II. REVIEW OF LITERATURE

Enormous work has been done on the action of all the carbamate insecticides in relation to the activity and inhibition of the enzyme acetylcholine esterase and its effect on the nervous system. Further, detailed reports are available for carbamate insecticides on the lethal doses for various pests and insects, their effects on human and their companion animals. All these have been well documented and legal regulatory rules formulated by the international committee for regulation of poisonous chemicals, finally accepted by the World Health Organization (WHO), way back in 1970’s (WHO/FAO, 1974). However, no consistent data could be established about the carcinogenicity, teratogenicity and mutagenicity of these insecticides, as there are contradictory reports from various researchers on these aspects. In vitro studies on these carbamates to evaluate the cytotoxicity of animal cells are very meager. It was observed that the carbamates were not used as therapeutic agent for cancer treatment.

2.1. BAYGON (Propoxur)

2.1.1. Effects on enzymes

Propoxur is a biologically active material because of its structural complimentarity to the active site of cholinesterase. As a cholinesterase inhibitor, propoxur behaves as a synthetic neurohormone that produces its toxic
action by interrupting the normal action of acetylcholinesterase so that the substrate acetylcholine accumulates at synaptic junctions. *In vivo* signs of poisoning are manifested by irritability, tremors, incoordination, convulsions, paralysis and death. While several other carbamate insecticides (carbaryl, landrin, and others) produce a transient anaesthetic effect following high dose administration, there is no such effect noted with propoxur (Vandekar *et. al.*, 1971). *In vivo* and *in vitro* studies have shown propoxur to be a potent inhibitor of various types of cholinesterase.

2.1.2. Neurotoxicity

Adult white leghorn hens were orally administered propoxur at levels ranging from 100 to 1000 mg/kg in a single oral dose or as a single i.p. dose at levels ranging from 25 to 100 mg/kg. In two of the three trials, propoxur (100 mg/kg) and atropine sulfate (50 mg/kg) were injected intraperitoneally prior to treatment. No neurotoxic signs of poisoning were noted during periods of up to six weeks of observation after treatment (Kimmerle, 1966).

2.1.3. Observations in man

Because of the widespread experimental views of propoxur and control through the auspices of WHO, some significant observations in humans are available (Dawson *et. al.*, 1964; Vandekar *et. al.*, 1968; Vandekar and Wilford, 1969). Dawson *et. al.*, (1964) showed that oral administration of 110 and 116
mg/person produced no signs of illness. The level of urinary phenols reached 140 ppm in the absence of clinical signs of poisoning. In persons engaged in spraying or other occupation exposure, urinary levels of 80 ppm were uniformly associated with illness. In another study, Vandekar et. al., (1971) administered 135 mg/person to a male volunteer (1.5 mg/kg bw) and within 20 minutes after ingestion described clinical signs of poisoning due to the carbamate. Significant erythrocyte cholinesterase depression was evident coinciding with clinical signs of poisoning, while plasma cholinesterase depression was not observed. Two hours after the ingestion of propoxur, there were no signs of poisoning and the rapid disappearance of symptoms was consistent with the rapid recovery of erythrocyte cholinesterase activity. Absorption and excretion of propoxur was very rapid as evidenced by measurement of urinary phenols which reached a maximum value within four hours of almost 200 ppm. Of the total phenol content excreted, 81% was found within five hours after administration.

2.2. CARBARYL

2.2.1. Effects on enzymes

As a carbamate compound, carbaryl is an inhibitor of cholinesterase (ChE) activity. A number of studies have been performed by Carpenter (1961) to assess the extent of ChE inhibition by carbaryl in mammals. A single oral dose of carbaryl of 560 mg/kg produced a 43% inhibition of the erythrocyte ChE in rats in 0.5 h, and a 30% inhibition in brain AChE. However, they
returned to normal in rats that survived 24 hrs after administration of the dose. Carbaryl did not depress plasma ChE significantly. Two groups of Beagle dogs were injected once, intravenous, with 10 or 15 mg/kg as an 8% solution in 95% alcohol. No significant effects were found on either erythrocyte or plasma ChE. On the 5th day, several administrations of the same doses depressed plasma ChE by 24% and erythrocyte AChE by 40%. It is doubtful that a long incubation time for the samples (2 hrs for plasma) played a role in the slight depression of ChE. Comparative data on ChE inhibition in the brain, plasma, and erythrocytes of rats that received single doses of carbaryl were reported by Mount and Oehme (1981).

