DISCUSSION
5. DISCUSSION

5.1. GENERAL CONSIDERATIONS:

Any detrimental effect produced by the exposure to developing organisms during embryonic stages of development can be either irreversible or reversible. Embryo-lethal lesions are incompatible with survival of the embryo and results in resorption. Irreversible lesions that are compatible with survival may result in structural or functional anomalies in living, and these are named as teratogenic. Persistent lesions that cause overall growth retardation or delayed growth of specific organ systems are generally referred as embryotoxic. For a chemical to be labelled as teratogen, it must significantly increase the occurrence of structural or functional abnormalities in offspring after it is administered into the female during pregnancy, or directly to the developing organism.

Most teratologists believe that any chemical administered under appropriate conditions of dose and time of development can cause some disturbance in embryonic development (Karnofsky, 1965; Staples, 1975). Metals too are no exception to this general understanding. Although, the metals play a significant
role in many complex biological events, their excess or deficiency are known to cause many animal and human disease. The presence of metals in living tissue can be attributed to the fact that either they are required for some essential metabolic function or they represent an accumulation from exposure whatever is the source. Schroeder (1960a) has put it simple: Any element not present in plants is not essential to man, and any element found in man but not in plants, is an environmental contamination. Thus, some 40 different elements may play significant metabolic roles in biological system (Ferm, 1972). Of these 40, thirteen may be considered to be normal structural or bulk elements (C,N,H,K, Na, Mg, Ca, Cl,S,P,O,Fe,I), and five are known to be essential trace elements in animal tissue (Mn,Co,Cu,Zn,Mo). Boron is essential for plants but its essentiality for animals is not clear. The remaining 21 elements have been found in animal tissues and have demonstrable metabolic activity but their biological essentiality has yet to be determined. Out of these, 14 are ubiquitous but may well have important metabolic activity (Rb, Li,Sr,Cs, Ba, Ti, V, Cr, Ni, F, Si, Se, Br, Al). Other (Ag, Au, Sn, Cd, Hg, Pb, Bi) are probable environmental contaminants. Three other elements (Be, W, Sb) are not normally found in plants or animal tissue but may be biologically active as competitors.
A number of these metals interact with a wide variety of organic molecules and most of their biological activity is due to their involvement with important intracellular enzyme systems. Most of the metals form the metal moiety of metalloenzymes. Their presence is a specific requirement for enzyme activity. If, in each case, the specific metal is replaced by a second metal, a decrease in catalytic capability is almost invariably observed. The biochemical reactivity of each metal varies considerably. For example, the copper metalloenzymes catalyse a variety of oxygen-dependent oxidoreductase reactions, while the zinc metallo-enzymes display a much broader spectrum of specificity. Some of these include hydrolase activity (alkaline phosphatase), NAD-dependent oxidoreductase and hydrolyase activity.

Only few general principles are available that contribute to understanding the pathophysiology of metal effects. Most metals affect multiple organ systems and the targets for toxicity are specific biochemical processes (enzymes) and/or membranes of cells and organelles (Goyer, 1984).

A definite correlation of the hardness of drinking water to congenital malformations has been established suggesting that metal content of the water
may be a contributory factor (Schroeder, 1960b). For these potentially important biological properties of metals surprisingly little is known about the role of metals in embryonic development. Since normal embryonic development is characterised by critical periods of protein synthesis during cell division and differentiation, it is obvious that these periods represent a time of optimal enzymatic activity. The very nature of cell and organ differentiation suggests that a wide variety of enzymes must be active during this period of growth, and it is not surprising that many of these enzymes may be sensitive to toxic levels of metals or competitive antagonism between metals.

5.2. TIME OF EXPOSURE- A FACTOR IN TERATOGENIC EFFECT:

Developing organisms undergo rapid and complex changes within a relatively short period. Consequently, the susceptibility of the organism to chemical insult varies dramatically within the narrow time span of the major developmental stages.

Before organogenesis, the important morphological events in the development are formation of a compact cellular mass and blastocyst. Development of the blastocyst involves differentiation of tropho-ectoderm and the inner cell mass (ICM) which differentiates into primary endoderm and ectoderm. The ICM gives rise to the embryo and the extra embryonic
membranes. A remarkable similarity exists in the timing of the part of development in different species irrespective of the total period of embryogenesis (Brinster, 1975).

At the time of blastocyst formation, there is a dramatic increase in cell division and metabolic capability. Both the total synthetic rate as well as the types of RNAs and proteins synthesized are markedly increased. The embryo during this period appears to be susceptible to lethality but rarely to teratogenicity with chemical insult. Severe toxicity is manifested by rapid death of the embryo, while less severe effects are measured by decrease in cleavage rates and arrested development (Brinster, 1975).

In chick embryo all the major organogenesis begins by 48 hours of incubation (Beaudoin, 1961). The organogenesis period is characterized by the division, migration, and association of cells into primitive organ rudiments. The basic structural templates for organization of tissue and organ are established on the molecular, cellular and morphological level. The most characteristic susceptibility of the embryo during the organogenesis period is the induction of structural birth defect, although these
are often accompanied by embryolethality. Within the organogenesis period, individual organ systems possess highly specific periods of vulnerability to teratogenic insult (Wilson, 1973). Administration of a teratogen in early period of organogenesis would result in high level of brain and dye defect, with intermediate levels of heart and skeletal defects, and a low level of urogenital defects. If the same agent is administered later, a different spectrum of malformation would appear. Consequently, the exact time of exposure has a strong influence on the final pattern of malformation.

Later part of development is marked by histogenesis, functional maturation and growth. Insult at these stages leads to a broad spectrum of effects that can be generally manifested as functional disorders and carcinogenesis. At least three factors contribute to this enhanced effect—high cellular replication rates, ontogeny of xenobiotic biotransforming enzymes and low immunocompetence.

