VI. DISCUSSION

The aims and objectives of the present study were to study the toxic effects of commercial formulations of the carbamate insecticides on *in vitro* cultured animal cells. The toxic effects of commercial preparations were then compared with the technical grade carbamate insecticides. The effect of gastric juice on the commercial formulations of carbamate insecticides was also undertaken in order to assess the changes that occur in these toxins during their passage through the stomach before entering the blood stream. The animal cells selected in the present study were hepatocytes (the main organ of detoxification), lymphocytes and spleenocytes (related to immunity). Percent hemolysis of erythrocytes was also taken up so as to see the degree of hypoxia that the toxins under study bring about upon their entry into the animal body. The effect of technical grade carbamate insecticides on tumor cell was studied inorder to evaluate the potency of these toxins as carinocidic agents. The tumor cells selected were mouse myeloma cells, horn cancer cells and squamous cell carcinoma.

Three carbamate insecticides viz. Baygon, Carbaryl and Carbofuran were used in the present study. The purpose of selection of these three insecticides was that they are closely related to each other in chemical structure and action. Further, *in vivo* experimental results and clinical case reports of these insecticides are varied. Clinical reports suggests that accidental and / or
suicidal consumption of the commercial preparations of these insecticides did not result in total fatality, in most of the cases the patients were saved and they recovered successfully back to normality. *In vivo* experimental results are varied, wherein it is reported that these insecticides are toxic at one instance but did not show such toxic effects at other instances. There are no consistent reports that these insecticides may cause any reduction in birth weight, body weight, growth, immunity, effects on internal organs and organ systems, reproduction or will lead to lameness or are mutagenic, teratogenic and carcinogenic (Siebert and Eisenbrand, 1974). Hence it was felt necessary to evaluate the exact degree of toxicity of these toxins by taking up *in vitro* experiments. Further, tumor cells were also included in the experiment in order to study, whether these insecticides can be used as drugs for the treatment of cancer or not. The study was also aimed to observe whether these drugs could be used on cancer vis-à-vis their effect on the other cells like hepatocytes, lymphocytes, spleenocytes and erythrocytes.

The purpose of selecting carbamate insecticides over organophosphates in the present study as agents for the treatment of cancer was that the inhibition of cholinesterase by carbamates is reversible. This inhibition can be selectively reversed by an appropriate dose of atropine. Hence these can be used as therapeutic agents by administering atropine before treatment with carbamates. Therefore, the chemicals when used in chemotherapy of cancer shall be safe and
less toxic as compared to organophosphates, because organophosphates have shown to be mutagenic, teratogenic and carcinogenic with varying effects on immunity, reproduction and causing permanent lameness.

The details of the commercial formulations of the three insecticides selected for the present study are given in table No. 2. Accordingly, it can be seen that the commercial preparation of baygon is in the form of Baygon spray that is an emulsified solvent. Carbaryl is commercially sold as Sevin that is a wettable powder in which carbaryl is entrapped within silica and clay dust. The commercial form of carbofuran used was Carbogran 3G, which is a mixture of chitin, cellulose, gelatin and collagen granules entrapping the carbofuran within it and it is neither soluble in any solvents nor wettable. Therefore the results obtained for the toxicity of these three commercial formulations of insecticides on the animals cells were according to their solubility. Baygon spray did show some toxic effects on the cells cultured in vitro, Sevin had little or no effect and Carbogran 3G had absolutely no toxic effects on the in vitro culture of the animal cells. Efforts were made to mimic the process of chewing (grinding) and digestion by the animals when it consumes the insecticide. Hence, the Carbogran 3G granules where grinded to fine powder and all the three insecticides were incubated with human gastric juice containing exogenously added chitinase, trypsin and collagenase enzyme. Even this process did not
make the commercial formulations equivalently toxic to the technical grade insecticides.

