast eight animals.

= $P < 0.05$; * = $P < 0.005$.

Table 18 : Effect of administration of Thioacetamide with or without a pretreatment with either vitamin E or *P.fraternus* on the cytochrome content of rat liver mitochondria.

GROUP	cytochrome concentration a +	b	c
Control	100 ±4	100 ± 1	100 ± 1
Thioacetamide	7 6 + 1 *	7 8 + 1	8 8 + 1
Vitamin.E	107 ± 1	85 ± 1	8 3 + 1
Vit.E +TAA	87 ± 1	96 ± 1	9 3 + 1
P.fraternus	96 ± 1	93 ± 1	97 ± 1
P.frat.+ TAA	9 2 + 2	85 ± 1	91 ± 1

Experimental details are same as described for Table 13.

Results are given as percent controls. 4 mg protein was used for each assay. mM extinction coefficients used was 16.0, 20.0 and 19.1 for cyt aa , cyt b and cyt c respectively. Concentration of each cytochrome was calculated as n moles per mg protein and expressed relative to control which was taken as 100. The control value for cyt aa , cyt b and cyt c was 0.243 ± 0.009, 0.27 + 0.001 and 0.375 + 0.002 n moles per mg protein respectively.

Values are represented as Mean ± SD of atleast eight animals.

= P < **0.05.**

Table 19 : Effect of administration of Thioacetamide with or without a pretreatment with vitamin E or *P.fraternus* on the phospholipid composition of liver mitochondria.

GROUP	Phosphatidyl choline	Phosphatidyl ethanolamine	Cardiolipin
Control	100 ± 12	100 ± 9	100 ± 6
Thioacetamide	58 + 4	72 + 3 *	56 ± 3
Vitamin.E	98 ± 6	91 + 1	92 + 1
Vit.E + TAA	84 + 1	86 + 1	107 + 1
P.fraternus	84 ± 1	87 + 5	86 ± 3
P.frat.+ TAA	89 + 6	91 + 3	92 ± 5

Experimental details are same as described for Table 13.

Results are given as percent controls. Phospholipids were separated by TLC using chloroform : methanol : water as solvent. Approximately 200 µl of the lipid extract was applied as a streak. The separated phospholipids were digested with perchloric acid (60%) and the concentration of inorganic phosphate was estimated according to Fiske-Subbarow method. The concentration of phospholipids are calculated as µg phospholipid phosphorous / gm tissue and expressed relative to control which was taken as 100. The control value for phosphatidyl choline, phosphatidyl ethanolamine and cardiolipin are 21.6 + 2.5, 16.7 + 1.5 and 13.3 ± 0.7 µg phospholipid phosphorous / gm tissue.

Values represented are Mean + SD of atleast six animals.

= P < 0.01 ; • • P < 0.005 ; ** = P < 0.001.

Table 20: Effect of administration of Thioacetamide on the levels of ATP, ADP, AMP and energy charge of liver homogenate.

GROUP	ATP	ADP	AMP	ENERGY CHARGE
Control	1.76 ± 0.06	0.82 ± 0.16	0.28 ± 0.02	0.76 ± 0.04
TAA	0.76 ± 0.005**	0.85 ± 0.12	0.57 ± 0.005**	0.58 ± 0.05$

Experimental details are same as described for Table 13.

Levels of ATP, ADP, AMP are expressed as μ moles per gm of liver wet weight.

$$\text{Energy charge} - \frac{[\text{ATP} + 1/2\ \text{ADP}]}{[\text{ATP} + \text{ADP} + \text{AMP}]}$$

Values are Mean ± SD of atleast six animals.

$ P = < 0.01 ; •• = P < 0.001.

Table 21: Effect of administration of Thioacetamide on the activities of liver and plasma transaminases.

	liver transaminases		plasma transaminases	
GROUP	Al AT	AAT	Al AT	AAT
Control	18 ± 2	8 4 + 6	0.056 + 0.005	0.4 ± 0.02
Thioacetamide	8 + 1 *	31 + 2**	0.23 *t* 0.002**	2.7 + 0.12**

Experimental details are same as described for Table 13.

Activities of transaminases are expressed as μ moles of NADH oxidised per hour per mg protein. A1AT : alanine aminotransferase, AAT : aspartate aminotransferase.

Values are Mean ± SD of atleast six animals.

• = P < 0.005 ; •• = P < 0.001.

GALACTOSAMINE :

The effect of *in vivo* administration of galactosamine hydrochloride (2.5g/kg bw) on respiratory chain enzymes and oxidative phosphorylation was studied. The rats were sacrificed at 12h, 24h and 36h (three time intervals) after the injection of galactosamine.

NADH oxidase: Fig. 17 shows the effect of galactosamine on NADH oxidase. Effect was studied at three different time intervals i.e., 12h, 24h and 36h after the administration of galactosamine. State 3 respiration decreased by 57%, 75% and 95% respectively at the end of 12h, 24h and 36h. State 4 respiration was stimulated by 22% and 45% at the end of 12h and 24h while at 36h state 4 respiration was equal to state 3 and could not be differentiated. RCR decreased by 65% ,82% and 83% at the end of 12h , 24h and 36h respectively. P/O ratio decreased by 21% at 12h and were completely abolished at 24h and 36h. In the presence of CCCP (an uncoupler), the RCR in control rats was 7.2 + 0.4, while in galactosamine treated rats (at the end of 36h) such stimulation was not seen showing that the mitochondria were uncoupled.

Succinate oxidase: Fig. 18 shows the effect of galactosamine on succinate oxidase. State 3 respiration decreased by 45%, 82% and 91% at the end of 12h, 24h and 36h respectively. State 4 respiration was increased by 39% at the end of 12h and at the end of 24h and 36h, state 4 and state 3 could not be differentiated. RCR was decreased by 61%, 73% and 74% at 12h, 24h and 36h respectively. P/O ratio were suppressed by 31% at 12h and completely abolished at 24h and 36h. In presence of CCCP control rats showed a RCR of 4.4 + 0.2, while in galactosamine treated rats such stimulation was not observed showing that the mitochondria are uncoupled.

Table 22 shows the effect of galactosamine on NADH dehydrogenase and succinate dehydrogenase. Activity of NADH dehydrogenase in galactosamine treated rats was decreased by 25%, 35% and 83% at the end of 12h, 24h and 36h respectively. As this enzyme is involved in the transfer of electrons from NADH to the prosthetic group of the flavoprotein [E-FMN to E-FMNH2], an inhibition in its activity shows that the entry of electrons on to site I of the electron transport chain was affected. Administration of galactosamine significantly decreased SDH activity by 20% and 47% at 24h and 36h respectively. At 12h the decrease in SDH was not significant (12%).

Table 23 shows the effect of galactosamine on succinate cytochrome C reductase and cytochrome oxidase. The activity of succinate cytochrome C reductase decreased by 57%, 60% and 66% at the end of 12h, 24h and 36h

respectively. The activity of cytochrome oxidase was inhibited by 56% at the end of 12h itself and was nearly the same even at 36h (63%).

Studies with submitochondrial particles : Table 24 shows the effect of galactosamine 36hr after administration, on different parameters in SMP. There was a significant decrease on NADH oxidase, NADH dehydrogenase, succinate dehydrogenase and cytochrome oxidase (43%, 71%, 33% and 56% respectively). In galactosamine treated rats reverse electron transport and membrane potential decreased by 64% and 38% respectively when compared to controls.

Lipid peroxide level : Table 25 shows that the lipid peroxide level in both liver homogenate and mitochondria was significantly increased and the increase was progressive (60%, 133% and 213% in homogenate and 48%, 78% and 145% in mitochondria at the end of 12h, 24h and 36h respectively).

Cytochrome content: Table 26 gives the cytochrome concentrations in galactosamine treated rats. Cytochrome aa_3 was significantly decreased by 52%, 59% and 65% at the end of 12h, 24h and 36h respectively. The cytochrome b content was also decreased significantly by 40%, 48% and 44% at the end of 12h, 24h and 36h respectively. The concentration of cytochrome c showed a progressive decrease i.e., 16%, 38% and 47% at the end of 12h, 24h and 36h respectively.

Phospholipid composition : Table 27 shows the effect of galactosamine on the phospholipid composition of mitochondria. There was a progressive decrease in the three classes of phospholipids studied. Phosphatidylcholine (PC) was decreased significantly (47%, 49% and 55% at the end of 12h, 24h and 36h respectively). Phosphatidylethanolamine was decreased significantly (41%, 51%, and 58% at the end of 12h, 24h and 36h respectively). Cardiolipin was also progressively decreased by 34%, 53% and 61% at the end of 12h, 24h and 36h respectively.

Studies on kidney mitochondria: Table 28 shows the effect of galactosamine on NADH dehydrogenase, succinate dehydrogenase and cytochrome oxidase in kidney mitochondria The effect was studied for 36hr after the administration of galactosamine. Administration of galactosamine showed no effect on any of the parameters studied in kidney mitochondria.

Experimental details :

Controls received saline.

Galactosamine group received galactosamine (2.5g/kg bw) and sacrificed at 12h, 24h and 36h.

Results are expressed as percent control. About 2.5 mg protein was used for each assay. Glutamate and malate were used to reduce matrix NAD^+ to NADH. State 3 ADP added respiration was calculated as n g atom of oxygen per min mg protein.

