CHAPTER : 4
DISCUSSION
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I : MORPHOLOGICAL STUDY

A number of many changes have observed in the liver as a result of toxic effects of chemicals, and there have been various attempts to organise the accumulated data. Liver is the first organ in which investigations of dose response relationships in chemicals carcinogenesis were carried out systematically (Druckrey, 1943). The determination of the induction and the total dose required for the production of liver tumours by different hepatocarcinogens led to the well known concept of Druckrey (1967) that the primary carcinogenic effects of all individual dose act additively to form the final tumour. In the present study also incidences of tumours were directly correlated with the total dose level as indicated by the duration of exposure. No liver tumour was developed in animals immediately after 2 month of BHC exposure. However tumours appeared after BHC treatment for longer duration of time and maximum tumour developed after 8 months of BHC treatment. The result of the present study indicates dose dependence relationship with incidence of tumours as suggested by Druckrey(1967) and James et al;(1992).

Liver weight % Body Weight (Lw % Bw)

Liver is the most affected organ when animals are exposed to chlorinated insecticides. Since these chlorinated insecticides are metabolised in the liver. Therefore, increase in liver weight presented as % of body weight observed in these experiments in Group II (BHC Treated), it may be an indicator to know overall effect of BHC on the liver. The increase in liver weight as % of body
weight after exposure of various hepatotoxic chemicals including organochlorine insecticides has been reported (Abston and Yarbrough, 1976) and the technical grade BHC also has been reported by Thakor Kashyap (1984). The toxic chemical effect on liver weight expressed as% of body weight is associated either with cellular damage or increased cell population and fatty infiltration. The increase in liver weight observed within initial few weeks exposure indicates that liver is involved at an early stage of exposure. These changes are more pronounced with longer duration of exposure and substantial cellular damage has taken place when duration of BHC exposure is beyond 6 months. In duration period of 6 to 8 months the Liver weight as % Body weight (Lw %Bw) was maximum. The change was very significant after 6 months exposure. At this stage neoplastic nodules were developed in liver. Thus, increase in liver weight may also reflect neoplastic development in liver. Eltze et al; (1977) and Karnik (1989) reported that liver undergoing neoplastic changes increased more markedly in size and wet weight. On the basis of this suggestions the BHC model as the liver tumour model was investigated, and effects of AP was seen on it. However, in the morphological changes in the liver lesion in the BHC with AP supplemented animals liver damage was not found in only BHC induced tumours condition.

II : HISTOMORPHOLOGICAL STUDY

The Liver is a multifunctional organ and the broad spectrum of the manifestations of its diseases often leads to diagnostic or therapeutic problems. One of the main functions of the liver is the protection of the body against intoxication caused by chemicals which pollute man's environment and invade our body as smoke particles and toxic gases, as dyes or preservatives, pesticides, disinfectants and last but not least, as drugs either therapeutically useful or useless.
The liver also plays a central role in intermediate metabolism as well as in the maintenance of the homeostasis of the whole organism. The vast majority of liver diseases induce alterations in a numerous parameters of the blood serum. Laboratory tests informative on various functions are essential in the diagnosis of liver diseases. The diagnosis is based on characteristic clinical, laboratory and histological findings.

**Anatomical Considerations**

The liver is well adapted anatomically for the role of extracting substances from the blood. It has a generous blood supply, three-quarters of which comes from the portal vein, and one-quarter from the hepatic artery. The microvascular unit of the liver, the acinus, consists of a microscopic mass of liver cells arranged around an axis, the portal tract, which contains terminal portal venule and hepatic arteriole, bile ductule, lymph vessels and nerves (Rappaport, 1982). The liver cells are arranged in inter-connecting plates one cell thick, amongst which run the hepatic sinusoids. Blood enters the sinusoids from the terminal portal venule and hepatic arteriole, and percolates out to the periphery of the acinus to exit via the terminal hepatic venule. While passing along the sinusoidal blood elements other than red cells come into intimate contact with liver cells by movement into the extra-sinusoidal wall which does not have a basement membrane, and contains numerous fenestrae.

The present study shows considerable changes in functional marker and growth behaviour of abnormal hepatocytes, at several stages of liver carcinogenesis after BHC treatment.

The most important characteristic of early Hepatocellular carcinoma (HCC) lesion due to BHC treatment has the following five characteristics
in varying degrees:

1. Increased cell density associated with the increase of nucleus : cytoplasm ratio
2. Increased eosinophilic staining affinity
3. Fatty changes and clear cells changes
4. Irregular thin trabecular pattern and
5. Acinar and pseudoglandular pattern

Among the various types of morphological changes in the liver during carcinogenesis, hyperplastic areas and nodules have been described as very important preneoplastic elements in the neoplastic transformation of liver parenchymal cells. (Farber, 1973, Tomoyuki Kitagawa 1976). These are focal nodular proliferation of somewhat altered liver cells appearing during the neoplastic phase. Thus, the neoplastic development is a multi-step process where early cellular events or changes finally lead to liver tumour developments.

In general cytoplasmic changes have been observed. There is increased basophilic or eosinophilic cytoplasmic staining which may be of diagnostic use (Kondo Y et al; 1986, and Kondo F et al; 1987). For unknown reasons, tumour cells have a remarkable tendency to undergo fatty changes or clear cells alterations, especially in small nodules. Consequently, small nodules in the liver with prominent fatty changes were detected as early hepatocellular carcinoma by Kojiro et al; (1991).

The clear cell variant of HCC (hepatocellular carcinoma) is characterized by tumour cells with a clear cytoplasm; which is due to the presence of abundant glycogen. It has been reported that HCC of the clear cells variant has a favourable prognosis compared with that of other variants. (Lai et al; 1979).
In this study BHC treated liver showed small to large hepatocytes with enlarged nucleus and bigger nucleoli. At a later stage these lesions turn in to preneoplastic nodules, similar to the one observed by Bannasch. (1976) and Williams (1976).

The preneoplastic lesion of the liver has been analyzed histologically and the reversibility or irreversibility of preneoplastic or neoplastic changes has been discussed extensively by Bannasch. (1978), and Ito et al; (1976). Bannasch et al; (1980) indicated the earliest changes in rat and mice liver, after exposing them to N-Nitrosomorpholine and Ethylnitrosourea respectively, where emergence of clear cells with intermediate cells took place also. He further mentioned that the appearance of basophilic cells during later stage of hepatocarcinogenesis indicated the final step towards neoplasia. The malignant nature of the commonly included nodular lesions in the absence of metastasis has been discussed by Vesselinovitch et al; (1978).

According to an earlier study hepatocellular carcinoma induced by Hexachloro cyclohexane, and it was found that the liver lesion in their early stage were reversible, but after 4 months of exposure the changes seen both histologically and histochemically, to be permanent or non reversible, because even on the discontinuation of BHC feeding, the changes not only persisted but also progressed further (Kashyap et al; 1979 and Nigam et al; 1981, 1986). After 4 and 6 months of BHC exposure many mesenchymal cells like lymphocytes, mononuclear cells, macrophages and even neutrophils infiltration in the neoplastic nodules. Degenerated liver cells were also been made by Ito et al; (1973) and Nigam et al; (1981), (1986). They have further suggested that the period between 3 to 5 months of exposure to BHC is the critical period when permanent alteration in the cell morphology occurs and those changes then persist and progress ultimately, leading to the development of well differentiated hepatocellular carcinoma.
The histological changes observed in the present study also reveals that the lesions in liver are multifocal in origin and progressive in nature. This finding supports the earlier observations (Ruebner 1976, Kashyap et al; 1979 and Nigam et al; 1981, 1984a, 1986).

