Chapter 3

Effects of Three Metallic Chloride Salts on the Mitotic Chromosomes of the Frog *Rana tigerina*

Interest in metallic pollutants in our country as elsewhere in the world has developed with the increase in industrialization and consequently in the consciousness that metals and metallic compounds form a major component of the chemical toxicants which pollute the soil, water and air in major cities. The hazards posed by metallic compounds are different in different organisms; most of them involve some form of genetic toxicities (Sharma, 1984). Mercury (Hg), Cadmium (Cd) and Nickel (Ni) are some of the major toxic metals in the industry and in environmental pollution. Severe chromosomal anomalies have been repeatedly observed in lymphocytes cultured from persons exposed to heavy metals in workers from the industry (Dekhnut *et al.*, 1973; Dekhnut and Leonard, 1975; Bauchinger *et al.*, 1976).

Mercury in all forms is a protoplasmic poison lethal to all species in higher concentration. This hazardous chemical is widely used in production of paints, chemicals, fungicides, insecticides and electrical equipment. Many industrial waste contain mercury and they are released into oceans, rivers, lakes and landfills leading to alarmingly high level of the compound in the immediate environment of man. In addition the potential for aquatic life to convert all forms of Hg to MeHg (Wood *et al.*, 1969) a more toxic form is a serious environmental problem. Test to determine the toxicity of twenty-two metals to amphibians revealed mercury to be most toxic to *Gastrophyrne carolensis* eggs (Birge *et al.*, 1979). Studies using *Rana pipiens* embryos at different stages showed that the death and serious developmental effects can be induced by trace amounts of mercury (Dial, 1976). Mercury compounds have been observed to exert harmful effect on physiological, biochemical and genetical system

in mammals (Venugopal and Luckey, 1978). In human populations, increased chromosomal aberration of lymphocytes have been observed in individuals with elevated blood mercury concentrations (Skerfving *et al.*, 1974). Toxic effects of mercury has also been reported on the mitotic chromosome of the fish *Boleophthalmus dussumieri* (Krishnaja and Rege, 1982).

The carcinogenic as well as mutagenic property of nickel compounds in mammalian systems are well established (Nishimura and Umeda, 1979; Sen and Costa, 1985; Haugen *et al.*, 1989; Chiocca *et al.*, 1991; Howard *et al.*, 1991). In mammals, nickel compounds cause chromosomal aberrations such as breaks, fragments, gaps and translocation etc. Amongst the chemicals studied by Sharma and Sobti (1989) Nickel chloride appears to have the least clastogenic potential as compared to other crystalline nickel compounds. Nickel chloride was unable to cause inversion in x-chromosome of *Anopheles stephensi* unlike Nickel nitrate and Nickel sulphate.

Cadmium chloride is used in photography, dying and calico-printing and in the manufacture of special mirrors. Less than 1ppm of this salt caused mortality of tadpoles or abnormal development and retarded growth in frogs (Ghate and Mulherkar, 1980). Several experiments *in vivo* (GIlliavod and Leonard, 1975; Rohr and Bauchinger, 1976) have reported the mutagenicity of cadmium. The results of these experiments are contradictory depending on difference of species, organs, or cadmium compounds used.

Though there is considerable literature on the effects of above three metallic salts on the mammalian genome, nothing is known about the mutagenic potential of these compounds on amphibians. In the current study an assessment has been carried out to examine the effect of sublethal concentrations of these metallic chloride salts on the mitotic chromosomes of the frog *Rana tigerina*. Bone marrow was taken as the hemopoietic target tissue for the analysis of chromosomal aberrations.

Materials and Methods

Animals: Medium sized frogs, *Rana tigerina* (Daudin) were collected from uncontaminated areas of villages of Dhaboi near Baroda. The animals were maintained in the laboratory in an aquaterraria, and were fed cockroaches twice a week.

