METHYLMERCURIC CHLORIDE TOXICITY:
IN VIVO EVALUATION OF TERATOGENICITY
AND CYTOGENETIC CHANGES IN MICE

A THESIS PRESENTED

TO

THE FACULTY OF GRADUATE STUDIES

UNIVERSITY OF MANITOBA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

BY

DENNIS CHARLES CURLE, B.Sc., M.Sc. (MANITOBA)

DEPARTMENT OF ANATOMY

HUMAN GENETICS

AUGUST, 1985

G78839

METHYLMERCURIC CHLORIDE TOXICITY:

IN VIVO EVALUATION OF TERATOGENICITY

AND CYTOGENETIC CHANGES IN MICE

BY

DENNIS CHARLES CURLE

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

© 1985

Permission has been granted to the LIBRARY OF THE UNIVERSITY OF MANITOBA to lend or sell copies of this thesis, to the NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film, and UNIVERSITY MICROFILMS to publish an abstract of this thesis.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

INDEX

Page No.

Acknowledgements 2

Abstract 6

Introduction 9

Literature Review 12

Materials and Methods 37

Results 48

Discussion 107

Conclusions 122

References 126

ACKNOWLEDGEMENTS

To Dr. M. Ray for his many hours of stimulating discussion, for his unending patience and for his encouragement, thank you.

I wish to express my sincere thanks and appreciation to Dr. T.V.N. Persaud for his interest, encouragement and expert guidance in completing this research.

I am most grateful to Dr. F.D. Bertalanffy and Dr. E.N. Larter, the other members of my thesis committee, for their expert guidance, empathy and patience.

To the individual members of the Anatomy Department who have been friends as well as colleagues. To Bert Sam for his technical assistance and friendship. Many thanks to Robert Carmichael for proofreading the manuscript.

I wish to thank Dean B. Barnard for her support and assistance in completing this research. I would also like to thank Luigina Girden and Wendy Lussier for their excellent typing support.

I am indebted to Amrat Jivan for his assistance in the preparation of the statistics. A very special thanks to Marilyn Carter in the preparation of the illustrations and graphics and to Grant Ball in the photography department.

Many thanks to my friends and colleagues who have tolerated me during my ups and downs during this study. Special thanks to Elizabeth Omeniuk for her friendship and assistance in my academic struggles.

CONTENTS

Page

1.0 INTRODUCTION 9

1.1 General problems...................................... 9
1.2 Purpose of Study 11

2.0 LITERATURE REVIEW 12

2.1 Mercury - History and uses............................. 12
2.2 Hazards of mercury contamination....................... 15
2.2.1 Human exposures and poisonings 15
2.2.2 Teratology studies 19
2.2.3 Light microscopical studies 23
2.2.4 Electron microscopical studies 23
2.2.5 Biochemical studies 24
2.2.6 Cytogenetic studies 26
2.2.6.1 Conventional analysis of chromosomes 26
2.2.6.2 Sister chromatid exchanges 28
2.2.6.3 Nucleolus organizing regions......... 33

3.0 MATERIALS AND METHODS 37

3.1 Animals 37

3.2 Treatments... 37
3.2.1 Fetal studies 37
3.2.2 C-mitotic studies 38

3.3 Teratological studies 38
3.3.1 Maternal weight changes........................ 38
3.3.2 Resorptions and fetal deaths................... 38
3.3.3 Fetal weights.................................. 39
3.3.4 Gross anatomical analysis (Wilson sections)....... 39
3.3.5 Skeletal analysis (skeletal staining)............. 39

3.4 Chemical analysis of mercury levels.................... 40
3.4.1 Maternal....................................... 40
3.4.2 Fetal 40

3.5 Light microscopy 40
3.5.1 Preparation of tissues 40
3.5.2 Examination of tissues......................... 41

3.6 Electron microscopy.................................... 41
3.6.1 Preparation of tissues 41
3.6.2 Examination of tissues......................... 42

Page

3.7 Cytogenetic studies 42
3.7.1 Preparation of slides 42
3.7.2 Conventional staining and chromosomal analysis 43
3.7.3 Sister chromatid exchanges (SCEs) staining and analysis 44
3.7.4 Nucleolus organizing regions (NORs) - staining and analysis 45
3.7.5 C-mitosis - processing of tissues and preparation of slides 45
3.7.6 C-mitosis - staining and analysis 46

3.8 Statistical analysis 46
3.8.1 Analysis of variance 46
3.8.2 Coefficient of correlation 47

4.0 RESULTS 48

4.1 Teratological studies 48
4.1.1 Maternal weight changes 48
4.1.2 LD_{50} of MMC for maternal mice 53
4.1.3 Total implantations 53
4.1.4 Resorption 56
4.1.5 Intrauterine death 56
4.1.6 Live fetuses recovered (normal and abnormal) 59
4.1.7 Fetal malformations (macroscopic and skeletal staining) 59
3.1.8 Mean fetal weights 67

4.2 Mercury levels 70
4.2.1 Maternal 70
4.2.2 Fetal 70
4.2.3 Comparison 70

4.3 Light microscopy 76
4.3.1 Fetal lung 76

4.4 Electron microscopy 80
4.4.1 Fetal lung and liver 80

4.5 Cytogenetic studies 83
4.5.1 Conventional chromosome analysis 83
4.5.2 Chromosome counts 87
4.5.3 Sister chromatid exchanges (SCEs) - analysis 91
4.5.4 Nucleolus organizing regions (NORs) - analysis 96
4.5.5 C-mitosis - analysis 100

Page

5.0 DISCUSSION 107

5.1 Methylmercuric chloride as an environmental pollutant and teratogen 107
5.2 Manifestations 109
5.2.1 Maternal weight changes 109
5.2.2 LD_{50} for maternal mice 110
5.2.3 Total implantations 110
5.2.4 Resorptions 111
5.2.5 Intrauterine death 111
5.2.6 Live fetuses recovered (normal & abnormal) 112
5.2.7 Fetal malformations 113
5.2.8 Mean fetal weights 113
5.2.9 Levels of maternal mercury 114
5.2.10 Levels of fetal mercury 115
5.2.11 Comparison of maternal and fetal mercury levels..... 115
5.2.12 Light microscopy 116
5.2.13 Electron microscopy 118
5.3 Cytogenetics 119
5.3.1 Conventional chromosome analysis 119
5.3.2 Chromosome counts 119
5.3.3 Sister chromatid exchange frequencies 120
5.3.4 Nucleolus organizing regions frequencies 120
5.3.5 C-mitosis 121

6.0 CONCLUSIONS 122

6.1 Manifestations of mercury toxicity 122

6.2 Mechanisms of mercury toxicity 124

7.0 REFERENCES 126

ABSTRACT

Mercury is a major environmental pollutant and a proven teratogen in man and animals. Its teratogenicity and effects on fetal chromosomes were investigated in mice. Various dose levels of methylmercuric chloride (MMC) 1000 ppm (5 mg through 30 mg/kg of body weight) were administered via an intragastric tube to pregnant ICR Swiss/Webster mice on day 9 of gestation. On day 18 of gestation the animals were killed and the fetuses removed. Fetal lung and liver sections were processed for light microscopy, electron microscopy and cytogenetic studies. Fetuses were also fixed in Bouin's solution for subsequent examination of the internal organs, using Wilson's technique. Mercury levels were determined in maternal blood and randomly selected fetuses. One fetus from each litter was processed for skeletal staining with Alizarin Red S.

Adult male mice were given a single intragastric dose of MMC at the previously mentioned dose levels. They were killed six hours later and duodenal tissue samples were removed and processed for light microscopic examination.

A significant increase in embryonic deaths and resorptions were observed at all dose levels. The incidence of fetal anomalies was significantly increased following maternal treatment with 10, 15, 20 mg/kg of MMC. Maternal weight between day 9 and day 18 of gestation decreased significantly. The LD_{50} of MMC in pregnant mice was determined to be 20

mg/kg in body weight; the LD_{100} was 30 mg/kg. A significant difference was observed between the mean fetal weights at the various dose levels. Levels of mercury were found to be significantly higher in treated animals and increased in a dose-related manner. Levels of mercury were also significantly increased in fetuses as the dosage increased. The levels of mercury were significantly higher in the fetuses than in the mothers at the same dosage, indicating a correlation between the levels of mercury in maternal mice and corresponding higher levels in their fetuses. There was a very pronounced dose-response relationship between dosage given and the maternal and fetal mercury levels detected.

Light microscopy of fetal lungs from MMC treated animals were hypoplastic and retarded in development. The severity of pulmonary changes increased with the dose-level of MMC. Electron microscopy of fetal lung and liver showed degeneration of mitochondria. Vacuolation and lysis of mitochondria increased in severity with the dose-level of MMC.

Cytogenetic studies revealed significant clumping of chromosomes in metaphase at all dose levels and the frequency of clumping increased as dosage increased. The euploidy number ($2n = 40$) of chromosomes per cell did not vary between the treatment groups and control groups. C-mitosis in duodenal tissue (similar to colchicine metaphase arrest) was observed at all dose levels and significantly increased as dosage increased. The frequency of sister chromatid exchanges (SCEs) increased significantly

as the dosage increased. The frequency of nucleolus organizing regions (NORs) per cell did not change significantly between the treatment groups and the control.

Clumping of chromosomes was prevalent and may be due to deactivation of the microtubular proteins following mercury binding. MMC is a firm binder of proteins which leads to denaturation and loss of biological activity. This may cause a defect in many enzymatic systems which would be subsequently inhibited.

The oxidative enzymes are very sensitive to metabolic poisons and would be strongly inhibited by mercury. Enzyme inhibition may account for the mitochondrial changes. Energy deficiencies, as a result of enzyme inhibition, would ultimately lead to entropy and eventually cellular death. Maternal treatment with MMC does appear to have caused subtle DNA damage in the offspring as indicated by the significant increase in SCE frequencies. However, such exposure to MMC does not appear to have cause any changes in the frequencies of NOR. The teratological mechanisms of MMC might be related to metabolic derangement in the fetus as a result of the Chromosomal changes.

1.0 INTRODUCTION

1.1 General Problems

Mercury is as fluid as water, heavier than lead and as brilliant as silver— an element of perfect singularity. This unique element has fascinated man for centuries. It has found use as a medicine, a pigment and is still used daily in numerous ways in our lives. However, due to industrial accidents our wonder is now mixed with fear and confusion about this engimatic element. The extent of the potential danger it poses for humans, alive and unborn, is just now beginning to unravel.

There is ever-increasing concern about the potential deleterious effects of environment pollutants on the embryo and fetus as numerous toxic substances, including mercury, find their way into our air, water and food supplies. The increasing pollution of the environment with mercury compounds is due in large part to its use in fungicides, pulp and paper industries and in dental preparations. This contamination has resulted in several occupational and epidemic poisonings in humans. Mercury accumulates in the aquatic food chain due to industrial effluent which is consequently converted into methylmercury by micro-organisms. Methylmercury concentrates in aquatic life and subsequently finds its way into the diet of humans. Methylmercury has a relatively long half-life in humans and can

readily cross the placenta accumulating in the fetus where it can exert harmful effects on the unborn child. Methylmercury is the most toxic of the mercury compounds and produces the most profound changes in the fetus and is a well documented chemical teratogen.

The mercurial triad has been well recognized and documented in humans. Mercury intoxication was brought into sharp focus as a serious health hazard by the Minimata tragedy in Japan. The earliest effects in humans seen after mercury intoxication are erethism, tremors in the appendages and ataxia. This triad is very often accompanied by weight loss in acutely poisoned individuals. Increased frequencies of congenital anomalies were observed in children of mothers accidentally poisoned with mercury. Experimental investigations with rodents, particularly mice, have indicated that methylmercuric chloride (MMC) is most certainly a teratogen of considerable strength, causing a high frequency of cleft palate, skeletal deformities and hydronephrosis.

The association of chromosomal aberrations with certain syndromes of congenital malformations has raised the question of a possible connection between abnormal chromosome complements and teratogenesis. Genes which control normal development could be altered and thereby contribute to abnormal development if adversely affected by teratogenic chemicals. The relationship between the

ability of a teratogen to produce embryotoxic response and to affect chromosomes has been investigated but not intensively pursued. Now, however, with the development and use of sophisticated cytogenetic probes it is possible to further investigate the intricacies of chromosome structure and it may now be possible to demonstrate the effects of a proven teratogen on fetal chromosomes.

1.2 Purpose of the Study

To elucidate the embryotoxic effects of methylmercuric chloride, a well documented chemical teratogen and environmental pollutant, on the fetuses of ICR Swiss-Webster mice. To determine whether an association or relationship exists between abnormal morphogenesis and chromosomal aberrations. This study may also provide insight as to the mechanisms of teratogenesis with respect to methylmercuric chloride, and also whether a correlation exists between the degree of teratogenicity and cytogenetic changes.

2.0 LITERATURE REVIEW

2.1 Mercury-History and Uses.

The story of mercury began over 4½ billion years ago as the earth was formed. It is an element 1.2 times heavier than lead, with a melting point of -38°F: consequently, it is usually liquid or a gas. It has, through the eons, worked its way throughout the earth's surface and now appears in rocks, soil, water, air and in living organisms.

The sulfide ore of mercury is a colorful rock known as cinnabar. Early man was attracted to it by its red color. When mixed with water he could use it to draw on cave walls. Archaeologists have discovered it in the ruins of ancient Egypt, Babylon and Pakistan where it had been used as a pigment in ancient paintings.

A Greek physician Diocorides wrote that cinnabar was "good for healing eye sores." He also noted that it healed burns and combated the outbreak of pustules but that it was dangerous to health if swallowed. Cinnabar was used in Roman festivals where it reddened the face of Jupiter. Romans also used it practically to separate gold from dust. Ancient Hindus believed it to be an aphrodisiac, and the Chinese saw immortality in its properties and made an elixir with it to drink. Alchemists sought to turn base metals into gold by using mercury and it was they who gave mercury its name - after the

fleet-footed Greek God. It can, therefore be appreciated that mercury has a long and somewhat mysterious past.

Modern man has also found a variety of uses for mercury and as mercury finds new industrial uses, even more incidents of poisoning occur. Mercury vapour and dust are most pernicious to humans and they deal a slow agonizing death manifesting in a mental stupor. Workers in the felt-hat industry dipped furs into vats of mercuric chloride solution to make them more pliable for shaping. These workers eventually exhibited a common triad of mercurial poisoning, tremors, ataxia and mental confusion. The Mad Hatter in "Alice in Wonderland" was patterned after such a typical mercury poisoned victim. "Hatters disease" as it was often known was also called the Danbury shakes named after that Connecticut hat making city.

Mercury poisoning has struck many occupational employees, such as miners, ammunition workers, thermometer fillers, laboratory dental technicians, grain treaters, pulp and paper workers and even policemen who used it as a finger printing compound. Dentists have long used 50% mercury amalgam in tooth fillings because mercury dissolves an alloy of silver and tin and this solidifies on application. There are more than 200 million tooth fillings each year in North American. This means a large portion of the population is exposed to mercury contamination.

Fluorescent light tubes that light offices, schools, and homes contain mercury. Seventy percent of America's lighting is provided by powerful street lamps which are activated by mercury vapours. Mercury is ubiquitous, it can be found in paints, floor waxes, furniture polish and even in fabric softeners. Mercury has been formulated into medicinal antiseptics such as mercurochrome and has been used widely as a diuretic. Farmers also use the chemical compound panogen, which contains mercury to treat seeds to prevent fungal growth.

In modern chemical industries mercury and its compounds play an important role. It is used to help make plastic, paper, clothing and camera film. For decades mercuric fulminate detonated conventional explosives and now with the atomic age plays a new role: participating in separation of lithium 6, an isotope involved in the fusion reaction of hydrogen bombs. Mercury and its compounds are omnipresent throughout the world. It is found on the moon where American astronauts used mercury batteries to power lights, or in our bodies where small batteries power cardiac pacemakers to keep hearts beating steadily.

2.2 Hazards of Mercury Contamination

2.2.1 Accidental Human Exposure and Poisoning

In 1953 in villages around Minimata Bay on the Japanese island of Kyushu, fishermen and their families began to fall victim to a strange and enigmatic disease. They began to suffer from neurological disorders for no known reason. The affected individuals all had one thing in common , they became ill after eating fish on a daily basis. Autopsies on the victims revealed similar findings, a reduction of neurons in the cerebrum and cerebellum. This would explain many of mercury's neurological symptoms: ataxia, convulsions, paresthesia, constriction of visual fields and dysphasia.

Analysis of fish and shellfish taken from the area indicated levels as high as 14 ppm of mercury. Further analysis showed levels as high as 140 ppm of mercury in humans who had eaten seafood containing methylmercury, the most toxic of all mercury compounds. The methylmercury had originated from effluent by the Chisso Corporation into Minimata Bay. Mercury was being used by this group in the manufacture of vinyl chloride, which is subsequently used in the plastic industry. The mercury pollutant was converted to methylmercury by micro-organisms in the Bay and absorbed by the marine life. The flesh of the marine life contaminated with methylmercury were then consumed by the fishermen and their families (1).

A short time later another example of mercury's deleterious effects on animals occurred in Sweden. It was noticed that birds were declining at an alarming rate. Swedish farmers had been treating their grain with the mercury compound panogen and this was polluting the environment. The seed eating birds were the first to vanish and then the birds of prey. Analysis of dead birds confirmed high levels of mercury in their tissues. Farmers had also been feeding treated grain to their domestic animals despite warnings of possible harmful effects (2).

In New Mexico, where a farmer fed his pigs grain treated with panogen, the hogs showed no ill effects from the contaminated grain. However, after slaughtering the hogs and eating their meat, disaster struck his family. All of the farmer's children began to suffer severe neurological symptoms, and a neonate was born with several congenital birth defects. These adverse effects were attributed to high levels of mercury in the pork (3).

Another outbreak of methylmercury poisoning in Iraq resulted when large numbers of people ate bread made from grain which had been treated with panogen. A dose response relationship was very evident as affected people showed overt signs of poisoning and not just mild symptomatic complaints. This was probably the largest and worst outbreak of mercury poisoning recorded to date.

Methylmercury poisoning in adults appears to follow a set pattern. The intake of methylmercury is very high during exposure and mercury concentration in blood is also very high, yet no signs or symptoms of mercury poisoning are experienced during the exposure period. About one month after exposure a mild paresthesia appears and several months later more serious signs such as ataxia and dysarthria appear, followed by visual disturbances, all of which are irreversible. Methylmercury inhibits protein synthesis in the brain before the acute onset of poisoning. It has also been observed that methylmercury tends to accumulate in neuronal cells of the cerebrum, and in the Purkinje and granular cells of the cerebellum (6).

The first indication of fetal mercury toxicity occurred after frequent abortions were noted in women undergoing mercury treatment for syphilis. Mercury was subsequently detected in the aborted fetuses of mercury treated mothers, indicating that mercury was crossing the placental barrier into the fetuses. The question then arose concerning the effects of mercury and its various compounds on fetuses after ingestion by the mother during pregnancy. The extent of maternal mercury intake during pregnancy, its distribution, placental transport, fetal uptake and concentration are all important considerations in understanding subsequent fetal toxicity (9, 10).

Three major epidemics of mercury poisoning and numerous individual cases of mercury poisoning during pregnancy have resulted in a large number of fetal and neonatal cases of mercury toxicity being reported. The majority of the children born alive suffered from cerebral palsy, ataxia, tremors, seizures and mental retardation. The fact that these infants had not ingested contaminated food would suggest that their neurological symptoms resulted from toxicity *in utero* (9-13). The fetus appears to be more sensitive to the effects of mercury than does the adult. This was seen in Minimata where 21 infants had severe neurological disorders associated with prenatal poisoning with methylmercury while the mothers showed no symptoms of mercury poisoning (9). Fetal exposures have also occasionally occurred through the intentional use of mercury compounds during pregnancy as a topical antiseptic, a germicidal and diuretic and for the treatment of syphilis.

Maternal mercury poisoning may also occur as a result of occupational hazards such as in dental offices, or doctors' offices. Exposure may also occur through industrial pollution of the environment and in agriculture through grain treatment. The major exposure to methylmercury however, occurs through consumption of contaminated fish by mothers. Elevated levels of mercury in

humans originate from contaminated waters. The water is transferred through the fishes' gills. Methylmercury becomes concentrated in the fish which is then consumed by humans.

Methylmercury has an excretion half-life of several hundred days in fish which allows for accumulations several thousands times greater than in surrounding water (11-13). Methylmercury is non-polar and can easily cross through membranes and accumulate in fetal tissues at levels much greater than in maternal tissues. This means elevated levels of methylmercury can accumulate within the fetus penetrating the lipid rich nervous system of the fetus (14).

2.2.2 Teratology studies.

The function of the mammalian placenta is to protect the developing fetus from external influences by enveloping it in a stable physical environment and by regulating transport of materials from the mother to the fetus. The placenta, in general, is quite efficient at excluding detrimental substances from entering the fetal blood stream while permitting the passage of essential materials. Methylmercury can cross the placental barrier, the blood- brain barrier, and will concentrate in the brain which was thought to be protected by these barriers. Methylmercury being stable and non-polar can penetrate through cell membranes because of it's lipid solubility accumulating in the lipid rich tissues of

the nervous system (14, 15). It is known that mercury has a great affinity for and attaches to sulfhydryl (sulfur containing) groups of cellular proteins and membranes, hence damaging normal cellular processes.

The effects of _in utero_ methylmercury exposure have been studied in many animal models. Rat, mouse, rabbit and hamster have been the most popular species used for experimental studies of maternal mercury exposure during gestation. Evidence has accumulated from animal studies which indicate that the rate of mercury transfer is much greater in the direction of the fetus from the mother than in the reverse direction. Such a phenomenon may contribute to the "trapping" of mercury in fetal tissues (14). Animal studies, as well as human cases, point to the fact that the fetus has a much greater affinity for methylmercury than the mother and that the fetus is more vulnerable to its toxic effects (15). Experimental work in rats and mice indicate that methylmercuric chloride is a chemical teratogen (16, 17).

Intrauterine death is a frequent result of congenital organomercurial intoxication and is usually manifested by fetal resorption or still-birth. Methylmercury when given during developmentally sensitive periods, particularly during the days of rapid organogenesis, may produce increased fetal mortality even at a relatively low dosage (16). An increased incidence of fetal

deaths and fetal malformations were observed after a single intraperitoneal injection of 8 mg/kg methylmercury on gestation day 8 in golden hamsters. The same dose on day 4 of gestation resulted in a much lower percentage of mortality and malformations while administration on day 10 caused a high rate of mortality but fewer malformations (18).

Congenital malformations are a very frequent embryotoxic manifestation of methylmercury's effect upon developing organisms. The most common malformations produced in mice have been cleft palate, skeletal defects, incomplete ossification of the sternebrae, hydronephrosis and hydrocephalus (16-19). A high incidence of cleft palate in fetuses following methylmercury exposure has been observed in all animal species but is especially prevalent in mice (16, 17). One hundred percent incidence of cleft palate was observed in the fetuses of ICR dams following administration of 25 mg/kg of MMC on day 10 of gestation, approximately 60% was seen at 20 mg/kg dose, 20% at 15 mg/kg dose diminishing rapidly to only 2% at 10 mg/kg dose (17). While the precise mechanism by which MMC induces cleft palate is not known, it has been suggested that protein synthesis may be inhibited and this may disrupt regulatory processes controlling closure of the lateral shelves of the palate (19, 20).

Clinical symptoms of mercury poisoning begin with a numbness felt in the extremities and this is followed by dysarthria, ataxia, dysphagia and finally constriction of the visual field (3). These clinical symptoms appear usually after a latency period of several months following ingestion and/or exposure to methylmercury. The severity of the symptoms depend upon the amount of mercury ingested and the length of the exposure. The neurological disorders are as a result of methylmercury's ability to penetrate the blood brain barrier and concentrate in the C.N.S. Methylmercury appears to have a propensity for neurons of the cerebrum and cerebellum manifesting in impairment of coordination of muscle movements and mental slowness (4).

The levels of mercury in the blood of fishermen in Minimata were generally less than 100 ppm, but some had blood levels in the 100-200 ppm range. This compares to blood levels of mercury of 250-400 ppm for adults studied in Iraq (5). It appears that it may not be the length of exposure to mercury that is important but more the total body burden of mercury. This can be seen in the two groups: the fishermen had relatively mild symptoms but long exposure, whereas the Iraq poisonings were of short duration but mercury levels in the contaminated food was very high (5).

2.2.3 Light Microscopic Studies

Neuropathological lesions resulting from fetal methylmercury poisoning have been investigated and reported by Japanese researchers (21). The most severe methylmercury induced congenital abnormalities are exencephaly, encephalocele and hydrocephalus (21). Light microscopical studies of the C.N.S. in congenitally affected animals reveal a variety of pathological lesions. Loss of neurons and cytoarchitectual rearrangement were seen in the fetuses exposed to 5 mg/kg methylmercury during gestational days 8-12 (17). Cerebellar lesions primarily involved the granular neuronal cell layers. Hypoplastic and atrophic Purkinje neurons were observed in rats following chronic methylmercury poisoning during their development resulting in ataxia (22). The decrease of cell numbers after methylmercury treatment produced a persistent hypoplasia. The mechanism of this hypoplasia appears to be related to the antimitotic effect of methylmercury (23).

2.2.4 Electron Microscopic Studies

Ultrastructural evidence of cellular damage in the brains of rats and mice whose mothers were fed doses of methylmercury during gestation has been reported (24). In the cerebellum, both Purkinje and granular cell neurons contain abnormal accumulations of lysosomes. Disorientation of rough endoplasmic reticulum was also seen in Purkinje cells (25-27). Abnormalities in myelin formation, with accumulation of large myelin ovoids and degenerating axons

were seen in the cerebellum of animals a year after they were exposed to a single prenatal administration of methylmercuric chloride via their mother (26, 27). Other reported neurological disorders caused by methylmercury poisoning relate to visual defects. The rod photoreceptors cells of the retina are very active in protein synthesis and are particularly susceptible to methylmercury intoxication. Normal protein synthesis was disrupted by mercury's toxic effects and may account for this sensitivity (27, 28).

The livers of male adult rats injected daily with 10 mg/kg MMC for 5 weeks were examined for ultrastructural changes (29). A moderate accumulation of glycogen and proliferation of smooth endoplasmic reticulum was found in hepatocytes 2 weeks after intoxication. Edema and other degenerate changes were observed in mitochondria of the liver 3-4 weeks after intoxication. Large cytosegresomes containing degenerated organelles were plentiful after 5 weeks of exposure. Pathological degeneration of some hepatocytes persisted throughout the recovery period probably representing the residual and recirculating effects of mercury in the liver (29).

2.2.5 Biochemical studies

Mercury compounds affect many cellular and subcellular systems. They have a high affinity for proteins, amino acids, purines, pyrimidine and nucleic acids (28, 30-34). This affinity and

subsequent binding to proteins causes denaturation and loss of biological activity. This may be explained by the interaction of mercury with these molecules, since it is known that mercury is a heavy metal and will cause protein coagulation. By attaching to sulfhydryl groups and disulfide bonds of cell membrane proteins, mercury compounds alter the selective permeability of membranes causing a loss of homeostasis.

Mitochondrial membranes interacting with mercury can result in suppression of oxidative phosphorylation, calcium transport and phosphate accumulation and this induces edematous changes in the mitochondria (30). These changes in mitochondrial function and structure are important ultimately since inhibition of oxidative enzymes will result in cell death (32).

Mercury may also interact with the phosphate and base groups of nucleic acid. This may cause denaturation and alterations of the intrinsic viscosity of DNA (34). Mammalian ribosomes are a favorite binding site for mercury since it may contain as many as 120 sulfhydryl groups for which mercury has a great affinity (35). This would strongly suggest that methylmercury exposure would inhibit important regulatory mechanism inherent to mRNA and rRNA, inhibiting active protein synthesis (36).

2.2.6 Cytogenetic studies

2.2.6.1 Conventional analysis of chromosomes

The association of chromosomal changes with certain syndromes of congenital malformations has raised the question as to the possible role of abnormal chromosome complements in teratogenesis. The relationships between the ability of a teratogen to produce embryotoxic responses and to affect chromosomes have been investigated but these studies have been limited to analysis of chromosome numbers and aberrations (37-39).

A teratogenic dose of 6-aminonicotinamide, administered to pregnant mice on day 13 of gestation, produced a high incidence of cleft palate in the fetuses with an increase of polyploidy and fragmentation of the chromosomes (39). In Sweden, chromosome analysis of human lymphocytes showed increased frequency of chromosome breakage associated with elevated levels of blood mercury (43). Animal studies using embryotoxic doses of x-irradiation, administered to pregnant rats on day 13 of gestation, found detectable chromosome abnormalities in the embryos of the treated pregnant rats. There was also a marked increase in the number of abnormal metaphases in cells from treated rat embryos within the first 24 hours after treatment (44).

Although the production of chromosomal aberrations in eukaryotic cells by ionizing radiation has been observed, no completely satisfactory theory of chromosomal aberration production has emerged. Ionizing radiation does produce chromosome aberrations which probably result from a double stranded DNA break, together with single-strand breaks and probably other DNA lesions. This is in contrast with most other chromosome breaking agents, which appear incapable of producing double-strand breaks (45). Less spectacular results were obtained following the administration of teratogenic doses of nitrogen mustard, chlorambucil, streptonigrin and hydroxyurea to pregnant rats. The embryos from rats treated with nitrogen mustard and chlorambucil showed a peak of less than 20% metaphase configurations with chromosome abnormalities. Streptonigrin produced less than 10% while hydroxyurea produced no detectable chromosome abnormalities in the embryos (38).

Methylmercury has been shown to cause chromosome breaks in plant tissues (46). It has also been shown to inhibit mitosis in plant cells causing a c-mitotic effect (47). It was also found that mercury compounds produce a similar c-mitotic effect on grasshopper chromosomes at concentration 1000 times lower than colchicine (48). The chromosome breaking effect has been attributed to a

direct reaction between mercury and the chromosome material, while the effects on cell division have been probably due to interaction of mercury with sulfhydryl groups of spindle proteins resulting in their deactivation (48-50). Several authors have concluded that even low level concentrations of mercury have an important effect upon aneuploidy and c-mitosis, but it appears to take higher levels exposures of mercury for the induction of chromosome aberrations (51, 52). Other studies utilizing conventional methods have shown that mercury exhibited a strong toxic effect on human leukocytes *in vitro*. The chromosomes became clustered (clumped) in the center of the cell as a dense lump (53). All of the formentioned studies have had their analysis of chromosomal aberrations restricted to the classical types such as structural changes (fragmentation, breaks, gaps, deletions), numerical changes and disruption of cell mitosis (54).

2.2.6.2 Sister chromatid exchange (SCEs)

In mammalian cells the presence of chromosomal aberrations in response to chemical agents had been used as a measure of chromosomal damage for many years. In the early 1970's more refined techniques were developed which sensitively detected the effect of chemicals on chromosomal DNA (55). The analysis of sister chromatid exchange frequencies is a very sensitive approach for assessing chromosomal damage. The technique has been used successfully to detect the degree of mutagenicity of

chemicals (56, 57). The majority of investigations were carried out using in vitro systems in cell cultures. The technique involves differential staining of sister chromatids which visually shows any exchange between sister chromatids. This technique has made it possible to distinguish sister chromatids from one another and is known as sister chromatid exchange (SCE) (56).

