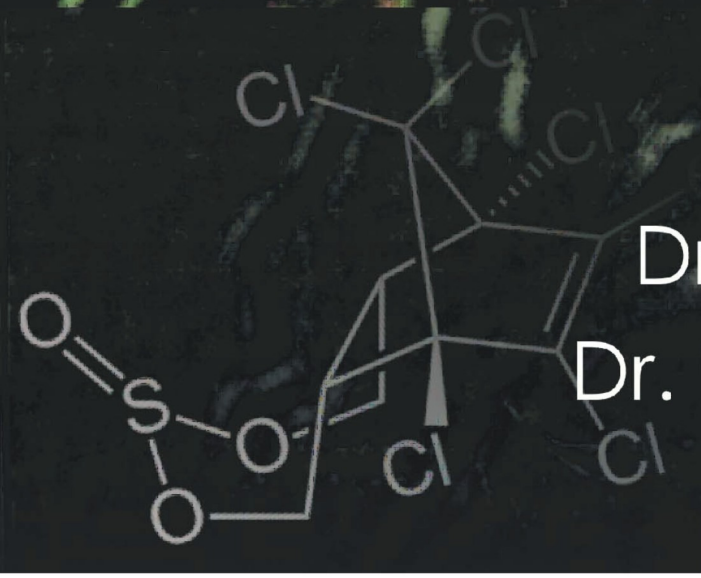
Experimental Evidences of

The Toxic Manifestations of Endosulfan



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The Toxic Manifestations of Endosulfan

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LIST OF ABBREVIATIONS

ALP	-	Alkaline phosphatase
ALT	-	Alanine aminotransferase
AST	-	Aspartate aminotransferase
b.wt.	-	Body weight
BUN	-	Blood urea nitrogen
CRE	-	Creatinine
F	-	Female
g	-	Gram
GGT	-	Gamma glutamyl transferase
Glu	-	Glucose
h	-	Hour
Hb	-	Haemoglobin
HCT	-	Haematocrit
M	-	Male
MCH	-	Mean Corpuscular Haemoglobin
MCHC	-	Mean Corpuscular Haemoglobin Concentration
MCV	-	Mean Corpuscular Volume
MNE	-	Micronucleated erythrocyte
n	-	Number of animals
NCE	-	Normochromatic erythrocyte
No.	-	Number
PCE	-	Polychromatic erythrocyte
RBC	-	Red Blood Corpuscles
T.Pro	-	Total protein
TE	-	Total erythrocyte
WBC	-	White Blood Corpuscles

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CHAPTER 1

1. INTRODUCTION

Endosulfan is a chlorinated hydrocarbon insecticide and acaricide of the cyclodiene subgroup, which acts as a poison to a wide variety of insects and mites on contact. Although it may also be used as a wood preservative, it is used primarily on a wide variety of food crops including tea, coffee, fruits, and vegetables, as well as on rice, cereals, maize, sorghum or other grains. Formulations of Endosulfan include emulsifiable concentrate, wettable powder, ultra-low volume (ULV) liquid, and smoke tablets. It is compatible with many other pesticides and may be found in formulations with dimethoate, malathion, methomyl, monocrotophos, pirimicarb, triazophos, fenoprop, parathion, petroleum oils, and oxine-copper. However, it is not compatible with alkaline materials. Technical Endosulfan is made up of a mixture of two molecular forms (isomers) of Endosulfan, the alpha- and beta-isomers (Arrebola *et al.* 2001).

1.1 Trade and Other Names

Trade or other names for the product include Afidan, Beosit, Cyclodan, Devisulfan, Endocel, Endocide, Endosol, FMC 5462, Hexasulfan, Hildan, Hoe 2671, Insectophene, Malix, Phaser, Thiodan, Thimul, Thifor, and Thionex.

1.1.1 Chemical Name

6,7,8,9,10,10- hexachloro-1,5,5a,6,9,9a-hexahydro–6,9-methano-2,4,3-benzodioxathiepine-3-oxide.

1.1.2 Synonyms

Endosulfan is produced commercially by different trade names which are as mentioned in the table given below:

1	1,2,3,4,7,7 – Hexachlorobicyclo (2.2.1) hepten - 5,6 – bioxymethylenesulfite
2	1,4,5,6,7,7 - Hexachloro - 5 - norbornene - 2,3 - dimethanol cyclic sulfite
3	6,7,8,9,10,10 - Hexachloro - 1,5,5a,6,9,9a - hexahydro - 6,9 - methano - 2,4,3 - benzodioxathiepin - 3 – oxide
4	6,9 - Methano - 2,4,3 - benzodioxathiepin, 6,7,8,9,10,10 – hexachloro – 1, 5, 5a, 6, 9, 9a - hexahydro – 3, 3, dioxide.
5	Sulfurous acid, cyclic ester with 1,4,5,6,7,7 - hexachloro - 5 - norbornene - 2,3 – dimethanol
6	Hexachlorohexahydromethano 2,4,3 - benzodioxathiepin - 3 - oxide Hildan
7	BIO 5,462
8	Benzoepin

9	Beosit	26	Niagara 5,462
10	Chlorthiepin	27	OMS 570
11	Crisulfan	28	Rasayansulfan
12	Devisulphan	29	SD - 4314
13	ENT 23,979	30	Thifor
14	Endocel	31	Thimul
15	Endosulfan	32	Thiodan
16	Endosulfan (ACGIH:OSHA)	33	Thiodan
17	Endosulphan	34	Thiofor
18	Endotaf	35	Thiomul
19	FMC 5462	36	Thionate
20	Goldenleaf tobacco spray	37	Thionex
21	HOE 2,671	38	Thiosulfan
22	Insectophene	39	Thiosulfan tionel
23	Kop – thiodan Malix	40	Thiotox
24	NCI - C00566	41	Thiotox (insecticide)
25	NIA 5462	42	Tiovel

1.2 Regulatory Status

Endosulfan is a highly toxic pesticide in EPA toxicity class I. It is a Restricted Use Pesticide (RUP). Labels for products containing Endosulfan must bear the Signal Words DANGER - POISON, depending on formulation.

1.3 Physical Properties

Sr. No.	Physical Properties	
1	Appearance	Pure Endosulfan is a colorless crystal. Technical grade is yellow-brown in color (Kidd, 1991).
2	Chemical Name	6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzadioxathiepin 3-oxide (Kidd, 1991).
3	CAS Number	115-29-7 (alpha-isomer, 959-98-8; beta-isomer, 33213-65-9)
4	Molecular Weight	406.96
5	Water Solubility	0.32 mg/L @ 22^0C (Kidd, 1991)
6	Solubility in other Solvents	Soluble in toluene and hexane (Kidd, 1991)
7	Melting Point	Technical material, 70-100^0C (Kidd, 1991)
8	Vapour Pressure	1200 mPa @ 80^0C (Kidd, 1991)
9	Partition Coefficient	Not Available
10	Adsorption Coefficient	12,400 (Wauchope *et al.*, 1992)

1.4 Chemical Class, Structure and Properties

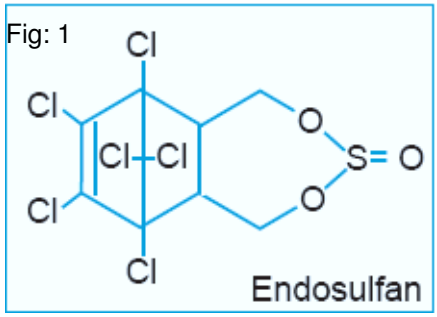
Endosulfan has been classified as a moderately hazardous chemical 5 and it has been classified as a Moderately Hazardous (class II) pesticide by World Health Organization (WHO, 1984, 2002).

The U S Environmental Protection Agency (EPA) classifies Endosulfan as Category Ib – Highly Hazardous. The European Union also rates it Highly Hazardous. However, World Health Organisation (WHO) classifies Endosulfan in Category II - Moderately Hazardous. Nevertheless classification of WHO was found to be inappropriate considering the classification followed in many countries and the available toxicological information. It has been alleged that the classification is based mainly on LD_{50} value for acute toxicity generated by the producer company (Romeo *et al.,* 2000). The Industrial Toxicological Research Centre (ITRC) in India, the nodal centre for the Regional Based Assessment of Persistent Toxic Substances (PTS) for the Indian Ocean region by the United Nations Environment Programme - Global Environment Facility (UNEP-GEF) classifies Endosulfan as Extremely Hazardous (Anon, 1989).

Chemical Formula - $C_9 H_6Cl_6O_3S$

Structural Formula (figure 1)

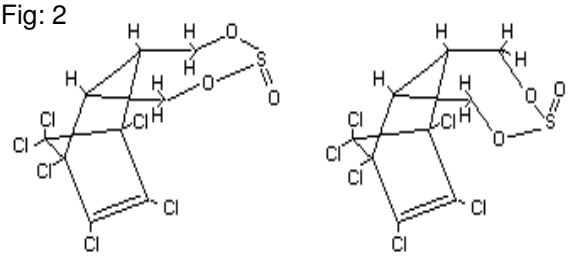
Fig: 1



In pure form Endosulfan exists as colourless crystals. But the technical product is brownish crystals with slight odour of sulphur dioxide. Technically Endosulfan is a mixture of two isomers- alpha-Endosulfan and beta-Endosulfan in the ratio of 7:3. Technical grade Endosulfan contains 94% alpha-Endosulfan and beta-Endosulfan and other related compounds like Endosulfan alcohol, Endosulfan ether and Endosulfan sulfate. Though Endosulfan is only very slightly soluble in water, it dissolves readily in xylene, chloroform, kerosene and most organic solvents and is a noncombustible solid. It is mixable with most fungicides and compatible with most pesticides (Anon, 1989).

Technical Endosulfan contains two stereoisomers, Endosulfans A and B (figure 2) in the proportion variously reported as from 4:1 to 7:3. The technical material is a 90-95 per cent pure mixture of the two isomers.

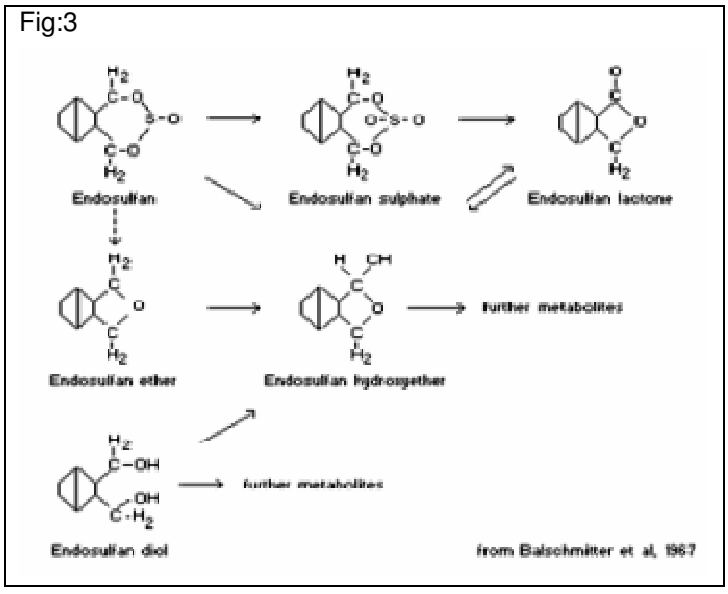
Fig: 2



1.4.1 Other Relative Chemical Properties Including Metabolites

Ballschmitter *et al.,* (1967) have considered all of the possible metabolites of Endosulfan which can hypothetically be formed on hydrolysis, oxidation or reduction. They have investigated Endosulfan metabolism in the mouse and rat using thin layer and gas chromatographic techniques and have identified five of the possible metabolites, including Endosulfan sulfate, diol, ether, hydroxyether and lactone as illustrated in the following figure 3:

Fig:3



1.5 Formulation

Various formulations of Endosulfan exist in the market. These formulations of Endosulfan include emulsifiable concentrate, wettable powder, ultra-low volume (ULV) liquid and smoke tablets.

1.6 Exposure Guidelines

ADI : 0.006 mg/kg/day (Lu, 2000)
MCL : Not Available
RfD : 0.00005 mg/kg/day (US EPA, 1995)
PEL : Not Available
HA : Not Available
TLV : 0.1 mg/m3 (8-hour) (American Conference of Governmental Industrial Hygienists, Inc, 1986)

1.7 Compositions and Chemical Structure of Endosulfan (selected for dissertation)

Endosulfan 35% EC	-	Insecticide
Batch Number	-	DNDO-170
Date of manufacture	-	June -2003
Date of Expiry	-	May 2005
Quantity	-	250ml
Manufactured by	-	Nothern Minerals limited
Daulatabad road, Gurgaon	-	122001 (Haryana)
Manufacturing License Number	-	3/73/PPH
Registration Number	-	V1-1375(3) (E.C.)-369

Chemical Composition

Endosulfan (active ingredient)	-	35 % (w/w)
Adjuvants	-	65 % (w/w)
		100 %

1.8 Justification for Selecting of Emulsifiable Concentrate

Most of the products of Endosulfan used for biocidal purposes are of emulsifiable concentrate. Solvents and/or emulsifiers used with Endosulfan in formulated products may influence its absorption into the system via all routes; technical Endosulfan is slowly and incompletely absorbed into the body whereas absorption is more rapid in the presence of alcohols, oils, and emulsifiers (Smith, 1991).

1.9 Production of Endosulfan

In many countries the production of Endosulfan is banned, but in India it is still produced. Though the production of Endosulfan is reducing gradually it was produced 4489, 3663 and 3657 tonnes in the year 2001-2002, 2002-2003 and 2003-2004, respectively (Ware, 1986).

Practically very little information is available of the volumes of production of Endosulfan. WHO estimated that the worldwide production of Endosulfan was 10,000 MT in the year 1984 (Anon, 2003), Current estimates of worldwide production or domestic formulations could not be located. Many countries that produced Endosulfan in the 1970's and 80's do not produce it any more. For instance, Endosulfan has not been produced in the

United States since 1982, but it is still used in chemical formulations. In USA, annual average amount of 626 tonnes is used (Michael, 2003). In European Union, Germany is the only producer of Endosulfan and it is manufactured at a single site, where 5000 TPA is produced. Vast majority of this is exported to South America and South East Asia (Michael, 2003). In EU (1999), 90% of the Endosulfan was used in Mediterranean area (Michael, 2003). Especially Spain, Italy, Greece and France are the major consumers. Endosulfan is produced mainly in Israel, India, China and South Korea (Michael, 2003) and India being one of the major producers of Endosulfan. Since 1996-97 it produces an average of 8206 MTPA totaling 41033 MT during 1995-20002. India exported 12180 MT during this period and consumed on an average 3599 MTPA. The UNEP-GEF report on PTS has identified some of the producing and importing countries but there is a large data gap. No information regarding stockpiles of Endosulfan could be located.

2. USES OF ENDOSULFAN

2.1 Agricultural Uses of Endosulfan

The agricultural use of Endosulfan is very diverse and it is being used with several other insecticides or used at different intervals pre and/or post plantation. However it is difficult to draw a distinct line between the effect of Endosulfan alone or in combination since Endosulfan is often being used with other pesticides to evoke desirable end result in different crop fields against diverse groups of pest population.

2.1.1 Description

Endosulfan is a non-systemic insecticide and acaricide with contact and stomach action. It is used in the control of sucking, chewing and boring insects and mites on a very wide range of crops, including fruit (including citrus), vines, olives, vegetables, ornamentals, potatoes, cucurbits, cotton, tea, coffee, rice, cereals, maize, sorghum, oilseed crops, hops, hazels, sugar cane, tobacco, alfalfa, mushrooms, forestry, glasshouse crops, etc. It also controls tsetse flies (Tomlin, 1994).

2.1.2 High Risk Circumstance of Poisoning

Accidental poisoning of children by Endosulfan stored in the home or garage, accidental exposure among formulating plant workers and suicide attempts have a high risk circumstance of poisoning. Additionally individuals with a history of convulsive disorders would be expected to be at increased risk from exposure (Mackison *et al.,* 1981).

2.1.3 Occupationally Exposed Populations

Factory workers involved in synthesis of Endosulfan, workers involved in formulating and dispensing Endosulfan and Public health workers involved in pest control are occupationally exposed populations.

2.2 Other Uses of Endosulfan

Endosulfan is being used as wood preservative in a variety of products with different compositions.

3. EXPOSURE OF ENDOSULFAN

3.1 Exposure Routes of Endosulfan

3.1.1 Oral

Ingestion occurs through accidental or deliberate ingestion or accidental ingestion of contaminated foodstuffs.

3.1.2 Inhalation

Endosulfan vapor is absorbed by inhalation.

3.1.3 Dermal

Endosulfan is readily absorbed after dermal contact, at a degree depending on the type of solvent used.

3.1.4 Eye

Eyes are exposed to vapors, dust and aerosols.

3.1.5 Parenteral

No data available on parenteral exposure.

4. KINETICS

4.1 Absorption by Route of Exposure

The percentage of Endosulfan absorbed after oral dosing would appear to have been moderate to high. Single oral doses of 0.3 mg Endosulfan and its two isomers administered to male Balb-c mice were not completely absorbed from the gastrointestinal tract but were excreted with the metabolites Endosulfan sulfate and diol in the faeces (IPCS, 1998a).

4.2 Distribution by Route of Exposure

The autopsy and toxicological findings in a fatal case caused by ingestion of Endosulfan dispersed in a colorless liquid containing about 55% of xylene (w/v) is reported by Bernardelli and Gennari (1987). The following concentrations of Endosulfan were found: blood 30 mg/L, gastric contents 0.5g in the total 50 mL, liver 20 mg/kg, kidney 2.0 mg/kg , brain 0.3 mg/kg, xylene (solvent) was detected only in stomach contents (0.4 g in the total 50 mL). When Endosulfan was fed to Balb-c mice in the diet at a concentration of 10 ppm for up to 49 days, the sulfate metabolite was detected in the liver and visceral fat of all animals. Both isomers and the sulfate and diol metabolites of Endosulfan were detected in the faeces, while the only Endosulfan product detected in the urine of these animals was the diol metabolite. After a single dose of 0.3 mg of ^{14}C-labelled Endosulfan to Balb-c mice the

highest concentrations followed, in rank order, by visceral fat > urine > small intestine > kidney > brain> expired carbon dioxide > blood (Deema *et al.,* 1966).

At the end of a 24 - month study in which NMRI mice were given diets containing 0, 2, 6, or 18 ppm technical - grade Endosulfan, the concentrations of Endosulfan and its main metabolites Endosulfan hydroxyether, sulfate, lactone, and diol were measured in the liver and kidneys. No Endosulfan was detected in either the liver or the kidney. In mice that were given 18 ppm Endosulfan, the concentrations of the hydroxyether, lactone, and diol metabolites were at or below the level of detection (0.02 ppm), while the Endosulfan sulfate concentrations were 0.1 to 0.2 ppm in kidney and 0.7 to 1.1 ppm in liver. The tissue concentrations of Endosulfan sulfate in mice at 2, 6 and 18 ppm, respectively, were: kidney, 0.2 to 0.4 ppm, 0.04 ppm and 0.1 to 0.2 ppm; and liver, 0.06 to 0.07 ppm, 0.12 to 0.45 ppm, and 0.7 to 1.1 ppm (Leist, 1989).