Three series of experiments were conducted using the following metallic salts:
Series A: Mercuric chloride
Series B: Nickel chloride
Series C: Cadmium chloride

The animals in each series were treated with two different sublethal doses (1 mg/kg body wt.; 3 mg/kg body wt., same dose was used in all the series) for three different treatment periods, ie., 6hrs, 24 hrs and 48 hrs. Five animals were used for each experiment while 15 animals were served as controls against all the experimental groups in a series.

Preparations of mitotic chromosomes were made from the bone marrow of all the animals after *in vivo* colchicine treatment. The technique used for preparation of cell suspension, hypotonic treatment, fixation and staining were described in detail in Chapter 1. Sixty metaphase cells were analysed from each animals. Statistical analysis of the data was carried out by Equality proportion test (Z test).

Mitotic index: Mitotic index was calculated using the following formula:
MI = (Number of cells in division/total number of cells counted) x 1000. The statistical analysis of data was done by Student's 't' test.

Results

Mercuric chloride: Cytogenetic effect of $HgCl_2$ on bone marrow cells after different periods of treatment are shown in table 1. Morphology of chromosomes was found to be altered. The incidence of hypodiploidy was more frequent after the treatment. Significant increase in aberrations was noticed in all the treatments. Major types of

Table 1. Frequency of chromosomal aberrations induced in the bone marrow cells of the frog by Mercuric chloride (ip)

Dose and Treatment period	No. of animals tested	No. of cells analysed	Numerical Anomalies				Structural anomalies						Total No.of aberrati-ons	Percentage Aberrations	Z Value
			Hypo-diploi-dy	Hyper-diploid-y	Poly-ploidy	Total	Chromatid		Chromosome		Others @	Total			
							Gap	Break	Gap	Break					
Control	15	900	24	0	3	27	0	5	0	4	6	15	42	4.66	-
1.5 mg/kg b.wt															
06 hrs	5	300	10	0	0	10	1	4	2	3	3	13	23	7.66	1.98
24 hrs	5	300	10	0	1	11	1	5	1	5	4	16	27	9.00	2.79
48 hrs	5	300	12	0	0	12	3	2	6	8	5	24	36	12.00	4.46
3.0 mg/kg b.wt															
06 hrs	5	300	9	0	1	10	1	3	1	7	3	15	25	8.33	2.39
24 hrs	5	300	14	0	1	15	3	10	2	8	7	30	45	15.00	5.98
48 hrs	5	300	15	1	0	16	2	12	2	8	3	27	43	14.33	5.65

Result is statistically significant when $Z \geq 1.96$

@ = Acentric fragment, dicentric chromosomes or abnormal chromosome configuration

Table 2. Frequency of chromosomal aberrations induced in the bone marrow cells of frogs by Nickel chloride (ip)

Dose and Treatment period	No. of animals tested	No. of cells analysed	Numerical Anomalies				Structural anomalies						Total number of aberrations	Percentage Aberrations	Z Value
			Hypodiploidy	Hyperdiploidy	Ploypploidy	Total	Chromatid		Chromosome		Others @	Total			
							Gap	Break	Gap	Break					
Control	15	900	30	0	0	30	0	1	0	3	6	10	40	4.44	-
1.5 mg/kg b.wt															
06 hrs	5	300	9	0	0	9	2	0	0	0	1	3	12	4.00	0.14
24 hrs	5	300	13	0	1	14	1	1	1	2	2	7	21	7.00	2.47
48 hrs	5	300	16	0	2	18	2	0	0	4	3	9	27	9.00	4.21
3.0 mg/kg b.wt															
06 hrs	5	300	8	0	0	8	1	0	0	3	3	7	15	5.00	0.57
24 hrs	5	300	14	0	0	14	1	0	1	4	4	10	24	8.00	3.36
48 hrs	5	300	17	0	0	17	2	1	1	4	2	10	27	9.00	4.21

Result is statistically significant when Z≥1.96

@ = Acentric fragment, dicentric chromosomes or abnormal chromosome configurations

Table 3 . Frequency of chromosomal aberrations induced in the bone marrow cells of frogs by Cadmium chloride (ip)