2.2.2. Effects on the immune system

Young mice weighing 10-12 g were infected with influenza by applying 3-4 drops (0.05 g) 1% influenza virus in physiological solution in the nose. The murine influenza virus strain A.P.R. 8 was used in the primary dilution 1:32. After 2-3 days, this group of 30 mice was treated orally with carbaryl in sunflower oil, at a dose of 500 mg/kg (a dose that killed 3 out of 10 mice). Two control groups with the same number of mice, one treated with carbaryl alone, and one infected, were compared with the experimental animals for survival, blood biochemistry, and pathomorphological changes. The greatest number of mice dying was in the experimental group. AChE depression was more pronounced and recovery slower and there was more histological changes in the
livers of mice infected and intoxicated by carbaryl (Moreynis and Estrin, 1965).

2.2.3. Effects in blood

Carbaryl affects the coagulation process. Hyper and hypocoagulation were reported in different studies (Hassan and Cueto, 1970; Gapparov, 1974; Lox, 1984; Krug and Berndt, 1985; Krug et al., 1988).

Gapparov (1974) studied the indices of blood coagulation in dogs after oral treatment with a daily dose of 2 mg carbaryl/kg body weight, over 5 months. A clearly manifested hypercoagulation was established, which was connected with a rise in the general coagulation activity of the blood, higher thromboplastic activity, and prothrombin content, increased number of thrombocytes, accelerated coagulation time, and increased activity of the fibrinostabilizing factor. Also noted was a decrease in the recalcification time of blood plasma, fibrinogen concentration, and free heparin quantity, together with an inhibition of the fibrolytic activity of the blood. The author interpreted all these changes as being connected with the arousal of the parasympathetic system. The blood coagulation time was considerably shortened in rabbits that were given, orally, a mixture of carbaryl (5 mg/kg), DDT (5 mg/kg), and parathion (0.5 mg/kg), for 222 days. This effect corresponded to increased levels of 5-hydroxy-3-indolacetic acid (5-HIAA) and 4-hydroxy-3-
methoxymandelic acid (VMA) in the urine, indicating an increased rate of metabolism of serotonin and catecholamine (Hassan and Cueto, 1970). The authors suggested that this effect was a manifestation of non-specific stress, since adrenocortical hormones shorten the coagulation time.

Carbaryl produced, *in vitro*, a dose-dependent increase in methaemoglobin (Met Hb) formation at 10 and 100 mg/litre, as well as decreases in reduced glutathion levels in the erythrocytes of Dorset sheep with low erythrocyte glucoso-6-phosphate dehydrogenase (G-6-PD), which is similar to humans who have G-6-PD deficiency. Carbaryl posed oxidative stress to G-6-PD-deficient red cells, probably due to its major metabolite alpha-naphthol (Calabrese and Geiger, 1986). Decreases in the K⁺ ion concentration in erythrocytes (with more than 24%) and in haematocrits (from 43.5 to 38%) were found by Sokur (1971), in rats fed carbaryl 0.05 LD50/day for 2 months.

In an *in vitro* study, Szczepaniak and Jeleniewicz (1980), found that carbaryl binds free blood amino acids (plasma and erythrocytes). These authors also performed a series of *in vitro* studies to investigate the effect of carbaryl on amino acids. A single application of 475 mg carbaryl/kg on 46 treated and 12 control rats produced significantly decreased amino acid values in the brain, except for valine and phenylalanine. All amino acids reached the control level after 72 and 120 hrs. Two hours after administration, erythrocyte amino acids
also decreased >50%. A slight decrease in erythrocyte amino acid concentrations was observed after 30 days with 95 mg carbaryl/kg administered orally. Blood serum amino acids in 24 rats decreased 4 h after a single application of 189.6 mg/kg. There was a larger decrease in valine, then in phenylalanine, alanine, aspergic acid, serine, and glycine.

The effects of carbaryl on the thermoresistance and fractional content of blood serum proteins was studied by Subbotina and Belonozhko (1968). A single dose of 150 mg carbaryl/kg administered to rabbits and multiple applications of 100 mg carbaryl/kg for 2 months showed that, with a single application of carbaryl, there was an increase in protein thermocoagulation from 28% on day 1 to 67% on day 7, and with multiple applications of carbaryl there was a lowering of albumin levels and a rise in globulins (mostly alpha-globulins) in serum, on day 10. These changes were reversible.