Following acute over-exposure, high Endosulfan concentrations can temporarily be found in the liver; the concentration in the plasma decreases rapidly (IPCS, 1984).

4.3 Biological half-life by route of exposure

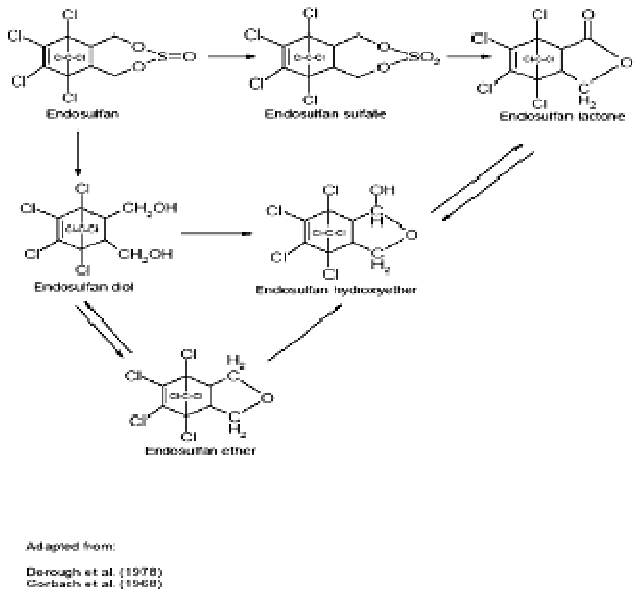
The half lives for urinary and faecal elimination for male and female rats were biphasic, with an earlier half life of 6 to 14 hour and a later half life of 33 to 67.5 hour (IPCS, 1998a).

4.4 Metabolism

Proposed Metabolic Pathway for Endosulfan

No information is available on the metabolism of Endosulfan in adult humans or children. Endosulfan is readily metabolized in animals following exposure (Deema *et al.,* 1966; Dorough *et al.,* 1978; Gorbach *et al.,* 1968). It exists in two stable stereoisomeric forms, which can be converted to Endosulfan sulfate and Endosulfan diol (WHO 1984). These can be further metabolized to Endosulfan lactone, hydroxyether, and ether. Figure 4 shows the pathway for the degradation of Endosulfan. Dorough *et al.,* (1978) indicated that the major portion of residues in the excreta and/or tissues consisted of unidentified polar metabolites that could not be extracted from the substrate, whereas the nonpolar metabolites, including sulfate, diol, α-hydroxyether, lactone, and ether derivatives of Endosulfan, represented only minor amounts. Excretion data from an acute dermal study in rats showed that, after 24 hours, a dose-related decrease in excretion occurred at higher doses, suggesting saturation of the metabolic pathway of Endosulfan (Hoechst, 1986).

Fig:4



High concentrations of Endosulfan sulfate were found primarily in the liver, intestine, and visceral fat, 24 hours after mice were exposed to a single dose of 14^{C}-Endosulfan (Deema *et al.,* 1966).

Xenobiotic Metabolism in animals is by oxidation and hydrolysis. When given to rats by various routes, Endosulfan is metabolized to the sulfate, diol, hydroxyether, lactone, ether, hydroxy Endosulfan carboxylic acid. Most Endosulfan metabolites are polar and yet to be identified (IPCS, 1998a).

4.5 Elimination and Excretion

After oral and intravenous administration of ^{14}C - Endosulfan to male and female Wistar rats at a dose of 2 or 0.5 mg/kg body weight respectively, >80% (intravenous) or 90% (oral) of the dose was eliminated in the urine and faeces within seven days; elimination however was essentially complete within 1 - 2 days (IPCS, 1998a).

^{14}C-Endosulfan (alpha or beta isomer) was rapidly excreted by female rats after a single oral dose of 2 mg/kg body weight or administration in the diet at a concentration of 5ppm. After a single dose, > 85% was excreted within 120 hours (> 70% after 48 hours), mainly in the faeces and to a lesser extent in the urine. After dietary administration for 14 days, followed by a 14 day recovery period, >72% of the administered dose was recovered. Biliary excretion of radiolabel in male rats given 1.2 mg/kg body weight as a single dose approached 50% for the alpha isomer and 30 % for the beta isomer over 48 hours. There appeared to be little enterohepatic circulation. The largest proportion of the radiolabel administered was metabolized to highly polar products, most of which could not be extracted from faeces (28%) or tissues (71%). Of the extractable fraction, unidentifiable polar

metabolites constituted 6.2 % in faeces and 13 % in urine. The apolar metabolites of Endosulfan identified in faeces and urine were the diol, the lactone, the alpha-hydroxyether, and the sulphate (Dorough *et al.,* 1978).

5. TOXICITY

5.1 Human Data

5.1.1 Adults

In general, the doses of Endosulfan, involved in cases of poisoning have been poorly characterized. In a summary of case reports (Lehr, 1996), the lowest reported dose that resulted in death was 35 mg/kg body weight; deaths have also been reported after ingestion of 295 and 467 mg/kg body weight, within 1hour of ingestion in some cases. Intensive medical treatment within 1 hour was reported to be successful after ingestion of doses of 100 and 1000 mg/kg body weight. The clinical signs in these patients were consistent with those seen in laboratory animals, dominated by tonic clonic spasms. In a case where a dose of 1000 mg/kg body weight was ingested, neurological symptoms requiring anti-epileptic theraphy was still required one year after exposure (IPCS, 1998a).

5.1.2 Children

No data available.

5.1.3 Relevant Animal Data

Acute oral LD_{50} for rats	:	80 mg/kg (IPCS, 1998b)
Acute oral LD_{50} for mice	:	14 - 35 mg/kg
Acute dermal LD_{50} for rabbits	:	290 mg/kg
Inhalation LC_{50} (1 hour) for rats	:	> 21 mg/L air
(4 hours) male rats	:	0.0345 mg/L
(4 hours) female rats	:	0.0126 mg/L (Tomlin, 1994)
NOEL	:	30 mg/kg (2 year feeding trials in rats)
NIOSH REL	:	Ca TWA 0.1 mg/m^3 (skin)
Acceptable daily intake (ADI)	:	0 - 0.006 mg/kg body weight.

5.2 Toxicity Types

5.2.1 Acute Toxicity

Endosulfan is highly toxic via the oral route, with reported oral LD_{50} values ranging from 18 to 160 mg/kg in rats, 7.36 mg/kg in mice, and 77 mg/kg in dogs (Kidd, 1991; Smith, 1991). It is also highly toxic via the dermal route, with reported dermal LD_{50} values in rats ranging from 78 to 359 mg/kg (Kidd, 1991; Smith, 1991). Endosulfan may be only slightly toxic via inhalation, with a reported inhalation LC_{50} of 21 mg/L for 1 hour, and 8.0 mg/L for 4 hours (Smith, 1991). It is reported not to cause skin or eye irritation in animals (Smith, 1991). The alpha-isomer is considered to be more toxic than the beta-isomer (Smith, 1991). Animal data indicates that toxicity may also be influenced by species and by level of protein in the diet;

rats which have been deprived of protein are nearly twice as susceptible to the toxic effects of Endosulfan (Smith, 1991). Solvents and/or emulsifiers used with Endosulfan in formulated products may influence its absorption into the system via all routes; technical Endosulfan is slowly and incompletely absorbed into the body whereas absorption is more rapid in the presence of alcohols, oils, and emulsifiers (Smith, 1991). Stimulation of the central nervous system is the major characteristic of Endosulfan poisoning (U.S. Agency for Toxic Substances and Disease Registry, 1990). Symptoms noted in acutely exposed humans include those common to the other cyclodienes, e.g., incoordination, imbalance, difficulty in breathing, gagging, vomiting, diarrhea, agitation, convulsions, and loss of consciousness (Smith, 1991). Reversible blindness has been documented for cows that grazed in a field sprayed with the compound. The animals completely recovered after a month following the exposure (Smith, 1991). In an accidental exposure, sheep and pigs grazing on a sprayed field suffered a lack of muscle coordination and blindness (Smith, 1991).

5.2.2 Chronic Toxicity

In rats, oral doses of 10 mg/kg/day caused high rates of mortality within 15 days, but doses of 5 mg/kg/day caused liver enlargement and some other effects over the same period (Smith, 1991). This dose level also caused seizures commencing 25 to 30 minutes following dose administration that persisted for approximately 60 minutes (Smith, 1991). There is evidence that administration of this dose over 2 years in rats also caused reduced growth and survival, changes in kidney structure, and changes in blood chemistry (U.S. Agency for Toxic Substances and Disease Registry, 1990; Smith, 1991).

5.2.3 Reproductive Toxicity

Rats fed doses of Endosulfan of 5 mg/kg/day for three generations showed no observable reproductive effects (Smith, 1991), but 5.0 mg/kg/day caused increased dam mortality and resorption (U.S. Agency for Toxic Substances and Disease Registry, 1990; Smith, 1991). Female mice fed the compound for 78 weeks at 0.1 mg/kg/day had damage to their reproductive organs (National Cancer Institute, 1978). Oral dosage for 15 days at 10 mg/kg/day in male rats caused damage to the semeniferous tubules and lowered testes weights (Hurt, 1991; Smith, 1991). It is unlikely that Endosulfan will cause reproductive effects in humans at expected exposure levels.

5.2.4 Teratogenic Effects

An oral dose of 2.5 mg/kg/day resulted in normal reproduction in rats in a three-generational study, but 5 and 10 mg/kg/day resulted in abnormalities in bone development in the offspring (U.S. Agency for Toxic Substances and Disease Registry, 1990; Smith, 1991). Teratogenic effects in humans are unlikely at expected exposure levels.

5.2.5 Carcinogenic Effects

In a long-term study done with both mice and rats, the males of both groups experienced

such a high mortality rate that no conclusions could be drawn (National Cancer Institute, 1978). However, the females of both species failed to develop any carcinogenic conditions 78 weeks after being fed diets containing up to about 23 mg/kg/day. The highest tolerated dose of Endosulfan did not cause increased incidence of tumors in mice over 18 months, and a later study also showed no evidence of carcinogenic activity in mice or rats (National Cancer Institute, 1978; Smith, 1991). Therefore, it infers that Endosulfan is not carcinogenic (IPCS, 1998a).

5.2.6 Organ Toxicity

Data from animal studies reveal that the organs most likely to be affected include kidneys, liver, blood, and the parathyroid gland (U.S. Agency for Toxic Substances and Disease Registry, 1990).

5.3 Genotoxicity of Endosulfan

5.3.1 Mutagenic Effects

Endosulfan is mutagenic to bacterial and yeast cells (U.S. Agency for Toxic Substances and Disease Registry, 1990). The metabolites of Endosulfan have also shown the ability to cause cellular changes (U.S. Agency for Toxic Substances and Disease Registry, 1990; Smith, 1991). This compound has also caused mutagenic effects in two different mammalian species (U.S. Agency for Toxic Substances and Disease Registry, 1990). Thus, evidence suggests that exposure to Endosulfan may cause mutagenic effects in humans if exposure is great enough.

Endosulfan was not mutagenic in *E. coli* or *S. typhimurium* (Fahrig, 1976; Moriya *et al.,* 1983). It did not induce mitotic conversion in *Saccharomyces cerevisae* (Fahrig, 1976). However, in one study technical grade Endosulfan was reported to induce reverse mutations, cross overs, and mitotic gene conversions in *Saccharomyces cerevisiae* (Yadav *et al.,* 1982).

Endosulfan did not induce chromosomal aberrations in bone marrow cells or spermatogonia of male rats treated with 5 daily oral doses of 11 to 55 mg/kg body weight (Dikshith and Datta, 1978).

An increased number of micronuclei induced in the bone marrow erythrocytes of mice treated with Endosulfan in the drinking water (43.3 mg/litre) for 2 consecutive days was not statistically significant (Usha Rani *et al.,* 1980). Negative results were observed in a dominant lethal test in mice (Canada, National Research Council, 1975).

5.3.2 Genotoxicity Issues

The Spanish conclusions about genotoxicity of Endosulfan were presented at the Working

Group on the Classification and Labelling of Dangerous Substances: Meeting on Pesticides-Health Effects (25-27 April 2001) as documents ECBI/11/01 and ECBI/11/01 Add. 1. This evaluation had taken into account all studies that were included both in the monograph and in the first addendum (July 2001). It was concluded that although Endosulfan was non-mutagenic *in vitro* and *in vivo* for somatic cells, it could not be precluded its mutagenicity for germ cells. In this sense, the notifier was requested by the ECCO 102-Peer Review Meeting to address the significance of published studies showing genotoxicity to germ cells.

5.4 Neurotoxicity

5.4.1 Mode of Action to the Nerve Cell

Chlorinated hydrocarbon insecticides act by altering the electrophysiological and associated enzymatic properties of nerve cell membranes, causing a change in the kinetics of Na^+ and K^+ ion flow through the membrane. Disturbances of calcium transport of Ca^{2+}-ATPase activity may also be involved, as well as phosphokinase activities (Hayes and Laws, 1991).

The cyclodiene compounds antagonize the action of the neurotransmitter gamma-aminobutyric acid (GABA), which induces the uptake of chloride ions by neurons. The blockage of this activity by cyclodiene insecticides results in only partial repolarization of the neuron and a state of uncontrolled excitation (Klassen and Watkins, 1999).

5.5 Interactions

The report of Arnold *et al.,* (1996) indicated that even estrogen mimics low potency, such as Endosulfan, could have important effects because of interactions with other chemicals. The estrogenic properties of combinations of chemicals were screened in a system in which the human estrogen receptor sequence is incorporated into the yeast genome. Combinations of two weak environmental estrogens, such as Dieldrin, Endosulfan, and Toxaphene, were 1000 times more potent in human estrogen receptor – mediated transactivation than any chemical alone. This result was not produced in another laboratory in which the same assay was used or in a uterotropic assay in which sexually immature rats were treated with Endosulfan or Dieldrin alone or in a combination of three successive days and the uterine mass weighed on the following day. Both chemicals were inactive in either assay, and there was no evidence of synergism (Ashby *et al.,* 1997). In a further study with the human estrogen receptor assay, however, 0.1 mmol/L Endosulfan increased the activity of beta-glycosidase (Ramamurthy *et al.,* 1997).

More doubt was cast upon the thesis of synergism by an independent study in which Endosulfan and dieldrin showed no additive effect in displacing ^{3}H-17-estradiol from rat uterine estrogen receptors or in inducing the proliferation of MCF-7 breast cancer cells. The weak proliferative potential described by Soto *et al.,* (1994, 1995) was, however, confirmed in this assay *in vitro*. Endosulfan or dieldrin alone at 3 mg/kg body weight per day or in combination, injected intraperitoneally daily for three days, did not stimulate

uterotrophic activity and had no effect on pituitary prolactin or other endocrine related end-points in immature female rats, indicating that these weakly estrogenic compounds did not interact in a synergistic fashion in binding to estrogen receptors or in activating estrogen receptors-dependent responses in mammalian tissues or cells (Wade *et al.,* 1997). The paper in which synergism was originally proposed was later withdrawn, since the results could not be reproduced, even in the same laboratory (McLachalan, 1997). Overall, these suggest that concomitant exposure to weakly estrogenic compounds probably does not result in reproductive toxicity related to estrogen action.

5.6 Fate in Humans and Animals

Endosulfan is rapidly degraded into mainly water-soluble compounds and eliminated in mammals with very little absorption in the gastrointestinal tract (Smith, 1991). In rabbits, the beta-isomer is cleared from blood plasma more quickly than the alpha-isomer, with reported blood half-lives of approximately 6 hours and 10 days, respectively (Smith, 1991), which may account in part for the observed differences in toxicity. The metabolites are dependent on the mixture of isomers and the route of exposure (Smith, 1991). Most of the Endosulfan seems to leave the body within a few days to a few weeks.

6. ECOLOGICAL EFFECTS

6.1 Effects on Birds

Endosulfan is highly to moderately toxic to bird species, with reported oral LD_{50} values in mallards ranging from 31 to 243 mg/kg (Hudson, 1984; Kidd, 1991) and in pheasants ranging from 80 to greater than 320 mg/kg (Hudson, 1984). The reported 5-day dietary LD_{50} is 2906 ppm in Japanese quail (Hill, 1986). Male mallards from 3 to 4 months old exhibited wings crossed high over their back, tremors, falling, and other symptoms as soon as 10 minutes after an acute, oral dose. The symptoms persisted for up to a month in a few animals (Hudson, 1984).

6.2 Effects on Aquatic Organisms

The effect of Endosulfan on non-target species can be swift and devastating. Farmers in Benin have observed birds and frogs dying following consumption of insects sprayed with Endosulfan (Ton *et al.,* 2000). According to one such farmer, "*fields smell awful two or three days after spraying because virtually every living thing has been killed and starts to rot*" (Myers, 2000).

Endosulfan is also extremely toxic to aquatic life. Research has shown that exposure to Endosulfan, even at sublethal doses (50% of LC_{50}), induces behavioural and biochemical changes in fish (Abu Zeid *et al.,* 2000).

Endosulfan is very highly toxic to four fish species and both of the aquatic invertebrates studied; in fish species, the reported 96-hour LC_{50} values were (in µg/L):

rainbow trout, 1.5; fathead minnow, 1.4; channel catfish, 1.5; and bluegill sunfish, 1.2. In two aquatic invertebrates, scuds (*G. lacustris*) and stoneflies *(Pteronarcys*), the reported 96-hour LC_{50} values were, respectively, 5.8µg/L and 3.3µg/L (Johnson, 1980). The bioaccumulation for the compound may be significant; in the mussel (*Mytillus edulis*) the compound accumulated 600 times to the ambient water concentration (U.S. National Library of Medicine, Hazardous Substances Data Bank, 1995).

Endosulfan runoff from cotton fields killed over 240,000 fish in Alabama (USA) in 1995, despite the pesticide reportedly having been applied according to label instructions (PAN-UPS, 1996). Similarly, mass fish deaths have been reported in India (PAN-UPS, 1996), Benin (Ton *et al.,* 2000), Sudan (Dinham, 1993), Germany (WHO/UNEP/ILO,1984) and Australia (www.mp.wa.gov.au/giz-watson/speeches/fitzroy.html).

Dr Michael Berrill of Ontario's Trent University conducted seminal research into the effects of Endosulfan on amphibians (Raloff, 1998). Frogs and toads hatched from eggs exposed to low Endosulfan concentrations exhibit a depressed "avoidance behaviour", increasing their likelihood of predation. Tadpoles exposed after hatching experienced elevated mortality, with death being considerably more likely for two-week old tadpoles than those just hatched. Symptoms of sub-lethal poisoning were also observed and included: hyperactivity, whip-like convulsions, temporary paralysis and slow growth rates. Berrill concluded that the hazard posed by Endosulfan is "*sufficiently great to warrant its replacement by less toxic alternatives wherever possible*." (Raloff, 1998) In a separate experiment with red-spotted newts, low-concentration exposure to Endosulfan impaired the pheromonal system, thereby disrupting mate choice and reducing mating success (Park, *et al.,* 2001).