Dose and Treatment period	No. of animals tested	No. of cells analysed	Numerical Anomalies				Structural anomalies						Total number of aberrations	Percentage Aberrations	Z Value
			Hypodiploidy	Hyperdiploidy	Ploypploidy	Total	Chromatid		Chromosome		Others @	Total			
							Gap	Break	Gap	Break					
Control	15	900	21	0	0	21	0	6	0	6	6	18	39	4.33	-
1.5 mg/kg b.wt															
06 hrs	5	300	9	0	1	10	1	3	0	2	3	9	19	6.33	1.96
24 hrs	5	300	12	1	0	13	2	5	1	2	5	15	28	9.33	3.27
48 hrs	5	300	12	0	1	13	2	6	4	4	3	19	32	10.67	4.65
3.0 mg/kg b.wt															
06 hrs	5	300	7	0	0	7	0	4	0	2	2	8	15	5.00	0.48
24 hrs	5	300	12	0	1	13	1	6	1	3	4	15	28	9.33	3.27
48 hrs	5	300	14	1	1	16	2	8	2	6	5	23	39	13.00	5.27

Result is statistically significant when Z≥1.96

@ = Acentric fragment, dicentric chromosomes or abnormal chromosome configuration

Table 4. Mitotic index recorded in the frog bone marrow after the treatments with Mercuric chloride ($HgCl_2$), Cadmium chloride ($CdCl_2$) and Nickel chloride ($NiCl_2$) at different concentrations. The data is presented as mean ± SE of 15 animals in control and 5 animals each in treatment groups.

	Control	1.5 mg/kg body wt.			3.0 mg/kg body wt.		
		6 hrs	24 hrs	48 hrs	6 hrs	24 hrs	48 hrs
$HgCl_2$	3.48 ± 0.07	2.80^{NS} ± 0.35	3.50^{NS} ± 0.11	3.42^{NS} ± 0.14	3.00^{NS} ± 0.28	3.57^{NS} ± 0.11	4.08^{*} ± 0.18
$CdCl_2$	2.93 ± 0.04	2.51^{*} ± 0.09	2.73^{NS} ± 0.09	2.11^{NS} ± 0.10	2.49^{*} ± 0.16	2.67^{*} ± 0.11	2.70^{NS} ± 0.21
$NiCl_2$	2.56 ± 0.03	2.52^{NS} ± 0.07	2.68^{NS} ± 0.08	3.17^{NS} ± 0.30	2.36^{NS} ± 0.24	2.61^{NS} ± 0.11	3.68^{*} ± 0.25

NS-Nonsignificant; * significant at 0.05 level

structural aberrations induced by $HgCl_2$ include, addition, deletion, colchiploidy, multiple breaks, chromosome condensation and pulverization. Chromosomes were found to be thicker and shorter, and often the sister chromatids were found separated out from the centromere (Plate 4). Mitotic index though decreased initially, showed an increasing trend, which was significant after 48 hrs with the higher dose (Table 4).

Nickel chloride: The data on the chromosomal aberations induced in the bone marrow cells of *R. tigerina* are given in table 2. Increase in chromosomal aberrations was significant in all the treatments except for 6 hrs. Various types of aberrations include chromosomal breaks, centromere breaks, fragmentation, colchiploidy, stickness, clumping etc. An increase in dicentric chromosomes was observed after 48 hours of treatments (Plate 5).

Mitotic index marginally decreased after 6 hrs treatment with both doses. However, after 24 hrs mitosis increased which was significantly noted after 48 hrs when compared with the control animals (Table 4).

Cadmium chloride: Clastogenic effects of Cadmium chloride was almost comparable to the other two metallic salts (Table 3, Plate 6). Frequency of chromosomal aberrations were significant after 24 hrs and 48 hrs. An increase in pulverization was observed after the treatments. However, unlike the other two agents, $CdCl_2$ caused a decrease in mitotic index after the treatments. Mitotic index decreased in all the treated groups and it was significant in all the treatment except with the higher dose for 48 hrs (Table 4).

