PATHOLOGICAL CHANGES

Light microscopic studies

Carbon tetrachloride, even in a single dose, produces hepatic necrosis and fatty infiltration in a variety of experimental animals and man (Rouiller, 1964). Gallagher (1962) has reported massive centrilobular necrosis and fatty infiltration in rats 24 hr after feeding CCl₄ (4 ml/Kg). Dinman et al. (1962) have demonstrated hydropic and granular degeneration immediately after exposure to 200 ppm of CCl₄ in the rabbit. Liver necrosis was not seen at this time. After 8 hr exposure to 200 ppm of CCl₄, typical mallory's body, polymorphonuclear infiltration and accumulation of fat was seen. Liver necrosis was very little at this time. Karyotic changes in liver cell nucleus, increase in intracellular fat and minimal inflammation in the centrilobular area were observed after 16 hr exposure to CCl₄. After 24 hr exposure to CCl₄, there was marked fatty infiltration and hepatocytes without nuclei or mallory's bodies were also seen. Regeneration with minimum infiltration in the centrilobular area was seen 72 hr after exposure.

Dinman et al. (1962) had observed hydropic cells in the centrilobular area 8 hr after 500 ppm of CCl₄ in the rabbits. After 36 hr, the reticular stroma immediately
about the central vein area had collapsed and infiltration of round cell was seen. Fatty infiltration, degeneration of centrilobular area and necrosis was observed 48 hr after exposure to 500 ppm of CCl₄.

Krishnan and Stenger (1966) had reported that rats sacrificed 24 hr after subcutaneous injection of CCl₄ (0.5 ml/Kg), showed a centrilobular zone of pale cells containing cytoplasmic droplets of fat. Swollen hydropic cells with dark nuclei were also observed. There was little necrosis or inflammation; peripheral zones were intact.

Castro et al. (1978) observed that 72 hours after CCl₄ injection (1 ml/Kg i.p) in rats an intense centrilobular necrosis was produced with hemorrhage and edema. The hepatic cells in the periportal zones were enlarged and the normal structure was lost.

Centrilobular necrosis in mice after single dose of CCl₄ (1 ml/Kg orally) was observed by Adam and Thorpe (1972). Midzone had large number of balloononed cells and severe lipid infiltration. Liver of the mouse 24 hr after two doses of CCl₄ with an interval of 7 days showed the exaggerated balloononing of the parenchymal cells around the centrilobular necrotic zone. Mice treated with CCl₄ at weekly intervals
for 3 weeks showed centrilobular necrosis similar to that seen after a single dose of CCl₄. But few scattered congested sinusoids were visible in the necrotic zone. The necrotic cells were surrounded by balloononed cells and fatty infiltration appeared in the midzone of the lobule. Scattered binucleated cells were also present in the cytoplasm.

A marked centrilobular necrosis 24 hr after administration of CCl₄ (1 ml/Kg bw i.p) in the rats has also been reported by Ferréyra et al. (1977). Hemorrhage and edema was present in the centrilobular zone. Periportal zones had enlarged hepatocytes and normal structural pattern was lost.

Rana (1977) studied histopathological changes in squirrels treated with CCl₄ (1 ml/Kg bw), on each alternate day for 15 days. Binucleated cells were present at an early stage of CCl₄ treatment. Later treatment caused formation of balloon cells, which were round in shape and had a reticular nucleus. Vacuoles were present in the centrilobular zone. Whole liver lobule was filled with balloon cells and hepatocytes had lost their structural integrity. Areas of centrilobular as well as perivascular necrosis were seen.

Pleurivacuolar degeneration, balloononing, pycnosis, necrosis of hepatocytes and loss of cytoplasmic granulation
have been shown by Strubelt et al. (1978), in rats sacrificed 24 hr after CCl₄ treatment (0.1 ml/Kg bw i.p). There was a moderate degree of fatty infiltration.

Yasuda et al. (1980) reported a marked centrilobular necrosis surrounded by balloon cells in the rats sacrificed 24 h after CCl₄ treatment (0.25 ml/Kg bw i.p). Necrosis in the centrilobular zone is produced in all specific (Cameron et al., 1937) and by all routes except for the direct injection into the portal venous system, which leads to massive general necrosis. Rouiller (1964) has reported that the necrosis is midzonal rather than centrilobular if the rats are pretreated with thyroid hormones.

**Electron microscopic studies**

The earliest electron microscopic evidence of cytotoxicity is the damage to the rough endoplasmic reticulum, as reported by a number of workers (Rouiller, 1964; Smuckler, 1969 and Stenger, 1970). Dinman et al. (1963) have reported that after 8-hr exposure of rabbits to 500 ppm vapour concentration of CCl₄ there was dilation of rough endoplasmic reticulum and its normal lamellar configuration was lost. After 16-hr exposure to 500 ppm of CCl₄, two types of changes were seen in the hepatocytes of the centrilobular area. One group of hepatocytes showed slight to moderate swelling of endoplasmic reticulum whereas the mitochondria
were normal in configuration. The other group of cells demonstrated swelling both of mitochondria and endoplasmic reticulum. After 48-hr exposure to 2000 ppm vapour concentration of CCl₄ in rabbits, Dinman et al. (1968) observed an increase in the number of lysozomes. Rough endoplasmic reticulum had either a linear or vesicular appearance. Mitochondria were swollen. Krishnan and Stenger (1966), treated rats with 0.5 ml/Kg of CCl₄ subcutaneously and they were sacrificed 24, 30 or 36 hr later. Electron microscopic studies revealed that centrilobular zone exhibited moderate glycogen depletion and dilatation of both rough and smooth endoplasmic reticulum. A few dark osmiophilic lipid bodies were also seen. Almost immediately after the administration of CCl₄ a decrease in the NADP content and drug-detoxifying enzyme activity of smooth endoplasmic reticulum with dilatation of the smooth endoplasmic reticulum has been reported by Reckinger (1967). Neldolesi (1968) studied changes in liver of rats 24 hr after CCl₄ (2.5 ml/Kg bw) was given by stomach tube. Large number of lipid droplets were present in the centrilobular parenchymal cells. Rough endoplasmic reticular elements were reduced in number; smooth endoplasmic reticulum was abundant and arranged in large masses of closely packed vesicles. Free and nonaggregated ribosomes were scattered in the cytoplasm. Mitochondria were swollen and often contained calcium-associated dense masses.
Peripheral zone exhibited mild fatty infiltration; mitochondrial lesions were infrequently seen. Few dark nuclei were localized in the peripheral and in the mid zone of the liver. Endoplasmic reticulum was dilated, resulting in the formation of hydropic cells. Castro et al. (1977), injected rats intraperitoneally with 1 ml/kg of CCl4 and observed that ribosomes were bound to the outer membrane of the nucleus envelope. Similar changes were also seen by Bruni and Porter (1965). In the studies of Castro et al. (1977) mitochondria were characterized by dark electron dense granules and cristae with double membranes. Numerous stacks of rough endoplasmic reticulum with ribosomes bound to their outer surface were evident, often bending and curving around the mitochondria. In another studies, Castro et al. (1978) using the same dose of CCl4 observed that ribosomes were few in number and most of the smooth endoplasmic reticulum was present in the vesiculated form. Golgi apparatus has been shown to undergo several types of changes (Shinozuka et al., 1971; Stein et al., 1972). It may show fragmentation and vacuolation in company with rough endoplasmic reticulum.

BIOCHEMICAL CHANGES IN LIVER

Enzyme changes

Histochemical examination reveals that CCl4 injury,
in addition to bringing about depletion of liver enzymes, cause subcellular translocation of enzymes.

A substantial fall in overall activity has been described for enzymes involved in glycolysis, citric acid cycle and for enzymes involved with glycogen metabolism. The activity of lactate dehydrogenase (LDH), malate dehydrogenase (MDH), aldolase (ALD), isocitric dehydrogenase (ICDH), all reached low values in the rabbit 8 and 16 hr after 2000 ppm of CCl₄ vapours. Friede (1960) found a low activity of sorbitol dehydrogenase (SDH) after CCl₄ poisoning, the reduction being inversely related to the extent of lipid peroxidation. Chakarvary and Chandicharan (1964) found a complete disappearance of SDH in rats treated with 0.3 ml/100 g bw of CCl₄ for 3 consecutive days. Reynolds (1967) isolate mitochondria from poisoned animals and showed that there is a loss of respiratory control 24 hr following intoxication. A relationship is evident between the decline in whole liver activity and the accompanying rise of the enzyme in the circulation e.g., for esterase and xanthine oxidase in rabbits exposed to daily doses of 52-100 ppm of CCl₄ for 10 days (Cornish et al., 1960), and glutamate dehydrogenase (Dinman et al., 1963), after 6 hr exposure to 500 ppm of CCl₄.

The most pronounced change in phosphatase enzymes elicited in CCl₄ poisoning is the drastic centrilobular
suppression of glucose-6-phosphatase (Glu-6-P) activity, which is marked within 2-4 hr after intoxication (Recknagel, 1960; Reynolds and Yee, 1968). The extent of inactivation of hepatic glu-6-p has been demonstrated with as low as 0.3 ml/Kg bw of CCl₄ (Klaassen and Plaa, 1969). Glucose-6-phosphatase is a microsomal bound enzyme which catalyzes the final step in hepatic glycogenolysis. Rapid destruction of glu-6-p is demonstrated with CCl₄ in isolated liver microsomal preparations together with extensive lipid peroxidation (Dianzani et al., 1966; Ghoshal and Recknagel, 1965b). Antioxidants are able to protect microsomal lipids from peroxidative reaction and also prevent the in vitro loss of glu-6-p activity due to CCl₄ (Ghoshal and Recknagel, 1965b). According to Cignoli and Castro (1971) lipid peroxidation and destruction of glu-6-p by CCl₄ are separate phenomena. The data on the inactivation of glu-6-p are consistent with the view that the endoplasmic reticulum represents the first site of damage by CCl₄. Chakarvarty and Chandicharan (1964) found a complete disappearance of the glu-6-p from the central zone but the peripheral portion contained some amount of it, when normal rats were treated with 0.3 ml/100 g bw of CCl₄ for 3 consecutive days.