In the present study early preneoplastic lesions seen after 2 months of BHC exposure consisted mainly of clear cells, acidophilic cells and cells with bigger nuclei. However these changes became reversible, because of BHC with AP treatment. 2 month BHC + AP supplemented animals (in group III) have not many changes corresponding to BHC alone. The hepatocytes appeared cytologically quite normal. Hepatic cells and radial pattern were not disturbed compared to 2 months BHC alone (group II).

In 4 months BHC treated animals liver showed many changes. The cellular alteration observed in liver ultimately progressed towards the development of neoplastic nodules. In such preneoplastic nodules, 4 types of cells were observed and they were clear cells, acidophilic cells, basophilic cells and vacuolated cells with some intermediate cells. The Preneoplastic nodules with predominant cellular alteration in 4 months BHC treated. The present finding supports the earlier observations and descriptions (Kashyap et al; 1979 and Nigam et al; 1981, 1984ab, 1986).

The above mentioned liver damages were less severe in animals supplemented with Andrographis paniculata for 4 months in group III compared to BHC alone for the same period. Fat depositions were observed and few clear cells were observed but preneoplastic nodules were not observed clearly in 4 months AP supplemented animals in group-III. Successive improvement at cellular level in hepatocytes may be due to the effects of AP.

In liver lesion changes after 5 to 6 month of BHC exposure. The changes occurred in mesenchymal cells like lymphocytes, mononuclear cells,
macrophages and even neutrophils infiltrated in the neoplastic observed as earlier described. Degenerative liver cells were also present of these mesencymal elements probably indicated that they play some role immunologically in restricting the growth. Some observations have also been made by Ito et al; (1973),(1976), Nigam et al; (1981), (1984). After 6 months of the BHC treated liver shows neoplastic nodules indicating in liver, 4 types of cells in the liver and basophilic cells were predominant.

On the other hand in liver lesion after 6 months AP supplementation (group III) morphological changes indicate, acidophilic, basophilic and few clear cells but neoplastic nodules were not seen as which were observed in 6 months BHC treatment.

In the present study after 7 to 8 months of BHC feeding most of the tumours are distinct and well differentiated hepatocellular carcinoma. After 8 months of BHC feeding, the liver architecture was totally destroyed, hepatocytes were totally turned, and trabecular structure was indicated. Thus, the association between the presence of trabecular nodular lesions and pulmonary metastasis demonstrated the malignant character of trabecular nodular lesions, reported by Tomatis et al; (1972), Turusov et al; (1973) and Nigam et al; (1981). Thus, it is quite evident that BHC induces three different types of lesions such as hyperplastic areas, neoplastic nodules and trabecular cell carcinoma and at the end of these, only trabecular carcinoma could metastasize.

It was further observed after 8 months of AP supplementation (Group III) in mice liver, that many distinct changes were indicated like acidophilic cells, basophilic cells, few clear cells and cell vacuolization. However neoplastic nodules were not visible as were observed in 6 months BHC alone (Group II) . Fully grown tumorous condition was observed when BHC was given for eight months. Contradictory to that when AP was supplemented with
BHC for the same duration the damage observed was less. This indicates that AP suppressed the toxic effect of BHC on liver to a certain extent.

III : ULTRA STRUCTURAL STUDY

The Ultrastructural study of the liver showed various changes in different Groups. The electron micrographs shows changes in BHC treated animals of Group II. The presence of glycogen, the proliferations of smooth endoplasmic reticulum and the excessive accumulation of fat. The another prominent changes in hepatocytes are seen in BHC treated for 6 to 8 months, where the shape and structure of mitochondria had altered considerably. The mitochondria were enlarged with lack of cristae, the cristae were broken with their membrane ruptured or disintegrated, and permeability has been disturbed in the innermembrane of mitochondria. The shift of the energy requirement from one side to other may be affected. The permeability disturb in inner membrane has suggested that the accumulation of the substances in to matrix which lead to swollen and disorganized cristae structure of mitochondria. The disorganized cristae may also altered the enzymatic pattern of the respective chain complexes. That is reflected in the parameter which declined SDH and ATPase activities in the present study in BHC treated mice in Group II.

The excessive accumulation of Glycogen was found within the cytoplasmic matrix in BHC treated hepatocytes. The large amount of glycogen was also seen enclosed in autophagic vacuoles during neoplastic nodule development. In later stages glycogen became ultimately granular. It is suggested that these changes may be due to enhanced glycogenesis or inhibition of glycogenolysis, which may be a consequence of a toxically induced enzyme deficiency. The reduced activity in present study of the
HEPATOCYTE

Specific toxic effect of the Carcinogen (BHC) Hexachlorocyclohexane

ACINUSPERIPHERAL CYTOTOXIC PATTERN
(Enhanced storage of glycogen, fatty deposition, dislocation and relative reduction of ergastoplasm, possible hypertrophy of the smooth ER and mitochondrial changes)

CELLULAR TRANSFORMATION
Progressive reduction of the accumulated glycogen, possible transitory fat accumulation, enhancement of ribosomes with transformation of rough ER into smooth and pronounced diffused proliferation of the smooth endoplasmic reticulum.

HEPATOMA
Phosphorylase and the Glucose-6-phosphatase suggest that these enzymes play an important role in the glycogenolysis and origin of carcinogen induced glycogenesis. The storage of glycogen in the hepatic cell may be due to enhanced glycogenesis or inhibition of glycogenolysis. This reduced activity of this enzyme glucose-6-phosphatase was also described earlier by Bannasch and Muller, (1964).

The most prominent change was observed in the cytoplasm in form of pronounced diffuse proliferation of smooth endoplasmic reticulum. This well-known change of the cytoplasmic fine structure is responsible for the acidophilic appearance of the respective hepatocytes, which were observed in light microscopy. The granular or rough ER also play an important role in hepatocarcinogenesis (Porter and Bruni 1959, Simard and Daoust 1966, Muller 1967, Bannasch 1968, Nigam et al; 1984ab). The rough ER showed structural changes such as the cisternae which were no longer parallely stacked and individual cisternae were often scattered throughout the cytoplasm, frequently losing their ribosomes. This study also confirms the earlier findings. The characteristic feature of hepatoma seen under EM in this study is the detached ribosomes, and changes of in ER and RER. These are valuable morphological criteria for the degree of cell differentiation.

The outstanding feature of the acidophilic cells is a pronounced proliferation of the agranular endoplasmic reticulum. Earlier study on BHC exposed mice showed hypertrophy of the smooth endoplasmic reticulum. It was always preceded by enhanced glycogen storage and thus indicative of a direct or indirect relation between a granular ER and glycogen in hepatocytes. It was also suggested that hypertrophy of agranular ER is the consequence of an impaired carbohydrate metabolism which was a common feature in hepatocarcinogenesis. Similarly concentric whorls of the endoplasmic reticulum are associated with a decreased in the synthesis of phospholipids and
that happens either by desynchronization of the synthesis of protein and phospholipids or due to some other mechanism involved in lipid protein interaction in hepatocytes exposure to various hepatocarcinogens. (Bannasch, 1968, Norback and Allen, 1969; Ortega et al; 1966, and Ito et al; 1973). Concentric arrangement of lamellar cisternae complex of the agranular reticulum as described in this study has also been reported earlier study on BHC by Norback and Allen (1969) and Ito et al; (1973). The formation of lamellar bodies may be one of the toxic effect of BHC.

Ultrastructurally when vacuolated cells were examined under EM, they showed a large amount of lipid droplets, Fat droplets in their cytoplasm. It is presumed that inhibition of protein synthesis and consequent impairment of lipoprotein formation resulted in accumulation of fat mainly triglycerides with ER and smooth membrane bound vacuoles, which coalesced to from large lipid droplets in cytoplasmic matrix (Donald 1966 and Mathews and Martin 1971 ). The loss of canalicular microvilli as observed is suggestive of abnormality in the transport and canalicular secretion of bile. Such changes have also been observed earlier in CCl₄ (Smuckler et al; 1962), ethionine (Herman et al; 1962) and diethylnitrosamine (Emmelot and Benedetti, 1960).