2.2.4. Effects on the liver and other organs

Several authors reported data on disturbances in the carbohydrate, protein-forming, and detoxicating functions of the liver. A single application of 300 mg carbaryl/kg in rats produced an increase in albumin and alpha-globulins, and a decrease in β and gamma-globulins (Zapko, 1970).
Kagan et. al., (1970), studied the effects of carbaryl on the liver of 180 rats and 18 rabbits. During an 11-month study, they gave a daily, oral dose of 38 mg carbaryl/kg to rats. After 1 month, they observed a rise in the alanine-aminotransferase and alkaline-phosphatase activities in serum, and a decrease in succinic dehydrogenase and glycogen in the liver. Doses of 0.76 mg/kg and 0.38 mg/kg, given in the diet to rabbits caused retention of bromosulfophthaleine in the blood. The researchers also reported a changed ratio in the protein fractions in serum and an increase in liver weight. The pathomorphological changes in the liver were destructive, necrobiotic, and proliferative.

An effect on the liver was also demonstrated in the study of Pavlova et. al., (1968) who found that, with acute and long-term exposures, carbaryl affected the oxidative processes in tissues, because of its direct action on the enzymes of cell respiration and possible disturbance in the membrane processes. They performed studies on rats treated with 0.2 LD50 for 3 days and on rats treated with 0.01 LD50 for 20 weeks. At the higher dose, there were decreases in the cytochromoxidase and succinedehydrogenase activities in the liver and brain mitochondria. A histochemical examination revealed a rather high, irregular activity of the cytochromoxidase in the heart, as well as increased succinic dehydrogenase activity. With the long-term dosing, the changes were not significant, but there was a lowering of the cytochromoxidase and
succinedehydrogenase activities in the heart mitochondria. Development of experimental cholesterol arteriosclerosis in rabbits was facilitated by the application of 20 mg carbaryl/kg for 2.5 months (Lukaneva and Rodionov, 1973). Changes were found in the following indices: general cholesterol, β-lipoproteins, ECG changes, and pathomorphological changes in the aorta and coronary vessels. Carbaryl (100 mg/kg body weight) given to dogs in their diet for 45 days caused disturbance in the secretion of the intestinal enzymes. There was an increase in enterokinase secretion, as well as in the excretion of alkaline phosphates and lipase into the intestinal juice. The no-observed-effect level (NOEL) for these effects was 700 μg/kg body weight, which corresponds to 7 mg carbaryl/kg diet. Three dogs were used in each group (Georgiev, 1967).

2.3. CARBOFURAN

2.3.1. Effects on enzymes

In a study to investigate the relationship between carbofuran metabolism in vivo and acetylcholinesterase inhibition, male Sprague-Dawley rats were given single intravenous or oral doses of \(^{14}\)C carbofuran at 50 μg/kg bw, and urine, faeces, and expired air were analysed for oxidative and hydrolytic metabolites; blood, plasma, and various tissues were analysed for carbofuran and its main oxidative metabolite, 3-hydroxycarbofuran. Erythrocyte acetylcholinesterase activity in vitro was used as an index of toxicity. From 41 to 47% of the administered dose was recovered as \(^{14}\)C-carbon dioxide after 8 h,
independently of the route of administration. About 15% of the dose was found in urine and <1% in faeces. 3-Hydroxy-carbofuran was formed rapidly and underwent enterohepatic circulation, resulting in an elimination half-life of about 64 min for all tissues; the elimination half-life of the parent compound was about 29 min. Rapid recovery of the erythrocyte acetylcholinesterase activity closely paralleled carbofuran metabolism, and the primary disposition of 3-hydroxycarbofuran in vivo was by metabolic conjugation (Ferguson et al., 1984).

In a range-finding study, groups of rabbits were treated dermally with carbofuran (technical-grade; purity, 96.9%) at doses of 0, 100, 300, or 1000 mg/kg bw per day over seven days; the contact time was 6 hrs/day. No clinical signs of toxicity and no local irritation were observed. The treatment did not affect body weight or food consumption. A 30% depression in plasma cholinesterase activity was measured in males at 100 and 300 mg/kg bw per day and a 47% reduction at 1000 mg/kg bw per day. Brain acetylcholinesterase activity was reduced by 26% in males at 100 mg/kg bw per day, 49% in those at 300 mg/kg bw per day, and 54% in those at 1000 mg/kg bw per day. These reductions were not statistically significant (Kedderis, 1985).

