CHAPTER - IV

Discussion
CHAPTER IV

Discussion

The aim of the present investigation was to demonstrate the toxicity of a fungicide mixture (Metalaxyl + Mancozeb; M + M) and to evaluate the degree of the toxic effect of it at tissue and cellular levels in a rat model. Hence, the combination of M + M was suitably suspended in distilled water at a dose level of 500 mg/kg and was given through oral intubation to the experimental groups. The LD$_{50}$ value of M + M combination was 5150 mg/kg body weight. The control animals were provided only with distilled water throughout the study. The duration of the treatment was 30, 60 and 90 days and for studying effects on hormonal imbalances of thyroid gland and testis, implantation loss due to mutagenic effects on male germ cells, on epididymal spermiogram and fertility, it was restricted to a maximum of 60 days. For recovery, animals were kept for a further period of 60 days to detect reversibility or persistence of toxic effects. At the termination of each treatment period, bone marrow smears were also made to assess the incidence of micronuclei and aberrations in the chromosomes. In vitro studies were also carried out on human lymphocytes in the absence and presence of metabolic activation to support in vivo genotoxicity findings (Appendix I). Various haematological, serum and tissue biochemical parameters were assayed. In addition, histopathological and gravimetric studies were also carried out to investigate the toxicity of this fungicide combination in orally fed and recovery groups.
Body weight is an extraordinarily sensitive and objective measure of health of the animals because growth integrates all the influences acting on it day by day. Reduction of body weights is known to have a considerable effect on other biological parameters (Schwartz et al., 1973; Scharer, 1977; Oishi et al., 1979). A significant reduction in the weekly average body weight from eighth week of the treatment period was evident in our study. It is in conformity with the work carried out with mancozeb in rats by others, who found a decline in absolute body weights (Szepvolgyi et al., 1989; WHO, 1994; Kackar et al., 1997a).

Organ weight analysis is also of a great importance in general toxicity studies and surveys have shown that, organ weights of rats were affected in 82% of the studies (Heywood, 1981). Assessing organ weights helps to focus the histopathological examination on target organs such as liver, kidney and testis. The weights of these organs are frequently altered by administration of xenobiotics. Metalaxyl fed rats showed no alterations in kidney weights (Kaloyanova et al., 1991). Thus in the present study, it is evident that reduction in kidney weight is probably attributed to mancozeb of the mixture which is in agreement with the studies conducted in rats with zineb, a dithiocarbamate (Przedziecki et al., 1969). Similarly, reduction in male reproductive organ weight is a sensitive endpoint in reproductive toxicity assessments including alterations in testis structure (Sprando and Collins, 1998). Testicular weights correlate with testicular toxicity, and a precaution at the early phase of development of a new chemical prior to any assessment of
male fertility (Heywood and James, 1978). Weights of the testis were significantly reduced by M + M treatment which corroborate with the findings of Lowy et al. (1979; 1980) who obtained a decrease in testicular weights in rats treated with thiram, a dithiocarbamate at the dose level of 450 mg/kg body weight. Thus reduction of testis weight in the present study could be attributed to a loss of spermatogenetic elements and reduced levels of androgen binding protein in the testis that is regulated by both follicle stimulating hormone and androgens (Tindall and Means, 1976; Rao, 1997; Rao and Sharma, 2001). Contrarily oral feeding of fungicide combination induced an increase in liver weights of rats. These data were correlated with the findings conducted with ethylenebis dithiocarbamates (Griffaton et al., 1975; Szepvolgyi, 1989). Thus, variations in body weight including kidney, liver and testis weights indicate that this combination fungicide affected general metabolism and growth of animals. Other general toxic effects on repeated oral administration of M + M for 90 days resulted in dyspnea, diarrhoea, salivation, nasal bleeding, hind limb paralysis which are in agreement with the findings of Trivedi et al. (1993).