Sister chromatid exchange involves the interchange of DNA between replication products at homologous loci, without changing the overall morphology of the chromosome (57). Autoradiographic procedures were first used to differentiate the sister chromatids, but poor visual distinction of the chromatids rendered this procedure unequivocal (59). Recent advances have made it possible to differentiate between chromatids without the use of radioisotopes and autoradiography (60, 61). These newer techniques were based on the premise that after two rounds of replication in the presence of 5-bromodeoxyuridine (BudR), a thymidine analogue, the BudR is incorporated into the DNA replacing thymidine (61). The chromosomes contain one chromatid in which DNA is unifiliarly substituted with BudR and one chromatid in which the DNA is bifiliarly substituted with BudR. This will allow cytological distinction to be made between the sister chromatids after staining. The two chromatids are visually distinguishable after staining with the fluorescent dye Hoechst followed by Giemsa stain. The unifiliarly substituted sister chromatid stains very darkly while the bifiliarly substituted chromatid will stain lightly

giving the chromosome a "harlequin-like" appearance. It has been speculated that the incorporation of BudR has loosened the DNA molecule making it less compact. This allows the fluorescent dye to bind with the DNA, subsequently blocking the Giemsa dye from staining bifiliarly substituted chromatids but not blocking Giemsa from staining unifiliarly substituted chromatids (57).

It has been found that SCEs occur as much as 200 times more frequently than do chromosome and/or chromatid breaks and therefore probably represent a much more sensitive indicator of chromosome damage induced by extrinsic agents (55, 62). Several possible repair mechanisms of SCE have been suggested; it is believed, that gaps formed in newly synthesized DNA are filled by recombination with complimentary DNA strands. That *de novo* DNA synthesis may take place in the gap rather than strand exchange has also been suggested as a possible mechanism. DNA strands from the unaffected sister chromatid may also be borrowed and function as a new template for replication at the gap to bypass the lesion (63).

It would seem that SCEs point to a direct relationship between DNA damage and DNA repair, with the observed exchange being the result of a repair mechanism (66). There appears to be several types of repair mechanisms by which the damage caused by hazardous agents can be ameliorated. Repair systems consist of highly specific enzymes with restricted substrate specificities. Photoreactivating enzymes revert one kind of damage: cyclobutane

pyrimidine dimers induced by ultraviolet light are reverted back to the original two separate thymine nucleotides. This type of repair is usually only seen in bacteria.

Excision repair is the most versatile repair mechanism and is the main repair system in mammalian cells. This system excises damaged DNA and replaces it with proper segments much like patch repair. The damage is firstly excised by an endonuclease and then repaired by replication by a polymerase and subsequently annealed by ligation (64). Post-replication repair, on the other hand, is activated during semi-conversative DNA replication and represents a modification of normal replication. This is really a transient perturbation of DNA replication that involves blocked replication resulting in gaps which are then filled in by *de novo* synthesis (65). Errors in DNA can be generated through faulty replication and/or repair which results in chromosome aberrations and SCEs. The incidence of SCEs in chromosome breakage syndromes has been studied and in some cases abnormal DNA repair processes have been identified in these disorders as the cause of SCEs.

Xeroderma pigmentosum was the first disease to be identified as a DNA repair deficiency disease. Fanconi anemia, Ataxia telangiectasia and Bloom syndrome are other disorders which are considered to be deficient in repair mechanisms and all are inherited as autosomal recessive disorders. Xeroderma pigmentosum normally exhibits a stable SCE level and the chromosome aberration

frequency. After exposure to ultraviolet light, however, the cells show a great increase in SCEs and chromosome aberrations. This may be due to the sensitivity of SCE production to small amounts of unexcised DNA damage resulting from excision repair defect (64).

Fanconi anemia patients exhibit no increase in SCEs but a marked increase in chromosome aberrations. Their cells are very sensitive to death caused by cross-linking agents (66). This disorder is due to a defect in DNA repair of cross-linked damage and may be acquired after heavy metal exposure as well as inherited.

Bloom syndrome, a disease which exhibits a sensitivity to sunlight, has a high incidence of SCEs and chromosomal aberrations. Associated with both the increased SCEs and chromosomal aberrations after ultra violet exposure is a defect in post-replication repair (67).

Ataxia telangiectasia exhibits a stable level of SCEs but a high incidence of chromosome aberrations. There is, however, an increased sensitivity to death caused by x-rays with an increase in SCE frequencies. It appears that some x-ray induced lesions are not excised and repaired and this may involve some defect in DNA excision repair (68).

Sister chromatid exchanges have proven to be very useful in confirming several chromosome fragility disorders (68). It has also been shown that all agents (physical and chemical) which have been shown to induce SCEs may also give rise to chromosome aberrations (68-71). Therefore, agents which induce strand breaks in DNA as well as agents which influence the DNA repair processes necessary for subsequent healing of breaks should be able to influence the SCE frequency (71, 73). Sister chromatid exchange technique utilizing BudR has been widely used in both *in vitro* and *in vivo* systems to test the mutagenicity of a large number of agents and has proven to be an accurate indicator of DNA damage (69-77). The exact nature of SCEs is yet to be unequivocally established but whatever its exact nature, a chemically induced increase in SCEs represents an alteration in chromosome structure which is not present if the chemical is absent (78). Hence, such changes must be viewed as potentially deleterious. There have been to date very few published *in vivo* studies which have utilized SCEs using BudR to estimate the effects of methylmercuric chloride on fetal chromosomes (79).

2.2.6.3 Nucleolus organizing regions (NORs)

The structural genes for 18S and 28S ribosomal RNA (rRNA) are clustered on specific chromosome sites, known as the nucleolus organizer regions (NORs). Such rRNA gene clusters are localized to the secondary construction regions of all five pairs of human

acrocentric chromosomes (80, 81). In mice, which have 20 pairs of acrocentric chromosomes, there are three to six pairs of chromosomes which have rRNA cistrons or NORs (85). Each NOR is located on the long arm of a chromosome near the centromere.

A silver nitrate-ammoniacal silver technique has been developed which detects nucleolus organizing regions (NORs) on metaphase chromosomes as spherical 'black bodies' (81, 82). This technique will demonstrate only NORs whose rRNA cistrons are active during interphase and not those that are suppressed (83). The silver nitrate technique has been used to study various mammalian species thus NORs are a convenient cytogenetic tool to study gene activity (86).

The nucleolus organizer appears to be vital for cellular life. The loss of NORs or mutation of necessary operator genes which controls the duplicate genes of NOR, prevents synthesis of 18S and 28S rRNA subunits. This will result in the death of the organism. Evidence of this function comes from the mutant NORs detected in the South African clawed toad (Xenopus laevis). The normal toad has two NORs, one on each of two particular chromosomes. Each cell will therefore contain two nucleoli if both NOR are expressed. One mutant has only one NOR and subsequently one nucleolus per cell, while another mutant strain has no NORs or nucleolus. The homozygous mutant is deficient in rRNA without which the organism cannot survive. Consequently, the anucleolar homozygote has less

nuclear and cytoplasmic RNA and is retarded in development causing its early death (87, 88).

Obviously, the nucleolus organizing region is essential for survival because the NOR is an active site for rRNA production, without which, the organism cannot exist for a protracted time period. Once the store of rRNA is exhausted in anucleolar nuclei and the nucleus is unable to replenish the supply, protein synthesis will cease, resulting in cellular death. The nucleolus must then be viewed as a detachable product of the chromosome rDNA (NOR), renewal at each successive cell cycle.

The cyclic behavior of the nucleolus is based on the premise that it must detach from the chromosome (rDNA) in order for clean anaphase segregation of the chromosomes producing chromatids (87). The loss of expression of rRNA cistrons (NOR) due to chromosome damage or mutation of the necessary operator gene would surely result in inhibition of 18S and 28S rRNA and place the cell's survival in jeopardy.

In has been observed in mice embryos that activation of rRNA cistrons (NOR's) occurred as early as the 2-cell stage. At the 4 cell stage 4-6 chromosomes had activity at their NORs and at the 8 cell stage all 6 chromosomes having NORs reacted to silver staining (89). The silver nitrate ammoniacal silver technique reflects the reactivity and staining of active rRNA cistrons (NORs) rather than just their presence (89). The activation of NORs depends not upon

a specific embryonic stage but rather the time interval following ovulation which indicates that initiation of NORs is regulated by specific molecules (90).

Presently, there are no published reports which would indicate the effects of teratogens on the expression and/or suppression of NORs. It is known that NORs are attenuated areas of the chromosome and may be susceptible to damage from the action of a teratogen (91). The nucleolus must be renewed each successive cell cycle, hence, could be preferentially damaged by chemically induced lesions to NOR. Therefore, the investigation of the activity and expression of NORs by the silver staining method in abnormal fetuses from methylmercuric chloride treated animals may prove to be of interest in determining if a relationship exists between the ability of a teratogen to cause recognizable alteration in NOR and abnormalities.

3.0 MATERIALS AND METHODS

3.1 Animals

ICR Swiss Webster mice were chosen for this study because it has been observed that the offspring of this strain has a particularly high incidence of cleft palates when treated with methylmercuric chloride during pregnancy (17). Male and female mice were caged together overnight and examined on subsequent mornings for copulatory plugs. A copulatory plug confirmed mating and this was then taken to be day 0 of gestation. The pregnant female was then housed alone and weighed at regular intervals during pregnancy.

3.2 Treatments

3.2.1 Fetal studies

Methylmercuric chloride (MMC) is a proven teratogen and is the test substance utilized in this study. Methylmercuric chloride (1000 ppm) was administered as a single dosage using an intragastric tube on day 9 at dose levels of 5, 10, 15, 20, 25 and 30 mg/kg of body weight. Control animals received a corresponding volume of isotonic saline.

On day 17 of gestation, 30 hours before killing the pregnant animals, a 50 mg tablet of 5-bromodeoxyuridine (BudR) was implanted subcutaneously in each mouse. The animals were injected with colchicine, 4 μg/g body weight intraperitoneally, four hours prior to killing. On day 18 of gestation, the pregnant mice were anesthetized and then laparotomized.

3.2.2 C-mitotic studies

Adult male mice of the ICR Swiss Webster strain were administered a single intragastric dose of methylmercuric chloride (1,000 ppm) at dose levels of 5, 10, 15, 20, 25 and 30 mg/kg of body weight. Control animals were given a corresponding volume of isotonic saline. Six hours after treatment the animals were anesthetized and killed. Duodenal tissue samples were removed and fixed in 10% neutral buffered formalin.

3.3 TERATOLOGICAL STUDIES

3.3.1 Maternal Weight Changes

The weight of each pregnant mouse was closely monitored and recorded at days 1, 9 and 18 of gestation. The difference in weight change was calculated between time of treatment and recovery of the fetuses. The average (mean) and standard deviation were calculated for each group of treatment and control. The weight change in the control group was used as a base line and the treatment groups were compared as a percentage to the control group. Statistical analysis, consisting of analysis of variance and coefficient of correlation between groups, was done to determine the level of significance.

3.3.2 Resorptions and Fetal Deaths

Following ether anesthetization and sacrifice of the pregnant animals on day 18 of gestation, the uterine horns were exposed.

The number of live and dead fetuses and resorption sites were recorded. All data relating to litter size, resorptions, fetal deaths and total number of implantation sites were noted and statistically analyzed by the analysis of variance and coefficient of correlation to determine the level of significance.

3.3.3 Fetal Weights

Fetuses that were recovered following laparotomy and hysterotomy were placed in Bouin's fluid. The fetuses were dissected free of the placenta and all fetal membranes and weighed. The data were recorded and statistically analyzed.

3.3.4 Gross Anatomical Analysis

The fetuses were subsequently examined for gross abnormalities. The fetuses were further examined for internal abnormalities by slicing the fetus into 1 mm cross sections (Wilson sections) and examined with a dissecting microscope (92).

3.3.5 Skeletal Staining

Randomly sampled fetuses from each litter (1 per litter) were used for skeletal analysis. The fetuses were eviscerated, dehydrated in 95% ethyl alcohol, stained with Alizarin Red S and cleared by 10% KOH to demonstrate the skeletal system (93).

3.4 CHEMICAL ANALYSIS OF MERCURY LEVELS

3.4.1 Maternal Analysis

Maternal mice were killed on day 18 of gestation and laparotomy performed. Blood from the mothers was collected by cardiac puncture, frozen and stored at -20°C. The samples were then analyzed for mercury levels using Hendzel and Jamieson's automated technique (94).

3.4.2 Fetal Analysis

From each litter, one fetus was randomly sampled, frozen and stored at -20°C. The fetuses were then analyzed for mercury levels (94). The data were collected from maternal and fetal samples and statistically analyzed .

3.5 LIGHT MICROSCOPY

3.5.1 Preparation of Tissues

Pregnant mice were treated with methylmercuric chloride, BudR tablet and colchicine. The fetuses were removed on day 18 of gestation and lungs were removed with the aid of a dissecting microscope. The lungs were immediately fixed for 48 hours in 10% neutral buffered formalin and subsequently processed in a

Histomatic processor and embedded in Paraplast. Five micra sections were cut on a AO Spencer rotory microtome. The sections were stained with Periodic Acid Schiff to demonstrate the presence of glycogen (95).

3.5.2 Examination of Tissues

The fetal lung tissues, which were stained with P.A.S., were examined with a Leitz Dialux 20 binocular microscope. Black and white photomicrographs were taken of lungs from each group with a Wild-Leitz MPS 45 - 35 mm camera mounted on the Leitz Dialux microscope.

3.6 ELECTRON MICROSCOPY

3.6.1 Preparation of Tissues

Lung and liver tissues were removed from fetuses on day 18 of gestation. The tissues were immediately fixed in 3% glutaraldehyde buffered at pH 7.4 using Millionig's buffer containing 10% dimethylsulfoxide and 7.5% sucrose for 60 minutes at 4°C (96). The tissues were then diced into small blocks with a razor blade and fixation continued for another 1-3 hours at 4°C. The tissues were then rinsed in Millionig's buffer and post fixed in 1% osmium

tetroxide buffered in 0.1m Sorensen's pH 7.4 for 2 hours. Tissues were then dehydrated with graded series of ethanol/methanol solutions and embedded in small gelatin capsules with Araldite.

The tissues were cured at 60°C for 18-24 hours. They were cut on an LKB ultratome III and gold to silver sections were taken up on copper 200 mesh grids, blotted and dried. The grids were stained in aqueous uranyl acetate and lead citrate.

3.6.2 Examination of Tissues

Thin stained sections were examined using a Philips-201 electron microscope. Black and white photomicrographs were taken from all groups.

3.7 CYTOGENETIC STUDIES

3.7.1 Preparation of Slides

All animals were treated with methylmercuric chloride or saline, BudR tablets and colchicine. Fetuses recovered on day 18 following laparotomy were dissected free of all fetal membranes and placed in sterile isotonic saline. The fetuses were dissected and various fetal tissues removed. Approximately 100 mg of fresh tissue was transferred to freshly made 1% sodium citrate (hypotonic solution)

and mildly agitated with a pasteur pipette to make a cell suspension and left for 15 minutes (97). Following hypotonic treatment the suspension was centrifuged for 5 minutes and a pellet collected. The pellet is then resuspensed in 3:1 methanol/acetic acid fixative for 30 minutes. The supernatant was poured off and the pellet was agitated to make a suspension. A few ml of suspension were decanted into a small dish and 0.5 ml of 60% acetic acid was added until a homogenous mixture was obtained (98). The cell mixture was then used to make chromosome spreads. Two ml of the mixture was drawn up into a pasteur pipette and transferred onto a clean warm microscope slide and then quickly drawn back into the pipette. This procedure was repeated several times on different regions of the slide. The cells migrate to the periphery of the drops and are fixed onto the warm slide and this allows for adequate metaphase preparations permitting chromosome identification. The slides were then dried and subsequently used for assessing different cytogenetic parameters.

3.7.2 Conventional Staining and Chromosome Analysis

Metaphase preparations were stained with 5% Giemsa stain made up in Gurr's buffer pH 6.8 for 5-7 minutes. The slides were then rinsed in Gurr's buffer twice and allowed to air dry. The slides were cleared with xylol and mounted with permount. One hundred cells were examined for clumping of the chromosomes from each of the treatment and control groups. Clumping was interpreted as a chromosomal aberration (54).

Further analyses of conventionally stained chromosomes to determine the number of chromosomes per metaphase, fragmentation, gaps and deletions of chromosome were carried out on 100 cells for each treatment and control group. The data were statistically analyzed.

3.7.3 Sister Chromatid Exchanges (SCEs) staining and analysis

The demonstration of SCEs required that cells be exposed to BudR continually during two consecutive cell cycles. Metaphase spreads were prepared as described previously and slides from each treatment group and control were stained in the fluorescent dye Hoechst 33258 for 12 minutes; they were washed in distilled water and mounted in distilled water then sealed with rubber cement. The slides were then exposed at a distance of 60 cms. to ultraviolet light for 12 hours. The coverslip was removed and the slides were incubated in 2x SSC for 1 hour at 60°C, then rinsed in distilled water and stained in 4% Harleco Giemsa made up in Gurr's buffer for 5 minutes. The slides were then rinsed in distilled water, air dried, cleared in xylol and mounted with permount.

This technique allows differential identification of the sister chromatids and microscopic determination of the sites and frequency of sister chromatid exchanges. Fifty metaphase cells were examined and the frequency of SCEs determined. Photomicrographs were prepared using Kodak Panatomic-X film. The data were statistically analyzed.

3.7.4 Nucleolus Organizing Regions (NORs)

Metaphase preparations were obtained using the method previously described. The development of a cytochemical staining reaction which gives improved visual identification of NORs was employed to stain slides from each treatment group and control group (83). The slides were flooded with 50% silver nitrate for 10 minutes and exposed to a heat lamp at a distance of 25 cms. After rinsing with distilled water the slides were flooded with 4 drops of ammoniacal silver nitrate and 4 drops of 3% formalin (pH 5.0) and exposed to the heat lamp until the cells turn a brownish-yellow color. The slides were then rinsed in distilled water, dehydrated with alcohol, cleared with xylol and mounted with permount.

The slides were examined with a Leitz microscope. The NORs were demonstrated as discrete black bodies on the chromosomes. Fifty cells from each dose level and control were examined, and the frequency of NORs was determined. Metaphase cells were photographed using Kodak Panatomic-X film. The data was statistically analyzed.

3.7.5 C-mitosis (processing tissue and preparation of slides)

Adult male mice of the ICR Swiss Webster strain were given methyl-mercuric chloride (1,000 ppm) administered intragastrically,at dose

levels of 5, 10, 15, 20, 25 and 30 mg/kg of body weight. The animals were killed six hours later. Tissue samples of the duodenum were taken and fixed immediately in 10% neutral buffered formalin for 48 hours (99). The tissues were processed, embedded in Paraplast and cut at 5 micra on the AO spencer rotary microtome.

3.7.6 C-mitosis (staining and analysis)

Tissue sections were stained with hematoxylin and eosin. The stained sections were examined with the Leitz microscope and 1000 cells in the crypts of the duodenum were counted at each dose level and from the control.

The cells arrested in a clumped metaphase condition were counted and frequency determined. Photomicrographs were prepared using a Wild-Leitz microscope and camera as previously mentioned. The data were statistically analyzed .

3.8 STATISTICAL ANALYSIS

3.8.1 Analysis of Variance - (ANOVA) was used to compare mean differences involving several independent variables.

The level of significance (acceptable level of probability) $p <$ 0.05 or less allowing rejection of the null hypothesis, meaning the results obtained were significantly different from control.

$p < 0.05$ * significant

$p < 0.01$ ** very significant

$p < 0.001$ *** highly significant

3.8.2 <u>Coefficient of Correlation</u> - is used to indicate the linear relationship between two variables; a measure of linear correlation: dose-response association.

$r = 0$ (no correlation)

$r = +1.000$ (positive correlation)

$r = -1.000$ (negative correlation)

The higher the correlation value (r) the greater the level of significance.

4.0 RESULTS

4.1 Teratological studies

Treatment with methylmercuric chloride at six dose levels to pregnant ICR Swiss-Webster mice produced significant findings as summarized in Table 1. A high percentage of intrauterine death, fetal resorptions and malformations was induced by treatment with methylmercuric chloride.

4.1.1 Maternal weight changes

Maternal weights were measured and recorded on day 1, 9 and 18 of gestation. Table 2 shows the maternal weight changes between time of treatment (day 9) and time of fetal recovery (day 18) for the six dose levels and the control group. The maternal weight change for each treatment group was compared to the maternal weight change for control group and was expressed as a percentage of the mean weight increase of the control (control weight increase being 100 percent). If the weight at sacrifice was greater than the weight at treatment it was expressed as a positive (+) change and if the weight was less at sacrifice than it was at treatment, it was then expressed as a negative (-) change, indicating a weight loss. All dose levels, except the two highest dose levels (25 and 30 mg/kg) showed a positive weight change. The positive weight change was greatest for the lowest dose level (5 mg/kg = +94.7%) but this steadily diminished

(20 mg/kg = +18.5%) until negative changes were observed at (25 mg/kg = −15.3%) and (30 mg/kg = −30.5%). Statistical comparison of data using analysis of variance indicated differences between the mean weight change in treatments and control were highly significant ($p < 0.001$). Figure 1 shows a linear relationship between dosage and maternal weight changes as illustrated by the coefficient of correlation value ($r = -0.98021$).

Table 1

Effects of methylmercuric chloride (MMC) administered via intragastric tube on day 9 of gestation in pregnant ICR Swiss Webster mice

Dosage (MMC)	Number of animals	Implantations		Live fetuses		Malformations		Intrauterine deaths		Resorptions	
		n	$\bar{x}$	n	(%)	n	(%)	n	(%)	n	(%)
Control	20	210	10.5	209	(99.6)	0	-	0	-	1	(0.4)
5 mg/kg	12	158	13.2	129	(82)	0	-	0	-	29	(18)
10 mg/kg	8	96	12.0	75	(78)	17	(22)	6	(6)	15	(16)
15 mg/kg	8	106	13.2	82	(77)	57	(70)	8	(8)	16	(15)
20 mg/kg	8	97	12.1	34	(35)	34	(100)	43	(44)	20	(21)
25 mg/kg	5	55	11.0	0	-	0	-	20	(36)	35	(64)
30 mg/kg	5	59	11.8	0	-	0	-	0	-	59	(100)

$\bar{x} \pm$ S.D.
11.97±1.01

$P<0.02$
$r = 0.8612$
(between all treatment groups and control)

Table 2

Maternal weight changes between the time of treatment (day 9) and recovery of the fetuses (day 18)

Dosage (MMC)	Weight change (gms) $\bar{x}$ ± S.D.	Percentage change
control	(+)46.17 ± 10.79	(+) 100%
5 mg/kg	(+)43.74 ± 9.31	(+) 94.7%
10 mg/kg	(+)34.76 ± 12.33	(+) 75.3%
15 mg/kg	(+)26.89 ± 9.34	(+) 58.2%
20 mg/kg	(+) 8.55 ± 7.48	(+) 18.5%
25 mg/kg	(-) 7.14 ± 7.82	(-) 15.3%
30 mg/kg	(-)14.08 ± 10.51	(-) 30.5%
	$P<0.001$ (between all treatment groups and control) $r = -0.98021$	

+ and - indicate a positive or negative maternal weight change between day 9 and day 18 of gestation. The percentage increase or decrease is also indicated as + or - as compared to the control weight change which is taken as a (+)100% positive increase.

FIGURE 1. Maternal weight changes, mean ± S.D. between day 9 and 18 of gestation following Methylmercuric chloride treatment.

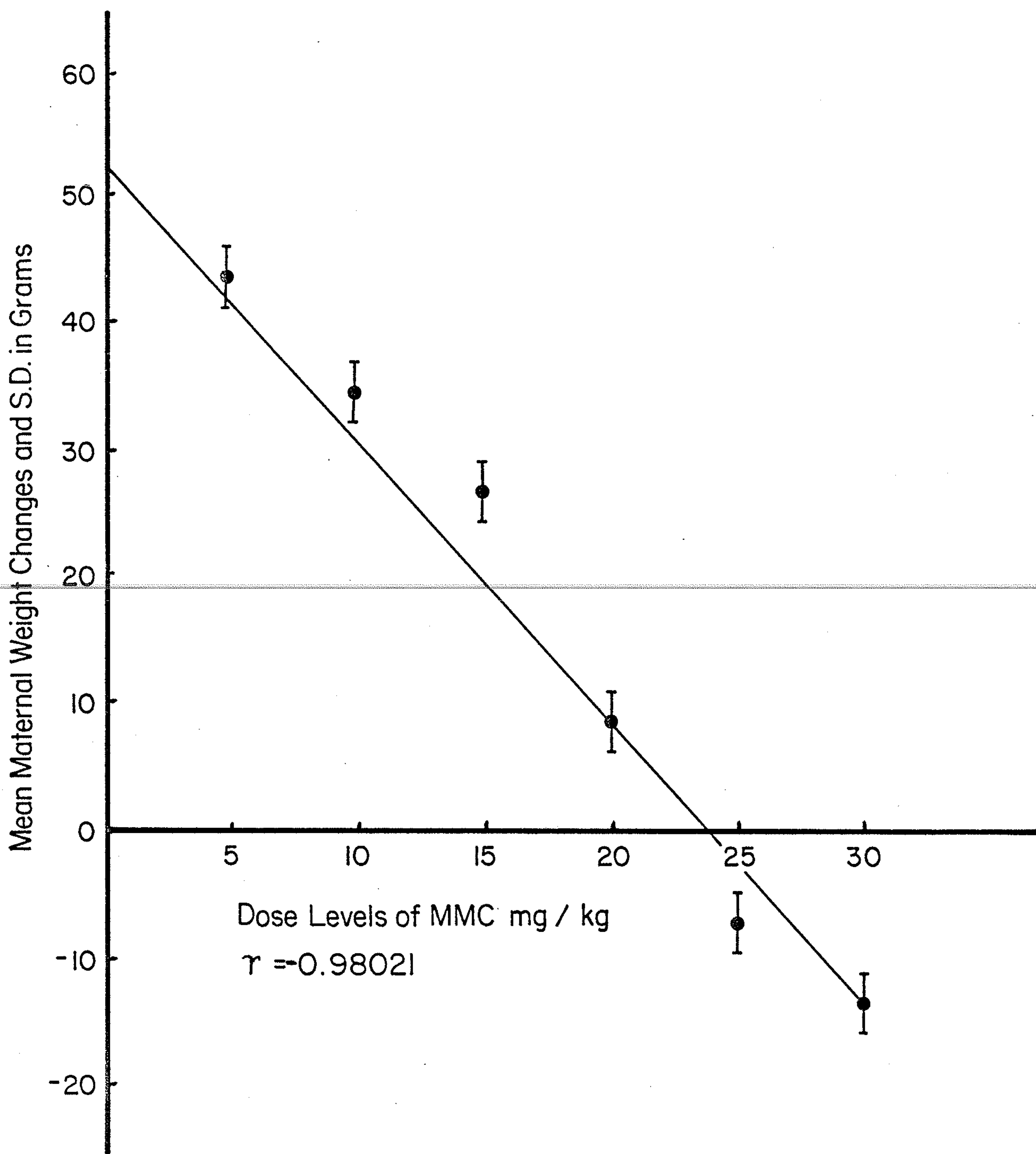


Fig. 1

4.1.2 LD_{50} of MMC in maternal mice

The LD_{50} dose of MMC in pregnant mice as shown in Table 3 is 20 mg/kg. Eighty seven and one half percent (87.5%) of the pregnant mice died after treatment with 25 mg/kg while 100% of the mice died after treatment with 30 mg/kg of MMC compared to 0% for control, 5 mg/kg and 10 mg/kg. Statistical analysis revealed a difference between the number of deaths in treated groups as compared to control resulting in a probability value of less than one percent ($p < 0.001$). Figure 2 shows that a linear relationship exists between increasing dosages of MMC and frequency of maternal deaths which is expressed by the coefficient of correlation value (r = 0.99217).

4.1.3 Total implantations

Table 1 shows the total number of implantations and the mean ($\bar{x}$) per group from treatments and control. The average number of implantations and standard deviation of the six treatment groups and control was $\bar{x} = 11.97 + 1.01$.

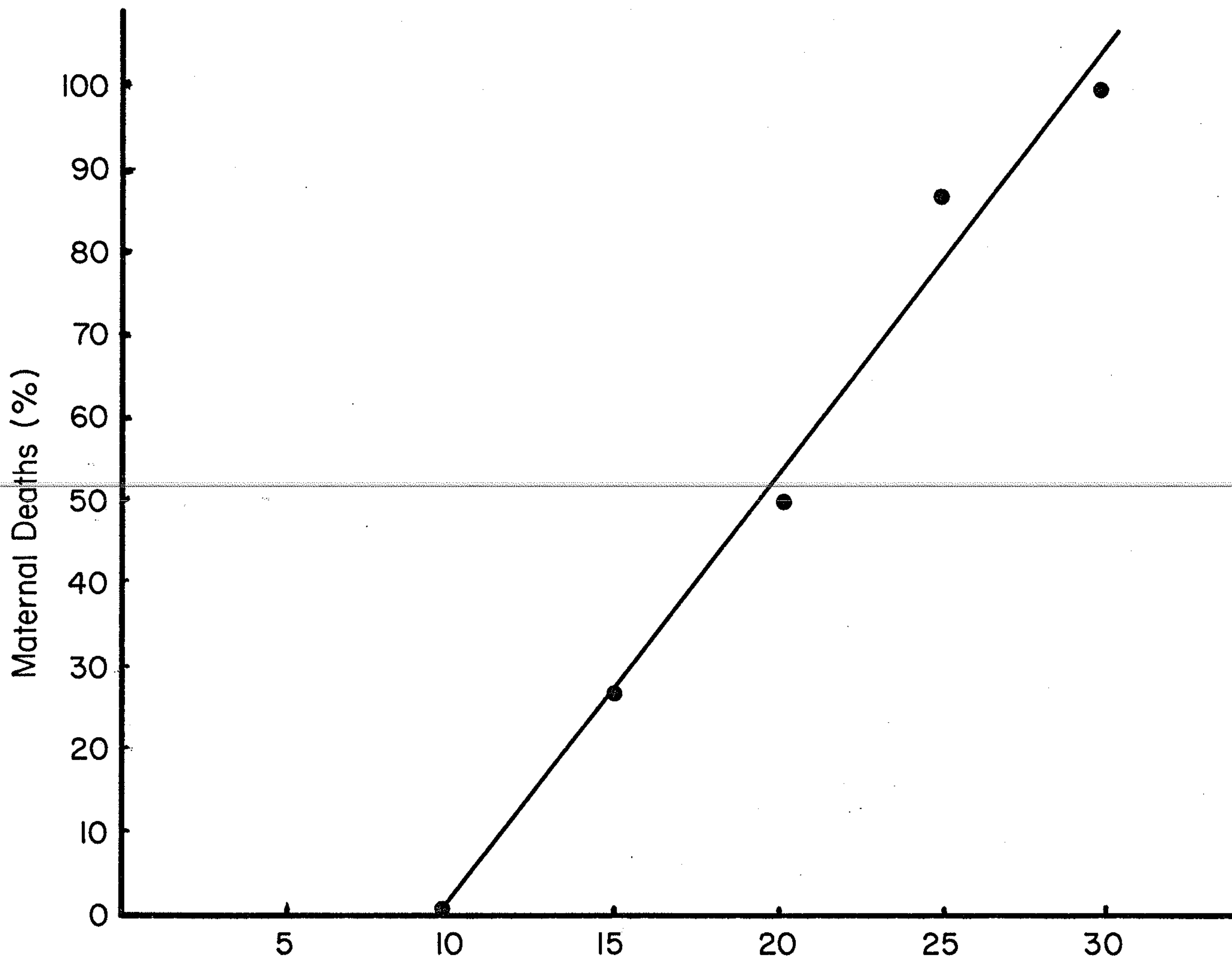
Table 3

LD_{50} dosage of MMC in pregnant ICR Swiss Webster mice

Dosage (MMC)	Number of pregnant animals	Number of deaths n	%
Control	12	0	(0)
5 mg/kg	10	0	(0)
10 mg/kg	10	0	(0)
15 mg/kg	11	3	(27.2)
20 mg/kg	16	8	(50.0)*
25 mg/kg	10	9	(87.5)
30 mg/kg	10	10	(100.0)

* LD_{50} = 20 mg/kg $p<0.001$ (between all treatment groups and control)

FIGURE 2. Maternal deaths following methylmercuric chloride treatment.