6.3 Effects on Other Organisms

It is moderately toxic to bees and is relatively nontoxic to beneficial insects such as parasitic wasps, lady bird beetles, and some mites (Kidd, 1991; U.S. National Library of Medicine Hazardous Substances Data Bank, 1995). Toxicity for bees is low to moderate (IPCS, 1984)

7. ENVIRONMENTAL FATE

7.1 Degradation of Endosulfan

Microbial degradation of Endosulfan may play an important role in detoxifying the Endosulfan- contaminated sites in the environment. There are a few reports on degradation of Endosulfan by different groups of microorganisms. However, recent reports indicated that microbial conversion of Endosulfan to Endosulfan diol by hydrolytic pathway is a detoxification process whereas Endosulfan sulfate was found to be a terminal degradation product. In this study, they reported the degradation of Endosulfan by a soil fungus *M. thermohyalospora* MTCC 1384 in culture medium under laboratory conditions (Shetty, *et al.,* 2000).

7.2 Breakdown in Soil and Groundwater

Endosulfan is moderately persistent in the soil environment with a reported average field half-life of 50 days (Wauchope, 1992). The two isomers have different degradation times in soil. The half-life for the alpha-isomer is 35 days, and is 150 days for the beta-isomer under neutral conditions. These two isomers will persist longer under more acidic conditions. The compound is broken down in soil by fungi and bacteria (Kidd, 1991). Endosulfan does not easily dissolve in water, and has a very low solubility (Kidd, 1991; Wauchope, 1992). It has a moderate capacity to adhere or adsorb to soils (Wauchope, 1992). Transport of this pesticide is most likely to occur if Endosulfan is adsorbed to soil particles in surface runoff. It is not likely to be very mobile or to pose a threat to groundwater. It has, however, been detected in California well water (Howard, 1991).

7.3 Breakdown in Water

In raw river water at room temperature and exposed to light, both isomers disappeared in 4 weeks (Howard, 1991). A breakdown product first appeared within the first week. The breakdown in water is faster i.e. within 5 weeks, under neutral conditions than at more acidic conditions or basic conditions i.e. within 5 months (Howard, 1991). Under strongly alkaline conditions the half-life of the compound is 1 day. Large amounts of Endosulfan can be found in surface water near areas of application (U.S. Agency for Toxic Substances and Disease Registry, 1990). It has also been found in surface water throughout the country at very low concentrations (Howard, 1991).

7.4 Breakdown in Vegetation

In plants, Endosulfan is rapidly broken down to the corresponding sulfate (Kidd, 1991). On most fruits and vegetables, 50% of the parent residue is lost within 3 to 7 days (Kidd, 1991). Endosulfan and its breakdown products have been detected in vegetables (0.0005-0.013 ppm), in tobacco, in various seafoods (0.2 ppt-1.7 ppb), and in milk (Howard, 1991).

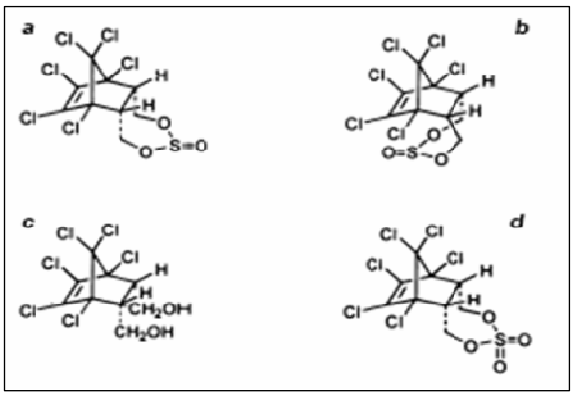


Figure 5: Degradation **(**Endosulfan isomers and its metabolites); ***a***, *α*-Endosulfan; ***b***, *β*-Endosulfan; ***c***, endodiol and ***d***, endosulfate.

8. ISSUES WITH ENDOSULFAN

8.1 Risks of Endosulfan

Pesticide safety is classified by the World Health Organization (WHO) according to the results of LD_{50} tests, which document the amount of a chemical required to kill 50 % of a population of laboratory rats. Under this system, Endosulfan is currently classified as Class II – Moderately Hazardous to human health. However, the United States' Environmental Protection Agency (EPA) rates Endosulfan as Category Ib – Highly Hazardous. LD_{50} data for Endosulfan are equivocal, with some published results indicating that the chemical should be in the WHO's Class Ib, according to the organization's own criteria. Evidence of the threats to human health posed by Endosulfan is abundant, and the chemical has been banned outright or severely restricted in a number of countries as a result (see box). Independent of LD_{50} results, these threats warrant the immediate upgrading of Endosulfan to WHO Class Ib.


Fig. 6

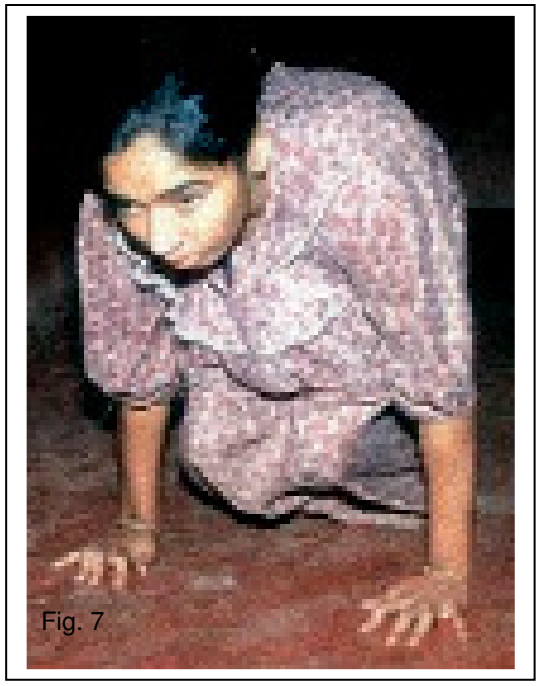


Fig. 7

Figure 6: A boy showing cerebral palsy. The boy cannot walk or talk. **Figure 7:** A lady showing acute epilepsy and severe neural disfunction. The village was exposed to aerial spraying of Endosulfan for over 15 years.

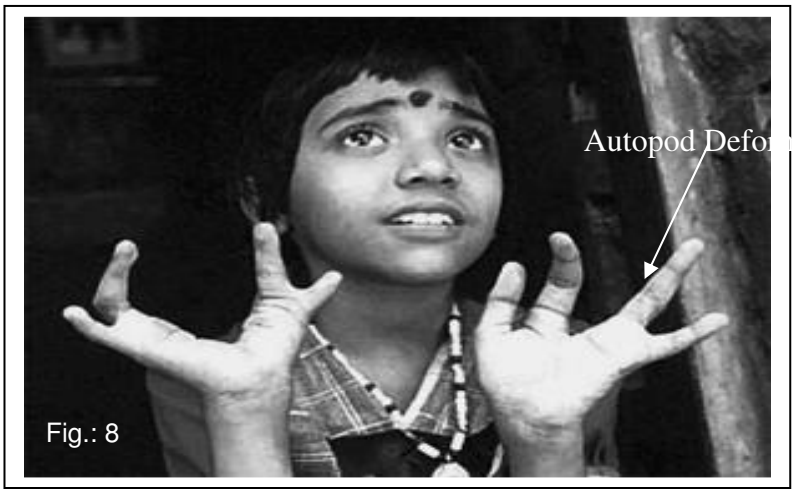


Figure 8: A girl exposed to Endosulfan shows autopod deformity

8.2 Other cases

Endosulfan has been linked to dozens of accidental deaths in the USA, Colombia, Benin, India, Malaysia, Sudan, and the Philippines (PANAP, 1996).

In the USA, Endosulfan exposure was linked to the death of one farmer and permanent neurological impairment of another (Brandt *et al.,* 2001).

In Benin's Borgou province, Endosulfan poisoning caused many deaths during the 1999/2000 cotton season. Official records state that at least 37 people died and a further 36 became seriously ill, although an independent report estimated that nearly 70 people actually lost their lives (Ton *et al.,* 2000). In 1999, a boy in Benin died after eating corn sprayed with Endosulfan (Myers, 2000).

In southern Sulawesi, Indonesia, Endosulfan was the leading cause of pesticide poisoning between 1990 and 1993. Of 153 reported poisoning cases, 32 were due to Endosulfan (PANAP, 1996).

In Sudan, in 1988, Endosulfan barrels washed in irrigation canals caused fish mortalities and three people died after drinking water from the canal (Dinham, 1993). In 1991, also in Sudan, 31 people died after eating food containing seed sprayed with Endosulfan (PAN-UK, 1995).

Colombia's Departmental Committee of Coffee Growers recorded 155 cases of poisoning due to pesticide exposure in 1994, most of which were due to Endosulfan (PAN-UK, 1995).

Pesticides Action Network North American reported that in 1993, 60 poisonings and one death occurred in Colombia due to Endosulfan use on coffee (PANUPS, 16th June 1994).

Chronic, sub-lethal effects of Endosulfan exposure manifested in experimental rats include liver enlargement, seizures and retarded growth (EXTOXNET. 1996).

The EPA states that "*available scientific literature suggests that Endosulfan may act as a potential endocrine disruptor*." This means that the chemical has the potential to interfere with normal hormone production and activity. Implications of endocrine disruption may include disruption of development, and promotion of certain types of cancer. A major concern, especially in developing countries, is that low protein diets may increase people's sensitivity to the effects of this pesticide (PANAP, 1996).

A further concern stems from the evidence that Endosulfan may cause mutagenic effects in humans if exposure is great enough; Endosulfan has been shown to be genotoxic to human cells under experimental conditions (Lu *et al.,* 2000).

In Kerala, India, Endosulfan has been linked to hundreds of deaths and disorders among cashew nut plantation workers and villagers (THANAL, 2001). In Kasaragod province, where aerial spraying of Endosulfan occurred for at least 15 years, alarmingly high levels of Endosulfan residues have been detected in the blood and breast milk of villagers and cancers and disorders of the reproductive and central nervous systems are very common. A survey of only 123 houses found 49 cancer cases, 43 psychiatric cases, 23 epileptics, 9 with congenital abnormalities and 23 with mental retardation (Joshi, 2001).

A case-controlled study comparing 170 children exposed to Endosulfan with 92 unexposed children found, among the former, significantly poorer academic performance, elevated prevalence of congenital abnormalities and learning difficulties, delayed puberty in boys, and very high levels of menstrual disorders in girls (Yadav and Jeevan, 2002).

Romeo Quijano (2000), Professor of Pharmacology and Toxicology (University of Philippines), recently led an investigation of health defects in Kasaragod District and stated that, "no other reasonable cause can explain the illnesses experienced by the people, except Endosulfan (THANAL, 2002).


Fig: 9


Fig: 10

Figure 9: Shows deformed cow from area of heavy Endosulfan use in Kerala, southern India. Endosulfan residues measured in cow milk and flesh in Kasaragod province were over 100 times the permissible level (Vankar *et al.,* 2001). **Figure 10**: shows that Endosulfan has caused mass mortalities of fish.

8.3 Incidence of Symptoms Linked to Endosulfan Exposure

In a study conducted by Yadav and Jeevan (2002) the people exposed to Endosulfan showed increased incidences of learning disability, congenital abnormalities, menstrual disorders, compared to people free from exposure.

8.4 A Persistent Problem

Like the widely banned pesticides DDT and dieldrin, Endosulfan is an organochlorine and, as such, is persistent in the environment. Endosulfan degrades relatively quickly in water (half life: 2-22 days) (PANAP, 1996) but persists longer in soil (half life: 60-800 days) (PANAP, 1996), and its major degradation product, Endosulfan sulphate, is not only more persistent but is equally toxic (Park *et al.,* 2001). The pesticide Endosulfan, bioaccumulates in humans and other animals (particularly in their liver, kidneys and fatty tissue). Experiments have shown Endosulfan to accumulate 600 times more to the ambient water concentration in mussels (*Mytillus edulis*) (PANAP, 1996).

Such persistent organic pollutants (POPs) are of concern because of their long-term subtle effects on hormones, the immune system, and reproduction. Because of Endosulfan's toxicity to fish, Canadian regulations discourage farmers from using Endosulfan near open water. However, aerial drifting of the pesticide can leave residues up to three meters beyond the perimeter of sprayed agricultural fields (Raloff, 1998). Ultra low volume Endosulfan products were banned in Australia, where spray drift had been resulting in residue problems for the beef industry (Cattle Council of Australia, 2001). Indeed, Endosulfan residues led to South Korea's rejection of Australian beef in the past (Myers, 1999). Similarly, in 1999, the European Union temporarily suspended imports of fish from Tanzania, Uganda and Kenya because of contamination with pesticides, including Endosulfan (European Commission,

Directorate-General Health & Consumer Protection, Directorate D – Food And Veterinary Office, 1999) Given the serious health concerns associated with Endosulfan exposure, it is highly worrying that a report by the International Programme on Chemical Safety stated that Endosulfan has been shown to persist on the hands of pest control operators for up to 31 days after exposure (WHO/UNEP/ILO, 1984).

9. WORLD-WIDE RESTRICTIONS ON ENDOSULFAN USE

(www.indiatogether.org/petitions/Endosulfan/worldwide.htm, Cattle Council of Australia, 2001)

9.1 Countries Where Endosulfan is Banned

Endosulphan is banned in Singapore, Tonga, Syria, Germany, USA, the Brazilian state Rondonia, UK, Sweden, Netherlands, Colombia, and the Indian state Kerala.

Endosulfan is severely restricted in: Australia, Bangladesh, Indonesia, Cambodia, Japan, Korea, Khazakhstan, Kuwait, Philippines, Lithuania, Sri Lanka, Taiwan, Thailand, Denmark, Yugoslavia, Norway, Finland, Russia, Venezuela, Dominica, Canada.

Endosulfan has been identified as a pesticide of concern due to health and environmental problems associated with its use in Ecuador, Mauritius and Paraguay (PRC, 1994).

10. REQUIREMENT AND RELEVANCE OF ASSAY METHODOLOGIES

(*In vitro and in vivo* screening of Endosulfan)

The requirement and relevance of assay methodologies selected for determination of genotoxic and systemic effects of pesticide / combination: *in vitro and in vivo* screening of Endosulfan is based completely on regulatory requirement, environmental concerns and controversies related to its toxicity and genotoxicity.

The safety assessment of new chemical substances includes the requirement for an assessment of genotoxic potencial based on the following guidelines:

1. International Conference on Harmonisation guidelines for testing of pharmaceuticals,
2. EU Technical Guidance Document for testing of industrial chemicals,
3. German BfR overview of strategies for testing of industrial chemicals,
4. UK Committee on Mutagenicity Guideline for testing of chemicals,
5. Food and Drug administration (FDA) Redbook,
6. Updated Recommended Strategy for Testing Oxidative Hair Dye Substances for their potential Muatgenicity/Genotoxicity,
7. Recommended Mutagenicity/Genotoxicity Tests for the Safety Testing of Cosmetic Ingredients to be included in the Annexes Council Directive 76/768/EEC and
8. FDA Guidance for Industry recommended Approaches to Integration of Genetic Toxicology Study Results.

Many assay systems have been developed and introduced for safety assessment of chemicals. More than a half of them are *in vitro* assay systems, therefore we can say that the field of genotoxicity started from the alternatives of animal experiments. Although there are many kinds of assay systems but none can detect chemical genotoxicity. Assays are generally endpoint specific, so we usually use several assays in combination referred to as "battery".

In addition to the regulatory guidelines (*viz.* OECD, ICH etc.) various workshops have been organized by professional scientists on Genotoxicity Testing (IWGT), International Association of Environmental Mutagen Societies (IAES) and its workshops (International Conferences on Environmental Mutagens i.e. ICEM) have given the following recommendations which is summarized briefly:

1. Bacterial Tests
2. Mammalian Cell Gene mutation Tests
3. In Vitro chromosomal aberration Tests
4. Bone marrow micronucleus and chromosomal aberration tests
5. Unscheduled DNA synthesis tests and
6. Germ cell tests

In conjunction to the above mentioned tests following tests are performed in present research work. Two most important endpoints are "gene mutation" and "chromosomal aberration". This battery was proposed for pharmaceutical drugs in international harmonization. This also includes gene mutation and chromosomal aberration *in vitro*, and one *in vivo* assay.

The experimental protocol of this work includes *in vitro* and *in vivo* tests to assess genotoxic and systemic effects of Endosulfan.

10.1 Mutagenic Assays

These assays are performed to assess mutagenicity (point mutations) in prokaryotic (Non – mammalian - *Salmonella typhimurium*) and eukaryotic (Mammalian – Chinese Hamster Ovary Cell lines) or Mouse lymphoma forward mutation assay.

10.1.1 Bacterial Tests (Ames assay)

There are inconclusive reports on its Genotoxic (Mutagenic) effects. As differences in the results of various investigators in different types of studies, *In vitro* and *In-vivo* studies were performed to assess Genotoxic and Systemic effects of Endosulfan (Refer Chapter-II for further details). An Ames test can be performed as Endosulfan falls in organochlorine group and there is no restriction or modification required as per OECD guideline 471.

10.1.2 Mammalian Cell Gene Mutation Tests

The Chinese hamster ovary cell/hypoxanthine-guanine phophoribosil transferase (CHO/HGPRT) assay has been widely applied to the toxicological evaluation of industrial and environmental chemicals.(refer Chapter-II for further details).

10.1.3 *In Vitro* Chromosomal Aberration Tests

This assay is performed to assess clastogenicity and anugenecity of the test compound by grossly examining the aberrations directly under the microscope (refer Chapter-II for further details).

10.1.4 Bone Marrow Micronucleus Tests

Clastogenicity and Aneugenicity Assays

For a test of clastogenicity *in vitro* chromosomal aberration test was performed in Chinese Hamster Ovary cell lines as *in vitro* assay and 28 days repetitive test for micronucleus assay was performed as *in vivo* assay (refer Chapter-II for further details).

10.2 *In Vivo* Screening of Endosulfan

In vivo screening includes an acute and sub acute exposure in mice. In case of acute test mice were treated once with the compound and observed for five days. This part of the study was referred to as dose selection study.

The second test applied included 28 days repetitive treatment with Endosulfan 35% EC. The study of 28 days repetitive treatment with Endosulfan 35% EC was evaluated for gross pathological symptoms after treatment, various biochemical parameters, hematological examinations, differential leukocyte counts, sperm morphology and 28 days micronucleus assay as a test for *in vivo* clastogenicity.

CHAPTER 2

1. EXPERIMENTAL DESIGN

1.1 MATERIALS

The system adopted in present thesis for assay includes various materials and assay procedures. Hence, has been discussed in detail under different sections.

1.1.1 Lab Ware and Instrument Details

For cell gene mutation test and chromosomal aberration test

Glass and plastic wares

Tissue culture flasks - 25-cm^2 culture flasks (Falcon)
Tissue culture dishes - 60 mm Petri dishes (Falcon) for Survival frequency
- 90 mm Petri dishes (Falcon) for mutation frequency
Cryovials - 1.0 ml plastic cryovials (Nalgene Nunc)

Autoclave the cryovials by drying method – for this maintain the cleaned vials at 20℃ for more than 10 hours (overnight) in oven.