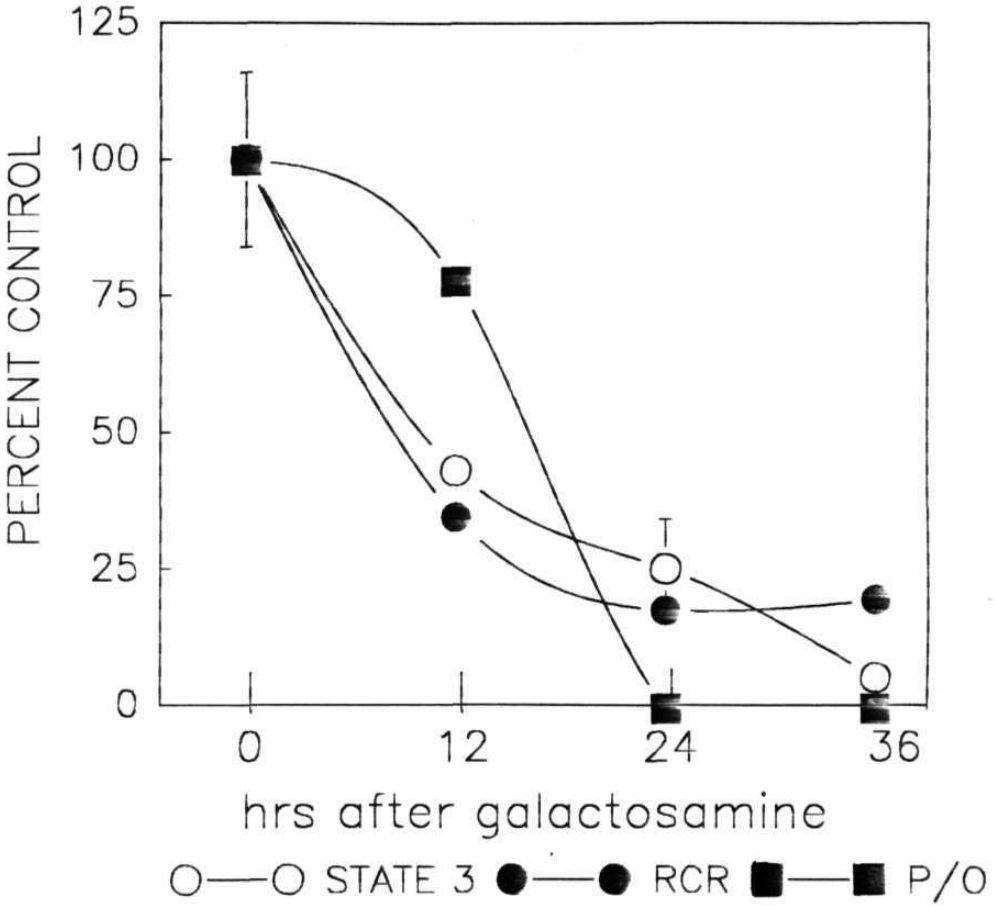
Respiratory control ratio (RCR) is the ratio of ADP stimulated to ADP exhausted respiration.

P/O = ADP/O ratio.

All the values are expressed relative to control which was taken as 100. The control value for state 3 ADP respiration, RCR and P/O ratio was 63 + 10, 5.7 + 0.2 **and** 2.94 + 0.02 respectively.

Values are Mean + SD of atleast three animals.

Fig. 17 Effect of administration of galactosamine on NADH oxidase



Experimental details :

Controls received saline.

Galactosamine group received galactosamine (2.5g/kg bw) and sacrificed at 12h, 24h and 36h.

Results are expressed as percent control. About 2.5 mg protein was used for each assay. Succinate was used as substrate. State 3 (ADP) added respiration was calculated as n g atom oxygen per min mg protein.

Respiratory control ratio (RCR) is the ratio of ADP stimulated to ADP exhausted respiration.

P/O = ADP/O ratio.

All the values are expressed relative to control which was taken as 100. The control value for state 3 ADP added respiration, RCR and P/O ratio was 73 + 9, 3.75 + 0.08 and 1.96 + 0.01 respectively.

Values are Mean + SD of atleast three animals.

Fig. 18 Effect of administration of galactosamine on succinate oxidase

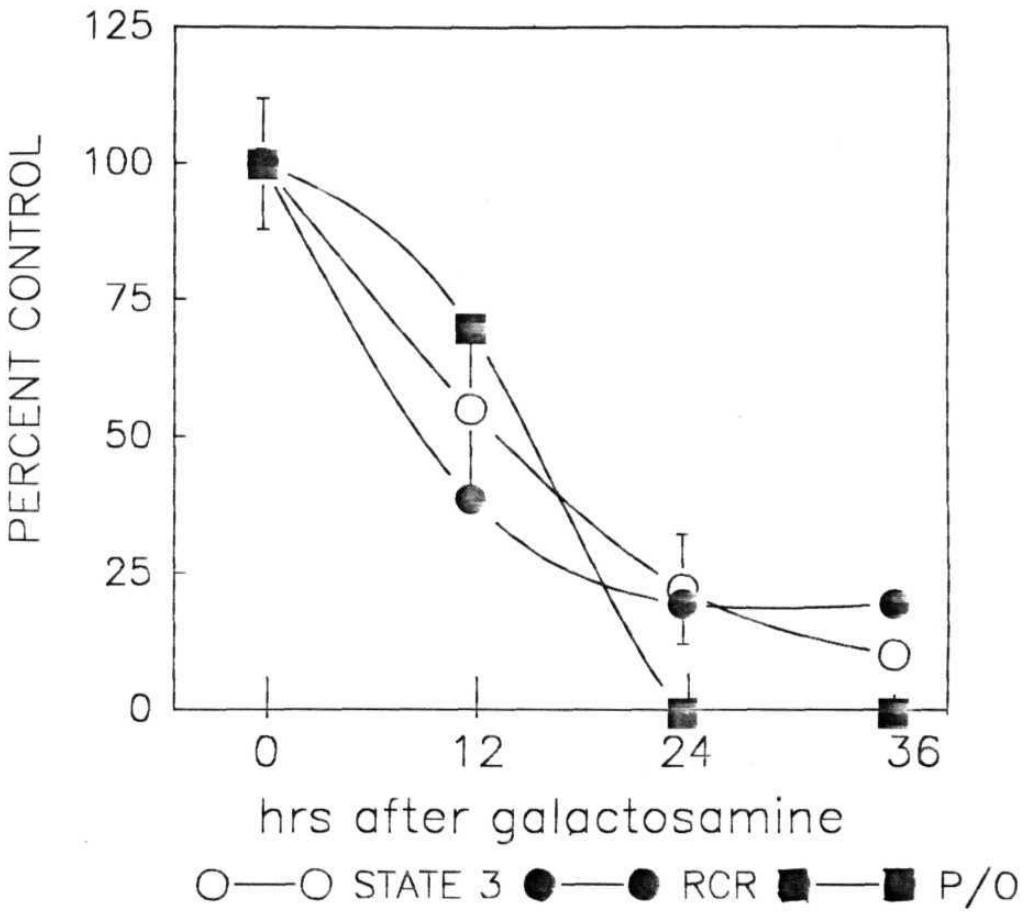


Table 22: Effect of administration of Galactosamine hydrochloride on a) NADH dehydrogenase and b) Succinate dehydrogenase of rat liver mitochondria.

GROUP	NADH dehydrogenase (a)	Succinate dehydrogenase (b)
Control	1725 ± 287	165 ± 8
Galactosamine hydrochloride		
12 hours	1300 ± 173	144 ± 29
24 hours	1125 ± 106 •	131 ± 4 ••
36 hours	300 ± 30 •	88 ± 12 •

Experimental details :Control group received saline, Galactosamine group received galactosamine (2.5g/kg bw) and rats were sacrificed at 12h, 24h and 36h later.

a) 20 µg protein was used for each assay. NADH was used as substrate. NADH dehydrogenase activity is expressed as NADH units per min per mg protein. (One NADH unit = One n mole of potassium ferricyanide reduced per min per mg protein).

b) 10 µg protein was used for each assay. Succinate was the substrate. SDH activity is expressed as SDH units per min per mg protein). (One SDH unit • one n mole of DCPIP reduced per min per mg protein).

Values are Mean ± SD of atleast three animals.

= P < 0.05 ; • • P < 0.005 ; •• - P < 0.001.

Table 23: Effect of administration of Galactosamine hydrochloride on a) Succinate Cyt.C Reductase and b) Cytochrome oxidase of rat liver mitochondria.

GROUP	Succinate Cyt.C reductase (a)	Cytochrome oxidase (b)
Control	68 ± 16	1641 ± 182
Galactosamine hydrochloride		
12 hours	29 ± 2@	723 ± 189
24 hours	27 ± 4@	484 ± 98**
36 hours	23 ± 2$	610 ± 79**

Experimental details are same as described for Table 22.

a) 20 µg protein was used for each assay. Activity is expressed as n moles of cytochrome C reduced per min per mg protein.

b)1.0 µg protein was used for each assay. Reduced cytchrome C was used as substrate. Cytochrome C oxidase activity is expressed as n moles of cytochrome C oxidised per min per mg protein.

Values are Mean ± SD of atleast three animals.

@ = $P < 0.02$; $ = $P < 0.01$; • = $P < 0.005$; •• = $P < 0.001$.

Table 24 : Effect of administration of Galactosamine hydrochloride on the activities of various enzymes in sub mitochondrial particles.

Enzyme	Control	Galactosamine
1)NADH oxidase	342 ± 12	193 ± 55®
2)NADH dehydrogenase	4200 + 300	1200 + 150
3)Succinate dehydrogenase	233 ± 28	157 ± 17@
4)Cytochrome oxidase	1552 ± 140	680 ± 90••
5)Reverse electron transport	1 7 + 2	6 + 3
6)Membrane potential	235 + 20	145 ± 4

Experimental details are same as described for Table 22.

1) 60 µg protein was used for each assay. Activity is expressed as n moles of NADH oxidised per min per mg protein.

2) 20 µg protein was used for each assay. NADH dehydrogenase activity is expressed as NADH units per min per mg protein. (One NADH unit = One n mole of potassium ferricyanide reduced per min per mg protein).

3) 10 µg protein was used for each assay. SDH activity is expressed as SDH units per min per mg protein. (One SDH unit = one n mole of DCPIP reduced per min per mg protein).

4)1.0 fig protein was used for each assay. Cytochrome C oxidase activity is expressed as n moles of cytochrome C oxidised per min per mg protein.

5) 360 µg protein was used for each assay. Activity is expressed as n moles of NAD reduced per min per mg protein.

6) 380 µg protein per ml was used for each assay. 1 mM succinate was added to energise the membrane and 0.3 µM CCCP was added to deenergise the membrane. Membrane potential is expressed as millivolts.

Values are Mean + SD of atleast six animals.

@ = P < 0.02 ; $ = P < 0.01 ; * = P < 0.005 ; •* = P < 0.001.

Table 25: Effect of administration of Galactosamine hydrochloride on lipid peroxide level in liver homogenate and in mitochondria

GROUP	LIPID PEROXIDE LEVEL	
	liver homogenate	mitochondria
Control	159 ±26	88 ± 11
Galactosamine hydrochloride		
12 hours	255 i 32@	131 ± 11$
24 hours	371 ± 43*	157 ± 24@
36 hours	499 ± 44	216 ± 26

Experimental details are same as described for Table 22.