Dose Levels of MMC mg / kg

$r = 0.99217$

LD_{50} = 20 mg/kg

Fig. 2

4.1.4 Resorptions

Both partial and complete resorptions were recorded from controls and experimental animals from all treatment doses. Table 1 shows that the number of total resorptions steadily increased from 0.4% in controls to 100% in 30 mg/kg treatment dose. The coefficient of correlation value (γ = 0.8612) and the probability value as determined by analysis of variance was less than two percent ($p < 0.02$). Figure 3 shows fetuses at various stages of resorption from three groups of animals, (A=25 mg/kg, B=20 mg/kg, C=15 mg/kg). All fetuses from mothers treated at the 30 mg/kg dose level were totally resorbed.

4.1.5 Intrauterine death

The frequency of intrauterine death in treated animals are shown in Table 4. Included in the total of fetal deaths were those that occurred late in the pregnancy which were not in a state of resorption and those fetuses which had died shortly after treatment and were partially resorbed. Figure 3 shows dead fetuses and partially resorbed fetuses compared to control fetuses which were recovered alive. The LD_{50} for fetuses was determined to be at 17 mg/kg treatment level and LD_{100} was found to be the 25 mg/kg treatment dose.

FIGURE 3. Three groups (A, B and C) of fetuses recovered from experimental animals on day 18 of gestation. Control fetuses are on the extreme left . Row A, fetuses from mothers treated with 25 mg/kg of MMC. Row B, fetuses recovered from mothers treated with 20 mg/kg of MMC. Row C, fetuses recovered from mothers treated with 15 mg/kg of MMC. Note varying degrees of resorption.

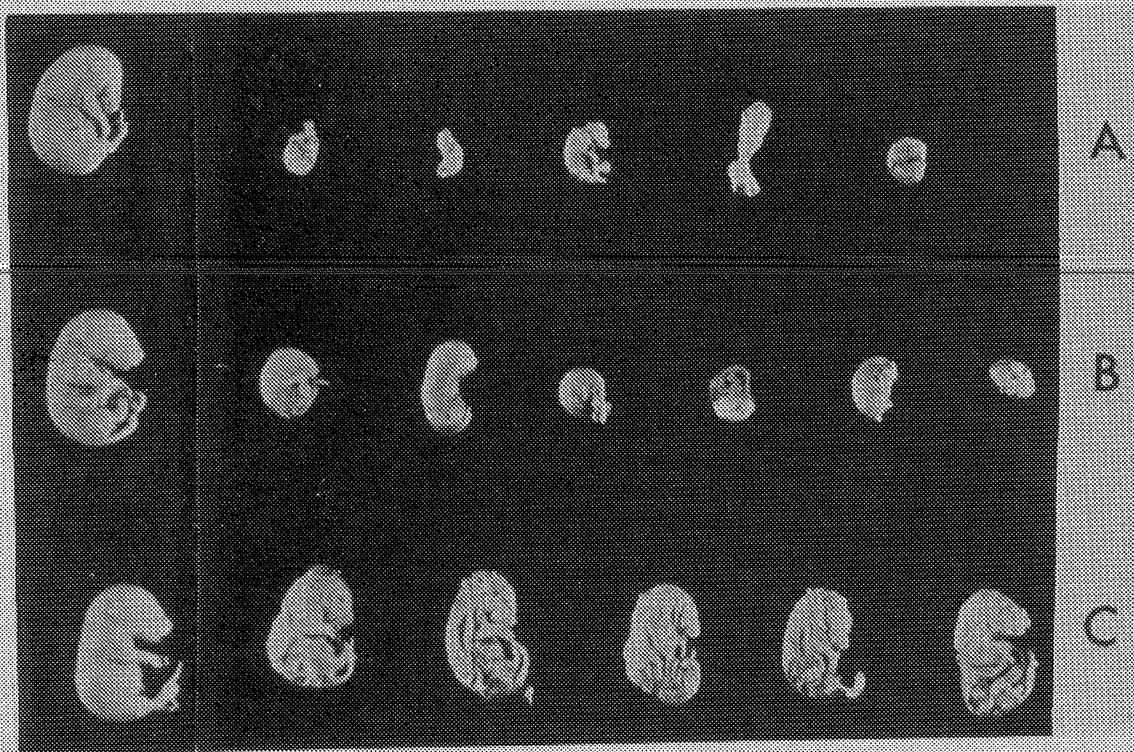


Fig.3

Table 4

Number of intrauterine deaths (early and late) observed in fetuses recovered at day 18 of gestation

Dosage (MMC)	Live fetuses n	(%)	Dead fetuses n	(%)
Control	209	(99.6)	1	(0.4)
5 mg/kg	129	(81.6)	29	(18.4)
10 mg/kg	75	(78.1)	21	(21.9)
15 mg/kg	82	(77.4)	24	(22.6)
20 mg/kg	34	(35.1)	63	(64.9)
25 mg/kg	0	(0)	55	(100)
30 mg/kg	0	(0)	59	(100)

17 mg/kg=LD_{50} $p<0.01$ (between all treatment groups & control)
25 mg/kg=LD_{100} $r=0.9477$

4.1.6 Live fetuses recovered (normal and abnormal)

Table 1 summarizes the number of live fetuses recovered at the time of sacrifice. The percentage of live fetuses recovered at sacrifice compared to implantation sites is also summarized in Table 1. The percentage of live fetuses steadily decreased from a value of 99.6% in controls, to 82% in 5 mg/kg, 78% in 10 mg/kg, 77% in 15 mg/kg, 35% in 20 mg/kg and 0% in 25 mg/kg treatment groups.

The fetuses recovered alive were fixed and subsequently examined for congenital malformations and recorded as either normal or abnormal. The dose range which produced congenital abnormalities in fetal mice is designated as the embryotoxic range. Table 5 shows that the dose range of MMC which produced congenital anomalies was between 10 mg/kg and 20 mg/kg. The ET_{50} (embryotoxic dose causing 50% abnormalities) was established at the 12 mg/kg level and the ET_{100} (embryotoxic dose causing 100% abnormalities) established at the 20 mg/kg dosage.

4.1.7 Fetal malformations (macroscopic and skeletal)

A high percentage of congenital anomalies occurred at the 10, 15 and 20 mg/kg dose levels. Figure 4 shows the presence of cleft palates in fetuses recovered at day 18 of gestation. Control and 5 mg/kg treatment groups did not show any evidence of cleft

palates (figure 4 A and B). Cleft palates were observed with increasing regularity, commencing initially at 10 mg/kg dose (22%) (Figure 4C). Seventy percent of the fetuses recovered from mothers treated with 15 mg/kg dose had cleft palates (Figure 4 D), while 100% of the fetuses examined from the 20 mg/kg treatment group displayed cleft palates (Figure 4 E).

Figure 5 shows other gross anomalies observed following treatment at the at 20 mg/kg dose level. Omphalocele is shown in (Figure 4 A and B) from the 20 mg/kg treatment level. The skeletal system, as revealed with Alizarin Red S showed only cleft palate in fetuses that were treated with 10, 15 and 20 mg/kg as seen in (Figure 6 C, D and E). Skeletal deformities were not evident in the control and 5 mg/kg treatment groups (Figure 6 A and B).

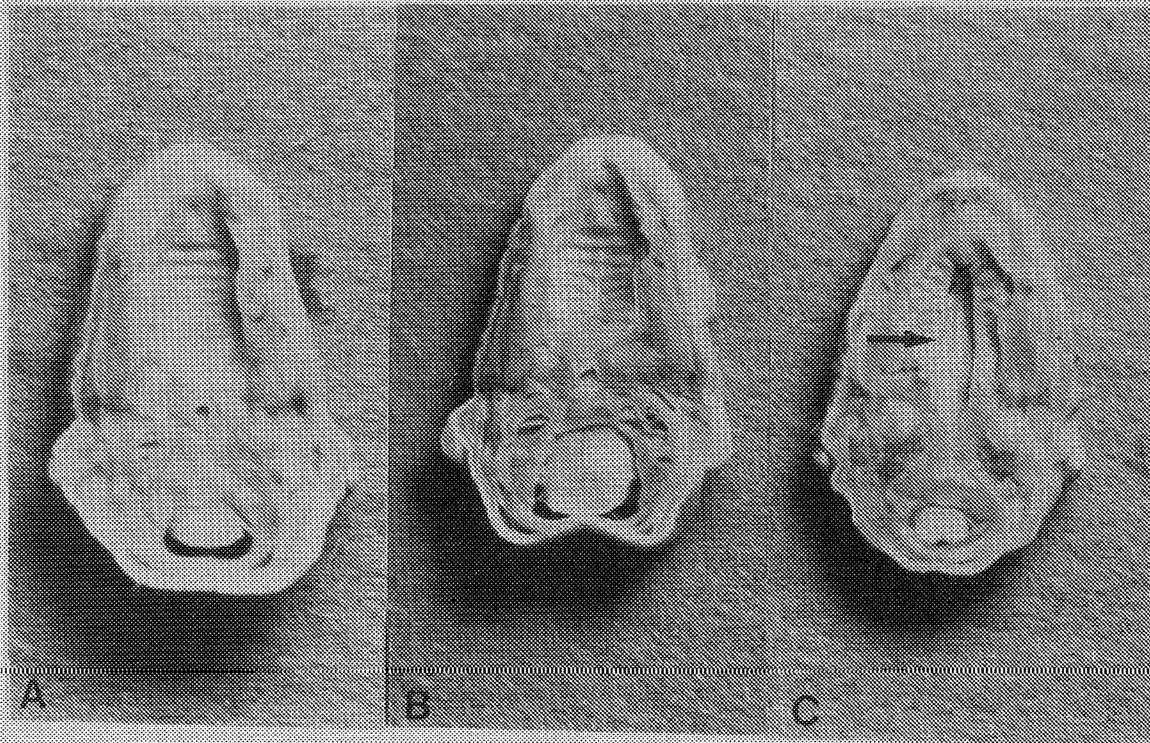
Table 5

Live fetuses (normal and abnormal) recovered on day 18 of gestation

Dosage (MMC)	Normal n	Normal (%)	Abnormal n	Abnormal (%)
Control	209	(100)	0	(0)
5 mg/kg	129	(100)	0	(0)
10 mg/kg	58	(77.4)	17	(22.6)
15 mg/kg	25	(30.5)	57	(69.5)
20 mg/kg	0	(0)	34	(100)
25 mg/kg	0	(0)	0	(0)
30 mg/kg	0	(0)	0	(0)

$p < 0.01$ (between all treatment groups and control)
$\Upsilon = 0.99225$

FIGURE 4. Five groups (A, B, C, D and E) of palates from gestational day 18 fetuses are shown. Control (A) and 5 mg/kg MMC (B) are normal. Cleft palates (arrows) in fetuses from 10 mg/kg (C), 15 mg/kg (D) and 20 mg/kg (E) treatment groups.



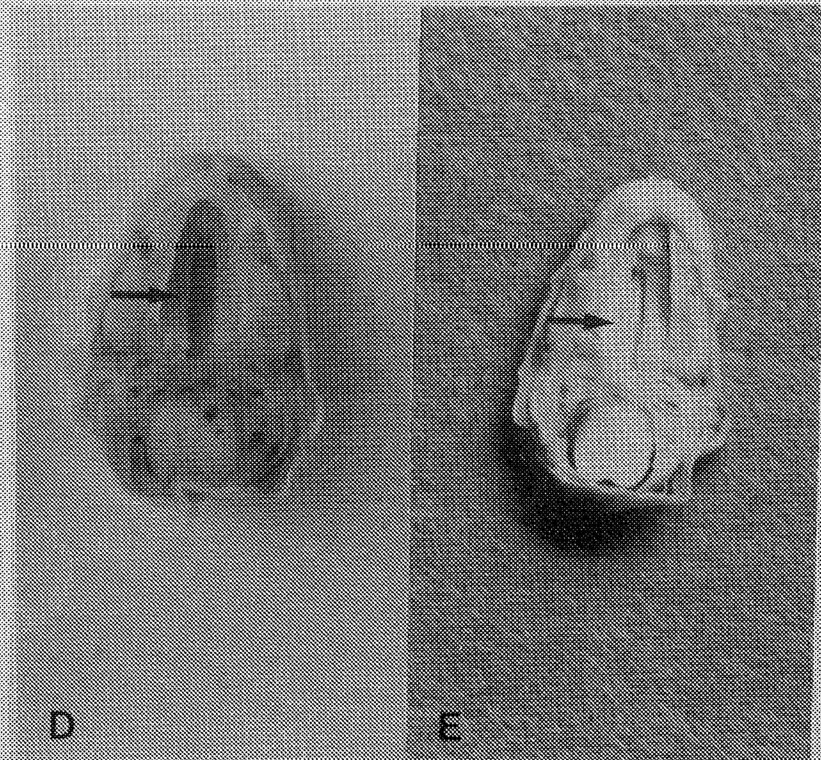
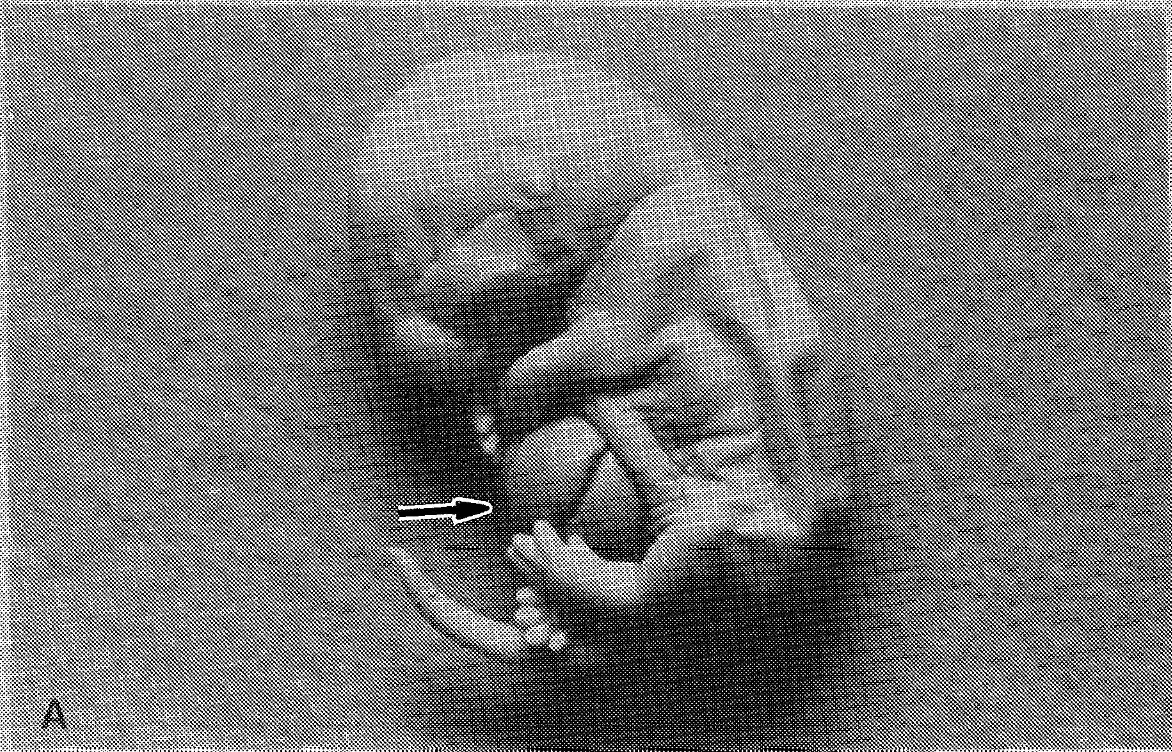


Fig.4

FIGURE 5. Two fetuses (A and B) from 20 mg/kg MCC treatment group showing the presence of omphaloceole (as indicated by the arrows).



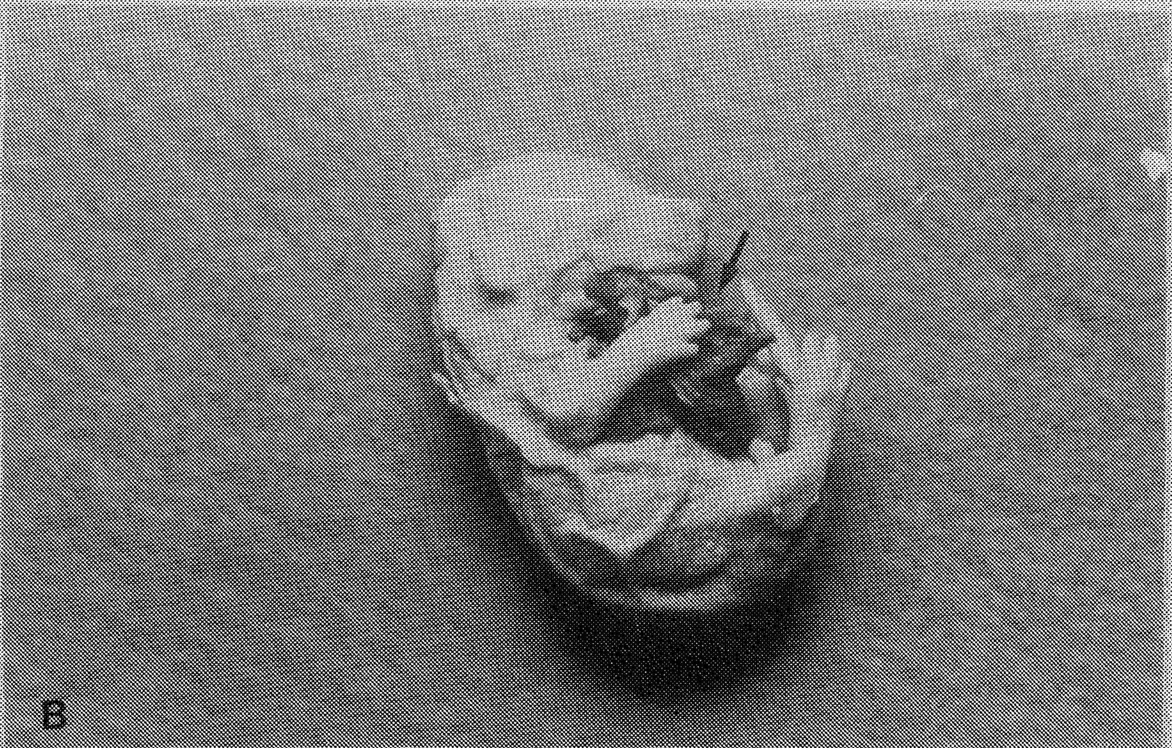
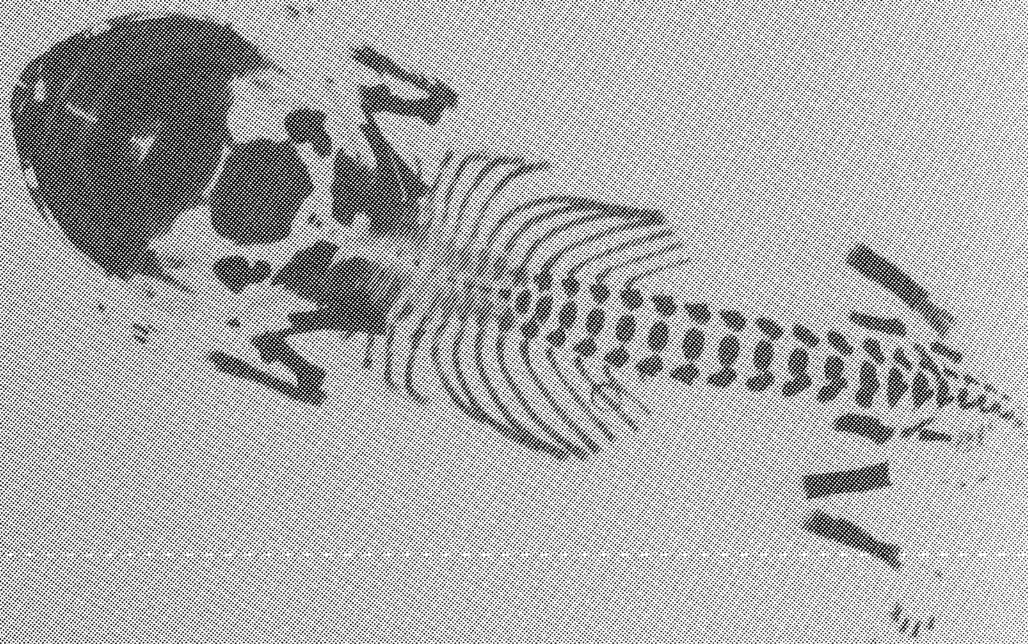
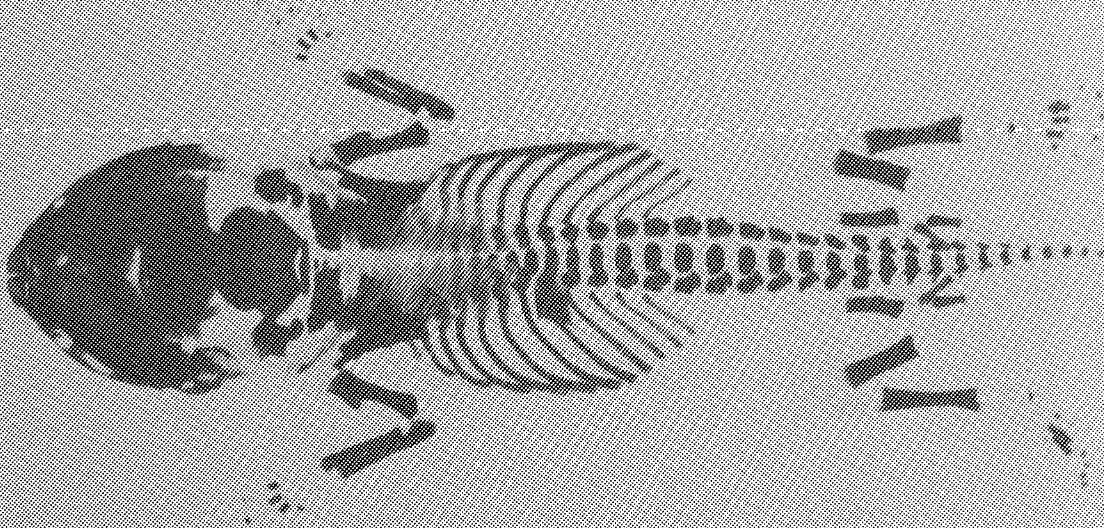


Fig. 5

FIGURE 6. Alizarin Red S stained skeletal system of 18 day fetuses from five groups (A, B, C, D and E). Control (A) and 5 mg/kg MMC groups of fetuses (B) showed no irregularities in the skeletal system. Fetuses from mothers treated with 10 mg/kg MCC (C), 15 mg/kg MMC (D) and 20 mg/kg MMC (E) all had cleft palate irregularities (as indicated by the arrows).

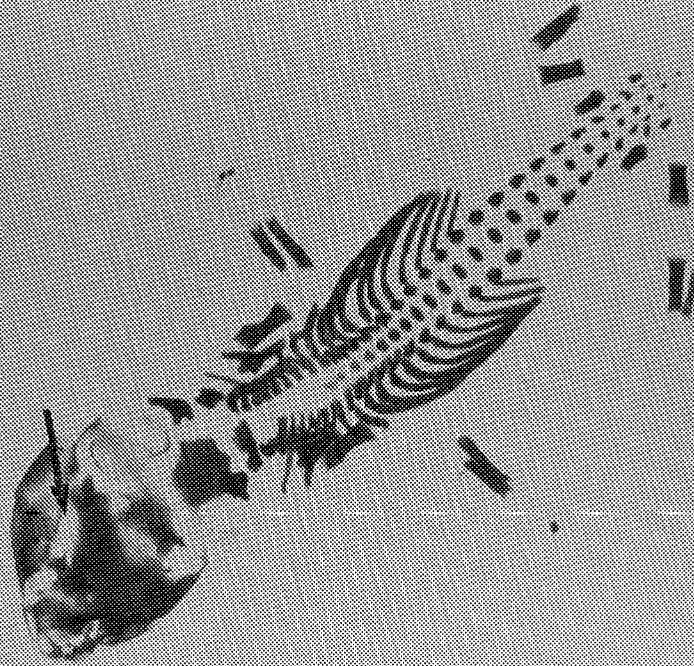


A

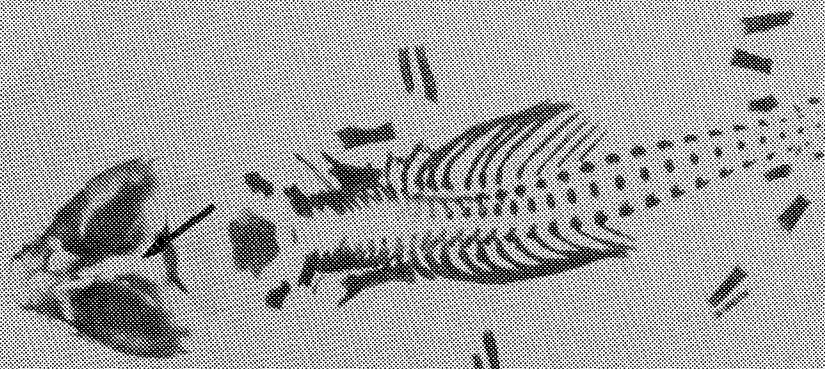


B

Fig. 6



C



D

Fig·6

Fig·6

4.1.8 Mean fetal weights

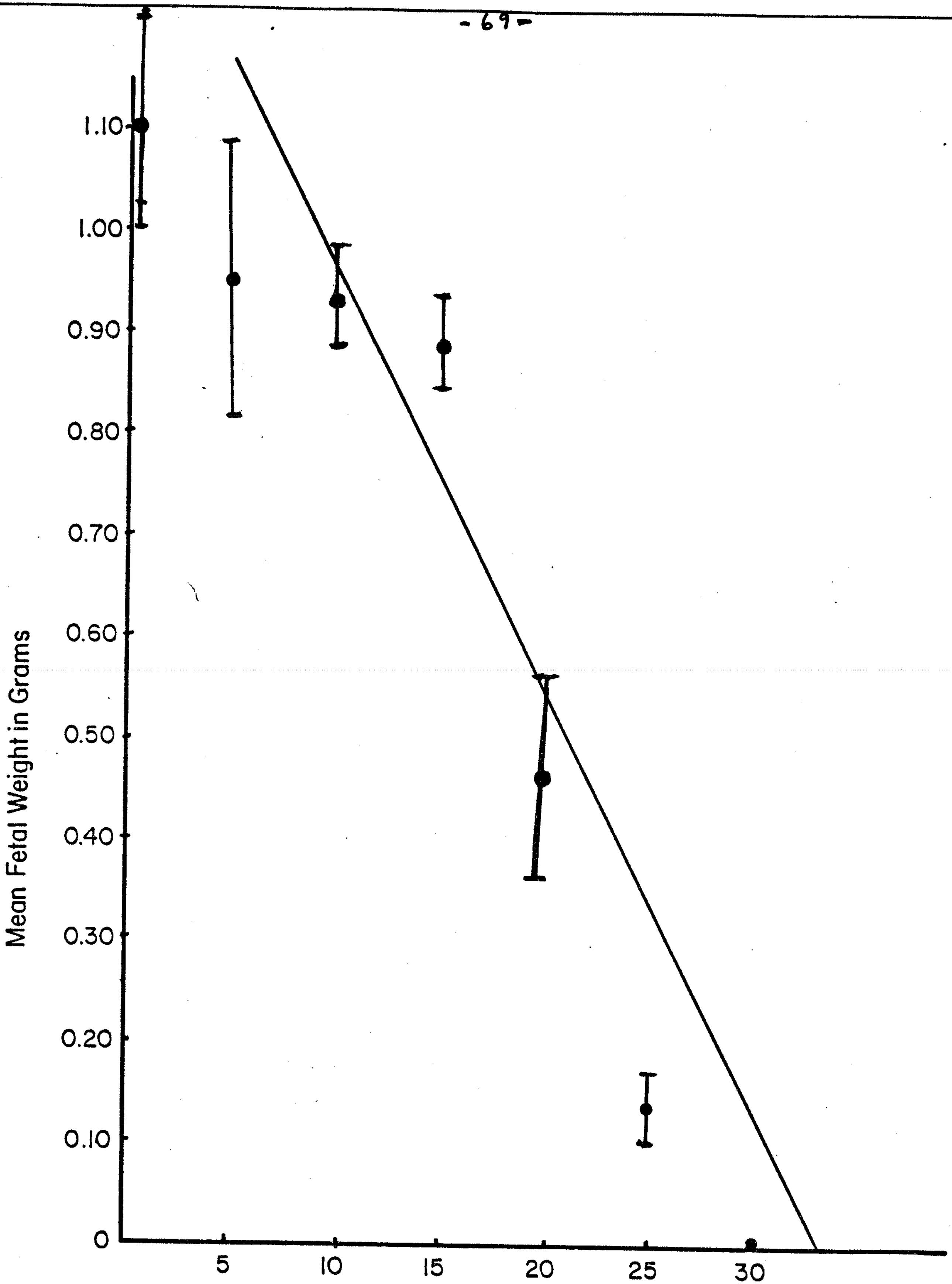
Fetal weights are summarized in Table 6 which shows the mean weight and standard deviation ($\bar{x}$ ± S.D.) for each treatment group and the control. No fetuses were recovered from the 30 mg/kg group due to total resorption. The mean weight of control fetuses was 1.10 ± 0.21 grams. The mean weight of fetuses recovered from the treatment groups decreased as the dose levels of MMC increased as seen in Figure 7. Fetuses from the 25 mg/kg treatment group weighed signficantly less (0.15 ± 0.06 grams) than those from control animals.

Table 6

Mean fetal weights and standard deviations on day 18 of gestation in the treatment and control groups

Dosage (MMC)	Fetal weights $\bar{x} \pm$ S.D.
Control	1.10 ± 0.21 gm
5 mg/kg	0.95 ± 0.36 gm
10 mg/kg	0.93 ± 0.11 gm
15 mg/kg	0.89 ± 0.08 gm
20 mg/kg	0.47 ± 0.19 gm
25 mg/kg	0.15 ± 0.06 gm
30 mg/kg	0
$p<0.005$ (between all treatment groups & control) r =0.98703	

FIGURE 7. Weight of fetuses (mean ± S.D.) at gestational day 18 following methylmercuric chloride treatment (5, 10, 15, 20, 25 and 30 mg/kg) and control.