Teratogenesis and carcinogenesis are viewed as graded responses of the embryo to injury, with teratogenesis representing the grosser response involving major tissue necrosis. Bolande (1977) has postulated that certain agents cause teratogenic damage
in early relatively undifferentiated embryos, combined carcinogenic-teratogenic damage in older embryos, and finally, carcinogenic damage alone in later part of development.

5.3. DOSE RESPONSE BEHAVIOR IN TERATOGENIC EFFECT:

The relationship between embryolethality, malformations, and growth retardation is quite complex and varies with the type of agent, the time of exposure and the dose. If an agent is administered at a single time point during organogenesis and at exposure levels not severely toxic, three diverse patterns of response are obtained (Neubert et al, 1980).

Some developmental toxicants can cause malformations of the entire embryo at exposure levels that do not cause embryolethality. If the dose is increased beyond that embryolethality can occur. Malformed embryos are often growth retarded and the curve for growth retardation is often parallel to and slightly displaced from the curve for teratogenic potency (Fig. 109A).

A more common dose-response pattern which has probably been encountered in this study with metals (mercury, lead, arsenic and zinc) involves
EMBRYOTOXIC RANGE OF DOSES

DOSE-RESPONSE PATTERNS FOR DIFFERENT TYPES OF DEVELOPMENTAL TOXICANTS.

FIGURE 109
embryolethality, malformations and growth retardation (Fig.109B). For agents producing this response pattern, exposure within the embryotoxic range of doses results in a combination of dead, malformed, growth retarded, and normal embryos. Depending on the teratogenic potency of the agent, lower doses may cause predominantly death (resorptions) or malformations. As the dosage increases, however, embryolethality predominates. Agents with high teratogenic potency would produce a pattern where the teratogenicity curve was to the left but still overlapping the embryolethal curve, while agents that were predominantly embryolethal would remain so throughout the range of doses. Growth retardation would precede both these outcomes or parallel the teratogenicity curve.

A third dose-response pattern consists of growth retardation and embryolethality without malformations (Fig.109C). The dose response curve for embryolethality in this case is usually steep, implying the existence of a sharp threshold for survival of the embryo. Growth retardation of surviving embryos usually precedes significant embryolethality. Agents producing this pattern of response would be considered embryotoxic or embryolethal, but not teratogenic (Neubert et al., 1980).
The existence of these three general patterns of response indicate that for some agents embryolethality and teratogenicity are different degrees of the same primary insult (Fig. 109B). For other agents, there is a qualitative difference in response, and the primary insult leads to embryolethality alone (Fig. 109C), or teratogenicity alone (Figure-109A).

If an agent with selective developmental toxicity is administered throughout the organogenesis period, it becomes difficult to identify the most sensitive target organs and to produce a consistent pattern of malformation. In addition, teratogenic effects induced by agents acting according to the pattern B can be marked by embryolethality with repeated dosing during organogenesis period. If the agent is administered at levels sufficiently toxic, then all responses can revert to pattern C, embryolethality or growth retardation, but rarely malformations.

5.4. TERATOGENIC/DEVELOPMENTAL TOXIC EFFECTS OF THE METALS (MERCURY, LEAD, ARSENIC & ZINC) IN CHICK EMBRYO:

5.4.1. EXTERNAL MALFORMATIONS:

MERCURY:

Injections of 5 ug/egg solution of mercuric
chloride in distilled water into the yolk sac of fertile leghorn eggs on 5th day of incubation resulted into the death of 35.54 percent embryos as observed on 18th day. The embryolethal effect is significantly high (P < .001) as compared to the control (Table-1). This observation is supported by the finding of Kojima (1970) who had also reported a very high percentage of death of chick embryo (85 percent) with this metal. Mclaughlin et al. (1963) has reported the death of all embryos with the injection of 0.5 mg of Hg Cl2, whereas reduced hatchability has been observed by a number of workers (Kuahara 1970, Kojima 1970). Methyl mercury has also been found to cause increase in the death and absorption of chick embryo (Greener and Kochen, 1983). A very high percentage (79.01%) of abnormal embryo were also observed.

The spectrum of malformations included stunting (72.39 percent), beak defect (22.11 percent), head swelling (hydrocephalus - 33.85 percent), meningocoele (13.54 percent), twisted neck (17.70 percent), toe defect (37.5 percent), and ectopic conditions (14.06 percent) (Table-2). The embryo besides being stunted present a significant high percentage of the deformities in head (hydrocephalus) and neck region (meningocele and twisted neck). These can certainly be attributed to the involvement of the developing
brain and the neural tube since the mercury is known to affect the developing central nervous system in chick embryo (Kojima, 1970; Ogunranti et al., 1986), in other animals (Morikawa, 1961; Matsumoto et al., 1967; Fugita, 1969; Khera and Tobacova, 1973; Sharpf and Hill, 1973) as well as in human (Takeuchi, 1961; Matsumota et al., 1965; Muro and Goyer, 1969; Irukayama, 1969).

Malformation in chick embryos as observed by Gilani (1974) after injecting methyl mercury solutions in various doses (0.0009 to 0.010 mg/egg) were reduced body size, small and twisted limbs, microphthalmia, exencephaly, short neck and everted viscera. The spectrum of malformations almost resembles with the one described in the present study except for the eye defects. Increased incidence of twisted or clawed limbs found in the chick embryos after mercury treatment could be due to the primary involvement of the nervous system.

The effect of mercury has been known to be profoundly influenced by species and strain differences (Spyker and Smithberg, 1972; Su and Okita, 1976), the duration of exposure, the dosage administered (Spyker and Smithberg, 1972; Harris et al., 1972; Fujuta et al., 1978), and the route of exposure. In
general, however, mercury has proved to be embryotoxic as observed in the present study also. Malformations are the common sequelae of experimental administration during organogenesis which may result from an interference in protein synthesis (Olson and Massaro, 1977). Continued presence/exposure of the metal however, results in lethal as well as organ toxicity.