The BHC treatment to the mice indicates gross morphological deformities in ultrastructural levels of liver. However these ultrastructural damage was gradual and observed from 4 months on word and was in extreme condition at 8 months treatment of BHC.

The ultrastructural changes in liver of mice from Group III, which was supplemented with AP along with BHC treatment. In 4 months AP supplemented, the hepatocytes showed an almost recovery at ultrastructural
level. The structure of the hepatocytes was almost normal corresponding to 4 months BHC treatment.

The 6 month AP supplementation in Group III, showed partial recovery. The electron micrograph of the hepatocyte has shown bile canal. This bile canal shows the presence of Microvilli. The size and structure of nucleus as well mitochondria were normal, Ribosomes were attached with RER. In 6months AP supplemented mice hepatocyte showed glycogen granules.

In 8 months AP supplementation in Group III showed slight recovery compared to the 8 months BHC treatment. The ultrastructural of hepatocytes showed clear nuclear membrane. The cell showed lamellated SER and RER, and the ribosomes were present on the RER. The SER found near the nucleus. The cell showed some fat droplets, vacuolization and glycogen clumping. These well-known changes of the cytoplasmic fine structure were responsible for fatty deposition of the respective hepatocytes observed in light microscopy.

These profound changes at an ultrastructural level after AP supplementation shows in the ultrastructure of liver and considerable recovery. The minute cell organelles reached almost to the structure observed in control condition of animals.
The Schematic Diagram showing the Interactions of Glucose, Lactate and Glycogen with other metabolites.
PROTEIN:

A significant decrease in protein content during hepatocarcinogenesis was also seen in various types of liver tumours (Smuckler and Arcasoy, 1969). Many earlier reports have shown changes in protein, after pesticide exposure (Takahashi et al; 1976). The present study also showed a decline in protein level after BHC exposure. In the initial month of BHC treatment (1st and 2nd months) the protein level does not decrease significantly, but after the 3rd month till the 8th month the level was significantly declined in Group II. The maximum reduction in protein content was observed at 6 to 8 months BHC exposure in Group II as compared to the control one. The probable explanation for the decrease in liver protein after BHC exposure may be that the protein content of the average hepatic cells during the stages of hepatocarcinogenesis is very low and this decrease takes place especially in the mitochondria, microsomes and the supernatant fluid. (Waber, 1961; Kashyap et al; 1979). Further, it has been suggested that the decline in protein content in the mitochondria is chiefly due to changes in the number of large granules in parenchymal liver cells. The result obtained by this study, a further increase in level of protein in BHC with AP supplemented, showed a considerable recovery as compared to the corresponding Group II. One may assume that AP plays a protective role in the defence mechanism.

SERUM GLUTAMATE PYRUVATE TRANSAMINASE (SGPT) AND
SERUM GLUTAMATE OXALATE TRANSAMINASE (SGOT):

The research publications from the observation of De Ritis et al; (1955) describing a significant rise in the activity of glutamate oxalate transaminase (GOT) [formerly known as aspartate amino transferase (AST)] in acute viral
hepatitis or liver damage, is considered one of the most important milestones in the progress of clinical enzymology. Raised glutamate pyruvate transaminase (GPT) levels [formerly known as alanine aminotransferase (ALT)] were detected subsequently in various hepatic disorders. Both transferases are considered among the most sensitive markers of hepatocellular injury. During changes in high concentrations in the intracellular space, they enter the blood serum and cause a characteristic increase. The persistent elevation of these serum enzymes activity or fluctuations of these levels develop chronic liver diseases.

The changes in the activity of SGOT are usually moderate in chronic liver disease, as hepatocellular injury (cytolysis, cellular necrosis) is less extensive. Permanently elevated SGOT levels in these conditions are indicators of active diseases. If the follow up tests do not demonstrate any significant change in SGPT levels, yet SGOT activity is progressively increasing, then the possibility of malignant transformation has to be considered in patients with known cirrhosis or chronic liver injury (Panteghini et al; 1984; Schmidt et al; 1990; Nemesanszky 1991). SGPT and SGOT have been extensively studied during acute or chronic intoxication of the livers, (Rees et al; 1960, Griffiths et al; 1961, Rana et al; 1988).

The result obtained in this extensive study indicates a significant increase of serum enzyme (transferases) SGPT and SGOT both were in BHC treated (Group II) as compared to control (Group I). On the other hand in animals which were supplemented with *Andrographis paniculata* in Group III, the activity of SGOT and SGPT both had decreased drastically. Simultaneous treatment of AP and BHC together caused significant recovery of the damage induced by exclusive BHC treatment. This effect is evident specially with AP because it caused drastic fall in transaminase activity in serum. These results are conform with the earlier work by Kaul et al; (1994). They have reported
that SGPT activity increased with CCl₄ damage liver and further the activity of SGPT increased with diterpenses of *Andrographis paniculata*.

The earlier researchers have used carbon tetrachloride (CCl₄) as a model for the studies on liver damage. They have shown that the rise in AST and ALT levels was reduced by Jigrin (herbal drug) indicating its hepatoprotective activity (Karunakar et al; 1997). Further evidence by Deshpande et al; (1998), they have shown that SGPT and SGOT level was decreased with protective treatment of turmeric. The marker enzyme SGPT and SGOT were increased with acute dose of CCl₄ and the level of these marker enzymes were also decreased with drug Acacia catechu by Jayasekhar et al; (1997).

SGPT and SGOT are reliable markers of liver function. Antioxidants have succeeded in restricting the efflux of transaminase from liver to blood. These observations conform improved liver function in protected mice.

**ALKALINE PHOSPHATASE (ALP) :-**

Alkaline Phosphatase (ALP) is one of the enzymes that have been studied since the beginning of clinical enzymology. At the outset, it was investigated mostly as a marker of liver and bone diseases. It is highly probable that liver injury was responsible for elevated alkaline phosphatase level.

Alkaline phosphatase is a hydrolytic enzyme and acts on phosphoric esters with liberation of inorganic phosphate from various substrates. It is endogeneous to the canalicular membranes of normal hepatocytes. It is considered to be a good marker for hepatic neoplasma.

In the present study alkaline phosphatase activity in liver tissue was found to be increased significantly in BHC treated Group II from 1 month to 8 months. The activity of ALP was very high after 4 months to 8 months BHC.
exposure, in 6 months to 8 months BHC exposure the level of alkaline phosphatase (ALP) was still higher. These increase was many fold as compared to control (Group I). Activity of alkaline phosphatase is mainly localised in hepatocytes lining canaliculi and sinusoidal membranes. When hepatocytes are damaged relatively less alkaline phosphatase gets released into the blood most probably coming from cells which are killed. At the time of bile duct obstruction, new alkaline phosphatase is synthesized in hepatocytes, much of which escapes causing increase in enzyme activity in liver. It is also reported that the ALP activity increased many fold in the neoplastic nodule of the liver after continuous feeding of technical grade hexachlorocyclohexane to in-bread swiss mice (Corcos et al; 1967). Corcos et al; have also summarised numerous reports dealing with histochemical changes occurring in the liver of different laboratory animals treated with sub-lethal dose of various already well known toxicants. They have also specified an increased in alkaline phosphatase activity. This ALP activity further reported an increase due to aflotoxin intoxicated rats, (Mietkiewski et al; 1970). The activity of this enzyme increased in many of the benign and malignant nodules (Essigmann and Newberne, 1981). Pugh et al; (1978) have described that this enzyme is a strong positive marker for induced mouse hepatic foci and nodular lesions. Recently Meada et al; (1993) have done an advance study on alkaline phosphatase activity and they have described hepatocellular carcinoma producing universal type of alkaline phosphatase. They have also studied the immunohistochemical observations of the carcinoma cells excluding the intestinal or placental type of ALP. The tissue extracts from the carcinoma area had much higher ALP activities than those of a noncancerous area. (Meada et al; 1993).