Dose Levels of MMC mg/Kg

r =-0.98703

Fig. 7

4.2 MERCURY LEVELS

4.2.1 Maternal

Maternal blood was collected by means of cardiac puncture and analyzed for levels of mercury. The mean values and standard deviations are shown in Table 7. Analysis of maternal blood indicated that the mercury levels steadily increased with the dose levels as seen in Figure 8. Control levels of mercury are negative while treatment groups show an increasing level of mercury in maternal blood.

4.2.2 Fetal

From each litter, one fetus was sampled at random and the level of mercury was determined. The results of mercury analysis are summarized in Table 8. Higher levels of mercury were seen in the treatment groups as compared to the control, as seen in Figure 9. No observations were possible from the 25 and 30 mg/kg treatment groups because of the complete resorption of fetuses.

4.2.3 Maternal and fetal mercury level comparison

Figure 10 compares the levels of mercury (PPM) in maternal blood to fetal tissues (PPM). The levels of mercury in the fetus as compared to the maternal level of mercury, at the same treatment dose, were approximately twice as high. This pattern was consistent in all treatment groups except for the 25 and 30 mg/kg treatment groups for which there was complete fetal resorption.

Table 7

Maternal mercury blood levels

Dosage (MMC)	Mercury levels (PPM) in maternal blood $\bar{x} \pm$ S.D.
Control	0.01 ± 0.00
5 mg/kg	0.83 ± 0.42
10 mg/kg	1.70 ± 0.26
15 mg/kg	6.67 ± 2.82
20 mg/kg	14.87 ± 2.87
25 mg/kg	70.67 ± 3.60
30 mg/kg	89.53 ± 2.73

$p<0.001$ (between all treatment groups & control)
r =0.9604

Table 8

Fetal mercury tissue levels

Dosage (MMC)	Mercury levels (PPM) in fetal tissue $\bar{x} \pm$ S.D.
Control	0.01 ± 0.00
5 mg/kg	1.07 ± 0.54
10 mg/kg	5.41 ± 1.94
15 mg/kg	17.47 ± 7.71
20 mg/kg	36.01 ± 7.45
25 mg/kg	----
30 mg/kg	----
$p<0.001$ (between all treatment groups & control) $r = 0.99898$	

FIGURE 8 Mercury levels (PPM) expressed as a logarithm in maternal blood following treatment with six different dosages of methylmercuric chloride also expressed as a logarithm.

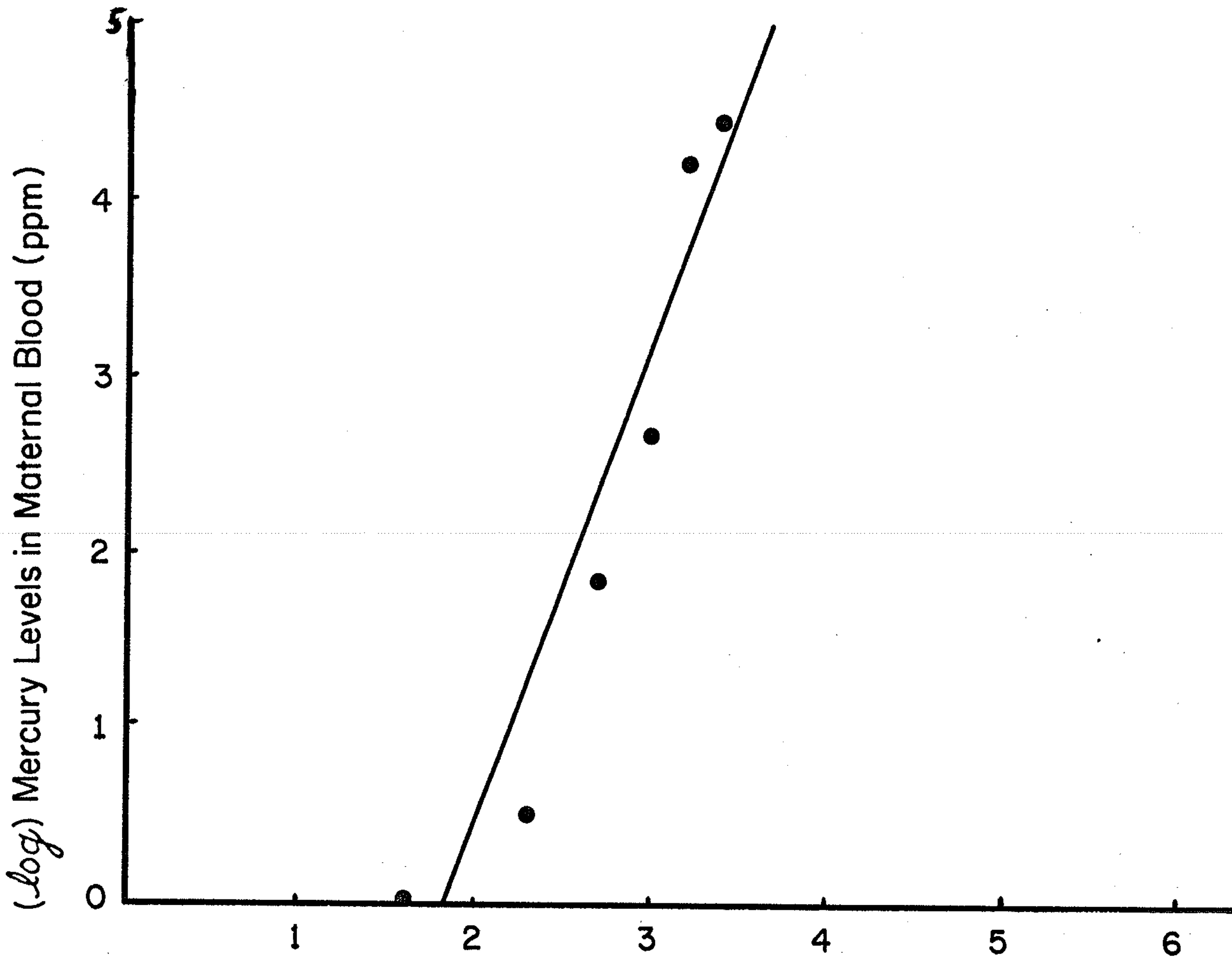


Fig. 8

FIGURE 9. Mercury levels (PPM) expressed as a logarithm in fetal tissues following maternal treatment with different dose levels of methylmercuric chloride, also expressed as a logarithm.

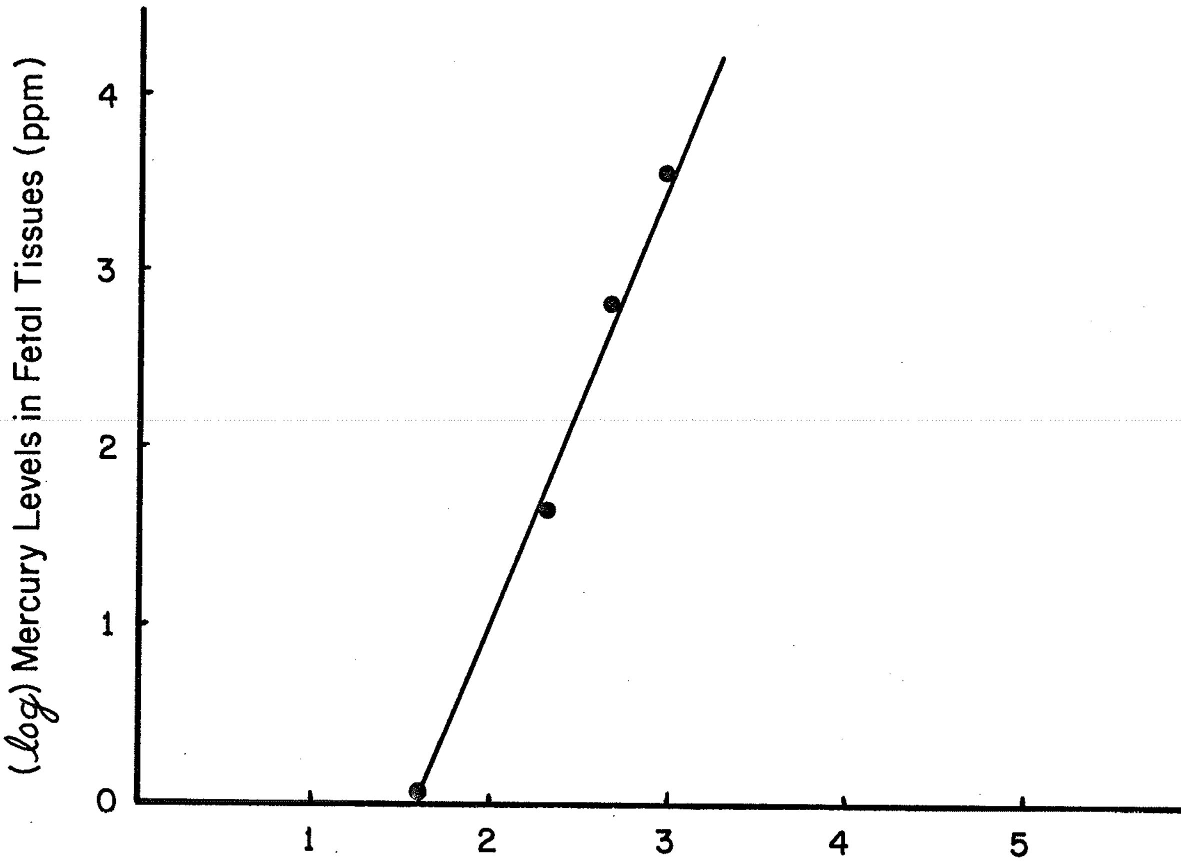
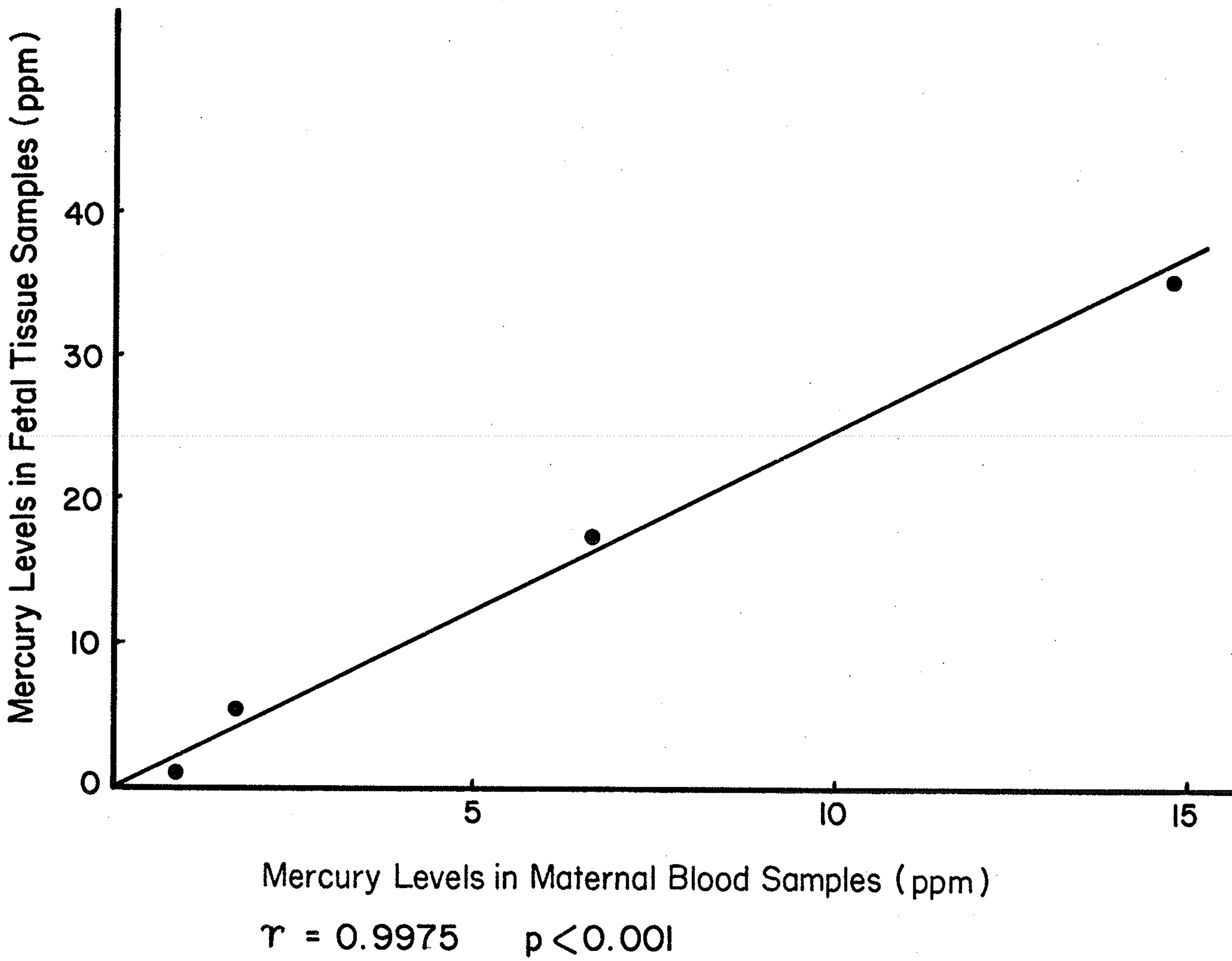


Fig. 9

FIGURE 10. Comparison of the amounts of mercury (PPM) in fetal tissues and the amount of mercury (PPM) in maternal blood at different dose levels.



r = 0.9975 p < 0.001

Fig. 10

4.3 LIGHT MICROSCOPY

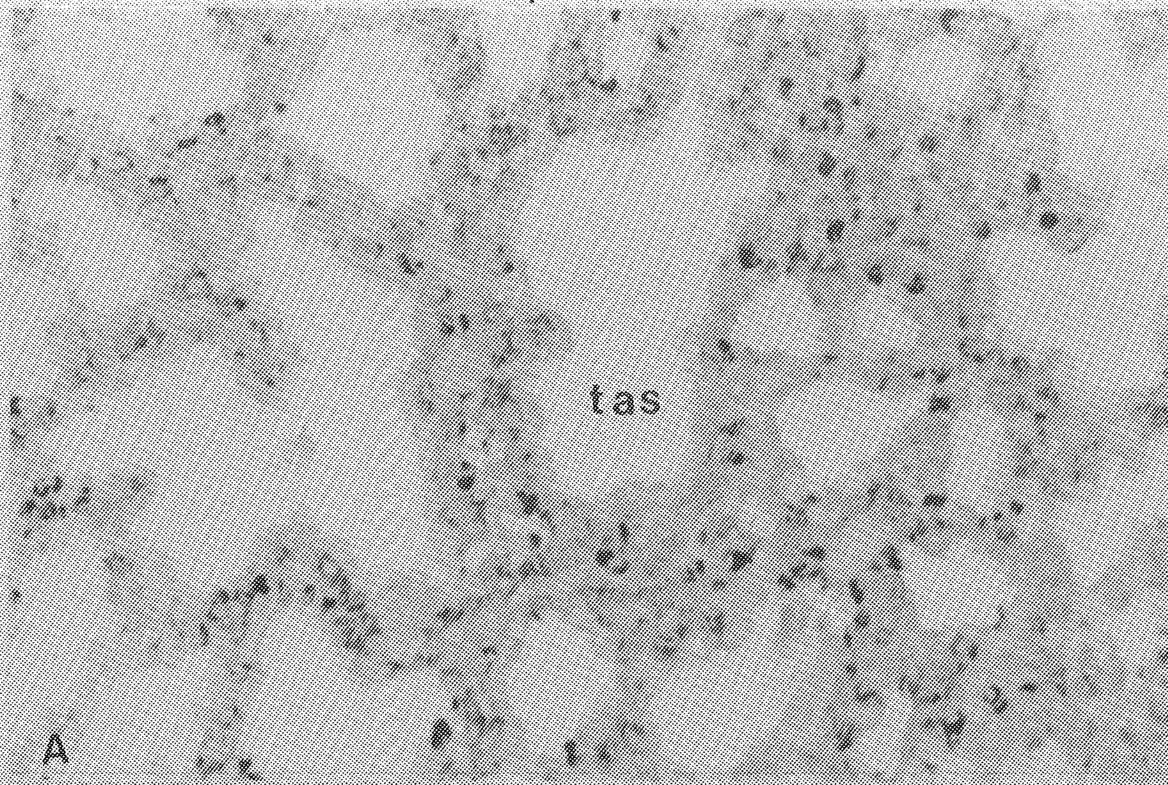
4.3.1 Fetal Lung

Fetal lung growth and development were morphologically studied using paraffin sections histologically stained with P.A.S., from fetuses recovered on day 18 of gestation. Figure 11 shows fetal lungs from control, 5, 10 and 15 mg/kg treatment groups. Morphological appearance of fetal lung was used to estimate its the relative maturity and development. The location and amount of glycogen was also used to determine the rate of growth and the relative developmental stage of the fetal lungs on day 18 of gestation.

Control lung was in the terminal air sac stage (tas), as can be seen in Figure 11A. The presence of numerous terminal air sacs (tas) confirm this morphological finding. Glycogen was not present in the flattened differentiating epithelial cells and was largely confined to interstitial mesenchymal cells in small amounts. The morphological appearance of lungs from animals of the 5 mg/kg treatment group showed the presence of some terminal air sacs (tas) and numerous unbranched alveolar tubules (t). There was a large amount of glycogen present in the epithelial cells of the alveolar

tubules, with lesser amounts in the epithelia of the terminal air sacs and the interstitial cells. Lungs from fetuses of the 5 mg/kg treatment group were in a transitional phase between the more mature terminal air sac stage and the less mature pseudoglandular stage. This phase is seen in Figure 11B. Fetal lungs obtained from the 10 mg/kg treatment group revealed a glandular appearance due to the presence of straight alveolar tubules (t) embedded in a thick mesenchyme. The epithelial lining of the alveolar tubules are columnar, filled with large amounts of glycogen. Morphologically these lungs appeared pseudoglandular as seen in Figure 11C. Fetal lungs from mothers treated with 15 mg/kg of methylmercury appeared to have still fewer alveolar tubules. These alveolar tubules are lined by columnar epithelium distended with copious amounts of glycogen, while the very thick interstitial mesenchyme appeared to have no glycogen. Morphologically, these lungs appeared immature as seen in Figure 11D and were in an early pseudoglandular stage consisting of widely spaced straight alveolar tubules embedded in a thick mesenchyme.

Figure 11. Paraffin sections stained with P.A.S. to demonstrate glycogen. Four groups (A, B, C and D) are shown. Control 11A shows the presence of terminal air saccules (tas) and very little glycogen in epithelial cells (glycogen stains darkly). The 5 mg/kg treatment group 11B shows a mixture of terminal are saccules (tas) and tubules (t). The epithelium of the airways are still filled with darkly stained glycogen. Figure 11C (10 mg/kg group) shows the presence of only tubules (t) lined by epithelium filled with glycogen. The 15 mg/kg (11D) reveal fewer tubules (t) lined by glycogen ladened epithelial cells, x 400.



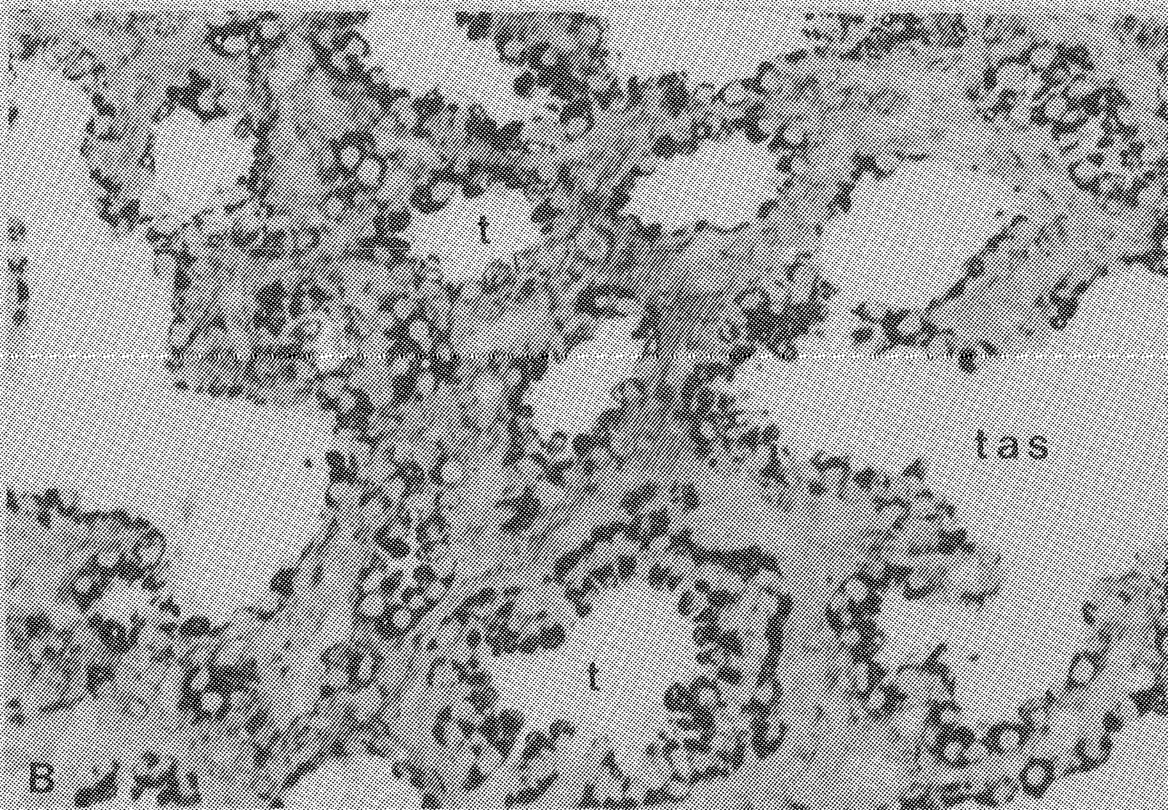
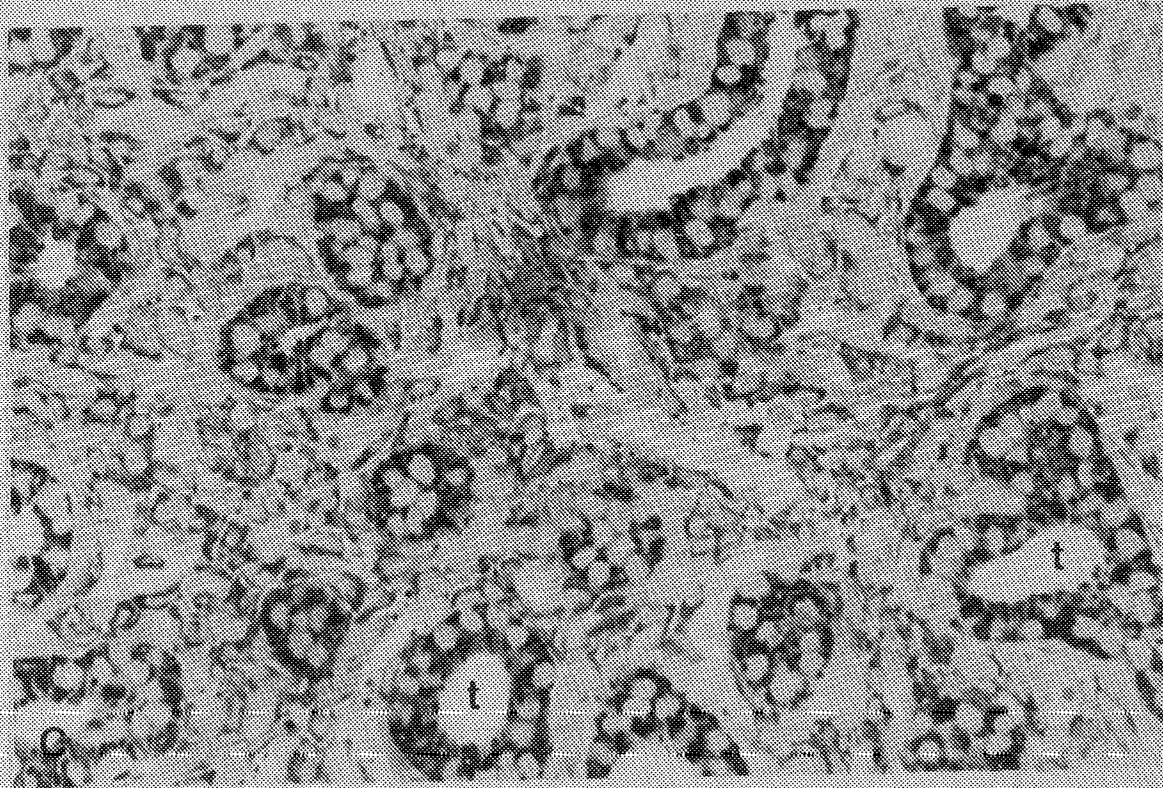


Fig. 11



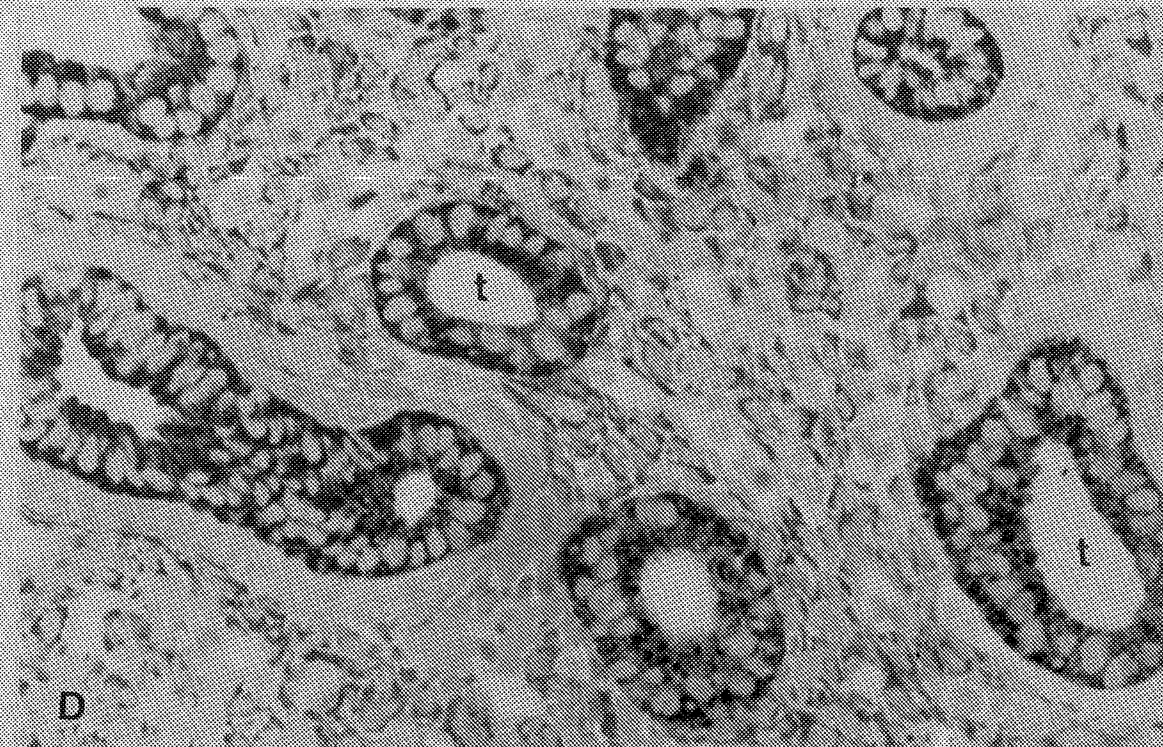


Fig.11

4.4 ELECTRON MICROSCOPY

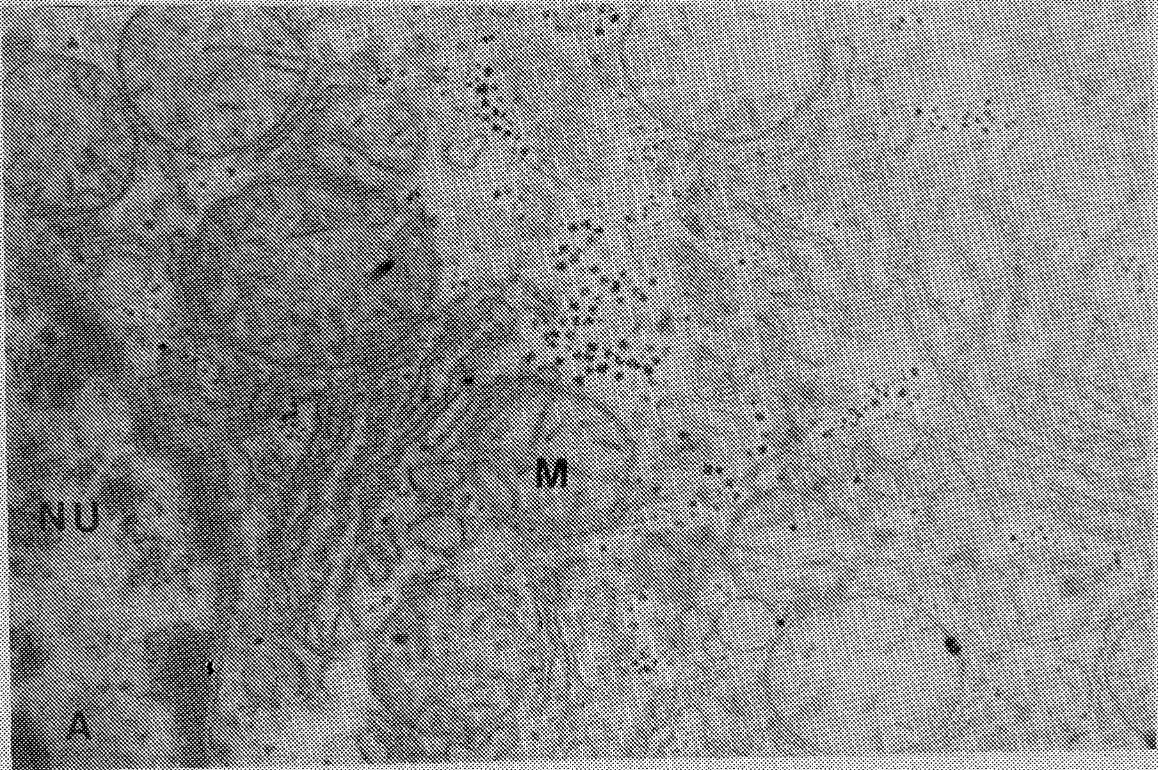
4.4.1 Fetal lung and liver

Electron microscopical examination of day 18, fetal lung following maternal treatment with MMC revealed several degenerative ultra-structural changes. Figure 12 shows photomicrographs from control animals and of three treatment groups (5, 10 and 15 mg/kg).

Control lungs appeared normal with no obvious ultrastructural changes in either the nucleus or cytoplasm as shown by Figure 12A. Fetal lungs examined from animals of the 5 mg/kg treatment group showed the presence of numerous small vacuoles in the mitochondria, as well as slightly pleomorphoric mitochondria. The remainder of the cellular organelles appear normal as shown in Figure 12B.