Following the administration of CCl₄, there is a prompt alteration in the structure and function of endoplasmic reticulum (Smuckler and Arcoasy, 1969). These
changes include a decrease in the capacity to make protein and a disruption of microsomal electron transport at least at the level of cytochrome p-450. The time course in the evolution of these changes and development of lipid peroxidation (measured by diene conjugation) are not entirely concomitant. The latter process is established by five minutes following oral feeding of CCl₄. During this period there is no alteration in nucleic acid formation, no changes in transmembrane potential nor in ATP concentration. The role of the peroxidation of the membranes according to the view of Smuckler and Arcasoy (1969) may have limited relationship to the altered protein synthesis. The microsomal electron transport chain shows a progressive deterioration starting with functions related to cytochrome p-450.

Certain enzyme activities in the liver or in some subcellular fractions may reach unexpectedly high values on exposure to CCl₄. Acute treatment with CCl₄ disrupts lysosomal membranes, evoking the release of enzymes contained in these organelles, amongst which are acid RNAase, acid phosphatase, β-glucoronidase, aroylsulphatase and phosphate-splitting enzymes e.g. phosphodiesterase and phosphomonoesterase (Alpers and Isselbacher, 1967).
Mitochondrially-linked enzymes may display a similar rise in activity, whenever CCl₄-induced membrane lesion occurs as demonstrated for carbamoylphosphatase synthetase (Rossi and McLean, 1963). Treatment with CCl₄ apparently enhances the synthesis of certain hepatic enzymes, probably applicable in terms of adrenocortical intervention requiring the mediation of glucocorticoids. Thus, CCl₄ stimulates hepatic tyrosine aminotransferase in the same manner as exogenous glucocorticoid administration (Murphy and Malley, 1969). The induction is abolished by prior adrenalectomy, hypophysectomy or by pretreatment with actinomycin D (Rossi and McLean, 1963; Magus and Fouts, 1968; Magus et al., 1972). The promoting effect of CCl₄ upon endogenous glucocorticoid supply may also explain the temporary rise in GPT and GOT activity in toxic injured liver (Dinman et al., 1963). Glucocorticoids are indeed known to have a crucial role of the homeostatic regulation of gluconeogenic enzyme levels. However, all the changes are not mediated through glucocorticoids, since CCl₄ acts antagonistically upon the hydrocortisone-mediated activation of tryptophan pyrolase reversing the inductive effect (Aleman and Gitler, 1962; Magus et al., 1972).

Within 1 hr of administration of CCl₄, activity of a number of smooth endoplasmic reticular enzymes is inhibited.
These include NADPH reductase, aminopyrene demethylase, aniline demethylase and cytochrome P-450 activity (Recknagel and Glende, 1973).

Ca-ATPase activity is inhibited in rats fed orally with 0.25 ml/100 g bw of CCl₄. Increasing concentrations of CCl₄ in the blood stream and in the liver inhibit the active efflux of calcium by repressing Ca-ATPase activity, leading to unbalanced, passive calcium influx and accumulation within the cells. Since this effect of CCl₄ on liver Ca-ATPase is presumably completely reversible, when CCl₄ is cleared from the blood and the liver, the inhibition reverts and the accumulated calcium is extruded from the cells (Izutsu and Smuckler, 1978).

**Other Biochemical Changes**

A significant fall in liver glycogen has been noticed after treatment with CCl₄ (0.3 ml/Kg bw injected intraperitoneally to the rats for 3 consecutive days (Chakarvarty and Chandicharan, 1964). Hickenbottom and Hornbook (1971) have reported that starved rats given CCl₄ (200 ug/Kg bw i.p) 24 hr previously had hepatic glycogen concentration lower than those of starved controls. In addition, the treated rats did not deposit liver glycogen when refed for 3 hr, a procedure which produced substantial accumulation
of glycogen in livers of control rats. These changes in hepatic glycogen metabolism produced by CCl₄ were associated with a decreased activity of glycogen transferase and of phosphorylase phosphatase. Depletion of liver glycogen is observed after 0.5 to 1.0 ml CCl₄/Kg bw injected intraperitoneally in mice fasted for 16 to 18 hr (Puspendran et al., 1977). Agostini and Alfisi (1979) also found depletion of liver glycogen in rats treated with 0.25 ml/Kg bw of CCl₄ injected daily for 6 days.

Rapid and prolonged depletion of liver glycogen is found after the administration of CCl₄ to rats (Stowell and Lee, 1950; Leduc and Wilson, 1958; Ashworth et al., 1963; Krishnan and Stenger, 1966). Rubinstein (1962) found that the initial loss of liver glycogen produced by administration of CCl₄ was associated with increased activity of plasma epinephrine and decreased catecholamine content of adrenal gland.

A steady increase in liver lactate has been reported by Puspendran et al. (1977) in mice treated with 0.5 ml to 1.0 ml CCl₄/Kg bw, which reaches a peak value at 4 hr. Even at the end of 24 hr, liver lactate levels of CCl₄ treated mice were twice those of the controls. There was a decrease in pyruvate concentration of both liver and plasma after CCl₄ treatment. Hickenbottom and Hornbook (1971) also found a higher concentration of lactate after 24 hr of CCl₄ treatment (200 ug/Kg bw i.p) in rats.
Livers of CCl₄-treated rats have been reported by Hickenbottom and Hornbook (1971) to have lower concentration of ATP and UDP-glucose in rats after 24 hr treatment with CCl₄ (200 μl/Kg bw i.p.). A decreased concentration of ATP after treatment with CCl₄ has been reported also by other investigators (Dianzani et al., 1966; Hyam et al., 1967; Smuckler et al., 1968; Wands et al., 1970). Exposure to CCl₄ results in an early and later sustained increase in hepatic calcium content (Izutsu and Smuckler, 1978).

An impairment of protein synthesis has been demonstrated by incorporation of leucine ¹⁴C into hepatic lipoprotein and glycine ¹⁴C into plasma albumin-fibrinogen and gamma globulin within one hour of CCl₄ treatment (Recknagel and Glende, 1973).

Increased synthesis of lipids has been demonstrated after CCl₄ poisoning (Recknagel and Glende, 1973). The lipid that accumulates in the liver may be the result of increased transport to the liver, increased synthesis by the liver or altered capacity to excrete lipids from the liver. It has also been suggested on the basis of transport experiments that there was no defect in the capacity of the liver to accept free fatty acid from the plasma, nor in the capacity to esterify it, but there is a decreased release
(Recknagel and Lombardi, 1961; Smuckler, 1968). Further studies have suggested that there is a correlation between the decreased release of lipids and a reduced capacity to make protein (Smuckler et al., 1961), specifically the carrier protein for lipid that permits export of lipids (Lombardi and Ugazio, 1965).

Administration of CCl₄ (0.04 ml/Kg bw) to mice causes a 2-fold increase in liver triglyceride concentration after 2 hr treatment (Schwets and Plas, 1969); simultaneous treatment with catecholamines does not significantly increase liver triglycerides. Benedetti et al., (1974) have reported a 3-fold increase in the liver triglyceride levels 4 hr after intraperitoneal injection of CCl₄ (250 μl/100 g bw). Pretreatment with vitamin E at 25 mg/100 g bw had a minor effect on the CCl₄-induced liver steatosis. However, they found a decrease of the liver triglyceride concentration in rats previously given vitamin E at 75 and 150 mg/100 g bw. Cawthorne et al., (1970) have observed that a large oral dose of vitamin E (200 mg/100 g bw) 48 hr before CCl₄ administration, rather than preventing CCl₄-induced triglyceride rise, markedly increases it.

Maling et al., (1974) have reported a rise in the liver triglyceride 24 hr after intraperitoneal injection of CCl₄.
(2.0 ml/Kg bw). Mancini and Kocsis (1974) treated rats intraperitoneally with 1.9 ml/Kg of CCl₄. The liver triglyceride was 9 times that of the controls and 2.5 times more than that with low doses of CCl₄ (0.08 ml/Kg). A much higher release than in controls, 24 hr after intraperitoneal injection of 0.5 ml/Kg bw of CCl₄, has also been reported by Sohel et al. (1974). In the studies of Agostini and Alfisi (1979), total lipids increased to 10 times the control values in rats treated with daily intraperitoneal injections of CCl₄ (0.25 ml/100 g bw) for 6 days.

**BIOCHEMICAL CHANGES IN SERUM**

**Enzyme changes**

The biochemical changes in blood reflect the histologic patterns of toxic hepatic injury. Carbon-tetrachloride serves as a model hepatotoxic agent, and has received a great attention in serum enzymology to ascertain involvement of the liver. The maintenance of the CCl₄-induced raised activities varies with the type of enzyme concerned. Transaminases persist in showing usually high serum activities for longer periods of time than oxidoreductases.