**LEAD:**

Experiment carried out in chick embryo by injecting lead into the egg produced (76.89 percent) malformation which included stunting (68.39 percent), beak defect (28.49 percent), head swelling (8.8 percent), twisted neck (2.59 percent), wing defect (32.12 percent), hind limb defect (47.66 percent), ectopia viscerum (38.86 percent) and abdominal oedema (11.91 percent)(Table-4). The earliest work by Franke et al. (1936) have also reported ectopia in chick embryo by injections of lead in sublethal doses.

Occurrence of head swelling (hydrocephalus) induced by lead in chick embryo in this study is supported by the finding of other workers (Cartizone and Gray, 1941; Butt et al., 1952; Karnofsky and Ridgway 1952; Gilani, 1973; Hirono and Kochen, 1973; King and Liu, 1974). They have described malformations of the
central nervous system in the form of hydrocephalus and anterior meningocele. However, the incidence of hydrocephalus described by King and Liu (1974) is much high (81 percent) as compared to present work (8.8 percent). The difference might be due to variation in the dose of lead injected.

Except for those of the central nervous system, the spectrum of malformation observed in this study closely resembles with those described by others (King and Liu, 1974; Anwer et al., 1988).

Embryolethal effect of lead in chick embryo has been found to be significant in this study (20.56 percent, P<001). However, it is slightly lower than those described by King and Liu (1974).

The exact cause of these effects of lead on developing embryo is difficult to predict, but it is most likely due to "lead-induced necrosis" in the early stages as suggested by Baker (1960). Changes in the central nervous system has been believed to be caused by abnormal alteration in the cerebral vasculature (Hirano and Kochen, 1973).
**ARSENIC:**

Solution of arsenic trioxide injected in the dose of 5 ug/egg into the yolk sac of fertile eggs on the 5th day of incubation resulted in the increased number of deaths of the chick embryos (Table-7). The percentage of death was found to be very high (45.56 percent) and statistically significant (P<.001). Earlier reports also present a high percentage of dose-related deaths of chick embryo by the injection of arsenic into the eggs (Franke et al., 1936; Ridgway and Karnofsky, 1952). This clearly indicates a high embryolethal potency of arsenic.

A wide range of serious malformations were found in the present study (69.83 percent)(Table-8). This included stunting (62.4 percent), beak defect (24 percent), wing defect (8.8 percent), hind limb defect (34.45 percent), ectopia viscerum (44.8 percent) and enlarged yolk sac (24.40 percent). The incidence of ectopia viscerum and hind limb defect are very high (except stunting) clearly indicating the involvement of ventral mesoderm in the developing embryo. Stunting, micromelia and abdominal oedema in chick embryo has also been described by Franke et al. (1936) and Ridgway and Karnofsky (1952). Ancel (1946) however, has described cause of spina bifida in chick embryo after treatment with organic mercury. Interestingly,
no abnormality of the nervous system could be detected in this series of experiment, although cases of exencephaly have been reported in hamster (Ferm and Carpenter, 1968) and mice (Hood and Bishop, 1972) after treatment with arsenic during pregnancy in the critical stage of embryogenesis. Presumably, this might be due to the specific effect of the teratogen used (sodium arsenate) in a particular species.

Moreover, the effect of arsenic is highly dependent upon its chemical form and oxidation state. Arsenite, arsenate, arsine gas, and organoarsenicals vary to a great extent on their effects. Trivalent arsenite is considered more toxic than pentavalent arsenate, although arsenate is the more common form. A number of sulfhydryl containing protein and enzymes systems have been found to be altered after exposure to arsenic. The most important manifestation at the tissue level is the inhibition of cellular respiration. Arsenate has long been known as an uncoupler of mitochondrial oxidative phosphorylation. The inhibitory effect of arsenate on mitochondria function has been observed, both in vitro (Crane and Lipman; 1953; Azzone and Ernster, 1961; Estabrook, 1961; Packer, 1961; Wadkins, 1961; Ter Welle and Slater, 1967) and in vivo (Brown et al., 1976; Fowler, 1975). The
Mechanism by which this occurs is thought to be related to competitive arsenate substitution for inorganic phosphate with subsequent formation of an unstable ester that spontaneously decomposes.

**ZINC:**

Zinc sulphate injected into the fertile eggs of white leghorn in the dose of 100 µg/egg on 5th day resulted into marked lethal effect (22.15 percent, significant level p<0.001). Embryolethal effect of the excess of zinc in chick embryo has also been reported by Jenccn (1975), and Shamal and Singh (1982). However, the incidence of mortality described by Shamal and Singh (1982) was much higher (50 percent) than those of the present study (22.15 percent). The difference can be attributed to the variation in the dose of zinc used. Increased mortality of the offspring has also been reported in rats possibly due to the destruction of B-cells of the islets of Langerhans (Underwood, 1962) by the excess of ZnCl2.

The present study showed a wide range of malformations (77.69 percent) in chick embryos (significant level P<.001). It included stunting (63.86 percent), beak defect (26.73 percent), head swelling
(22.27 percent), wing defect (30.69 percent), twisted hind limb (38.61 percent), abdominal swelling (14.85 percent), and ectopic conditions (17.82 percent) (Tables-10 & 11). The spectrum of malformations is almost the same as described by Shamal and Singh (1982) though, the total percentage is much higher (77.69 percent). Difference may be due to the dose of zinc used for injections into the eggs.