Fetal lungs from animals of the 10 mg/kg treatment group, as seen in Figure 12C, showed prominent vacuolation in the mitochondria and also degenerating sworls within the mitochondrial vacuoles. The mitochondria are pleomorphoric and markedly degenerated, having assumed a rather bizarre shape. Other cellular organelles do not appear to have been adversely affected at this stage by MMC. At the highest dose level (15 mg/kg), fetal lungs appeared to be more adversely perturbed by MMC. The mitochondria, as seen in Figure 12D, underwent lysis having lost their smooth contour. The mitochondria were degenerated and scalloped in appearance. The electron microscopic appearance of fetal liver displayed similar ultrastructural changes as was seen in the fetal lungs.

Figure 12. Electron micrographs of fetal lung from control and three treatment groups (A, B, C and D). Control (12A) shows normal ultrastructural organelles, Nucleus (NU) and mitochrondria (M), x 22,610. The 5 mg/kg treatment group (12B) shows the presence of numerous small vacuoles (arrows) in the mitochondria, x 22,610. Group C (10 mg/kg) shows large pheomorphoric mitochondria with large vacuoles (arrows) and sworls, x 22,610. Group D (15 mg/kg) shows degenerating mitochondria (M) which are undergoing lysis, x 22,610.



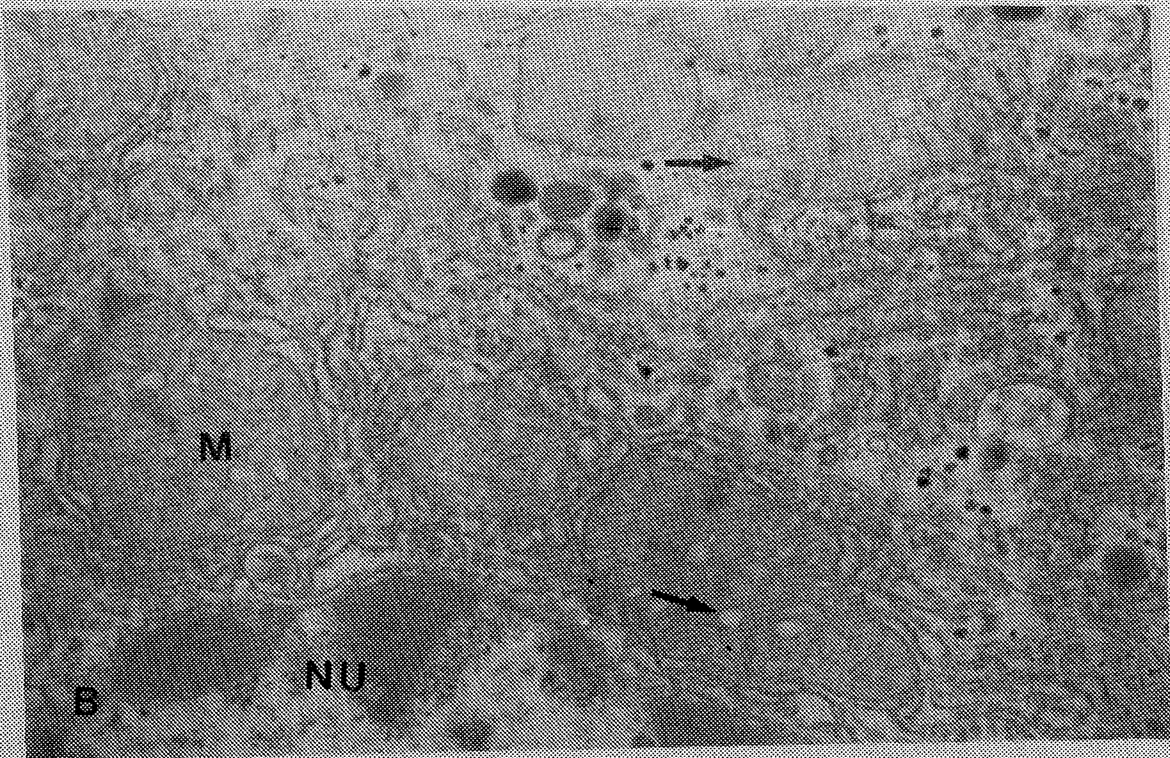
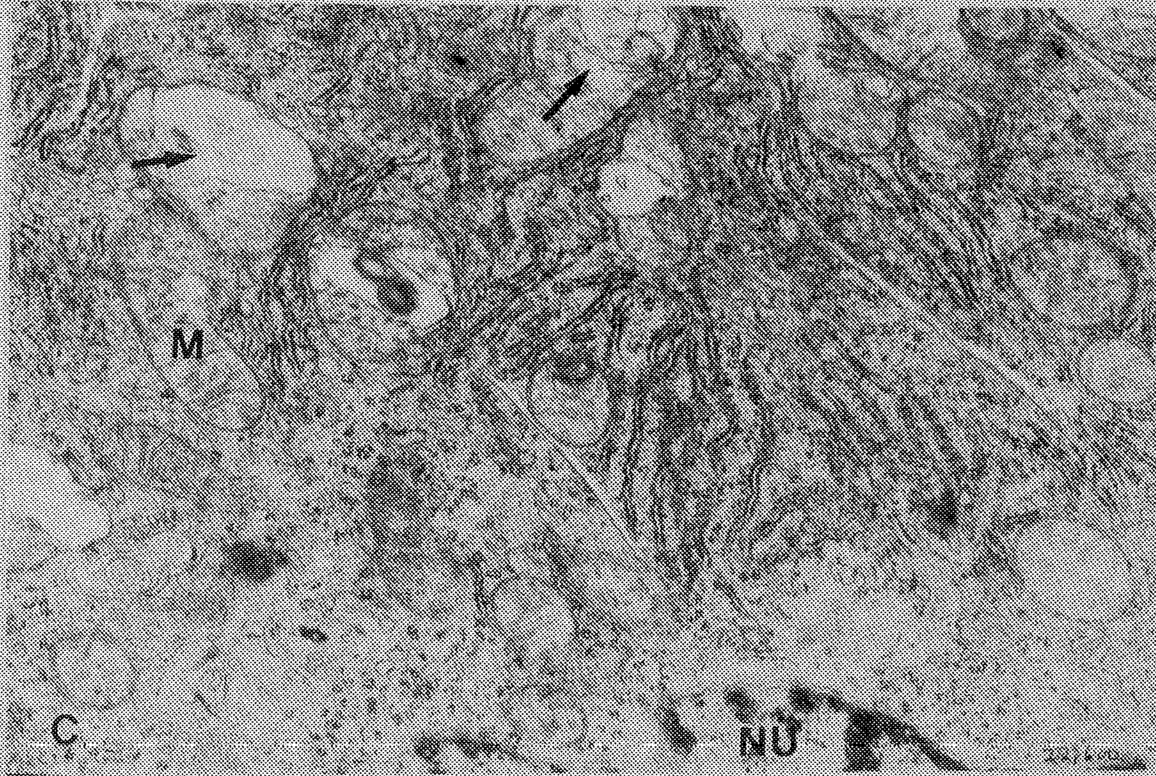
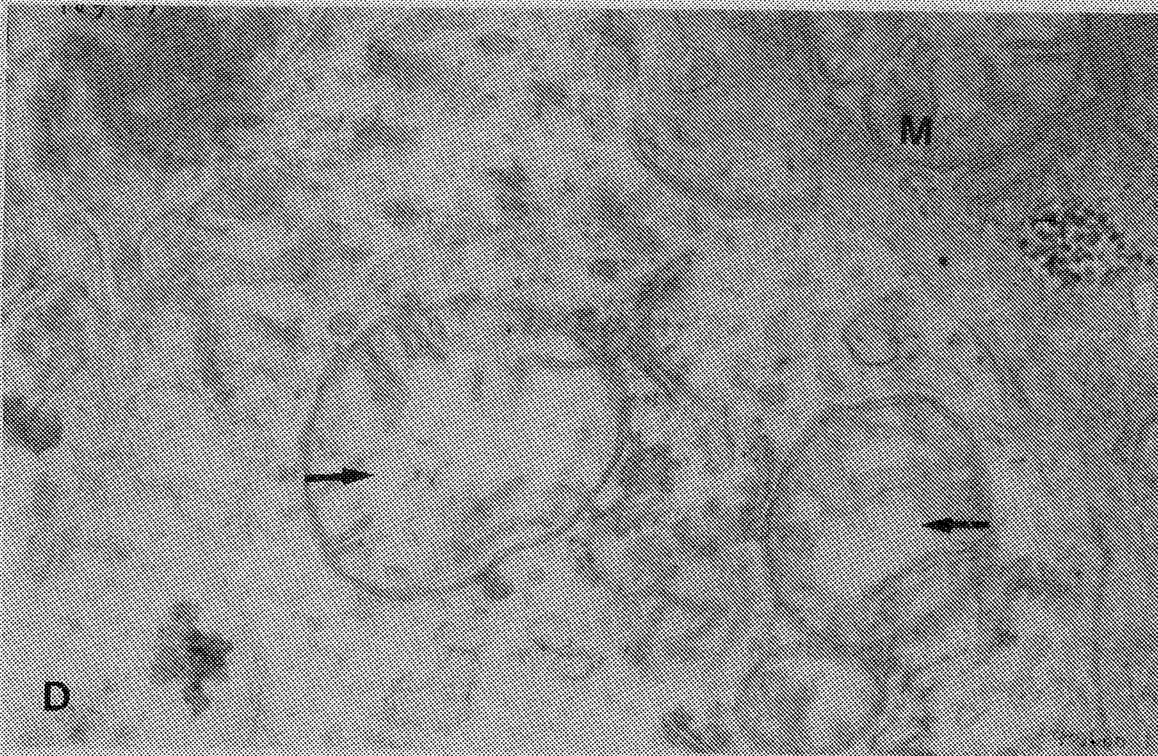


Fig.12





Fig·12

4.5 CYTOGENETIC STUDIES

4.5.1 Conventional chromosome analysis

Conventional chromosome analysis of metaphase spreads was done to detect the presence of chromosome aberrations. This was performed by noting the number of clumped metaphases among 100 metaphases examined from each treatment group (5, 10 and 15 mg/kg) and the control.

The results are summarized in table 9, which shows there was no clumping of chromosomes in the control. The results from the treated groups showed extensive clumping of metaphase chromosomes which is a type of chromosome aberration. Table 9 shows that treatment at the 5 mg/kg dose level resulted in 70% of the metaphases being clumped, 10 mg/kg dose level had 81% of the metaphases clumped, and 15 mg/kg induced 85% clumping of metaphase chromosomes. Metaphase clumping of chromosomes as seen in Figure 13 makes it more difficult to analyze chromosomes for other types of aberrations. Figure 14 shows the number of clumped chromosomes at different treatment doses of MMC.

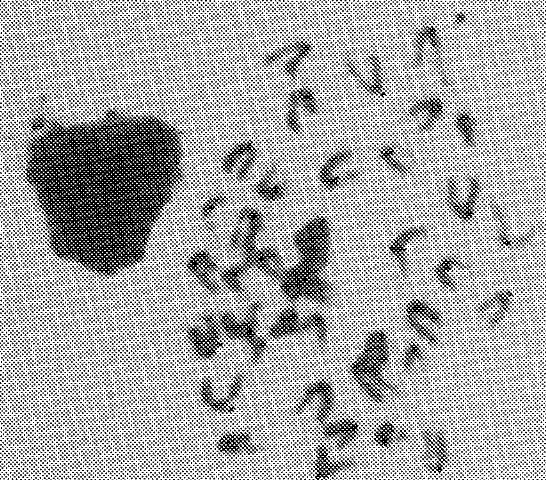
Table 9

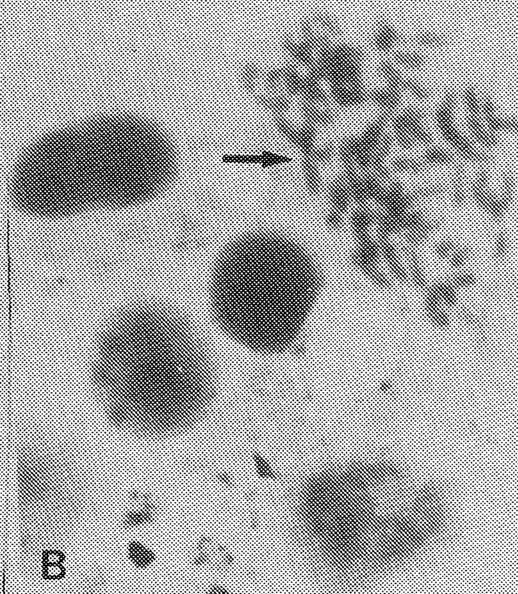
Number of clumped chromosomes (aberration) per 100 cells counted in treatment groups and control

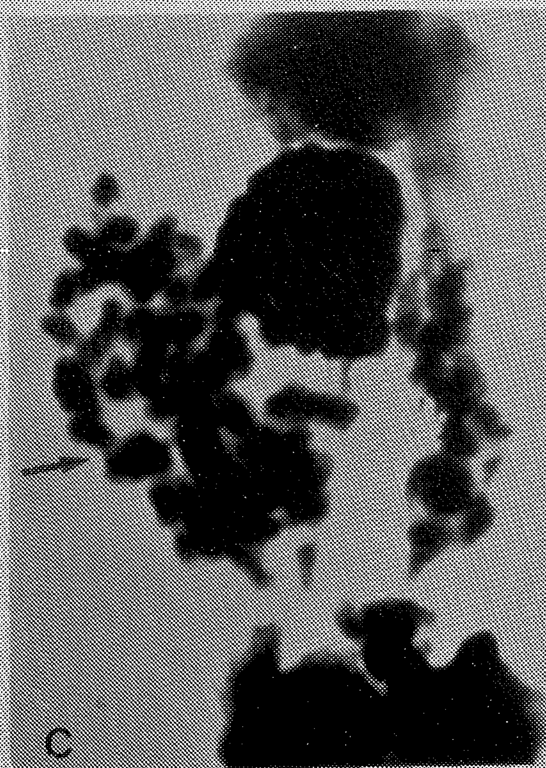
Dosage (MMC)	Number of cells counted	Clumped chromosomes	
		n	%
Control	100	0	(0)
5 mg/kg	100	70	(70)
10 mg/kg	100	81	(81)
15 mg/kg	100	85	(85)

$p<0.005$ (between all treatment groups & control)
$\tau=0.94022$

FIGURE 13. Four groups (A, B, C and D) are shown, control (13A) shows metaphase spread without clumping. The 5 mg/kg (13B) treatment group shows moderate clumping of chromosomes while 10 mg/kg (13C) and 15 mg/kg (13D) treatment groups shows more severe chromosome clumping, x 1,000.







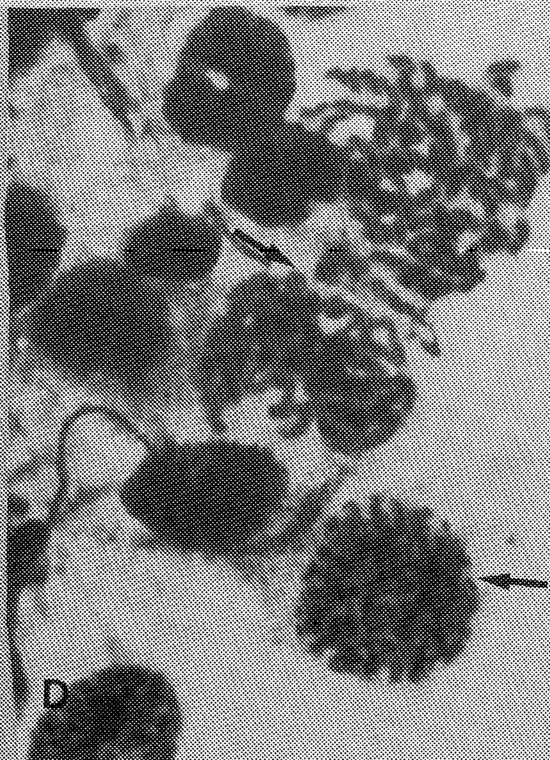
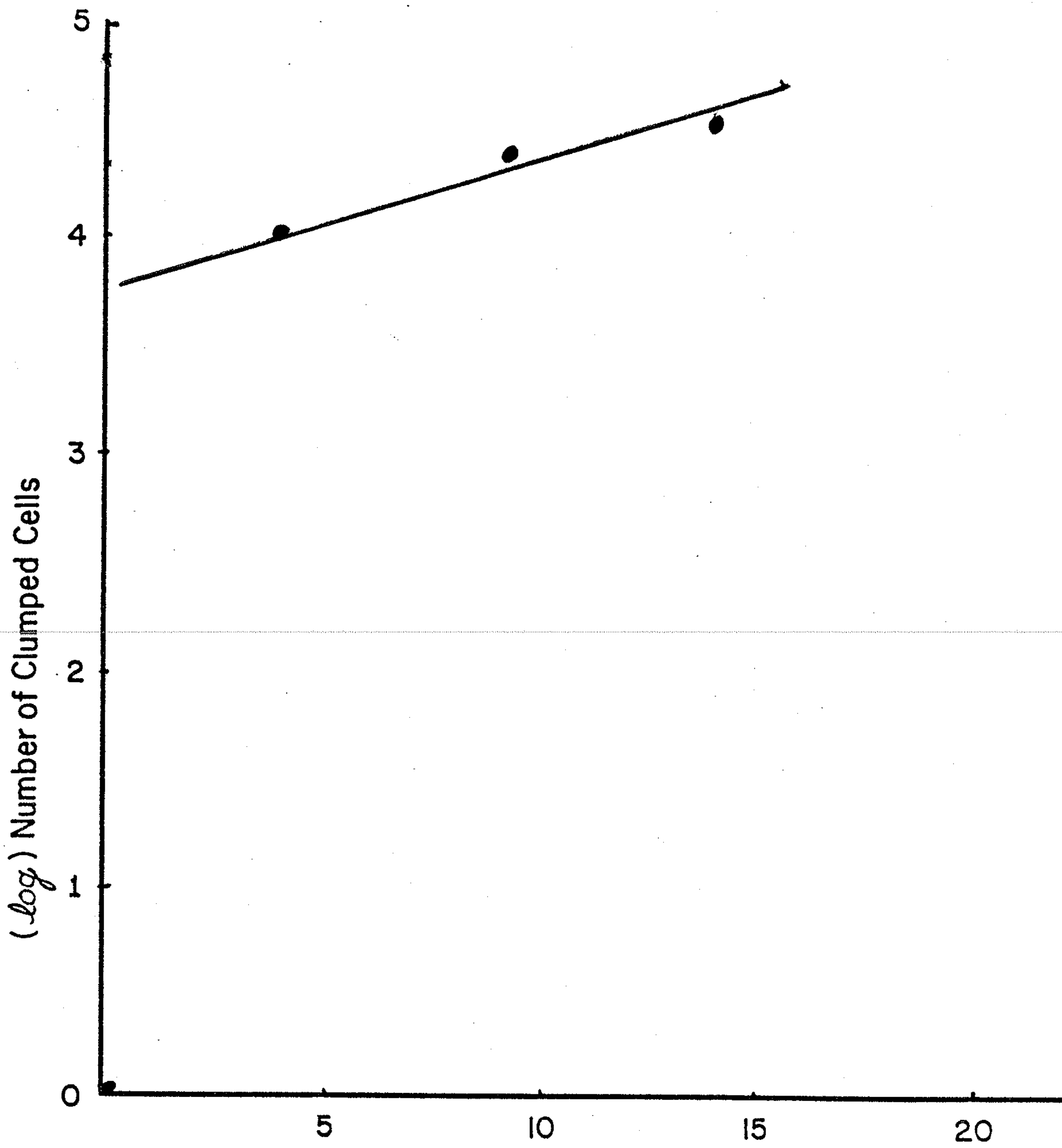


Fig.13



Dose Levels of MMC mg / kg

$r = 0.99022$

(log) = logarithm

Fig. 14

FIGURE 14. Total number of clumped metaphases expressed as a logarithm at different dose levels of MMC also expressed as a logarithm.

4.5.2 Chromosome counts

Totaling of chromosome numbers from 100 metaphase cells was done in each treatment group (5, 10 and 15 mg/kg) and the control to determine the mean number of chromosomes per genome in each of the groups. Chromosomes were also examined for any structural chromosome aberrations.

Table 10 summarizes the results of the chromosome counting. The total number of chromosomes varied between 3,972 ($\bar{x}$=39.72) in the 10 mg/kg treatment group and 4,008 ($\bar{x}$ = 40.08) in the 5 mg/kg treatment group.

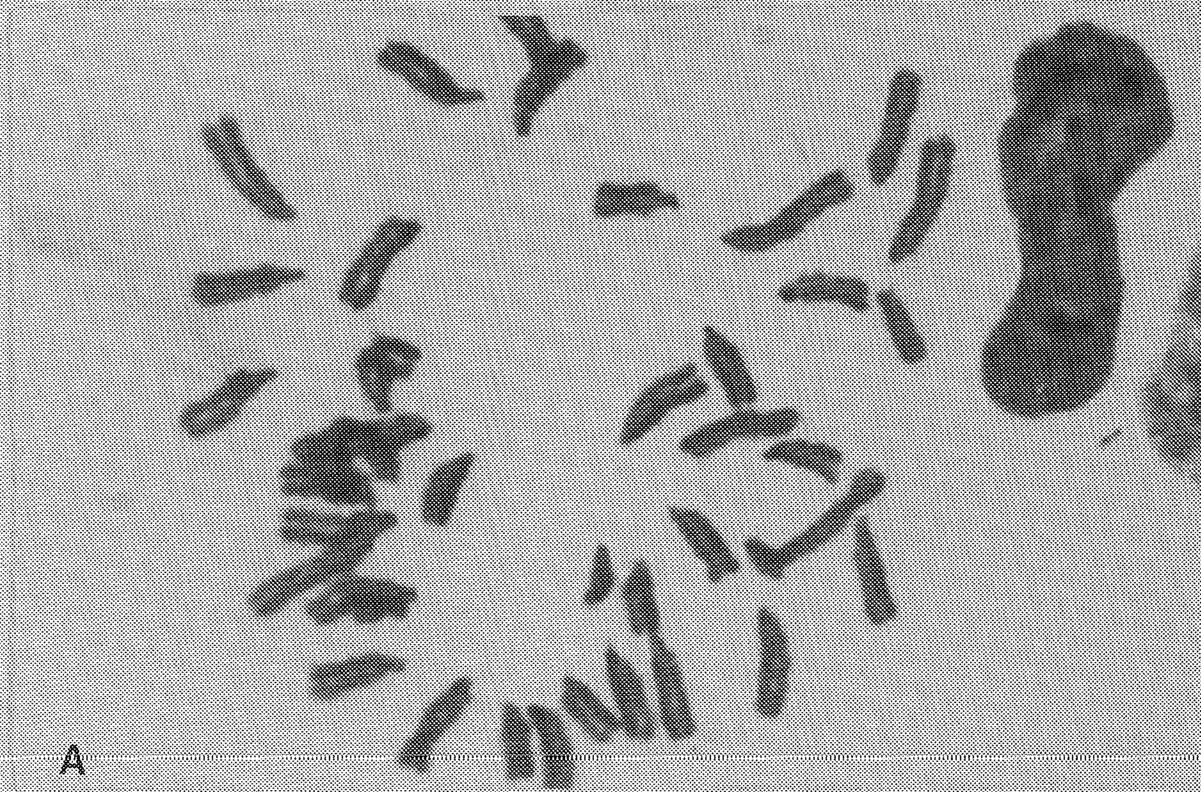
Figure 15 shows metaphase chromosomes from each group, no numerical or structural aberrations were detected.

Table 10

Mean chromosome number per metaphase

Dosage (MMC)	Number of cells counted	Total chromosome number	$\bar{x}$ of chromosomes per cell
Control	100	3994	39.94
5 mg/kg	100	4008	40.08
10 mg/kg	100	3972	39.72
15 mg/kg	100	3986	39.86
$p>0.95$ (not significant for any group)			

FIGURE 15. Four groups (A, B, C and D) showing the total number of chromosome per genome. Control (15A) and three treatment groups, 5 mg/kg (15B), 10 mg/kg (15C) and 15 mg/kg (15D) do not show any numerical differences, x 1,000.



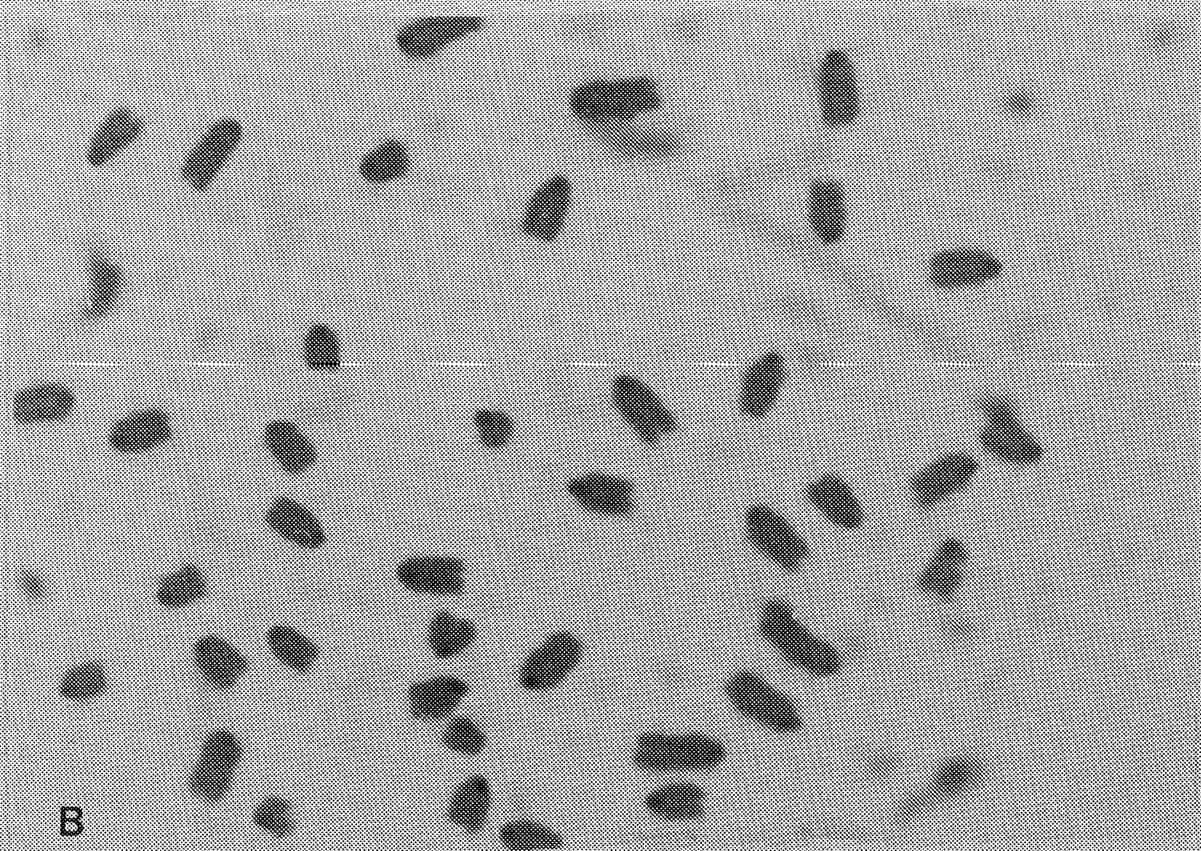
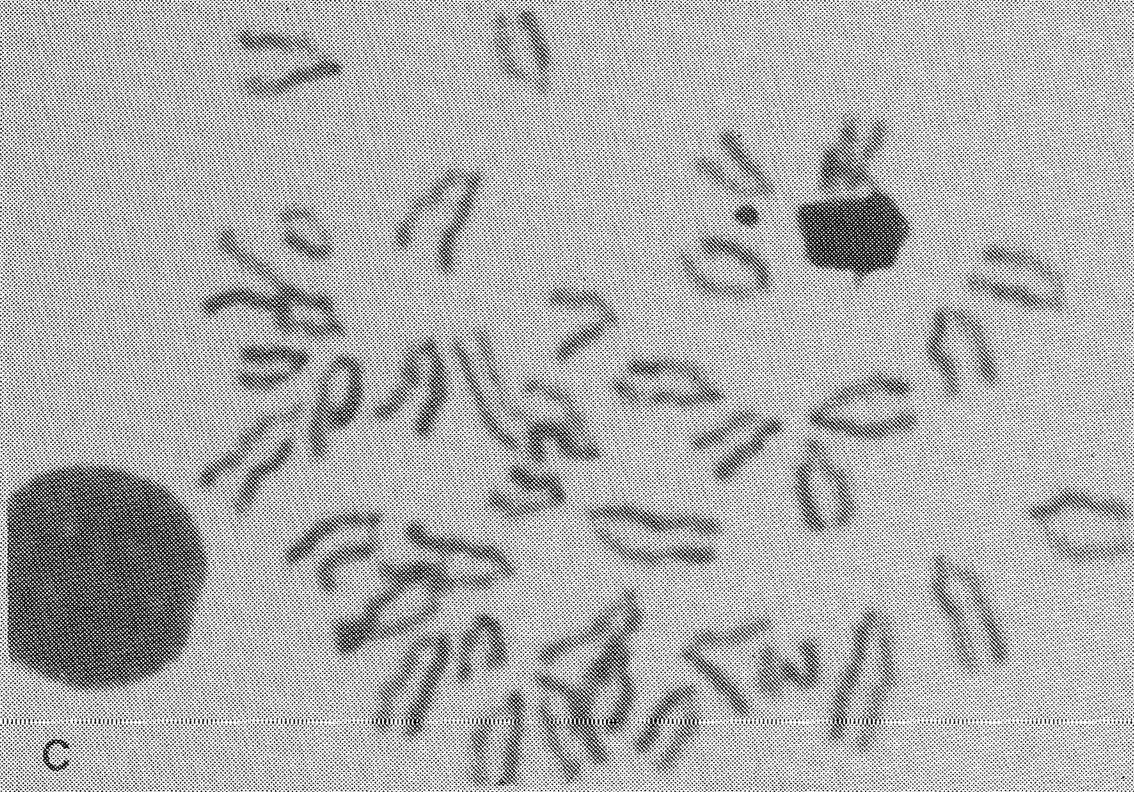