An increase in serum glutamic-oxaloacetic transaminase (SGOT) activity following administration of CCl₄.
has been reported by a number of workers. The rise in SGOT in the rats has been observed with an intraperitoneal dose of 0.25 ml/100 g bw (Friedman and Lapan, 1964), 0.1 ml/100 g bw through intraperitoneal route (Rubinstein, 1962) and 0.01 ml/100 g bw i.p. (Strubelt et al., 1978). Exposure to daily doses of as low as 52-100 ppm vapours for 10 days brings about a rise in the SGOT (Cornish et al., 1960). Rise in SGOT has also been reported in rabbits exposed for 2 hr to 500 ppm (Fox et al., 1962) and for 6 hr to 200-500 ppm of CCl₄ vapours. Dinman et al. (1962) have observed that rise in SGOT is proportional to liver pathology. Earlier rise in SGOT is followed by decline, but further increase is observed during regeneration. Dinman et al., (1963) have reported rise in SGOT immediately after exposure of rabbits to 500 ppm of CCl₄ vapours for 6 hr and before the onset of necrosis.

Sinha and Saran (1972) found the peak rise in SGOT 24 hr after 0.5 ml/Kg bw of CCl₄ by intragastric route. Rise in SGOT following CCl₄ has also been reported in sheep (Alemu et al., 1977).

An increase in serum glutamic pyruvate transaminase (SGPT) activity has been reported in different species by a number of investigators. An elevation in SGPT in the rats
by CCl₄ has been observed with an intraperitoneal dose of 0.1 ml/Kg bw (Strubelt et al., 1978) and 1.2 ml/Kg bw (Engelina et al., 1974). Repeated weekly administration of CCl₄ is reported by Sinha and Saren (1972) to increase the levels of SGPT with 0.5 ml/Kg of CCl₄ through intragastric route, the peak levels being obtained at 24 hours. The height of the peak decreased with each successive dose of the CCl₄. There is a rise in the levels of SGPT in rabbits exposed to 200 to 500 ppm of CCl₄ vapours for 6 hr (Dinman et al., 1962). The rise is proportional to liver pathology and precedes the onset of necrosis (Dinman et al., 1963). Rise in SGPT following administration of CCl₄ (0.5 ml/Kg bw) by the intragastric route has also been observed in sheep (Alemu et al., 1977).

A significant decrease in the serum alkaline phosphatase activity has been demonstrated in sheep 2 days after treatment with 0.5 ml/Kg bw of CCl₄ (Alemu et al., 1977). The rise of serum glutamate dehydrogenase (GDH) has been reported to be delayed as compared to the other serum enzymes in rats following administration of CCl₄ (Zimmerman et al., 1965). In rabbits exposed to 2000 ppm of CCl₄, a significant decrease in liver activity has been observed prior to the rise of serum GDH (Dinman et al., 1968). Maximum serum GDH enzyme activity has been observed earlier at 500 ppm than at 200 ppm of CCl₄ vapour (Dinman et al.,
1962). In sheep serum GDH activity is seen to rise, on day 4th after dosing with CCl₄ (0.5 ml/Kg bw orally), to attain a peak on day 6th and to return to normal on day 9th (Alemu et al., 1977).

Serum aldolase activity following exposure to CCl₄ vapours in concentration of 100 ppm, 200 ppm and 500 ppm causes a maximum increase in rabbits at 36 hr, 24 hr and 16 hr respectively (Dinman et al., 1962 and 1968). An eight-fold rise in aldolase has been demonstrated in the sheep after oral administration of 0.5 ml/Kg of CCl₄ (Alemu et al., 1977). It is maximum on the 2nd day and persists for as long as 10 days after administration of CCl₄. The increase in enzyme is observed even with a mild degree of liver injury and therefore it can provide an early test for liver damage (Korsurd et al., 1972).

Carboxytetrachloride increases the levels of serum phosphohexose isomerase (PHI). A significant rise has been observed in rabbit immediately following exposure for 8 hr to vapour concentration of 500 ppm (Dinman et al., 1962), and also on exposure to 200 ppm for 6 hr (Dinman et al., 1963). The increase in activity is dose-dependent and higher values are obtained with 500 ppm than with 200 ppm.

Increase in lactate dehydrogenase (LDH) activity has been observed with exposure to CCl₄ vapours in concen-
trations varying from 100 ppm to 2000 ppm (Dinman et al., 1962, 1968). Maximum values in the rabbit are obtained at 16 hr after exposure to concentrations of 2000 ppm and 36 hr after exposure to 100 ppm. With concentrations of 200 and 500 ppm, peak values are obtained at 24 hr after exposure. Zimmerman et al. (1965) have reported maximum values 12 hr after exposure.

A rise in serum isocitric dehydrogenase (ICDH) has been reported in rats administered with 2.0 ml/Kg of CCl₄ orally. After 8-hr exposure to vapour concentrations of 200 ppm and 500 ppm in the rabbit, a marked increase in serum ICDH has been reported (Dinman et al., 1962), the magnitude of rise being the same with both the concentrations. ICDH attains a peak at 24 hr after exposure to CCl₄ (Zimmerman et al., 1965). Serum ICDH following exposure of rabbits to 2000 ppm of CCl₄ has been shown to have either concomitant elevation in serum and cells or no change in cell levels inspite of considerable increase in serum levels (Dinman et al., 1968).

Single acute dosage of CCl₄ causes a serval-fold rise of sorbitol dehydrogenase (SDH) over the basal level (Curtis et al., 1972). Alemu et al. (1977) have found an increase in SDH activity in sheep. The enzyme activity was markedly elevated on day 4th and returned to normal levels by day 6th.
Dinman et al. (1968) have reported an increase in the serum malate dehydrogenase (MDH) levels in the rabbits exposed to 100 and 2000 ppm vapour concentrations of CCl₄. Levels of MDH reach a peak 36 and 24 hr after exposure to concentration of 100 ppm and 2000 ppm of CCl₄ respectively. An increase in MDH has been observed by the same workers (1962 and 1963) in the rabbits exposed for as short as 6 hr to 200 or 5000 ppm vapour concentrations of CCl₄. Maximum rise was observed after 24 hr. Zimmerman et al. (1965) have observed that MDH levels attain a peak at 36 hr after administration of various doses of CCl₄. Marked rise of MDH levels has been reported by Cornish et al. (1960). A significant rise in MDH after administration of CCl₄ is indicative of an acute hepatotoxic lesion (Lee and Bernstein, 1968).

Cornish et al. (1960) have reported a fall in serum esterase values in rats exposed to CCl₄ vapours in concentrations of 1000 and 15000 ppm for 4 hr. Lowering in pseudocholinesterase and arylesterase has been observed in rats with various doses of CCl₄ (Brauer and Root, 1946; Takahashi et al., 1970; Curtis et al., 1972). The non-specific arylesterase, however, shows an initial rise of enzyme activity followed by a fall to subnormal levels in animals administered CCl₄ through subcutaneous route or
A marked rise in the levels has been reported in rats after administration of 0.25 ml/100 g bw of CCl₄ intraperitoneally (Friedman and Lapan, 1964).

Alteration in serum xanthine oxidase (XOD) levels evoked by CCl₄ treatment under different exposure conditions has been reported by various investigators (Affonso, 1955; Mitidieri and Affonso, 1966).

An increase in the plasma alanineα-Ketoglutarate transaminase (AKT) activity has been reported in rats by Murphy and Malley (1969), after oral administration of CCl₄ (0.5 ml to 2 ml/Kg). Elevation of plasma AKT was not detected until after 5 hr of CCl₄ administration. Repeated oral doses of CCl₄ (1 ml/Kg) administered to rats twice a week for four weeks resulted in higher plasma activity than in the controls; the increase was small as compared to that observed at 24 hr after a single dose of CCl₄.

Dinman et al. (1962) found no change in serum leucine aminopeptidase activity after 8-hr exposure to 500 ppm vapour concentration of CCl₄. But at 16 hr a rise in serum enzyme activity was reported.

**Other biochemical changes**

An increase in the levels of blood glucose has been
reported in the rats and mice after CCl₄ intoxication. An increase in the blood glucose was seen in the first 1 and 4 hr, with a peak at 2 hr and levels returning to normal by 4 hr, in rats administered CCl₄ (1 ml/Kg) through intraduodenal route (Rubinstein, 1962). A significant rise in blood sugar has also been reported in rats after treatment with CCl₄ at the rate of 0.3 ml/100 g bw for 3 consecutive days (Chakravarty and Chandicharan, 1964). Puspenderan et al. (1966, 1977) have observed significant alterations in plasma glucose levels in mice after CCl₄ injection at a dose of 0.5 ml/Kg bw. Mice were starved 16 to 18 hr before CCl₄ injection. Although, glycogen depletion was rapid in starved mice, changes in plasma glucose levels became significant only 2 hr after CCl₄ injection. Glucose appeared in greater quantities in plasma of mice refed for either 1 or 2 hr and then treated with CCl₄. The rise in blood glucose has been shown to be concomitant with increase in serum epinephrine levels in rats (Rubinstein, 1962).