In comparison to other trace metals zinc has been reported to be less toxic. Many of the ill effects attributed to the zinc excess by early investigators may actually be due to other contaminating elements such as lead, cadmium or arsenic (Heller and Burke, 1927). However, the teratogenic effects in chick embryo described above are certainly the manifestation of zinc excess. The exact mode of action is difficult to ascertain from the present study. Biogenic activity in the chick embryo might be affected by the decreased activity of liver catalase and cytochrome oxidase which are known to occur in zinc excess (Van Reen, 1966). Many metals are enzyme inhibitor and cause hypoxic damage and cell death (Peter, 1948). Zinc excess might be exibiting teratogenic and lethal effects through the above mechanism.
5.4.2. EFFECTS OF THE METALS ON DEVELOPING SKELETAL SYSTEM IN CHICK EMBRYO:

Alizarin red-s staining of the skeletons of embryos treated with different metals used in this experiment demonstrates a clear effect on the developing osteoid tissue. Except for the severity not much difference could be observed in the nature of lesions caused by different metals. The lesions were underdevelopment of the skull bones, defective thoracic cage and rudimentary ribs with improper ossification, defective vertebral column, defective scapulae and pelvic bones thinning and shortening of long bones, and unossified phalanges (Figures- 40 - 51). Such reports in chick embryo are obscure in the available literature. Ancel (1946) has reported spina bifida in chick embryo with arsenic. However, there are plenty of observations in other animals where metals have caused similar lesion. Thus, mercury has been found to cause skeletal anomalies mostly the abnormalities of ribs (delayed calcification) in rat (Scharpf and Hill, 1973; Okita and Jacobson, 1974). Lead has been reported to cause malformations localized to sacral and tail vertebrae in golden hamster (Ferm and Carpenter, 1967; Ferm and Ferm, 1971), and incomplete and delayed ossification in mice (Mclellan et al., 1974). Similarly, arsenic produced vertebral defects
in rat and mice (Ferm et al., 1971) and skeletal defects (ribs and crania) in mice, rat and hamster (Hood and Bishop, 1972).

The exact mechanism of the above lesion is difficult to infer from the present study but, the mesenchymal damage in the early stage of bone development is obvious. The difference in the severity of effects might be due to the different rate of diffusion or migration of the agents to reach the developing embryo. The yellow yolk is about 8 times as viscous as white and the migration of substances through the yolk depends upon the relative density of the yolk and that of the substance injected (Schlesinger, 1958).

5.4.3. ANOMALIES OF THE INTERNAL ORGANS (LIVER AND HEART) INDUCED BY METALS:

A number of morphological aberrations of liver and heart were encountered during dissection of the embryos treated with different metals during development. Thus with mercury 38.02 percent (liver 8.45%, heart 5.63%, both liver and heart 23.14%), with lead 25.73 percent (liver 8.0% heart 4.41%, both 13.23%), with arsenic 46.61 percent (liver 6.77%, heart 9.32%,
both 30.5%), and with zinc 17.59 percent (liver 4.62%, heart 2.77% both 10.18%) anomalies were observed. Maximum anomalies of the organs were found in embryos treated with arsenic and the minimum were with zinc.

The anomalies of liver found in this investigation were hypertrophy, suppressed left lobe, divided lobes and hypertrophy with large gizard, and that of heart were generalized enlargement, ectopia and notching or total seperation of ventricles (Figure-39).

Some teratogens are known to produce anomalies of the internal organs. Singh and Raju (1974) reported anomalies of the viscera in 90 percent of rat foetuses after cyclophosphamide treatment. In chick embryo also, involvement of heart and liver were reported after treatment with chlorambucil (Singh et al., 1974) and mitomycin (Singh and Singh, 1976). Impiramine has also been found to affect the internal organs in rabbit (Robson and Sullivan, 1963), and in chick embryo (Singh et al., 1978). In the present series of experiment with metals involvement of liver and heart were detected in fairly high percentage (mercury 38.02%, lead 25.73%, arsenic 46.61%, zinc 17.59%). These anomalies can be compared to some extent with those caused by other agents proving the belief that
apparently similar defects may be produced by agents of dissimilar nature (Landauer, 1953; Duraiswami, 1955). The metals used in the experiment may have acted at different times on the same series of developmental events, or entirely different events, all of which were necessary for the natural development of an organ or embryo as a whole. Further, it is clear that these diverse teratogenic agents must work primarily by interfering with some normal biochemical or metabolic activities of the developing embryonic cells and tissue resulting into abnormal development.

5.4.4. HISTOPATHOLOGICAL LESIONS PRODUCED BY METALS (MERCURY, LEAD, ARSENIC & ZINC) IN DEVELOPING CHICK EMBRYO:

Histopathological changes in different tissues of developing chick embryo have been reported by many workers in the past. Thus, Hall (1972) has induced achondroplasia in developing chick bones by thallium. Changes which were observed are necrosis in the cartilage and decrease in bone growth. Necrosis and haemorrhages in the myocardium have been reported after treatment with imipramine (Singh et al., 1978). Mitomycin-C (Kury and Craig, 1967), Chlorambucil (Singh et al., 1974), and lithium (Singh and Singh, 1990) were found to damage developing liver of chicks. The changes were degeneration of the hepatic cells, dilatation and congestion of the sinusoids. In the present series of experiment with metals tissue damage
has been observed in bones (femur and humerus), liver, heart and kidney proving their toxic effect even after the period of organogenesis in chick embryos.

The changes observed in bones were disorganisation and thickening of the lamellae, areas of necrosis and degeneration in unossified mesenchymal tissue, and large spaces in the chondrified tissue (Figures-53, 73, 85 & 97). Liver showed widespread damage and distortion of lamellar pattern with mercury and arsenic, thickening of sinusoidal wall and dilatation of biliary channel (Figures-54, 74, 86 & 98). Inflammatory reaction and hydropic degeneration was detected giving the evidence of toxic myocarditis in the heart (Figures-75, 87 & 99). Kidney lesions included thickening and cellular proliferation in the glomerulus, and degeneration with necrosis of the tubular cells (Figures-56, 76, 88 & 100).

A comparison of the severity of damage revealed that mercury and arsenic are more toxic to the tissue than lead and zinc. Zinc was found to be least damaging in the dose used in the present study.