The prime aim of haematological studies is to identify chemicals, which can exert toxic effects on the cellular constituents of blood, namely erythrocytes, leucocytes, platelets and the differential leucocyte cell (DLC) counts namely lymphocyte, neutrophil, eosinophil, basophil and monocyte. To assess the effects on red blood cells, it is usual to perform RBC count, haemoglobin determination and measuring the packed cell
volume (PCV) or haematocrit. Such parameters provide information on the estimation of the number of circulating red cells, the oxygen carrying capacity of the blood. Anemic hypoxia results from a decreased concentration of functional haemoglobin, reduced number of red cells or chemically induced alterations in haemoglobin. Acute damage to red cells or to their content of haemoglobin can result in an impairment of oxygen transport and secondary peripheral hypoxia. Thrombocytes or platelets are the first line of defence against accidental blood loss. Thrombocytopenia may be manifested by hemorrhagic disorders. An abnormally increased number of circulating platelets (thrombocytosis) has not been associated with chemical exposure. When total granulocyte count falls, it leads to granulocytopenia. Devoid of neutrophils leads to neutropenia. Granulocytopenia is the most common manifestation of chemically induced bone marrow damage (Rifkind et al., 1980). In our study RBC counts and haematocrit values were reduced by 60 and 90 days treatments indicating probable severe anemic and hypoxic conditions induced by this combination fungicide. This condition was correlated with a concomitant decline in haemoglobin levels in 90 days fed rats. Such results were also reported by several researchers in mancozeb and metiram treated rats. The trend was same with respect to platelet count, where platelet count was significantly decreased on 60 and 90 days of treatment in comparison to the control group indicating the loss of defensive mechanism of blood (WHO, 1994).
Contrarily, WBC counts were higher in 60 and 90 days treated rats in this study. Leucocytes have the most complex organization among the formed elements. These differ from other blood cells in that these perform important functions outside the vascular compartment. Phagocytosis is one of the defensive mechanisms against foreign or extraneous material. Phagocytes are subdivided into granulocytes (neutrophils, eosinophils, basophils and monocytes/macrophages). Neutrophils are the most active phagocytes and eosinophils are less active and eosinophilia occurs in some allergic diseases and infestations with large parasites. Basophils seem to be related to tissue mast cells and release histamine and other mediators in response to immunologic stimuli. Lymphocyte count was significantly increased after 90 days of treatment. Contrarily, neutrophil count was significantly low and basophil, monocyte and eosinophil counts were unaltered. This data reflects on altered function of differential leucocytes and defensive functions by M + M treatment. Thus, variations in blood cell counts would indicate the effect of this combination on bone marrow/haematopoietic cells. Many reports are available on the effect of pesticides on formed elements of blood including man (WHO, 1994). However, these changes were recovered after 60 days of the withdrawal of the M + M feeding to rats.