Fig.15



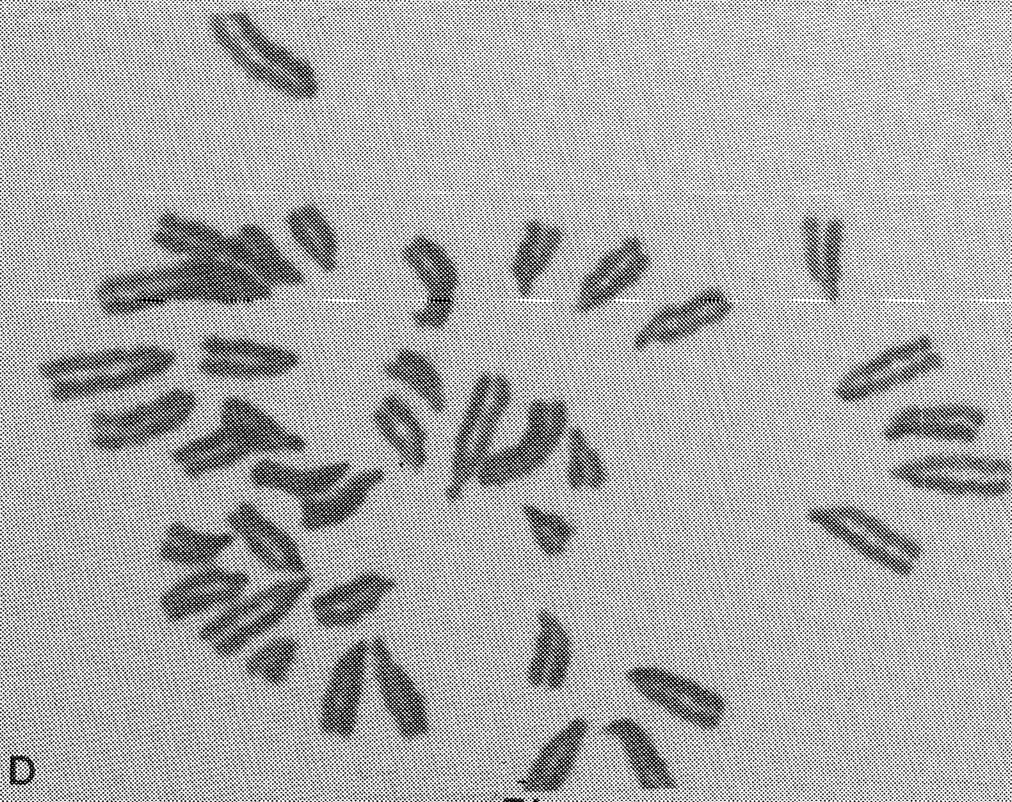


Fig.15

4.5.3 Sister chromatid exchange analysis (SCEs)

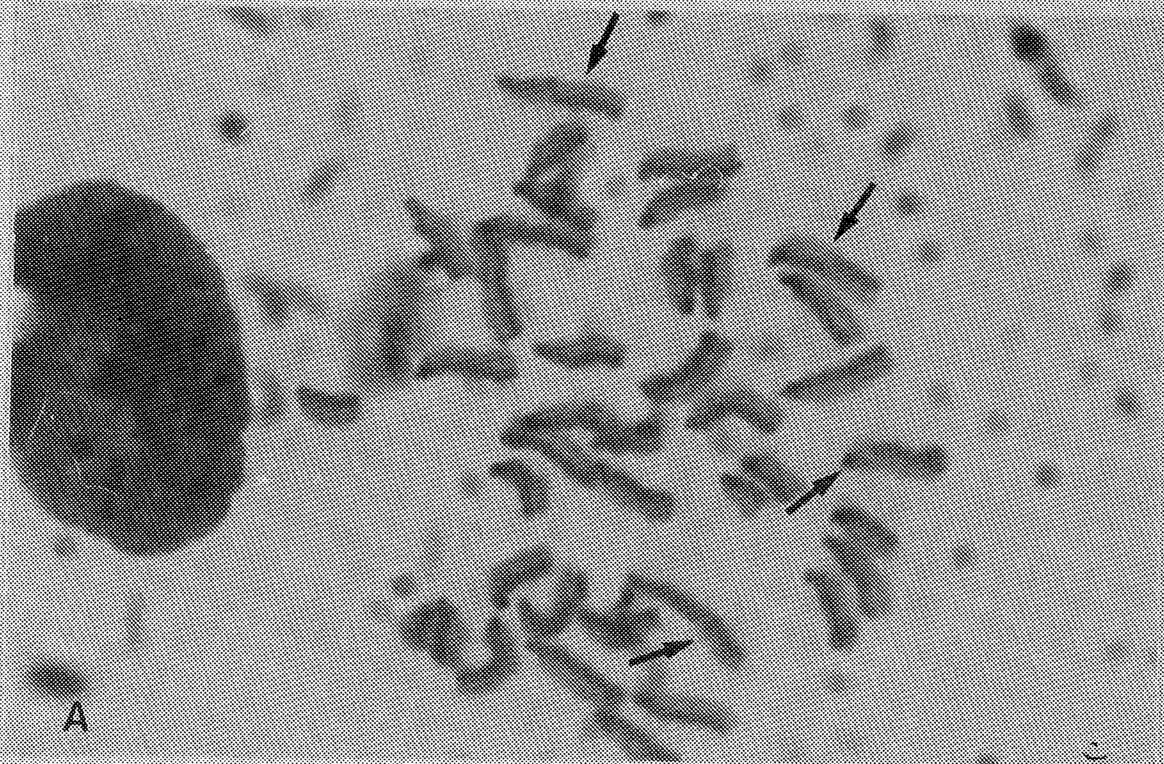
Fifty metaphase cells were examined from each treatment group and the control, with the frequency of SCEs shown in table 11. Control metaphases had the lowest frequency of SCEs, 4.4±1.01, 5 mg/kg treatment had a frequency of 5.0±1.63, 10 mg/kg treatment had a frequency of 7.04±1.04 and 15 mg/kg treatment had the highest frequency of SCEs at 7.76±1.23. Figure 16 shows SCEs in the treatment groups and the control. The total number of SCEs at different treatment doses increases as the dosage of MMC increases as seen Figure 17.

Table 11

Frequency of SCEs in control, 5, 10 and 15 mg/kg treatment doses

Dosage (MMC)	Number of metaphase cells counted	Number of SCEs	SCEs/cell $\bar{x}$ ± S.D.
Control	50	220	4.4 ± 1.01
5 mg/kg	50	250	5.0 ± 1.63
10 mg/kg	50	352	7.04 ± 1.04
15 mg/kg	50	388	7.76 ± 1.23
		$p<0.02$ (between all treatment groups & control) $r=0.9748$	

FIGURE 16. Sister chromatid exchanges (SCEs) are shown in four different groups (A, B, C and D). Some of the SCEs are indicated by arrows. Control (16A) show a few SCEs, while treatment of animals with 5 mg/kg (16B), 10 mg/kg (16C) and 15 mg/kg (16D) show increased frequency of SCEs, x 1,000.



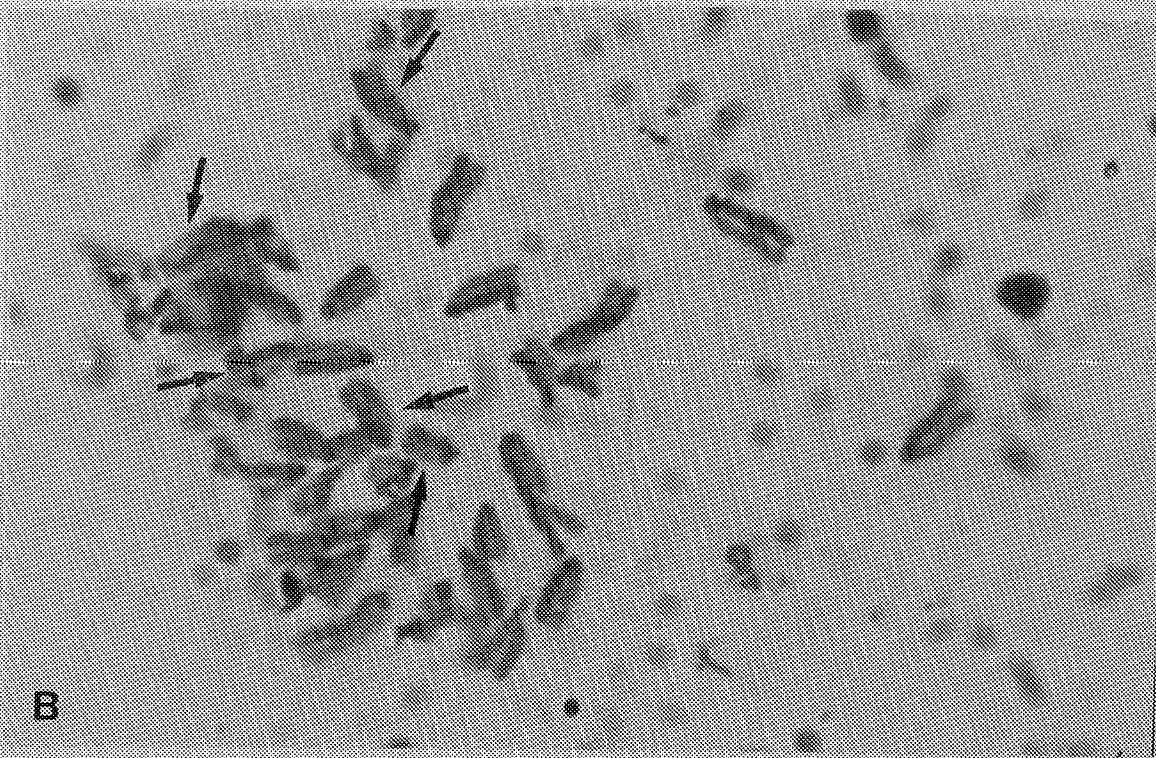
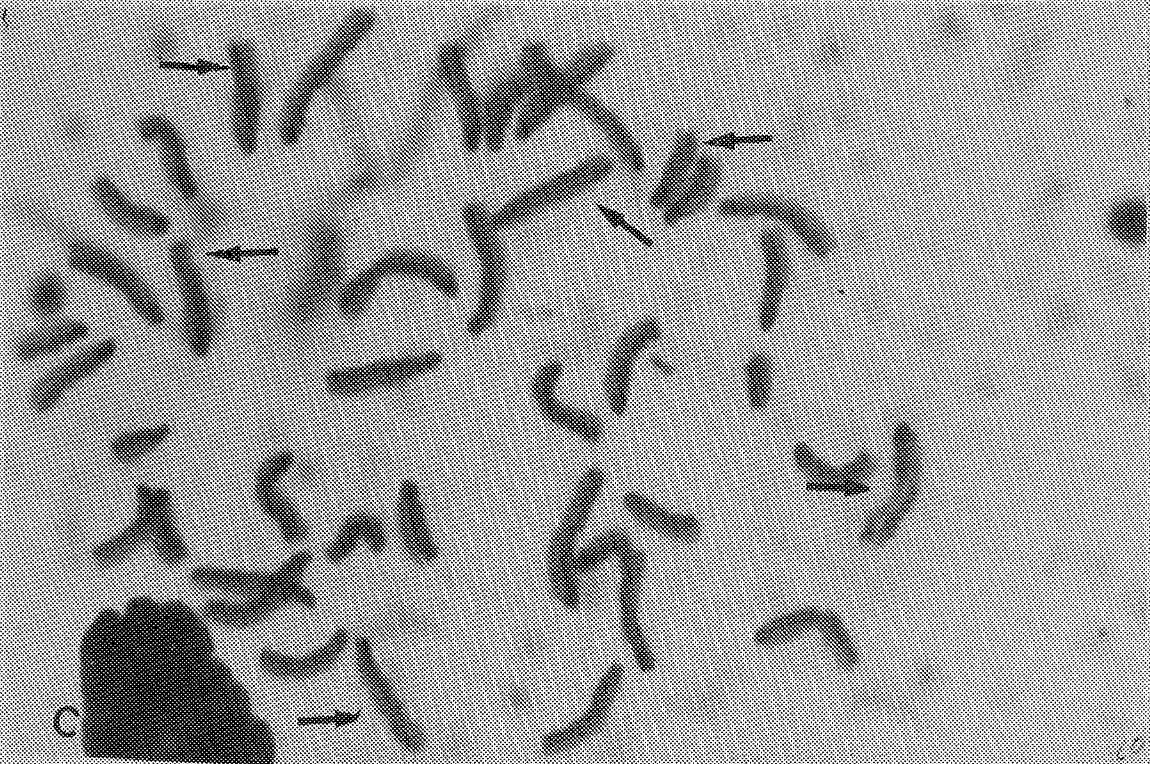


Fig.16



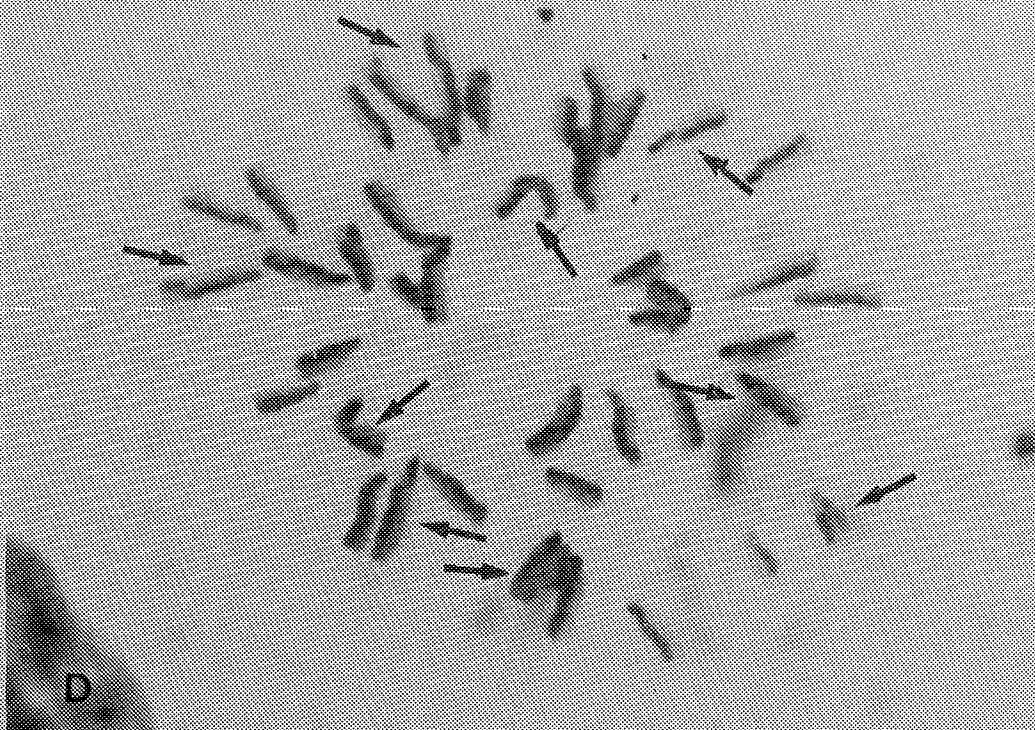


Fig.16

FIGURE 17. Total number of SCEs counted in 50 metaphase cells at different dose levels of MMC treatment.

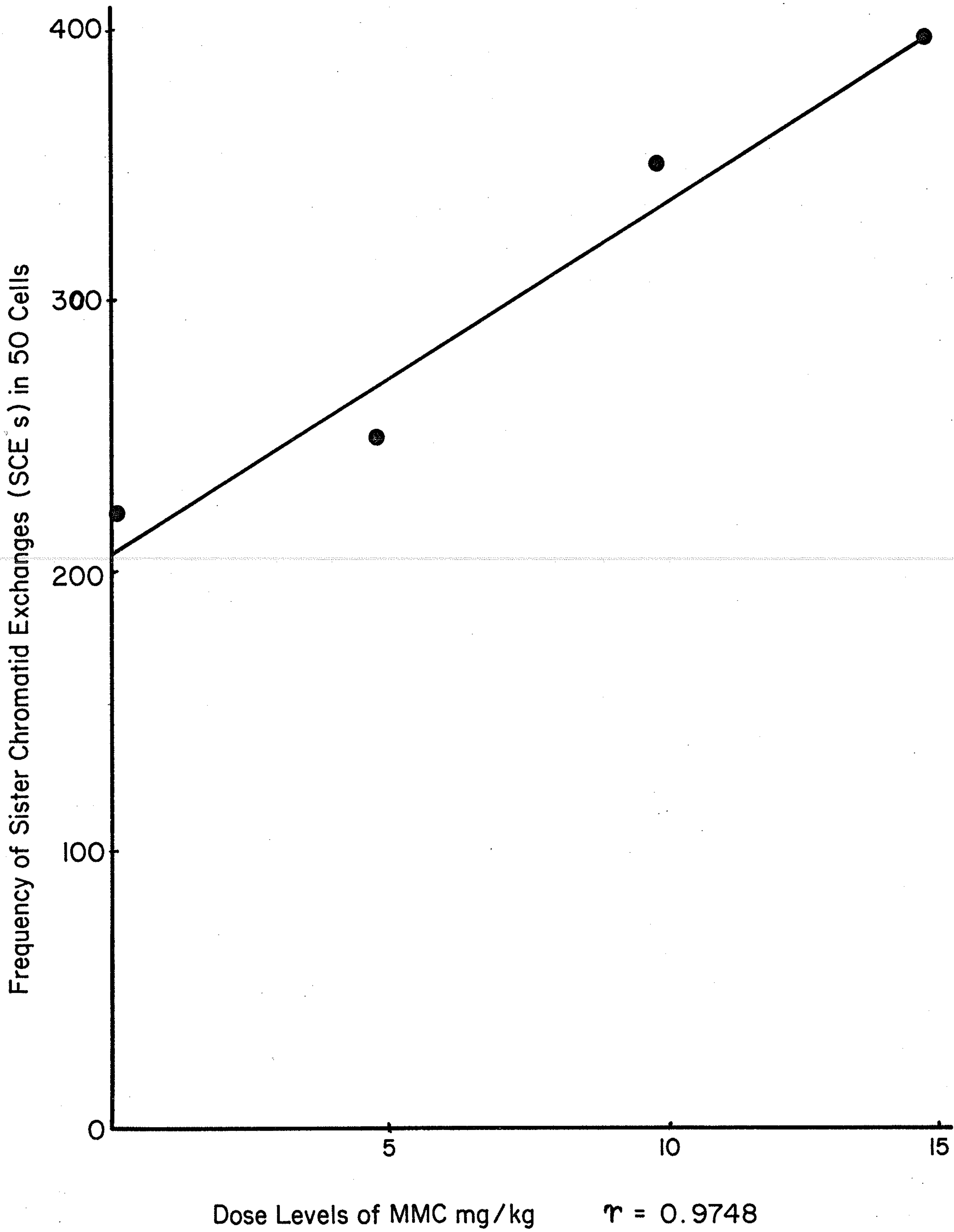


Fig. 17

4.5.4 Nucleolus organizing regions (NORs)

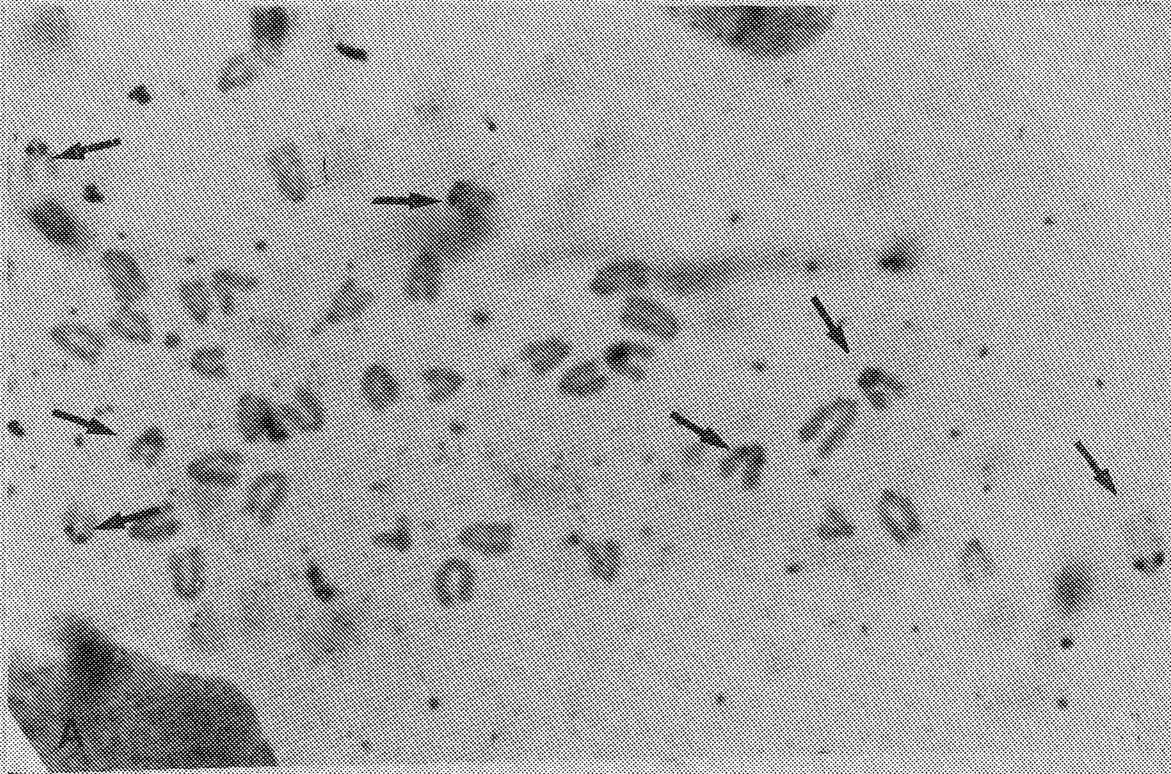
Darkly silver-stained NORs were located on several pairs of acrocentric mouse chromosomes. In mice there are usually 3-6 pairs of NORs that are active at any one time. Table 12 summarizes the results from counting the number of NORs observed in 50 metaphase cells per group. The average number of NORs per metaphase cell varied between 7.44±1.10 in 10 mg/kg treatment group to 6.76±1.67 in the 15 mg/kg treatment group. Paired black stained NORs are seen in Figure 18, which includes the control and three treatment doses.

Table 12

Frequency of NORs in control, 5, 10 and 15 mg/kg treatment doses

Dosage (MMC)	Number of metaphase cells counted	Number of NORs	NORs cell $\bar{x} \pm$ S.D.
Control	50	356	7.12 ± 1.04
5 mg/kg	50	360	7.20 ± 0.40
10 mg/kg	50	372	7.44 ± 1.10
15 mg/kg	50	338	6.76 ± 1.67
$p>0.80$ (not significant for any treatment groups & control) $x^2=0.8345$ (chi-square for all groups & control and is not significant)			

FIGURE 18 Nucleolus organizing regions (NORs) are shown in four groups (A, B, C and D) as pairs of black bodies (arrows). Control (18A), maternal treatment with 5 mg/kg (18B), 10 mg/kg (18C) and 15 mg/kg (18D). Note the similar number of NORs per genome, x 1,000.



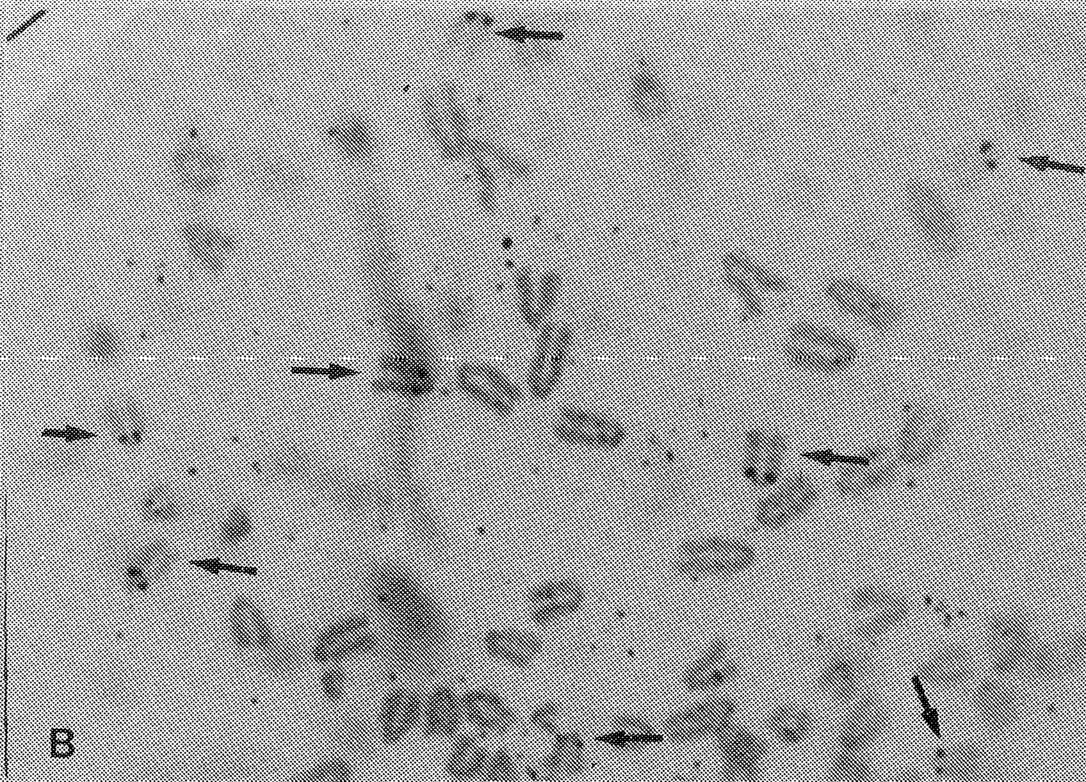
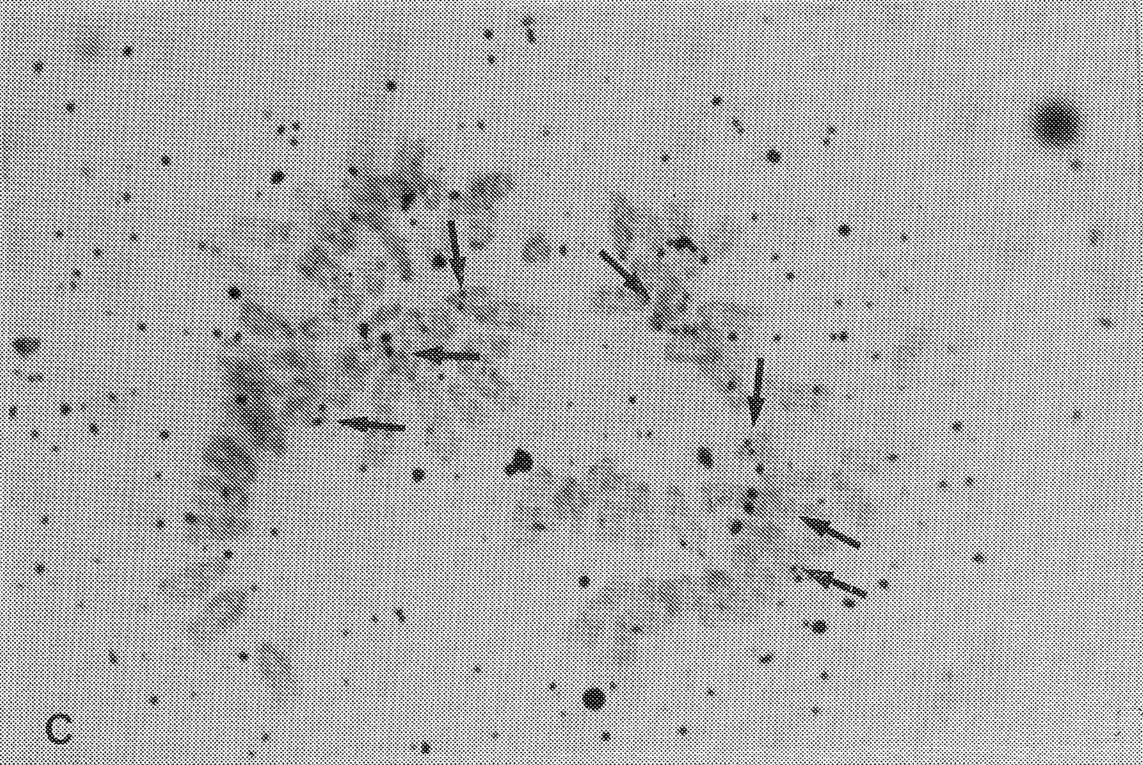


Fig. 18



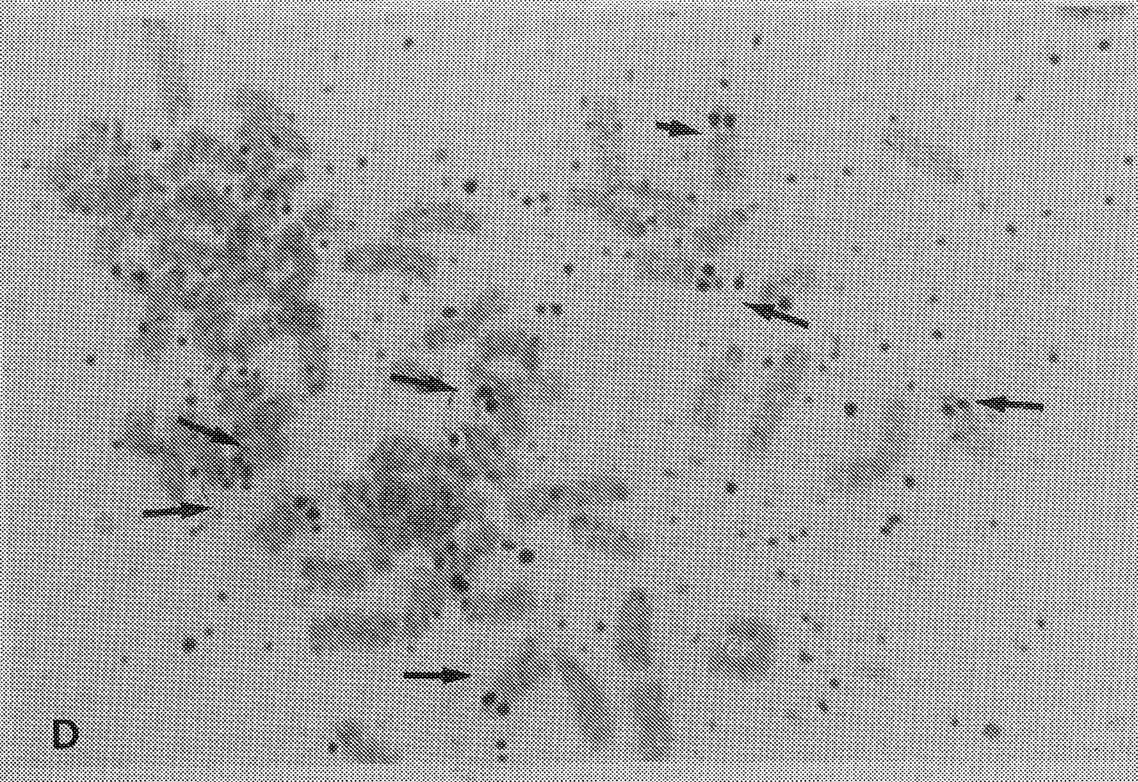


Fig.18

4.5.5 C-Mitosis analysis

Light microscopical examination was done on male adult mouse duodenal tissues from control and six treatment doses (5, 10, 15, 20, 25 and 30 mg/kg). One thousand cells were examined from each group, observing the number of cells suspended in metaphase. Table 13 summarizes the results of cells suspended in metaphase in a clumped condition. A relatively low percentage of cells were observed in all stages of mitosis in the control (9.4%). The percentage of cells arrested in metaphase increased as the treatment dose increased, 11.1% in 5 mg/kg treatment as compared to a value of 22.6% in the 30 mg/kg treatment. There was a consistent increase of metaphase arrest observed as dosage increases.

Figure 19A and 19B shows duodenal cells from control and the treatment groups. Mitotic cells observed in control tissues were clearly passing though metaphase into anaphase without mitotic arrest. The treatment groups showed all mitotic cells arrested in metaphase with complete clumping of the chromosomal material. Vacuolation was present and seen as empty spaces in the duodenal tissue. It was first evident at 15 mg/kg treatment dose and increased with the dose of methylmercury.

Figure 20 compares the increase in percentage of metaphase cells following treatment with different dose levels of MMC.