The selection of cells was dependent upon the fact that lack of consideration of \textit{in vivo} biokinetics when estimating the toxicity of a compound from \textit{in vitro} cytotoxicity experiments can lead to misinterpretations. If a compound never actually reaches the type of cells \textit{in vivo}, which are used \textit{in vitro} because of rapid metabolism in other tissues, then the cytotoxicity of the compound for this cell type is irrelevant for its toxicity \textit{in vivo}. Even if the toxicity for the target cells is properly determined \textit{in vitro}, overestimation of the toxicity of the compound \textit{in vivo} can occur if only a small proportion of a dose which is expected to be toxic is absorbed and/or distributed to the target cells. Alternatively, the toxicity of a compound can be underestimated if it is concentrated in certain organs, tissues or cells. Thus, keeping this in view, the cells mainly involved in the detoxification of the insecticides viz. hepatocytes, and those in the blood circulation i.e. lymphocytes and erythrocytes were selected for the present study. The reason for including spleenocytes (which are the cells of secondary lymphoid system) in this study was that they are also related with the immunity of the animal as that of lymphocytes, though it is doubtful that the carbamates ingested or injected into the animal may or may not reach spleen in the same proportions at that of liver.
The difference in the toxicity of these insecticides (both commercial and technical grade) observed on various animal cells selected were due to the difference in the activities of these cells. Hepatocytes are the main cells involved in the detoxification of the insecticides; hence the toxic effects of such insecticides were not so profound on hepatocytes. Whereas, lymphocytes and spleenocytes have shown considerable toxic effects. The most surprising outcome of the present study was the effect of these insecticides on the tumor cells, wherein the tumor cells either failed to establish a cell monolayer in presence of the insecticides or ceased to grow when carbamates were added after establishing the culture. Thereby opening a new era of chemotherapy for cancer treatment. The results obtained for each and every parameter are discussed in detail hereunder.

6.1. OBSERVATION FOR THE FORMATION OF MONOLAYER

Sevin and Carbogran 3G did not effect the establishment or destruction of cell monolayer. When baygon spray was used at the beginning (0 hour) of the culture, there was no formation of cell monolayer till 24 hours in all the three types of cells used viz. hepatocytes, lymphocytes and spleenocytes. However, owing to the detoxifying nature of hepatocytes due to the presence of cytochrome P450 enzyme system, all the propoxur was metabolized and hence hepatocytes started growing after 24 hours and were able to establish cell monolayer. Various degrees of cell deformities and destruction were observed
when Baygon spray was introduced into the culture after establishment of the monolayer. The toxic effects exerted were more on lymphocytes and spleenocytes than hepatocytes.

Owing to the strength of their toxicity, the technical grade carbamates prevented the formation of monolayer for almost 48 hours even in the culture where hepatocytes were inoculated, thereafter it took another 48 hours for the establishment of hepatocyte cell monolayer. Lymphocytes and spleenocytes however, failed to establish cell monolayer indefinitely due to the death of the cells.

Tumor cells failed to establish cell monolayer in presence of technical grade carbamates till 72 hours. This inhibition in the growth and multiplication of tumor cells clearly indicated that carbamate insecticides were potent inhibitors of cellular metabolism (both energy production and protein anabolism). However, the cell population did not cease completely owing to their high survivability rates under stress. The most striking observation was that among the surviving population the cells, many were abnormal cells. These findings should definitely be an inspiration for taking up further research in elucidating the possibility of using carbamate insecticides as chemotherapeutic agents for cancer.
6.2. CELL VIABILITY USING TRYPAN BLUE DYE EXCLUSION

The culture bottle had pieces of glass slides inside and one slide was taken out of the flask, each at intervals of 24, 48, and 72 hours, stained with trypan blue and observed for normal and abnormal cells under microscope and are reported as average percentage of abnormal cells per microscopic field. The abnormal cells counted in the above data included; cells with nucleus expelled out, with disintegrated membrane, ruptured cells, swollen cells, small cells which represented the ceased growth and completely lysed cells i.e. those cells which got stained with trypan blue.

Greater resistance power and detoxifying action of hepatocytes resulted in lower or negligible number of abnormal cells when commercial formulations were used. However, use of technical grade insecticides had about 24.00±0.35 % (with baygon and carbofuran) to 26.75±1.43 (with carbaryl) abnormal cells at 600 ppm after 72 hours as compared to control with 12.75±0.41% abnormal cells.