Centrifuge tubes

50 ml (B.D. Falcon)
15 ml glass centrifuges
15 ml plastic centrifuges (autoclavable)
Round bottom test tubes with lid (10 ml and 15 ml for dose dilutions)

Media bottles

50 ml, 100 ml, 250 ml, 500 ml, 1000 ml Scott Duran (autoclavable)

Pipettes

5.0 ml, 10 ml calibrated glass pipettes and 5.0 ml plastic pipettes
Cryocan (for temporary storage).
Cryocan (Tarsons) is used if freezer of -1℃ is not available for freezing the cells. The vials with cells are inserted to pre-chilled (in freezer) cans filled with isopropyl alcohol. Follow all the procedure in ice bath. Keep the vials at -20℃ overnight and store in liquid nitrogen tank next day.

1.1.2 Miscellaneous Instruments

- Centrifuges (REMI-R 8 C and Cooling centrifuge REMI 60C)
- Cryofreezer (Deep freezer (Galaxy)
- Electronic Weighing Balance (Ohus, Adventurer®)
- Filter assembly (Millipore)
- Horizontal Laminar air flow
- Liquid Nitrogen tanks
- Millipore filter (0.22 µm)

- pH meter (Cyber Scan)
- Pipette Aid
- Test tubes (glass)
- Water Baths (Horizontal Shaking water bath)

1.1.3 Details of chemicals

β-Nicotinamide Adenine Dinucleotide Phosphate- 1.0g
$C_{21}H_{27}N_7O_{17}P_3Na$ [1184-16-3], Fw = 765.4
Sigma Chemical Co. (N-0505)

β-Nicotinamide Adenine Dinucleotide Phosphate- 1.0g
$C_{21}H_{26}N_7O_{17}\ Na_2\ P_3$, [1184-16-3], Fw = 787.37
Himedia (RM 392)

Foetal Bovine Serum
(Himedia)® (RM 1112)

Penicillin Benzyl Sodium Salt
$C_{16}H_{17}N_2O_4SNa$, Fw = 356.4
Himedia (RM 132) million units/vial

L-Histidine-5g
L-α- Amino- β[4-imidazolyl] propionic acid
$C_6H_9N_3O_2.HCl.H_2O$, [5934-29-2], Fw = 209.6

Cyclophosphamide-1g
Cyclophosphamide monohydrate
[6055-19-2]
Sigma Chemical Co. (C-0768)

Trypsin 1:250
[9002-07-7]
From Porcine Pancreas, trypsin activity-1800 BAEE units/mg solid
Chymotrypsin activity 2 BTEE units/mg solid
Sigma Chemical Co. (T-4799)

Tetracyclin-5g
Tetracyclin hydrochloride, Sigma Aldrich
Acromycin
$C_{22}H_{24}N_2O_8.HCl$, Fw. : 480.9
Himedia (RM 219)

Methotrexate hydrate
[59-05-2], $C_{20}H_{22}N_8O_5$, FW. – 454.4
Sigma Chemical Co. (M-8407)

Mitomycin-C- 2.0mg
[50-07-7], From: Streptomyces caespitosus
1 Vial contains 2mg of mitomycin-C and 48mg NaCl
Sigma Aldrich Co. (M-4287)

d- Biotin (Vitamin H) -100mg
[58-85-5], $C_{10}H_{16}N_2O_3S$, FW.- 244.3
Sigma Aldrich Co. (B-4501)

Colchicine-1.0g
$C_{22}H_{25}NO_6$, FW. -366.45
Plant Growth Hormone

Heparin Sodium Injection I.P. (25000 IU in 5ml)
Derived from mucosa
Beparine®, Biological E. Ltd. (India)

Benzo[a]pyrene
[50-32-8], FW- 252.32
Sigma Aldrich Inc., 3050 Spruce Street, St. Louis, M063103 USA
314-771-5765 (B 1760-1G)

Lectin -5.0mg
From – phaseolus vulgaris (Red Kidney Bean)
[9008-97-3]
Essentially salt- free lyophilized powder,
Sigma Aldrich Co. (L-9132)

RPMI- 1640 = 10.3g/l
With L- glutamine without Sodium bicarbonate
Himedia (AT-028)

MEM Eagle – 10.1g/l
With L- glutamine without Deoxyribonucleosides, Ribonucleosides and Sodium bicarbonate (α- modification)
Himedia (AT-081)
Dulbecco's Modified Eagle Medium -13.4g/l
With L- glutamine, 4.5g glucose/l and sodium pyruvate, without Sodium bicarbonate
Himedia (AT-007)

Nutrient Mixture F-12 (HAM's) -10.63g/l
With L- glutamine without Sodium bicarbonate
Himedia (AT-025)

Nutrient Mixture F-12 HAM,- 11.1g/l
Kaighn,s modification (N3520-10× 1l)
With L- glutamine without Sodium bicarbonate
Sigma Aldrich Inc.
9- Aminoacridine (Aminoacrine) (A-1135) - 5g

$C_{13}H_{10}$ N_2·HCl, FW = 230.7, [52417-22-8]
Sigma Chemical Co. (A-1135)
Room temperature

2-Aminoflurine (A- 9031) - 5g
$C_{13}H_{11}N$, [153-78-6], FW = 181.2

Sodium Azide- 25g
NaN_3, [26628-22-8], FW-65.01
Sigma Chemical Co. ((S-2002)

D- Glucose- 6 – phosphate monosodium salt
[54010-71-8], $C_6H_{12}O_9PNa$, FW-282.1

HEPES – 25g
(N- [2-Hydroxyethyl] piperazine- N′-[2-ethane sulfonic acid] sodium salt)
[75277-39-3], $C_8H_{17}N_2O_4SNa$, FW. 260.3
Sigma Aldrich Co. (H- 3784)

DimethylSulphoxide (DMSO)- 100 ml
Hybri-Max ®, C_2H_6OS, FW. 78.13 [67-68-5], Endotoxin tested, Hybridoma tested
Sigma Chemical Co. (D2650)

2-Aminofluorene -5g
$C_{13}H_{11}N$, FW. -181.24, [153-78-6]
A5, 550-0 (Aldrich®)

Glycine (Aminoacetic acid)-100g
$C_2H_5NO_2$, FW. 75.07
Sigma Ultra (G -7403)
9, 10- Dimethyl- 1, 2- Benz- Anthracene
[57-97-6],1g, $C_{20}H_{16}$, FW. – 256.3
Sigma Chemical Co.

Ethylmethanesulfonate
[62-50-0], $C_3H_8O_3S$, FW. - 124.16, Specific density- 1.206
Sigma Aldrich Inc. (M 0880-10G)

Sodium ammonium phosphate tetrahydrate
[7783-13-3], Na $NH_4HPO_4.{}_4H_2O$, FW.209.07, d- 1.544
Sigma Aldrich Inc., (S4172-250G)

Ethylenediamine tetra acitic acid (EDTA), 50g
Disodium salt; Dihydrate
$C_{10}H_{14}N_2O_8Na_2.2H_2O$, [6381-92-6]
FW. 372.2
Sigma Chemical Co.
Potassium Chloride (KCl)- 250g
[744-40-7], FW. - 74.55, cell culture tested

Sigma Chemical Co. (P-5405)

Sodium Chloride (NaCl)- 500g
[7647-14-5], FW. 58.44
Cell Culture tested
Sigma Aldrich Co. (S-5886)

Potassium phospate monobasic
[7778-77-0], KH_2PO_4, FW. 136.1
Sigma Aldrich Co. (P-5655)

D- (+) Glucose (Dextrose; Corn sugar)-100g
$C_6H_{12}O_6$, [492-62-6], FW. 180.2
Cell culture tested,
Sigma Aldrich Co. (G-7021)

Sodium Bicarbonate ($NaHCO_3$), 500g
[144-55-8], FW. 8.01,
Cell culture tested
Sigma Aldrich Co. (S-5761)

Citric acid monohydrate, 500g
$C_6H_8O_7.H_2O$; [5949-29-1], FW. 210.14
Sigma Aldrich Co. (C7129)

Sodium phosphate (Na_2HPO_4)
Dibasic anhydrous [7558-79-4], FW. 142.0, Sigma Aldrich Co. (S-5136)
Sodium pyruvate
Pyruvic acid, sodium salt
C_3H_3ONa, FW. 110.05
Himedia (RM-082)

Tri- Sodium Citrate- LR
$Na_3C_6H_5O_7.H_2O$, FW. 294.10
S.D Fine- Chem Ltd. Mumbai-400 025
Glycerol -500g
$CH_2OH.CHOH.CH_2OH$, FW., 92.09
Qualigens® Fine chemicals (24505), A division of Glaxosmithkline pharma ltd.
Dr. Anne Besant Rd., Mumbai- 400 025.

Ethanol Alcohol AR
Made in China Distributed by K. Raj & Company, Mumbai

Giemsa's Stain Powder-25g
Qualigens Fine Chemicals (39382)

Trypan blue (M.S.)
$C_{34}H_{24}N_6NaO_{14}S_4$, FW. 960.82
S.D. Fine Chemicals

Difco ™ Nutrient Agar (213000)- 500g
Becton Dickinson and & Co. Sparks MD- 21152 USA. 38800 Le Pnt de claix, France.
Composition of Difco Agar

Beef Excract	3.0g/l
Peptone	5.0g/l
Agar	15.0g/l

Final pH 6.8±0.2
Suspend 23g of the powder in 1l of water, mix thoroughly. Heat with frequent agitation and boil for one minute to completely dissolve the powder. Autoclave at 121℃ for 15 minute.

Nutrient Broth No. -2 (Oxoid), CM 67
Composition 'Lab – Lenco' powder 10.0g/l,
Peptone 10.0g/l, NaCl 5.0g/l
pH 7.5±0.2 at 25℃
Procedure: Add 25g to 1l of distilled water & sterilize by autoclaving at 121℃ for 15 minute.

Magnesium chloride ($MgCl_2.6H_2O$) - 500g
FW. – 203.3
Ranbaxy Laboratories Ltd.
Chemical division SAS nagar- 160055.

Magnesium sulphate Pure (17517)
$MgSO_4.7H_2O$ (246.47g/ml)

1.1.4 Media and Reagents
For cell gene mutation and chromosomal aberration test
0.1N HCl
As all the acids are supplied in 12N (the received HCl was 11.6N) concentration therefore a 1.0 ml of normal HCl was diluted with 11of ml distilled water to get 1.0N and diluted further (1/10) to get the 0.1N concentration.

1N NaOH
Weigh 4g of NaOH to 10 ml of distilled water to prepare 1N NaOH.

Penicillin Stock Solution (1,00,000 IU/litre) Hi-media
Reconstitute 1 vial (10million IU) of Penicillin benzazolium Sodium salt with 10 ml of sterile distilled water and add to 1litre of respective culture media before filtration.
Storage: Stock solution can be filtered sterile and can be stored at 2-8℃ for 6 months.

Streptomycin (Stock solution, 100 µg/litre**)**
To 50 mg of streptomycin sulphate add 50 ml of sterile distilled water (Stock solution) and add the above 1 ml of the above stock solution to 1 litre of respective media before filtration.

α-MEM (Modified Eagles Medium) with Nucleotides and without Nucleotides
Add 1 vial of α-MEM with Nucleotides/ α-MEM without Nucleotides Culture Media to 1 litre of sterile distilled water shake the flask till medium is completely gets dissolved, add Sodium(2200 mg) bicarbonate and Penicillin and Streptomycin stock Solutions, mix well and

filter by using 0.22 µm Nitrocellulose filter (Millipore). After filtration store the media in media bottles in sterile condition and seal by wrapping with paraffin.
Note: Add Sodium Bicarbonate solution as per the instruction of the manufacturer.
Storage: Culture media can be stored at 2-8℃ for 1 month as glutamine concentration decreases with time; in few cases it can be stored for a week or so.
Note: Check at regular intervals the change in pH of the media.

Foetal Calf and Foetal Bovine Serum (Hi-media)
Readymade foetal calf and foetal bovine serum has been used as supplement to culture media as it provides growth factors and other nutrients.

Phosphate Buffered Saline
Solution A
Add 6.8g KH_2PO_4 to 500 ml of distilled water.
Solution B
Add 1.0g of NaOH to 250 ml of distilled water.
Mix 50 ml of solution A, 22.4 ml of Solution B and 27.6ml of distilled water to get 100 ml of buffer solution. Adjust the pH 6.8 by adding one or the other solution.

Standard Buffer Solutions (pH 4.0, 7.0, 10.2)
Add standard buffer tablets of pH 4.0, 7.0 and 10.2 respectively to sterile (boiled for 10 minutes and cooled) to get pH of 4.0, 7.0 and 10.2 strength.

Fixative Solution
Methanol
Acetic Acid (glacial)
Mix the above solutions (Acetic Acid: Methanol) in 1:3 ratio. Prepare fresh and chill before use.
Hypotonic Solution (0.75M KCl solution)
Add 0.56g of potassium chloride to 100 ml of distilled water adjust the pH 7.4 and filter with Whatman filter paper No.1.

Colchicine Solution
To 250 ml of sterile distilled water add 10 mg of colchicine and filter with 0.22µm filter.
Glycerol – McIlvaine's buffer
Solution A
Dissolve 5.68g of Na2HPO4 in distilled water, make up to 100 ml (0.4M).
Solution B
Dissolve 4.2g of Citric acid in distilled water, make up to 100 ml (0.2M).
For preparing 40 ml of Glycerol –McIlvaine's buffer mix 11.37 ml of Solution A with 8.63 ml of Solution B. Adjust the pH 5.5 then add equal volume of glycerol to the above mixture.

Sterile solution
70% Isopropyl Alcohol and Ethanol are used for maintaining sterility procedures.

Lens Cleaning Fluid
Mix 80 ml Petroleum ether (non-anaesthetic) to 20 ml of methanol to get 100 ml of lens cleaning fluid.

6-Thioguanine (2.5mg)
(A-4660, Sigma) Hybri-Max®, 50X Lyophilized powder, approx. 98%, Hybridoma Tested.
Reconstitute contents of vial with 10 ml sterile cell culture medium. When reconstituted to 10 ml each vial contains 1.5×10^{-3}M 2-Amino-6-mercaptopurine. Dilute to 500 ml. Store solution at 2-8℃.

HAT Media Supplement (50X)
It is used for removal of mutants (HGPRT $^{-}$) in CHO K1 cell lines. (H 0262, Sigma) Hybri-Max®
Reconstitute contents of vial with 10 ml sterile cell culture medium. When reconstituted to 10 ml each vial contains 5×10^{-3}M hypoxanthine, 2×10^{-3}M aminopterine and 8×10^{-4}M thymidine.
Working stock: 10 ml of above solution was added to 490 ml of α-MEM (without Nucleotides). Store solution at 2-8℃ for approx 5 days, for long storage aliquot and freeze at -20℃.

For Ames test
Preparation of reagents
Top Agar preparation
For cytotoxicity and mutagenicity
Add Bacto Agar (B-D-Difco) - 0.6g
NaCl - 0.6g
Histidine/Biotin Solution - 10 ml
Distilled water - 90 ml
For genotype confirmation
Add Bacto Agar (Difco) - 0.6g
NaCl - 0.6g

Weigh 0.6g Bacto agar and 0.6g NaCl and add 90 ml of distilled water warm in the autoclave till it gets mixed completely. Add pre-warmed, sterile histidine/biotin solution to top agar in round bottom flask and mix well. As histidine/biotin solution is light sensitive, so to protect it from photo-degradation, perform the procedures in dark (away from direct light) and cover the round bottom flasks with aluminum foil. Keep the round bottom flask on heating mantle or warm at regular intervals with water and dispense 2.0 ml of top agar into autoclavable glass tube (10ml) with the help of graduated glass pipette equipped with pipette aid. Autoclave the glass tube and store at room temperature till use.

For genotype confirmation test perform the analysis with plain medium (without histidine and biotin).

Minimal Glucose Agar (MGA) preparation
For cytotoxic and mutagenic assay
Bacto Agar - 7.5g
(Oxoid Nutrient Agar can be used for master plate preparation solubility as per the instruction of the label on the bottle)
Vogal boner medium - 10ml
Glucose solution (10%) - 25ml
To 7.5g of Bacto agar add 450 ml of distilled water in round bottom flask (500ml, cap.) autoclave and maintain at 60℃ in shaking waterbath. Add prewarmed, sterile vogal boner medium and glucose solution. Mix the above solution completely and pour (about 20 - 25 ml) it on to autoclaved petriplates (under Laminar Air Flow). After getting mixture solidified in the

MGA-plates, invert the plates and incubate (individually, don't pile one above the other) in bacteriological incubator (at 37℃). After 24 hour of incubation observe the plates for presence of contamination before use. Plates can be packed in autoclavable plastics and stored in refrigerator for two months. Again plates should be examined for moisture contents and presence of bacterial colonies.

For genotype confirmation assay

Minimal Glucose Agar (MGA) enriched with histidine

Bacto Agar	-	7.5g
Vogal boner medium	-	10ml
Glucose solution (10%)	-	25ml
Histidine (0.5%)	-	4.0ml

Minimal Glucose Agar (MGA) enriched with biotin

Bacto Agar	-	7.5g
Vogal boner medium	-	10ml
Glucose solution (10%)	-	25ml
Biotin (0.01%)	-	4.0ml

Minimal Glucose Agar (MGA) for ampicillin resistant

Bacto Agar	-	7.5g
Vogal boner medium	-	10ml
Glucose solution (10%)	-	25ml
Histidine (0.5%)	-	4.0ml
Biotin (0.01%)	-	4.0ml
Ampicillin solution (8 mg/ml)	-	1.5ml

Minimal Glucose Agar (MGA) for tetracycline resistant

Bacto Agar	-	7.5g
Vogal boner medium	-	10ml
Glucose solution (10%)	-	25ml
Histidine (0.5%)	-	4.0ml
Biotin (0.01%)	-	4.0ml
Tetracycline solution (8 mg/ml)	-	0.125ml

To 7.5g of Bacto agar add 450ml of distilled water in round bottom flask (500ml, cap.) autoclave and maintain at 60℃ in shaking waterbath. Add pre-warmed, sterile vogal boner medium and glucose solution. Mix the above solution completely and pour (about 20-25ml) it on to autoclaved petriplates (under Laminar Air Flow). After mixture gets solidified in the MGA-plates, invert the plates and incubate (individually, don't pile one above the other) in bacteriological incubator (at 37℃). After 24 hour of incubation observe the plates for presence of contamination before use. Plates can be packed in autoclavable plastics and stored in refrigerator for two months. Again plates should be examined for moisture contents and presence of bacterial colonies.

Vogal- Bonner (VB salts) medium E (50X)

Use: for preparation of GM agar plates

Warm distilled water	-	65ml
Magnesium sulphate	-	1.0g

Citric acid monohydrate	-	10g
Potassium phosphate, Dibasic, anhydrous (K_2HPO_4)	-	50g
Sodium ammonium phosphate ($Na_2NH_2PO_4.4H_2O$)	-	17.5g

Glucose solution

Use: for preparation of GM agar plates

Dextrose powder	-	225g
Distilled water	-	50ml

To 25g of dextrose powder add 250ml of distilled water dissolve it and dispense an aliquot of 25ml into small conical flasks (100ml, cap.) and autoclave. Glucose solution can be stored for a period of 3 months at below 8℃.