Toxic effects of metals in different organs has been widely reported in animals and human being. Mercury (methyl) is known to cause renal damage and
necrosis (Oliver et al. 1951). It accumulates in the liver readily and causes severe hepatopathological changes (Takahashi et al. 1971, Chang and Yamaguchi, 1974; Desnoyers and Chang, 1975). Damaging effects of lead on kidney and liver have been reported in detail (Goyer, 1971; Wedeen et al., 1975). It causes degeneration of tubules, cellular swelling and necrosis of proximal convoluted tubules in the kidney. Arsenic is known to cause fatty change in the myocardium of the heart (Anderson, 1985). Finner and Calvery (1939) has observed marked degeneration in liver and kidney of rats and dogs after feeding the diet containing arsenic. Zinc content of liver, kidney and pancreas has been found to be markedly increased in animals fed on zinc containing diet (Miller et al., 1978). However, all the changes described above, as a result of toxic effects of metals, were in fully developed and grown organs. Not much of the difference could be recognized in the nature of histopathological lesion as observed in the present investigation in developing chick embryos. Obviously, the lesion could be attributed to the toxic effect of mercury, lead, arsenic and zinc present throughout the period of incubation within the shell of the eggs, but the exact mechanism is difficult to interpret from this experiment.
5.5. COMPARATIVE ANALYSIS OF THE TERATOGENIC EFFECTS OF MERCURY, LEAD, ARSENIC AND ZINC IN DEVELOPING CHICK EMBRYO:

Considering the comparative values of the lethal and teratogenic effects of the metals, as presented in the table-13, arsenic was found to be the most lethal metal. It showed the highest percentage of death of the chick embryos (45.56 percent) followed by mercury, zinc and lead. No significant difference could be detected between the lethal effects of lead and zinc with the similar dose (100 ugm/egg) of injection used for both. Lead, mercury and zinc are reported to be moderately less toxic to chick embryos, producing survival rates of 74-83 percent when distributed into the yolk sac at a concentration of 0.001 ppm (Birge and Roberts, 1976). This report is in concurrence with the findings of the present investigation in which all the three metals have shown survival between 64.45 to 77.84 percent.

All metals produced a wide spectrum of teratogenic effects in the chick embryos. Mercury, lead and zinc have shown 73.01 percent, 76.89 percent and 77.69 percent of abnormal embryos, respectively. No significant difference could be found among these
# COMPARISON OF LETHAL AND TERATOGENIC EFFECTS INDUCED BY MERCURY, LEAD, ARSENIC AND ZINC.

<table>
<thead>
<tr>
<th>PERCENTAGE OF EMBRYO</th>
<th>MERCURY</th>
<th>LEAD</th>
<th>ARSENIC</th>
<th>ZINC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAD EMBRYO</td>
<td>35.54%</td>
<td>20.56%</td>
<td>45.56%</td>
<td>22.15%</td>
</tr>
<tr>
<td>SERVIVING EMBRYO</td>
<td>64.45%</td>
<td>79.43%</td>
<td>54.74%</td>
<td>77.84%</td>
</tr>
<tr>
<td>ABNORMAL EMBRYO</td>
<td>79.01%</td>
<td>76.89%</td>
<td>69.83%</td>
<td>77.69%</td>
</tr>
</tbody>
</table>

RELATIVE FATALITY - ARSENIC > MERCURY > ZINC = LEAD
RELATIVE ABNORMALITY - ZINC = LEAD = MERCURY > ARSENIC

TABLE - 13
MALFORMATIONS IN THE DEVELOPING CHICK EMBRYO INDUCED BY METALS.

HEAD SWELLING

- M: 33.85%
- L: 8.8%
- A: NIL
- Z: 22.27%

STUNTING

- M: 72.39%
- L: 68.39%
- A: 62.4%
- Z: 63.86%

BEAK DEFECT

- M: 22.9%
- L: 28.49%
- A: 24%
- Z: 26.73%

MENINGOCELE

- M: 13.5%

TWISTED NECK

- M: 17.7%
- L: 2.59%

ECTOPIA VISCIERUM

- M: 14.06%
- L: 38.86%
- A: 44.8%
- Z: 17.82%

WING DEFECT

- M: NIL
- L: 32.12%
- A: 8.8%
- Z: 30.69%

ABDOMINAL DEDEMA

- L: 11.91%
- Z: 14.85%

HIND LIMB DEFECT

- M: 37.5%
- L: 52.58%
- A: 34.45%
- Z: 38.61%

ENLARGED YOLK SAC

- A: 26.4%

M = MERCURY
L = LEAD
A = ARSENIC
Z = ZINC

FIGURE - 111
three metals with regard to teratogenic effects. However, arsenic presented a significantly low percentage of abnormality (69.83\%, P<.001) when compared with mercury, lead and zinc.

Evaluating the type of defect, all the four metals used in the present study have caused a high rate of growth retardation or stunting (Figure-110). Mercury showed a very high percentage of head swelling (hydrocephalus) and, arsenic that of ectopia viscerum and abdominal oedema in chick embryos. This indicates the primary sites of involvement of these two metals (Figure-111). However, the above observations are in slight contradiction to the report by Birge and Roberts (1976) according to which the spectrum of defects caused by different metals in chick embryo do not show any substantial variation. Other abnormalities such as beak defects, wing defects and hind limb defects were present at more or less similar level without any significant difference.

5.6. INTERPRETATIONS OF COLLAGEN, GLYCOGEN, ALKALINE AND ACID PHOSPHATASE CHANGES:

COLLAGEN:

Collagen tissue in the developing bone of metal treated chick embryos showed a generalised decrease, both in quantity and density indicating interference
in the bound connective tissue (Table-14). Collagen fibres appeared broken and disintegrated with creation of empty spaces. Out of the four metals used in the present investigation zinc induced least effect although, its presence has been found to be essential in collagen synthesis and epithelial repair (Miller et al., 1965; Oberleas et al., 1971; Van Rij and Rories, 1980). This may be due to the fact that zinc deficiency is more detrimental than its excess.