Decreased activity of Alkaline phosphatase in present study Group III evokes changes in the liver. The highly decreased ALP activity indicates relative modification of cellular functioning. A decline in enzyme level in hepatic cells suggests protective effect on the liver. This finding is in
conformation with earlier reports by Zimmerman et al; (1970). The level of ALP decreased in AP supplemented Group III, this result may suggest that *Andrographis paniculata* restores plasma membrane permeability including the repair of injured hepatic cells. Kaul et al; (1994) also reported ALP activity was decreased with administration of diterpenses of *Andrographis paniculata* on CCl₄ damaged liver. Similar to reports has been found in the case of silymarin (Ramellini et al; 1976). These observations suggest that AP is a more effective drug on acute hepatic condition leading even to tumorous condition.

**GLUCOSE-6-PHOSPHATASE (G6PASE) :-**

The glucose-6-phosphatase (G6Pase) is an important compound being at the junction of several metabolic pathways (glycolysis, gluconeogenesis, the HMS, glycogenesis and glycogenolysis). The G6Pase is one of the key gluconeogenic enzyme that exert a rate-limiting role in the production of glucose from lactate and other glucogenic precursors. This enzyme activity is located exclusively in organ capable of glucogenic precursors. The role of G6Pase enzyme in synthesis as well as hydrolysis of Glucose-6-phosphate in the liver is well documented (Nordlie, 1979). Hepatic microsomal enzyme glucose-6-phosphate, is an important enzyme in the regulation of carbohydrate metabolism.

The G6Pase is also considered to be one of the marker for hepatocarcinogenesis (Farber, 1973). Many data are available on G6Pase activity in fully developed tumours (Friedrich-Freksa et al; 1969; Hezfeld et al; 1972). G6Pase depletion has been reported after administration of a number of hepatotoxins and carcinogens (Ashmore et al; 1959, Grasso et al; 1974), like diethylnitrosamine (Schaver 1966 and Eltze et al; 1977).

The hepatic microsomal enzyme G6Pase is important in the regulation of carbohydrate metabolism, which is decreased in BHC treated mice (Group
II). This enzyme has shown a many fold decline from 5 to 8 months exposure of BHC. A decrease in the activity of Glucose-6-phosphatase can be expected to have severe consequences on the organised metabolism of normal liver cells which has a key role to play in maintaining the blood sugar by gluconeogenesis. This finding conforms with the earlier work by Karnik et al; (1981). They have studied on gluconeogenic enzyme and glycogenolysis showed a negative correlation with BHC induced hepatocarcinogenesis and also described an indirect correlation between glycogen accumulation and decrease in the activity of G6Pase, and hypertrophy of smooth endoplasmic reticulum (SER) in hepatocytes. Similarly, in the experimental studies on insecticides, the activity of G6Pase decreased in BHC treated mice (Nigam et al; 1981).

In the present work not only was the effect of BHC exposure was seen at different time intervals, but the recovery of this enzyme after supplementation of *Andrographis paniculata* was also observed. The activity of G6Pase significantly increased in Group III. The results of the present study shows that the oral feeding of AP extract to BHC treated mice stimulates to regenerate the microsomal enzyme, which decreased due to the BHC toxicity. Vijaylakshmi et al; (1998) have shown the changes in glucose metabolizing enzyme. They have reported increased G6Pase activity due to Siddhadrug.

Kataria et al; (1997) have reported that this enzyme G6Pase activity had decreased activity in CCl₄ treated rats but the drug Liv-52 significantly stimulated G6Pase activity in response to CCl₄ toxicity.

On the basis of changes in the activity of hepatic enzymes G6Pase, it seems that AP (*Andrographis paniculata*) provides certain amount of protections and has the capacity to correct liver disfunctions. However, the mechanism of action of this ayurvedic drug AP in restoring the liver functions appears to be different.
**PHOSPHORYLASE :-**

Phosphorylase is a very important enzyme for the liver in glycogenolysis. The significant reduction in glycogen phosphorylase activity in BHC treated animals in 2 to 8 months in Group II indicates decrease in glycogenolysis and may cause deposition of glycogen in liver. The result of previous workers in the literature indicates an unusual accumulation of glycogen after induction of liver cancer by various carcinogens (Epstein et al; 1967, Scherer et al; 1972, Bannasch et al; 1974, Williams et al; 1976, Gasso and Gray 1977, Kuhlmann, 1978). Karnik et al; (1981) have also reported similar changes after BHC treatment of mice. Reduction in G6Pase activity in BHC treated animals in Group II also reveals reduced dephosphorylation and release of glucose from cells. Karnik et al; (1981) also reported decreased in G6Pase activity during hepatocarcinogenesis induced by BHC. It is known that the decrease in activity of the G6Pase has been observed by Weber (1961) and phosphorylase by Hadjiolov and Dancheva (1958) and since long it is observed that hepatoma is also manifested during the precancerous phase.

The supplementation of AP in Group III animals increased the level of phosphorylase and G6Pase corresponding to Group II. Such increase in activity of phosphorylase and G6Pase suggests the improved glycogenolysis in liver.

**ADENOSINE TRIPHOSPHATASE (ATPase) :-**

ATPase is an important group of mitochondrial enzyme, which controls the energy metabolism of the body and its cation transport. ATP has been called the energy currency of the tissue, for it can be spent and remade again and again. Thus ATP is used in the cells to promote various cellular functions such as membrane transport, synthesis of proteins, phospholipids, cholesterol and a great host of other substances for maintaining normal structure and functions of the tissue. The energy rich ATP is broken down to ADP and...
inorganic phosphate (ip) which is catalysed by ATPase to release its energy rapidly and almost explosively whenever needed in the tissue.

The decline in the ATPase activity observed in present study in Group II, retards the energy metabolism of the liver. The ATPase showed a significant decline in the activity after BHC administration in experimental Group II. A highly significant decline was observed from 2nd month to 8th month and a gradual decline was observed in Group II as compared to the control (non treated) Group I. Similarly Bhatt et al; (1981) have done work on histochemical changes in ATPase distribution during BHC induced Hepatocarcinogenesis in inbred swiss mice. They have proved that the neoplastic and tumours have shown less ATPase activity as compared to normal animals. An earlier reported by Kalengayi and Desmet (1975) have observed decreased activity of ATPase in the aflatoxin B1 induced liver tumour in rats. Pugh et al; (1978) have shown decrease in ATPase activity during 2-acetylamino fluorene induced hepatocarcinogenesis. Total ATPase activity showed decline suggesting a reduced utilisation of ATP produced in the cell. Toskulko and Glinsukon (1988) have reported increased accumulation of Ca\(^{2+}\) inside mitochondria causing mitochondrial dysfunction and reduction in hepatic ATP content.

Another reason for the decline in ATPase activity in present study group II is due to the decline in important antioxidant like GSH, which was observed in the same group. It has been suggested that GSH act as a protective agent for SH groups of ATPase, and a loss in GSH leads to the disulphide formation, thus contributing to the inactivation of the enzyme through conformational changes. This could lead to less availability of ATP for energy requiring biosynthetic processes, which are of paramount importance for continuous supply of nutrients. As there is more demand for reduced glutathione, most of energy in the form of glucose enters in the HMP shunt to generate NADPH so that reduced atmosphere can be maintained. Therefore productivity of ATPase is reduced.
In this study, further ATPase activity increased significantly in Group III (BHC with AP supplemented) as compared to the Group II (BHC treated). This may be due to the prevention of oxidative damage in the tissue. Other reasons may be increase of number of active ATPase sites, which would contribute towards maintaining total ATPase level in tissue.