Lymphocytes and spleenocytes exhibited a greater degree of cell destruction and deformities with commercial formulation of Baygon spray (66.00±1.87% lymphocytes and 63.00±0.94% spleenocytes) but not with Sevin (24.75±1.95% lymphocytes and 21.00±1.27% spleenocytes) or Carbogran 3G (18.00±2.15% lymphocytes and 16.50±1.95% spleenocytes). This difference in the action was due to the fact that baygon spray was soluble and reached the
target cells to exert its action. Whereas Sevin and Carbogran 3G were neither soluble nor available in the media to act upon the cells in the culture. However technical grade of all the three carbamates used had very adverse effects on the cell culture wherein the abnormal lymphocyte cells were as high as 84.25±1.95% with carbaryl, and spleenocytes had a highest of 76.50±2.19% abnormal cells again with technical grade carbaryl.

Tumor cells were acted upon by technical grade carbamate insecticides only at 400 ppm. The histological nature of horn cancer cells have a very thickened cellular membrane and the monolayer formed contained huge amounts of the protein keratin which does not allow diffusion of materials across itself. Hence even the technical grade of the insecticides could not disrupt the horn cancer cells to that greater extent as compared to the other two types of tumor cells studied (Table No. 9). The cells maximally affected were those of squamous cell carcinoma with carbaryl and myeloma cells lines were affected intermittently to that of the other two.

Thus, the present study has demonstrated that hepatocytes were much resistant even to the technical grade insecticides whereas lymphocytes and spleenocytes were susceptible to some extent. The remarkable finding was that, not only the inhibition in growth and multiplication but destruction of tumor cells was also possible with these insecticides, hence can be tried as anti-tumor agents.
6.3. PROTEIN CONCENTRATION IN THE CULTURE SUPERNATANT

The amount of protein in the culture supernatant was estimated using ultraviolet absorbance at 210 nm. The supernatant was collected at the end of the experiment and diluted 1000 fold and the concentration calculated from the extinction coefficient of proteins at 210 nm.

The general trend observed for the protein concentration in the culture supernatant was that the carbamate insecticides inhibited the protein synthesis in almost all the cells studied. However the effect of commercial formulations was far less than that of technical grade insecticides. Among the three carbamates studied carbaryl exerted the maximum inhibitory effect on protein synthesis followed by baygon and carbofuran had the least effect, as was the case with other parameters studied.

Comparison between the type of cells used indicated that hepatocytes were least effected cells owing to their ability to detoxify, followed by lymphocytes and the cells effected maximally were spleenocytes. Among the tumor cells horn cancer cells were the least effected followed by myeloma cell lines and the maximally effected cells were that of squamous cell carcinoma.

The observation presented in table No. 12, clearly shows that in hepatocytes, carbamates inhibited the protein synthesis and hence the protein
concentration in 200 ppm treated sample was less than that of control. However, the increase in the concentration of proteins in the culture supernatant in 400 ppm and 600 ppm treatment samples was due to the death of the cells and release of the cellular contents into the media and not the proteins secreted by the hepatocytes. Lymphocytes and spleenocytes exhibited a higher degree of inhibition of protein synthesis owing to the fact the cells were unable to detoxify the carbamates. Hence even in 600 ppm treated cultures, the death of the cells did not release much protein.

The results presented in table No. 13, show that in tumor cells there was an increase in the concentration of proteins in the culture media in treatment groups as compared to control. This is because technical grade carbamates were used in tumor cells hence it resulted in excessive cell destruction. As expected, myeloma cells were excessively involved in protein synthesis as compared to squamous cells and the least amount of protein synthesis in horn cancer cells, hence the supernatants of these cells showed the same trend of protein concentration after the respective cell destruction i.e. myeloma cells had the highest proteins in the media and horn cancer cells had the least amount of proteins in the culture media.
6.4. LACTIC ACID PRODUCTION AND LEAKAGE

Mammalian cells, under typical conditions, convert most of the glucose consumed into lactic acid that has adverse effects on cell growth. Lactic acid production reduces the buffering capacity of the medium and leads to a drop in pH, which is detrimental to the growth of cells in the culture. There is an inverse relationship between pH and lactic acid concentration; as lactic acid increases, pH decreases. This process is limited to the buffering capacity of the media in vitro. Once bicarbonate is exhausted, lactic acid accumulates. Higher lactate concentrations were postulated to cause negative feedback of glycolysis. Glucose diffusion into the cell layer also becomes limiting (Jensen and Trager, 1978).