About 4 mg protein from 10% liver homogenate and mitochondria was used for each assay. Tetra methoxy propane was used as an external standard. Level of lipid peroxide is expressed as n moles of MDA formed per 100 mg protein.

Values are Mean ± SD of atleast four animals.

@ = P < 0.02 ; $ = P < 0.01 ; * = P < 0.005 ; ** = P < 0.001.

Table 26 : Effect of administration of Galactosamine hydrochloride on cytochrome content of rat liver mitochondria.

GROUP	cytochrome concentration a + a	b	c
Control	100 + 7	100 ± 8	100 ± 2
Galactosamine hydrochloride			
12 hours	48 ± 14*	60 ± 12$	84 ± 11
24 hours	41 ± 7**	52 ± 4 *	62 ± 2**
36 hours	36 + 7 *	56 + 4 *	53 ± 1 **

Experimental details are same as described for Table 22.

Results are given as percent controls. 4 mg protein was used for each assay. mM extinction coefficients used was 16.0, 20.0 and 19.1 for cyt aa , cyt b and cyt c respectively. Concentration of each cytochrome was calculated as n moles per mg protein and expressed relative to control which was taken as 100. The control value for cyt aa , cyt b and cyt c was 0.146 ± 0.01, 0.25 ± 0.02 and 0.263 ± 0.004 n moles per mg protein respectively.

Values are represented as mean ± SD of atleast three animals.

$ = P < 0.01 ; * = P < 0.005 ; ** = P < 0.001.

Table 27 : Effect of administration of Galactosamine hydrochloride on the phospholipid composition of liver mitochondria.

GROUP	Phosphatidyl choline	Phosphatidyl ethanolamine	Cardiolipin
Control	100 ± 6	100 ± 14	100 ± 11
Galactosamine hydrochloride			
12 hours	53 ± 4**	59 ± 7@	66 ± 6$
24 hours	51 ± 4**	49 ± 9$	47 ± 2*
36 hours	45 ± 3**	42 ± 6 *	39 + 1*

Experimental details are same as described for Table 22.

Results are given as percent controls. Phospholipids were separated by TLC using chloroform : methanol : water as solvent. Approximately 200 µl of the lipid extract was applied as a streak. The separated phospholipids were digested with perchloric acid (60%) and the concentration of inorganic phosphate was estimated according to Fiske-Subbarow method. The concentration of phospholipids are expressed as µg phospholipid phosphorous / gm tissue and expressed relative to control which was taken as 100. The control value for phosphatidyl choline, phosphatidyl ethanolamine and cardiolipin are 24.9 ± 1.4, 17.5 ± 2.5 and 12.3 + 1.3 µg phospholipid phosphorous / gm tissue.

Values represented are mean + SD of atleast three animals.

@ = P < 0.02 ; $ = P < 0.01 ; * = P < 0.005 ; ** = P < 0.001.

Table 28: Effect of Galactosamine hydrochloride on a) NADH dehydrogenase b) Succinate dehydrogenase and c) Cytochrome oxidase of kidney mitochondria.

GROUP	NADH dehydrogenase (a)	Succinate dehydrogenase (b)	Cytochrome Oxidase (c)
Control	1200 + 150	145 ± 10	1100 ± 65
Galactosamine hydrochloride			
36 hours	1266 ± 56	125 ± 12	1090 ± 50

Experimental details are same as described for Table 22.

a) 20 μg protein was used for each assay. NADH was used as substrate. NADH dehydrogenase activity is expressed as NADH units per min per mg protein. (One NADH unit = One n mole of potassium ferricyanide reduced per min per mg protein).

b) 10 μg protein was used for each assay. Succinate was the substrate. SDH activity is expressed as SDH units per min per mg protein. (One SDH unit = one n mole of DCPIP reduced per min per mg protein).

c) 1.0 μg protein was used for each assay. Reduced cytchrome C was used as substrate. Cytochrome C oxidase activity is expressed as n moles of cytochrome C oxidised per min per mg protein.

Values are Mean ± SD of three animals.

ETHIONINE :

The effect of administration ethionine (100 mg/ 100 gm bw for four days) on structural and functional properties of liver mitochondria was examined. Vitamin E was administered prior to ethionine and its protective effect on all the parameters was studied.

NADH oxidase : In the presence of NAD^+ linked substrates (glutamate plus malate) state 3 respiration decreased by 43% in ethionine treated rats. RCR and P/O ratio decreased by 56% and 24% respectively (Fig. 19). Addition of CCCP stimulated respiration and gave a RCR of 7.0 + 0.2 in controls while in ethionine treated rats no such stimulation was observed showing that the mitochondria were uncoupled. Administration of vitamin E alone did not show any significant effect on rate of respiration, RCR and P/O ratio. In the group in which vitamin E was given prior to ethionine administration, the amount of inhibition (induced by ethionine) was relieved completely on state 3 respiration while RCR and P/O ratio were relieved by 84% and 50% respectively.

Succinate oxidase : Fig.20 shows that in ethionine treated rats the state 3 respiration was inhibited by 41% as compared to that of controls. RCR and P/O ratio were inhibited by 51% and 38% respectively in ethionine treated rats. Administration of vitamin E alone stimulated state 3 respiration and RCR by 18% and P/O ratio was inhibited by 8%. These changes were insignificant. Addition of CCCP stimulated respiration and gave a RCR of 4.47 + 0.2 in control rats while no such stimulation was seen in ethionine treated rats showing that the mitochondria were uncoupled. Administration of vitamin E prior to ethionine treatment relieved the inhibition (induced by ethionine) by 88%, 90% and 68% on rate of respiration, RCR and P/O ratio respectively.

Table 29 and 30 show the effect of ethionine in the presence and absence of high circulating vitamin E levels on NADH dehydrogenase, succinate dehydrogenase, cytochrome oxidase and succinate cytochrome C reductase. There was a significant decrease in the activities of NADH dehydrogenase, cytochrome oxidase and succinate cytochrome C reductase in ethionine treated rats compared to controls. The decrease was 31%, 36% and 45% respectively. Succinate dehydrogenase did not show any effect either by ethionine alone or vitamin E administration. The administration of vitamin E prior to ethionine treatment relieved the inhibition induced by ethionine. The percent inhibition relieved was 74% for NADH dehydrogenase, 92% for cytochrome oxidase and 80% for

succinate cytochrome C reductase. Administration of vitamin E alone did not show any significant change on these parameters.

Studies with Submitochondrial particles : Table 31 shows the effect of ethionine on the activities of enzymes in submitochondrial particles. A significant inhibition was observed by ethionine administration on NADH oxidase, NADH dehydrogenase and cytochrome oxidase (22%, 20% and 39% respectively). There was a 33% decrease in the rate of transfer of electrons through site I in the reverse direction. This change is not statistically significant. The membrane potential in ethionine treated rats was significantly decreased (26%).

Lipid peroxide level : Table 32 shows the effect of ethionine alone or in the presence of high levels of vitamin E on the lipid peroxide level in homogenate and mitochondria. From the table it can be seen that the lipid peroxide level increased by 72% and 37% in liver homogenate and mitochondria respectively. In the rats in which vitamin E alone was given the lipid peroxide level increased by 7% in mitochondria and there was no change in liver homogenate as compared to controls. In the group in which vitamin E was given prior to ethionine administration, the increase in the lipid peroxide level was 11% and 7% in the homogenate and mitochondria which was insignificant. This study shows that vitamin E can block the ethionine induced lipid peroxidation and maintain the lipid peroxide level to almost control levels.

Cytochrome content : Table 33 shows the effect of administration of ethionine on the cytochrome content. The concentrations of cytochromes aa_3 and b were decreased but not significantly (by 20% and 16%) while there was no change in the concentration of cytochrome c. In the group in which vitamin E was given alone the concentration of cytochromes aa_3, b and c were decreased by 5%, 12% and 11% respectively. By administration of vitamin E prior to ethionine treatment, the inhibition induced by ethionine was relieved completely on cytochromes aa_3 and b (125% and 119% respectively).

Phospholipid composition : Table 34 shows the effect of ethionine on the composition of the mitochondrial phospholipids. In ethionine treated rats phosphatidyl choline decreased by 23% but was not significant. A significant decrease was seen in cardiolipin (44%). The concentration of phosphatidyl ethanolamine was not effected by ethionine treatment.

Adenine nucleotides : Table 35 shows the effect of the administration of ethionine on the levels of ATP, ADP, AMP and energy charge of the cell. ATP levels decreased significantly (57%) accompanied by a significant increase in

AMP levels (110%). There was no significant effect on the ADP levels. Energy charge decreased significantly by 29% in ethionine treated rats.

Transaminases : Liver damage was assessed by measuring the activity of alanine and aspartate aminotransferases in controls and ethionine treated rats in both liver and plasma (Table 36). In experimental animals alanine aminotransferase decreased significantly by 47% in liver while the activity in plasma increased significantly by four folds (416%). In liver aspartate aminotransfearse decreased significantly (by 52%) accompanied by significant increase (457%) in the plasma levels.

Experimental details :

Control group recived saline.

Ethionine group received ethionine (100mg/100 g bw) for four days.

Vtamin E group received vitamin E (20IU/day/rat) for 20 days.

Vit.E + ethionine group received ethionine after a pretreatment with vitamin E.

Results are expressed as percent control. About 2.5 mg protein was used for each assay. Glutamate and malate were used to reduce matrix NAD^+ to NADH. State 3 (ADP added) respiration was calculated as ng atom of oxygen per min mg protein.