Serum biochemical parameters are important for diagnosis of liver and kidney functions. A battery of these parameters including serum transaminases, protein, cholesterol, glucose, creatinine and blood urea
nitrogen (BUN) were assessed. Biochemical assays provide a relatively simple method for screening populations for potential liver necrosis caused by occupational or environmental toxins. Liver damage could be diagnosed by monitoring serum enzyme values such as alanine aminotransferase (ALT) or glutamate-oxaloacetate transaminase (GOT) and aspartate aminotransferase (AST) or glutamate pyruvate transaminase (GPT). The importance of serum enzymes particularly GOT and GPT in the diagnosis of liver diseases was well established in human beings (Goetz, 1980). The GOT and GPT levels are elevated in hepatic diseases and also due to exposure to various chemicals and drugs (Lynch et al., 1964; Hanke and Piotrowski, 1980). Wolf et al. (1967) stated that GPT involves increased transamination of aminoacids during gluconeogenesis and production of urea, thus resulting in elevated serum urea levels. The GOT and GPT levels in the serum were significantly higher in M + M treated animals after 60 and 90 days of treatment in comparison to the respective control group. After 60 days of post treatment no recovery was observed and these levels remained higher only. A rise in GOT and GPT levels could be due to the effect of M + M on hepatocytes causing permeability alterations and leakage of lysosomal enzymes enhancing the release of these enzymes into circulation (Oser, 1976). Funakoshi (1995) studied the effects of dithiocarbamates along with cadmium on these enzymatic activities and observed elevated levels of GOT and GPT indicating the toxicity of liver. These elevations in serum clinical parameters were in conformity with histopathological findings of liver in this study.
Serum protein levels were found to be significantly lowered in treated animals after 90 days as compared to control group. This could be related to retardation of protein synthesis gradually in liver, increased protein catabolism in treated groups. Cholesterol is a major component of cell membranes and acts as the substrate for steroid hormone formation in adrenals and gonads. It is present in the plasma mainly in the form of esterified fatty acids. As the body cannot breakdown the sterol nucleus, cholesterol is either excreted as such in bile or converted to bile acids and then excreted. The serum cholesterol level was also significantly decreased during 60 and 90 days of treatment periods. The reduction in serum cholesterol could possibly be due to either a decrease in the synthesis and / or an increase in the breakdown of lipids due to proliferation of peroxisomes. Reduction of cholesterol levels has also been reported in previous toxicity studies conducted on certain pesticides which induced hepatic peroxisomal proliferation (Cohen and Grasso, 1981; Reddy and Lalwani, 1983; Kawashima et al., 1984). The peroxisomal proliferators possess hypolipidemic property and play a role in lipid metabolism (Locke et al., 1989) because proliferation of peroxisome increases in fatty acid beta-oxidation and also induces hepatocellular carcinomas in rats (Lalwani et al., 1981; Reddy and Lalwani, 1983). These observations suggest that M + M causes chemical injury to vital organs such as liver and may interfere with carbohydrate and lipid metabolism in treated rats. Contrarily, the serum glucose level was found to be elevated significantly after 90 days of treatment. It probably shows hyperglycemic condition induced by M + M or the
stress induced during hyperadrenal activity and gluconeogenesis in the liver (Goyal et al., 1986). Measurement of bilirubin in blood helps to assess hepatic anion transport, although the liver transports many other anions, including bile salts (Smith, 1971; Klaassen and Wattkins, 1984; Erlinger, 1988). Total bilirubin content was found to be significantly higher by 60 and 90 days of treatment with M + M and 60 days after post treatment as compared with control group which is in harmony with the findings cited by WHO (1994).

Urea is the main end product of nitrogen metabolism and constitutes about 80% of the nitrogen found in urine. It originates in the liver, being derived from the aminoacids and the amount produced varies with protein catabolism. Dietary intake of protein has a considerable effect upon urea production and generates considerable variances in plasma levels. Creatinine is the anhydride of creatine, which is found particularly in skeletal muscle. Creatine synthesis occurs in the liver and pancreas from which it is transported through blood stream to various tissues. Blood urea nitrogen (BUN) and serum creatinine concentrations help to assess the potential effects of a compound on glomerular filtration rate. Serum creatinine and BUN levels were significantly increased by treatment with M + M and in recovery group as compared to control animals. This situation demonstrated that liver and kidney functions were affected and reflected on the alterations induced in their normal histological features. These effects were in conformity with the studies on rats and dogs treated with mancozeb.
(Kampmeier and Haag, 1954; WHO, 1994). Thus, clinical chemistry parameters were affected in all experimental animals including withdrawal group manifesting persistent toxicity at milder level except for serum glucose, protein and cholesterol levels, which showed a recovery in this study.