Discussion

The present study reveals that the three heavy metal salts used in the experiment have clastogenic effects in the frog bone marrow; however, the percentage of aberration differs. The mitotic indices recorded after the treatment in most cases do not conform to that recorded in the case of mammals.

Plate 4. Chromosomal aberrations induced by Mercuric chloride (ip) in the bone marrow of the frog, *Rana tigerina* (all x 1075).

Figure 1. Abnormal chromosome configurations.

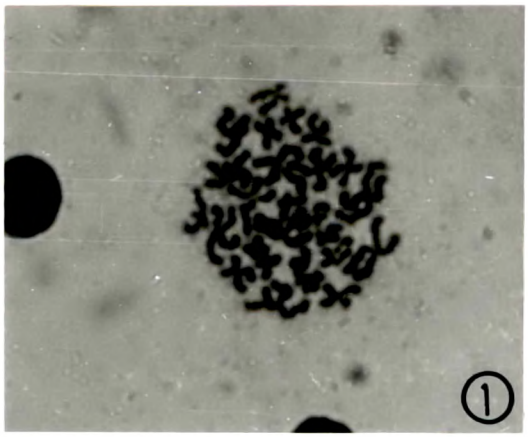
Figure 2. Short and thick chromosomes with an abnormal arm (arrow).

Figure 3. Highly condensed chromosomes. Arrow points to the separation of sister chromatids.

Figure 4. Chromosome condensation and pulverization.

Figure 5. Chromosomal deformalities. Arrow points to an abnormal chromosome with aberrant arms.

Figure 6. Severe chromosomal aberrations.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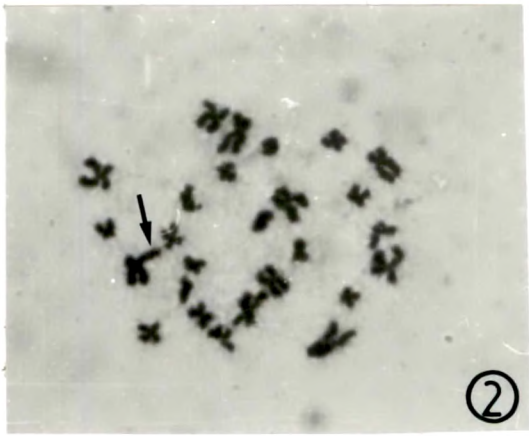
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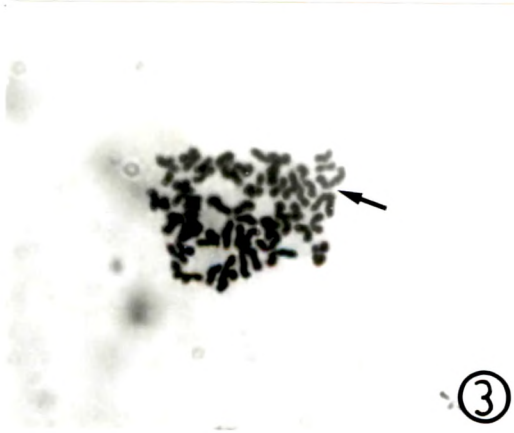
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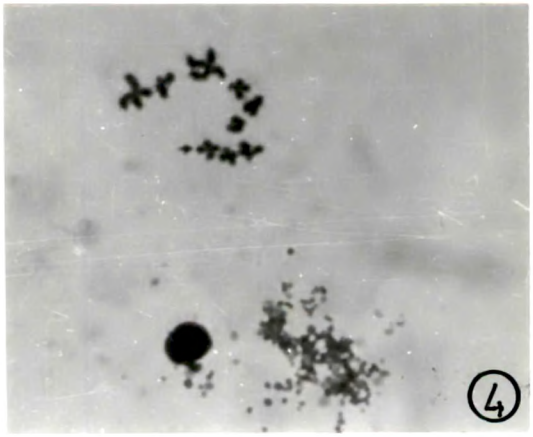
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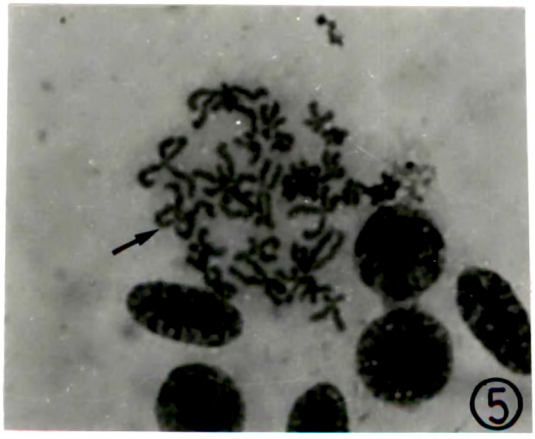
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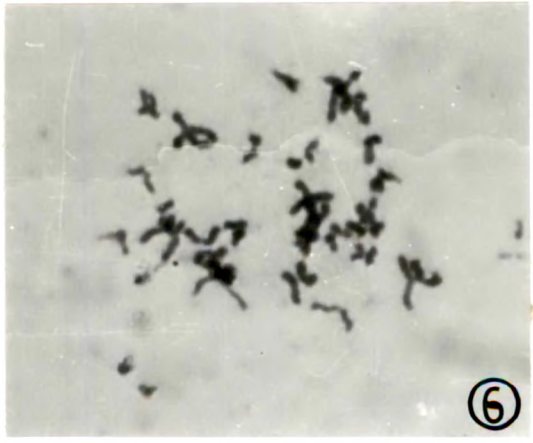
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Plate 5. Chromosomal aberrations induced by Nickel chloride in the bone marrow of the frog, *Rana tigerina* (all x 1075).

