VII. SUMMARY

Commercial formulations of the carbamate insecticides; propoxur (Baygon spray), carbaryl (Sevin) and carbofuran (Carbogran 3G) were tested for their toxicity on in vitro cultured hepatocytes, lymphocytes and spleenocytes isolated from sheep. Technical grade chemicals of these three insecticides were also used to test their in vitro toxicity on normal animal cells (hepatocytes, lymphocytes and spleenocytes) and also on tumor cells (myeloma cell lines, horn cancer cells and squamous cell carcinoma). Each of these insecticides were inoculated at 200, 400 and 600 ppm for 24, 48 and 72 hours. However tumor cells were incubated only at 400 ppm for 24 hours. The percentages of abnormal cells in the primary cell culture were enumerated as a measure of the toxicity of the insecticides. The extent of toxicity caused by these insecticides on the cells cultured in vitro was further confirmed by estimation of proteins, lactic acid and lactate dehydrogenase in the culture supernatant as an index of cell leakage. Squamous cell carcinoma cells were further subjected to the measurement of the viable cell number using neutral red and proteins in the cell monolayer using methylene blue, in an ELISA reader. Osmotic fragility of erythrocytes, following exposure to technical grade carbaryl was also studied in order to assess the degree of anaemia, resulting upon exposure to carbamates.

Isolation of hepatocytes was carried out by trypsinisation, followed by centrifugation. Lymphocytes were isolated from heparinized whole blood,
centrifuged, and layered onto histopaque in a capillary tube, again centrifuged and the interface ring containing mononuclear cells was collected. Spleenocytes were isolated by gently teasing the spleen to loosen up the cells. Referral myeloma cell lines were obtained from National Centre for Cellular Sciences, Pune. Horn cancer and squamous cell carcinoma were collected from the clinical complex of Veterinary College, Bidar during surgical excision from bullocks. Isolation of the tumor cells was done by trypsinisation, followed by centrifugation. All the cells isolated were adjusted to a cell population of $4 \times 10^5$ cells/ml in RPMI – 1650 media without serum. Ten milliliters (10 ml) of this media with cells was taken into 50 ml sterile culture bottles containing glass slides measuring $4 \times 1 \text{ cm}^2$ and incubated at $37^\circ \text{C}$ for the primary cell culture. Periodic observations under an inverted tissue culture microscope were made to see the formation of monolayer. The carbamate insecticides (both commercial formulations and technical grade) were inoculated 24 hours after the formation of the monolayer, at 200 ppm, 400 ppm and 600 ppm for 24, 48 and 72 hours.

The carbamate insecticides were prepared as follows; original baygon spray concentrate is 5000 ppm (5%), 0.4 ml of this solution was directly added to 10 ml of the culture media in the culture bottle to give a final concentration of 200 ppm, 0.8 ml to give 400 ppm and 1.2 ml made 600 ppm. An amount of 10 mg of Sevin was mixed with one litre of water that made 1000 ppm carbaryl. Similarly, 1.7 gms of Carbogran 3g was taken in one litre of water to make
1000 ppm carbofuran. An amount of 2 ml of each of the stock solutions (1000 ppm) was directly added to 10 ml of the culture media in the culture bottle to give a final concentration of 200 ppm. Similarly, 4 ml of stock was added to make 400 ppm and 6 ml was added to make 600 ppm. Technical carbamates were prepared using their solubilities in water. About 250 mgs of each of the carbamates was poured separately, each in one litre of water, allowed to dissolve for an hour with constant stirring and left overnight for settlement of undissolved chemical. The upper one-third solution was collected separately in different bottles that formed a solution of 2000 ppm of baygon, 1200 ppm of carbaryl and 700 ppm of carbofuran solution. Finally these solution were diluted appropriately to make 200, 400 and 600 ppm solution for use in the present study.