Treatment with M + M to rats indicated that the liver function was altered, as all biochemical parameters of this tissue showed variations. The decreased protein level induced by M + M feeding to rats might be correlated with the pattern of hepatic structural alterations and exhibited a positive correlation with changes in biochemical parameters, signifying the utility of these biochemical tests in early diagnosis of hepatic damage in toxic state and explains the probable arrest of protein synthesis of the liver in this study. But in our report, the glycogen levels were significantly increased by M + M treatment and its level remained higher upto the end of recovery period as compared with respective control animals. The significant accumulation of glycogen in the liver might be due to depression of glycolysis by enolase-mediated inhibition (Dousset et al., 1987). The enhancement of glycogen might also be due to reduction in the activity of phosphorylase, an enzyme which catalyses the conversion of glycogen into glucose-1-phosphate. All these changes might be related to alterations in catecholamines, which regulate carbohydrate metabolism (Patel and Chinoy, 1997; Rao and Patil, 2000).
In many tissues alkaline phosphatase (ALP) is attached to cell membranes, suggesting an association between acid phosphatase (ACP) activity and membrane transport (Chakaravarty and Sreedhar, 1982). In the liver, ALP activity is localized on those parts of the cell membrane of the parenchyma cell adjoining the biliary canaliculus and the sinusoid. The enzyme, ACP is more prominent in lysosomes and released in more quantities during stress or toxic conditions. The ALP and ACP levels were significantly increased by M + M after 60 and 90 days treatment indicating that fungicide combination has altered the liver phosphatases. At normal levels of alkaline phosphatase production, the hepatic cell can excrete this phosphatase into the biliary tract. The observed alterations in alkaline phosphatases might be due to an inhibition of bile flow and/or a failure of the secretory functions of the liver cells. It might also be due to an increased production of the enzyme by the cells of the biliary duct (Griffiths et al., 1961). The changes in acid phosphatase activity might be due to an effect of these fungicides on the lysosomes. Increased acid phosphatase activity in rats after pesticide treatment has been reported by Galdhar et al. (1978) to support our data. These altered biochemical profiles are also correlated with histological changes generated by this combination in our study, thus giving an importance of these tests in early diagnosis of liver toxicity. The liver pathological changes exhibited sinusoidal distention, marked hepatocellular degeneration in the form of vacuolar changes and focal hyperaemia. These changes were persistent even after withdrawal of treatment with minimal recovery. However, few parameters like ALP
and ACP showed a recovery but others were persistent showing M + M toxicity. All these tissue biochemical changes could explain the altered status of this organ.

In kidney, levels of protein, ACP and ALP were evaluated after 60 and 90 days of treatment period and 60 days after post treatment in rats. Protein levels in the kidney were significantly declined in M + M treated animals throughout the treatment and post-treatment periods. But phosphatases viz., ALP and ACP levels were significantly increased by M + M treatment. Exposure to a multitude of chemicals and pharmaceuticals has been reported to increase ALP (Young et al., 1975) and it depicts degree of cellular damage taking place in this tissue (Passow, 1961) and ACP reflects on increased lysosomal activity during toxic conditions. Proximal tubule is most vulnerable to chemical nephrotoxicants (Grantham, 1982; Mc Kinney; 1982). In the present study, treated animals showed structural alterations in kidney including diffused hyperaemia, glomerular retraction and tubular degeneration even after withdrawal of the M + M treatment indicating that in a combination, these fungicides act as a nephrotoxicant. Thus, the results were similar to those noted in liver and testis biochemically and histologically.