For genotype confirmation assay

Histidine Solution (0.5%)

Histidine	-	0.5g in 100ml of distilled water

Biotin Solution (0.01%)

Biotin	-	10mg in 100ml of distilled water

Nutrient agar

Add 14g of nutrient agar to 450ml of distilled water, autoclave and prepare nutrient agar plates by pouring 20-25ml of agar to petriplates.

For Cytotoxicity and Mutagenicity Assay

Oxoid Nutrient Broth

Oxoid Nutrient Broth is used for growing the bacterial cultures.

Add 2.5g of Oxoid nutrient broth number-2 to 100ml of distilled water; dispense it into in aliquots of 10ml and autoclave.

0.5 mM Histidine Biotin Solution

Biotin	-	12.215mg
Histidine	-	10.48mg in100 ml of distilled water

Dissolve biotin solution in boiling distilled water, after it gets dissolved completely add histidine to it, autoclave the above solution and store it in amber coloured bottle to protect it from direct sunlight.

Co-factor mix (a)

Co-factor mix is used for the preparation of S9 mix for metabolic activation.

D-glucose-6 phosphate	-	0.80g
β- NADP	-	1.75g
$MgCl_2$	-	0.90g
KCl	-	1.35g
Na_2HPO_4	-	6.40g
NaH_2PO_4	-	1.40g
Distilled water	-	450ml

Dissolve the above constituents one by one and filter it with 0.22µm nitrocellulose filter and dispense into aliquots of 9.5, 9.0and 7.0ml for the preparation of 5, 10 and 30% S9 mix (v/v)

and store it in amber coloured bottle to protect it from direct sunlight below 0ºC in the freezer for long term storage.
This co-factor mix is generally used for Ames test.

Co-factor mix (b)

150mM KCl	-	1.5ml
Glu-6 PO_4 (180mg/ml)	-	1.5ml
β-NADP (25mg/ml)	-	1.5ml
S9 fraction	-	3.0ml

pH of the above mixture was adjusted with 1N NaOH and it is referred as the culture medium with 5% metabolic activation for 52.5ml of plain culture medium. In case of without metabolic activation system add equal amount of 150mM KCl. This co-factor mix is generally used for studies including cell lines or primary cell lines.

0.2M Sodium Phosphate buffer

Sodium phosphate buffer is used as vehicle for negative control and as supplement in case of without metabolic action.

Sodium phosphate monobasic	-	38g in 50ml of distilled water (solution A)
Sodium phosphate dibasic	-	8.64g in 300ml of distilled water (solution B)

To 264 ml of Solution B add 36 ml of solution A and adjust the pH 7.4 ± 0.2 by adding respective solution (A or B).

1.2 S9 PREPARATION

Acclimatization

Acclimatize 8 weeks of rat (Wistar Rat) at 22±3ºC and at a relative humidity of 60-62 for a minimum period of five days, feed and water were provided *ad libitum.* The animals weighed 213 and 222g.

Preparation of Aroclor

A day before treatment weigh 200mg of Aroclor -1254 into sterile glass vial with micropipette as it is very viscous and add 1.0ml of corn oil to the vial, pipette it till it dissolves completely (prepare the volume as per the requirement).

Treatment

The animals were treated with Aroclor-1254 intraperitonially at the dose concentration of 500mg/ kg body weight and observed for five days.

Sample collection

The animals were sacrificed after five days by cervical dislocation. The animals were fasted overnight (12 hours) while water was provided *ad libitum* before sacrifice. The abdominal area, were wiped with ethanol and the skin was removed with sterile scissors. With another scissors the peritoneum was cut and the body cavity was opened. The liver was removed, taking care to avoid any cut into organs other than liver. Liver was removed aseptically and collected into beaker containing equal volumes of 0.15 MKCl.

1. Immediately and aseptically transfer the liver into a preweighed beaker containing 0.15MKCl (30ml) and note the weight of the beaker containing liver and KCl record the difference (11.0ml).

2. Give 4 washes with KCl and in the finally add 3 volumes of KCl to liver (i.e. for 1g of liver add 3ml of KCl, therefore to a 11g of liver add 33ml of KCl).
3. Chop the liver with help of sterile scissors or scalpels and transfer the contents into 50 ml homogenizing tube insert the Teflon rod and cover with sterile foil.
4. Homogenise the liver in a 50ml glass tube fitted in ice jar by increasing the rpm slowly.
5. After homogenization transfer the homogenate into 50ml centrifuge tube (Falcon) maintained on ice.
6. Centrifuge the contents at 9000g (at 10400 RPM and 9000 RCF) for 10 minutes.
7. Aseptically transfer the supernatant into 1ml cryovials into 1.0ml and 0.5ml volumes maintained on ice.
8. Immediately transfer the vials into the freezer (Heto mini freeze, - 80ºC) till the sample gets frozen.

Store the sample at the upper portion of the liquid nitrogen (gas phase) overnight and then store at liquid phase of nitrogen.

Sterility test

Sterility test was performed to assess any contamination present in the sample by streaking a loop full of S9 to the nutrient agar plates in duplicates. The plates were incubated for 48 hours at 37ºC. Colony forming units were not observed after incubation.

Efficiency test

Efficiency of the newly prepared S9 was checked by treating the S9 with a known mutagen (2-Aminofluorine, 20µg/ml). This batch of S9 was compared along with concurrent positive and negative controls of older batch. The Enzyme content was estimated indirectly by estimating for protein concentration.

***Salmonella typhimurium* TA-100**			
	No. of colonies		Mean and Std. deviation
	Replicate-1	Replicate-2	
Negative control (New S9)	131	135	133 ± 8.48
Negative control (Old S9)	139	127	133 ± 2.82
Positive control (New S9)	3648	3280	3198 ± 313.95

Protein Estimation

The protein concentration was estimated by using Lowry's kit (KT 18, Bangalore Genie Private limited).

Preparation of Standard

One vial of Standard (Bovine serum Albumin) was reconstituted to 1.0ml of distilled water (5 mg/ml). 100µl of this was further diluted to 1.0ml using 900µl of distilled water (0.5mg/ml).

Complex forming reagent

20ml of solution-II was added to 0.2ml of solution-I.

Preparation of calibration curve

The standards of different concentrations were prepared as follows:

Volume of standard (µl)	Actual weight (µg)	Volume of distilled Water (µl)	Final volume (µl)
0	0	200	200
20	10	180	200
40	20	160	200
80	40	120	200
120	60	80	200
160	80	40	200

Two ml of complex forming mixture was added to each tube, mixed and kept for 10 minutes. 200µl of solution-III was added to each tube, mixed using a cyclomixture and kept for 30 minutes. The O.D. was measured at 660nm using a photoelectric colorimeter.A concentration curve was constructed by plotting O.D. on Y- axis against standard protein (µg/tube). The equation derived from the graph was Y= 0.007+ 0.0077

Preparation of Sample

A 40µl aliquot of S9 fraction was added to 760µl of distilled water (0.05µl/ml), 100µl of this stock was added to 100µl of distilled water (5µl of S9). Two ml of complex forming mixture was added to this, mixed and kept undisturbed for 10minutes.

A 200µl aliquot of Solution-III was added, mixed using a colourimeter and kept for 30 minutes. The O.D. was measured at 660nm using a photoelectric colorimeter.

OD (Y) = 1.2

Y = 0.007 X + 0.0077

Sample concentration = X/ V mg/ml

Where X = value of graph in µg = 170.33

V = volume of sample in µl = 5µl

Therefore sample concentration = 170.33 ÷ 5mg/ml

Actual protein Content	Optical Density
Blank(0)	Adjusted to 0
10	0.085
20	0.15
40	0.26
60	0.48
80	0.54

A similar (32.6mg/ml of protein) result was also obtained by Eva Rasmussen (1992) by using adult male Wistar rats, treated with Aroclor-1254, dissolved in corn oil, and was injected i.p. 5 days before sacrifice and S9 was prepared as described by Lake (1987).

1.3 Composition of Culture Media

All defined classical cell culture media generally consists of four basic chemical groups: amino acids, carbohydrates, inorganic salts and vitamins.

Amino acids
Amino acids (essential and non-essential) are required for protein synthesis. Essential amino acids cannot be synthesized by the cell and must be supplemented exogenously in the formulation. Non essential amino acids, on the other hand, depend on the metabolism of individual cells and are not required in the formulation. However, a formulation that provides nonessential amino acids may minimize the metabolic burden of the cell, thus allowing the cells to proliferate more rapidly or to produce a desired end product more efficiently.

Carbohydrates
Glucose is the most common carbohydrate used in mammalian cell culture. It provides the major energy or carbon source for biosynthesis. Through glycolysis, glucose is broken down to pyruvate, which is converted to essential metabolites in the citric acid cycle. Some media also contain sodium pyruvate as a carbon source. Galactose, which metabolises to lactic acid and acts at a slower rate, is sometimes substituted or used with glucose. This prevents excessive lactic acid accumulation and the resulting pH shift.

Inorganic salts
Inorganic salts are essential to cell growth and maintenance. They provide major ions in the form of sodium, magnesium, potassium, calcium, phosphate, chloride, sulphate, and bicarbonate. Inorganic salts also help to maintain the cellular membrane by controlling the osmotic pressure. Additionally, they act as buffers to protect cells from sharp pH fluctuations due to metabolic waste products.

Vitamins
Vitamins are generally included in all formulations and function as catalysts or substrates to facilitate or control certain metabolic functions. Most cells require B vitamins other vitamins or co-enzymes may be required by some cells and are therefore, included in cell culture media.

Other Components
Most cell culture media contain a pH indicator (most commonly phenol red) that allows visual observation of pH change in the media due to cell metabolism or environmental factor. Other organic or inorganic components are often included in cell culture media to provide for specific nutritional or other requirement affecting cell growth. As serum free and protein free media gain wider use, an increasing number of components once contributed by a serum supplement are being replaced by chemically defined components.

1.4 TEST SYSTEMS
The term test system refers to various biological systems on which the experiment is being performed (i.e. the test item is being tested) and endpoint results are made. The assay includes various methodologies which involves prokaryotic cell system to eukaryotic cell and finally whole animal. The various test systems are described in their respective sections.

2. METHODS
Based on review of the available literature the toxicity of Endosulfan is very clear and well understood and much work has been done by many investigators. It has been found that mice are the most sensitive animal among rodent species. The mutagenicity/genotoxicity of Endosulfan is still not much clear there are conflicting reports. In short term exposures it is less genotoxic. Further, there are cases showing malformations and other anomalies in man

and animals. To compensate all these factors more effort has been done on mutagenicity of Endosulfan and it is also regulatory requirement for *in vitro* and *in vivo* screening.
For this various methods were used to study genotoxic and systemic effects of Endosulfan. As there are several assay methodologies involved these methodologies are described individually. These methods are given below:

Methods for Genotoxic effects

- Reverse Mutation Test - (assay type: *In vitro* screening assays)
- Forward Mutation Test - (assay type: *In vitro* screening assays)
- Clastogenicity Test (chromosome aberration test) - (assay type: *In vitro* screening assays)
- Bone marrow micronucleus test- (assay type: *In vivo* screening assays)

Systemic toxicity assays (In *vivo* screening assays)

- 28 days toxicity test -(assay type: *In vivo* screening assays)
- Sperm abnormalities test -(assay type: *In vivo* screening assays)
- Biochemical tests -(assay type: *In vivo* screening assays)

2. METHODS FOR GENOTOXIC EFFECTS

Reverse Mutation Assay

2.1. Ames Test

The Ames *Salmonella* microsome mutagenicity assay evolved over the years from the initial screening of a number of histidine mutants which led to selection of mutants that were highly sensitive to reversion by a variety of chemical mutagens (Ames *et.al*., 1971, 1973, Levin *et.al*., 1982a, 1982b and Maron *et.al.,* 1983). As bacteria are unable to metabolize chemicals via cytochrome P450, unlike mammals and other vertebrates, a key component for making the bacterial mutagenicity test useful was the inclusion of an exogenous mammalian metabolic Activation system (Ames *et.al*., 1973 and Malling *et.al.,* 1971). At the same time, the development of the plate incorporation assay protocol to replace spot test or liquid suspension procedures was a major contributing factor to the success of the Ames test because it made the test easier to perform and reduced its cost.

2.2 Screening of histidine mutants and development of the plate incorporation assay.

Studies performed to identify and map the genes responsible for histidine biosynthesis produced a large number of spontaneous, radiation -, and chemical-induced mutants of *Salmonella typhimurium* LT-2 (Whitfield *et.al.,*1966). Some of the mutants contained single base changes (base-pair substitution mutants), and others contained additions or deletions of one or more bases (frameshift mutants). It was later realized that some of this mutant strains could be used to identify and characterize mutagenic chemical by their ability to revert to wild-type (histidine-independence) in the presence of mutagens. In 1966, Ames and Whitfield (Ames *et.al*., 1966) proposed a set of histidine mutant strains for screening chemical for mutagens using a spot test procedure that was previously used by Szybalski (Szybalski *et.al.,* 1958) and Iyer and Szybalski (Iyer *et.al.,* 1958) for mutagen screening with an *E. Coli* strain .The spot pest consists of applying a small amount of the test chemical directly to the center of a selective agar medium plate seeded with the test organism. As the chemical diffuses in to the agar a concentration gradient is formed. A mutagenic chemical will give rise to a ring of revertant colonies surrounding the area where the chemical was applied. If the chemical is toxic, a zone of growth inhibition will also be observed (Ames

et.al., 1971). Fig. 1 depicts a spot test with methylmethane sulfonate and *Salmonella* strain TA100.

In 1973, Ames *et al.,* developed the plate incorporation assay procedure which is more sensitive and quantitative than the spot test. The procedure consists of adding the buffer or S9 mix, the histidine dependent bacteria (10^8) and test chemical to 2ml of top agar containing biotin and a trace amount of histidine (0.05 mM each). The mixture is than gently mixed and poured on glucose minimal (GM) agar plates. When the top agar has solidified the plates are incubated in an inverted position in a 37℃ incubator for 48 h at which time the histidine revertant colonies are counted.

When the histidine dependent bacteria are grown on glucose Minimal (GM) agar plate containing a trace amount of histidine, only those cells that revert to histidine independence (His^+) are able to form colonies. The small amount of histidine allows all the plated bacteria to undergo a few cell divisions; in many cases, these His^+ revertants easily scored as colonies against the slight background growth. The number of spontaneously induced revertant colonies is relatively constant for each strain. However, when a mutagen is added to the plate, the number of revertant colonies per plate is increased, usually in a dose related manner.

The plate incorporation test does not permit the enumeration of the total number of surviving cells because of bacterial growth on the plate and competing toxicity due to the chemical treatment. Also, because of the extra cell divisions that take place after adding the bacteria to the plate, it is not possible to estimate the number of cells at risk for mutation. Therefore, the mutation values obtained can only be expressed as number of mutants /amount of chemical added.

A plate test that can provide quantitative toxicity information is the “treat–and-plate" suspension procedure but takes longer to perform than the plate incorporation assay. In this procedure, the bacteria are washed free of growth medium and re-suspended in non-nutrient growth medium and treated with the test substance for various time intervals. Separate samples of the bacteria are then plated on selective medium for mutant determination and on complete medium for survival determination .The results establish the mutation frequency by calculating the number of mutants per surviving fraction of bacteria (Green *et.al.,* 1976). However, the plate incorporation assay offers the advantage that limited steps are required to expose the bacteria to the test chemical with no need for washing or re-suspending the bacteria prior to or after treatment. In addition, the bacteria are allowed to undergo a few cell divisions in the presence of the test substance, which increases their sensitivity to mutation induction.

2.3 Metabolic activation systems

2.3.1. Oxidative metabolism

Some carcinogenic chemicals, such as aromatic amines or polycyclic aromatic hydrocarbons, are biologically inactive unless they are metabolized to active forms. In humans and lower animals, the cytochrome-based P450 metabolic oxidation system, which is present mainly in the liver and kidneys, is capable of metabolizing a large number of these chemicals to DNA-reactive, electrophilic forms. Some of the intermediate metabolites are potent mutagens in the Ames *Salmonella* assay. Since bacteria do not have this metabolic capability, an exogenous mammalian organ activation system needs to be added to the petriplate together with test chemical and the bacteria. For this purpose, a rodent metabolic

activation system was introduced into the test system (Ames *et.al*., 1973, Garner, *et.al.,* 1972, Malling *et.al.,* 1971, Miller *et.al*., 1971, Smith *et.al*., 1966) The metabolic activation system usually consists of a 9000×g supernatant fraction of a rat liver homogenate (S9 microsomal fraction), which is delivered to the test system in the presence of NADP and cofactors for NADPH-supported oxidation (S9 mix) (Maron *et.al.,* 1983). To increase the level of metabolizing enzymes, the animals are pretreated with the mixed-function oxidase inducer Aroclor- 1254. Other inducers, such as Phenobarbital and β-naphthoflavone, can also be used.

The mixed function oxidase enzymes (S9 fraction) can also be obtained from animal species other than rat such as mouse, hamster, guinea pig and monkey and organs other than liver such as kidney (Ishida *et. al.,* 1987, Matusushima *et.al.,* 1980) and from human liver (Shimada, *et.al.,* 1988). In comparative studies in which coded compounds were tested, induced and un-induced Syrian hamster or mouse liver S9 offered no overall advantage over Arocolor-1254 induced rat liver S9 for the induction of mutagenesis (Dunkel *et.al.,* 1984 and 1985 and Hawarth *et.al.,* 1983). However, there are chemicals that may be more efficiently detected as mutagens with rat, mouse, or hamster liver S9 (Dunkel, *et.al.,* 1984, Dunkel *et.al.,* 1985 and Zeiger *et.al.,* 1988). A detailed procedure for the preparation of metabolic activation mixture is given above in the section of materials and methods for the preparation of S9 mix.

2.3.2 Reductive metabolism

The metabolic activation system can also consist of reductive enzyme system for classes of chemicals containing azo and diazo bonds. Reduction of chemical substances can occur in mammals, including humans, by anaerobic intestinal micro flora, and very likely by mammalian reductases in the intestinal wall or in the liver. Two types pf reductive in vitro metabolic activation systems have generally been used, those based on a liver homogenate supplemented with FMN and those that are based on rat intestinal microflora preparation. However, assay based on reductive metabolism was not performed.

2.4 The Test System

The *Salmonella* tester strains TA 1535, TA 1537, TA 98, TA 100 and TA 102 are the recommended strains and used as test system for detecting bacterial reverse mutation tests. These *Salmonella* strains were procured from DRDE Gwalior, subcultured and maintained at Jai Research Foundation, Vapi (India).

2.4.1 Genotypes

The genotyopes (***Salmonella*** strains) used in the experiment were as follows:

Strain	bio,chlD, uvrB, gal	LPS defect	Plasmid
TA 1535	Base pair mutations	*rfa*	No plasmid
TA 100		*rfa*	With plasmid
			pKM101
TA 98	Various frame shifts mutations	*rfa*	pKM101
TA 1537		*rfa*	No plasmid

TA 102	Transitions/transversions and DNA cross linking agents	*rfa*	No plasmid pKM101, pAQ1

All strains are histidine dependent by virtue of a mutation in the histidine operon. Additional mutations/genetic alterations that have made the tester strains more sensitive to chemical mutagens are listed below.