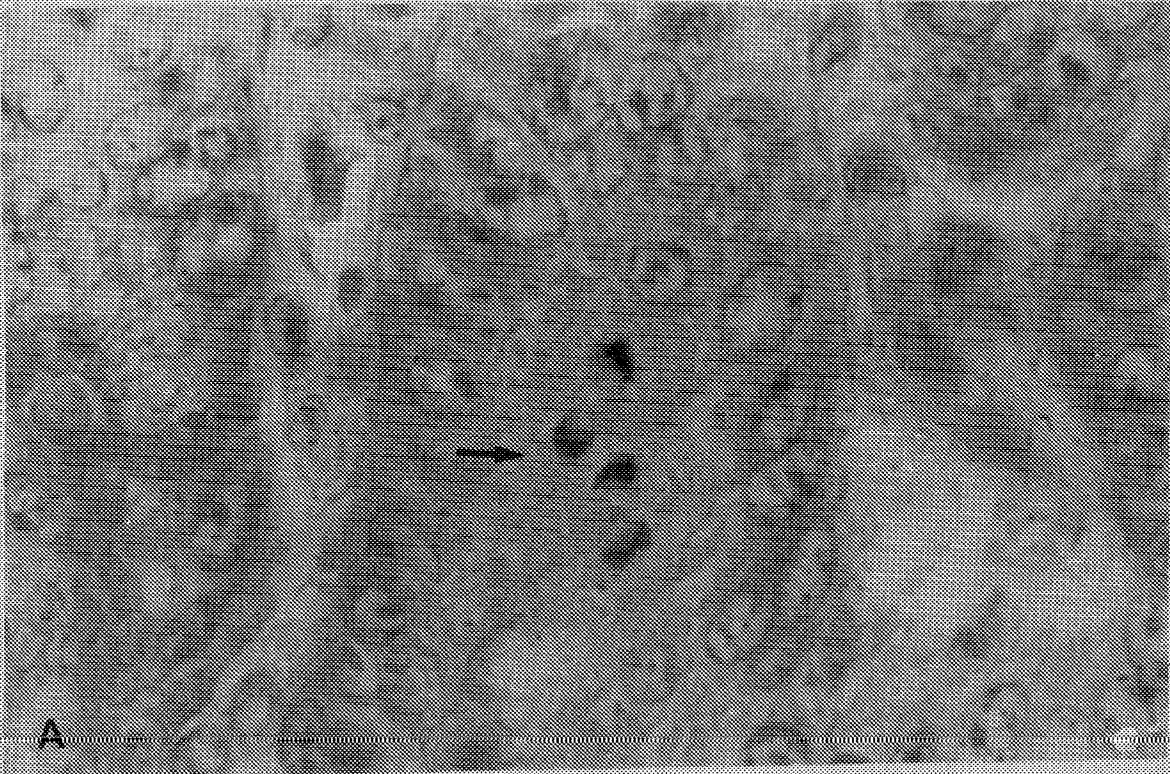
Table 13

Frequency of duodenal cells suspended in metaphase in control and six dose levels

Dosage (MMC)	Total number of cells counted	Cells in metaphase	Cells in interphase	Cells suspended in metaphase (%)
Control	1000	94	916	(9.4)
5 mg/kg	"	111	849	(11.1)
10 mg/kg	"	157	843	(15.7)
15 mg/kg	"	183	817	(18.3)
20 mg/kg	"	190	810	(19.0)
25 mg/kg	"	212	792	(21.2)
30 mg/kg	"	226	774	(22.6)

$p<0.001$ (between all treatment groups & control)
$r=0.9784$

FIGURE 19. Seven groups (A, B, C, D, E, F, and G) of duodenal tissues demonstrating mitotic cells in paraffin sections stained with hematoxylin and eosin. Control (19A) shows two mitotic cells in anaphase (arrow); the 5 mg/kg (19B) treatment group shows several mitotic cells arrested in metaphase (arrows). Figure 19C (10 mg/kg) shows several mitotic cells arrested in metaphase. Figure 19D (15 mg/kg) also shows numerous clumped mitotic cells (arrows). Figure 19E (20mg/kg), 19F (25 mg/kg) and 19G (30 mg/kg) show numerous arrested mitotic cells (arrows) and lyzed cells which appears as vacuoles (V), x 1,000.



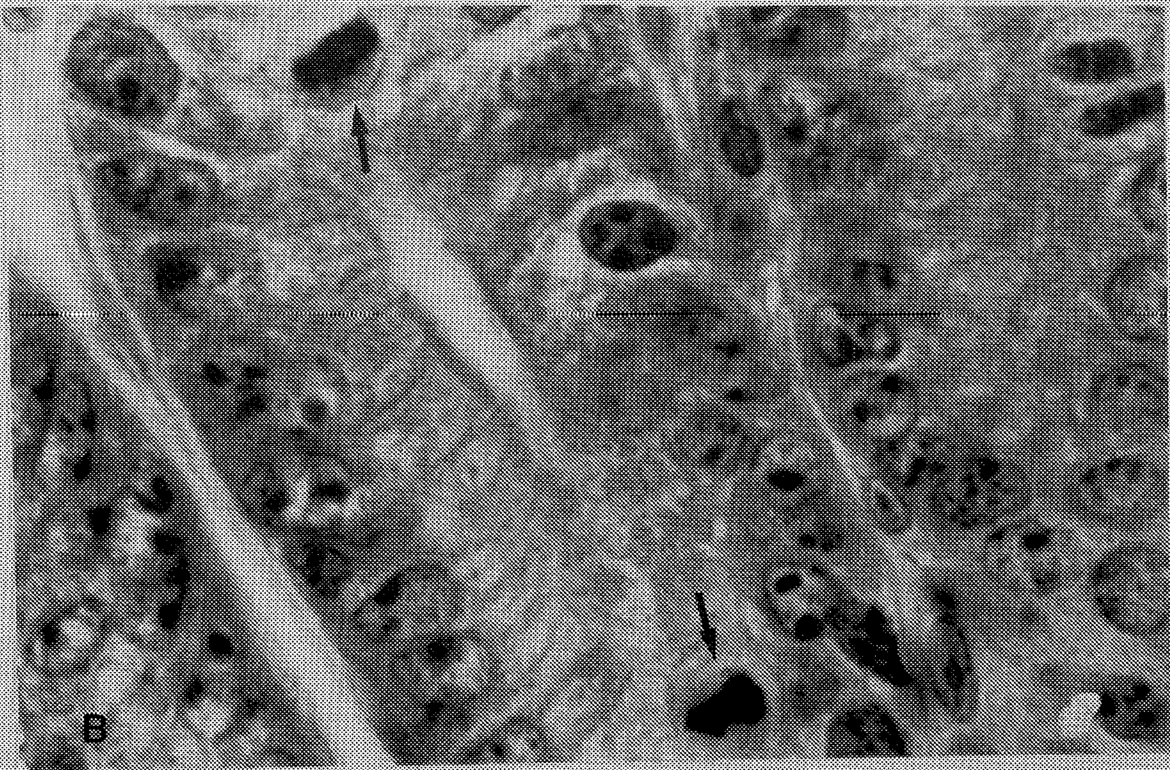
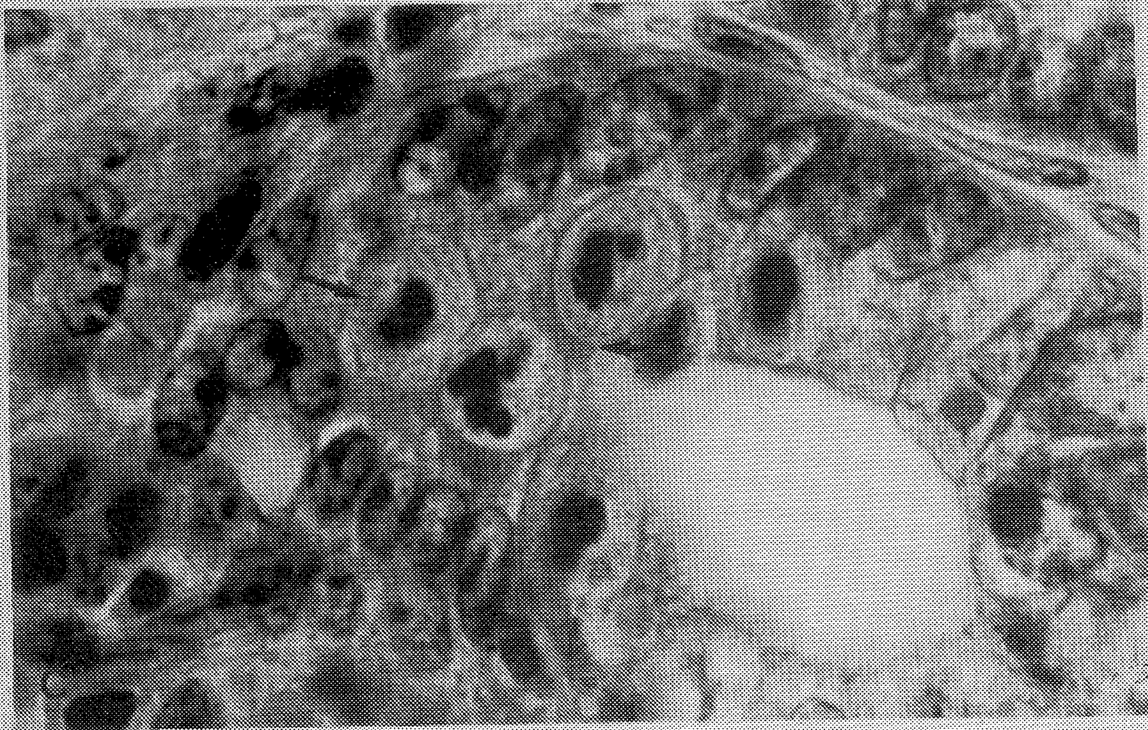


Fig.19



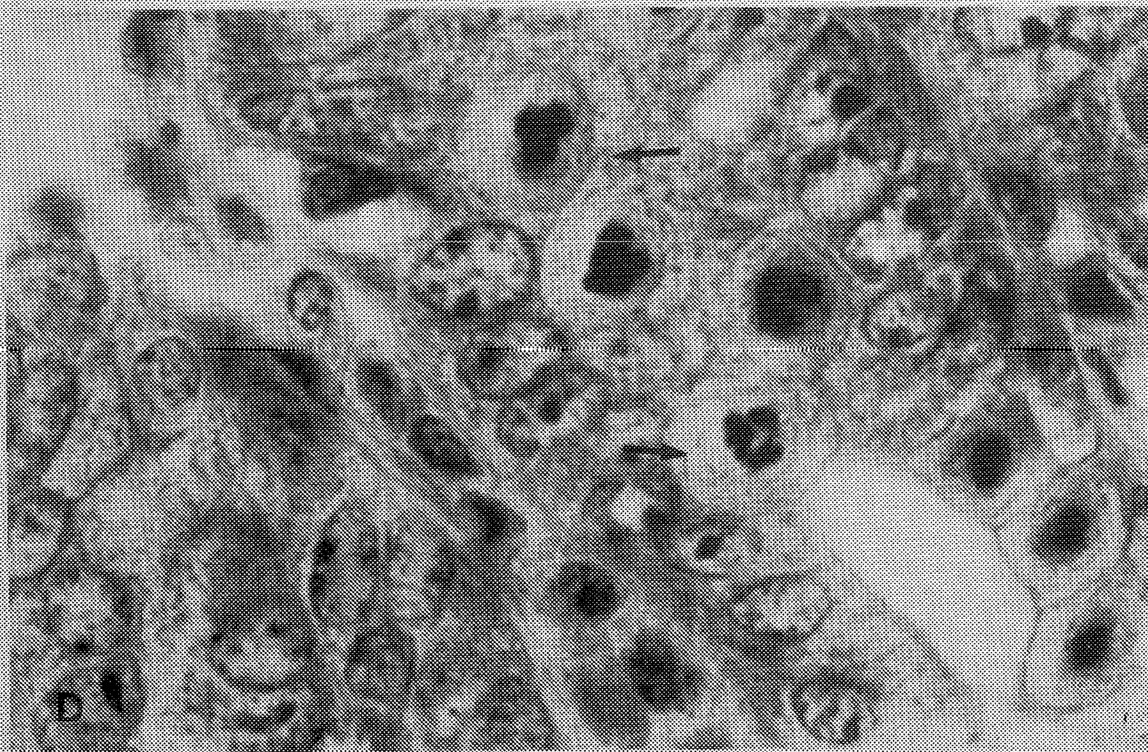
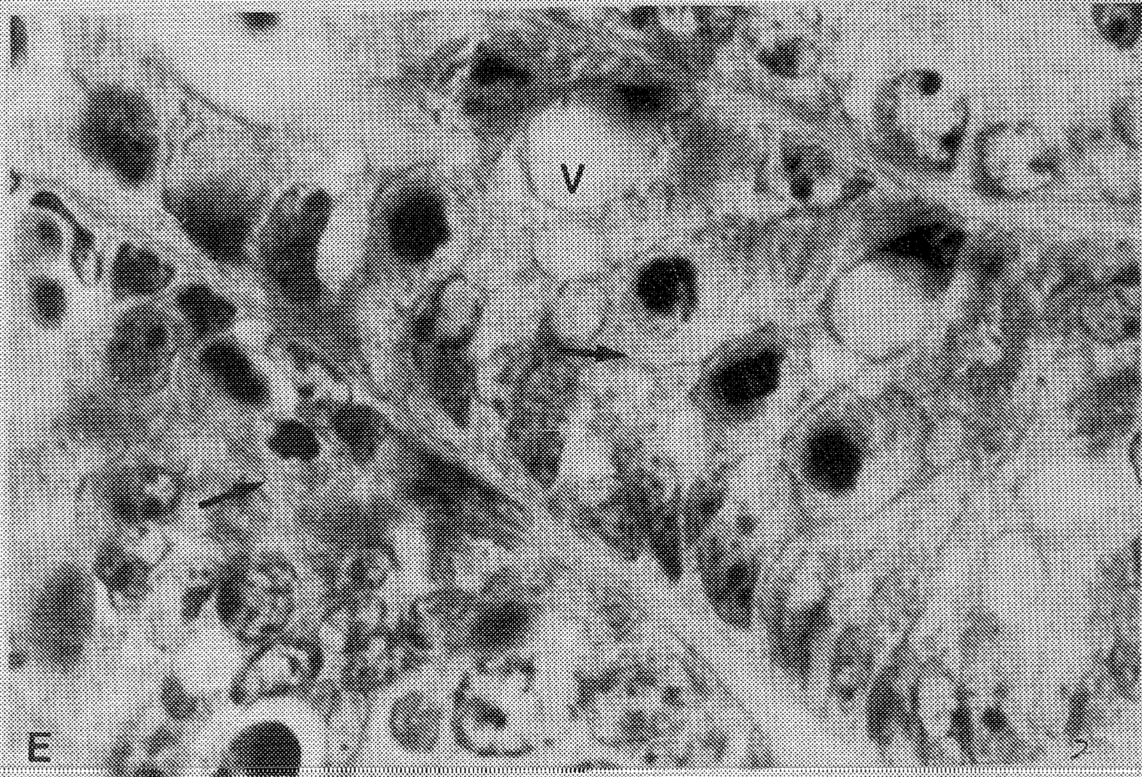


Fig.19



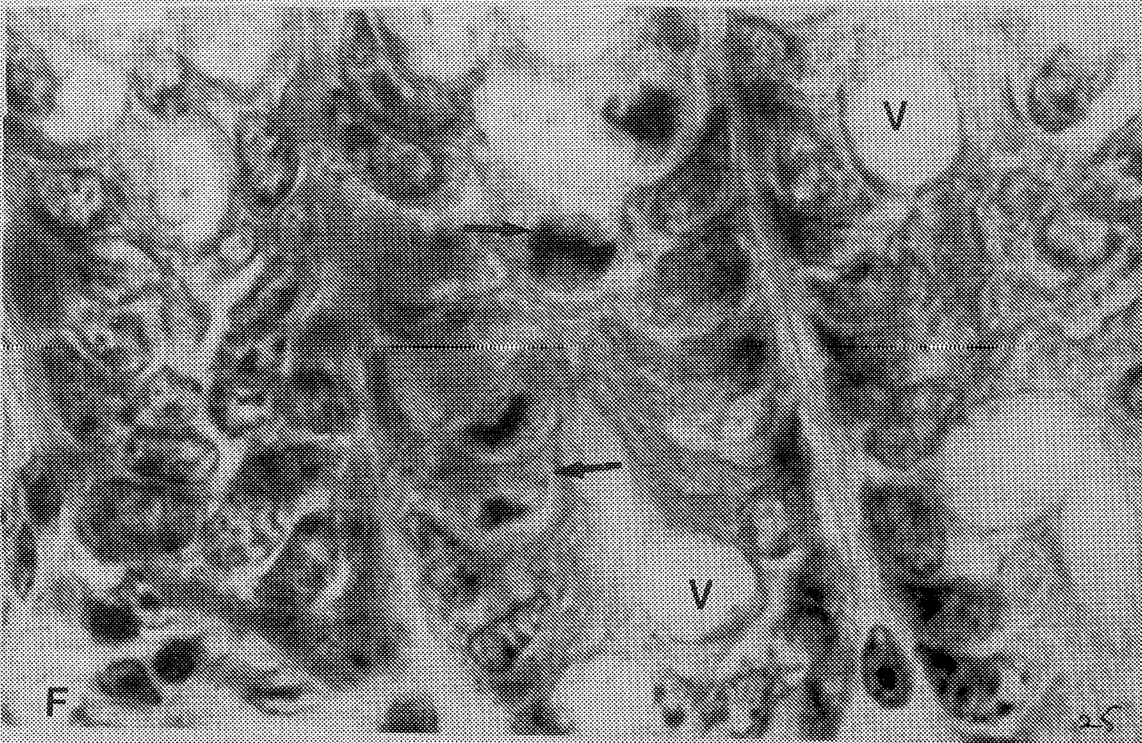


Fig.19

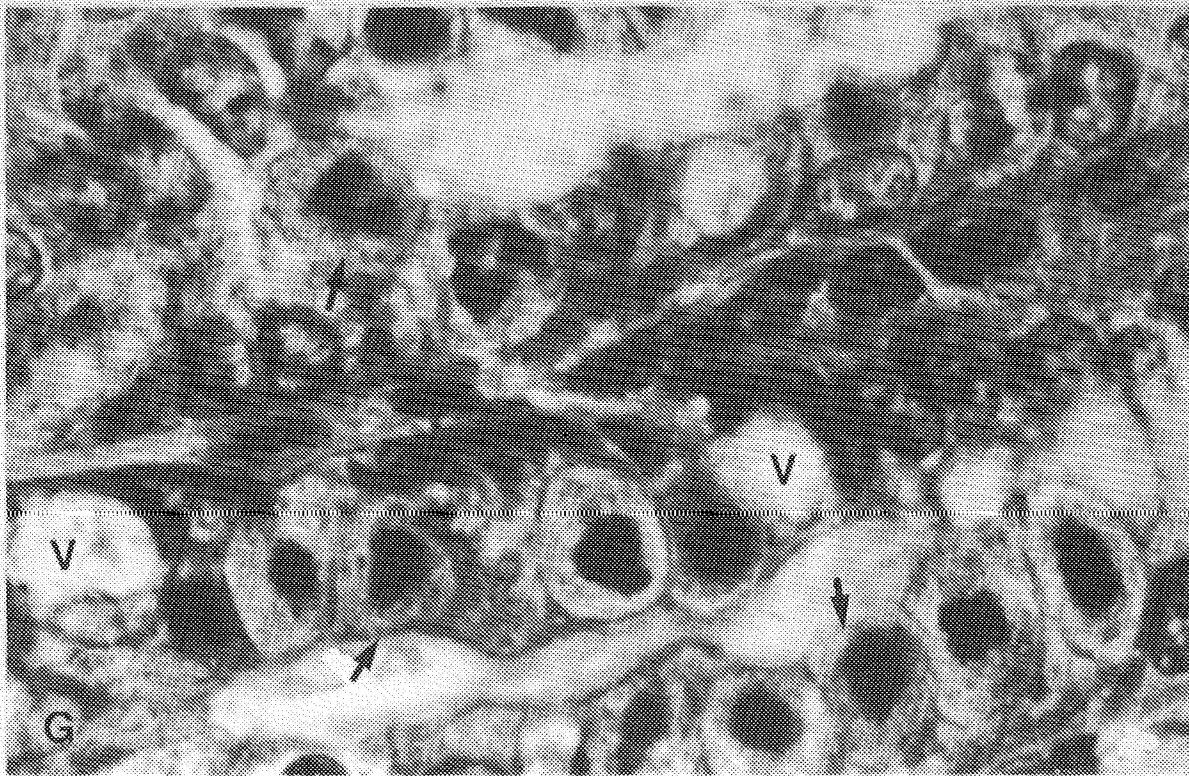
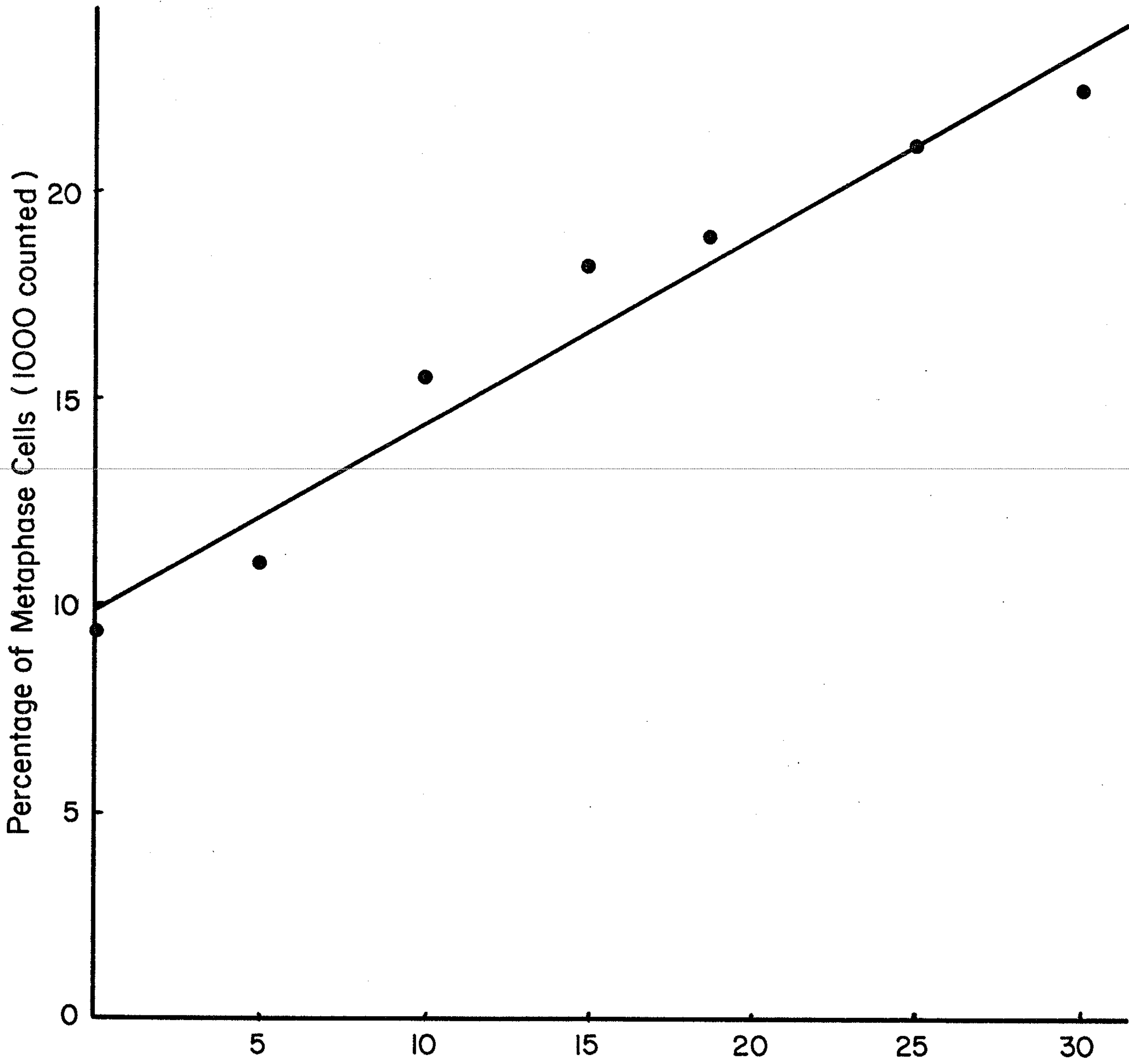


Fig.19

FIGURE 20. Percentage of mitotic cells observed following treatment with different dose levels of MMC.



Dose Levels of MMC mg/kg

$r = 0.9784$

$p < 0.001$

Fig. 20

5.0 DISCUSSION

5.1 Methylmercuric chloride as an environmental pollutant and teratogen

Methylmercury is a world-wide major environmental pollutant and its toxic effects are well documented. During the past decade, several outbreaks of mercury poisoning having occurred (1-6,100). There is ample evidence that organic mercury can harm the developing fetus (17). The Minimata tragedy and others have provided tangible evidence of its harmful effects during pregnancy (7, 9, 10,& 14).

The short term toxic effects of methylmercury on plants, animals and man have been studied and its harm to living organisms has generated justified concern. Even more attention is being focused on the prospects of long-range consequences (chromosomal damage) which are largely unknown. Mercury is widely distributed in the earths crust, sea, ground and rain water; all phyla naturally contain traces varying with local extensive industrial and agricultural usage which has significant effects on its redistribution in specific regions. Methylmercury can pass the placental barrier and cause damage to the neonate which may not be evident at birth. Affected infants develop mental and motor disabilities, manifested in retardation, cerebral palsy and seizures. These problems arise often without maternal clinical signs of poisoning.

Experimental studies signified that methylmercury is highly teratogenic in rats, mice, hamsters, cats and also in chickens (101). Mice showed the highest incidence of abnormalities in the offspring of all the species studied. Cleft palate was the most common abnormality observed in mice but growth retardation was also prevalent in fetal mice. A high incidence of skeletal anomalies was also observed in fetuses from treated mothers (16-20).

5.2 Manifestations

5.2.1 Maternal weight changes

Maternal weights increased between day 9 and 18 of gestation except for the two highest dose levels, where there was an actual net loss of weight as indicated by a negative value at the 25 and 30 mg/kg dose levels. Statistical comparison (ANOVA) showed that the differences between the means of the treatment groups and the control were significantly different (P<0.001). The maternal weight increased more slowly in the treated animals; however, at the 5 mg/kg dose level it increased almost to that of the control. The 10, 15 and 20 mg/kg dose levels showed weight increases that were reduced and 25 and 30 mg/kg dose levels showed actual weight losses. The animals from the two highest dose levels weighed significantly less at the time of sacrifice when compared to their weight at the time of treatment; this drastic change in weight is probably due to the high incidence of fetal resorptions at these dose levels. A linear relationship exists between maternal weight changes and dosage of methylmercury. The coefficient of correlation ($r = -0.98021$) shows a high degree of correlation between increasing dose levels and reduced maternal weight increases between day 9 and 18 of gestation. The probability value ($p < 0.001$) indicates a causal dose/response association as shown in Figure 1.

5.2.2 LD_{50} dose of MMC in maternal mice

The LD_{50} dose for pregnant mice after treatment with methylmercuric chloride was found to be 20 mg/kg body weight, as shown in Table 3. The lethal dose which killed 100% of the animals treated was found to be 30 mg/kg dose level, also shown in Table 3. These results indicate that methylmercury is lethal for maternal mice in the upper range of the treatment groups. The probability of differences between the number of maternal death in treatment groups and the control was highly significant. Figure 2 compares the percentage of maternal deaths to the increasing dosage of methylmercuric chloride. The coefficient of correlation indicates (r = 0.9217) a very definite association between methylmercuric chloride dosage and the high frequency of maternal deaths. The r value indicates a causal dose-response relationship demonstrating that methylmercuric chloride is responsible for maternal deaths and is lethal. Death is due to the denaturating effect of mercury on the proteins of the cell.

5.2.3 Total Implantations

The total number of implantation sites and the average number of implantations per group ($\bar{X}$) is shown in Table 1. The average for all groups, treatments and control, was 11.97 ± 1.01. The difference between the means for individual groups did not vary significantly from the total mean number of implantations. These results indicate that treatment with MMC on day 9 gestation does not significantly influence the number of implantations per litter.

5.2.4 Resorptions

Methylmercuric chloride induced a significant increase in the incidence of resorptions as indicated by the probability value ($p < 0.02$). The coefficient of correlation ($r = 0.8612$) showed a dose-related response between increasing dose levels and frequencies of resorptions indicating that MMC is embryolethal at all dose levels. The embryolethal dose, where 50% of embryos were resorbed (LD_{50}), was determined to be 17 mg/kg while the dosage required to cause a 100% resorption rate was determined to be 25 mg/kg of MMC. It would appear that 15 mg/kg of body weight of methylmercuric is the limiting dose level that can be administered to the mother before the level becomes lethal to the fetuses.

5.2.5 Intrauterine death

Depending upon the time of fetal death, two different manifestations of methylmercury poisoning can occur. If fetal death occurs shortly after administration of MMC, the dead fetuses will have been resorbed by the time of maternal sacrifice. This tends to occur at the higher dose levels of MMC. The 25 mg/kg dose caused a 64% partial resportion rate, and a

36% rate of intrauterine death without any resorption. This is probably ascribable to fetal death occurring briefly prior to sacrifice of the mothers and harvesting of the fetuses. The results shown in Table 4 indicate that methylmercury had very significant effects on the total number of intrauterine deaths following treatment.

A significant increase in the incidence of fetal deaths ($p < 0.01$) was also recorded. The LD_{100} dose for fetuses was determined to be at the 25 mg/kg dosage. Correlation of coefficient result (γ = 0.9477) showed a dose-response association which is highly significant. This demonstrates that the number of intrauterine deaths increased significantly with augmenting dosages of MMC, confirming a causal relationship between dose and response.

5.2.6 Live fetuses recovered (normal and abnormal)

A high percentage of fetuses was recovered alive in the control and 5 mg/kg treatment groups. The proportion of fetuses recovered alive decreased with augmenting dosages of MMC, until none were recovered alive at the 25 mg/kg dose level and beyond. Among the live fetuses recovered, a significantly high percentage of malformations ($p < 0.001$) was observed following treatment with 10, 15 and 20 mg/kg of MMC. The 20 mg/kg dose induced 100% malformations in the live fetuses. These results establish the embryotoxic range of MMC to be between 10 - 20 mg/kg of body

weight. The coefficient of correlation between dosage and malformations was highly significant ($r = 0.99225$). These results clearly indicate fetal malformations are induced *in utero* by methylmercuric chloride in a dose-response manner.

5.2.7 Fetal malformations (macroscopic and skeletal)

The incidence of fetal malformations, especially cleft palates were markedly increased in 10 through 20 mg/kg treatment doses. It was statistically highly significant ($p < 0.001$). Omphalocele was also present at the 20 mg/kg treatment level. Skeletal staining revealed the presence of cleft palates at three dose levels (10, 15 and 20 mg/kg). The coefficient of correlation between dosage and fetal anomalies was highly significant ($r = 0.99225$) in a dose-response association thus indicating MMC to be highly teratogenic in fetuses of ICR Swiss Webster mice.