Observations made in the present study for the production and release of lactic acid was in accordance with the observations of Jensen and Trager, loc. cit. The amount of lactic acid in hepatocyte culture supernatant was almost negligible. It was not due to non production of lactic acid but because of the ability of the hepatocytes to convert lactate produced by glycolysis to glucose by gluconeogenesis. The observation was in accordance with the observations made in the present study for the change in the colour and pH of the culture media. The colour exhibited by the media was rose pink with a pH of 7.5. This clearly indicates that lactic acid produced was completely utilized without releasing much into the media. Whatever was released due to damage to the
cells was reabsorbed by the surviving cells and converted to glucose. In case of
death of the cells, the enzymes of gluconeogenesis remained active in the media
and hence lactic acid could be converted to glucose even after the death of the
cells due to effect of the insecticides. Further, the increase in pH to 7.5 of the
culture media of hepatocytes after 72 hours may be attributed to the release of
bicarbonate ion produced by the action of the enzyme carbonic anhydrase on
water and carbon dioxide evolved by the metabolism of the cells.

Lymphocytes and spleenocytes do not have the ability of converting
lactic acid to glucose, hence their culture supernatant had considerable high
amounts of lactic acid produced by the metabolism of the cells and released as a
result of damage to the cells. However, the inhibitory effect of the insecticide
carbaryl on lactate dehydrogenase and other enzymes of glycolysis was evident
from the reduction in the reduced amount of lactic acid produced by the cells in
which carbaryl was incubated. Baygon and carbofuran also had some inhibitory
effect on the production of lactic acid but it could not be appreciated due to
release of lactic acid by the cells as a result of damage to the cells.

Owing to their faster anaerobic metabolic rates tumor cells produced
enormous amounts of lactic acid, ranging from 72.07±5.18 in horn cancer cell
culture to 95.24±1.70 in myeloma cell culture. Presence of carbamate
insecticides reduced the production and release of lactic acid into the medium,
even though there was cell damage and death. This was again due to the inhibition of the enzymes of glycolysis and especially lactate dehydrogenase. The percent inhibition observed due to carbaryl in the release of lactic acid as compared to control in myeloma cell, horn cancer cell and squamous cell carcinoma culture was 64.30%, 65.85% and 41.55% respectively.

Due to the inhibitory effects of carbamate insecticides on the production and release of lactic acid this parameter could not be considered for assessing the degree of cell damage, but effective for the study of metabolic activity of the cells.

6.5. LACTATE DEHYDROGENASE (LDH) LEAKAGE IN THE CULTURE SUPERNATANT

The leakage of lactate dehydrogenase (LDH) into the culture media was measured as an index of cell damage due to the toxic effects of the carbamates. But, it was found that the levels of LDH obtained in the present study were not in accordance with the degree of cell death and/or cell damage. This was due to the inhibitory effect of the carbamate insecticides on enzyme activity and glycolytic enzymes in particular. Parafita and Fernandez (1984), in their in vivo experiments, reported that lactic dehydrogenase activity was reduced by 38% in presence of 1.0 mmol carbaryl/litre of drinking water. In the present study, similar results were obtained, wherein the presence of carbaryl and other carbamate insecticides in the culture media inhibited the activity of LDH to
varying degrees, hence the results were non consistent and could not be correlated to the cell death or deformity. However, the data formed a good basis for the toxic effects of carbamates on enzymes and decreased utilization of glucose by the cells resulting in various degrees of cell deformities and cell deaths.

As observed for other parameters above, the commercial formulation of carbamate insecticides, except baygon spray did not exert their toxic effects in relation to LDH leakage. However, technical grade carbamates, especially carbaryl produced a very high degree of inhibition in the activity of the enzyme and also its synthesis from the DNA. The exact degree of inhibition in the activity of the enzyme could not be calculated because of the fact that cell damage and cell death must have leaked sufficiently high amounts of LDH but due to the presence of carbamates in the media the estimation process must have been interrupted.