2.3.2. Neurotoxicity

In a 28-day range-finding study, groups of five male and five female Sprague-Dawley CD rats were maintained on a diet providing technical-grade
carbofuran (purity, 98.6%) at concentrations of 0, 50, 200, 500, 1000, 3000, or 6000 ppm, equivalent to 0, 2.5, 10, 25, 50, 150, and 300 mg/kg bw per day. Two males receiving 6000 ppm died. Dose-related clinical signs that were noted at doses >200 ppm in animals of each sex consisted of exophthalmia, splayed hindlimbs in females at 200 ppm, tremors and staggered gait at 500 and 1000 ppm, and loss of muscle control and ataxia at 3000 and 6000 ppm. Treatment related clinical signs seen in animals at doses >500 ppm were decreased locomotion, dehydration, lacrimation, and unthriftiness. Body-weight gain was reduced at concentrations >50 ppm among males (marginal at 50 ppm) and at > 200 ppm among females (marginal at 200 ppm). Necropsy showed no treatment-related gross lesions (Freeman, 1994). The NOEL was 50 ppm, equivalent to 2.5 mg/kg bw per day.

2.3.3. Observations in humans

Carbofuran was reported to have induced sensitization in a patch test in 30 farmers with contact dermatitis (Sharma and Kaur, 1990). Poisoning was reported in three female farm workers who threw carbofuran granules onto a coffee plantation in Jamaica. The signs of poisoning reported included vomiting, lassitude, nausea, and hypersalivation. Cholinesterase activity was not determined in these patients (Coleman et. al., 1990).
2.4. USE OF IN VITRO CELL CULTURE FOR THE STUDY OF VARIOUS ORGAN SYSTEMS

2.4.1. Hepatocytes

*In vitro* liver preparations are increasingly used for the study of hepatotoxicity of chemicals. In recent years their actual advantages and limitations have been better defined. The cell models, slices, and mainly primary hepatocyte cultures, appear to be the most powerful *in vitro* systems, as liver-specific functions and responsiveness to inducers are retained either for a few days or several weeks depending on culture conditions. Maintenance of phase I and phase II xenobiotic metabolizing enzyme activities allows various chemical investigations to be performed, including determination of kinetic parameters, metabolic profile, interspecies comparison, inhibition and induction effects, and drug-drug interactions. *In vitro* liver cell models also have various applications in toxicology: screening of cytotoxic and genotoxic compounds, evaluation of chemoprotective agents, and determination of characteristic liver lesions and associated biochemical mechanisms induced by toxic compounds. Extrapolation of the results to the *in vivo* situation remains a matter of debate. Presently, the most convincing applications of liver cell models are the studies on different aspects of metabolism and mechanisms of toxicity. For the future, there is a need for better culture conditions and differentiated hepatocyte cell lines to overcome the limited availability of human liver tissues. In addition, strategies for *in vitro* analysis of potentially toxic chemicals must be better defined.
Wu and Vesonder (1977), dispensed 1.1 million of liver cells per well of a 24-well culture plate. After culturing in a humidified incubator over night, colonies of polygonal shaped cells were observed. After washing off the Medium 199 and reincubated in Krebs solution containing either lactate or fructose for 30 min or longer, these cells released glucose into the incubation solution. Liver cells so isolated from 12 to 18 day embryonated eggs and cultured over night maintained the ability of gluconeogenesis. Cells isolated from the younger embryos (i.e., 14 day incubated eggs) could be further cultured in Medium 199 for 4 days and maintained capacity of gluconeogenesis. However, cell cultures from 18 day old embryos reduced the efficiency to produce glucose from lactate or fructose after further culture for 4 days, due to an extensive propagation of fibroblasts which readily consume the newly formed glucose. The liver tissues of old embryos contain more differentiated fibroblasts, thus make the parenchymal cells isolated less homogenous.