Carbontetrachloride (0.5 ml/Kg orally) has been shown to cause a slight increase in the blood cholesterol in the sheep between 1/4 and 8 days after treatment (Alemu et al., 1977). A decrease in the serum free fatty acids has been reported in the rabbits following exposure to CCl₄ vapours in concentration of 500 ppm for 2 hr (Fox et al., 1962).
A marked decrease in the concentrations of total proteins has been reported in rats by CCl₄ at a dose of 0.25 ml/100 g bw injected simultaneously for 6 days (Agostini and Alfisi, 1979). However, no significant alterations in the levels of total serum proteins were seen in sheep following CCl₄ treatment (Alemu et al., 1977). Agostini and Alfisi (1979) have observed a 59% decrease in the serum albumin over control rats injected subcutaneously for 6 days with 0.25 ml/100 g bw of CCl₄. But Alemu et al. (1977) could not find a significant change in concentrations of albumins, globulins and albumin-globulin ratio in sheep after 0.5 ml/Kg of CCl₄.

Plasma lactate levels are elevated after treatment with CCl₄. A dose of 0.5 ml/Kg i.p. causes a greater rise in the levels of plasma lactate in mice refed for 1 hr than in the case of either the fasted animals or those refed for 2 hr (Pushpendran et al., 1977).

Serum epinephrine levels have been reported to increase after 1 ml/Kg bw of CCl₄ given intraduodenally to rats (Rubinstein, 1962). The epinephrine level was three times the control value after 2 hr and returned to normal by the 4th hr.

Serum cyanocobalamin was reported by Dinman et al. in (1962) to rise rabbits exposed to vapour concentrations of
either 200 or 500 ppm of CCl₄ for 6 hr. An early rise with a maxima at 8th hr was followed by a sharp drop within the normal range at 16 hr and a mimima at 72 hr accompanied by a rise in bound cyanocobalamin.

MECHANISM OF HEPATOXICITY ACTION OF CARBON TETRACHLORIDE

Several theories have been proposed for the underlying mechanisms of CCl₄-induced hepatotoxicity.

The mitochondrial hypothesis

According to an early view (Christie and Judah, 1954) the mitochondrial damage plays a key role in the pathogenesis of the CCl₄ hepatotoxicity. This was based on the observation that carbon tetrachloride alters the permeability of the mitochondria and the activity of the enzymes involved in the Kreb's cycle is diminished. In addition, others (Dianzani, 1954; Dianzani and Bahr, 1954), had demonstrated an uncoupling of oxidative phosphorylation in mitochondria. Shift of electrolytes, particularly calcium ion, was shown to occur early in the intoxication. These observations led to the conclusion that mitochondrial effects were probably the cause of the fatty liver. Late appearance of mitochondrial degeneration in the pathological sequence (Recknagel and Glende, 1973) and early appearance of fat accumulation or damage to the endoplasmic reticulum were serious objections to the mitochondrial theory. The
eventual role of the mitochondrial changes in the development of necrosis has yet to be investigated fully (Lyachovich et al., 1971).

**Catecholamine hypothesis**

The catecholamine hypothesis (Calvert and Brody, 1960; Brody et al., 1961) attributed the effect of CCl₄ to the stimulation of central nervous system followed by a marked increase in the catecholamine levels in the blood. Catecholamine discharge mediated by the stimulation of the pituitary adrenal system could give rise to hepatic vasoconstriction leading to cell necrosis. In addition Brody et al. (1961) had proposed that catecholamines give rise to excessive mobilization of free fatty acids from the fat depots induced by their lipolytic effects. The experimental evidence rested primarily on the results obtained with the adrenergic blocking agents, ganglionic blocking agents, spinal cord transection, adrenalectomy and thyroidectomy. Several objections against this theory came from various lines of investigations.

a) Some of preventive measures have only a limited range of action, being confined to either counteraction of triglyceride accumulation (as in the case of adrenalectomy) or a decrease in the necrogenic action as in the pretreatment with nerve blocking agents (Brody, 1963) or antihistamines
(Rees et al., 1961). This emphasizes the dual nature of CCl₄ hepatotoxicity in which cellular necrosis and lipid infiltration constitute separate manifestations (Recknagel, 1967; Judah et al., 1970; Recknagel and Glende, 1973).

b) The effectiveness of spinal cord transection in preventing necrosis due to CCl₄ varies with the levels of transection (Larson and Plaa, 1963a,b; 1964a,b).

c) Cervical cordotomy at the level of C₇ results in hypothermia which affords protection to the CCl₄-treated animals. Hypothermia induced by immersion of normal rats in cold water also results in a protective effect comparable to that of cordotomy (Larson and Plaa, 1965). Moreover, cordotomy fails to produce an ameliorating effect in rats kept at normal body temperature.

d) Hepatic lesions can be induced by carbon tetrachloride in immunologically sympathectomized animals (Larsen et al., 1965).

e) Liberation of catecholamines in the CCl₄-induced fatty liver is of small magnitude and the transient nature (Schotz and Recknagel, 1960; Rubinstein, 1962; Maximchuk and Rubinstein, 1963). Moreover, it is now established that it is not excessive supply of fatty acids to the liver but the
impaired hepatic triglyceride secretion which plays a major role in the pathogenesis of hepatocellular lipid deposition (Robinson and Seaskins, 1962; Heimberg et al., 1965).

f) The hepatonecrotic effect of $\text{CCl}_4$ does not appear to be associated with diminished blood flow through the liver and the subsequent centrilobular hypoxia (Sigel et al., 1967).

g) Larson and Plaa (1965) have shown that large infusion of norepinephrine, epinephrine or mixture of these substances do not result in lesions similar to those produced by $\text{CCl}_4$.

In conclusion, there is much evidence to support the view that the vascular role attributed to $\text{CCl}_4$ via release of catecholamines must be rejected as a primary cause of hepatic injury.

**Depression of protein synthesis as the basis for carbon-tetrachloride hepatotoxicity**

Inhibition of protein synthesis in rat liver has been demonstrated by Smuckler and coworkers (1961 and 1962). Impairment of protein synthesis has been demonstrated by decreased incorporation of leucine $^{14}$C into hepatic lipoprotein (Seaskins and Robinson, 1963), and of glycine
$^{14}$C into plasma albumin, fibrinogen and gammaglobulin within one hour of administration of CCl$_4$ (Smuckler and Benditt, 1962). The synthesis of $\beta$-lipoproteins, notably the very low density lipoproteins (VLDL), shows an early failure in CCl$_4$ poisoning. It is proposed that the effect of CCl$_4$ in reducing synthesis of lipoproteins apoprotein may be the initiating event responsible for the production of fatty liver (Seaskins and Robinson, 1963). This is supported by the observations that several known inhibitors of protein synthesis e.g. ethionine and puromycin also cause hepatic lipid accumulation (Farber et al., 1964; Judah, 1969). On the other hand, the failure of the protein inhibitors such as actinomycin D (McLean et al., 1965) and the hepatotoxic agent thioacetamide to induce fatty infiltration weakens the hypothesis that defective protein synthesis is a preliminary step in the production of fatty liver.

**Hepatic triglyceride secretion**

According to this hypothesis CCl$_4$-induced steatosis is due to a defect in the liver mechanism for secretion of triglycerides. Fat accumulates because its transport out of the liver is blocked. Blockage of transport of lipid may result from disruption of the mechanism for coupling triglycerides to the appropriate apoprotein to form the lipoprotein carrier molecule (Becknagel, 1967).
and Glende, 1973), defective synthesis of apolipoprotein (Recknagel, 1967; Judah et al., 1970; Dianzani, 1975; Farber, 1975) and perhaps of defect in transport of lipoprotein across the plasma membrane (Judah et al., 1970). A delay in the appearance of radioactivity in the plasma triglycerides pool of CCl₄-treated rats when injected with (¹⁴C)-palmitate, provides the evidence for impairment of hepatic fat secretion (Frank et al., 1961; Maling et al., 1964).

Fatty liver by orotic acid was first reported in rats by Standerfer and Handler, (1955). The fat which accumulates is largely triglyceride. Windmueller (1964), had found that the mechanism by which dietary orotic acid induces a fatty liver involves a defect in the synthesis or secretion of lipoprotein by the liver. Moreover, the fatty liver induced by feeding of orotic acid is now being attributed to defective coupling of the lipids with lipoprotein-apoprotein, rather than to impaired protein synthesis (Roheim et al., 1965). These facts exclude the causative role of inhibited protein synthesis in the pathogenesis of toxic fatty liver by CCl₄.