A deletion mutation through the *uvrB–bio* genes, in all strains, except the strain TA 102. The *uvrB* deletion mutation eliminates the accurate excision repair mechanism, thereby allowing more DNA lesions to be repaired by the error prone DNA repair mechanism

All the tester strains are analyzed for their genetic integrity and spontaneous mutation rate before conducting the main experiment or cryopreservation of the strain. The cultures inoculated in Oxoid Nutrient Broth No-2 incubated overnight at 120rpm and 37±1°C. Following steps were followed for the genotype confirmation test:

2.4.2 Tests for verifying the genotypes present in the strain

2.4.2.1 Histidine dependence

A 100µl aliquot of overnight culture was mixed in plain top agar (without histidine and biotin), mixed and poured over minimal glucose agar plate with excess of biotin (without histidine), because all the *Salmonella* strains used are histidine dependent.

2.4.2.2 Biotin dependence

A 100µl aliquot of overnight culture was mixed in plain top agar (without histidine and biotin) mixed and poured over minimal glucose agar plate with excess of histidine (without Biotin), because all the *Salmonella* strains used are biotin dependent.

2.4.2.3 Histidine and Biotin dependence

A 100µl aliquot of overnight culture was mixed in plain top agar, mixed and poured over minimal glucose agar plate with histidine and biotin, because all the *Salmonella* strains used are histidine and biotin dependent.

2.4.2.4 *rfa marker*

A 100µl aliquot of overnight grown culture was mixed in plain top agar mixed and poured over nutrient agar plate. Sterile paper disks (punched pieces of Whatman filter paper No. 1 autoclaved) were placed in the nutrient agar plate and a drop (10µl) of crystal violet solution was dropped on the disc. All the *Salmonella* strains were checked for a zone of growth inhibition surrounding the disc.

2.4.2.5 *uvr B* deletion

A 100µl aliquot of overnight culture was mixed in plain top agar, mixed and poured over minimal glucose agar plate with histidine and Biotin. After few minutes of pouring all the plates were kept over a stand 33cm below the ultra violet source inside the laminar air flow, lids were removed from the plates and a sterilized acrylic strip was kept on only half of the plate exposing the other half. All the plates were exposed for *uv* for 6 seconds i.e. strains TA1535, TA1537 and strains TA 98, TA100 TA102. The lids were replaced to their respective plates. Because the deletion mutation stretches across the *bio-uvrB* region of the chromosome and cannot revert to wild type there is a deletion mutation through the *uvrB – bio* genes in all strains, except TA102. The *uvrB* deletion mutation eliminates the accurate excision repair mechanism, thereby allowing more DNA lesions to be repaired by the error prone DNA repair mechanism.

2.4.2.6 Presence of plasmid pKM101 (Ampicillin resistance)

A 100µl aliquot of overnight culture was mixed in plain top agar, mixed and poured over minimal glucose agar plate for Ampicillin resistance with an excess of histidine, Biotin and Ampicillin.

2.4.2.7 Presence of plasmid pAQ1 and plasmid pKM101 (Ampicillin+Tetracycline resistance)

A 100µl aliquot of overnight culture was mixed in plain top agar, mixed and poured over minimal glucose agar plate for tetracycline resistance with an excess of histidine and Biotin and trace amount of Ampicillin and tetracycline.

Observation Table for genotype confirmation test

Strain	Dependency			Markers		Resistancy	
	Biotin	Histidine	Biotin and Histidine	*rfa*	*uvr B*	pKM101 Ampicillin	pAQ1 Tetracycline
TA 1537	NG	NG	G	ZI	NG	NG	NG
TA 1535	NG	NG	G	ZI	NG	NG	NG
TA 98	NG	NG	G	ZI	NG	*G*	NG
TA 100	NG	NG	G	ZI	NG	G	NG
TA 102	G	NG	G	ZI	*G*	G	G

Note: NG = No growth, G = Growth, ZI = Zone inhibition

Results

The above results indicated that all the strains of *Salmonella typhimurium* had retained their genetic characteristics and were suitable to be used for the main study.

2.4.3 Spontaneous mutant frequency

Apart from genotype markers the revertant frequency of the test system were checked before treatment on minimal glucose agar plates which is referred as spontaneous mutant frequency of that particular test system. A 100µl aliquot of overnight culture was mixed in top agar enriched with histidine and biotin, mixed and poured over minimal glucose agar plate. The revertant colonies were enumerated.

2.4.3.1 Spontaneous Revertant Frequency

The following table shows literature values of various strains:

Strain	Revertant Frequency	
	Absence of Metabolic activation	Presence of Metabolic activation
TA 1537	5-20	5-20
TA 1535	5-20	5-20
TA 98	20-50	20-50
TA 100	75-200	75-200
TA 102	200-400	200-400

The above mentioned range may vary in the experiment and are the acceptable range of mean revertants/plate.

2.5 Assay procedures

The test was carried out by following two assay procedures

- Plate incorporation assay
- Pre incubation assay

Before conducting the treatment various test dilutions of S9 mix were prepared by mixing the S9 fraction with cofactors in their respective concentration and maintained on crushed ice, top agar was warmed in autoclave till melting and after that maintained at 45ºC and all the Petri dishes were labeled with their respective test concentrations code, strain code etc. All the positive controls were prepared and stored in the freezer well in advance.

2.5.1 Planting of Culture

The bacterial cell cultures were inoculated by scrap method where in the frozen cultures were scraped by sterile spatula and inoculated in culture medium (Oxoid Nutrient Broth No. 2). The cultures were incubated overnight for 12-16 hours period at temperature of 37℃ and 120±10 rpm.

2.5.2 Measurement of Growth

Before conducting the test, bacterial cell population was enumerated by reading its Optical density (O.D.) at 660nm on photoelectric colorimeter.

For measuring O.D. a one ml of Oxoid nutrient broth was diluted to cuvettes filled with 4ml of distilled water mixed thoroughly, adjusted for blank 0. Similarly one ml of each bacterial culture was diluted to 4.0ml of distilled water and O.D. was measured against the control respectively for each culture.

2.5.3 Storage

The bacterial cell culture were also prepared in advance and stored in refrigerator till its use.

2.5.4 Treatment Procedures

Two different treatment procedures were adopted in the assays which were as follows:

2.5.4.1 Plate incorporation assay

A 100µl aliquot test substance or solvent (DMSO), 500µl of S9 mix or Phosphate buffered saline (in case of without S9 mix) and 100µl of overnight grown culture was mixed in molten top agar maintained at 45ºC containing trace amounts of histidine and biotin, mixed and poured over minimal glucose agar plate enriched with histidine and Biotin. The plates were gently rotated to make a uniform spread and allowed to solidify. After some time the plates were inverted and incubated in the bacteriological incubator at 37ºC for 48 hours.
Culture inoculated at 6:30pm, culture flasks were removed at 8:30 am, after overnight incubation of 14 hrs the optical density was read at 660nm.

2.5.4.2 Pre incubation assay

With few exceptions it is believed that this assay is more sensitive than the plate incorporation assay, because short-lived mutagenic metabolites may have a better chance reacting with the tester strains in the small volume of preincubation mixture, and the effective concentration of S9 mix in the preincubation volume is higher than the plate.

A 100µl test substance or solvent (DMSO), 250µl of S9 mix or Phosphate buffered saline (in case of without S9 mix) and 100µl of overnight grown culture was mixed in molten top agar maintained at 45ºC containing trace amounts of histidine and biotin, mixed and poured over minimal glucose agar plate enriched with histidine and biotin. The plates were gently rotated to make a uniform spread and allowed to solidify. After some time the plates were inverted and incubated in the bacteriological incubator at 37ºC for 48 hours.
Culture inoculated at 6:00 pm, culture flasks were removed at 8:30 am, after overnight incubation of 14:30 hrs the optical density was read at 660nm.

2.5.5 Positive Controls

The positive controls used both in plate incorporation and pre incubation assay were as follows:

<table>
<tr><th>Sr. No</th><th>Positive control</th><th>Solvent</th><th>Strain</th><th>Con. µl/plate</th><th>Metabolic activation</th></tr>
<tr><td>1</td><td rowspan="2">Sodium Azide</td><td rowspan="3">Distilled water</td><td>TA 1535</td><td>0.5</td><td rowspan="5">Without metabolic activation</td></tr>
<tr><td>2</td><td>TA 100</td><td>5.0</td></tr>
<tr><td>3</td><td>Mitomycin-C</td><td>TA 102</td><td>0.5</td></tr>
<tr><td>4</td><td>2-Nitrofluorine</td><td rowspan="4">DMSO</td><td>TA 98</td><td>5.0</td></tr>
<tr><td>5</td><td>9-Aminoacridine</td><td>TA 1537</td><td>50</td></tr>
<tr><td>6</td><td>2-Aminoflourine</td><td>TA 98
TA 100,
TA 102</td><td>20</td><td rowspan="2">With metabolic activation</td></tr>
<tr><td>7</td><td>2-Aminoanthracene</td><td>TA 1537,
TA 1535</td><td>5</td></tr>
</table>

2.5.6 Observation

The plates were observed for back ground lawn inhibition and enumeration of revertant colonies.

2.6 Dose selection (toxicity determination)

Dose Selection tests included solubility, precipitation and Cytotoxicity tests which were as follows.

2.6.1 Solubility test

As Endosulfan (35% EC) is hydrophobic in nature, DMSO was selected as a suitable organic solvent. Endosulfan gives pinkish white precipitates and gets dissolved by further dilution with culture media. Endosulfan (500µl) was first diluted with DMSO (500µl) after that it was diluted serially with the culture media. In case of EC emulsifiable concentrate, distilled water can also be used but DMSO gives more suitable solution.

Dose Selection (Cytotoxicity) tests were carried out for determination of maximum applicable doses used in various experimental protocols. This test indicates toxicity to the particular test system and justifies the dose selection procedures. Different types of cytotoxic parameters were performed for conducting their final study protocols.

2.6.2 Precipitation test

Ames precipitation tests were carried out at the 5.0µl/plate by adding the test dilution to the molten top agar and poured onto the plate. A precipitation interfering in counting of the colonies was not observed.

2.6.3 Cytotoxicity (Lawn Inhibition/diminution test)

Cytotoxicity was assayed qualitatively by testing background lawn inhibition observed under the microscope with 10X and 40X magnifications.

Determination of toxicity in the Ames *Salmonella* Test requires the evaluation of characteristics of the final population on the minimum glucose agar plate after the 48 hour incubation instead of a quantitative survival determination. These characteristics are as follows:

- Thinning of the background lawn, accompanied by a decrease in the number of revertant colonies.
- Absence of background lawn (i.e. complete absence of growth)
- Presence of pinpoint non-revertant colonies (generally in conjunction with the absence of background lawn).

For determination of toxicity background lawn inhibition or reduction in number of revertant colonies are considered in case of Ames cytotoxicity test. This parameter is useful for determination of doses for Ames reverse mutation test. A decrease (diminution) in background lawn indicates toxicity at that concentration. In the present investigation lawn inhibition test was performed in strains TA 98 and TA 100 (Refer Table: 1 chapter 3)

The experimental procedures were same as the main study. Toxicity determination is an important factor for selecting doses for the main study.

2.7 Criteria for data acceptance

The following criteria for data acceptance (David *et. al.*, 1980) were followed in the assay:

1. Each chemical should be tested at the maximum concentration compatible with the test procedure. The compound should be tested up to a clearly apparent toxic level or 5mg/plate or ml.
2. Occasionally the compounds have extremely low levels of solubility. If solubility is found, toxic levels of the compound should be screened for, even if it exceeds obvious limits of solubility. Care should be exercised in scoring plates with considerable precipitate, especially if colony discrimination is reduced.
3. Appropriate positive and solvent control should be conducted with each assay. When possible a positive control agent should be matched with the test agent by chemical class. A range of negative control mutant colony counts or mutant frequencies appropriate for the tester strain should be defined and not exceeded.
4. Although not always necessary or relevant, a metabolic activation system, such as an S9 microsome mix (Ames *et. al*., 1975) is recommended.

2.8 Analysis of Results and Criteria for Evaluation

2.8.1 Statistical analysis

Several approaches were used in statistical analysis purpose as per the quality of the data is concerned to increase the sensitivity of the data obtained.

In case of Ames test revertant frequency (frequency of reverse mutation) is demonstrated as revertant frequency/plate, the data generated was in duplicate plates therefore the analysis was not analyzed by ANOVA followed by Dunnet's multiple comparison test. The data was analyzed by linear trend test, where a relationship between mean revertant frequency/plate v/s concentrations was analyzed (Maron and Ames, 1983). The stronger the relationship with increasing concentration was considered positive.

2.8.2 Biological (Analysis) Evaluation

Further the data was analyzed qualitatively by fold increase. Relative fold value (increase/decrease) was calculated by dividing mean revertant data of treatment v/s control were calculated.

The formula is as follows:

$$\text{Relative Fold Value} = \frac{\text{Mean counts in treatment}}{\text{Mean counts in vehicle control}}$$

2.8.2.1 For the strain TA 98, TA 100 and TA 102

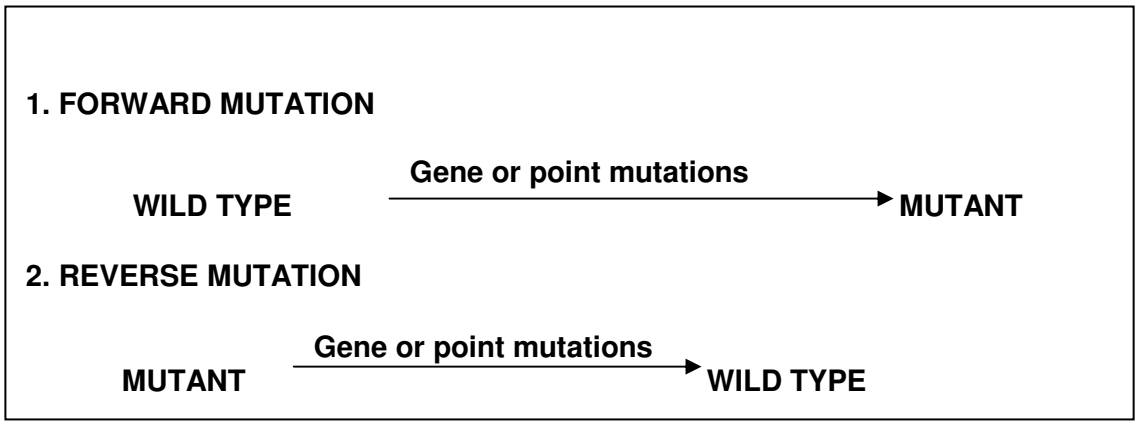
The test article can be considered positive if it produces at least 2- fold increase in the mean revertants/plate of at least one of these tester strains over the mean revertants/plate of the appropriate vehicle control.

2.8.2.2 For the strain TA 1535 and TA 1537

For a test article to be considered positive it should produce at least 3-fold increase in the mean revertants/plate of at least in one of these strains over the mean revertants/plate of the appropriate vehicle control.

METHODS FOR GENOTOXIC EFFECTS

3. Forward Mutation Assay



Gene mutation assays are basically of two types - one type where a mutation in the gene locus changes its phenotype form normal to mutant this is known as forward mutation assay (eg. CHO-HGPRT Assay) and another type in which mutation is being created in the genome to asses certain specific point mutations. These mutants revert to normal phenotype when there is any mutation generated in that particular locus because of treatment (eg. Ames test).

3.1 CELL- GENE MUTATION TEST (CHO- HGPRT ASSAY)

The Chinese Hamster Ovary cell/Hypoxanthine-guanine Phosphoribosil Transferase (CHO/HGPRT) assay has been widely applied to the toxicological evaluation of industrial and environmental chemicals.

CHO/ HGPRT assay detects forward mutations of the X-linked hypoxanthine – guanine phosphoribosil transferase (hgprt) locus (coding for the enzyme, HGPRT) in Chinese Hamster Ovary (CHO) cells.

CHO cells have been widely used in studies of somatic cell genetics, biochemistry, biophysics, molecular biology, physiology and cellular biology for over two decades. Recent genetic studies have been performed with either the CHO line, which contains 21chromosomes or its derivative, CHO-K1 line, which has 20 chromosomes (Kao and Puck, 1968). The cells are perhaps the best characterized mammalian cells genetically and are readily synchronized by various physical and chemical means. They exhibit a routine cloning efficiency of more than 80% in a reasonably defined medium on a glass or plastic substratum or in suspension, with a population doubling time of 12-14 hours (Hsie *et al*., 1975; Kao and Puck, 1968). The CHO-K1 BH_4 subclone, which has a relatively low spontaneous mutation frequency at the hypoxanthine–guanine phosphoribosil transferase (hgprt) locus, has been used extensively for studying mutagen-induced cytotoxicity and gene mutation (Hsie *et al*., 1975; Kao and Puck, 1968).The relatively stable and easily recognizable karyotype has made the CHO cell line and its derivative one of the best choices for studies on the effects of gene mutagens at the chromosome and chromatid level as well as studies of gene mutation and cytotoxicity. There are differences among the cell lines employed and a number of general characteristics are critical for performance of the assay.

- The cloning efficiency (CE) of the stock cultures should not be less than 70%. The cloning efficiency of untreated experimental cultures should not be less than 50%.
- Cultures in logarithmic phase of growth should have a population doubling time of 12-16 hours.
- The modal chromosome number should be 20 or 21, as is characteristic of particular subclone used.
- Cultures should be free of microbial and mycoplasma contamination.

These cell properties that are critical for the assay should be routinely monitored as part of the quality control regimen. As maintenance of proper number of cells in the culture flasks is necessary, cells must be counted before seeding to their respective treatment.

3.1.2 Cell counting from haemocytometer

Cells are counted manually from haemocytometer through following method:

- Clean the surface of the slide and coverslip with 70% isopropyl alcohol.
- Mix the cell sample thoroughly, pipetting vigorously to disperse any cell clumps and collect about 20µl (15µl is an ideal volume) into the tip of a Pasteur pipette or pipettor. Do not let the fluid rise in a pasture pipette or else cells will be lost in the upper part of the stem.
- Transfer the cell suspension immediately to the edge of the haemocytometer chamber, and let the suspension run out of the pipette and be drawn under the coverslip by capillary action. Don't overfill or under fill the chamber or else its dimensions may change, due to alterations in the surface tension; the fluid should run only to the edges to the grooves.
- Fill the second chamber.
- Blot off any surplus fluid (without drawing under the coverslip).
- Select a 10X objective, and focus on the grid lines in the chamber.
- The haemocytometer consists of nine 1 mm squares. One of the 1mm squares represent a volume of $0.1mm^3$ of 10^{-4} ml. Using the 10X objective, count the number of cells in a 1mm square area (WBC chambers)
- Count the cells that lie on the top and left hand-lines of each square, but not on the bottom or right-hand lines.
- For routine subculture, attempt to count between 100 and 300 cells/ mm^2; the more the cells that are counted the more accurate the count becomes. For more precise quantitative experiments, 500-1000 cells in all squares should be counted.
  (a) If there are very few cells (<100/mm^2), count one or more additional squares (each $1mm^2$) surrounding the central square.
  (b) If there are too many cells (>1000/mm^2) count only five small squares (each bounded by three parallel lines) across the diagonal of the larger ($1mm^2$) square.