\( \text{o-Hydroxyphenylacetaldehyde} \) (\( \text{o-HPA} \)), the product of coumarin 3, 4-epoxide, was synthesized and its contribution to the hepatotoxic effects of coumarin in the rat was determined. The relative toxicity of coumarin and \( \text{o-HPA} \) were initially assessed in Chinese hamster ovary K1 (CHO K1) cells, a cell line that does not contain cytochrome P450. In CHO K1 cells, \( \text{o-HPA} \)-mediated toxicity greatly exceeded that of coumarin. CHO K1 cell viability, determined via the reduction of 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), was decreased by 95 and 6% in cultures containing
\(o\text{-HPA}\) and coumarin (4 mM), respectively. Coumarin and \(o\text{-HPA}\) were then incubated in metabolically competent primary rat hepatocyte cultures. Cell viability was determined via the reduction of MTT, and lactic dehydrogenase (LDH) release was used as a measure of cytotoxicity. Concentration-dependent decreases in cell viability and increased LDH release were observed using 0.2 to 0.8 mM \(o\text{-HPA}\) and coumarin, with coumarin being consistently less toxic than \(o\text{-HPA}\). Cell viability was decreased by 11 and 50% at 0.5 mM coumarin or \(o\text{-HPA}\), respectively. Hepatocyte LDH release increased 5-fold after a 6-h exposure to 0.8 mM \(o\text{-HPA}\), corresponding to a greater than 90% loss of cell viability in these cultures. In contrast, 0.8 mM coumarin decreased cell viability by 60%, an effect likely due to the conversion of coumarin to coumarin epoxide and \(o\text{-HPA}\). Furthermore, 3-hydroxycoumarin (0.8 mM), which is not a product of coumarin epoxidation, had no effect on cell viability or hepatocellular LDH release. These studies demonstrate that metabolically active rat hepatocytes convert coumarin into toxic metabolites, and strongly suggest that \(o\text{-HPA}\) and coumarin 3,4-epoxide mediate the toxicity of coumarin in rodents \textit{in vivo}. (Stephanie \textit{et. al.}, 2000).

2.4.2. Spleenocytes

Spleenocytes isolated were a mixture of B and T lymphocytes with viability greater than 95% as determined by trypan blue exclusion. The cells had undergone blastogenesis in response to mitogens such as phytohemagglutinin P and concavalin A (ConA).
2.4.3. Erythrocytes

Different researchers reported that osmotic fragility of red blood cells may be caused by physiological (Mokken et. al., 1992, Smith et. al., 1997) or pathological (Brewster et. al., 1976, Wu et. al., 1998) reasons. Low osmotic resistance may lead to intravascular hemolysis, which may cause a reduction of the erythrocyte life span and hence lead to anemia (Oyewale et. al., 1997) if not treated.

p,p'-Dichlorodiphenyl sulfone has been tested for its ability to oxidize hemoglobin to methemoglobin in human erythrocytes in vitro. This compound did not produce a substantial increase in the percentage of methemoglobin detected, relative to untreated erythrocytes (Kramer, 1972).

Some of the observations made by Ferguson (1999), about the effects of primaquine on erythrocytes in vitro / in vivo are (1) extraerythrocytic factors, including hepatic drug metabolism, are important for hemolysis in vivo. (2) Although high concentrations of primaquine may cause lysis of G6PD-deficient erythrocytes in vitro, normal cells are equally susceptible. (3) Sequestration of deformed cells by the reticuloendothelial system (the spleen) in vivo is probably essential for the observed hemolytic effect. However, absence of metabolic potentiation of primaquine in the in vitro system may also account for this discrepancy. (4) Metabolites of several hemolyzing agents (including
primaquine) are more effective than the parent compounds in causing increased mechanical fragility of erythrocytes in vitro. (5) Active metabolites produced principally in the liver must be sufficiently stable to reach the erythrocyte before the red cell can be injured. (6) Additional intraerythrocyte catalytic activities probably play a role in the pathogenesis of hemolysis. Several hemolytic drugs can interact with hemoglobin to generate low levels of hydrogen peroxide, which, like organic hydroperoxides, may result in oxidation of GSH via erythrocyte glutathione peroxidase. (7) The glutathione transferases are a potentially important group of enzymes, which has hitherto not been suspected of playing a role in G6PD-deficient hemolysis. Quinones may lead to both oxidation and depletion of GSH, independently of possible epoxide formation.