Protein, ACP, ALP, hydroxysteroid dehydrogenases (HSDs) and cholesterol levels in testis were estimated and were found to be significantly altered by this treatment. Mishra et al. (1994) and Kackar
et al. (1997a) observed such alterations in dithiocarbamate treated male rats. Elevated ALP level due to M + M treatment might be an indicative of cytopathologies or 'leaching out' of the enzyme from the spermatogonia, spermatocytes, spermatids and sperm (Sudhir et al., 1996). Increase in the values of ALP suggests severe metabolic disturbance in the testes of treated rats (Vhemes, 1986). The enzyme, ACP is a histochemical marker for determining the site and pattern of distribution of lysosome in a cell (Manocha et al., 1975). Increase in the concentration of the enzyme could be explained by an increased lysosomal activity or due to necrotic changes in germ cells. The total protein levels were declined and are correlated with regressive changes of testis morphology. Cholesterol augmentation in contrast to reduction in 3β and 17β HSDs levels explained an inhibition of the testicular androgen biosynthesis (Mann and Lutwak - Mann, 1981; Knobil et al., 1988). It is further followed by a fall in circulating androgens in this study. These tissue biochemical alterations were consistent with testicular histological changes exhibiting disruption of germinal epithelium with vacuolization, loss of sperm in tubular lumen contributing to oligospermic condition, subcapsular oedema and atrophy of seminiferous tubules leading to androgen deficiency state. Atrophy of Leydig cells was also noted. At the end of recovery period, only certain biochemical parameters exhibited a recovery followed by partial revival in testis histology.
Any chemical that produces an adverse effect in experimental animal studies is assumed to pose a potential reproductive hazard to humans. This assumption is based on comparison of data for known reproductive toxicants (Thomas, 1981; Hemminki and Vineis, 1985; Meistrich, 1986; Working, 1988; Chapin, 1988; Zenick and Clegg, 1989; Russell et al., 1990; Kimmel et al., 1990; Takahashi and Matsui, 1993). When mancozeb given at the dose level of 140-1400 mg/kg body weight twice in a week for 4.5 months, it affected both reproductive and endocrine structures leading to decreased fertility (WHO, 1994). While an evaluation of the usefulness of some potential indicators of reproductive toxicity in the rat, Blazak et al. (1985) examined sperm production rate, epididymal sperm numbers, transit time and motility in groups of F 334 strain animals of different ages. It is known that the epididymis is the main organ for maturation of sperm and its function is under the strict control of circulating androgens (Blaquier et al., 1970; Rao, 1997; Rao and Sharma, 2001). Hence, the epididymal sperm profiles--viz., sperm motility, sperm count and sperm viability exhibited alterations in our study as its physiological function was affected by this fungicide poisoning and histoarchitecture of the epididymis also showed regressive changes. The cauda epididymal sperm motility in M + M treated animals for 60 days exhibited a reduction in the percentage of motile sperm indicating loss of their membrane properties. Similarly, other fungicides are also known to inhibit sperm motility (WHO, 1994). These results are in agreement with the findings reported by Ivanova-Chemishanska et al. (1973), who observed a rapid loss of mobility and changed resistance in spermatozoa of rats treated with dithiocarbamates.
Moreover, viability of the cauda epididymal sperm also showed a reduction in the percentage of live sperm by M + M treatment. This condition reflects on maturational defect of sperm in the epididymis, as it has become hostile for sperm survival as result of oral-feeding of combination fungicide to rats. Moreover, mice treated with dithiocarbamates resulted in a significant abnormal sperm heads (Zdzienicka et al., 1982; Reddy and Prasad, 1986). Fungicide treatment also brought a significant decrease in sperm reserves in the cauda epididymis of rats. It indicated antispermatic effect of this treatment on the testis, as it is the site of production of sperm. Moreover, observations of our study also revealed a loss of testicular function as mentioned earlier. All these effects manifested alterations in androgen function due to this combination fungicide treatment and further it is evident by a fall in circulating testosterone levels in this present study. These affected sperm parameters were probably