The formation/inhibition/destruction of the monolayer was studied by observing the culture bottles under an inverted tissue culture microscope. Abnormal cells were counted by removing the microslides from the culture bottle at regular intervals of 24, 48 and 72 hours and staining with trypan blue. The results are presented as percentage of abnormal cells per microscopic field. Morphological changes observed by microscopy were features like enucleated cells, lysed cells, granulated cells, altered cell membrane, granulated nucleus without rupture of plasma membrane, swollen cells and cells with decreased size due to cessation of growth. Statistical analysis was done as per the method.
given by Snedecor and Cochran (1994). Further, the exact viable cell numbers of squamous cell carcinoma cells were measured using neutral red and proteins using methylene blue in an ELISA reader. The culture supernatant was collected after 72 hours of cell culture for the estimation of protein, lactic acid and lactate dehydrogenase as a measure of cell function/leakage/toxicity. Proteins were estimated by using ultraviolet absorbance at 210 nm. Lactic acid and lactate dehydrogenase were estimated colorimetrically.

The results indicated that, among the three commercial preparations of carbamate insecticides used; only Baygon spray exhibited statistically significant toxic effects, whereas Sevin and Carbogran 3G did not show any toxic effect. This difference in the effect of the insecticides was not due to their chemical nature but because of the fact that propoxur in Baygon spray was present in a soluble form and hence could effect the cells in the culture. Carbaryl in Sevin was lavigated in silica and clay crystals and carbofuran in Carbogran 3G was encapsulated in chitin/cellulose/gelatin granules, hence were not soluble in any of the solvents, therefore could not reach the cells in culture to effect them. Further, among the three cell types used, hepatocytes, owing to their detoxifying ability did not experience much toxicity of even baygon spray. However lymphocytes and spleenocytes did get effected by baygon spray but not by Sevin or Carbogran 3G. The percentage of abnormal hepatocytes due to the effect of baygon spray at 600 ppm after 72 hours increased to 16.5±0.56
from 12.00±0.71 in control. The only significant toxic effect observed was on lymphocytes and spleenocytes by baygon spray wherein the percentage of abnormal cells in 600 ppm treatment group after 72 hours were 66.00±1.87 and 63.00±0.94 percent respectively as compared to 17.50±0.56 and 15.50±1.25 percent in control. Previous work on similar aspects could not be traced for comparison. However, 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.

Similar trend was observed for the protein concentration in the culture supernatant where commercial formulations of the carbamates were used. Baygon spray had inhibitory effects on the protein synthesis at the initial concentrations of 200 and 400 ppm but at 600 ppm due to increased cellular destruction the protein levels in the supernatant showed an increase. The amount of protein in Sevin and Carbogran 3G inoculated culture supernatant did not differ from that of control. Lactic acid and lactate dehydrogenase exhibited the same trend wherein they were found high in Baygon spray culture supernatants but no difference in values between control and treated groups in Sevin or Carbogran 3G treated culture supernatants in all the three types of cells studies.
The results obtained with technical grade carbamate insecticides were very exiting. In general, it was observed that owing to their detoxifying action, hepatocytes resisted the toxic effects, even of the technical grade carbamates. However lymphocytes and spleenocytes did succumb to the technical grade chemicals. The most striking point was that these insecticides not only inhibited the growth of tumor cells but they also caused varying degrees of cell deformity /destruction and cell death.

In hepatocyte culture, the extent of cell deformity was just twice that of control when technical grade insecticides were used. There was a greater inhibitory effect on protein concentration in the culture supernatant, with carbaryl effecting to the maximum extent. Lactic acid and lactate dehydrogenase concentration did not tally with the degree of cell damage, indicating that the carbamates inhibit the cellular metabolism. Lactic acid showed an increase from 1.87±0.50 in control to 4.57±0.97, 1.19±0.50 and 8.12±1.33 mg/dl in 600 ppm baygon, carbaryl and carbofuran treated groups respectively. LDH release in hepatocytes with technical grade carbamates was 69.93±4.24 (U/L) in control and 89.91±3.53, 34.13±2.46 and 72.43±2.97 in 600 ppm treatment with baygon, carbaryl and carbofuran respectively.

Lymphocytes showed greater extent of cell damage as compared to hepatocytes with technical grade carbamate insecticides. The abnormal lymphocytes were 84.25±1.95% when technical grade carbaryl was inoculated
in the culture. The secretion of proteins by lymphocytes into the culture media was decreased due to the presence of the insecticides, which was evident by the fact that even at 600 ppm level the excessive cell death did not release much of protein. Lactic acid and LDH levels also showed a fall in the concentration as compared to control, due to inhibition of cellular metabolism by carbamates.