2.4.4. Lymphocytes

In a study by Georgian et. al., (1985) the cytogenetic effects of Maleic hydrazide (MH) at different concentration and treatment periods were investigated in peripheral blood culture. For this purpose, 75, 300, 500, 1000 µg/ml concentrations of MH dissolved in distilled water were used. Human lymphocytes were treated with MH at different concentrations for 24 and 48 hours. Chromosome abnormalities were observed such as chromatid gap, chromatid break, isochromatid gap and isochromatid break. Results were evaluated in two ways. If gaps are accepted as chromosome aberrations (CA), at all the groups MH significantly induced CA except 75 µg/ml and 48 hour
treatment group, compared to control. If not, it was observed that MH is not affective. MH also decreased mitotic index (MI) depending upon the increasing concentrations and treatment periods.

The herbicide metolachlor was evaluated for genotoxic potential. Metolachlor did not induce micronuclei in mice, however at 40 mg/kg it significantly decreased the percentage of polychromatic erythrocytes, which is a cytotoxic effect. Metolachlor did not induce chromosomal aberrations in human lymphocytes in vitro, but 2.0 mg/ml culture medium resulted in cytotoxicity, decreasing the mitotic index significantly. The indirect exposure test was carried out by adding plasma from metolachlor-pretreated rats to the human lymphocyte cultures. There was no indication of clastogenicity by metolachlor metabolites. On the other hand, plasma of cyclophosphamide-pretreated rats had a significant clastogenic effect (Cesar and Iris, 1991).

The frequencies of chromosomal aberrations (CA) and micronuclei (MN) in peripheral blood lymphocytes of 40 workers at a phosphate fertilizer factory in North China were studied. HF and SiF₄ are the main air pollutants and small amounts of dust containing fluoride, NH₃ and SO₂ were also present in the factory. It was shown that the chemicals caused an increase in both CA and MN. The mean frequencies per 100 metaphase of major CA type (chromosome rings, translocations, and dicentrics) of the workers and the non-exposed
permitted the isolation of cells with high yields in physiological solutions by perfusing the liver using a glass tube and a polythene pestle and finally filtering through nylon of increasing mesh size to remove the strains of connective tissue and clumps of cells.

2.5.1.2 Use of Chelators

Chelators are used as a chemical method of cell separation, as metal ions, particularly calcium, are known to play a role in cellular adhesion (Howard 1967). The method involves removal of $\text{Ca}^{++}$ and $\text{K}^+$ from the liver by perfusing and back-perfusing with calcium binding agents like citrate (Anderson, 1953), EDTA (Coman, 1954), EGTA (Seglen, 1973).

2.5.1.3 Enzymatic methods

Trypsin was the first enzyme used for the isolation of hepatocytes through digestion of intercellular binding proteins. Other proteolytic enzymes like trypsin, papain, lysozyme, pronase, neuraminidase and pepsin were used subsequently but were found to be ineffective. Later, Howard (1967) incubated the tissue with collagenase and hyaluronidase followed by mechanical treatment, which did not result in a sufficiently high yield. Later Berry and Friend (1969) introduced a major development, describing the first intact liver perfusion method. In this method, they have used 0.05% collagenase and 0.08%...
hyaluronidase. Different investigators have contributed many technical modifications, such as perfusion and calcium concentration (Seglen, 1973; Jeejeebhoy and Phillips, 1976), enzyme concentration and types of enzymes (Berry and Friend 1969; Clarke et. al., 1974; Seglen, 1976), pH, oxygenation and buffer composition (Berg et. al., 1972, Seglen 1973,) and perfusate rate (Capuzzi et. al., 1971, Seglen, 1973). Among the different pioneers who have contributed in many ways for the development and refinement of the technique of isolation, Seglen (1976), is credited for establishing the so-called two-step collagenase perfusion method. This technique involves, perfusing the liver with a divalent cation-free buffer followed by proteolytic digestion of the matrix with collagenase, with the maintenance of the temperature, pH and oxygenation. With the introduction of this method, several workers began to use Ca\textsuperscript{++} along with collagenase (Barnabei et. al., 1974, Baur et. al., 1975). Seglen (1976) has used Ca\textsuperscript{++} removal followed by addition of calcium for the effective enzymatic dispersion. The development of these techniques enabled different investigators to isolate hepatocytes from different sources with certain modifications. Hepatocytes were isolated from mouse, guinea pig, hamster, rabbits, pigs and baboons (Carona et. al., 1973; Elliot and Pogson, 1977; Klaunig et. al., 1981; Panburn et. al., 1981; Maslansky and Williams, 1982). Refinement of the techniques of isolation has also led to isolation of human hepatocytes. The isolation of human hepatocytes with high yield and viability was also reported by enzymatic perfusion of liver (Guguen-Guillouzo et. al., 1982, Strom et. al.,
1982). The method used was simply an adaptation of the two-step rat liver perfusion procedure. A new modified method of human fetal liver perfusion was developed by Habibullah et. al., (1990).