The enzymatic hydrolysis of ATP by ATPase is an ubiquitous property of cells, which is important for intracellular transfer of energy. Michell (1966) proposed a chemiosmotic system operating in mitochondria and postulated that ATPase, as a part of such system, plays an important role in ATP synthesis during oxidative phosphorylation.

**ACID PHOSPHATASE (ACPase) :-**

Hydrolytic enzyme such as acid phosphatase is the prime candidate for the tissue reorganization and tissue repair. Intracellularly acid phosphatase is restricted to the membrane bound vesicle lysosomes. ACPase is one such enzyme group, which is concerned with uptake of glucose and its metabolism in cytoplasm.

In the present study activity of ACPase was highly increased in Group II (BHC exposure). Rise in acid phosphatase (ACPase) activity can be due to effect of BHC toxicaion on hepatocytes causing permeability alterations, leakages of lysosomal enzyme, as well as, lysis of the cell causing enhanced release of this enzyme. Increased syntheses of lysosomal enzyme occur as a response to increased cell degeneration and other pathological liver injuries. Tung et al; (1972) and Singh et al; (1987) and Kohle (1994) have reported that ingestion of aflatoxin-contaminated feed caused increased activity of acid phosphatase.
However the activities of ACPase showed significant decline in the present study in Group III (BHC with AP supplementation) as compared to BHC treated alone Group II. This may be because the prevention of hepatic damage condition of ACPase in the body is related to tissue repair and hydrolysis. The prevention of oxidative damage to liver tissue explains the normalization or decreased ACPase value observed in AP supplemented Group III.

**SUCCINATE DEHYDROGENASE (SDH):**

Succinate dehydrogenase is a key enzyme of mitochondrial krebs cycle. Succinate dehydrogenase is NAD$^+$ linked dehydrogenases and is normally very active in the liver, and is mainly concerned with aerobic oxidation of acetyl co-A and generation of ATP. According to Putilina and Eschanko (1969) among the krebs cycle dehydrogenase; SDH is more active than any other enzyme.

In the present study the activity of SDH showed a significant decline during BHC treatment. It can be due to reduction in oxygen transport to the tissues. While the BHC exposure from 1 to 8 months in Group II causes gradual decrease in SDH activity, on the later stage of 6 to 8 months BHC treatment, the maximum decline was recorded. This decline may be associated with tumour development. In this study, the hepatocytes of the BHC exposed mice were studied ultrastructurally, and show swollen mitochondria and lack of cristae. Our data corroborates with the finding on histoenzymology study on SDH activity in BHC induced rat liver by Nigam et al; (1981). Inhibition of liver mitochondria and electron transport flow by aflatoxin has been reported in the rat (Doherty and Campbell 1972, 1973) Roy (1968) and has also reported decreased activity of SDH in mitochondrial swelling during aflatoxicosis.

The SDH activity was found increased in Group III in the present study. The supplemented group III (BHC + AP) showed an appreciable recovery in
SDH activity. The SDH activity was increased in group III as compared to BHC treated group II. This may be due to extensive changes in liver beyond the limits of recovery.

**ORNITHINE CARBAMOYL TRANSFERASE** :

Ornithine carbamoyl transferase enzyme is also called as ornithine transcarbamoylase. It catalyzes the second reaction in the pathway of urea synthesis, namely the synthesis of citrulline from ornithine and carbamoyl phosphate. OCT is present in liver mitochondria of ureotelic animals. OCT is also found associated with carbamoyl phosphate synthetase I in the mitochondrial matrix. OCT catalyzes the nucleophilic addition of ornithine to the carbamoyl group of carbamoyl-p to produce citrulline. During this reaction, the $\delta$-NH$_2$ group of ornithine attaches itself to the carbamoyl group of carbamoyl-p and the phosphate group (pi) is released. (Lehninger et al; 1993).

This enzyme catalyses the following reactions :-

$$\text{Carbamoyl - P} \quad \text{pi}$$

Ornithine $\longrightarrow$ citrulline

Ornithine carbamoyl transferase (OCT)

Ornithine, which is regenerated in cytosol in the 5$^{th}$ reaction of urea cycle, is transported in to the mitochondria matrix by a specific “transport protein” in the inner mitochondrial membrane. Similarly citrulline in mitochondrial matrix is also transported across the inner mitochondrial membrane to the cytosol by a specific transport protein. (Chatterjea and Shinde 1995)
Biosynthesis of Urea or ornithine urea cycle

The reactions of urea cycle in five sequential steps:
1. Synthesis of carbamoyl – phosphate
2. Synthesis of Citrulline
3. Synthesis of argininosuccinate
4. Cleavage of arginino – succinate
5. Cleavage of arginine to form ornithine and urea
OCT is involved in urea synthesis, is exclusively found in liver and is virtually not active in any other tissue. So, it is considered as an important hepato-specific enzyme. In liver diseases, the enzyme level is remarkably elevated to 10 to 200 folds in patients with acute viral hepatitis, depending on the severity and also in those with other forms of hepatic necrosis, Relatively slight increase occurs in obstructive jaundice, cirrhosis of liver, metastatic carcinoma etc. (Chatterjea and Shinde, 1995).

Ornithine carbamoyl transferase was reported to be increased in workers with more than 5 years of occupational exposure to organochlorines. (Michail et al; 1972). Thakore (1984) have also studied in human workers exposed to BHC of both handler groups and non handler groups, and the increase was of statistically significant level in OCT.

The results obtained in this study indicates a highly increased activity of OCT in the liver in BHC treated mice (Group II). This is due to Hepatocellular damage or increased permeability of liver cells. The OCT activity gradually increases after BHC treatment from 1 to 8 months as compared to the control group.

On the contrary in Group III which was BHC exposed and supplemented with AP, there was a significant decrease observed in the OCT activity as compared to the BHC treatment alone (Group II). The OCT activity which is decreases significantly in liver functioning, shows a remarkable recovery in AP supplemented mice.

**LIPID PEROXIDATION (LPO) :-**

Lipid peroxidation is a complex process whereby polyunsaturated fatty acids or unsaturated lipids undergo reaction with molecular oxygen to yield
lipid peroxides. The initiation and propagation reaction, are as follows:

\[ \text{LH} \rightarrowdot \text{L} \]

Oxidizing species (Fatty acyl radical)

\[ \text{LOO} \]

(Lipid hydroperoxide)

\[ \text{LH+LOO} \]

(Lipid peroxy radical)

The semistable Lipid peroxides ultimately resolve into malondialdehyde (MDA) as the major product, dien conjugates and gaseous hydrocarbons (Tappel, 1975).

The peroxidation of lipid is a natural phenomena and occurs on its exposure to oxygen (Bowers and Jacob, 1978). Free radicals forms as the byproduct of many biochemical reactions, (Electron transport chain, catabolic steps in mitochondria, microsomal reaction etc.), initiate this phenomena in a chain reaction. Recently, Free radical induced lipid peroxidation has gained much importance because of its involvement in several pathological changes such as aging, wound healing, oxygen toxicity, inflammation and liver disorders (Siraj and Mufti, 1998). The protection of the cell membrane from the lipid peroxidation could prevent, cure or delay the aforesaid pathologies.