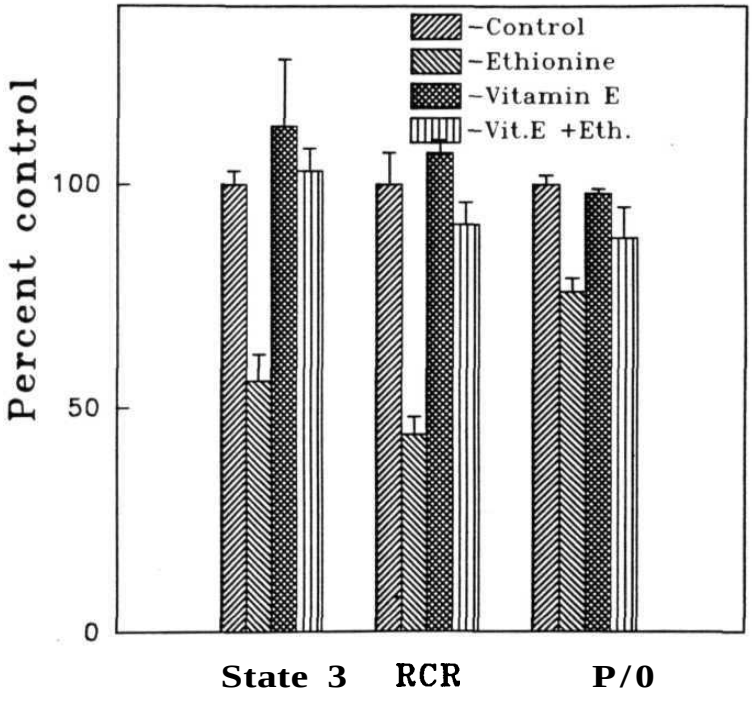
Respiratory control ratio (RCR) is the ratio of ADP stimulated toADP exhausted respiration.

P/O = ADP/O ratio.

All the values are expressed relative to control which was taken as 100. The control value for state 3_{ADP} respiration, RCR and P/O ratio was 62 + 2, 4.4 $\pm$ 0.3 and 3.03 + 0.04 respectively.

Values are Mean + SD of atleast 8 animals.

Fig.19 Effect of administration of Ethionine with or without pretreatment with vitamin E on NADH oxidase.



Experimental details :

Control group recived saline.

Ethionine group received ethionine (100mg/100 g bw) for four days.

Vitamin E group received vitamin E (20IU/day/rat) for 20 days.

Vit.E + ethionine group received ethionine after a pretreatment with vitamin E.

Results are expressed as percent control. About 2.5 mg protein was used for each assay. Succinate was used as the substrate. State 3 (ADP added) respiration was calculated as ng atom of oxygen per min mg protein.

Respiratory control ratio (RCR) is the ratio of ADP stimulated to ADP exhausted respiration.

P/O = ADP/O ratio.

All the values are expressed relative to control which was taken as 100. The control value for state 3 ADP respiration, RCR and P/O ratio was 64 + 6, 3.06 + 0.05 and 2.1 + 0.01 respectively.

Values are Mean + SD of atleast 8 animals.

Fig.20 Effect of administration of Ethionine with or without pretreatment with Vitamin E on Succinate oxidase.

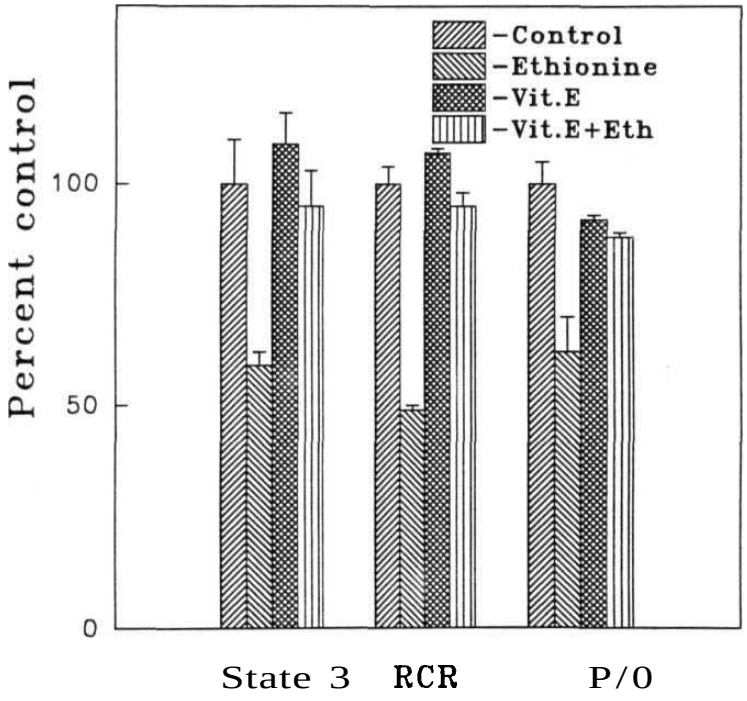


Table 29 : Effect of administration of Ethionine with or without a pretreatment with Vitamin E on a) NADH dehydrogenase and b) Succinate dehydrogenase of liver mitochondria.

GROUP	NADH dehydrogenase (a)	Succinate dehydrogenase (b)
Control	2033 + 152	154 ± 11
Ethionine	1400 ± 99*	142 ± 25
Vitamin E	2120 ± 75	165 ± 8
Vit.E + Ethionine	1880 + 90	142 ± 25

Experimental details : Control group received saline, Ethionine group received ethionine (100mg/100g bw) for four days, Vitamin E group received vitamin E (20IU/day/rat) for 20 days, Vit.+ ethionine group received ethionine after a pretreatment with vitamin E.

a) 20 µg protein was used for each assay. NADH was used as substrate. NADH dehydrogenase activity is expressed as NADH units permin per mg protein. (One NADH unit • one n mole of potassium ferricyanide reduced per min per mg protein).

b) 10 µg protein was used for each assay. Succinate was the substrate. SDH activity is expressed as SDH units per min per mg protein. (One SDH unit = one n mole of DCPIP reduced **per min per** mg protein).

Values are Mean ± SD of atleast four animals.

• = $P < 0.005$.

Table 30 : Effect of administration of Ethionine with or without pretreatment with Vitamin E on the activities of a) Succinate Cyt.C reductase and b) Cytochrome oxidase.

GROUP	Succinate Cyt.C reductase (a)	Cytochrome oxidase (b)
Control	66 + 2	1506 ± 61
Ethionine	36 + 5	961 + 33
Vitamin E	59 + 2	1610 + 120
Vit.E + Ethionine	60 + 3	1460 ± 131

Experimental details are same as described for Table 29.

a) 20 fig protein was used for each assay. Activity is expressed as n moles of cytochrome C reduced per min per mg protein.

b) 1.0 fig protein was used for each assay. Reduced cytochrome C was used as substrate. Cytochrome C oxidase activity is expressed as n moles of cytochrome C oxidised per min per mg protein.

Values are Mean ± SD atleast of four animals.

•• = $P < 0.001$.

Table 31: Effect of administration of Ethionine on the activities of various enzymes in sub mitochondrial particles.

ENZYME	CONTROL	ETHIONINE
1)NADH oxidase	342 ± 12	268 ± 18
2)NADH dehydrogenase	4200 ± 300	3360 ± 150@
3)Succinate dehydrogenase	233 ± 28	269 ± 12
4)Cytochrome oxidase	1552 + 340	942 + 107#
5)Reverse electron transport	15 ± 3	1 0 + 2
6)Membrane potential	197 + 12	145 + 7•

Experimental details are same as described for Table 29.

1) 60 fig protein was used for each assay. Activity is expressed as n moles of NADH oxidised per min per mg protein.

2) 20 μg protein was used for each assay. Activity is expressed as NADH units per min per mg protein. (One NADH unit = one n mole of potassium ferricyanide reduced per min mg protein).

3) 10 fig protein was used for each assay. Activity is expressed as SDH units per min per mg protein. [One SDH unit = one n mole of DCPIP reduced per min per mg protein).

4) 1.0 μg protein was used for each assay. Activity is expressed as n moles of cytochrome C reduced per min per mg protein.

5) 360 fig protein was used for each assay. Activity is expressed as n moles of NAD reduced per min per mg protein.

6) 380 fig protein per ml was used for each assay. 1 mM succinate was added to energise the membrane and 0.3 fiM of CCCP was added to deenergise the membrane. Membrane potential is expressed as millivolts.

Values are Mean 1 SD of atleast six animals.

= $P < 0.05$; @ = $P < 0.02$; • • $P < 0.005$.

Table 32 : Effect of administration of Ethionine on lipid peroxide level in homogenate and mitochondria with or without a pretreatment with Vitamin E.

	LIPID PEROXIDE LEVEL	
GROUP	liver homogenate	mitochondria
Control	122 +8	96 ± 8
Ethionine	210 ± 20*	132 ± 10$
Vitamin E	122 ± 8	8 0 + 5
Vit. E + Ethionine	136 ± 12	103 + 8

Experimental details are same as described for Table 29.

Approximately 2-3 mg protein from 10% liver homogenate and mitochondria was used for each assay. Tetramethoxy propane was used as an external standard. Level of lipid peroxide is expressed as n mol MDA formed per 100 mg protein.

Values are Mean + SD of atleast six animals.

$ = P < 0.01 ; • = P < 0.005.

Table 33 : Effect of administration of Ethionine with or without a pretreatment with vitamin E on cytochrome content of rat liver mitochondria.

GROUP	cytochrome concentration		
	a + a_3	b	c
Control	100 ± 10	100 + 8	100 ± 7
Ethionine	80 ± 20	84 + 8	107 + 4
Vitamin E	95+10	80 + 4	89+11
Vit.E + Ethionine	105 ± 5	103 +8	93 ± 7

Experimental details are same as described for Table 29.

Results are given as percent controls. 4 mg protein was used for each assay. mM extinction coefficients used was 16.0, 20.0 and 19.1 for cyt aa_3, cyt b and cyt c respectively. Concentration of each cytochrome was calculated as n moles per mg protein and expressed relative to control which was taken as 100. The control value for cyt aa cyt b and cyt c was 0.2 *t* 0.02, 0.25 + 0.02 and 0.28 ± 0.02 n moles per mg protein respectively.

Values are represented as Mean 1 SD of atleast six animals.

Table 34 : Effect of administration of Ethionine on the phospholipid composition of rat liver mitochondria.

GROUP	Phosphatidyl choline	Phosphatidyl ethanolamine	Cardiolipin
Control	100 ±6	100 ± 15	100 ± 14
Ethionine	77 + 15	103 ±16	56 ± 6@

Experimental details are same as described for Table 29.

Results are given as percent controls. Phospholipids were separated by TLC using chloroform : methanol : water as solvent. Approximately 200 µl of the lipid extract was applied as a streak. The separated phospholipids were digested with perchloric acid (60%) and the concentration of inorganic phosphate was estimated according to Fiske-Subbarow method. The concentration of phospholipids are expressed as µg phospholipid phosphorous / gm tissue and expressed relative to control which was taken as 100. The control value for phosphatidyl choline, phosphatidyl ethanolamine and cardiolipin are 20.5 ± 1.2, 14.7 ± 2.2 and 13.1 ± 2.1 µg phospholipid phosphorous / gm tissue.