2.5.2. Parameters for the assessment of viability, membrane integrity and detoxifying capacity of hepatocytes

2.5.2.2. Lactate dehydrogenase (LDH)

Lactate dehydrogenase (E.C:1.1.1.27) is the enzyme that catalyses the reversible reaction of pyruvate to lactate during the process of anaerobic oxidation in glucose metabolism (Latner and Skillen, 1968). The enzyme is located mainly in cytoplasm. The enzyme exists in five genetically distinct molecular forms in mammals (isozymes). LDH is considered as the most important representative of enzymes liberated into the medium during cellular damage. The serum levels of this enzyme, produced by these damaged cells, play an important ancillary role in the diagnosis of the diseases e.g. acute hepatitis, myocardial infarction, anemias, malignant disease, infections, and to assess cytotoxicity and membrane integrity (Burgner et. al., 1978, Rivedal and Sonner 1979, Trommer et. al., 1979, Rikans and Cai 1992, Rivas et. al., 1993).

Different investigators have also revealed the fact that, hepatocyte viability can be measured in terms of lactate dehydrogenase release by isolated hepatocytes (Boudjema et. al., 1991, Horiuti et. al., 1992, Vreugdenhil et. al., 1992, Srinivas et. al., 1993). The leakage of LDH is also reported to be an
expression of structural membrane damage (Berg and Blix 1973). Cell death in isolated hepatocytes was estimated by the release of LDH (Tinton et. al., 1993, Neuman et. al., 1993, McGrath et. al., 2001). Ellouk-Achard et. al., (1995) have used LDH leakage as a marker of membrane integrity. Lahiri et. al., (1995) have used cytoplasmic LDH as a marker of viability in irradiated rat hepatocytes. From the above reports, it is well evident that lactate dehydrogenase plays a pivotal role in the determination of the membrane integrity.

2.6. CANCER CHEMOTHERAPY

Coley’s toxins are a treatment for cancer devised by William Coley in 1890s. The toxins are, the fluids derived from a bacterial culture of two microorganisms. The inoperable cases of sarcoma were treated with an artificially-induced erysipelas infection. It was done by mixing the attenuated strains of *Streptococcus pyogenes* (the cause of erysipelas) with another bacterium that lives in the human gut, called *Serratia marcescens*. The fluid derived from the bacterial culture was then injected into the patient, into and around the tumors. This approach was far more successful, then available. These results compare favorably to even today’s methods in some cancers. The toxins were said to be successful with sarcomas (including lymphosarcomas), and their beneficial effect may extend to all cancers that originate in mesodermal tissue (including kidney and ovarian cancer).
Kaposi's sarcoma is very rare and is usually treated initially with radiation therapy, few patients have been treated with chemotherapy and no randomized, prospective trials have compared with one agent to another. Several workers have used single-agent vinblastine at a weekly dose of approximately 0.1 mg/kg (Solan et al., 1981). Almost all the patients had good to excellent response. In most cases, patients required prolonged courses of therapy, up to several years, to maintain a partial response. Doses of vinblastine were titrated in individual patients to maintain a WBC count above approximately 3000. Follow-up after completion of therapy was not presented.

Some of the other drugs used in cancer chemotherapy and their actions are DMSO - (dimethyl sulfoxide) - it "deactivates" cancer cells, especially when used with many other cytotoxic (cell-killing) drugs used in chemotherapy. Hydrazine Sulfate - a super-oxidizer, has been used widely as an effective cancer fighter, however many chemotherapy drugs will counteract its effects.