- To calculate the concentration of the cells, first calculate the average of all $1mm^2$ areas counted and apply this formula:

$$C = n/v$$

Where C = cell concentration in cells/ml
n = average number of cells
v = Volume counted

Thus, $C = n \times 10^4$ or calculate the number of cells in four WBC chambers and multiply with 2500. This will give number of cells/ml.

3.2 Solubility

Determine solubility of the test article by dissolving it in a proper solvent before treatment. Commonly used solvents in order of preference are medium, water, phosphate buffer for water soluble chemicals and dimethyl sulfoxide, ethanol and acetone for chemicals which are not soluble in distilled water. Generally for nonaqueous solvent, concentrations should not exceed 1% and should be constant for all samples.

3.3 Precipitation and pH test

Media bovine serum was replaced with plain medium (without foetal pH 7.3) final pH was measured from the supernatant taken out during the removal with treatment medium. Precipitation was not observed by naked eye but very fine globules were found in medium when observed under inverted microscope, which was slightly higher in the high dose group. The pH of media were as follows:

Treatment group	Without metabolic activation	With metabolic activation
NC	7.38	7.36
DMSO	7.39	7.35
$0.175\alpha_1$	7.27	7.25
$0.35\ \alpha_2$	7.20	7.22
$0.525\alpha_3$	7.21	7.19
PC	6.9	7.0

3.4 Mutagenesis assay procedures

The mutagenesis protocol can be divided into 3 phases: mutagen treatment, expression and selection.

3.4.1 Mutagen treatment and experimental procedure

3.4.1.1 Culture preparation

A fresh vial of CHO cells was removed from liquid nitrogen and kept in water bath at room temperature (37°C). Inoculate the cells in a test tube and wash by pipetting with complete medium with 5% serum twice by centrifuging the cells at 1000 rpm for 10 minutes. Remove the supernatant and suspend the pellet with media containing serum. The cells were finally inoculated in the $25cm^2$ culture flasks in complete medium (α- MEM with nucleosides 080) with 20% serum. The cells were subcultured twice at the intervals of three days. During third subculture the cells were treated with HAT medium (10ml HAT dissolved in 490ml of α- MEM without nucleosides 081) containing 5% serum for 48 hours.

3.4.1.2 Mechanism of HAT treatment

HAT Medium (Hypoxanthine Aminopterin Thymidine medium) is a selection medium for mammalian cell culture, which relies on the combination of the aminopterin, a drug that acts as a folate metabolism inhibitor by inhibiting dihydrofolate reductase, with hypoxanthine and thymidine, which are intermediates in DNA synthesis. The trick is that Aminopterin blocks DNA "*de novo*" synthesis, which is absolutely required for cell division to proceed, but the other components provide cells with the raw material to evade the blockage (the "salvage

pathway"), provided that they have the right enzymes, which means having functional copies of the genes that encode them. The enzyme dihydrofolate reductase which produces tetrahydrofolate (THF) by the reduction of dihydrofolate is specifically blocked by aminopterin. THF acting in association with specific proteins, can receive single carbon units that are then transferred to specific targets.

One of the important targets for cellular reproduction is thydimine synthase enzyme that creates thymidine monophosphate (TMP) from deoxyuridine monophosphate (dUMP). By additional phosphorylation reactions TMP can be used to make thymidine triphosphate (TTP), one of the four nucleuotide precursors that are used by DNA polymerases to create DNA. Without the THF required to convert dUMP, there can be no TTP, and DNA synthesis cannot proceed further unless TMP can be produced from another source. The alternative source is that thymidine present in HAT medium can be absorbed by the cells and phosphorylated by thymidine kinase (TK) into TMP.

The synthesis of IMP, (precursor to GMP and GTP) also requires THF, and also can be bypassed. In this case hypoxanthine-guanine phosphoribosyltransferase (HGPRT) enzyme reacts with hypoxanthine absorbed from the medium with PRPP, liberating pyrophosphate, to produce IMP by a salvage pathway.

Thus the use of HAT medium for cell culture is a form of artificial selection for cells containing working TK and HGPRT. Thus a cell lacking HGPRT is resistant to 6-Thioguanine (6-TG) and azaguanine. Thus with one of the two drugs, followed by HAT medium, will yield revertant colonies.

3.4.1.3 Cell plating (Day 0)

The day of plating is considered as "day- 0". Cells should be in exponential phase when plated for treatment. Several media (e.g.F12, α-MEM) that are known to be optimum for cell growth can be used. Cells should be seeded at appropriate cell density to allow exponential growth as well as quantitation of induced responses. A common practice is to plate 0.5×10^6 cells in a $25cm^2$ flask a day before treatment.

3.4.1.4 Treatment (Day 1)

Treatment can be performed both in the presence and absence of exogenous metabolic activation (S9). For both the treatments the cells were washed with Phosphate buffered saline and appropriate amount of media was added. In case of with metabolic activation serum was not added to the complete media while in case of without S9 media containing 5% serum was added. The test substance was added in their required volume. The day of treatment is considered as "day 1".Following test dilutions were made

500µl (Endosulfan 35% EC) + 500µl (DMSO) = 175mg/ml (a)
100µl (a) +900µl (media) =17.5mg/ml (b)
100µl (b) +900µl (media) = 1.75mg/ml (c)
100µl (c) +900µl (media) = 0.17 mg/ml (d)
100µl (d) +900µl (media) = 0.0175mg/ml (e)
i.e.17.5µg/ml

1) 50µl was added to 4.95ml medium with 5% serum
[C] = 0.175µg/ml α_1
2) 100µl was added to 4.90ml medium with 5% serum
[C] = 0.35 µg/ml α_2
3) 150µl was added to 4.85ml medium with 5% serum

For without metabolic activation 10µl of Ethyl Methane Sulphonate (EMS) was dissolved to 990µl of plain media and from this stock, 50µl was added to the culture flasks with 4.95ml media.

For with metabolic activation 12mg of Benzo (a) pyrene [B (a) P] was dissolved to 12 ml of DMSO, from this stock 30µl was added to the culture flasks with 4.97ml media. For negative control plain media were used for both with and without S9 mix. Flasks were prepared in duplicate for each treatment concentration. To metabolic activation (S9) flasks, media was replaced with complete media with nucleoside containing 5% serum after exposure period (5 hours) and incubated in humidified CO_2 (5%CO_2:95% air)incubator at 37±1℃. In case of without metabolic activation media was not replaced with complete media and was kept for incubation till the exposure period.

3.4.1.5 Plating for Survival Frequency (Day 2)

At appropriate exposure interval (i.e. after 24 hrs.) media were removed and the cells were washed with phosphate buffered saline and trypsinised. Cells were counted with haemocytometer and 200-400 cells were seeded in duplicate plates for survival frequency and 2-3×10^5 cells were inoculated to the flasks for determination of mutation frequency. Both flasks and petridishes were incubated in humidified CO_2 (5%CO_2:95% air) incubator at 37±1℃ for seven days.

3.4.1.6 Subculture of mutation frequency flasks

Flasks for mutation frequency were subcultured at regular intervals (every alternate day) to maintain proper cell population and to avoid cell-cell communication.

3.4.1.7 Selection for mutation Frequency

Cells after three to four subcultures were selected for mutation frequency. The cells were trypsinised and counted with haemocytometer and cell concentrations were adjusted by serial dilution. Around 1-2×10^5 cells were seeded (inoculated) to 90mm Petri dishes for determination of mutation frequency in duplicates for each concentration. Twenty ml media without nucleoside (α-MEM) containing 6-Thiogunine (6-TG) as selective agent (refer section of media and reagent preparation) pipetted over the dispensed cells for cell dispersion.

Similarly cells 200-400 cells were inoculated (seeded) in duplicate plates of 60mm diameter for determination of clonable cells at the time of selection. In this case the cells were flooded with 5.0ml of complete media without nucleoside and without selective agent (refer section of media and reagent preparation). Both the Petri dishes (for mutation frequency and clonable cells) were incubated in humidified CO_2 (5%CO_2:95% air) incubator at 37±1℃ for 9-10 days.

3.4.2 Observation of cells for mutation frequency

On 10^{th} day the Petri plates for both mutation frequency and clonable cells were removed from incubator, media was removed, and fixed with 3.4% formalin in Phosphate buffered Saline for 10 minutes. The plates were inverted, dried and stained with 0.4% methylene blue solution for 10 minutes. After staining the plates were rinsed with distilled water and again dried by inverting it on tissue paper. The colonies were observed and counted by using labomed zoomer microscope.

3.4.2.1 Observation criteria

A clonal population showing more than 50 cells was considered as a colony and counted manually by spotting with marker pen from backside of the Petri dish. The same criteria were followed both for counting colonies for viability assay and mutant frequency.

3.4.2.2 Data Calculation and Analysis

To determine mutation frequency following things should be determined.

(1) Absolute Cloning Efficiency (ACE)

$$ACE = \frac{\text{Number of colonies formed}}{\text{Number of cells plated}}$$

(2) Relative Cloning Efficiency (RCE)

$$RCE = \frac{\text{Absolute Cloning Efficiency of Treatment}}{\text{Absolute Cloning Efficiency of Treatment}}$$

(3) Mutation Frequency (MF)

$$MF = \frac{\text{Number of Mutant Colonies}}{\text{Number of Mutant Colonies}}$$

(4) Number of clonable cells = Number of cells plated at selection× ACE at selection

3.5 DATA ANALYSIS

3.5.1 Statistical Analysis

Due to possibility of fluctuation, only samples with fewer than 10^5 viable cells after treatment should be used with caution for data analysis. Judgment on mutagenicity should be made based on the following:

- Dose response relationship.
- Significance of response (in comparison to the negative control).
- Reproducibility of the results.

Exact Statistical analysis is difficult because the distribution of the number of mutant colonies depends on the complex process of growth and death after mutagen treatment. While other appropriate methods can be used, two commonly used appropriate methods are as follows:

- A weighted regression analysis where the weights are proportional to the observed number of mutant colonies divided by the square of the observed mutant frequency (Hsie *et. al.,* 1975). This weighing scheme was derived by assuming that the variance of the observed mutant frequency is a constant multiple of that which would occur if the number of mutant colonies on each selection plate has a Poisson distribution. A test compound is considered to exhibit a mutagenic response if the slope of the mutant induction as a function of test concentrations is greater than zero at the 0.01 level according to the *t*-test (Tan and Hsie, 1981).
- A power transformation procedure with which the observed mutant frequency is transformed using the formula

$$Y = (X+A)^B$$

where Y =transformed mutant frequency; X = Observed mutant frequency, and A, B =constants

Data transformed by this method appears to satisfy the assumptions of homogenous variance and normal distribution (Snee and Irr, 1981). Comparison to negative control values

and dose response relationships are examined with Student's *t*-test and an analysis of variance (ANOVA) using the transformed values.

3.5.2 Biological Analysis

Data can be analysed biologically by two fold rule where a two fold increase in mutant frequency is observed from the background mutation frequency (Spinner *et al*., 1994).
Data were not analysed by the above methods as there were no obvious difference between vehicle control and treatment groups.

3.6 FORWARD MUTATION (CHO/HGPRT) ASSAY – MAIN STUDY

3.6.1 Experiment performed

To determine the mutagenic property of Endosulfan 35% EC mitogenic concentration of Endosulfan was selected as the dose for mutagenicity experiment.

2.6.2 Culture preparation

The cells were subcultured twice at the intervals of three days. During third subculture the cells were treated with HAT medium (10 ml HAT dissolved in 490 ml of α- MEM without nucleuosides 081) containing 5% serum for 48 hours.

3.6.3 Plantation of culture (day 0) without metabolic activation

After 48 hours of HAT exposure the cultures were trypsinised, subcultured and cells were counted in haemocytometer. A concentration of 3.6×10^5 cells per flasks, were adjusted. Haemocytometer counts were194 and 196.

Mean = 195
Count = 195×2500
= 487500cells

A total of 6 flasks were prepared (NC-1, VC-1, α_1-1, α_2-1, α_3-1, PC-1).

3.6.4 Treatment (day 1)

The day of treatment is considered as day "1". Following test dilutions were made

500µl (Endosulfan 35% EC) +500µl(DMSO) = 175mg/ml (a)
100µl(a) +900µl (media) =17.5mg/ml (b)
100µl(b) +900µl (media) =1.75mg/ml (c)
100µl(c) +900µl (media) =0.175mg/ml (d)
100µl(d) +900µl (media) =0.0175mg/ml (e)

i.e.17.5µg/ml
1) 50µl was added to 4.95ml medium with 5% serum
[C] = 0.175 µg/ml $\boldsymbol{\alpha_1}$
2) 100µl was added to 4.90ml medium with 5% serum
[C] = 0.35 µg/ml $\boldsymbol{\alpha_2}$
3) 150µl was added to 4.85ml medium with 5% serum
[C] = 0.525 µg/ml $\boldsymbol{\alpha_3}$

[A] Culture plating for survival frequency
Without metabolic activation before selection (day 2)

Cultures were washed with DPBS and trypsinised and prepared in α-MEM with nucleotides containing 10% serum.
Plates were prepared in duplicate as follows:

Negative Control (-S9)
Haemocytometer counts were 79 and 92 cells/chamber =85.5x2500
=21, 3750 cells/ml
A 1000µl aliquot was diluted with 1.137ml of culture media (i.e.100, 000 cells/ml)
0.5ml cells+ 4.5ml media i.e. 10,000 cells/ml
0.5ml cells+ 4.5ml media i.e. 1000cells/ml
A 200µl aliquot was added to 60mm Petri dishes containing 5.0ml of culture medium i.e. 200 cells/dish were seeded for survival frequency and for mutation frequency 1.0ml of aliquot was added to culture flasks.

Vehicle Control (-S9)
Haemocytometer counts were 64 and 59 cells/chamber =61.5×2500
=15, 3750 cells/ml
A 1000µl aliquot was diluted with 0.537ml of culture media i.e. 100,032 cells/ml
0.5ml cells+ 4.5ml media i.e. 10,003.2 cells/ml
0.5ml cells+ 4.5ml media i.e. 1000.3 cells/ml
A 200µl aliquot was added to 60mm Petri dishes containing 5.0ml of culture medium i.e. 200 cells/dish were seeded for survival frequency and for mutation frequency 1.4ml of aliquot was added to culture flasks (i.e. 215250 cells/ml).

Treatment group (-S9) α_1
Haemocytometer counts were 76and 72 cells/chamber =74x2500
=18, 5000cells/ml
A 1000µl aliquot was diluted with 0.85ml of culture media i.e. 100,000 cells/ml
0.5ml cells+ 4.5ml media i.e. 10,000 cells/ml
0.5ml cells+ 4.5ml media i.e. 1000 cells/ml
A 200µl aliquot was added to 60mm Petri dishes containing 5.0ml of culture medium i.e. 200 cells/dish were seeded for survival frequency and for mutation frequency 1.5ml of aliquot was added to culture flasks.

Treatment group (-S9) α_2
Haemocytometer counts were 46 and 44 cells/chamber = 45x2500
=112500 cells/ml
A 2000µl aliquot was diluted with 0.250ml of culture media i.e. 100,000 cells/ml
0.5ml cells+ 4.5ml media i.e. 10,000 cells/ml
0.5ml cells+ 4.5ml media i.e. 1000 cells/ml
A 300µl aliquot was added to 60mm Petri dishes containing5.0ml of culture medium i.e. 300 cells/dish were seeded for survival frequency and for mutation frequency 2.0ml of aliquot was added to culture flasks.

Treatment group (-S9) α_3
Haemocytometer counts were 66, 97, 68 and 86 cells/chamber =79.25x2500
= 198125 cells/ml
= 396250 cells/ml of culture.
A 2000µl aliquot was diluted with 1.962ml of culture media i.e. 100,012 cells/ml

0.5ml cells+ 4.5ml media i.e. 10,001cells/ml
0.5 ml cells+ 4.5ml media i.e. 1000 cells/ml

A 400µl aliquot was added to 60mm Petri dishes containing 5.0ml of culture medium i.e. 400 cells/dish were seeded for survival frequency and for mutation frequency 1.5ml of aliquot was added to culture flasks.

Positive Control (-S9)

Haemocytometer counts were 47, 41, 45 and 47 cells/chamber =45x2500
=112500 cells/ml

A 1000µl aliquot was diluted with 0.125ml of culture media
i.e. 100,000 cells/ml
0.5ml cells+ 4.5ml media i.e. 10,000 cells/ml
0.5ml cells+ 4.5ml media i.e. 1000 cells/ml

A 200µl aliquot was added to 60mm Petri dishes containing 5.0ml of culture medium i.e. 200 cells/dish were seeded for survival frequency and for mutation frequency 2.0ml of aliquot was added to culture flasks.

3.6.5 Plantation of culture (day 0) with metabolic activation before selection

Subcultured CHO-cell cultures were trypsinised, subcultured and cells were counted in haemocytometer. A concentration of 3.6×10^5 cells per flasks, were adjusted. Haemocytometer counts obtained were 117 and 118.

Mean =117.5
Count=117.5×2500, 1.4ml was added
=411250 cells

A total of 6 flasks were prepared (NC-1, VC-1, α_{1}-1, α_{2}-1, α_{3}-1, PC-1)

Treatment (day 1) 26.08.04

The day of treatment is considered as day 1. Following test dilutions were made

500µl (Endosulfan 35% EC) + 500µl(DMSO) =175mg/ml (a)
100µl(a) +900µl (media) =17.5mg/ml (b)
100µl(b) +900µl (media) =1.75mg/ml (c)
100µl(c) +900µl (media) =0.175mg/ml (d)
100 µl(d) +900µl (media) =0.0175mg/ml (e)
i.e.17.5µg/ml
Cultures were washed with DPBS prior to treatment

1) 50 µl was added to 4.95 ml medium without serum
[C] = 0.175µg/ml $\boldsymbol{\alpha_1}$
2) 100 µl was added to 4.90 ml medium without serum
[C] = 0.35µg/ml $\boldsymbol{\alpha_2}$
3) 150 µl was added to 4.85 ml medium without serum
[C] = 0.525µg/ml $\boldsymbol{\alpha_3}$

After 5.0 hrs of treatment the cultures were again washed with DPBS and fresh media containing 10% serum were added to all the flasks and kept back for incubation.

[B] Culture plating for cell survival frequency
(day 1) with metabolic activation before selection

Cultures were washed with DPBS and trypsinised and prepared in α-MEM with nucleuotides containing 10% serum.

Plates were prepared in duplicate as follows

Negative Control (+S9)

Haemocytometer counts were 68 and 59 cells/chamber = 63.5×2500
=158750 cells/ml

A 1000 µl aliquot was diluted with 0.587ml of culture media
i.e. 100,031 cells/ml
0.5ml cells+ 4.5ml media i.e. 10,003.1cells/ml
0.5ml cells+ 4.5ml media i.e. 1000.3cells/ml

A 200µl aliquot was added to 60 mm Petri dishes containing 5.0ml of culture medium i.e. 200cells /dish were seeded for survival frequency and for mutation frequency 1.4ml of aliquot was added to culture flasks (222250cells/ml).