Values are represented as Mean ± SD of atleast four animals.

@ = $P < 0.02$.

Table 35: Effect of administration of Ethionine on the levels of ATP, ADP, **AMP** and energy charge of liver homogenate.

GROUP	**ATP**	ADP	AMP	ENERGY CHARGE
Control	1.85 + 0.07	0.84 ± 0.14	0.28 ± 0.01	0.76 + 0.05
Ethionine	0.81 ± 0.05	0.89 + 0.07	0.59 ± 0.04	0.54 ± 0.03

Experimental details are same as described for Table 29.

Levels of ATP, ADP, and AMP are expressed as μ moles per gm of liver wet weight.

$$\text{Energy charge} = \frac{[\text{ATP} + 1/2\ \text{ADP}]}{[\text{ATP} + \text{ADP} + \text{AMP}]}$$

Values are Mean ± SD of atleast six animals.

* = $P < 0.005$; ** = $P < 0.001$.

Table 36: Effect of administration of Ethionine on the activities of liver and plasma transaminases.

	liver transaminases		plasma transaminases	
GROUP	Al AT	AAT	Al AT	AAT
Control	20 ±1	90 ± 4	0.06 ± 0.01	0.42 ± 0.04
Ethionine	10.6 ±1	43 ± 2	0.31 ± 0.02	2.34 ± 0.12

Experimental details are same as described for Table 29.

Activities of transaminases are expressed as μ moles of NADH oxidised per hour per mg protein. A1AT : alanine aminotransferase, AAT : aspartate aminotransferase.

Values are Mean ± SD of atleast six animals.

•• = $P < 0.001$.

DISCUSSION

Acute liver failure in animals can be developed either i) by excision of liver (hepatectomy), (Lee and Fisher, 1961) or ii) by administration of a specific hepatotoxin (Keppler *et al,* 1968 ; Gazzard *et al,* 1975 ; Zimmerman *et al,* 1989). Carbon tetrachloride, acetaminophen, aflatoxin, thioacetamide (TAA), ethionine and galactosamine are widely used to induce hepatic failure. There are differences in the action of these drugs. CCl_4 and TAA require to be converted into toxic metabolites within the liver via the microsomal P-450 system (Blei *et al,* 1992) while galactosamine and ethionine are known to induce metabolic deficit. Galactosamine is a direct acting hepatotoxin (Keppler *et al,* 1968;Chojkier and Fierer,1985) and galactosamine induced liver injury resembles human viral hepatitis in its morphologic and functional features (Keppler and Decker, 1969). Heavy metals like mercury, lead and hepatotoxins (acetaminophen, carbon tetrachloride etc) cause cell death by elevating intracellular calcium levels (Kass *et al,* 1991 ; Nicotera *et al,* 1992).

Membrane lipid peroxidation is an important pathophysiological event in a variety of diseases and stress conditions. It is observed as a consequence of an intense iron dependent production of oxygen radicals or after administration of toxic drugs such as CCl_4 or acetaminophen both *in vivo* or *in vitro.*

Lipid peroxidation (LPO) results in a cascade of degenerative process from membrane denaturation to tissue damage. Biological membranes that are rich in polyunsaturated fatty acids are highly susceptible to free-radical catalysed oxidation reactions. LPO has been demonstrated to occur in isolated mitochondria, lysosomes and microsomes (Tappel, 1972). In mitochondria about 90% of the fatty acids in the phospholipids are PUFAs, present mainly in PC and PE, which account for approximately 80% of the phospholipids and are susceptible to LPO (Daum, 1985). The cell has built up an impressive set of system for protecting the integrity of their membranes including vitamin E which is the major natural lipophilic antioxidant. However, under pathological conditions these systems can be overwhelmed and non - toxic drugs would be required for preventing the deleterious effects of lipid peroxidation.

In the present study the effect of vitamin E (a biological antioxidant), colchicine and an aqueous extract of *Phyllanthus fraternus* were employed to see their protective action against CCl_4 induced liver damage. Vitamin E and *P.fraternus* were used independently as protective agents against TAA, and vitamin E for ethionine induced liver damage. Vitamin E, is known to be an antioxidant as well as stabiliser of biological membranes (Lucy, 1972). α-tocopherol is present both in the inner and outer mitochondrial membrane and is significantly higher in the outer membrane compared to inner membrane (Thomas *et al,* 1989). The normal range of vitamin E concentration in mitochondria is 0.2-0.3 n mol/mg protein (Lang and Packer, 1987;Thomas *et al,* 1989). Dietary supplementation or depletion of vitamin E for several weeks produces animals

with markedly different levels of endogenous vitamin E in the membranes of fast turnover tissues such as liver. Experimental vitamin E deficiency in animals results in severe pathological conditions such as cardiomyopathy (Sylven and Glavind, 1976 ;Liu *et al,* 1985). In vitamin E deficient rabbits the P/O ratio with succinate as substrate decreased from 1.7 to 1.1 (Tappel, 1972). Studies of ethanol induced hepatic injury (Diluzio and Hartman, 1969) have demonstrated damage to mitochondria concurrent with LPO and this damage could be prevented by vitamin E and other lipid antioxidants (Hartroft and Porta, 1967). α-tocopherol is a lipid soluble, chain breaking antioxidant capable of scavenging free radicals to protect mitochondria against reactive oxygen species generated by mitochondrial electron transport chain. However, oxidative stress destroys vitamin E and may cause its local depletion rapidly within minutes (Niki *et al,* 1988 ;Thomas *et al,* 1989). Thomas *et al* (1993) have reported that vitamin E deficiency in rats, leads to increased oxidative stress and a characteristic myopathy accompanied by decreased activity of complex I and IV of electron transport chain in muscle. It is reported that vitamin E protects against galactosamine induced toxicity (Sclafani *et al,* 1986).

P.fraternus, a medicinal plant which is known for its use in traditional medicine was used to see the protective effect against CCl_4 and TAA induced liver injury, An aqueous extract of the plant has been shown to be effective in the treatment of jaundice (Ramanan and Sainani, 1961 ; Thygarajan *et al,* 1977), diabetes (Ramakrishnan *et al,* 1982) and genito-urinary infections (Chopra *et al,* 1956). Phyllanthin and hypophyllanthin which have been identified as lignans (Row *et al,* 1964) have been shown to protect against CCl_4 and galactosamine induced toxicity produced in primary cultured rat hepatocytes. Recently it has been shown that the administration of an aqueous extract of *P.fraternus* along with alcohol prevented most of the liver damages induced by chronic alcoholism in rats (Thomas, 1994).

Colchicine, an alkaloid of *Colchicum autumnale* has been used for treatment against gouty arthritis (Flower *et al,* 1985) and it has also been used to reverse the experimental and clinical cirrhosis (Mourelle *et al,* 1981). Liver cirrhosis induced by CCl_4 was prevented by pretreatment with colchicine (10 μg/ day/rat) for seven days (Mourelle *et al,* 1987). Colchicine has been used as a drug for inhibition of lipid peroxidation and these studies have been limited to the plasma membrane (Mourelle *et al,* 1987). Mourelle (1989) has reported that colchicine prevents D-galactosamine induced hepatitis probably by its ability to inhibit lipid peroxidation.

When the control for selective membrane permeability of the mitochondrial membrane was lost, swelling was observed due to the excess transfer of solutes and thus the mitochondrial volume increased. This change was followed by observing the decrease in absorbance at 520 nm. Administration of CCl_4, TAA and ethionine induced swelling of mitochondria shown in Fig.7. Mitochondrial

swelling is known to be caused by energy deficiency state (Halestrap *et al,* 1993) and probably the decrease in ATP synthesis observed in the present study on administration of these toxins could have caused the observed swelling in mitochondria.

Mitochondrial membranes contain relatively large amounts of PUFAs in their phospholipids and are prime sites of LPO mediated damage (Fleischer and Rouser, 1965). Studies by Mcknight *et al* (1965) and Hunter *et al* (1964) have shown that LPO induces swelling and lysis leading to disintegration of mitochondria. *In vitro* lipid peroxidation of isolated rat liver mitochondria by ferrous ion brought about mitochondrial swelling, a decrease in membrane potential and alterations in membrane proteins (Castilho *et al,* 1994). Zhang *et al* (1990) have reported that all the complexes of mitochondrial electron transport chain are vulnerable to the damage by oxygen free radicals *in vitro.*

Transaminases are used as marker enzymes of liver damage. Aspartate aminotransferase mediates the reversible transfer of a-amino group from aspartate to α-ketoglutarate resulting in the production of glutamate and oxaloacetate. Alanine aminotransferase mediates the transfer of a- amino group from alanine to a- ketoglutarate forming pyruvate and glutamate. Administration of CCl_4 , TAA and ethionine resulted in a significant decrease in the activities of these enzymes in the liver (Tables 9,21 and 36). This decrease is probably due to the leakage of the enzymes from the liver into the extracellular compartment. If this extracellular compartment is in dynamic exchange with the blood, then the enzyme activities will increase in the blood (Mukherjee, 1990). It was observed that on administration of CCl_4 , TAA and ethionine the levels of these enzymes were increased in the plasma (Tables 9,21 and 36). This is probably due to the leakage of the enzymes from the liver cell during the toxicity induced by the drugs. With galactosamine also a similar progressive increase in plasma alanine aminotransferase and aspartate aminotransferase are reported (Reeba, 1995).

In mitochondria, the respiratory chain is stoichiometrically related to that of ATP synthesis (Pozzan *et al,* 1979). If the reducing substrate is glutamate or malate 3 mol of ATP per pair of electrons are synthesized If the substrate is succinate 2 mol of ATP are synthesized. The relative effects of all the four hepatotoxins on each parameter was discussed together even though the effect of each hepatotoxin was described separately in other sections. This approach helps to compare the toxicity by these hepatotoxins and the protective effect obtained on the toxicity by various protective agents. NADH oxidase which uses the electron carriers of all three sites of the electron transport chain was inhibited by 93%, 95%, 59% and 43% in CCl_4 , galactosamine, TAA and ethionine treated rats respectively (Tables 37,39,40 and 42). This indicates that there is an inhibition in the transfer of electrons through all three sites. RCR and P/O ratio were also significantly decreased by the administration of these hepatotoxins. Administration of vitamin E or *P.fratermus* or colchicine prior to CCl_4 relieved

Table 37: Protective effect of vitamin E or *P.fraternus* or colchicine on CCl_4 induced mitochondrial dysfunction.