Synthesis of collagen takes place within the fibroblast which secretes it in a soluble form to be deposited extracellularly. The polypeptide chain of collagen-pro-α chains, are formed on the ribosomes with N and C terminal extensions peptides. An important feature of synthesis is the conversion of proline and lysine residues on growing polypeptide chains to hydroxyproline and hydroxylysine residues. This enzymic hydroxylation requires F++, O2, ascorbic acid and alfa-Ketoglutarate. Glycosylation of some of the hydroxylysine residues then takes place. Pro-α-chains are converted into pro-collagen by the formation of disulphide bonds and starts assuming the trihelical structure. Procollagen molecules are stabilised by hydroxyproline and secreted via the Golgi apparatus. Outside the cell the N and C extension peptides which
# TABLE - 14

## A. Collagen Tissue (Von Gieson's Stain)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Mercury</th>
<th>Lead</th>
<th>Arsenic</th>
<th>Zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone</td>
<td>N</td>
<td>↓↓</td>
<td>↓↓</td>
<td>↓↓↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

## B. Glycogen (P.A.S. Staining)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Mercury</th>
<th>Lead</th>
<th>Arsenic</th>
<th>Zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>N</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑±</td>
</tr>
<tr>
<td>Kidney</td>
<td>N</td>
<td>↑±</td>
<td>↑</td>
<td>↑↑</td>
<td>N</td>
</tr>
</tbody>
</table>

Showing the changes in collagen and glycogen after metal treatment.
do not assume the helical form, are removed in some types of collagen by procollagen peptide. This alters the properties of the molecule which precipitates as tropocollagen. The tropocollagen molecule align side by side, probably in fives, staggered at a quarter of their length to produce a fibril.

An enzyme collagenase is secreted at the sites of wounds/damaged sites which splits the fibres and the fragments are ingested by macrophages. In a healing wound, lysis occurs in an early stage to clear up the damaged collagen. Lysis is continued for sometime in the process of wound remodelling.

Any imbalance between the collagen synthesis and lysis, as has been reported in sepsis (toxaemia), deficiency of protein or lack of oxygen, is bound to damage the tissue collagen (Anderson, 1985). Depletion and the damage to collagen in chick embryo due to the toxic effect of metals might be caused by upset in both synthesis as well as lysis. Synthesis could be affected by the interference of metallic ions in enzymic hydroxylation of proline and lysine and, of course, lysis is always expected at any site of damage in an attempt of regeneration or re-synthesis.
GLYCOGEN:

In the study conducted by Hall (1972) in developing chick bones and cartilage after treatment with thallium no abnormal accumulation of glycogen was found in bone and cartilage. However, Ogunranti et al., (1986) have reported an increase in the PAS positive substances in the liver of developing chick embryo after giving injections of mercuric chloride. In the present investigation also, mild to severe increase in the glycogen was detected in liver and kidneys of chick embryos treated with mercury, lead and arsenic (Figure-64, 72, 80, 84, 92 & 96). However, zinc showed almost no change in PAS positive material. The alteration was more apparent in liver than kidney (Table-14).

The exact cause of such change is difficult to infer from this study and needs further biochemical study of enzyme system involved in the glycogenesis.

ALKALINE PHOSPHATASE:

The enzyme, alkaline phosphatase, causes hydrolytic splitting of monoesters of the phosphoric acid in an alkaline medium (Young, 1946) and also functions as a phosphotransferase; that is, it transfers the phosphate radical from one molecule to another. In
early mammalian embryos, the enzyme is present in only small quantities and in a diffuse state, except that it seems to be always present in the nuclei of the cells (Danielli, 1953). However, no report could be found regarding its distribution in chick embryo under normal condition.

It appears in large quantities, but only in few tissues, at the onset of differentiation. It is found to be concentrated in the subcutaneous tissue of the embryo in the cells concerned with the development of the subcutaneous connective tissue layer. A little later, alkaline phosphatase is found in cartilage and in the hair papillae (Hardy 1952). In the later stages of development, the enzyme is very abundant in the periosteum and matrix of the bone.

In the present work activity of the alkaline phosphate has been studied in bone and liver of developing chick embryo under the toxic influence of metals. In the developing bones its activity has been found to be increased in mercury and arsenic treated embryos. Mild increase was observed with lead while, zinc present a normal level of activity (Table-15). However, Hall (1972), in one of his studies on the effects of thallium on chick embryo, has
reported absence of alkaline phosphatase from the necrotic chondrocytes in the cartilage.

The bone damage is rapidly followed by intense osteoblastic activity and an irregular mosaic of bone grows. This repairative mechanism with proliferation of osteoblast result in increased production of alkaline phosphatase (Woodard, 1953; 1959; Gutman, 1959). The increase in the activity of alkaline phosphatase in the damaged bones of chick embryo as a result of toxic metals (Mercury and arsenic) might be due to repairative (osteoblastic) phenomena in the later period of incubation. Lead and zinc, however, cause least damage and hence, less or no repair to the bony tissue during the development.

Increase in the activity of alkaline phosphatase in the liver of chick embryo after metal treatment (mercury, arsenic and lead) could be due to the varying degree of hepatic damage. Alkaline phosphatase has been reported to be increased in various types of liver damage in human beings due to metastases (Mendelsohn and Bodansky, 1952; Shay and Siplet, 1954; Zimmerman and West, 1963), infiltrations (Brem, 1955), Drugs (Smetana, 1963; Zimmerman, 1963) and chronic liver diseases (Paterson and Losowsky, 1967).
ACID PHOSPHATASE:

Slight to moderate degree of increase was observed in the activity of acid phosphatase in the developing bones of chick embryos after treatment with mercury, arsenic and lead. Liver also presents a mild increase in the enzyme in mercury and arsenic treated chick embryos (Table-15).

Acid phosphatase is capable of catalyzing the hydrolysis of various phosphate esters in acidic pH. It is present in many tissues but the greatest amount is found in human prostate gland (Kutscher and Worner, 1936, Cantarow, 1965).