Lipid peroxidation (LPO) could be prevented

a. by reducing the formation of free radicals
b. by destroying the free radicals already formed
c. by Supplying a competitive substrate for unsaturated lipids in the membrane and
d. by accelerating the repair mechanism of damage cell membrane

Many natural and synthetic antioxidant are in use to prevent the lipid peroxidation (Homsby and Crivello, 1983).

In this study the drug *Andrographis paniculata* has been used for its protective response. BHC treated animals (group II) liver showed a highly significant increase of lipid peroxidation as compared to the control animals (group I). The LPO levels drastically increased during 4 to 8 months of BHC exposure in Group II, this LPO level gradually increase month by month. LPO product (TBARS) could mainly arise from damaged kuffer cells. The increased TBARS of liver indicated enhanced lipid peroxidation due to tissue injury and failure of the antioxidant defence mechanism, which prevents that formation of excess free radicals (Boyld 1981). GSH plays a protective role in tissue by detoxification of xenobiotics. The significant decrease in liver GSH in Group II in the present study may be a consequence of enhanced substrate utilization by glutathione peroxidase. In fact there is direct correlation between GSH depletion and enhanced lipid peroxidase (Comporti et al; 1991). Significant decrease activity of lipid peroxidation was observed in the present study in AP supplemented group III as compared to the BHC exposure alone group II. The present result clearly indicated protective response afforded by *Andrographis paniculata* against hepatic damage by BHC. Present reports also support the significant enhancement in the GSH level in AP supplemented group III.

The other work on Jigrin as a hepatoprotective drugs also inhibited or reversed the increased level of LPO in alcohol – Carbon tetrachloride treated rats (Karunakar et al; 1997).
Earlier reports claim diterpenses from *Andrographis paniculata* to have shown significant depletion of lipid peroxidation and enhanced GSH level against CCl₄ damaged liver (Kaul et al; 1994). This finding further supports our observation and the result of the current study is comparable to the observation of Pandey et al; (1994), Suja et al; (1997), who worked with a different herbal extracts.

The findings of this study indicate remarkable decrease in LPO in AP supplemented group III. This may be due to accelerating the repair mechanism of damaged cell membrane. This certainly indicates that AP contain certain substances which are capable of preventing lipid peroxidation a natural deleterious process.

**GLUTATHIONE (GSH) :-**

Glutathione (GSH), a tripeptide enzyme (L-glutamyl – L-cysteinyl – glycine), being an intracellular reductant, takes part in a number of biological functions, viz. catalysis metabolism and cellular transport etc. The compound has a protective action against free radicals reactive oxygen species (ROS) like peroxidase and other toxic compounds.

Glutathione present in the erythocyte and several tissue. GSH helps in keeping the enzyme in an active state by preventing the oxidation of their – SH (Sulphydryl) groups to –S-S (disulphide) groups. GSH also protects the liver by detoxicating the foreign substances by forming mercapturic acids, which are excreted from the body.

Results from this investigation show that the diminution of glutathione is more in the liver of BHC treated animals. The GSH activity drastically decreased at the end of treatment, and this trend was gradually decreased month by month. There was a highly significant decreased activity of GSH in Group –
II in liver tissue as compared to Group - I. This indicates that high molecular weight aggregation with sulphydryl oxidation, which result in the impairment of membrane functions, could be a typical factor in tissue.

The diminished GSH levels in Group II could be attributed to other enzymes involved in glutathione metabolism.

- The accumulation of oxidized glutathione (GSSG), whose reduction to glutathione (GSH) by glutathione reductase (GR) is hampered. The lowered activity of GR in Group II in this study supports the above mechanism.
- The elevated activity of the mercapturic acid pathway enzymes [Glutathione S-transferase (GST) and Gamma Glutamyl transpeptidase (γ-GTP)], which degrade the conjugated GSH, may also be one of the factors responsible for lowered activity of GSH levels in liver of the BHC treated.

Further this study reports significant increased GSH level in the liver of Group III (BHC with AP supplemented) as compared to the Group II (BHC treated). This is indicative of the fact that in AP supplemented group III in liver tissue, the Sulphhydryl groups are maintained in the reduced state to a greater extent as compared to Group II. Thus supplementation of *Andrographis paniculata* (AP) during tumorous (severe liver damage) condition has elevated the GSH levels which in turn helps in maintaining the liver tissue damage.

The result is supported by Kaul et al; (1994). They have shown that effect of diterpenes from *Andrographis paniculata* (AP) was investigated on the Glutathione and on other antioxidant enzyme in CCl₄ treated mice. The deplecation of GSH by CCl₄ and the level of GSH was also in turn increased by treatment of diterpenes of AP (Kaul et al; 1994).
The protective effect of glutathione was assessed against cytotoxic antitumour agent like cyclophosphamide, methotrexate, S-Fluorouracil (CMF) and X-ray irradiated rats (Murali Krishnan et al; 1996).

GSH has been found in abundance in human tumours, particularly in drugs as well as radiation resistant tumours. The role of glutathione in various biological functions has been reviewed by Wang et al; (1992) and Meister A. (1994). The idea is, that the synthesis of GSH plays an important role in drug and radiation resistance (Moore et al; 1989).

A mention may be made here that the normal mice, which were given only AP extracts (Group IV) have an increase amount of GSH level even than the control ones. This is a suggestive condition that AP has an antioxidative property.

**GLUTATHIONE REDUCTASE (GR) :-**

Glutathione reductase (GR) is well known for its ability to keep the cellular concentration of reduced GSH high by catalyzing the conversion of oxidized glutathione (GSSG) to the reduced form (GSH). GR possesses flavin adenine dinucleotide (FAD), a prosthetic group, which transforms GSSG to GSH

\[
\text{GR} \quad \text{GSSG + NADPH} \longrightarrow 2 \text{GSH + NADP}
\]

Glutathione reduction (GR) reaction.

The result obtained by this study indicate a highly significant decreased activity of GR in liver in BHC treated group II as compared to the control group I. The GR activity has shown gradual decrease from, 1 month to 8 months BHC exposure as compared to the control animals liver. On the other
hand in Group III (BHC with AP supplemented) the activity of GR significantly increased as compared to the Group II (BHC treated, with a subsequent increase in the GSH levels.

Thus the increased activity of GR in Group III implies that there is an attempt to protect the liver tissue from oxidative damage by regenerating GSH from its oxidized form (GSSG). Increased GR activity in Group III was substantiated with a parallel increase in GSH levels.

Similarly, GR and GSH activity was observed increase in the liver with diterpenses of *Andrographis paniculata* (AP) against CCl₄ treated mice (Kaul et al; 1994).

**GLUTATHIONE S-TRANSFERASE (GST):**

Glutathione S-Transferase plays a key role in the metabolism of chemical carcinogens, mutagens and various anti-cancer drugs. This enzyme functionally might be involved in both the development of hepatocellular carcinoma and in determining the anti-cancer drug sensitivity of such tumours (Murray et al; 1993).

Glutathione S-transferase is a prevalent enzyme of the cytosol and is considered to play a key role in the detoxication process. Detoxification of electrophiles by their conjugation with the sulphur of glutathione, is catalyzed by glutathione S-transferase. It is the initial reaction of the mercapturic acid pathway.

\[
\text{GST} \\
\text{Glutathione - SH + electrophile X} \rightarrow \text{Glutathione - S - electrophile + HX}
\]

Reaction of glutathione S-transferase (GST)
Glutathione S-transferase activity is low in healthy individuals. Among the two isoenzymes of GST, the cationic fraction has been shown to be a highly sensitive marker of hepatocellular integrity. Though the tendency of alteration usually parallels that of the aminotransferases (AST, ALT), the changes in the activity in some times were more pronounced e.g. in acute viral hepatitis, fulminant hepatitis, acute liver injury caused by drugs (Donald et al; 1996). Elevated total GST activity is prevalent in chronic alcoholics. High value has also been observed in primary or secondary malignancies of the liver (Elmer Nemesanszky, 1996).