contributed to a reduction in fertility of these treated rats. Other insecticides like DDT and carbaryl are also known to affect sperm parameters (WHO, 1970). Carter and Laskey, (1982); Gray (1994) and Carter et al. (1987) observed an inhibitory effects of fungicides like carbendizm, vinclozolin and benomyl on fertility of male rats. However in our study, the mating rate of M + M treated animals were not affected throughout the experimental period. Similarly, no significant change was observed with respect to copulatory index in M + M fed animals for 60 days. The copulation index is a very practical parameter for assessing mating behaviour and libido in rats. Sexual behaviour is complicated involving central and peripheral nervous system, neural and
endocrinological activities and it is particularly difficult to identify loss of libido using other parameters in rats (Zenick and Goeden, 1988). However, the litter size of the M + M treated animals were significantly declined as compared to the control animals. Thus, all these altered parameters of fertility revealed effect of fungicide on reproduction and were partially reversible after cessation of feeding. Results of dominant lethal study have revealed that increase in early and late fetal deaths in untreated females mated with M + M treated male rats is primarily due to chromosome breakages indicating male germ cell mutation. The broken chromosomes are normally lost resulting in aneuploidy condition termed monosomy (Green et al., 1987). Monosomy as well as trisomy of autosomes which are formed due to non disjunction may also have resulted in the death of embryo. Thus early deaths in this study is due to monosomy and late death due to trisomy. This significant alteration in reproduction effect in male germinal cells was well confirmed with the results of endocrine disruption testis and thyroid gland. As carbamates are known to exert such effects, mancozeb component might probably be the one for manifestation of reproductive toxicity in our study (Short et al., 1976; Zenick et al., 1994). There was no dose related effects on rats treated with metalaxyl technical with regard to mating performance, pregnancy rate, litter size and reproduction effects such as embryotoxicity and teratogenicity (FAO, 1982). No evidence of dominant lethal effect, embryonic deaths was obtained in the progeny of male mice treated with metalaxyl and found to be not a carcinogen in mouse (FAO, 1982).
Dithiocarbamates including mancozeb manifests toxic effects on thyroid physiology and histology (WHO, 1994). Ethylenethiourea (ETU) forming dithiocarbamates such as mancozeb, maneb, ziram, zineb, mitram inhibit thyroid peroxidase and leads to hypothyroidism (EPA, 2002; Hill, 1998; Hurley, 1998) and results in hypothyroidism, manifested by decreased $T_3$ and $T_4$ levels, which in turn induce an increase in TSH levels via negative feed back mechanisms. In our study, follicular epithelium is intact filled with colloid in control animals whereas, animals treated with $M + M$ showed depletion of colloid from acini with an increased interacinar space, hyperplasia and focal atrophy. These changes might be contributed to a loss of endocrine function of this gland. Further our study also revealed an increase in plasma TSH levels followed by decrease in serum $T_3$, $T_4$, $fT_3$ and $fT_4$ levels indicating inhibitory action of this combination fungicide. This may be attributed to a loss of enzyme activity required for the synthesis of thyroid hormones and also negative feed back due to increased levels of TSH in treated rats. This is in agreement with findings of Blackwell-Smith et al. (1953) who observed hyperplasia in thyroid gland of rats treated with dithiocarbamates. Rats treated with mancozeb further exhibited hyperplasia of follicular cells with loss of colloid (Przedziecki et al., 1969; FAO/WHO, 1971; Ivanova- Chemishanska et al., 1971; 1975). These changes in the thyroid gland of rat exposed to mancozeb were also due to ethylenethiourea and carbon disulphide which are the main metabolites of mancozeb (Graham and Hansen, 1972; O'Neil and Marshal, 1984). However, withdrawal group revealed a partial recovery.
Reduced levels of thyroxine and histopathological changes were observed after exposure to mancozeb at single oral dose of 500 mg/kg in rats (Kackar et al., 1997b). Fungicides of ethylenebisdithiocarbamates group affected the thyroid gland and chromosomal aberration in lymphocyte genome among heavily exposed workers (Steenland et al., 1997).