The commercial formulation baygon spray when used with hepatocytes, the increase in percentage of LDH leakage was about 28 % wherein the change in the enzyme activity was from 71.60±5.33 (U/L) in control to 91.57±3.82 (U/L) in 600 ppm treatment group. Sevin and Carbogran 3G caused a marginal increase of 9 % and 6 % respectively in the activity of LDH in hepatocyte culture supernatant at 600 ppm levels as compared to controls. The results were
in accordance with the findings of Parafita and Fernandez (1984). The percentage of LDH leakage observed in hepatocyte culture supernatant where 600 ppm of technical grade carbamates was used was 28% for propoxur, -51% for carbaryl and 4% for carbofuran as compared to control. It is interesting to note that the reduction in the activity of the enzyme is not due to reduction in the leakage from the cells but, due to non availability of enzyme active sites for action, as they were reversely inhibited by the carbamates. This indicated that propoxur is less effective in inhibition of the activity of the enzyme as compared to control, whereas carbaryl is a very potent inhibitor of the enzyme LDH with less than half of the enzyme activity being inhibited and carbofuran is equally strong inhibitor of the enzyme activity, therefore can cause various degree of cell deformity and cell death as LDH activity is a direct indication of utilization of glucose by the cells.

The results obtained for the activity of the enzyme LDH in tumor cell culture supernatant are very much helpful in proposing carbamates as chemotherapeutic agents in the treatment of cancer. Again, as noted above among the three carbamates, carbaryl exerted the maximum degree of inhibition in enzyme activity; where the figures were 33% less than control in myeloma cell culture supernatant, 27% less in horn tissue as well as squamous cell carcinoma cell culture supernatants as compared to control. Goldman, et. al., (1964) reported a total LDH activity of 463 U/L in culture media and cells of
neoplastic tissue inoculated at a concentration of $4 \times 10^3$ cells cultured in closed mouth culture bottles. In contrast to this report, the results obtained in the present study were very low (33% less activity of LDH) as compared to the percentage of abnormal cells due to the effect of carbaryl on myeloma cells which had 82% more abnormal cells than control. It could thus be concluded that reduction in the activity of LDH observed in the present study was due to (1) Inhibition of the enzyme itself; (2) Inhibition of the other enzymes of glycolytic pathway thereby resulting in non availability of the substrate “pyruvate” for LDH thus, inhibiting the production of LDH from the gene and (3) Direct inhibition of the gene producing the enzyme LDH.

6.6. OSMOTIC FRAGILITY OF ERYTHROCYTES

The purpose of undertaking the study of osmotic fragility of erythrocytes due to the effect of carbamate insecticides in the present research work was to assess the degree of anemia that could result by the use of these insecticides as cancer chemotherapeutic agents.

The osmotic fragility (OF) test measures the stability of erythrocytes by testing the cells' ability to withstand hemolysis in decreasing concentrations of saline solution. The OF test is a rough index of red cell surface-to-volume ratio. When erythrocytes are placed in a hypotonic solution, water osmotically enters the cells and causes them to swell. The cell reaches a point where the membrane
starts to leak, and, finally, the cell bursts and releases hemoglobin. Damage to the red cell membrane also allows hemoglobin to escape from the cells but does not cause osmotic swelling. A positive correlation exists between abnormal OF test results and decreased survival time for red cells. The mean OF expresses the concentration of NaCl in which 50 percent hemolysis occurs. Increased as well as decreased osmotic fragility of erythrocytes have been described in different types of anemia in human medicine, dog and cat.

There are many factors that influence the osmotic fragility of erythrocytes viz., Mechanical damage of erythrocyte membrane, red blood cell age (Bowlder et al., 1981), changes in red blood cell shape, hemococoncentration and acidosis, elevation of body temperature, exercise stress and catecholamines (Brewster et al., 1976), lysolecithin (hemolytic agent released by the contracting spleen into circulation), peroxidation of the erythrocyte membrane, any factor inhibiting or attenuating glycolysis can cause erythrolysis as glycolysis is the most important source of energy in red cells.