VALUES ARE GIVEN AS PERCENT CONTROL.

PARAMETER	CCl_4	Vit. E + CCl_4	P.frat. + CCl_4	Colc. + CCl_4
NADH oxidase	7	85 (84)	88 (87)	82 (81)
Succinate oxidase	35	83 (74)	79 (68)	84 (75)
Cytochrome oxidase	49	104 (108)	77 (55)	88 (76)
NADH dehydrogenase	65	86 (60)	90 (71)	95 (86)
Succinate dehydrogenase	24	115 (120)	99 (99)	90 (87)
Succinate cytochrome C reductase	32	80 (71)	75 (63)	82 (74)
Cyt.aa_3	66	105 (114)	87 (62)	83 (50)

Results are expressed relative to control value which is taken as 100 (not shown in Table). The data for cytochrome oxidase (Table 2) for CCl_4 group and P.fraternus + CCl_4 group can be expressed as 49 and 77 respectively. The percent inhibition is 51 (100-49) and 33 (100-77) respectively. The relief on CCl_4 induced inhibition by P.fraternus was (51-33 = 28) 28. The percent relief was calculated as 100/51 x 28 = 55, which is shown in parenthesis.

CCl_4 group : 4g/kg body weight was administered orally and the animals were sacrificed 24 later.

Vitamin E +CCl_4 : Rats were treated with vitamin E (20IU/day/rat) for 20 days, then administered CCl_4 (4g/kg bw) and sacrificed 24h later.

P.fraternus + CCl_4 : Rats were given an aqueous extract of *P.fraternus* (100mg dry powder /kg bw) for fivr days, then administered CCl_4 (4g/kg bw) and sacrifced 24 h later.

Colchicine + CCl_4 : rats were given colchicine (10μg/day/rat) for seven days, then administered CCl_4 (4g/kg bw) and sacrificed 24 h later.

Table 38: Protective effect of vitamin E or *P. fraternus* on CCl_4 induced mitochondrial dysfunction.

VALUES ARE GIVEN AS PERCENT CONTROL.

PARAMETER	CCl_4	Vit. E + CCl_4	*P. frat.* + CCl_4
Cardiolipin	42	77 (60)	61 (33)
Phosphatidyl choline	62	72 (26)	76 (37)
Phosphatidyl ethanolamine	53	75 (47)	83 (64)
LPO level	231	145 (66)	118 (86)
ATPase(stimulation with DNP)	11	90 (89)	Not done

Experimental details are same as described for Table 37.

Values given in parenthesis indicate the percent relief calculated as explained in the legend for Table 37.

Table 39 : Effect of Galactosmaine administration on mitochondria.

VALUES ARE GIVEN AS PERCENT CONTROLS.

PARAMETER	CONTROL	GALACTOSAMINE
NADH oxidase	100	5
Succinate oxidase	100	10
Cytochrome oxidase	100	37
NADH dehydrogenase	100	17
Succinate dehydrogenase	100	53
Succinate cytochrome C reductase	100	34
Cardiolipin	100	39
Phospahtidyl choline	100	45
Phosphatidyl ethanolamine	100	42
Cyt.aa_3	100	36
Cyt.b	100	56
Cyt.c	100	53
LPO level	100	245

Control : Rats were given saline.

Galactosamine : Rats were given galactosamine (2.5g/kg bw) and sacrificed 36h later.

Values given in parenthesis indicate the percent relief calculated as explained in the legend for Table 37.

Table 40 : Protective effect of vitamin E or *P. fraternus* on Thioacetamide induced mitochondrial dysfunction.

VALUES ARE GIVEN AS PERCENT CONTROL.

PARAMETER	TAA	Vit. E + TAA	P.frat. + TAA
NADH oxidase	41	104 (107)	86 (76)
Succinate oxidase	53	91 (81)	75 (47)
Cytochrome oxidase	63	82 (51)	89 (70)
NADH dehydrogenase	39	83(72)	87(79)
Succinate cytochrome C reductase	46	76(56)	71(46)

TAA : Rats were given TAA (25mg/100g bw) for four days and sacrificed 24h later.

Vit.E +TAA : Rats were treated with vitamin E (20IU/day/rat) for 20 days, then given TAA (25mg/100g bw) for four days and sacrificed 24h later.

P.frat. +TAA : Rats were given an aqueous extract of *P. fraternus* (100mg powder/kg bw) for five days, then given TAA (25mg/100g bw) for four days and sacrificed 24h later.

Values given in parentheses indicate the percent relief calculated as explained in the legend for Table 37.

Table 41: Protective effect of vitamin E or *P. fraternus* on Thioacetamide induced mitochondrial dysfunction. VALUES ARE GIVEN AS PERCENT CONTROL.

PARAMETER	TAA	Vit. E + TAA	P.frat. + TAA
Cardiolipin	56	107 (116)	92 (82)
Phosphatidyl choline	58	84 (62)	89 (74)
Phosphatidyl ethanolamine	72	86 (50)	91 (68)
Cyt.aa_3	76	87 (46)	92 (67)
Cyt.b	78	96 (82)	85 (32)
LPO level	131	108 (74)	100 (100)
ATPase (stimulation with 2,4-DNP)	26	68 (57)	Not done

Experimental details are same as given in Table 40.

Values given in the parenthesis indicate the percent relief calculated as explained in the legend for Table 37.

Table 42 : Protective effect of vitamin E on Ethionine induced mitochondrial dysfunction.

VALUES ARE GIVEN AS PERCENT CONTROL.

PARAMETER	Ethionine	Vit. E + Ethionine
NADH oxidase	56	103 (107)
Succinate oxidase	59	95 (88)
Cytochrome oxidase	64	97 (92)
NADH dehydrogenase	69	92 (74)
Succinate cytochrome C reductase	55	91 (80)
LPO level	138	107 (82)
Cyt.aa_3	80	105 (125)
Cyt.b	84	103 (119)

Ethionine : Rats were given ethionine (100mg/100g bw) for four days and sacrificed 24h after the last dose.

Vit.E + Ethionine : Rats were given vitamin E (2 0IU/day/rat) for 20 days, then ethionine (lOOmg/lOOg bw) was administered for four days and sacrificed 24h after the last dose.

Values given in parenthesis indicate the percent relief calculated as explained in the legend for Table 37.

the inhibition (induced by CCl_4) on NADH oxidase by 84%, 87% and 81% respectively (Table 37). Prior administration of vitamin E or *P.fraternus* relieved the inhibition induced by TAA on NADH oxidase by 107% and 76% respectively (Table 40). Administration of vitamin E prior to ethionine relieved the inhibition on NADH oxidase completely (Table 42). Prior administration of these protective agents relieved the inhibition (induced by these toxins) on RCR and P/O ratio by more than 90%. Succinate oxidase was inhibited by 65%, 91%, 47% and 41% in CCl_4 , galactosamine, TAA and ethionine treated rats respectively (Tables 37,39,40 and 42). This indicates that there was an inhibition in the electron transfer at site 2 or / and site 3. Prior treatment with vitamin E or *P.fraternus* or colchicine relieved the inhibition (induced by CCl_4) by 74%, 68% and 75% respectively (Table 37). In case of TAA, prior administration of vitamin E or *P.fraternus* relieved inhibition (induced by TAA) by 81% and 47% respectively (Table 40). Administration of vitamin E prior to ethionine relieved the inhibition by 88% (Table 42). On addition of CCCP (uncoupler), a stimulation in respiration was seen in control rats (with both substrates) while in rats treated with CCl_4, TAA , ethionine and galactosamine no stimulation was observed showing that mitochondria were uncoupled. The mitochondrial respiration is tightly coupled to oxidative phosphorylation in intact cells of normal tissues (Tzagoloff and Meyers, 1986). Tightly coupled mitochondria always have high RCR and P/O ratio. Decreased RCR and P/O ratio indicate the damage of mitochondrial membrane, thereby rendering the membrane leaky to other ions or biomolecules that leads to uncoupling of mitochondria. Increased permeability of the mitochondrial membrane leads to uncoupling of oxidative phosphorylation (Soussi *et al,* 1990). In isolated liver mitochondria fatty acid hydroperoxides, intermediate products of LPO cause permeability changes in the mitochondria leading to an uncoupling of oxidative phosphorylation (Masini *et al,* 1994).

Our results are in agreement with those of Jikko *et al* (1984) who reported a decrease in RCR in mitochondria isolated from rats with CCl_4 induced cirrhosis. Moller and Dargel (1984) have also shown that RCR decreased on TAA treatment. Administration of D,L-ethionine to rats produces partial or total uncoupling of pancreatic mitochondrial respiration with the absence of morphologic evidence of mitochondrial injury (Wilson *et al,* 1986). Studies by Ouchi *et al* (1984) with galactosamine showed that there was a decrease in state 3 respiration and ATP synthesis.

Studies on ATPase activity in CCl_4 and TAA treated rats also confirmed that the mitochondrial membrane integrity was lost by these hepatotoxins. The ATPase activity in tightly coupled mitochondria is very low and the activity is elicited by the addition of uncouplers or by treatments that damage membrane and lead to uncoupling. The results in Tables 38 and 41 show that there was a significant decrease in the stimulation of ATPase by 2,4-DNP (11% and 26% stimulation in CCl_4 and TAA treated rats respectively compared to 100%

stimulation in controls). The data is expressed here relative to control which was taken as 100. In rats which were administered vitamin E prior to CCl_4 or TAA treatment the ATPase activity was stimulated (by 90% and 68% respectively) showing that stimulation on ATPase activity which was lost by CCl_4 or TAA was recovered by prior treatment with vitamin E (Tables 38 and 41).