**Lipid peroxidation theory.**

It was believed earlier that the toxic effect of CCl₄ was through the injury caused to the lipid membranes
of the hepatocytes by virtue of its property as a very strong lipid solvent. Ghoshal and Recknagel (1965), Recknagel and Ghoshal (1966) and Slater (1966) had expressed doubt on the suggestion that the unmetabolized CCl₄ was responsible for the CCl₄ hepatotoxicity. It was pointed out that many organic, non-polar compounds such as ether and nonchlorinated hydrocarbons having solubility similar to CCl₄ had no hepatotoxic properties. Butler (1961) and Wirtschafter and Cronyn (1964) proposed that it was not the unmetabolized CCl₄ but a free radical, probably CCl₃, formed by homolytic cleavage of CCl₄ which is responsible for the injury. That the lipid solvent property of CCl₄ cannot explain its hepatotoxicity was supported by the observations that its toxic action was largely on the liver although high concentrations were obtained in blood, heart and bone marrow (Recknagel and Glende, 1973). Smuckler (1965) and Smuckler and Benditt (1965) had made the observations that polysomes and ribosomes obtained from liver of intact rats by use of media containing CCl₄ did not show alterations in ultrastructure and inhibition of amino acids incorporation. On the other hand, such changes were observed in cell organelles derived from rat liver damaged by CCl₄ in vivo suggesting that direct action by solvation of structural lipids could not explain the toxic action of CCl₄. Ghoshal
and Recknagel (1965) observed that on incubation of rat liver microsomes at neutral pH and in the presence of ascorbic acid, glucose-6-phosphatase activity, which is membrane bound is rapidly destroyed and the microsomal lipids are peroxidized. If, on the other hand, lipoperoxidation was prevented by a chelating agent such as EDTA or an antioxidant like α-tocopherol, there was no loss of enzyme activity. Under these circumstances it was estimated that the amount of CCl\textsubscript{4} required to destroy glucose-6-phosphatase activity in the absence of lipoperoxidation was twenty times greater by weight than the entire quantity of microsomal lipid present in the \textit{in vitro} system. It was estimated by Ghoshal and Recknagel (1965) that this ratio of CCl\textsubscript{4} to microsomal lipid was at least 200 times greater than the absolute upper maximum that could be reached \textit{in vivo}, even with lethal doses. The fact that glucose-6-phosphatase activity of a liver of the rat poisoned with CCl\textsubscript{4} was markedly depressed within the first 2 to 4 hr further suggested that CCl\textsubscript{4} toxicity depends on its chemical reactivity and not on solvent action. There is now considerable experimental evidence that the toxic action of CCl\textsubscript{4} is mediated mainly through a metabolite. The degree of hepatotoxicity is influenced by factors which alter the metabolism of CCl\textsubscript{4}. Thus, \textit{in vivo} agents such as phenobarbital and DDT that increase
the drug metabolizing activity of the endoplasmic
reticulum enhance the toxicity of CCl$_4$ (McLean and
McLean, 1966; Seawright and McLean, 1967; Garner and
McLean, 1969; Seawright et al., 1972; McLean, 1975).
Recknagel and Glende (1973) have reported that only
those species are susceptible to hepatic injury by CCl$_4$
who are able to metabolize it. They have further made
the observation that the ability to metabolise CCl$_4$ by
rats is developed only after they are one week old and
this can be linked to the higher resistance of newborn
rats. The fact as observed by Recknagel and Glende (1973)
that a small dose of CCl$_4$, which interferes with the ability
to metabolise the drug makes the liver resistant to a
subsequent dose of CCl$_4$, further supports the view that
the toxicity of CCl$_4$ is mediated through a metabolite.
In addition to the above, Slater (1966) had pointed out
that the lesion is located in the centrilobular region
of the liver where the concentration of the CCl$_4$
metabolizing enzymes is high, while its highest concentration in
blood reaching the liver exists in the portal region.
There was, therefore, much evidence from different studies
which supported the original suggestion (Butler, 1961;
Wirtschafter and Cronyn, 1964) that free radicals of CCl$_4$
were involved in its hepatotoxicity. The sequence of events
leading to the formation of free radicals from CCl$_4$ are as
follows:-
Carbontetrachloride penetrates into the liver and interacts with an initial sensitive site located in the endoplasmic reticulum. Reynolds and Moslen (1974) have suggested that CCl₄ may interact with two or more loci of the smooth endoplasmic reticulum. Homolytic cleavage of CCl₄ yields free radicals of CCl₃ and Cl. CCl₃ is later metabolized to CHCl₃ and finally to CO (Recknagel and Glende, 1973). Hexachloroethane in small amounts is also produced by condensation of two molecules of trichloromethyl free radicals. Free radical generation involves the interaction of CCl₄ with cytochrome P-450 (Reynolds, 1967). The reduction of CCl₄/cytochrome P-450 complex is an essential step in the activation of CCl₄. Cytochrome P-450 reductase in a NADPH dependent reaction brings about the activation (Villarreal et al., 1975). Recknagel and Glende (1974) have provided evidence in favour of the involvement of cytochrome P-450. Chopra et al. (1972), on the other hand, have suggested an involvement of earlier stage of the microsomal electron transport system. The reactions involved in the homolytic cleavage of CCl₄ to free radicals are represented below:

\[
\text{NADPH} \rightarrow \text{Cytochrome P-450 (in endoplasmic reticulum)} \\
\text{Reductase} \\
\text{CCl}_4 + e^- \rightarrow \text{CCl}_4^- \rightarrow \text{CCl}_3^+ + \text{Cl}^-
\]
Although there is ample evidence that the toxic effects of $\text{CCl}_4$ are mediated largely through its metabolism and release of free radicals, the possibility of some contribution by the unmethylated $\text{CCl}_4$ cannot be ruled out. Several workers (Zimmerman, 1968; LePage and Dorling, 1971; Dorling and Page, 1972; Rufeger and Frimer, 1976), have suggested this possibility based on different lines of evidence. Zimmerman and Mao (1965) and Zimmerman et al. (1966) have reported immediate effects of $\text{CCl}_4$ on cells which were probably unable to metabolize $\text{CCl}_4$. Toxic effects of $\text{CCl}_4$ have been demonstrated on mitochondria (Recknagel and Lombardi, 1961) and on isolated membrane (Rufeger and Frimer, 1976). Cellular injury by $\text{CCl}_4$, though of a minor nature, has also been demonstrated in species such as the chickens and ducks which are not able to metabolize $\text{CCl}_4$ (Reynolds, 1972).

Several workers have provided evidence that the cellular changes in the liver by $\text{CCl}_4$ do not result from the direct effect of $\text{CCl}_3$ radical on cellular structure and functions but through the formation of lipid peroxides which magnify the effect of the initial free radicals. Diluzio (1963, 1964) and Diluzio and Costales (1964) were the first to suggest that the formation of lipid peroxides associated with the breakdown of the membranes
of different cell constituents including the mitochondria constituted the basic mechanism of CCl₄-induced hepatotoxicity. The basis for this was the observation by D'Luzio (1964) that antioxidants like butylated hydroxy toluene, butylated hydroxy anisole and propyl gallate provided protection against the triglyceride accumulation in the liver by ethanol and CCl₄. According to the lipoperoxidation hypothesis, carbon tetrachloride poisoning initiates an intrahepatic process of destructive lipoperoxidation. The hypothesis has received much support from the work of Recknagel and coworkers (Ghoshal and Recknagel, 1965, 1966; Recknagel and Ghoshal, 1965, 1966a,b, 1966c; Recknagel, 1967; Recknagel and Glende, 1973). According to these workers and Slater (1966, 1972) the free radicals of CCl₄ lead to peroxidation of the unsaturated lipids of the endoplasmic reticulum which leads to the generation of secondary free radicals derived from the lipids of the membranes, thus setting up a chain reaction. The damage to the other organelles of the cells is due to these secondary free radicals. The polyenoic long chain fatty acids contained in the membrane lipoidal components are believed to constitute the main target for such reactions due to the susceptibility of methylene double bonds to free radical attack. The mechanism is similar to that known for the rancidity of
stored foods (Holman, 1954). Antioxidants protect stored foods against spoilage of rancidity by interrupting the self propagating chain of peroxidative lipid breakdown. Recknagel and Ghoshal (1966b) have advanced several arguments in support of the hypothesis. The endoplasmic reticulum of the liver cells is known to be the primary subcellular organelle involved in the early stages of liver damage. Further the membrane components of the endoplasmic reticulum are thin, delicate, lipoprotein sheets, the lipodal centers of which are composed of ordered arrangements of the fatty acid side chains of complex lipids, mostly phospholipids. They have further pointed out that a large fraction of the total liver phospholipid fatty acids are polyenic, with high content of arachidonic acid. Recknagel and Ghoshal have further hypothesized that the normal tendency of the methylene bridges of the unsaturated fatty acids side chains to free radical attack is prevented by many agents including normal levels of α-tocopherol and other antioxidants. This protection cannot be sustained when the free radicals of carbon tetrachloride initiate many changes of autocatalytic peroxidative breakdown of the fatty acids. They are of the view that the initial action of the free radicals of CCl₄ on lipid peroxidation of the membrane results in all the manifestations of CCl₄ hepatotoxicity such as morphological
changes in the endoplasmic reticulum, loss of drug
metabolizing activity, loss of glucose-6-phosphatase
activity, loss of protein synthesis and loss of the
capacity of liver to form and excrete low density beta
lipoproteins. Support for the lipid peroxidation hypothesis
has been provided by several experimental studies. Recknagel
and Ghoshal (1966b) observed that peroxidation of rat liver
microsome system in vitro results in reduction of the
arachidonic acid as estimated by gas-liquid chromatographic
analysis. Further, it has been demonstrated by Holtkamp
and Hill (1951) that after administration of carbon tetra-
chloride in vivo the arachidonic acid of rat liver microsomal
lipids is reduced by 20% in 90 minutes and 33% in 24 hrs.
Recknagel and Ghoshal (1966b) observed diene conjugation
absorption in rat liver microsomal lipids as early as
90 min after carbon tetrachloride administration. The
appearance of diene conjugation absorption is the result
of the shift of the organic free radical electron after
peroxidative breakdown of the polyunsaturated fatty acids.