Spleenocytes had a similar trend as that of lymphocytes, with technical grade carbamates, wherein the abnormal cells in carbaryl treated group were 76.50±2.19 % more than the control. Protein (gm %), lactic acid (mg/dl) and LDH (U/L) recorded at 600 ppm with carbaryl (control) were 33.50±2.06 (10.00±0.71), 12.69±1.42 (21.32±2.33) and 20.81±1.38 (28.81±1.38) respectively.

Tumor cells recorded the most exciting results. Among the three types of tumor cells studied, horn cancer cells owing to their tougher membrane nature were more resistant to the technical grade insecticides, followed by myeloma cell lines and finally the most effected cell types were squamous cell carcinoma cells. It was observed that when technical grade insecticides were used there was inhibition in the formation of monolayer or cell growth and multiplication. Upon further exposure of tumor cells to the chemicals, there was cell deformities/destruction and even cell death. Varying degrees of inhibition of cellular metabolism was also observed. The abnormal cells with 400 ppm carbaryl at 24 hours were 49.75±3.90 for squamous cell carcinoma as compared
to 8.50±0.56 for control. Myeloma cells and horn cancer cells showed 45.25±3.23% (11.25±1.98 in control) and 36.75±3.75% (11.75±1.29 in control). The protein, lactic acid and LDH were significantly decreased in the treated groups (especially carbaryl treated groups) indicating inhibition in cellular metabolism.

The percent hemolysis of sheep erythrocytes without carbamates, initiated at 0.45% NaCl which reached 50% at 0.40 percent NaCl concentration. Complete hemolysis was seen at 0.35 percent NaCl. Baygon treated erythrocytes showed initial, 50% and complete hemolysis at 0.55 (29.76%), 0.45 (51.01±0.65%) and 0.35 % NaCl respectively i.e., a little earlier as compared to the control. Carbaryl treated erythrocytes showed initial, 50% and complete hemolysis at 0.65% NaCl (20.95%), 0.55 % and 0.45 % concentration of NaCl i.e., carbaryl treated erythrocytes were more fragile as compared to the control (0.35%), baygon (0.40%) or even carbofuran (0.40% NaCl) treated erythrocytes. Carbofuran exhibited less toxic effects on the fragility of erythrocytes as compared to baygon and carbaryl, where in the initial, 50% and complete haemolysis was seen at 0.50, 0.45 and 0.40 percent NaCl respectively.
VIII. CONCLUSION

It can be concluded, from the results obtained in the present study, that among the three commercial formulations of carbamate insecticides used viz., Baygon spray (Propoxur), Sevin (Carbaryl) and Carbogran 3G (Carbofuran) only Baygon spray exerts its toxic effects on animal cells to a considerable extent. Whereas, Sevin and Carbogran 3G are totally non toxic due to the fact that the active ingredient in these preparations are present in non soluble complex forms. Clinical case reports in humans, as published by Hayes and Vaughan, 1972 that deaths occurred by accidental or suicidal consumption of Baygon spray but not in case of Sevin or Carbogran 3G consumption are comparable to the present findings. However, the above report by the same authors also highlighted that workers in factories manufacturing these chemicals, those employed in the store houses, where the carbamates are stored and the farmers who are regularly using these formulations are at a higher risk of toxic effects of not only Baygon spray but even that of Sevin and Carbogran 3G. This is due to the fact that the factories manufacturing the commercial formulations of these carbamates use the pure form of the active ingredient; hence the workers are at higher risk, due to inhalation or contact with skin. Those working in the store houses and farmers using them are exposed to the active ingredients released by the microbial action upon the commercial preparations of these insecticides. The microbes degrade the "TRADE SECRET" ingredient in the stored commercial formulations thereby releasing
the active ingredient as fine dust that can be inhaled by the workers or comes in contact with the skin and gets absorbed, thereby causing the toxic effects.