Figure 1. Abnormal chromosome morphology. Arrow points to a break of an arm.

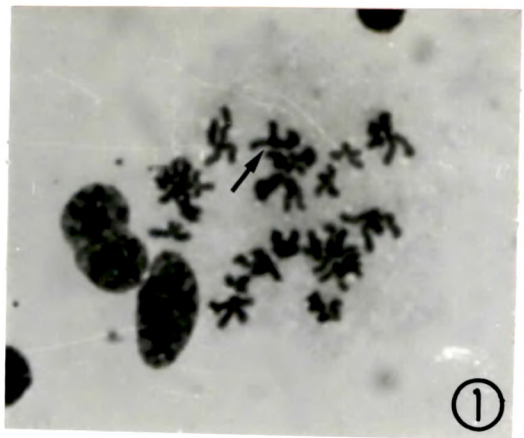
Figure 2. Clumping of chromosomes.

Figure 3. An unknown fragment (arrow).

Figure 4. Stickiness of the chromosomes and ring formation (arrow).

Figure 5. Severe chromosomal breaks.

Figure 6. Arrow shows a dicentric chromosome.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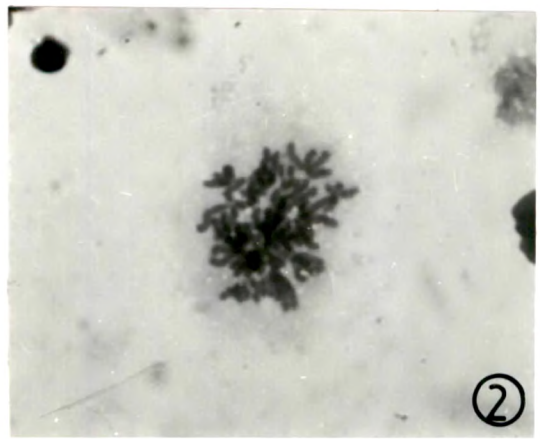
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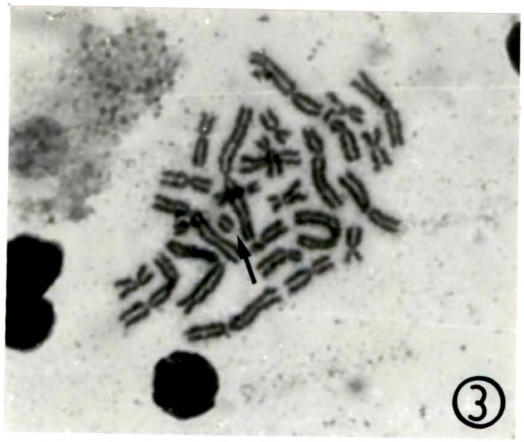
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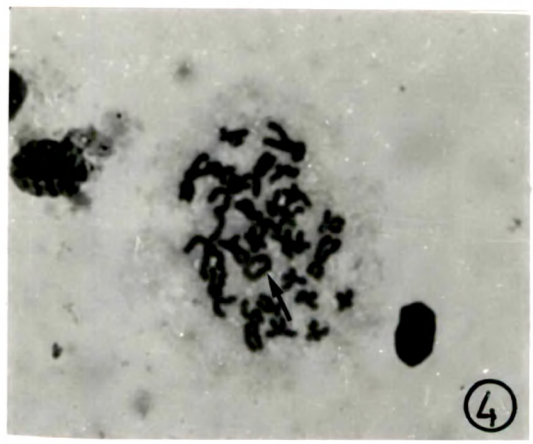