Comporti et al. (1965) have reported that upto 2 hours
after administration of carbon tetrachloride to rats, the
rate of malonaldehyde production by whole liver homogenate
was increased. Malonaldehyde results from the decomposition
of labile organic peroxides, cleared from polyenoic acid
chain. Ghoshal and Recknagel (1965a,b) and Recknagel and Ghoshal (1966b) found that in the microsomal supernatant fraction of rat liver as little as 0.3 µl of carbon tetrachloride accelerated production of malonaldehyde. Carbon tetrachloride had no pro-oxidant effect on lipoperoxidation in the microsome fraction of rat liver in absence of supernatant fraction. It also had no effect on microsome supernatant fraction of rat brain or rat kidney. Further evidence for lipid peroxidation hypothesis is provided by the protective effect of several agents including free radical scavengers and others which are believed to act through their antioxidant properties. Some of these are α-tocopherol (Gallagher, 1962; Meldolesi, 1968; Comporti and Benedetti, 1972; De Ferreyra et al., 1975), phenothiazines (Rees et al., 1961; Serratoni et al., 1969), DPPD (Gallagher, 1962; De Ferreyra et al., 1975; Comporti et al., 1974), propylgallate (Ugazio and Torrielli, 1968). Vitamin E in high doses has been reported to prevent steatosis caused by CCl₄ administration (Comporti and Benedetti, 1972; Benedetti et al., 1974). Agents with active thiol groups such as cystamine form stable adduct compounds with CCl₄ generated radicals and are, therefore, protective against toxicity (Castro et al., 1972; Corongiu et al., 1974).

The main features of the lipid peroxidation hypothesis are as follows:
The free radicals liberated from CCl₄ in the endoplasmic reticulum attack the polyenoic long chain fatty acids in the membranes. The methylene double bonds in the fatty acids are particularly susceptible to free radicals. In the process of lipoperoxidation peroxyl free radicals are liberated, which in turn attack other lipid constituents and thus set up a chain reaction (Seawright, 1967; Castro et al., 1968; Recknagel et al., 1974). This leads to steatosis by impairing lipoprotein transport system. The continuation of the process with further peroxidative injury of the mitochondrial, plasma and lysosomal membrane leads to necrosis. In addition to above, immediate injury to the plasma membrane by non-metabolized CCl₄ results in loss of electrolytes and enzymes from the cells and entry of ions from the extracellular space. The sequence of events in the process of hepatic injury are represented below:

(1) Effect of CCl₄

a) CCl₄ ----> Plasma membrane ----> cells

injury

(Rise of Ca⁺⁺, Na⁺⁺;
(solvation of lipids)

(Fall of K⁺, enzymes and coenzymes)

----> Cell necrosis
b) $\text{CCl}_4 \rightarrow$ Endoplasmic $\rightarrow$ Depressed protein synthesis $\rightarrow$

\[ \text{reticulum} \]

Reduced lipoprotein $\rightarrow$ Lipid release inhibited $\rightarrow$

 fatty infiltration in hepatic cells

2. Effect of free radicals of $\text{CCl}_4$

c) $\text{CCl}_4 \rightarrow$ Endoplasmic

\[ (\text{CCl}_3) \rightarrow \text{target enzymes (E)} \rightarrow \text{inactivated} \]

\[ \text{E} \rightarrow \text{CCl}_3 \] (Inactivated enzymes)

d) $\text{CCl}_3 \rightarrow$ Disruption of lipid protein linkage $\rightarrow$

Depressed lipoprotein $\rightarrow$ Depressed release $\rightarrow$

of lipid from cells

Fatty infiltration in hepatic cells

e) $\text{CCl}_3 \rightarrow$ Plasma membrane $\rightarrow$ Cells

injury

(Rise of $\text{Ca}^{++}$ and $\text{Na}^{++}$ and fall of $\text{K}^+$, enzymes and coenzymes)

Cell necrosis

3. Effect of lipoperoxidation

f) $\text{CCl}_3$ $\rightarrow$ membrane lipids ($\text{PH}$) $\rightarrow$ $\text{CHCl}_3 + P^*$

$P^* \rightarrow$ target enzymes

(Polymerization of $P^*$ $\rightarrow$ OInactivation of enzyme)

(enzyme proteins)

g) $P^* + O_2 \rightarrow$ POO$^*$

h) POO$^*$ $+$ PH $\rightarrow$ $P^*$ $+$ POOH

i) PooH $\rightarrow$ Distruption of membranes (microsomal, lysosomal, mitochondrial)

$\rightarrow$ Cell necrosis

j) $P^* + O_2 \rightarrow$ POO$^*$ (chain reaction)
Although, there is much evidence in favour of the occurrence of lipid peroxidation there are many experimental facts which conflict with the proposed mechanisms linking lipid peroxidation to the different manifestations of hepatotoxicity of $\text{CCl}_4$. One of the requirements of the proposals is that there is one main step common to the eventual processes of necrosis and lipid accumulation. It is to be expected from this that factors which modify this step should bring about parallel changes in necrosis and lipid accumulation. As pointed out by Cawthorne et al. (1970), on the basis of several experimental facts by many workers, necrosis, lipid accumulation and death are caused by processes that are separable and may be different in origin. Some of the facts supporting their conclusion are as follows:

Many agents which are believed to have antioxidant properties protect against liver necrosis without having any influence on lipid accumulation (Rees et al., 1961; Bangham et al., 1962; Fox et al., 1962). On the other hand, L-asperigine has been found to provide protection against the accumulation of lipids but not against necrosis. Vitamin E increases survival without affecting the hepatic lesions. BHT reduces fat accumulation and increases survival but has little effect on liver necrosis. There is
also evidence against any connection between lipid-peroxidation and hepatic microsomal functions in the early stages of CCl₄ damage (Smuckler et al., 1967). Although, DPPD was found by Castro et al. (1968) to reduced liver necrosis, it did not prevent the impairment of microsomal cytochrome P-450 or ethylmorphine demethylation. On the other hand, they had reported prevention of necrosis and impairment of cytochrome P-450 and ethylmorphine demethylase by SKF 525-A without any effect on hepatic fat accumulation. Dingell and Heimberg (1968) had reported that α-tocopherol as well as DPPD did not protect against the inhibitory effect of CCl₄ on hexobarbitone oxidase and aminopyrene demethylase. Sasame et al. (1968) have ruled out the role of lipid-peroxidation in the early impairment of microsomal enzymes by CCl₄. A decline in CCl₄-induced hepatic protein synthesis by DEPD and α-tocopherol could not be demonstrated by Alpers et al. (1968). In view of the observations that the substances which are known to have antioxidant effects vary on their effects on the CCl₄-induced hepatotoxicity suggesting different mechanisms of action, Cawthorne et al. (1970) have concluded that there is no single primary step subject to antioxidant control. Another important fact emphasized by Cawthorne et al. (1970) is that vitamin E did not reduce the hepatic lesions under conditions in which
it reached a high level in the liver. The effect of vitamin E could be demonstrated when it is administered 48 hours or 24 hrs before CCl₄ and not when administered 6 hr earlier. Similarly, the effects of ethoxyquin and BHT were maximal when these were hardly detectable in the liver.

Cawthorne et al. (1970) have further brought to light the fact that the decrease or increase in CCl₄ toxicity by factors which inhibit or stimulate respectively the activity of the microsomal drug processing enzymes is not true in all cases. McLean and McLean (1966) had shown that starvation increased the toxicity of CCl₄ in male rats, whereas Kato and Gillete (1965) had found that starved male rats had decreased aminopyrene demethylase and hexobarbitone oxidase activity. Ethoxyquin, DPPD, BHT and vitamin E, tended to increase rather than decrease hepatic aminopyrene demethylase activity. In addition, ethoxyquin protected against CCl₄ toxicity in rats that had been previously sensitized by the drug processing enzyme inducers such as DDT, although the antioxidant raised processing enzyme activity as much as with the inducers (Cawthorne et al., 1970).

On the basis of the above findings Cawthorne et al. (1970) have postulated that there are two specific metabolic
steps, one toxifying and the other detoxifying CCl₄.
The resultant toxicity under given set of circumstances will depend on the balance of these two processes. In support of this suggestion they refer to the effects of ethoxyquin and BHT which require about 48 hr to produce their effect, and which is consistent with the idea of an enzyme induction. For both the substances, there was a marked tendency for single doses to be more effective than multiple doses against fatty accumulation. This was explained on the basis of enzyme induction effect of the first dose and competition with CCl₄ for the enzymes by the subsequent doses. They further suggest that very high doses of vitamin E and BHT, especially if given fairly soon before the CCl₄ markedly increase hepatic triglyceride accumulation since they compete for processes concerned in the detoxification of CCl₄. Cawthorne et al. (1970) on the other hand, suggest the possibility of altered CCl₄ toxicity by substances through mechanisms other than those concerned with the drug processing enzymes. In this connection they refer to the work of Rapin (1967) that the protective effect of oxygen (Glynn and Himsworth, 1948), might be though the action of oxygen on regeneration of liver and not necessarily due to a competitive reaction between oxygen and endogenous microsomal process (Slater, 1966).
Similar could be the mechanism of vitamin E on survival as suggested by the work of Butturini et al. (1955), and Maros et al. (1966). In explaining the protective effect of 3-methylcholanthrene on bromobenzene hepatotoxicity, Reid et al. (1971) consider the possibility that the 3-methylcholanthrene administration increases the overall rate of \(^1^4\)C-bromobenzene metabolism in rats but reduces the metabolic pathway leading to the formation of a toxic metabolite capable of binding covalently to tissue macromolecules. Gawthorne et al. (1973) have considered the possibility that ethoxyquin and 3-methylcholanthrene might effect CCl\(_4\) hepatotoxicity by similar mechanisms.