Among the three cell types used, hepatocytes, owing to their detoxifying ability did not experience much toxicity of even Baygon spray. However lymphocytes and spleenocytes did get affected by Baygon spray but not by Sevin or Carbogran 3G. Hence, Sevin and Carbogran 3G did not effect on the immunity of the animal, however Baygon spray could reduce the immunological response due to excessive cell destruction and death. Further, there should be no effect on hepatocyte metabolism in presence of any of these three commercial preparations of insecticides. Therefore, it could be concluded that the household pesticide Baygon spray should be preserved very carefully at home, especially from children. The other two preparations i.e., Sevin and Carbogran 3G should not be stored for longer periods either in the store houses or at farmers residence.

As for the technical grade carbamates tested in the present study, carbaryl had the most deteriorating effects on the animal cells, followed by baygon and carbofuran had the least toxicity among the three carbamates studied. Carbaryl effected not only the cellular integrity but also cellular metabolism. This was evidenced by the fact that carbaryl treated samples had higher degree of cellular deformity, destruction and death in addition to inhibition in the formation and release of proteins, lactic acid and lactate
dehydrogenase by the cells. The technical grades of other two carbamates viz.,
baygon and carbofuran also had profound toxic effects, but not to the extent as
that exhibited by carbaryl. Hence, it could be concluded that the toxic effects of
these three carbamate insecticides as reported by previous works (Gosselin,
1984), that they are mutageic, teratogenic, carcinogenic, causes anaemia,
reduces reproductive capabilities and induces immunosuppression may most
probably, seem to be due to their inhibitory effects on cellular metabolism.
However, paralysis and/or deaths occurred by these insecticides are due to their
inhibitory effects on the enzyme acetylcholine esterase and not directly due to
inhibition of the cellular metabolism, though it can be a contributory factor to
some extent.

Hepatocytes, again owing to their detoxifying ability did not experience
much toxicity of technical grade insecticide as compared to lymphocytes and
spleenocytes that were effected to about 70 %. This might induce reduction in
the immunological response *in-vivo* due to excessive cell destruction, death and
inhibition of cellular metabolism. Erythrocytes were also tested for the
hemolytic effects of these chemicals, wherein it was observed that the fragility
of RBC reduces due to exposure to carbaryl, thereby resulting in some degree of
anaemia and hypoxia. However, exposure of the technical grade insecticides to
man and the animals is limited due to the fact that they are neither easily
available in the market nor are they used in the original form, and hence are at
no or least risk. However, workers in the factories/laboratories using the
technical grade insecticides are to be cautious about their handling, wherein they have to avoid contact with the skin and prevent inhalation.

The most striking outcome of the present study was the toxicity of carbamates on tumor cells. Carbamate insecticides, not only inhibit the growth of tumor cells but they also cause tumor cell destruction and death. Therefore, this study opens a new option for testing the feasibility of using carbamates, and carbaryl in particular for the treatment of cancer. It seems, from the review of literature that no such attempt has been made to use carbamate insecticides in the treatment of cancer. While treating cancer with carbamates, the toxic effects on other cell types can be overcome by preinjecting the antidote; atropine sulphate as shown by Kimmarle (1996) in his experiments, wherein he used 50 mg/kg atrophine sulphate prior to intra peritoneal dose of 100 mg/kg propoxur without any neurotoxic signs.
RECOMMENDATIONS FOR FUTURE STUDY

1. The exact nature of *in-vitro* effects of carbamate insecticides on each of the animal cell types needs to be evaluated.

2. The chemistry and degree of inhibition of the cellular metabolism by the insecticides has to be studied.

3. Extraction of active ingredients from commercial formulations of carbamates and their effect on the cells cultured *in vitro*.

4. Effect of hepatocyte culture supernatant on other cells cultured *in vitro* with commercial formulations and technical grade carbamates.

5. Effect of *in vitro* hepatocyte co-culture with other cells and incubated with carbamates.

6. Feasibility of use of carbamates, especially carbaryl in the treatment of cancer has to be worked out *in vitro*.

7. *In vivo* experiments for the treatment of peripheral tumors with carbamates especially in cattle and dogs has to be conducted.

8. In the event of use of carbamates in the treatment of cancer, the effect of the same on other body cells/organs are to be monitored in addition to the use of atropine as antidote in the prevention of the toxicity to other cells or organs.

9. Development of hepatocyte cell lines by using carbamates has to be worked out.