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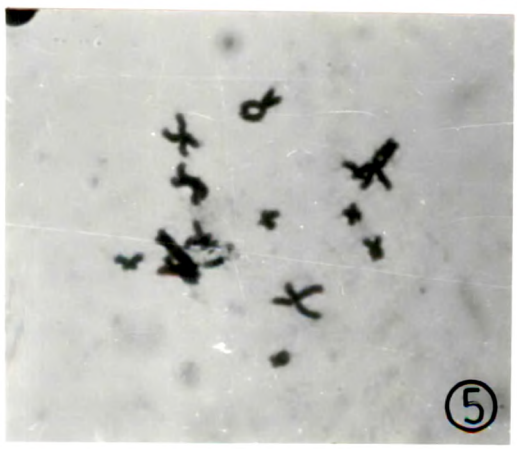
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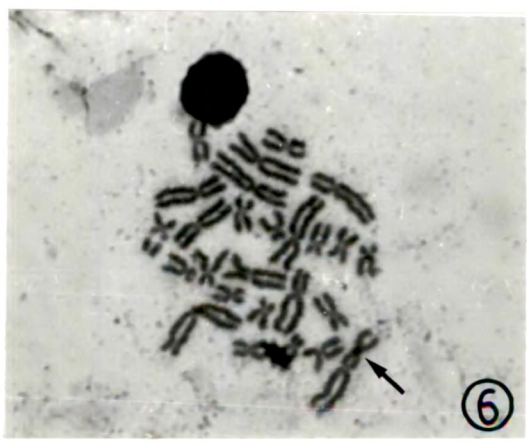
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Plate 6. Chromosomal aberrations induced by Cadmium chloride in the bone marrow of the frog, *Rana tigerina* (all x 1075).

Figure 1. An abnormal chromosome (arrow head). Arrow points to a chromatid gap.

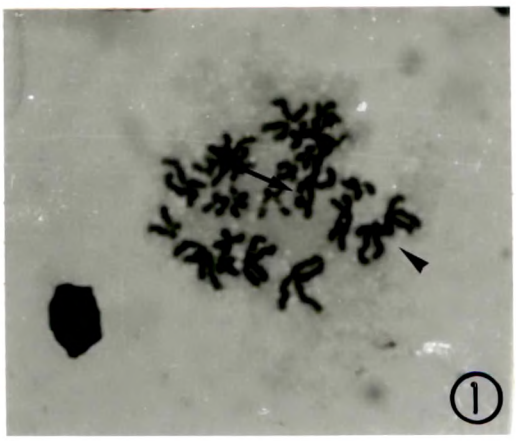
Figure 2. Abnormal chromosome configuration.

Figure 3. Pulverization of chromosomes.

Figure 4. Stickiness.

Figure 5. Break of an arm (arrow).