Evidence against lipid peroxidation as an essential step in CCl\(_4\) hepatotoxicity comes from a recent report by Toranzo et al. (1978). They have demonstrated that GXF, GF\(_1\) and Swiss mice do not exhibit increased lipid peroxidation in their liver microsomes in response to CCl\(_4\) administration although severe liver injury is caused. Similar observations had been reported earlier by Diaz Gomez et al. (1975) and Villarruel et al. (1976), in respect of Strain A/2 male mice. Hornbrook et al. (1976) had also confirmed the findings on the A/2 mice. On the basis of these observations Toranzo et al. (1978), feel that lipid
peroxidation does not play a key role in liver injury induced by CCl₄ \textit{in vivo}, though there is a strong evidence for the importance of this process on many effects induced by CCl₄ \textit{in vitro}.

Gravela \textit{et al.} (1979) reported that the free radical scavenger promethazine and the metabolic inhibitor/SKF 525A added to isolated liver cells have a strong inhibitory effect on the CCl₄ -- induced malonaldehyde production. These substances have no protective effects against the decrease in protein and lipoprotein secretions caused by CCl₄. The authors suggest that the CCl₄-induced blocks in protein and lipid secretion are not mediated through the peroxidative stimulus produced by CCl₄.

**FACTORS INFLUENCING CCl₄ HEPATOTOXICITY**

**Diet**, age, sex and endocrine status all modify CCl₄-induced hepatotoxicity.

**Diet**

Early studies have demonstrated that starvation, a low carbohydrate intake or a high-fat diet increase susceptibility to CCl₄ toxicity (Von Gett ingen, 1964; Drill, 1952; Rouiller, 1964). These observations remain unchallenged. The role of intake and body stores of protein, however, has been less clear. At midcentury, Shaffer \textit{et al.} (1946) held the idea that protein depletion
enhanced CCl₄ hepatotoxicity and that severity of the injury paralleled the extent of protein depletion. Recent studies by McLean and his associates (McLean and McLean, 1966; Seawright and McLean, 1967; Garner, and McLean, 1969; McLean, 1975; Seawright et al. (1972) and Aterman and Yuce (1975) have shown that a protein-free diet actually leads to a decrease rather than an increase in the toxic effect of CCl₄. The protection afforded by protein deprivation, according to these workers, results from the decrease in activity of the enzyme system that metabolizes the agent and a consequent inhibition of conversion of CCl₄ to toxic metabolites (McLean and McLean, 1966; Seawright, 1967; Garner and McLean, 1969; McLean, 1975). Korsurd et al. (1976) have also been unable to find the protective effects of no or low protein intake. The much longer period of protein deprivation could lead to deprivation of tissue protective factor, e.g. sulphhydryl molecules (Ferreyra et al., 1975; Korsurd et al., 1976) and thereby diminish the benefit derived from depressed activity of the cytochrome P-450 system. The adverse effects of a carbohydrate depleted diet have been attributed to the accompanying high-fat content of the diet which perhaps increased the concentration of CCl₄ in the liver (Drill, 1952; Rouiler, 1964) or to the lack of the protective effects of liver glycogen stores (McLean and McLean, 1966).
Sex

Studies of sex differences in rats have shown that males are more sensitive than females, a difference consistent with the greater activity of mixed function oxygenase in the male rat (Drill, 1952; Von Oettingen, 1964; McLean et al., 1965).

Age

The neonatal rat is far less susceptible to CCl₄ (Recknagel and Glende, 1973) toxicity than the adult. The resistance of the young rats disappears as the P-450 metabolizing system for foreign compounds matures. The regenerative response of aging rats to CCl₄-induced hepatic injury also is less vigorous than that of younger ones (Post et al., 1957). Rats less than about 6 days old fed with carbon tetrachloride produce little or no signs of necrosis (Dawkins, 1963). Susceptibility appears in the animal when they are seven days old. It is well known that mechanisms for chemical biotransformation and excretion are not fully developed at birth (Fouts and Adamson, 1959; Fouts, 1973; Knaasen, 1972, 1973, 1975; Hook and Hewitt, 1977).

Endocrine factors

Pituitary, hypothalamic, adrenal and gonadal effects apparently include the role of (a) fat accumulation induced
by some hormonal changes (Rouiller, 1964), b) the altered hepatic blood flow and/or body temperature (Larson and Plaa, 1965) induced by neuroendocrine factors of the protective or other modifying effects of glucocorticoid activity (Selye, 1971) and d) other complex and insufficiently delineated factors. Endocrine disturbances that lead to fatty liver appear to enhance toxicity to CCl$_4$, perhaps by permitting increased uptake and storage of the toxin in the liver (Schwarzmann, 1957). Transection of spinal cord delays but does not reduce the severity, of CCl$_4$ poisoning (Larson and Plaa, 1965). Presumably the depression of body temperature decreases hepatic blood flow and delivery of CCl$_4$ to the liver (Larson and Plaa, 1965), as well as metabolic conversion of CCl$_4$ to a metabolite (Recknagel and Glenda, 1973). Glucocorticoids and ACTH have been reported to intensify CCl$_4$ toxicity and adrenalectomy to reduce it (Rouiller, 1964).

Experimental diabetes has been found to enhance susceptibility of animals to CCl$_4$ toxicity (Hanasono et al., 1975). The effect of diabetes in animals is believed to be due to some associated metabolic disturbance, since it is reversed by insulin. Hypothyroidism protects against the CCl$_4$ hepatotoxicity and hyperthyroidism enhances it (Schwarzmann, 1957; Calvert and Brody, 1960, 1961; Larson et al., 1965; Vessel et al., 1975). This may be related
to the decreased and increased activity respectively of mixed function oxygenase system. Since an inverse relationship exists between endogenous levels of thyroid hormone and monoamine oxidase, it may be assumed that the beneficial action of low thyroid function is associated with enhanced removal of biogenic amines (Zile and Lardy, 1959; Goodkind et al., 1961). Conversely thyroxine administration, by reducing MAO activity (Zile and Lardy, 1959), would operate to prolong the retention of catecholamines in the body thus potentiating the toxicity of $\text{CCl}_4$.

**Vitamin E**

Hove (1948) was the first to report the protective effect of Vitamin E on $\text{CCl}_4$ mortality in rats. The observations were on young male rats treated with daily i.p. injections of 1 mg of vitamin E for 21 to 71 days in animals given 2 ml/Kg of $\text{CCl}_4$ intraperitoneally. An i.p dose of 25 mg $\alpha$-tocopherol was found to markedly increase survival of $\text{CCl}_4$ treated animals by Hove and Hardin, 1951a). Lower doses (5 mg) of $\alpha$-tocopherol were not effective. Same authors (1951b), have been unable to demonstrate prevention of fatty liver in rats by dietary supplement of 0.01% of $\alpha$-tocopherol for 56 days in rats given weekly subcutaneous injections of $\text{CCl}_4$ in 0.05 ml doses.

Administration of 100 mg of vitamin E daily to female rats for 16 to 18 days prior to a single oral dose of 0.5 ml
$CCl_4$ has been reported to reduce fat accumulation (Krone, 1952). With no effect on necrotic degeneration, although liver regeneration was found to be accelerated. Hartman et al. (1952) could demonstrate only a marginal reduction in liver fat accumulation when 10 mg of vitamin E per day was given to rats which were simultaneously given subcutaneous doses of 5 ml of $CCl_4$/Kg daily for 9 days. According to the observations of Butturini et al. (1955) prior administration of $\alpha$-tocopherol is protective more against necrosis than against fatty infiltration due to $CCl_4$. Fat accumulation was increased if vitamin E was administered simultaneously with $CCl_4$. Vitamin E also increased the regeneration of liver.

Gallagher (1961) has reported full protection against mortality due to 4 ml/Kg of undiluted $CCl_4$ by stomach tube given to female Wistar rats by 95 I.U. of $\alpha$-tocopherol acetate given intraperitoneally. Slight protection was also seen with 95 I.U. of $\alpha$-tocopherol, 24 and 6 hr and with 19 I.U. of $\alpha$-tocopherol, 72, 48, 24 and 6 hr before $CCl_4$ administration. There was no protection with 95 IU of tocopherol given together or 5 hr after $CCl_4$ toxicity. Else and Pesaresi (1963) had reported the reduction in fat accumulation and necrosis in liver by 100 mg of vitamin E in a single intramuscular dose 48 hr before i.p injection of 2.5 ml/Kg of $CCl_4$. 

Diluzio (1964) has demonstrated a lowering of fatty accumulation due to CCl₄ administration by intravenous injection of 50 mg/Kg of vitamin E, 24 hr before CCl₄ administration, but not when given simultaneously with CCl₄. McLean (1967) observed that administration of \( \alpha \)-tocopherol acetate at 60 mg doses 24 hr before administration of CCl₄ (2.5 ml/Kg) gives only slight protection of to liver. There was no effect on liver necrosis. The slight effect of vitamin E could not be demonstrated when the animals were fasted for 18 hr before administration of CCl₄. El-Kateb \& al. (1965) found no protection against mortality or liver damage with three 33 mg doses of tocopherol given intramuscularly for 3 successive subcutaneous doses of 8-75 mg/Kg of CCl₄ but found some protection with or single 100 mg dose administered 3 days before.

A marked reduction in the hepatic lesion was demonstrated by Meldolesi (1968), by intraperitoneal injection of 125 mg/Kg of \( \alpha \)-tocopherol 48, 24, 2 hr before oral administration of 2.5 ml/Kg of CCl₄.

Blendermann and Friedman (1968) did not find any effect of vitamin E on a number of CCl₄-induced alterations of carbohydrate and fat metabolism in rats. Vitamin E was
fed at the dose of 100 mg/Kg for 5 weeks and then CCl₄ (2 ml/Kg) was fed orally.