The carbamate insecticides tested in the present study were all potent inhibitors of not only glycolysis but many other enzymes of cell metabolism. Therefore, all the drugs rendered erythrocytes more fragile to the changes in the osmolality of the solution in which they were taken into. Carbaryl affected the erythrocytes most followed by baygon and carbofuran. Similar results were
reported by Fouad and Afaf (1990) wherein the carbamates methomyl and trichlorofon significantly produced a reduction in the number of erythrocytes (due to increased fragility), haemoglobin level and haematocrit value in *in vivo* experiments on birds. Willi *et. al.*, (1989) reported an increase in osmotic fragility of the erythrocytes due to infection by hemotrophic *Mycoplasma* species in Swiss cats.

Inspite of the fact that these insecticides made erythrocytes more fragile to changes in the various physiological parameter, they can still be used as chemotherapeutic agents very safely because the inhibition by these drugs is transient and reversible.

### 6.7. PARAMETERS FOR FURTHER STUDY

A preliminary study was made on the following aspects:

1. Extraction of active ingredients from commercial formulations of carbamates and their effect on the cells cultured *in vitro*.
2. Effect of hepatocyte culture supernatant on other cells cultured *in vitro* with commercial formulations and technical grade carbamates.
3. Effect of *in vitro* hepatocyte co-culture with other cells and incubated with carbamates.
4. Development of hepatocyte cell lines in presence of carbamates.
To conclude, the extraction of carbamate insecticides from commercial formulation using gastric juice was not achieved in Toto, as the companies manufacturing these products do not reveal the complete ingredients to the public and state it to be their "TRADE SECRET". For instance, carbogran 3G granules were totally insoluble in any of the solvents that can be conveniently used for cell culture work. Upon trying to trace out the make up of the granules, the company did not reveal the composition and kept it as their trade secret. However, efforts were made to solubilize the granule by dissolving the contents of the granule. With the presumption that the granule must be made up of chitin, cellulose or gelatin, the enzyme chitinase, cellulose and proteases were added to the gastric juice so as to extract the active carbamate insecticide. A reasonably high degree of success was obtained wherein 50 to 60 % of the active ingredient was extracted as assessed by measurement of the extinction coefficient of carbofuran. Carbaryl in sevin was ligated as silica and clay dust, hence was neither soluble nor extractable. Only baygon was available in a soluble form, hence could effect the cells in vitro. Clinical case reports in humans, that deaths occurred by accidental or suicidal consumption of baygon spray but not in case of sevin or carbogran 3G consumption (Hayes and Vaughan, 1972) are comparable to the present findings.

Culture of normal animal cells and tumor cells with hepatocyte culture supernatant containing carbamates had more deteriorating effects due to the combined effect of the carbamates and their metabolites released by
hepatocytes. However hepatocyte co-culture had less deteriorating effects owing to the fact that hepatocytes detoxify the carbamates.

Repeated dilution culture of hepatocytes, in presence of carbamates, resulted in the increased survivability of hepatocytes at each dilution, indicating that carbamates had brought about chromosomal aberrations resulting in better survivability of the cells in vitro. Therefore experiments are to be carried out to evaluate the possibility of using carbamates in the development of hepatocyte cell lines.

The efficacy of carbamates in the treatment of cancer in animals, especially peripheral tumors like horn cancer, squamous cell carcinoma, canine venereal transmissible tumors (CTVT) and mammary gland tumors is to be undertaken for the evaluation of these drugs. Carbamates can be conveniently used in large animals for cancer chemotherapy as it has been reported (Sigma chemical company’s leaflet enclosed with the carbamates) that about 250 mg/kg body weight intravenous is non toxic to cattle. This works out to be 250 grams of carbamate can be safely injected at the site of peripheral tumor in cattle and if atropine sulphate is preinjected the dose can further be enhanced. Thereby giving a good drug for peripheral cancer treatment in cattle, as presently effective drugs for peripheral tumors are not available for Veterinary use. Similarly, it has been reported that in dogs large doses of carbamates are non toxic, hence can be tried for treatment of CTVT.