Figure 6. Multiple breaks.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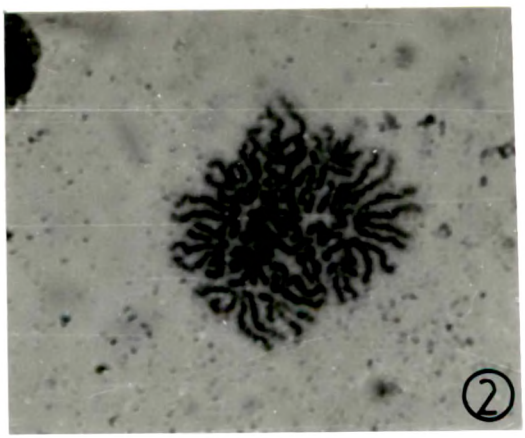
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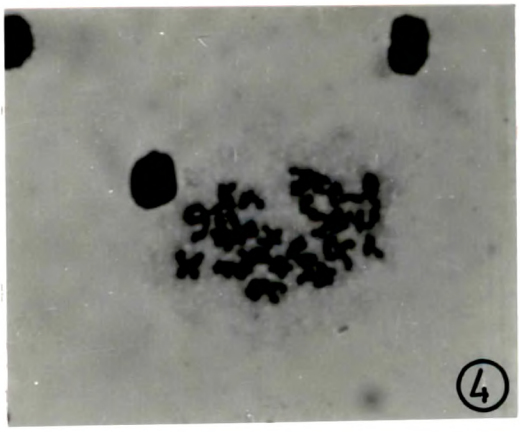
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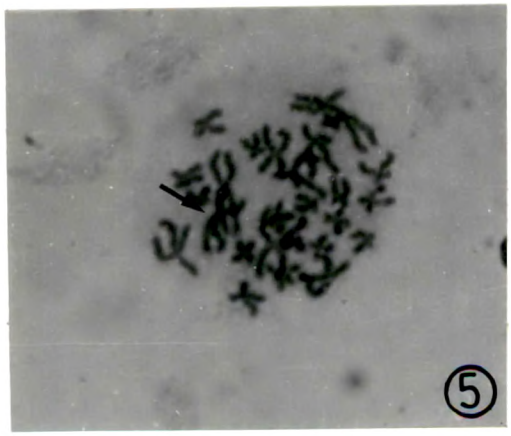
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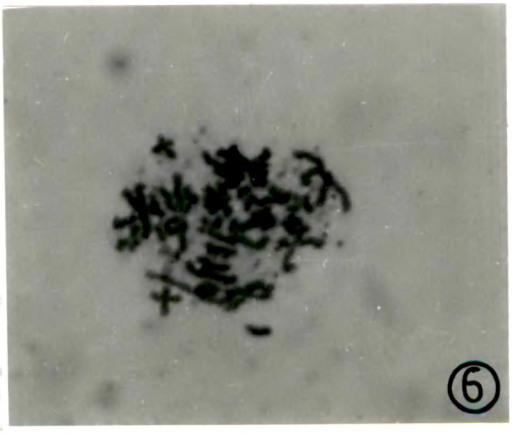
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A number of *in vivo* and *in vitro* studies have suggested that mercury compounds produce chromosomal abnormalities and genetic effects in mammals (Fiskesjo, 1970; Kato *et al.*, 1976; Verschaeve *et al.*, 1979 a, b; Howard *et al.*, 1991) and fish (Krishnaja and Rege, 1982). In the present study a significant increase in the incidence of chromosomal aberration was observed in the mitotic chromosme of the frog *Rana tigerina* after administration of Mercuric chloride at different doses.

Generally following chronic exposure to subtoxic doses, a decrease in mitotic frequency and an increase in the number of chromosomal abnormalities are observed after the treatment with heavy metals (Sharma and Talukder, 1987). However, in the present experiment an increase in the mitotic index was recorded after the administration of $HgCl_2$. This could be due to the specificity of action of Mercuric chloride in the bone marrow cells of frog. Mercury, is known to inhibit the cell growth at lower concentration while at higher concentration it promotes cell growth and development (Chang *et al.*, 1974). However, the actual mechanism of this dose-dependent effect is not yet understood. Costa *et al.*(1982) have reported an S phase specific cell cycle block produced by various inorganic metals including mercury.