Green et al. (1969) have demonstrated that the increased production of MDA during incubation of liver microsomes plus supernatant fraction of liver depends on vitamin E status of the animals. The effect of CCl₄ on the production of MDA in vitro studied in 5 months old rats. The volume of CCl₄ varied between 1 and 20 ul. In vitamin E deficient rats the production of MDA was rapid and unaffected by CCl₄. When the rats had been given vitamin E deficient diet supplemented with 70 ppm of D- tocophenol acetate from weaning, the rate of MDA production in vitro was much slower, and no prooxidant effect of CCl₄ was observed. When vitamin E deficient rats were given a very small dose (400 ug) of D- tocophenol 24 hr before being killed, there was slightly diminished production of MDA.

The effect of CCl₄ (2.0 ml/Kg orally) on ATP levels in rat liver was studied by Green et al. (1969) at intervals of 3 to 68 hr. The primary lesion leading to necrosis and fat accumulation after CCl₄ treatment occurred many hours before the eventual slight decrease in ATP. Although the levels of ATP were somewhat higher in vitamin E deficient rats, vitamin E did not prevent the slight decline in ATP that took place. These observations led the authors to
suggest that lipid peroxidation was not the primary event in CCl₄ poisoning since ATP was known to be highly sensitive to peroxidation.

The effect of CCl₄ on the metabolism of (¹⁴C) D-α-tocopherol in the rat also was studied by the same authors. A single intraperitoneal injection of CCl₄ (2.0 ml/Kg) did not decrease the destruction of α-tocopherol in the liver after 24 hours. Three smaller daily doses of CCl₄ (0.25 ml/Kg) also did not increase α-tocopherol catabolism. On the contrary, significantly more α-tocopherol was found in the livers of rats treated with CCl₄. This shows the CCl₄ does not increase lipid peroxidation in vivo. Increased survival of male rats, but not female rats was demonstrated by Cawthorne et al. (1970) by three oral doses, each 450 mg/Kg of vitamin E, given 24, 48 and 72 hr before oral administration of 3.5 ml/Kg of CCl₄ in males and 8.5 ml/Kg in females. Single oral doses of 450 mg/Kg 24 hr and 72 hr before CCl₄ were protective in males but decreased survival in females. There was no significant decrease in CCl₄-induced fat accumulation in the liver with any of the doses of vitamin E. Increased hepatic fat accumulation and mortality was observed with 2000 mg/kg of vitamin E administered orally 6 hr before (CCl₄).
Benedetti et al. (1974) have reported a decrease in the peroxidative reaction and fat accumulation in the liver in male rats given vitamin E orally in doses of 750 and 1500 mg/Kg 24 hr after oral administration of 2.5 ml/Kg of CCl₄. Microsomal concentration of α-tocopherol correlated with the decrease of microsomal lipid peroxidation and decrease in liver fat accumulation. Impairment of microsomal glucose-6-phosphatase and incorporation of ¹⁴C from ¹⁴CCl₄ into liver microsomal lipids was not affected by vitamin E treatment. Extent of microsomal lipid peroxidation did not correlate with the liver triglyceride accumulation by vitamin E pretreated rats given 0.2 ml/Kg or 0.025 ml/Kg of CCl₄. Deferreyra et al. (1975), were unable to detect any effect on lipid peroxidation by administration of α-tocopherol in male rats 40 min or 3 hr after 2.5 ml/Kg of CCl₄.

Ghoshal (1976) has reported an antioxidant role of vitamin E in CCl₄-induced hepatotoxicity. Rats were divided into 3 groups, each group being given (100, 200 or 400 mg/Kg bw of d1-α-tocopherol intragastrically. CCl₄ was given intragastrically, in the dose of 1 ml/100 g bw. They were killed at different time intervals from 0 to 24 hr. Tocopherol content reached maximum levels in mitochondrial and microsomal fractions 5 hr after the administration of 20 mg of vitamin. The α-tocopherol
content of the supernatant fraction remained stationary at a very low level. Four hours after feeding α-tocopherol to rats, the vitamin content in the mitochondrial fraction was maximum at a dose of 100 mg/Kg of α-tocopherol. At the same time, the α-tocopherol level in the microsome fraction was considerably higher with a dose of 400 mg/Kg. Increasing the dose of vitamin from 100 to 400 mg/Kg did not increase the α-tocopherol content of supernatant fraction.

Lipid peroxidation in the microsomes was not affected by oral administration of 600 mg/Kg of α-tocopherol given 4 hr prior to oral dosage of 1 ml/Kg of CCl₄. There was a significant reduction of α-tocopherol in the microsomal fraction of the CCl₄-treated animals.

Taylor and Tappel (1976) reported that the effect of dietary vitamin E on in vitro NADPH-dependent microsomal lipid peroxidation and the activation of microsomal lipid peroxidation by CCl₄. The rate and extent of in vitro NADPH-dependent microsomal lipid peroxidation were affected considerably by dietary vitamin E. The rate of lipid peroxidation measured by O₂ uptake, and the extent of lipid peroxidation by the amount of malonaldehyde that accumulated in 30 min, were both diminished as the amount of dietary vitamin E increased. A significant reduction of CCl₄-induced
increase in SGOT by administration of 750 mg/Kg of \(\alpha\)-tocopherol was reported in rats by Yasuda et al. (1980).

Pesh-Imam and Recknagel (1977) demonstrated a decrease in the vitamin E content of liver after 3 and 12 hr oral administration of 4 ml/Kg of CCl\(_4\).

**Ethoxyquin (6-Ethoxy 2,2,4-trimethyl-1,2 Dihydroquinoline)**

Cawthorne et al. (1970) reported protective effect on CCl\(_4\)-induced mortality, liver necrosis and fat accumulation in liver by administration of 3 daily oral doses of 300 to 500 mg/Kg of ethoxyquin given to male and female rats before oral administration of 3.5 ml/Kg of CCl\(_4\) in males and 8.5 ml/Kg in females. Single oral doses of 500 mg/Kg of ethoxyquin given 72 or 48 hr before CCl\(_4\) were also effective, but not when given 24 or 6 hr before CCl\(_4\). Administration of ethoxyquin by intraperitoneal route was also effective.

Cawthorne et al. (1973) have demonstrated that the rate of metabolism of CCl\(_4\) by liver homogenates of rat is not affected by ethoxyquin pretreatment.

**NN\(^{4}\)-Diphenyl-p-Phenylenediamine (DPPD)**

Reduced mortality by DPPD against CCl\(_4\) toxicity has been reported by Gallagher (1961). Complete protection against death was achieved in female rats with 3 intraperitoneal
injections of 100 mg DPPD at 48, 24 and 9 hr before oral administration of 4 ml/Kg of CCl₄. More than 50% protection against mortality was given by a single dose of 100 mg DPPD either 48 hr before or with CCl₄. DPPD in doses of 50 or 10 mg were also protective. The histological appearance of the liver in DPPD-treated animals was normal as compared to the control CCl₄-treated animals, which showed centrilobular necrosis and fatty change in the liver. Cawthorne et al. (1970) also reported increased survival in male and female rats given 3 daily doses of 600 mg/Kg of DPPD or a single dose, 72 h before oral administration of CCl₄ in doses of 3.5 ml/Kg in males and 8.5 ml/kg in females. No effect was seen with single doses of DPPD given 6 to 48 h before CCl₄. Lipid accumulation in the liver was also reduced. Multiple doses of DPPD decreased the liver necrosis but single doses were less effective. Dianszani and Ugazio (1973) demonstrated that the administration of 600 mg/Kg of DPPD intraperitoneally to male Wistar rats reduces the accumulation of lipids in the liver treated with 2.5 ml/Kg of CCl₄ administered orally. The animals were killed 4 hr after administration of CCl₄. It was further observed that there was no alteration in the non esterified fatty acid in the plasma. Administration of DPPD was found to inhibit the double bond shift in UV spectrum of microsomal lipids observed 30 min after CCl₄ treatment. Deferreyra et al. (1975)
reported that administration of 600 mg/Kg of water suspensions of DPPD significantly lowered concentration of CCl₄ in the liver when the latter was given intraperitoneally. The effect was particularly pronounced at 6 hr. No change in the concentrations of liver CCl₄ was found with oral administration of CCl₄. A change in CCl₄ concentration in the liver could also not be demonstrated with oil suspensions of DPPD. Purified DPPD or technical grade DPPD (Kodak) was shown to significantly decrease the irreversible binding of ¹⁴C to microsomal lipids. DPPD profoundly decreased the extent of CCl₄ activation to CCl₃ radicals.

Butyl hydroxy toluene (BHT)

Butylated hydroxy toluene (BHT) in oral doses of 400-600 mg/Kg administered to rats 72 hr before CCl₄ has been reported to be protective against CCl₄ toxicity (Cawthorne et al., 1970). Mortality, liver necrosis and fat accumulation in the liver were decreased. There was a large increase in liver weight after 48 hr. Oral doses given 6 to 24 hr before CCl₄ increased the CCl₄-induced rise in liver triglycerides. No protection was observed with intraperitoneal administration of BHT.

Sodium selenite

Sodium selenite given in doses of 0.5 mg/Kg at 72 hr and 0.25 mg at 48, 24 and 6 hr respectively in female rats
before 2.7 ml/Kg of CCl₄ was reported to have protective value against CCl₄-induced mortality (Gallagher, 1961).