5.2.8 Mean fetal weights

Administration of MMC on day 9 of gestation at the various dose levels produced a significant reduction in mean fetal weights by time of recovery on day 18 of gestation. The fetuses from all treatment groups weighed significantly less than those of the control (30 mg/kg treatment group had no fetuses available due to

total resorption). Table 6 shows the p value ($p < 0.005$) which is highly significant and reveals that the reduction in mean weight of fetuses is due to the dosage of MMC. There is a direct dose-response causal relationship between decreased mean fetal weights and treatment of maternal mice with MMC. Figure 7 indicates a high negative correlation between dose and response which is highly significant as confirmed by the coefficient of correlation ($\Upsilon = -0.98703$). The reduced fetal weight is probably due to lower mitotic growth rates in the affected fetuses.

5.2.9 Levels of maternal mercury

A significant increase of mercury levels was detected in treated animals compared to controls ($p < 0.001$). Figure 8 compares the levels of mercury (PPM) in maternal blood to dose levels of MMC and a high correlation between dose and response is evident ($\Upsilon = 0.9604$). This highly significant value confirms a direct relationship between dose levels and maternal blood levels. The two highest dose levels (25 and 30 mg/kg) have much higher mercury levels than the lower treatment doses, probably due to the fact that the fetuses which contained mercury were resorbed back into the maternal system and thereby elevating it even higher relative to the lower dose levels. The levels of mercury obtained in maternal mice, especially at the 20, 25 and 30 mg/kg dosages are similiar to the range of mercury blood levels seen in Maninata fishermen.

5.2.10 Levels of fetal mercury

Methylmercury levels in fetuses were determined and recorded as shown in Table 8. A highly significant increase of mercury levels was detected in fetuses of treated animals compared to fetuses from control animals ($p < 0.001$). No fetuses were available for analysis at the two highest dose levels due to resorption. Figure 9 shows the correlation between dosage and levels of mercury (PPM) in the fetuses. The coefficient of correlation value (γ = 0.99898) indicates an extremely close relationship between dosage and fetal mercury levels. This value is highly significant and clearly indicates a direct association between dose and response.

5.2.11 Comparison of maternal and fetal mercury levels

Mercury levels were found to be approximately twice as much in fetuses than in the mothers following maternal treatment at any dose level. Figure 10 establishes a correlation between the maternal and fetal mercury levels which is highly significant ($p < 0.001$) and directly related to dosages administered as shown by the coefficient of correlation value (γ = 0.9975). These values demonstrate that mercury is accumulating in the fetuses at about twice the concentration level as in the mothers for each treatment

dose. The dam showed little or no effect of mercury toxicity, whereas the fetuses were found to be most susceptible to mercury toxicity, on account of its teratogenic effects.

5.2.12 Light microscopy (fetal lung)

The growth and development of the fetal lungs can be estimated by its morphological appearance and by the amount and location of intracellular glycogen. The lungs of fetuses recovered on day 18 of gestation were found to be at different stages of growth and development. Control lungs appeared most mature and developed as they were in the terminal air sac stage. The epithelial lining was flattened and contained little intracellular glycogen. The interstitial mesenchyme was relatively thin and contained some intracellular glycogen. There were no alveolar tubules present or cuboidal epithelial cells filled with glycogen. This type of respiratory morphology is associated with relatively mature and well developed fetal lung just before parturition.

Fetal lungs from the 5 mg/kg treatment group revealed a less well developed appearance. Both terminal air sacs and the more immature alveolor tubules were present. The epithelium was not as advanced in differentiation as determined by its cuboidal shape

with abundant intracellular glycogen. The interstitial mesenchyme was thicker than in the control with some intracellular glycogen present. These findings suggested that these lungs are in a transitional phase between the more mature terminal air sac stage and the less mature pseudoglandular stage.

Fetal lungs from the 10 mg/kg treatment group appeared to be delayed in growth and development compared to those from 5 mg/kg treatment group and the controls. They exhibited a distinct pseudo-glandular appearance with numerous alveolar tubules but no terminal air sacs. The alveolar tubules were lined with undifferentiated cuboidal epithelium filled with intracellular glycogen. The very thick intracellular mesenchyme had little or no glycogen. These findings suggest a relatively immature pseudoglandular lung, delayed in growth and development, for a fetus of this gestational age.

Lungs examined from the 15 mg/kg treatment group had a hypocellular alveolar tubular pseudoglandular appearance. There were fewer alveolar tubules than in the 10 mg/kg group which gave a hypocellular appearance to the lung. The mesenchyme was thick with no glycogen present, while the epithelia cells were undifferentiated and distended with glycogen. The morphological appearance of the lung was that of an immature, underdeveloped lung not consistent with a fetal mouse lung just prior to parturition as was seen in the control.

These histochemical and morphological results indicate that methylmercury has a perturbing effect on fetal lung growth and subsequent development. The immature appearance of the lungs from the treatment groups compared to the control support the contention that lung growth and development are retarded by methylmercury. The degree of retardation of fetal lung growth and development paralleled the increasing dosage of MMC. The highest treatment dose (15 mg/kg) examined revealed the most retardation of pulmonary growth and development. The intermediate dose (10 mg/kg) caused less retardation of growth and development while the lowest dose (5 mg/kg) had the least effect. However, all three treatment groups appeared less developed compared to the controls.

5.2.13 <u>Electron microscopy (fetal lung and liver)</u>

Electron microscopical studies of fetal lung and liver revealed that mitochondrial degeneration was the salient morphological change seen in fetal tissues examined from dams treated with methylmercuric chloride. Initial damage was seen as mild vacuolation in mitochondria at the lowest dosage group (5 mg/kg). Mitochondrial ultrastructure damage was progressive as apparent in the intermediate treatment group (10 mg/kg); large vacuoles with sworls were seen inside pleomorphic mitochondria. The highest dose level (15 mg/kg) caused actual mitochondrial destruction as indicated by edema and lysis. The progressive

destruction and loss of mitochondria would lead to entrophy and eventually irreversible cell necrosis.

5.3 Cytogenetics

5.3.1 Conventional chromosome analysis

Chromosomal clumping is considered to be a major type of chromosomal aberration (54). The results summarized in Table 9 indicate that clumping increased significantly ($p < 0.005$) in the treatment groups compared to the control which showed no clumping. The relationship between dose and clumping as seen in Figure 14 ($\gamma = 0.94022$) is very significant indicating a causal dose-response association. Chromosome clumping, identified as an aberration, caused severe technical problems and all cytogenetic procedures were frought with great difficulties.

5.3.2 Chromosome counts

Chromosome counts were done on 100 metaphase cells to determine the average number of chromosomes per cell. These results summarized in Table 10 revealed that there was no significant variation ($p < 0.95$) between control and treatment groups. Chromosome

aberrations (structural) were not detected in the metaphase cells examined. It therefore appears that the euploidy and gross structural integrity of chromosomes was not affected by methylmercuric chloride toxicity.

5.3.3 Sister chromatid exchanges (SCEs) frequencies

Sister chromatid exchange frequency in treatment groups increased significantly ($p < 0.02$) from control levels. Table 11 summarizes the results of SCEs frequencies and an elevated number of SCEs was detected in all the treatment groups. The increase in SCEs appeared to be dependent upon increasing dosages of MMC. There was a significant increase in SCEs as the dosage of MMC increased which followed a dose-response relationship. SCEs represent subtle DNA damage and repair (69), and a dose-response association would suggest that methylmercury enhances subtle DNA damage and/or lack of DNA repair in some manner. Increased DNA damage may eventually lead to an increased mutation rate and this may have serious ramifications.

5.3.4 Nucleolus organizing regions (NORs) frequencies

Table 12 shows the total number of NORs counted in 50 metaphase cells from each treatment and control groups. The average number of NORs in control and treatment groups were not significantly different as determined by the Chi square analysis ($p > 0.80$).

Treatment with methylmercuric chloride does not appear to have any effect on NOR activity and expression in fetal mice.

5.3.5 C-mitosis

Table 13 shows the total number of mitotic cells seen in the duodenum of adult male mice treated with methylmercuric chloride. The control shows (Figure 19A) mitotic cells in anaphase which means mitosis was not perturbed and arrested in metaphase. All treatment groups, as seen in Figure 19B through 19G show mitotic cells arrested in metaphase with none passing through to anaphase indicating that mitosis would be inhibited after MMC treatment.

The degree to which mitosis was inhibited can be determined by correlating the number of arrested mitotic cells with dosages of MMC. The coefficient of correlation value ($r = 0.9784$) shown in Figure 21 indicates a linear relationship between increasing dose levels of MMC and increasing frequency of arrested mitotic cells (C-mitosis). This increase in C-mitois of treatment groups is highly significant ($p < 0.001$) compared to untreated animals. Vacuolation of duodenal tissue which was present at the higher dose levels of MMC are probably caused by the arrest of mitotic cells and subsequent lysis due to the extended length of time the cell was suspended in metaphase. Eventually, the entire mucosa of the duodenum may be destroyed by arrest of mitosis and subsequent lysis.

6.0 CONCLUSIONS

Administration of methylmercuric chloride to pregnant mice on day 9 of gestation, at doses of 5, 10, 15, 20, 25 and 30 mg/kg, revealed the following:

6.1 Manifestations of mercury toxicity

Teratogenesis

1. Treated pregnant mice did not gain as much weight as the controls between the time of treatment and sacrifice. The high dose levels resulted in no weight gain or an actual weight loss.

2. LD_{50} dose for maternal mice was determined to be 20 mg/kg and LD_{100} dose was established at 30 mg/kg proving methylmercury's lethality.

3. The mean number of implantation sites was not affected by methylmercury treatment on gestation day 9.

4. Methylmercuric chloride significantly increased the incidence of fetal resorptions.

5. The incidence of intrauterine death was significantly increased.

6. An increased incidence of congenital malformation followed methylmercury's administration at 10, 15 and 20 mg/kg confirming its teratogenicity.

7. Methylmercury treatment caused a significant reduction in fetal weight at sacrifice revealing a slower growth rate in treated animals.

Mercury analysis

8. Levels of mercury in maternal blood were greatly elevated in treated animals compared to the controls.

9. Levels of mercury in fetuses were approximately twice as high as maternal levels. This occurs because of the sequestering of mercury from the mother to the fetus with the fetus serving as a mercury "sink" or "trap".

Light Microscopy

10. Light microscopy revealed that methylmercury inhibited the growth and development of fetal lungs.

Electron microscopy

11. Lungs and liver of treated fetuses showed evidence of cellular damage. In particular the mitochondria which revealed varying levels of vacuolation.

Cytogenetics

12. Chromosome clumping in fetal tissue was a constant feature following methylmercury treatment suggesting a reduced growth rate in fetuses.

13. Chromosome numbers per metaphase in fetuses was not affected by methylmercury treatment.

14. Chromosome aneuploidy and structural aberrations in fetuses was not observed at any of the MMC dose levels.

15. Sister chromatid exchange frequencies were increased significantly in all dose levels indicating methylmercury might influence DNA damage or repair of fetal chromosomes.

16. Nucleolus organizing region frequencies were not affected by methylmercury treatment.

Treatment of adult male mice with varying dose levels (5, 10, 15, 20 25 and 30 mg/kg) of methylmercuric chloride, killed six hours later revealed the following:

C-mitosis

1. A greatly increased number of duodenal cells arrested in metaphase (C-mitosis) causing mitotic disruption.

2. Vacuolation of the duodenal cells indicating cell necrosis and autolysis occurring at the higher dose levels.

6.2 Mechanisms of mercury's toxicity

Mercury has long been known as a binder of proteins forming strong mercaptide bonds with sulfhydryl groups and other anionic protein sidechains resulting in denaturation and deactivation of protein molecules.

Mercury's affinity for biopolymer anions is a basic mechanism of mercury's toxicity resulting in a multifold of reactions between the cell's protein and mercury.

Specific mechanisms

1. Inhibition of enzymes is a subcellular mechanism caused by chelate formation with mercury. Poisoned enzyme systems result in a reduced energy supply and lead to entropy (32,102).

2. Protein synthesis is inhibited resulting in a decreased rate of cell growth and development (102,103).

3. Membranal changes are due to denaturation of its protein moiety causing edema and vacuolation. Mitochondrial degeneration probably result from membrane changes and enzyme deactivation.

4. Mitotic arrest (C-mitosis) is caused by mercury binding to spindle proteins inhibiting polymerization of the microtubules.

5. Chromosomal alterations such as clumping and increased SCE frequency may be due to mercury binding with nucleoproteins (104,105).

6. Increased formation of free radicals may lead to increased DNA damage. Intrinsic enzyme systems (superoxide dismutase) provide protection against free radical formation. This protection is conceivably lowered by mercury inhibiting this enzyme system. Increased free radical formation may be an important subtle mechanism of mercury's toxicity on the cell (105).

REFERENCES

1. Lofroth, G.: Methylmercury - A review of health hazards and side effects associated with the emission of mercury compounds into natural systems. Working group on Environmental Toxicol. University of Stockholm, Sweden. (1970).

2. Clegg, D.J.: Embryotoxicity of mercury compounds. Mercury in man's environment (Royal Society of Canada, ed.) Royal Society of Canada, Ottawa. 141 (1971).

3. Clarkson, T.W.: Methylmercury toxicity to the mature and developing nervous system; possible mechanisms. Biological aspects of metals and metal related diseases, Raven Press, New York. (1983).

4. Nelson, N. Byerly, T.C., Kolbye, A.C., Kurland, L.T., Shapiro, R.E., Shibko, S.I., Stickel, W.H., Thompson, J.E., Van Dun Berg, L.A. and Weissler, A.: Hazards of mercury. Environ. Res. 4: 1 (1971).

5. Clarkson, T.W.: Quantative measures of the toxicity of mercury in man. Trace elements in human health and disease, Vol. II (A.S. Prasad, ed.) Academic Press, New York. 453 (1976).

6. Yoshino, Y., Mozai, T., and Nakao, K.: Biochemical changes in the brain of rats poisoned with an alkylmercury compound with special reference to the inhibition of protein synthesis in brain cortex slices. J. Neur. Chem. 13: 1223 (1966).

7. Harada, Y.: Clinical investigations on Minimata disease and congenital Minimata disease. Minimata Disease, ed. M. Kateuma, Kumomoto University Press, Kumomoto. 92 (1968).

8. Berlin, M., and Gibson, S.: Renal uptake, excretion and retention of mercury. Arch. Envir. Health 6: 617 (1962).

9. Koos, B.J., and Longo, L.D.: Mercury toxicity in the pregnant woman, fetus and new born infant. Am. J. Obstet. Gynecol. 126: 390 (1960).

10. Afona, J. and DeAlvarez, R.: Effects of mercury on human gestation. Am. J. Obstet. Gynecol. 80: 145 (1960).

11. Goldwater, L., Jacobs, M., and Ladd, A.: Absorption and excretion of mercury in man: I. Arch. Environ. Health 5: 537 (1962).

12. Ladd, A., Goldwater, L. and Jacobs, M.: Absorption and excretion of mercury in man.: II Arch. Environ. Health 6: 480 (1963).

13. Jacobs, M., Todd, A., and Goldwater, L.: Absorption and excretion of mercury in man. III Arch. Environ. Health 6: 631 (1963).

14. Reubl, K., and Chang, L.: Effects of methylmercury on the development of the nervous system. A review: Trace elements in human health and disease. Ed. A.S. Prasad. Academic Press New York. (1978).

15. Mansour, M., Dyer, N., Hoffman, L., Davies, J., and Brill, A.: Placental transfer of mercuric nitrate and methylmercury in the rat. Am. J. Obstet. Gynecol. 119: 557 (1974).

16. Fuyuta, M., Fujimoto, T., and Hirata, S.: Embryotoxic effects of methylmercuric chloride administered to mice and rats during organogenesis. Teratology 18: 353 (1978).

17. Fuyuta, M., Fujimoto, T., and Kiyofuji, E.: Teratogenetic effects of a single oral administration of methylmercuric chloride in mice. : Acta anat. 104: 356 (1979).

18. Harris, S., Wilson, J. and Prinz, R.: Embryotoxicity of methylmercuric chloride in golden hamster. Teratology 6: 139 (1972).

19. Inouye, M. and Murakami, U.: Teratogenic effect of orally administered methylmercuric chloride in rats and mice. Congen. anomal. Kyoto 15: 1 (1975).

20. Olson, F., and Massaro, E.: Effects of methylmercury on murine fetal amino acid uptake, protein synthesis and palate closure. Teratology. 16: 187 (1977).

21. Murakami, U.: The effect of organic mercury on intrauterine life. Adv. Exp. Med. and Biol. 27: 301 (1972).

22. Harada, Y.: Congenital minimata disease. In Tsubaki and Irukayama (Eds), Minimata disease, Elsevier, Amsterdam - New York, 209 (1977).

23. Sager, P., Aschner, M., and Rodier, P.: Persistent, differential alterations in developing cerebellar cortex of male and female mice after methylmercury exposure. : Develop. Brain. Res. 12: 1 (1984).

24. Chang, L. and Hartmann, H.: Ultrastructural studies of the nervous system after mercury intoxication. I. Pathological changes in the nerve cell bodies. Acta Neuropath (Berl.) 20: 122 (1972).

25. Jacob, J., Carmichael, N., and Cavangh, J.: Ultrastructural changes in the nervous system of rabbits poisoned with methylmercury. Toxicol. and appl. pharm. 39: 249 (1977).

26. Miyakawa, T., and Deshimaru, M.: Electron microscopical study of experimentally induced poisoning due to organic mercury compound. Acta. neuropath. (Berl.). 14: 126 (1969).

27. Miyakawa, T., Deshimaru, M., Sumiyoshi, S., Teraoka, A., Udo, N., Hattori, E., and Tatestsu, S.: Experimental organic mercury poisoning - Pathological changes in peripheral nerves. Acta. Neuropath. (Berl.) 15: 45 (1970).

28. Braekevelt, C.: Morphological changes in rat retinal photoreceptors with acute methylmercury intoxication.: Elsevier, North Holland, Inc. J.G. Hollyfield (Ed.). The structure of the eye. 123 (1982).

29. Desnoyers, P., and Chang, L.: Ultrastructural changes in the liver after chronic exposure to methylmercury. Envir. Res. 10: 59 (1975)

30. Yoshikazu, Y., Toshyi, M., and Nakao, K.: Biochemical changes in the brain of rats poisoned with an alkylmercury compound, with special reference to the inhibition of protein synthesis in brain cortex slices. J. Neurochem. 13: 1223 (1966).

31. Egawa, K.: Studies on the biosynthesis of nuclear basic proteins. J. Exp. Med. 39: 67 (1969).

32. Brubaker, P., Lucier, G. and Klein, R.: The effects of methylmercury on protein synthesis in rat liver. Biochem. Biophys. Res. Comm. 44: 1552 (1971).

33. Hughes, R., Belser, R., and Brett, W.: Behavioral impairment produced by exposure to subclinical amounts of methylmercury chloride. Envir. Res. 10: 54 (1975).

34. Carrabine, J., and Sundaralingam, M.: Mercury binding to nucleic acids. Crystal and molecular structures of 2:1 complexes of uracil-mercuric chloride and dihydrouracil - mercuric chloride. Biochem. 10: 292 (1971).

35. Steinert, P., Baliga, R., and Munro, H.: Available sulphydryl groups of mammalian ribosomes in different functional states. J. Mol. Biol. 88: 895 (1974).

36. Webb, J.: Enzyme and metabolic inhibitors. vol. 2, 729-985, Academic Press, Lond. (1966).

37. Finley, W.: Effects of drugs on chromosome structure. Am. J. Clin. Nutr. 28: 521 (1975).

38. Soukup, S., Takacs, E., and Warkany, J.: Chromosome changes in embryo treated with various teratogens. J. embryol. exp. morph. 18: 215 (1967).

39. Ingalls, T., Ingenito, E., and Curley, F.: Acquired chromosomal anomalies induced in mice by injection of a teratogen in pregnancy. Science. 141: 810 (1963).

40. Skalko, R., Pachard, D., Schwendimana, R., and Raggio, J.: The teratogenic response of mouse embryos to 5-bromodeoxyuridine. Teratology 4: 87 (1971).

41. Skalko, R., and Gold, M.: Teratogenicity of methotrexate in mice. Teratology 9: 159 (1974).

42. Matsumoto, H., Koya, G., and Takeuchi, T.: Fetal minamata: a neuropathological study of two cases of intrauterine intoxication by a methylmercury compound. J. Neuropath. Exp. Neurol, 24: 563 (1965).

43. Skerfving, S., Hansson, K., and Lindsten, J.: Chromosome breakage in humans exposed to methylmercury through fish consumption. Arch. Environ. Health 21: 133 (1970).

44. Soukup, S., Takacs, E., and Warkany, J.: Chromosome changes in rat embryos following x-irradiation. Cytogenetics 4: 130 (1965).

45. Bender, M., Griggs, G., and Bedford, J.: Mechanisms of chromosomal aberration production. III. Chemicals and ionizing radiation. Mut. Res. 23: 197 (1974).

46. Ramel, C.: Genetic effects of organic mercury compounds. I. Cytological investigations on album roots. Hereditas 61: 208 (1969).

47. Ramel, C.: Genetic effects - In Mercury in the Environment. CRC Press. Cleveland (Ohio) 169 (1972).

48. Klasterska, I., and Ramel, C.: The effect of methylmercury hydroxide on meiotic chromosomes of the grasshopper. Stethophyma grossum. Hereditas 88: 255 (1978).

49. Sager, P., Doherty, R., and Olmsted, J.: Interaction of methylmercury with microtubules. Abst. 19033, 24 annual meeting. American Society for Cell Biology (1984).

50. Gruen, L.: Stoichiometry of the reaction between methylmercury iodide and soluble sulfides. Anal. Chim. Acta. 50: 299 (1970).

51. Verschaeve, L., Kirsch-Volders, M., Susanne, C., Groetenbriel, C., Haustermans, R., Lecomte, A., and Roossels, D.: Genetic damage induced by occupationally low mercury exposure. Environ. Res. 12: 306 (1976).

52. Umeda, M., Katsue, S., Hirose, K., and Mamora, S.: Cytotoxic effect of inorganic, phenyl, and alkyl mercuric compound on Hela Cells. Japan. J. Exp. Med. 39: 47 (1969).

53. Fiskesjo, G.: The effect of two organic mercury compounds on human leukocytes in in vitro. Hereditas 64: 142 (1970).

54. Klasterska, L., Natarajan, A., and Ramel, C.: An interpretation of the origin of subchromated aberrations and chromosomal stickiness as a category of chromatid aberration. Hereditas 83: 153 (1976).

55. Latt, S.: Sister chromatid exchanges, indices of human chromosome damage and repair. Detection by fluorescence and induction by mitomycin C. Proc. Nat. Acad. Sci. 71: 162 (1974).

56. Latt, S.: Microfluorimetric detection of DNA synthesis in human chromosomes. Proc. Nat. Acad. Sci. 70: 395 (1973).

57. Wolff, S., and Bodycote, J.: The production of harlequin chromosomes by chemical and physical agents that disrupt protein structure. Mol. Hum. Cytogenet. Acad. Press, N.Y.: 335 (1977).

58. Lehmann, A.: Postreplication repair of DNA in UV irradiated mammalion cells. J. Mol. Biol. 66: 319 (1972).

59. Taylor, J.: Sister chromatid exchanges in tritium labelled chromosomes. Genetics, 43: 615 (1958).

60. Zakharov, A., and Egolia, N.: Differential spiralization along mammalian mitotic chromosomes. I. BudR revealed differentiation in Chinese hamster chromosomes. Chromosoma (Berl.) 38: 341 (1972).

61. Perry, R., and Wolff, S.: New Giesma method for the differential staining of sister chromatids. Nature (Lond.). 251: 156 (1974).

62. Kato, H.: Mechanisms for sister chromatid exchanges detected by Brdu-labelling method. Nature 251: 70 (1977).

63. Galloway, S.: What are SCEs? DNA repair processes, Nichois, W. and Murphy, D. (EDS.) Symposia specialists. Inc. N.Y. (1977).

64. Cleaver, J.: DNA damage, repair systems and human hypersensitive diseases. J. Environ. Path. and Toxicol. 3: 58 (1980).

65. Perry, P. and Evans, H.: Cytological detection of mutagen-carcinogen exposure by sister chromatid exchange. Nature. 258: 121 (1975).

66. Fujiwara, Y., Tatsumi, M., and Saski, M.: Cross-link repair in human cells and its possible defects in Fanconi's anemia cells. J. Mol. Biol. 113: 635 (1977).

67. Chaganti, R., Schonberg., S., and German, J.: A many fold increase in sister chromatid exchanges in Bloom's syndrome lymphocytes. Proc. Nat. Acad. Sci. 71: 508 (1974).

68. Galloway, S.: Ataxia telangiectasia. The effects of chemical mutagenesis and x-rays in sister chromatid exchanges in blood lymphocytes. Mut. Res. 45: 343 (1977).

69. Kato. H.: Induction of sister chromatid exchanges by chemical mutagens and its possible relevance to DNA repair. Exptl. Cell Res. 85: 239 (1974).

70. Kato, H.: Mechanisms for sister chromatid exchanges and their relation to production of aberrations. Chromosoma (Berl.) 59: 179 (1977).

71. Lambert, B., Hansson, K., Lindsten, J. and Sten, M.: Bromodeoxy-uridine - induced sister chromatid exchanges in human lymphocytes. Hereditas. 83: 163 (1976).

72. Kanda, N., and Kato, H.: In vivo sister chromatid exchange in cells of various organs of the mouse. Chromosoma (Berl.) 74: 229 (1979).

73. Schneider, E., and Bickling, C.: Aging and sister chromatid exchange. Cytogenet. cell. Genet. 26: 61 (1980).

74. Allen, J. and Latt, S.: Analysis of sister chromatid exchange formation in vivo in mouse spermatogonia as a test system for environmental mutagens. Nature 260: 449 (1976).

75. Basler, A.: Sister chromatid exchanges in vivo in Chinese hamster embryonic liver cells exposed transplacentally to Brdu. Cytogenet. cell Genet. 24: 193 (1979).

76. Bloom, S. and Hsu, T.: Differential fluorescence of sister chromatids in chicken embryos exposed to 5-Bromodeoxyuridine. Chromosoma (Berl.) 51: 261 (1975).

77. Bauknecht, T., Vogel, W., Bayer, U. and Wild, D.: Comparative in vivo mutagenicity testing by SCE and micronucleus induction in mouse bone marrow. Hum. Genet. 35: 299 (1977).

78. Alvarez, M. and Cimino, L. and Pusateri, T.: Induction of sister chromatid exchanges in mouse fetuses resulting from maternal alcohol consumption during pregnancy. Cytogenet. Cell Genet. 28: 173 (1980).

79. Gebhart, E.: Sister chromatid exchange and structural chromosome aberration in mutagenicity testing. Hum. Genet. 58: 235 (1981).

80. Henderson, A., Warburton, D. and Atwood, K.: Location of ribosomal DNA in human chromosome complement. Proc. Acad. Sci. 69: 394 (1972).

81. Evans, H., Buckland, R. and Pardue, M.: Location of the genes coding for 18s and 28s ribosomal RNA in the human genome. Chromosoma (Berl.) 48: 405 (1974).

82. GoodPasture, C. and Bloom, S.: Visualization of nucleolus organizer regions in mammalian chromosomes using silver staining. Chromosoma (Berl.) 53: 37 (1975).

83. Bloom, S. and GoodPasture, C.: An improved technique for selective silver staining of nucleolar organizer regions in human chromosomes. Hum. Genet. 34: 199 (1976).

84. Miller, D., Dev, V., Tontravahi, R. and Miller, O.: Suppression of human nucleolus organizer activity in mouse-human somatic hybrid cells. Cell Res. 101: 235 (1976).

85. Henderson, A., Eicher, E., Yu, M. and Atwood, K.: The chromosomal location of ribosomal DNA in the mouse. Chromosoma 49: 155 (1974).

86. Ray, M.: Nucleolar organizing regions of the Chinese hamster and CHW cell line chromosomes. Cytobios 25: 37 (1979).

87. Roeder, R., Reeder, R. and Brown, D.: Multiple forms of RNA polymerase in *Xenopus laevis*. Their relationship to RNA synthesis *in vivo* and their fidelity of transcription *in vitro*. Cold spring Harbour Symp. Quant. Biol. 35: 727 (1970).

88. Brown, D., and Littra, E.: RNA synthesis during the development of *Xenopus laevis*, the south African clawed toad. Mol. Biol. 8: 669 (1964).

89. Engel, W., Zenzes, M. and Schmid, M.: Activation of mouse ribosomal RNA genes at the 2-cell stage. Hum. Genet. 38: 57 (1977).

90. Hansmann, J., Gerbauer, J., Bihl, L. and Grimm, T.: Onset of nucleolus organizer activity in early mouse embryogenesis and evidence for its regulation. Exptl. Cell Res. 114: 263 (1978).

91. Schwarzacher, A., Mikelsaor, A. and Schnedl, W.: The nature of the Ag-staining of nucleolus organizing regions. Electron and light microscopic studies on human cells in interphase, mitosis and necrosis. Cytogenet. Cell Genet. 20: 39 (1976).

92. Wilson, J.: Congenital malformations produced by injecting Azo blue into pregnant rats. Proc. soc. Exp. Biol. med. 85: 319 (1954).

93. Dawson, A.: A note on staining of the skeleton of cleared specimens with Alizarin Red S. Stain Tech. 1: 123 (1926).

94. Hendzel, M., and Jamieson, D.: Determination of mercury in fish. Anat. Chem. 48: 926 (1976).

95. Pearce, A.: Histochemistry, 3rd edition, 475-576. Churchill Ltd. London, (1968).

96. Sabatini, D., Bensch, K., and Barrnett, R.: Cytochemical and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J. Cell Biol. 17: 19 (1963).

97. Meredith, R.: A simple method for preparing meiotic chromosomes from mammalian testes. Chromosoma (Berl.) 26: 254 (1969).

98. Evans, E., Burtenshaw, M., and Ford, C.: Chromosomes of mouse embryos and newborn young. Preparation from membranes and tail tips. 47: 229 (1972).

99. Bertalanffy, F.: Mitotic rates and renewal times of the digestive tract epithelia in the rat. Acta. Anat. 40: 130 (1960).

100. Shepard, D.A.: Methylmercury poisoning in Canada. Can. Med. Assoc. J. Ottawa, 114: 463 (1976).

101. Gilani, H.S.: Congenital abnormalities in methylmercury poisoning. Environ. Res. 9: 128 (1975).

102. Brubaker, P., Klein, R., Herman, S., Lucier, G., Alexander, L., and Long, M.: DNA, RNA and protein synthesis in brain, liver, and kidneys of asymptomatic methylmercury treated rats. Exper. mol. Path. 18: 263 (1973).

103. Thrasher, J., and Adams, J.: The effects of four mercury compounds on the generation time and cell division in Tetrahymena pyriformis, WH14. Environ. Res. 5: 443 (1972).

104. Gruenwedel, D., and Davidson, N.: Complexing and denaturation of DNA by methylmercuric hydroxide. J. Mol. Biol. 21: 129 (1966).

105. Singh, S., Toles, J., and Reimer, D.: In vivo induction of sister chromatid exchanges (SCEs) during aging in genetic strains of mice. Genetics Society of Canada (abstracts) Annual Meeting, 107: 100 (1984).