Three month dietary toxicity of mancozeb in rats showed decreased T4 and increased TSH levels at 1000 ppm. Combined chronic and carcinogenicity with mancozeb in rats at 750 ppm exhibited enlarged thyroid with follicular cell carcinomas, adenomas and nodular hyperplasias, lower circulating T4 and elevated T3 and TSH levels. In a study conducted for potential tumorigenic and toxic effects in prolonged dietary administration to rats with mancozeb revealed lesser T4 levels. In Beagle dogs T3 and T4 were decreased with increase in serum cholesterol at 40 mg/kg for 52 weeks orally (EPA, 2000). The metabolite ethylenethiourea of ethylenebisdithiocarbamates was found to distort the humoral activity of thyroid gland by inhibition of dopamine-beta-hydroxylase (Laisi et al., 1985). Rate of deaths from thyroid, bone and prostate cancer were higher among white men in a region of Minnesota where mancozeb was used. Male rats fed with higher levels of mancozeb over a long time showed an increase in
number of benign and cancerous tumours in thyroid gland (Gandhi and Snedeker, 2000).

To assess genotoxic potential of M + M, micronucleus test and chromosomal aberration analysis were done in bone marrow cells. The most commonly conducted *in vivo* cytogenetic assay is the bone marrow micronucleus assay (Schmid, 1976; Mac Gregor et al., 1987). It helps to detect the induction of micronuclei by the chemical as a result of chromosomal breaks and abnormal chromosomal segregation in the polychromatic erythrocytes (PCE) of bone marrow i.e., young RNA-containing erythrocytes. Micronuclei arise from chromosomal fragments or chromosomes that are not included in the daughter nuclei during cell division (Evans, 1959; Heddle and Carrano, 1977). Micronucleus formations are cell lethal events in proliferating cell populations. Hence their presence indicates tissue dysfunction and possibly death of the organism when the incidence is frequent (Salamone and Heddle, 1983). The percentage of micronucleated erythrocytes in our report was significantly increased only by 90 days of treatment with M + M, indicating the genotoxic damage. These values were comparable with results of Mitomycin C, a positive control agent. This effect be attributed to the persistent nature of the chemical or its delayed effect or due to its metabolites formed in the test system. Hrelia et al. (1996) found that single intraperitoneal injection of 75-300 mg/kg metalaxyl had no effect on the frequency of micronuclei, in murine polychromatic erythrocytes and found to be non-carcinogenic and gave negative results in a battery of genotoxic tests. But it was noted that
thiram, a dithiocarbamate produced a significant increase in micronucleated polychromatic erythrocytes of male mice at the dose level of 50 mg/kg body weight in support of our data (IARC, 1976). Total erythrocytes which includes polychromatic and normochromic erythrocytes and their ratio in 30, 60 and 90 day M + M treated animals were not different in our investigation. Hence, it is obvious that in M + M combination, probably mancozeb was responsible for induction of micronuclei by inducing genotoxicity in bone marrow cells.

The *in vivo* cytogenetic assay of bone marrow cells also helps to detect chromosomal aberrations (Preston et al., 1981). The percentage of structural and numerical aberrations was significantly higher in M + M treated animals after 90 days of treatment when compared with the control group animals. The parameters included were mitotic index, incidence of chromatid and chromosomal aberrations including dicentric, fragment, polyploidy and aneuploidy condition and the results were in conformity with the observations of chromosome analysis in rats treated with dithiocarbamates at the dose level of 100 mg/kg body weight (Kurinny and Kondratenko, 1972; Hedenstedt et al., 1979). Zdzienicka et al. (1984) recorded chromosomal aberrations in mice and Philinskaya (1971), in human lymphocytes *in vitro* treated with ziram. Similarly chromosomal aberrations including chromatid fragments were recorded in bone marrow analysis of male rats administered with propylene bisdithiocarbamate (WHO, 1994). It was observed that mean incidence of aberrant metaphases was also more in
cultured lymphocytes of workers in zineb, a dithiocarbamate manufacturing unit. Jablonicka (1989) recorded a significant increase in the frequencies of cells with structural chromosomal aberrations in the peripheral lymphocytes of humans treated with mancozeb. Similar observation was recorded by Dolara et al. (1994) when dithiocarbamates was mixed with other pesticides. However, literature available on metalaxyl studies do not give any evidence for genotoxic effect. But, when metalaxyl was analyzed for cytotoxic effects and transforming properties under *in vitro* condition in BALB/c 3T3 strain mice, it induced only cell transformation (Perocco et al., 1995). However, the aberrations were not significant by 30 and 60 days of treatment with M + M combination. Thus from the present study, it is clear that M + M in combination has a potential to induce structural and numerical aberrations in rat bone marrow. Similarly study on human blood cultures (Appendix I) also indicated mild reduction in mitotic indices and increased frequencies of chromatid and chromosomal aberrations to support *in vivo* observation of combination fungicide induced genotoxic effect.

From these data, it is evident that the combination fungicide manifested variations in a number of biochemical tests of blood, tissues like liver, kidney and reproductive organs as well as fertility parameters in rats. Fungicides are known to exert their effects by binding with cellular components thereby manifesting toxic effects on the physiology and
histomorphology of tissues. These changes were reversible at varied levels. Moreover, this combination has a potential to induce genotoxicity including in human blood cultures.

Thus, this combination fungicide has potential to generate toxic effects in animal (rat) model, including genetic defects in vivo and in vitro conditions.