The other drugs used in cancer chemotherapy are Cisplatin, Tamoxifen for relapse of ovarian cancer, Gemcitabine for pancreatic cancer, Temozolomide in recurrent malignant glioma, Docetaxel, Paclitaxel, Gemcitabine and Vinorelbine for the treatment of non-small cell lung cancer. Fludarabine for B-cell chronic lymphocytic, Taxanes for breast cancer, Topotecan for ovarian cancer.
In vitro and in vivo studies with the drug combination thioTEPA and cyclophosphamide (CPA) were carried out (Teicher et. al., 1988) using the MCF-7 human breast carcinoma cell line and the EMT6 mouse mammary carcinoma cell line. In vitro, survival curves were essentially linear. The EMT6 cell line was less sensitive to thioTEPA than the MCF-7 cell line, with concentrations which reduce cell survival to 10% of 440 and 140 microM, respectively. Simultaneous and immediate sequential treatments with these drugs produced supra-additive cell killing of both cell lines, although the magnitude of the supra-additivity was greater in the MCF-7 cell line than in the EMT6 cell line. Both of these drugs appeared to be as effective as thiol-depleting agents as is diethyl maleate. In the EMT6 tumor in vivo, the maximally tolerated combination therapy (5 mg/kg x 6 thioTEPA and 100 mg/kg x 3 CPA) produced about 25 days of tumor growth delay which was not significantly different than expected for additivity of the individual drugs. Tumor cell killing by thioTEPA produced a very steep, linear survival curve through 5 logs. The tumor cell survival curve for CPA out to 500 mg/kg gave linear tumor cell kill through almost 4 logs. In all cases, the combination treatment tumor cell survivals fell well within the envelope of additivity. Both of these drugs were somewhat less toxic toward bone marrow cells by the granulocyte-macrophage colony-forming unit in vitro assay method than to tumor cells. When bone marrow is the dose-limiting tissue, there is a therapeutic advantage to the use of this drug combination.
Alexander et al. (1987) reported that Recombinant human tumor necrosis factor (rHTNF) alone had no effect on L929 tumor cells at 100 units/ml for 20 hrs of continuous exposure. However, under the same conditions, rHTNF markedly enhanced the cytotoxicity of adriamycin, actinomycin D, 4’-(9-acridinylamino)-methanesulfon-m-anisidide, teniposide (VM 26), and etoposide (VP 16), all targeted at DNA topoisomerase II. The rHTNF had a minimally enhancing effect on the cytotoxicity of bleomycin, hydroxyurea, and 1-beta-D-arabinofuranosylcytosine and no effect on the cytotoxicity of cis-platinum, mitomycin C, vincristine, and vinblastine, all chemotherapeutic drugs with dose-related cytotoxic effects on L929 cells but mechanisms of action which do not appear to involve topoisomerase II. Treatment with rHTNF first and then topoisomerase-targeted drugs yielded no enhanced cytotoxicity, whereas pretreatment with drug followed by rHTNF yielded marked enhancement of cytotoxicity. Topoisomerases have previously been implicated in cell kill phenomena following treatment with certain chemotherapeutic agents (Tewey, 1984). The data suggest that the lethality to the cell from topoisomerase-targeted drug treatment is increased by rHTNF in vitro. He suggested that rHTNF may be a useful adjuvant to this class of drugs which has well-known antitumor activity.

Nischal and Dhasmana (2004), used Bevacizumab in the treatment of cancer. Dona et al., (2004), analyzed the role of hyperforin (St. John’s Wort) in

The herbal chemotherapy supports formulations which are made to assist the body to deal with the poisons in cancers. The herbs found to be most helpful are - Chamomile, St Mary's Thistle, Alfalfa, Rosehips, Thuja, Parsley, Fennel, Blue Flag, Ginger and Liquorice. Also the Bach Flowers, Walnut, Wild Oat, Sceranthus, Honeysuckle and Crab Apple are included. It is also appreciable that these herbs cannot possibly do any damage to the health or be detrimental to the effectiveness of the chemotherapy itself which is suggested by a few oncologists now-a-days who feel that anything which makes healthier might somehow work against the poisons. The fact is that the poisons do their work and the herbs assist the body by minimizing the side effects.

2.7. USE OF CARBAMATES IN THE TREATMENT OF CANCER

The available literature scanned, indicates that there has been no attempt either in India or Abroad, to use these carbamates for the treatment of cancer.