Mercury residues can be accumulated by most aquatic biota (Cox *et al.*, 1975). In Yugoslavia, mercury residues were measured in a number of amphibians species from sites with varying levels of contamination (Byrne *et al.*, 1975). Indiscriminative use of mercury in industry and agriculture and subsequent pollution of aquatic habitats would have deleterious effect on the genome of aquatic vertebrates, especially amphibians.

Though there are contradictory reports regarding the mutagenic property of cadmium chloride in mammalian systems, the results of the present experiment indicate that $CdCl_2$ is mutagenic in the bone marrow cells of frog. A significant decrease in the mitotic index was generally noticeable after the treatment indicating the inhibitory effect of this compound on cell cycle. The cells exposed to cadmium chloride showed cell cycle specific block in the mitotic prophase. An increasing number of cells arrested in this phase that eventually died or were detached (Sharma and Sobti, 1989). Costa *et al.* (1982) found the block in the S phase when the cell cycle

was monitored by flow cytometry. An increased number of pulverization and a decreased mitotic index observed in the present study is amenable to the above fact.

There are conflicting reports regarding the clastogenicty of nickel compounds in mammalian system. Nickel chloride has been considered to be less toxic than other nickel compounds (Sharma and Sobti, 1989). However, Nishimura and Umeda (1979) reported that various nickel compounds including $NiCl_2$ get incorporated in the cells and they also have the inhibitory effects on the synthesis of protein, RNA and DNA. In the present experiment, a significant increase in the incidence of chromosome aberration was observed in the frog bone marrow after treatment with the Nickel chloride. The major type of aberrations were chromatid break, fragmentation, abnormal configuration of chromosomes, increased stickness and dicentric chromosomes. Increase in chromosomal aberrations was almost dose and time dependent. Though no significant increase was noted in the aberrations after 6 hours with both the doses, the increase was found to be maximum after 48 hours. The mitotic index though slightly decreased after 6 hrs with both the doses, an increase trend was noted afterwards which was significant after 48 hrs.

It was speculated that binding of nickel and other carcinogenic metal cations to the nucleotide bases renders the DNA susceptible to strand scission or to culmination of bases by an unknown nuclease resulting in mutation during the repair process (Sharma and Sobti, 1989). Ciccarelli and Waterhahr (1981) proposed that Ni may initiate DNA damage by forming a covalent Ni-DNA complex which appears to be associated with histone proteins. Howard *et al.* (1991) reported the $NiCl_2$-induced chromosomal aberrations and sister chromatid exchanges in CHO cells. Further, Chicocca *et al.* (1991) established the mutagenic potential of nickel providing the first example of a defined nickel-induced mutation in mammalian gene. It is therefore certain that whatever may be their mode of action, the nickel compounds have definitely a hazardous impact on almost all types of cells so far studied. The present study indicate the mutagenic potential of this Nickel chloride in amphibians.

A comparative assessment of the toxicity of the above three heavy metal salts, after the administration of equal quantity in the frogs shows that Mercuric chloride is the most toxic compound. Cadmium chloride was less toxic than that of mercury

while Nickel chloride was the least toxic of all. The type of aberrations observed in chromosomes were almost similar in all cases. However, an increase in dicentric chromosome was noticed after treatment with Nickel chloride. The C-mitosis was found occurring in all the treatment though it was predominantly found after the treatment with Mercuric chloride. An increased number of pulverization was found after the treatment with cadmium chloride. A dose and time depended increase in the incidence of chromosome aberration was generally noticeable after the treatments. The mitotic index was shown an increasing trend after the treatment with $NiCl_2$. High dose of $HgCl_2$ caused increased mitosis while the treatment with $CdCl_2$ caused only decrease in the mitosis. Thus it appears that though the type of aberration caused by these heavy metals are almost similar the intensity and the mechanism of action of these compounds widely differs; the mutagenicity of these chemicals on amphibian species may also be different from that of mammals.