Measurement of MDA (secondary product of LPO), a useful method for determination of LPO products (Sawicki *et al,* 1963; Placer *et al,* 1966) showed a significant increase in both liver homogenate and mitochondria with all the four hepatotoxins. The increase was observed in the order of galactosamine > CCl_4 > ethionine > TAA (245%, 231%, 138% and 131% respectively, Tables 39,38,42 and 41). LPO is a free radical phenomenon and induces a series of alterations in the structure and function of cellular membranes (Kale, 1990). Prior treatment with vitamin E or *P.fraternus* brought down the lipid peroxide level in CCl_4 (by 145% and 118% respectively, Table 38) and in TAA treated rats (by 108% and 107% respectively, Table 41). In ethionine treated rats prior treatment with vitamin E brought down the LPO levels by 107% (Table 42). This accounts to a recovery of 66% and 86% for vitamin E and *P.fraternus* respectively in the case of CCl_4. When TAA was used as hepatotoxin the recovery by vitamin E or *P.fraternus* was 74% and 100%. The recovery by vitamin E in the case of ethionine was 82%. *P.fraternus* appears to protect from these hepatotoxins (with respect to hepatotoxin induced accumulation of lipid peroxide) better than vitamin E. Hu *et al* (1992) reported that zinc had a protective effect on galactosamine induced liver damage. The effect may be due to inhibition on LPO and stimulation on protein synthesis.

NADH dehydrogenase, which feeds electrons to the electron transport chain was decreased by 35%, 83%, 61% and 31% in CCl_4 , galactosamine, TAA and ethionine treated rats respectively (Tables 37,39,40, and 42). Membrane phospholipids are essential for the functioning of mitochondrial NADH dehydrogenase (Hatefi and Stigall, 1976). The observed decrease in NADH dehydrogenase may limit the rate of electron flow through site I and effect energy conservation in this region of the respiratory chain. Administration of vitamin E or *P.fraternus* or colchicine prior to CCl_4 relieved the inhibition (induced by CCl_4) on NADH dehydrogenase by 60%, 71% and 86% respectively (Table 37). Prior administration of vitamin E or *P.fraternus* relieved TAA induced inhibition by 72% and 79% respectively (Table 40). Administration of vitamin E prior to ethionine relieved the inhibition by 74% (Table 42).

Rats were allowed to recover on their own after administration of a single dose of CCl_4 (4g/kg bw). Mitochondria were isolated at the end of 48hrs and 96hrs and different enzymes were studied. NADH oxidase, succinate oxidase, SDH and cytochrome oxidase showed complete recovery by 96hrs, while the inhibition on NADH dehydrogenase was still present (28%) at the end of 96hrs. This part of the electron transport chain has been shown to be susceptible for the

damage by organic solvents such as CCl_4 (Krahlenbuhl *et al,* 1989) and ethanol (Cederbaum *et al,* 1974 ; Schilling and Reitz, 1980).

The activity of SDH was significantly decreased by 76% and 47% in CCl_4 and galactosamine treated rats (Tables 37 and 39) while in TAA and ethionine treated rats it did not show any significant change. Administration of vitamin E or *P.fraternus* or colchicine prior to CCl_4 relieved the inhibition by 120%, 99% and 87% respectively (Table 37). The decrease in succinate oxidation can be related partly to the decrease in succinate dehydrogenase activity. It is involved in the oxidation of succinate to fumarate and the electrons released are transferred to the electron transport chain. A decrease in electron flow at source to complex II can lead to the decreased activity of succinate oxidase.

Another enzyme which was affected significantly by the hepatotoxins was cytochrome oxidase, which is the terminal enzyme in the mitochondrial respiratory chain and contains two atoms of haem (a and a_3) and two atoms of copper as the main redox component of the cytochrome oxidase complex (Hartzell *et al,* 1978, Azzi, 1980). The activity of cytochrome oxidase was decreased by 51%, 63%, 37% and 36% in CCl_4 , galactosamine, TAA and ethionine treated rats respectively (Tables 37,39,40, and 42). Administration of vitamin E or *P.fraternus* or colchicine prior to CCl_4 relieved the inhibition (induced by CCl_4) by 108%, 55% and 76% respectively (Table 37). Prior treatment with vitamin E or *P.fraternus* relieved the inhibition (induced by TAA) on cytochrome oxidase by 51% and 70% respectively (Table 40). Administration of vitamin E prior to ethionine relieved the inhibition by 92% (Table 42).

Respiratory process involves the transport of electrons via cytochromes to molecular oxygen and changes in cytochrome concentrations will affect the transport of electrons via respiratory chain and thereby alter the energy production of mitochondria. The concentration of cytochrome aa_3 which serves as an electron carrier between cytochrome c and oxygen was decreased by 34%, 64%, 24% and 20% in CCl_4, galactosamine, TAA and ethionine treated rats respectively (Tables 37,39,41, and 42). Administration of vitamin E or *P.fraternus* or colchicine prior to CCl_4 relieved inhibition on cytochrome aa_3 by 114%, 62% and 50% respectively (Table 37). Prior treatment with vitamin E or *P.fraternus* relieved the inhibition (induced by TAA) by 46% and 67% respectively (Table 41). Administration of vitamin E prior to ethionine administration relieved the inhibition induced by ethionine on cytochrome aa_3 by 125% (Table 42). Earlier studies (Tappel, 1972) have demonstrated that in isolated electron transport chain LPO causes damage to cytochrome aa_3 , b and c.

Studies by Moller and Dargel (1984) have shown that phospholipids (PC, PE and CL) are essential for providing proper polar and spatial arrangement necessary for optimal activity of membrane bound enzymes and carriers. A small change in lipid components may cause marked changes in the activity and efficiency of enzymes or carriers. PC and PE have been shown to be essential for

the normal functioning of cytochrome oxidase in ethanol fed baboons (Arai *et al,* 1984). Cardiolipin plays a special role in the activity of several enzymes localised in the inner mitochondrial membrane (Daum, 1985) including the enzymes at site I and site HI of the respiratory chain. The enzymes at site II lack the requirement of cardiolipin (Nicolay and Kruijff, 1987). Effect of these hepatotoxins on phospholipids of mitochondria (PC, PE and CL) was studied. PC decreased by 38%, 55%, 42% and 23% in CCl_4, galactosamine, TAA and ethionine treated rats respectively (Tables 38,39, 41 and 34). Administration of vitamin E or *P.fraternus* prior to CCl_4 relieved the inhibition (induced by CCl_4) on PC by 26% and 37% respectively (Table 38) while in TAA treated rats the induced inhibition was relieved by 62% and 74% respectively (Table 41). PE decreased by 47%, 58% and 28% in CCl_4 , galactosamine and TAA treated rats respectively (Tables 38,39 and 41). Administration of vitamin E or *P.fraternus* relieved the inhibition induced by CCl_4 on PE by 47% and 64% respectively (Table 38) while in TAA treated rats the inhibition was relieved by 50% and 68% respectively (Table 41). Significant effect was seen on cardiolipin, which is specifically abundant in the inner mitochondrial membrane. It decreased by 58%, 61%, 44% and 44% in CCl_4 , galactosamine, TAA and ethionine treated rats (Tables 38,39,41 and 34). Prior administration of vitamin E or *P.fraternus* relieved the inhibition on CL (induced by CCl_4) by 60% and 33% respectively (Table 38) while in TAA treated rats the inhibition was relieved by 116% and 82% respectively (Table 41). Cytochrome oxidase is dependent on cardiolipin for maximum activity (Soussi *et al,* 1990). CL is a polyunsaturated phospholipid located in the inner mitochondrial membrane and under conditions of high oxidative stress, it is prone to LPO due to the high degree of unsaturation. It has also been reported that CL, activates the cytochrome chain and influences several mitochondrial carrier systems including ATP synthase (Awasthi *et al,* 1971; Spencer *et al,* 1976; DeKruijff *et al,* 1981; Rietveld *et al,* 1987; Powell *et al,* 1990). This shows that the decreased activity of cytochrome oxidase was partly or solely due to the decreased CL in the membrane.

Arai *et al* (1984) have reported that in ethanol fed baboons the decrease in cytochrome oxidase activity was associated with changes in the lipid composition of the mitochondrial membranes accompanied by a decrease in the concentration of cytochromes. Studies by Paradies *et al* (1994) have shown that in hypothyroid rats the activity of cytochrome oxidase is decreased significantly (30%) and the content of cytochrome aa_3 is decreased by 15%. The depression in cytochrome oxidase activity was related to a decrease in the cardiolipin. In the present study also the decrease in cytochrome oxidase activity can be attributed to decreased levels of both phospholipids and cytochromes of mitochondria. Previous studies (Hernandez-Munoz *et al,* 1992) have shown that chronic treatment with CCl_4 resulted in a significant decrease in phosphatidylserine and cardiolipin accompanied by an increase in cholesterol levels. A key role for cardiolipin in controlling membrane fluidity has been suggested by Ellingson *et al* (1988).

Decreased level of cardiolipin may play a role in alteration of membrane fluidity. Chronic treatment with TAA resulted in marked decrease in PC, PE and CL leading to a decreased 20:4 / 18:2 ratio, which was responsible for alterations of the mitochondrial membrane fluidity (Moller and Dargel, 1984). Administration of D-galactosamine to rats caused a depression of total phospholipid content in the mitochondria] membrane (Sire *et al,* 1986). They reported that PC, PE and CL decreased by 21%, 29% and 40% on galactosamine administration. *In vitro* exposure of rat lung mitochondria to CCl_4 , inhibits PC synthesis and this inhibition was found to be accompanied by a dramatic loss in cellular ATP, a cofactor required by some enzymes involved in PC synthesis (Ma *et al,* 1989). As ATP is essential for the synthesis of these phospholipids, an impairment in oxidative phosphorylation (caused by hepatotoxins) may put a limit on the synthesis of these rapidly turning over phospholipids.

Succinate cytochrome C reductase activity decreased by 68%, 66%, 55% and 45% in CCl_4 , galactosamine, TAA and ethionine treated rats respectively (Table 37,39, 40 and 42). Administration of vitamin E or *P.fraternus* or colchicine relieved the inhibition (induced by CCl_4) by 71%, 63% and 74% respectively (Table 37). Prior treatment with vitamin E or *P.fraternus* relieved the inhibition induced by TAA by 56% and 46% respectively (Table 40). In ethionine treated rats the inhibition induced by ethionine on this enzyme was relieved by 80% (Table 42) on prior treatment with vitamin E. This decrease in succinate cytochrome C reductase activity may be explained partly due to a decrease in the content of cytochrome b (44%, 22% and 16% in galactosamine, TAA and ethionine treated rats respectively, Tables 39,41 and 42). In CCl_4 treated rats the decrease in the enzyme activity could be partly due to a change in the lipid environment of the mitochondrial membrane. The decrease on this enzyme could also be due to a decrease in the rate of succinate oxidation.