Many osteoblastic and osteolytic lesions of the bone have been reported to be associated with increase in the acid phosphatase activity (Gutman et al., 1940). In hepatic lesions also such as viral hepatitis, cirrhosis or metastatic cancer there are reports of increase in the acid phosphatase. The enzyme is most likely said to be liberated from the necrotic liver cells (Wolf and Williams, 1973). The increase in acid phosphatase activities in bone and liver of chick embryo growing under the influence of toxic metals can be reasoned out on the basis of the above reports.
### TABLE -15

#### A. Alkaline Phosphatase Activity (Gomori’s Method)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Mercury</th>
<th>Lead</th>
<th>Arsenic</th>
<th>Zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone</td>
<td>N</td>
<td>↑↑</td>
<td>↑</td>
<td>↑↑</td>
<td>↑±</td>
</tr>
<tr>
<td>Liver</td>
<td>N</td>
<td>↑↑</td>
<td>↑</td>
<td>↑↑↑</td>
<td>↑</td>
</tr>
</tbody>
</table>

#### B. Acid Phosphatase Activity Modified Gomori

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Mercury</th>
<th>Lead</th>
<th>Arsenic</th>
<th>Zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone</td>
<td>N</td>
<td>↑↑</td>
<td>↑</td>
<td>↑↑</td>
<td>N</td>
</tr>
<tr>
<td>Liver</td>
<td>N</td>
<td>↑</td>
<td>N</td>
<td>↑</td>
<td>N</td>
</tr>
</tbody>
</table>

#### C. DNA/RNA (Trevan & Sharrock’s Mod. Methyl Green Pyronin Staining)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Mercury</th>
<th>Lead</th>
<th>Arsenic</th>
<th>Zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>N</td>
<td>↓</td>
<td>↓±</td>
<td>↓</td>
<td>N</td>
</tr>
</tbody>
</table>

Showing the changes in alkaline phosphatase, acid phosphatase and DNA/RNA after metal treatment.
5.7. ROLE OF PROTEIN SYNTHESIS- DNA & RNA:

The site of action of an agent at the cellular level could be on deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or protein synthesis. The actions in a cell set in motion by the genes is broadly defined as the translation of genotype into phenotype. This essentially consists of transcription of a specific DNA code sequence into the complimentary sequence of bases in a RNA molecule. The messenger RNA then becomes the template on which aminoacids are synthesized into proteins. Ultimately, therefore, each specific DNA base triplet specifies the particular amino-acid, and the sequence of base triplet will specify the sequence of aminoacids in the final polypeptide product, the protein. There was known drugs or chemical interruption of this sequence at molecular level which can affect the final outcome.

DNA replication can be affected directly by alkylating agents which may alkylate either the phosphate group or the bases, or may hydrolyse the sugar-phosphate bond and thus break the DNA backbone. The antibiotic mitomycin leads to depolymerization of DNA with a rapid loss of its biological activity (Kury and Craig, 1967). As an indirect action on DNA,
its thymine can be replaced by 5-bromouracil through exposure to its nucleoside resulting in a transient mutation. Actinomycin affects the DNA replication by preventing it to act as a template. It has also been reported to interfere with the RNA synthesis (Barnes, 1968).

Metals are known to inhibit the DNA, RNA AND protein synthesis. Thus reduction in RNA and protein synthesis have been reported in the nerve cell after mercury intoxication (Yashino et al., 1966; Carnagh and Chen, 1971; Chang et al., 1972, 1973; Sugano et al., 1975). Decrease in the DNA content has been found in the astrocytes after mercury poisoning in the human beings (Choi et al., 1980). Lead has been known to depress the cell growth, DNA content and protein synthesis (SKREB and HABAZIN-NOVAK, 1975; Kussell et al., 1978; Dubreuil et al., 1979). Zinc also plays an important role in protein synthesis, DNA and RNA metabolism (Somers and Underwood, 1969a; Macapinlac et al., 1968; Fernandez-Madrid et al., 1973).

Qualitative study of DNA and RNA by staining method in the liver revealed mild to moderate decrease after treatment of chick embryo with mercury, lead, and arsenic (Table-15). Although the exact mechanism
is difficult to ascertain, there are reasons to believe that the inhibition of DNA and RNA synthesis in rapidly proliferating embryonal tissue could be a significant initiating factor in the patho-mechanism of teratogenesis. This is supported by the investigations carried out by numerous workers as reported above.

Sites of high proliferative activity in the embryo are susceptible to cell death after exposure to cytotoxic teratogen, and the frequency and pattern of the malformation are related to the localization and extent of necrosis in the embryonic tissue (Scott, 1977). Therefore, location of necrosis of the tissue is also an important factor. Depression or damage to DNA alone may not produce malformation unless accompanied by extensive necrosis (Ritter, 1977; Kochhar et al., 1978). Histopathological observations of the different tissue/organ of the chick embryo in the present investigation has revealed necrosis as a prominent feature. Hence, combination of DNA and RNA synthesis and tissue necrosis could be a more likely cause of malformation reported by metal.

5.8. MECHANISM OF TERATOGENESIS:

Metals usually produce toxicity in most animals when given chronically in sufficient dose.
Teratogenicity, on the other hand, cannot always be correlated with the dose. Some metals do not produce any teratogenic effect at sublethal dose, and the teratogenic potential of other metals can be altered by changing the species and the time and method of administration.

The specific mechanism of many teratogenic events still remains ill-understood. Form (1971) suggested two hypotheses, however, that help to explain why some teratogenic act differently from others. One hypothesis is that the teratogen is nonspecific i.e., the characteristics of a malformation depend primarily on the organogenetic event in progress at the time of the insult. Thus, a wide spectrum of malformations are expected from a single teratogenic stimulus given at different periods of critical embryogenesis. The other hypothesis suggests that certain teratogens might well prove to be site specific and induce malformations only in certain developing organs systems. This site specific mechanism implies that a specific organ-teratogen relationship exists which could best be explained by interference with a particular physiologic event of development. With reference to this metals should be a good example of site-specific teratogen as they enter into a variety of rather specific enzymatic reactions. Incidentally,
the results of the present work on the teratogenic effects of mercury, lead, arsenic and zinc revealed the possible involvement of both the hypotheses. A single dose injections of the four metals showed a wide spectrum of malformations in chick embryo. However mercury showed higher specificity for central nervous system while arsenic with ectopia viscerum and other abdominal defects.