Li-C, 1993 has suggested that GST can be used as an enzyme marker for hepatic cancer and preneoplastic lesions, and described possible role of hepatic carcinogenesis induced by chemical carcinogens.

The result from this study shows increased activity of GST observed in BHC treated mice model. The activity of GST was gradually increased in BHC exposed animals, as compared to the control. During 4 to 8 months (fully tumour development time) in BHC exposed animals the activity of GST has shown drastic increase. This is indicative of increased stress due to toxin in the liver tissue. Thus, activation of GST indicates removal of increased toxins like hydrogen peroxidase, especially intracellular produced membrane lipid hydroperoxide which can impair membrane function and damaging electrophiles, from the extra cellular source.

In animals which were BHC treated but supplemented with *Andrographis paniculata*, (Group III) the activity of GST value were shown less as compared to only BHC treated (Group II). Hence the amount of noxious substances present within the liver tissues of AP supplemented group II are less than that in BHC treated group II. The lowered activity of GST in Group III as compared to Group II supports above mentioned fact.
\textbf{\textit{γ-Glutamyl Transpeptidase (γ-GTP):}}

The membrane bound glycoprotein enzyme γ-Glutamyl transpeptidase (an enzyme of γ-glutamyl cycle) is of major importance in glutathione metabolism. It initiates GSH degradation. It can catalyze 3 types of reactions: (a) transpeptidation in which the γ-glutamyl moiety is transferred to an acceptor, (b) auto-transpeptidation, in which the γ-glutamyl moiety is transferred to GSH to form γ-glutamyl GSH and (c) hydrolysis, in which the γ-Glutamyl moiety is transferred to water. Glutathione, oxidised glutathione (GSSG), S-substituted glutathione and other γ-glutamyl compounds are substrates for this enzyme (Meister and Anderson, 1983).

γ-Glutamyltranspeptidase (γ-GTP) is found in the microsomal and cellular membrane function of cells with intense secretory and excretory functions. Its activity is high in the cells of bile canaliculi liver, kidney and pancreas. Through a high sensitive marker, γ-GTP is an enzyme with only moderate liver specifically. Its activity can equal or exceed the upper reference limit in liver disorders of any aetiology. The determination of γ-GTP levels is therefore a valuable ‘screening test’ with a high negative predictive value for liver disease (Elemer Nemesanszky. 1996).

γ-Glutamyl transpeptidase formerly known as γ-glutamyl transferase, is the most sensitive indicator of liver disease and is a useful marker in patients with liver metastases (Rosalki, 1975). A number of drugs and chemicals are known to increase γ-GTP activity by the induction of microsomal enzymes, such as barbiturates, antidepressants, anticonvulsant and contraceptives (Kim et al; 1977).
The present study showed the significant increase activity of \( \gamma \)-GTP after BHC exposure. During 4 month to 8 months BHC exposure the \( \gamma \)-GTP activity indicated many fold increase. The tumorous condition was observed after BHC exposure for 6 to 8 months. However by that time, significant elevation with many fold increased \( \gamma \)-GTP activities was observed as compared to control mice.

Such multifold increase in chemically induced rat hepatoma has been reported earlier (Fiala et al; 1972). After chronic treatment and until the development of hepatocellular carcinoma (HCC), a 20 to 60 fold increase in liver \( \gamma \)-GTP was reported which is comparable to the \( \gamma \)-GTP was reported which is comparable to the activity measured in fetal rat liver (Cameron et al; 1978). Earlier work in immunohistochemical and enzyme histochemical studies on \( \gamma \)-GTP in rat liver has used during 3-me-DAB hepatocarcinogenesis (Suzuki et al; 1987).

Thus the result of this study, showing elevated levels of \( \gamma \)-GTP in liver tumours induced by BHC, conform in principle elevation in several rat hepatomas induced by 3-me-DAB, N-OH-2AAF and 2-diacetyl amino flurence (Taniguchi et al; 1975, Fiala et al; 1976, and Harada et al; 1976).

Result from this investigation shows the significant increase in activity of \( \gamma \)-GTP in Group – II as compared to Group I, but the \( \gamma \)-GTP activity had significant decrease, as shown in supplemented group III. Oral administration of AP with BHC treated mice showed reduction in \( \gamma \)-GTP activity; thus it could be revealing the membrane stabilising activity of AP. This implies that the supplementation of AP is indicative of the improvement of liver. The hepatoprotective property of silymarin has been related to the inhibition of \( \gamma \)-GTP, and the decrease of \( \gamma \)-GTP activity was reported (Muriel et al; 1990, 1992).
ENZYMATIC & NONENZYMATIC REACTIONS

\[ \text{SOD} \quad \text{HABER-WEISS} \quad \text{PEROXIDASE} \]

\[ \text{CATALASE} \]

\[ \text{H}_2\text{O}_2 + \text{H}_2\text{O}_2 + \times - \text{AH}_2 + \text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \]

\[ + \text{O}_2 \]
GLUTATHIONE PEROXIDASE (GSH-Px):

Glutathione peroxidase enzyme is found in the cytoplasm and in the mitochondria. This enzyme which is unusual in that it contains the trace element of selenium.

Biochemically, \( \text{H}_2\text{O}_2 \) is detoxified by two major enzymatic mechanisms: one which uses catalase and the second using a dienzymatic redox cycling of the sulphhydryl compound glutathione (GSH) by glutathione peroxidase (GSH – Px), which catalyzes the reaction:

\[
\text{GSH-Px} \quad \text{H}_2\text{O}_2 + \text{GSH} \quad \underset{\text{GSH-Px}}{\longrightarrow} \quad \text{2 H}_2\text{O} + \text{GSSG}
\]

And Glutathione reductase (GR), which catalyzes:

\[
\text{GR} \quad \text{NADPH} + \text{H}^+ + \text{GSSG} \quad \underset{\text{GR}}{\longrightarrow} \text{NADP}^+ + 2\text{GSH}
\]

Thus, the co-operative activity of GSH-Px seen with GSH is consistent with a detoxifying role of this enzyme. It allows the levels of \( \text{H}_2\text{O}_2 \) to be driven very low, yet it prevents a sudden influx of \( \text{H}_2\text{O}_2 \) from drawing down GSH levels to where it becomes unavailable for other reactions.

In this investigation the GSH-Px activity in Group II was gradually decreased during 4 to 8 months BHC treatment. (Group II) as compared to the control (Group I).

The levels of Glutathione peroxidase decreased in 3-methyl – 4-dimethylaminoazobenzen induced hepatocellular carcinomas than those in the normal control rat liver (Lertprasertsuke et al; 1990).
The result from this investigation further shows that the activity of GSH-Px significant increased in AP supplemented Group – III. This indicates that the protective mechanism in the liver tissue was hampered more in Group III as compared to Group II. Thus the enzymes of the antioxidant scavenger system are themselves affected due to the increased level of the H$_2$O$_2$ during hepatic damage (tumorous) condition.

Since the GSH levels are maintained at higher levels in Group III as compared to Group II, the GSH-Px activity is more pronounced in the BHC with *Andrographis paniculata* supplemented group as compared to only BHC treated group.

This result supports the finding of Kaul et al;(1994). They determined the Glutathione and other related antioxidant enzyme in CCl$_4$ treated mice, the deplecation of GSH-Px activity in CCl$_4$ treated mice liver and further the increase of GSH-Px activity also by treatment of diterpenes extracted from *Andrographis paniculata* (Kaul et al; 1994).

The previous work showed that on the two flavonoids compounds, increased the activity of GSH-Px, GR and GSH in the liver tissue and other tissue (Deniel et al; 1998).