Reverse electron transport was decreased by 64%, 53%, 65% and 33% in CCl_4, TAA, galactosamine, and ethionine treated rats (Tables 3,15,24 and 31). This enzyme gives information about the ability of transfer of electrons through site I (in reverse direction). SMP have a right side out membrane, so substrates need not be transported to matrix for their oxidation. If the hepatotoxins have an effect on the transport of a substrate across the mitochondrial membrane, less substrate is transported and lower oxidation rates are found, even though the enzymatic machinery for its oxidation is normal. On the contrary, if the decreased oxidation is due to the decrease in oxidation machinery and the transport is not affected, one expects a similar oxidation rate both in SMP as well as mitochondria. In CCl_4 treated mitochondria NADH dehydrogenase, SDH and cytochrome oxidase were decreased by 35%, 76% and 51% respectively (Table 37) while in SMP a more or less similar inhibition was observed (17%, 53% and 45% respectively, Table 3). In galactosamine treated mitochondria (at the end of 36h), NADH dehydrogenase, SDH and cytochrome oxidase were decreased by 83%,

47% and 63% respectively (Table 39) while in SMP they were inhibited by 71%, 33% and 56% (Table 24). In TAA treated mitochondria, NADH dehydrogenase and cytochrome oxidase were decreased by 61% and 37% (Table 40) while in SMP a 49% and 61% inhibition was observed (Table 15). In ethionine treated mitochondria, NADH dehydrogenase and cytochrome oxidase decreased by 31% and 36% respectively (Table 42) while in SMP they were decreased by 20% and 39% respectively (Table 31). Studies using flourescent probes and electron paramagnetic spectroscopy revealed that chronic ethanol consumption induces modification in the fluidity of mitochondrial membrane which may result in alterations of metabolite transport across the mitochondrial membrane (Waring *et al,* 1982 ; Harris *et al,* 1987).

Incorporation of [^{35}S] methionine into mitochondrial protein products was decreased in CCl_4 treated rats showing that the administration of CCl_4 inhibited the mitochondrial protein synthesis (Fig.8) and the observed decrease in the various enzymes of the respiratory chain can also be attributed to the decreased mitochondrial protein synthesis. It has been reported earlier that CCl_4 inhibits protein synthesis (Smuckler *et al,* 1961).

Kinetic studies on cytochrome oxidase activity showed that the K_m of the enzyme in CCl_4 treated rats increased by 55% and the V_{max} decreased by 44% (Fig. 10). This explains the significant decrease in the activity of cytochrome oxidase by CCl_4 . SDS-PAGE of the purified enzyme in control and experimental rats revealed that four subunits of the enzyme (I,II,III and VI) were decreased significantly in CCl_4 treated rats when compared to controls (Fig. 12). Capaldi *el al* (1983) have reported that two subunits of the enzyme i.e., I and 11 contain the oxidation-reduction centres of the enzyme. Chemical cross linking (Briggs and Capaldi, 1978 ; Bisson *et al,* 1980 ; Fuller *et al,* 1981) and competitive binding experiments (Millet *et al,* 1982, 1983) have shown that the substrate, cytochrome c, occupies a high- affinity binding site on subunit 11. Studies by Prochaska and Fink (1987) have shown that subunit III is a membrane spanning polypeptide and is involved in vectorial proton translocation. The role of subunit VI is not clearly known. It is known that it is buried or shielded from both the hydrophilic and hydrophobic milieu.

Studies on kidney mitochondria showed that only CCl_4 had a significant effect (on NADH dehydrogenase, SDH and cytochrome oxidase activities, Table 11), while galactosamine (Table 28), TAA and ethionine did not have any significant effect on kidney mitochondria. Renal toxicity of CCl_4 can be explained as a consequential effect. It is well known that galactosamine affects specifically liver alone (Keppler *et al,* 1968).

Membrane potential (AT) is the main component of the proton motive force (Mitchell and Moyle, 1969) and has significant control over mitochondrial respiration (Brand, 1990a, 1990b ;Murphy, 1990). It is altered by changes in mitochondrial lipid and protein composition (Hoch, 1988 ;Hafner *et al,* 1988;

Murphy, 1990). Membrane potential was decreased by 31%, 24%, 38% and 26% in CCl_4, TAA, galactosamine, and ethionine treated rats (Tables 3,15,24 and 31). Under normal conditions, the transfer of electrons through the respiratory chain leads to the vectorial translocation of protons from the matrix to the other side of the inner mitochondrial membrane. Any disruption in the generation of the proton gradient will have adverse effects on mitochondrial energy production, in particular on ATP synthesis, because this proton gradient is the driving force for the formation of ATP from ADP.

The levels of ATP decreased in CCl_4, TAA and ethionine treated rats accompanied by a concomitant increase in ADP levels (Tables 8, 20 and 35). Energy charge of the cell also decreased significantly in CCl_4, TAA and ethionine treated rats. Energy charge, designated by Atkinson (1970) as (ATP+1/2 ADP)/ ATP+ADP+AMP, plays an important role as regulatory effectors of many enzymatic reactions and provides with a very sensitive intracellular control mechanism. The energy charge value reflects the balance between the energy yielding and energy utilising reactions and is maintained at 0.85 - 0.9 in normal cells. The energy charge strongly resists any deviations from 0.85, but if it decreases, the ATP generating sequences are accelerated and ATP requiring sequences are slowed down Adenylate energy charge level is a measure of the energetic state of the cell. It has been found to be decreased in livers of CCl_4 administered rats indicating a reduced ATP production in accordance with earlier reports (Jikko *et al*, 1984).

In studies conducted by Lambert and Wright (1984), cirrhotic patients showed a reduction in hepatic energy charge as a result of reduction in cellular ATP levels accompanied by an elevation in the levels of AMP. They also observed mitochondrial dysfunction with deterioration of the clinical status of the patients (Wright, 1980 ; Ozawa *et al,* 1973). Ozawa *et al* (1974) has stated that cirrhosis appears to decrease respiratory enzymes in liver mitochondria, leading to a reduction in ATP synthesis. Hyperbilirubinaemia is also known to inhibit mitochondrial oxidative phosphorylation (Cowger, 1971).

Administration of ethionine also induces a decrease in the intracellular ATP content both in the cytosol and mitochondria of fed and fasted rat hepatocytes (Lavoinne *et al,* 1983). Ouchi *et al* (1984) have reported that in galactosamine treated rats ATP, ADP and total adenine nucleotide contents decreased significantly.

The effects of these hepatotoxins appears to be due to the increased lipid peroxidation which leads to the loss of the integrity of mitochondrial membrane. Lipid peroxidation also leads to the decreased levels of phospholipids in the membrane, which significantly decreased the transfer of electrons through the electron transport chain. The decreased rate of ATP synthesis (due to the loss in the integrity of the mitochondrial membrane) together with the decreased rate of respiration leads to a significant decrease in the cellular ATP levels. The

decreased ATP levels in turn leads to the decreased protein synthesis which might be responsible for decreased level of cytochromes in the mitochondria, which will further decrease the rate of respiration.

Administration of vitamin E, or an aqueous extract of *P.fraternus* or colchicine reversed most of the hepatotoxin induced damages.

SUMMARY

In the present study it was observed **that** :

1) On administration of hepatotoxins (CCl_4, TAA and ethionine) the activities of aspartate and alanine transaminases in plasma increased significantly showing that the liver was damaged.

2) NADH oxidase which gives information on the ability of transfer of electrons through all three sites was decreased significantly in rats treated with CCl_4, TAA, galactosamine and ethionine .

3) Succinate oxidase which gives information on the electron transfer through site II and III was **also** decreased significantly on administration of CCl_4, TAA, galactosamine and ethionine .

4) RCR which is an index of membrane integrity was decreased by more than 50% on administration of these hepatotoxins (CCl_4, TAA, galactosamine and ethionine) showing that the **integrity** of mitochondria was damaged. P/O ratio, an index of the efficiency of the system to conserve energy in the form of ATP, was decreased significantly with all the hepatotoxins studied.

5) NADH dehydrogenase which feeds electrons to the respiratory chain at site I was decreased significantly in CCl_4. TAA, galactosamine and ethionine treated rats.

6) Succinate dehydrogenase, which feeds electrons to the respiratory chain at site II was decreased significantly in CCl_4 and galactosamine treated rats.

7) Cytochrome oxidase which gives information about electron carriers at site III of the respiratory chain was decreased significantly in CCl_4, TAA, galactosamine and ethionine treated rats.

8) Studies on succinate cytochrome C **reductase** which gives information about site II of the electron transport chain was decreased significantly in CCl_4, TAA, galactosamine and ethionine treated rats.

9) The ATPase activity which is generally very low in tightly coupled mitochondria, was found to be very high in CCl_4 and TAA treated mitochondia showing that the mitochondria were uncoupled.

10) Lipid peroxide level (index of membrane damage) was increased significantly in liver homogenate and mitochondria of CCl_4, TAA , galactosamine and ethionine treated rats.

11) Reverse electron transport which gives information about site I of electron transport chain was decreased significantly on administration of CCl_4, TAA and galactosamine treated rats.

12) The concentrations of cytochromes (aa_3, b and c) which serve as electron carriers were decreased sigificantly on administration of CCl_4, TAA galactosamine and ethionine treated rats.

13) The concentrations of phospholipids phosphatidyl choline, phosphatidylethanol amine and cardiolipin were also decreased significantly on administration of CCl_4, TAA, galactosamine and ethionine treated rats.

14) Studies on purified cytochrome oxidase from CCl_4 treated rats showed that four subunits of the enzyme were decreased significantly. Kinetic studies of the purified enzyme showed that the K_m had increased significantly while V_{max} showed a sigificant decrease.

15) There was a significant decrease in the energy charge of the cell due to the adminstration of CCl_4, TAA and ethionine.

16) Among the four hepatotoxins studied only CCl_4 showed a significant effect on kidney mitochondria.

Administration of the protective agents like vitamin E (biological antioxidant) or *P.fraternus* or colchicine prior to the hepatotoxin reversed most of the effects induced by these hepatotoxins. It can be concluded that the administration of the hepatotoxins damage the membrane integrity, decrease the rate of electron transport through various segments of the electron transport chain and decrease ATP synthesis These effects are due to the production of free radical which will lead to lipid peroxidation and membrane damage. All most all these damages can be reversed by prior administration of vitamin E or *P.fraternus* or colchicine.

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