Teratogenic mechanism covers a series of events beginning with the first stimulus exerted by the teratogen to the final outcome of the malformation. Significant difference exists in the mode and rate of absorption, transport, storage and the biomechanism of the degradation of teratogens. The initial event in the teratogenesis may be brought about by a teratogen by itself or its metabolic byproduct. A large number of factors initiate the abnormal development at the cellular or subcellular levels. These include genetic mutations, chromosomal aberrations, mitotic interference, altered nucleic acid integrity or function, lack or precursors and substrates etc., altered energy sources, enzyme inhibition, fluid osmolytic imbalance and changed membrane characteristics (Wilson, 1977).

Metals may act in one or more than one ways to start the invisible changes which in turn may cause
excessive cell death and necrosis, reduced rate of proliferation, failed cell interactions, reduced biosynthesis, impeded morphogenetic movements, tissue disruption etc. These abnormal events lead to too few cells or cell products to support normal morphogenesis or functional maturation (Singh, 1982).

5.1.1. EMBRYONIC DIFFERENTIATION AND SUSCEPTIBILITY TO MALFORMATION:

The embryo during the early period of developmental stages i.e. cleavage, blastocyst and the germinal layers stage consist of cells which are probably alike in having the same susceptibilities and metabolic needs, and therefore, mostly react alike to any teratogenic agent. Insult during this stage results in either the death of all the cells or there is no developmental defect at all.

With the beginning of first chemical differentiation at the time of appearance of primitive streak, synthesis of new varieties of RNA, needed for organogenesis, starts. The embryo at this stage suddenly becomes vulnerable to most of the teratogenic agents (Wilson, 1973). The chemical differentiation is followed by visible differentiation of tissues and organ primordia, when the appropriate RNAs begin
to code for the enzymatic and structural proteins characteristic of specific tissues and organs. Malformations are most easily produced soon after the structural differentiation of the target organs begins.

5.8.2. MORPHOGENETIC AND TISSUE INTERACTIONS:

Synchronised morphogenesis involving proliferation, aggregation, migration and organisation of differentiating cells in an embryo and the tissue interaction is a pre-requisite for the normal development (Grobstein, 1956; Moscona and Garber, 1968). Any experimental or otherwise interference in these interactions leads to abnormal development.

In one of the reports by Das and Singh (1979) on chick limb buds mesodermal damage has been observed 8 hours after the administration of cyclophosphamide while AER (apico-ectodermal ridge) was not affected. The AER in the early stage of limb development give signals to the underlying mesoderm for the differentiation of proximal part of limb (Figure-112 A & B). Mesoderm damaged at this stage recovers later on, but at that time the AER is giving signals for distal limb differentiation which results in phocomelia (Wolff, 1966). The two tissues have to be of similar
A scheme showing the morphogenetic interaction between the apical ectodermal ridge (AER) and the subjacent mesoderm in the limb bud.

FIGURE -112-A
NORMAL

BLEB
EARLY STAGE

BLEB
LATER STAGE

THE LIMB BUD DIFFERENTIATION OCCURS BY INTERACTION BETWEEN APICAL ECTODERMAL RIDGE (AER) SUBJACENT MESODERM.

FIGURE-112-B.
age or developmental stage in order that the limb morphogenesis proceeds normally (Hampe, 1966). However, mesodermal maintenance factor (MR) has its own role in the reciprocal interaction and responsible for the maintenance of AER (Zwilling, 1974). Considering the limb malformations in chick embryos induced by metals in the present study, involvement of these factors can not be ruled out. However, this needs a further study for the specific effects of metal on limb morphogenesis before a final conclusion is made.

5.8.3. REDUCED PROLIFERATIVE RATE:

Proliferative activity of the cell has been found to be reduced due to the cytotoxic effects of many cells. This results in reduced cell number which in turn causes teratogenic effects.

5.8.4. TISSUE NECROSIS:

Histopathological examination of the various tissue revealed a widespread necrosis and cell death in the metal treated chick embryo. Numerous teratological reports have shown that physical or chemical insult to the developing embryo produces, within a few hours or days obvious signs of cell necrosis in tissues destined to be malformed (Scott, 1977). Hence tissue
necrosis might have been a major event to cause malformations in chick embryos after mercury, lead, arsenic and zinc treatment.

5.8.5. SUSCEPTIBILITY OF CELLS TO NECROSIS:

Cells do not respond uniformly to cytotoxic agents. This may be due to differential distribution of the drug, permeability of the cells to the agent or the amount of intracellular binding that may vary between sensitive and resistant cells. The intrinsic cell difference is a major factor in determining the toxicity to a cell. In turkey embryos, massive cell death leading to malformation was found in nervous system and mesenchymal cells, and on the transplantation of tissue to healthy embryos, again the same tissues (nervous and mesenchymal) were affected (Tahara and Kosin, 1967). Severe vacuolations in the ectodermal cells have been reported after 6-aminonicotinamide treatment without any effect on mesodermal and endodermal component (Nuebert et al., 1971). Further it has been pointed out by Newbert et al., (1971) that nutritional status of the cell is also important in the sensitivity to a cytotoxic agent. Cells away from the source of nutrition die early.
The capacity of the cells to regenerate after cytotoxic effect of a teratogenic agent is a significant process and it modifies the final manifestation (Jelinek and Dostal, 1975). In the present investigation the difference in the severity as well as nature of the final outcome of the embryogenesis in the form of malformations observed with the metals used could be explained on the basis of the factors described above. However, a definite version needs further critical study of the various intrinsic factors of the cells altered under the influence of metals during the embryonic period.