**SUPEROXIDE DISMUTASE (SOD)** :-

Superoxide Dismutase (SOD) has been recognised to play an important role in body defense mechanisms against the deleterious effects of oxygen free radicals in biological system. (Fridovich, 1975 and Kellogg et al; 1977).

Superoxide is rapidly removed by the metalloenzyme superoxide dismutase (SOD), which is present in the cytosol and in the mitochondria of all cells that use oxygen. SOD is capable of catalysing dismutation of O$_2$ into H$_2$O$_2$. H$_2$O$_2$ itself is toxic to the cells. The H$_2$O$_2$ formed is eliminated by
catalase or glutathione peroxidase. Thus the role of SOD is to protect against the deleterious effects of $O_2$.

$$\text{SOD}$$

$$2H^+ + O_2^- + O_2^- \rightarrow H_2O_2 + O_2$$

Reaction of superoxide dismutase.

The results of the present study show that the activity of SOD in Group II (BHC treated) significantly decreased as compared to the control group I. Activity of SOD showed a gradual decrease during BHC exposure i.e. from 1 month to 8 months.

These results support that of earlier work on the administration of single ip dose of organochlorine, and in the observation of decreased SOD activity in liver (Janquira et al; 1993). The activity of SOD in the liver and kidney of mice treated with aflatoxin for was significantly lower than those in the control animals (Verma and Nair 1999).

The results from this investigation further indicate that the AP supplementation (Group III) has higher activity of SOD compared to the BHC treated non supplemented (Group II).

Most abundant oxidative free radicals generated in living cells are superoxide anions ($O_2^-$) and derivatives, particularly the highly reactive and damaging hydroxyl radical, which appears to act via peroxidation of membrane lipids. The superoxide is inactivated by SOD, the only enzyme known to use a free radical as a substrate. However, the free radical a scavenging activity of SOD, is effective only when it is followed by increase in CAT(catalase) or GSH-PX(Glutathione peroxidase) activity. Since SOD generates hydrogen peroxide as a metabolite, which is more toxic than oxygen radicals and has to
be removed by CAT or GSH-Px activity it is essential if beneficial effects from increase in SOD activity is to be expected.

Likewise, studies done on antioxidant enzyme system in liver, such as principle protective enzyme SOD also increased in liver due to treatment of diterpenses of *Andrographis paniculata* (AP) Kaul et al; 1994.

Similarly quercetin and several other flavonoids were shown to have potent super oxide anion scavenging activity (Chen et al; 1990).

Hence the water extract of AP has a considerable contribution in regulating the SOD activity.

**CATALASE :-**

Catalase is widely distributed in nature. It is found in all aerobic microorganisms in the animal cells. A catalase activity of mammalian tissues varies greatly. It is highest in the liver and the kidney. The detoxication of hydrogen peroxide (H₂O₂) is carried out by the enzyme catalase. H₂O₂ is removed very efficiently since catalase is present in the same organelles, as the oxidase, which produce it and the turnover number of the enzyme is very high. Catalase split hydrogen peroxide into water and oxygen: the oxygen is liberated or used to oxidize a variety of compounds. In the later case, catalase acts as a peroxidase and this reaction can serve a double purpose of detoxicating the other compounds as well as hydrogen peroxide (Plummer David, 1989).

\[
\begin{align*}
\text{Catalase} & : 2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O}_2 + \text{O}_2 \\
\text{Peroxidase} & : \text{H}_2\text{O}_2 + \text{CH}_3\text{CH}_2\text{OH} \rightarrow 2\text{H}_2\text{O} + \text{CH}_3\text{CHO}
\end{align*}
\]
In this study, in BHC treated mice liver (Group II) showed highly significant decrease activity of the enzyme catalase compared to the control mice liver (Group I). The activity of catalase was gradually decreased from 1 month to 8 months BHC treated mice liver.

On the other hand in BHC with AP supplemented Group III the activity of catalase was significantly observed as compared to the BHC treated alone Group II. This shows that AP possibly plays a pivotal role in defence mechanism. The superoxide anion is converted to H$_2$O$_2$ and the higher levels of H$_2$O$_2$ trigger the catalase activity by which H$_2$O$_2$ is eliminated from the liver tissue more effectively in Group III as compared to Group II.

ANTIOXIDANT PROPERTY OF ANDROGRAPHIS PANICULATA

Many plants products are known to exert antioxidative effects by quenching various free radicals and single form of molecular oxygen as mentioned by Aruna and Shivaram krishnan(1990). In the present study while studying for effects of *Andrographis paniculata* (AP) on liver tumour the attempt has also been made to know the antioxidant effect of AP. This plant extract activate antioxidant enzymes that catalyse the reaction of oxidants in diseased liver (BHC treated).

The GSH/GST detoxification system is an important part of cellular defence against a large array of injurious agents. The tripeptide, reduced GSH is essential to maintain structure and function integrity of the cells. The maintenance of GSH levels depends on the activities of various enzymes GR, GST. Since GR effects reduction of GSH, the level of this enzyme is also of
importance in detoxification of peroxides (Dhuley 1999). An effective defence against oxidative damage is the GSH cycle, which included oxidation of GSH to GSSG during detoxification of peroxidation by GSH-Px and GST, and further reduction of GSSG to GSH by GR.

The initiating free radicals such as O₂ and H₂O₂ will scavenged by SOD or CAT and GPx respectively. These enzymes activities will there by inhibit the production of secondary, more damaging free radicals, such as OH⁻³ ones. LPO has started, endogenous antioxidants will interfere with the chain propagation, as they are more easily oxidised than polyunsaturated fatty acids (Fohle 1982, Meister 1982).

Apart from its direct free radical scavenging properties and ability to conjugate with several electrophilic intermediates that are capable of initiating lipid peroxidation (Jacoby, 1978).

Here the lipid peroxidation reflects the interaction between molecular oxygen and polyunsaturated fatty acids and result in the oxidative deterioration of the later. The natural targets for such reaction are the biological membranes, therefore oxidative damage to these membranes result in an impairment of cellular and subcellular functions, change in permeability and morphological alterations. Free radicals disrupt the equilibrium of biological system by damaging their constituent molecules, leading eventually to cell death. The inhibitory effects of Andrographis paniculata on lipid peroxidation can also related to the membrane stabilizing activity as observed in electron microscopic study.

SOD is a principle protective enzyme dismutase O₂ to H₂O₂ and oxygen, H₂O₂ produced can the be decomposed enzymatically by CAT (catalase) or by GSH-PX. GSH-PX not only decomposes H₂O₂ but is also capable of interacting with lipid peroxidation. In the present study observed marked
increased in SOD, catalase and GSH-PX (glutathione peroxidase) activities in AP supplemented animals showed the adaptive nature of the system against damaging effects of superoxide radicals in liver as brought out by severe hepatic damage by BHC. Decreased activities of GSH, GR, CAT, SOD and GSH-PX in BHC treated mice may increase their susceptibility to oxidative injury. However, the over expression of the antioxidant molecules with aqueous extract of *Andrographis paniculata* is indicative of their ability to reactive hepatocellular antioxidant defence in the liver. Kaul et al; (1994) reported that effects of diterpenses from *Andrographis paniculata* on antioxidant defence system and lipid peroxidation. They have shown the adaptive nature of the system against damaging liver as brought about by CCl₄. Compos et al; (1987) and Chander et al; (1992) have reported, that hepatoprotective plant principle possess antioxidative activity also.

The result of the present study suggest that the antioxidant effects elucidated by aqueous extract of *Andrographis paniculata* is possibly due to their ability to activate antioxidant enzymes that catalyse the reduction of oxidants and their use for hepatic damage condition may be beneficial.