Vehicle Control (+S9

Haemocytometer counts were 81and 94 cells/chamber =87.5x2500
=218750cells/ml

A 1000 µl aliquot was diluted with 1.187ml of culture media
i.e. 100,022 cells/ml
0.5ml cells+ 4.5ml media i.e. 10,002cells/ml
0.5ml cells+ 4.5ml media i.e. 1000cells/ml

A 200µl aliquot was added to 60mm Petri dishes containing5.0ml of culture medium i.e. 200cells /dish were seeded for survival frequency and for mutation frequency 1.0ml of aliquot was added to culture flasks (i.e. 218750cells/ml).

Treatment group (+S9) α_1

Haemocytometer counts were 70and 88 cells/chamber = 79×2500
=197500cells/ml

A 1000µl aliquot was diluted with 0.9750ml of culture media i.e. 100,000 cells/ml
0.5ml cells+ 4.5ml media i.e. 10,000cells/ml
0.5ml cells+ 4.5ml media i.e. 1000cells/ml

A 200µl aliquoi was added to 60 mm petridishes containing5.0ml of culture medium i.e. 200cells /dish were seeded for survival frequency and for mutation frequency 1.2ml of aliquot was added to culture flasks.

Treatment group (+S9) α_2

Haemocytometer counts were 95,106,112 and 79 cells/chamber =98×2500
=245000cells/ml

A 1000 µl aliquot was diluted with 1.450 ml of culture media i.e. 100,000 cells/ml
0.5ml cells+ 4.5ml media i.e. 10,000cells/ml
0.5ml cells+ 4.5ml media i.e. 1000cells/ml

A 300µl aliquot was added to 60mm petridishes containing5.0ml of culture medium i.e. 300cells /dish were seeded for survival frequency and for mutation frequency 0.8ml of aliquot was added to culture flasks.

Treatment group (+S9) α_3

Haemocytometer counts were 307and 310cells/chamber =308.5×2500
=771250cells/ml

A 1.0 ml cells were diluted with 6.712 cells were recounted in haemocytometer and 49 and 45 cells/chamber were observed = 47×2500

=117500cells/ml

A 1000µl aliquot was diluted with 0.175ml of culture media

i.e. 100,000 cells/ml

0.5ml cells+ 4.5ml media i.e. 10,000cells/ml

0.5ml cells+ 4.5ml media i.e. 1000cells/ml

A 200µlaliquot was added to 60mm petridishes containing5.0ml of culture medium i.e. 200cells /dish were seeded for survival frequency and for mutation frequency 2.0ml of aliquot was added to culture flasks.

Positive Control (+S9)

Haemocytometer counts were 65,45,36 and 37 cells/chamber =45.75×2500

=1114375cells/ml

A 1000 µl aliquot was diluted with 0.143ml of culture media i.e. 100,065 cells / ml

0.5ml cells+ 4.5ml media i.e. 10,006.5cells/ml

0.5ml cells+ 4.5ml media i.e. 1000.6cells/ml

A 200µl aliquot was added to 60mm petridishes containing 5.0mlof culture medium i.e. 200cells /dish were seeded for survival frequency and for mutation frequency 2.0ml of aliquot was added to culture flasks.

Metabolic Activation (S9 treatment)

Preparation of S9 mix

S9 mix was prepared as per the protocol of 'O' Neal,

Following components were added

GLU-6-PO_4	-5mM
Sodium phosphate	-50mM
NADP	-4.0mM
KCl	-30mM
MgCl	-10mM

The above components were prepared and added to 10 mM $CaCl_2$ solution, a white precipitation was observed, and were filtered through 0.22µm filter. S9 treatment was given early in the morning 7.30 am after washing with PO_4 buffer, 1.0ml of S9 was added to 9.0ml of cofactor mixture and final volume was made up to 50ml by adding 40ml of culture media without serum. A 50µl of 0.6mg/ml of B (a) P was added to the positive control flask. Five hours after the treatment media was removed and replaced with complete medium. Five hours after this cell are trypsinised and subcultured.

3.6.6 Plating for Survival frequency and mutation frequency at selection (without metabolic activation)

Negative Control (-S9)

Haemocytometer counts were 104 and 124 cells/chamber =114×2500

=285000cells/ml

A 1000µl aliquot was diluted with 1.85ml of culture media i.e. 100,000 cells/ml

0.5ml cells+ 4.5ml media i.e. 10,000.0cells/ml

0.5ml cells+ 4.5ml media i.e. 1000.0cells/ml

A 200µl aliquot was added to 60mm Petri dishes containing 5.0ml of culture medium i.e. 200cells /dish were seeded for survival frequency and for mutation frequency 0.5ml of aliquot was added to culture flasks (142500cells/ml).

Vehicle Control (-S9)

Haemocytometer counts were 98and 84 cells/chamber =91x2500
=227500cells/ml
A 1000µl aliquot was diluted with 1.275ml of culture media i.e. 100,000 cells/ml
0.5ml cells+ 4.5ml media i.e. 10,000cells/ml
0.5ml cells+ 4.5ml media i.e. 1000cells/ml
A 200µl aliquot was added to 60mm Petri dishes containing 5.0ml of culture medium i.e. 200cells /dish were seeded for survival frequency and for mutation frequency 0.5ml aliquot was added to culture flasks (i.e. 113750cells/ml)

Treatment group (-S9) α_1
Haemocytometer counts were 102 and 98 cells/chamber =100×2500
=250000cells/ml
A 1000µl aliquot was diluted with 1.500ml of culture media i.e. 100,000 cells/ml
0.5ml cells+ 4.5ml media i.e. 10,000cells/ml
0.5ml cells+ 4.5ml media i.e. 1000cells/ml
A 200µl aliquot was added to 60mm Petri dishes containing5.0ml of culture medium i.e. 200cells /dish were seeded for survival frequency and for mutation frequency 0.5ml aliquot was added to culture flasks.

Treatment group (-S9) α_2
Haemocytometer counts were 139 and 156 cells/chamber =147.5×2500
=368750cells/ml
A 1000µl aliquot was diluted with 2.687ml of culture media i.e. 100,013 cells/ml
0.5ml cells+ 4.5ml media i.e. 10,001cells/ml
0.5ml cells+ 4.5ml media i.e. 1000cells/ml
A 200µl aliquot was added to 60mm Petri dishes containing 5.0ml of culture medium i.e. 200cells /dish were seeded for survival frequency and for mutation frequency 0.4ml aliquot was added to culture flasks.
Treatment group (-S9) α_3
Haemocytometer counts were 60 and 63 cells/chamber = 61.5×2500
=153750cells/ml
A 1.0 ml of above cell suspensions were diluted with .537ml of culture medium
i.e.100, 032 cells /ml of culture.
0.5ml cells+ 4.5ml media i.e. 10,003cells/ml
0.5ml cells+ 4.5ml media i.e. 1000cells/ml
A 400µl aliquot was added to 60mm Petri dishes containing 5.0ml of culture medium i.e. 400cells /dish were seeded for survival frequency and for mutation frequency 2.0ml aliquot was added to culture flasks.

Positive Control (-S9)
Haemocytometer counts were 27 and 25 cells/chamber =26×2500
= 65000cells/ml
0.5ml cells+ 2.5ml media i.e. 10,833cells/ml
0.5ml cells+ 4.5ml media i.e. 1008.3cells/ml
A 400µl aliquot was added to 60mm Petri dishes containing5.0ml of culture medium i.e. 403cells /dish were seeded for survival frequency and for mutation frequency 2.9ml of aliquot was added to culture flasks.

3.6.7 Plating for Survival frequency and mutation frequency at selection (with metabolic activation)

Negative Control (+S9)

Haemocytometer counts were 110 and 112 cells/chamber =111×2500
=27, 7500cells/ml

A 1000µl aliquot was diluted with 1.775ml of culture media i.e. 100,000 cells/ml
0.5ml cells+ 4.5ml media i.e. 10,000.0cells/ml
0.5ml cells+ 4.5ml media i.e. 1000.0cells/ml

A 200µl aliquot was added to 60mm Petri dishes containing 5.0ml of culture medium i.e. 200 cells /dish were seeded for survival frequency and for mutation frequency 1.0ml aliquot was of cell were added to culture flasks (27, 7500 cells/ml).

Vehicle Control (+S9)

Haemocytometer counts were 81 and 74 cells/chamber =77.5x2500
=19, 3750 cells/ml

A 1000µl aliquot was diluted with 0.937 ml of culture media i.e. 100,025 cells/ml
0.5ml cells+ 4.5ml media i.e. 10,002 cells/ml
0.5ml cells+ 4.5ml media i.e. 1000 cells/ml

A 200µl aliquot was added to 60mm Petri dishes containing 5.0ml of culture medium i.e. 200 cells /dish were seeded for survival frequency and for mutation frequency 1.0ml of aliquot was added to culture flasks (i.e. 193750cells/ml).

Treatment group (+S9) α_1

Haemocytometer counts were 151 and 140 cells/chamber =145.5×2500
=363750cells/ml

A 1000µl aliquot was diluted with 2.637ml of culture media i.e. 100,013 cells/ml
0.5ml cells+ 4.5ml media i.e. 10,001 cells/ml
0.5ml cells+ 4.5ml media i.e. 1000 cells/ml

A 200µl aliquot was added to 60mm Petri dishes containing 5.0ml of culture medium i.e. 200 cells /dish were seeded for survival frequency and for mutation frequency 0.5ml of aliquot was added to culture flasks = 181875.

Treatment group (+S9) α_2

Haemocytometer counts were 208 and 238 cells/chamber =223×2500
=557500cells/ml

A 1000µl aliquot was diluted with 4.0ml of culture media the culture was rediluted and counted 63 and 68 = 65.5 i.e. 163750 cells/ml

A 1000µl aliquot was diluted with 0.637ml of culture media = 100030
0.5ml cells+ 4.5ml media i.e. 10,003cells/ml
0.5ml cells+ 4.5ml media i.e. 1000cells/ml

A 200µl aliquot was added to 60mm Petri dishes containing 5.0ml of culture medium i.e. 200cells /dish were seeded for survival frequency and for mutation frequency 0.8ml of aliquot was added to culture flasks = 131000.

Treatment group (+S9) α_3

Haemocytometer counts were 44and 39 cells/chamber = 41.5×2500
=103750cells/ml
0.5ml cells+ 4.5ml media i.e. 10,375cells/ml
0.5ml cells+ 4.5ml media i.e. 1037cells/ml

A 200µl aliquot was added to 60mm Petri dishes containing 5.0ml of culture medium i.e. 207cells /dish were seeded for survival frequency and for mutation frequency 2.0ml of aliquot was added to culture flasks = 207500.

Positive Control (+S9)

Haemocytometer counts were 149 and 138 cells/chamber =143.5×2500
=358750cells/ml

A 1000µl aliquot was diluted with 2.587ml of culture media = 100013
0.5ml cells+ 2.5ml media i.e. 10,001cells/ml
0.5ml cells+ 4.5ml media i.e. 1000cells/ml

A 300µl aliquot was added to 60mm Petri dishes containing 5.0ml of culture medium i.e. 300cells /dish were seeded for survival frequency and for mutation frequency 0.5ml of aliquot was added to culture flasks = 179375.

CLASTOGENICITY TEST - CHROMOSOME ABERRATION TEST

4. CHROMOSOME ABERRATION TEST

This method includes gross chromosomal aberration changes in structure of the chromosomes directly observing under the microscope.

4.1 Significance of this Assay

The purpose of *in vitro* chromosomal aberration assay is to determine whether the test compound is a clastogen which introduces structural changes in chromosomes. This test belongs to a standard test battery for genotoxicity testing for pharmaceuticals recommended by the fourth international conference on Harmonization and other regulatory guidelines. The assay was performed as per the procedure described by Galloway *et al.,* (Galloway *et al*., 1994, 1985 and 1987) with some modifications.

The chromosomal aberration study was planned for conduct of dose selection and main study as the basic design described by various guidelines.

4.2 Test System

Chinese Hamster Ovary cell line (CHO cell line) was selected as a test system to analyze cytotoxic and cytogenetic damage, as number of chromosomes are less (2n=20-21) and the metaphases are clearly identified easily .The cell cycle time for CHO cells is about 12-14 hours (Latt *et.al.,* 1981, Preston *et.al.,* 1981, Dean and Danford *et.al.*, 1985) so a fast growing and dividing population of cells was obtained and exposed.

CHO cells were pretreated with HAT (Hypoxanthine, Aminopterin and Thyminidine) medium to select non-mutant cells (i.e. to avoid mutant cells) from the population a week prior to the treatment.

CHO-K1 cells are frequently used for *in vitro* chromosomal aberrations or micronucleus studies; it is having a mutant p53 sequence which lacks the G1 check point (Hu *et. al.,* 1999), therefore becomes more sensitive compared to normal peripheral blood lymphocyte cultures.

The CHO-K1 cell lines were obtained from NCCS, Pune (India) and maintained at Jai Research Foundation, Vapi (India).

4.3 DOSE SELECTION ASSAY FOR CHROMOSOMAL ABERRATION

This assay was performed to select a dose where Endosulfan is effective for any cellular or cytogenetic damage to the cells under exposure. In this assay cytotoxic effects of the compound were evaluated for conducting the final experiment. The performed experiments include solubility test, culture preparation, treatment, harvesting, slide preparation. The parameters chosen for selection of the doses for main study were solubility, precipitation, culture confluency and mitotic index.

4.3.1 Solubility Test

Solubility test was performed prior to treatment of the cells. Endosulfan (35% EC) is emulsifiable concentrate by nature, it is sparingly soluble in distilled water and gives precipitates with distilled water. In this case Dimethyl sulphoxide (DMSO) was selected as a suitable organic solvent. Endosulfan gives pinkish white precipitates and gets dissolved by further dilution with culture media. Endosulfan (500 µl) was first diluted with DMSO (500 µl) after that it was diluted serially with the culture media. In case of EC emulsifiable concentrate distilled water can also be used but DMSO gives more suitable solution.

4.3.2 Precipitation test

As described above turbidity and precipitations affecting conductivity of the assay were not observed with unaided eye. Only cloudiness was observed in the culture tubes when tested separately.

4.3.3 Experimental Procedure for Confluency and Mitotic Index

4.3.3.1 Seeding of Cells

Approximately 5×10^5 cells were inoculated (into a medium of α-MEM with 10% serum) into 14 petriplates (Φ =60 mm) a day before (24 hours) treatment with the test compound. The cell numbers were counted to the exponentially growing (80% confluent) cell population with the help of Neubauer's chamber (haemocytometer).

4.3.3.2 Test Conditions

The cells were washed with phosphate buffer to provide serum free normal medium.

The cells were treated with the test substance 24 hours after seeding. The test dilutions of Endosulfan used were 0.0445, 0.178, 0.712, 2.848, 11.387 and 45.557µg/ml of culture respectively. The test dilutions were prepared in the empty culture flasks or test tubes and diluted further by addition of culture media. Concurrent negative and vehicle controls were also maintained. All the culture flasks were treated in duplicate.

4.3.3.3 Treatment

Treatment was performed for short as well long term exposure periods. For short term the cells were treated with the test substance 24 hours after seeding. The test dilutions of Endosulfan used were 0.0445, 0.178, 0.712, 2.848, 11.387 and 45.557µg/ml of culture medium respectively. The test dilutions were prepared in sterilized test tubes and diluted further by addition of culture media. Concurrent positive and negative controls were also maintained.

a) **Composition of S9 mix**

Culture media	-	1.65ml
PO_4 buffer	-	2.50 ml
K^+- Mg^+ salt solution	-	0.10 ml

Glu-6 PO_4	-	0.05ml
β-NADP	-	0.20 ml
S9 fraction	-	0.50 ml

Complete media was used in the absence of S9 mix (250 µl) and also in negative control. Concurrent vehicle and negative controls were maintained, while vehicle control was treated with 1% DMSO.

b) Exposure period

Cells were exposed for short term duration only. By this the cells were exposed for a short time interval to expose a G_0 stage of a single cell cycle (12-18 hours).

A modification in the S9 preparation was performed as per the reference (J. Clements, 2000). The cells were exposed to above test dilutions for a short period of time (4.0 hours) with presence and absence of metabolic activation. Five ml of S9 fraction was dissolved in 45 ml of media. Therefore the concentration of S9 in media is 0.01% and S9 mix is 0.1%.

4.3.3.4 Culture Confluency Test

The exposed petridishes were observed under inverted microscope at 10X and 40X, magnification. Precipitation (presence of globules of endosulfan) was observed in 45.57 and 11.39µg/ml treated petridishes, while petridishes treated with 2.848 µg/ml of Endosulfan showed less number of globules of endosulfan both in the presence and absence of metabolic activation (S9 mix).

Eighteen hours after the treatment, petriplates were again examined under the inverted microscope. Petriplates treated at 45.57, 11.39 and 0.712 µg/ml exhibited complete sloughing off of cells. While some globules of Endosulfan were still found at 0.712 µg/ml treated culture plates, other lower dilutions also appeared normal in case of absence of metabolic activation (-S9 mix). In case of metabolic activation (+S9 mix) 47.57,11.39,2.848,0.712 and 0.178 µg/ml showed 100%, 100%, 20% and 2.0% dead cells approximately when observed under inverted microscope. Vehicle and negative control plates appeared normal.

4.3.3.5 Harvesting

A modification in the concentration of colchicine (decreased) and hours of exposure (increased) to get more and more metaphase cells and hence increase the sensitivity of the assay.

Eighteen hours after exposure all the cells were treated with colchicine at a concentration of 0.4µg/ml after morphological observations. Four hours after colchicine treatment the cells were washed with phosphate buffer and trypsinised. After trypsinisation the cells were collected in the centrifuge tubes and centrifuged at 1500 RPM for 10 minutes and treated with hypotonic KCl (0.56%) for about 40 minutes. The cells were fixed with 1:3 acetomethanol and two washes of fixatives were given, pellets were retained following removal of supernatant. The cells were refixed with acetomethanol, suspended and stored in refrigerator.

4.3.3.6 Slide Preparation

Twelve hours after storage, the cells were centrifuged at 1500 RPM for 10 minutes, supernatants were discarded by leaving 0.5ml of fixative with pellet. Three to four drops of cells were dropped on ice chilled slide held at an angle of 45º. Slides were air dried and stained with 5.0% Giemsa (prepared in Sorenson's buffer, pH-6.8).

The slides were mounted with dextran-plastisizer xylene (Depex or DPX) and examined using light microscopy.

4.3.4 Observations

Slides were observed for Mitotic Index to find toxicity.

a) Mitotic Index

"Mitotic index is a parameter which indicates dividing nature of the cells under exposed conditions". For calculation of mitotic index a population of minimum 1000 cells (including cells in division) were screened for the presence of cells in division (metaphases etc.) and calculated by following formula:

$$MI = \frac{\text{number of cells in division}}{\text{total number of cells}} X100$$

For mitotic index cells are observed under 40X magnification and cell are counted serially from lower to upper sides irrespective of presence and absence of metaphase cells.

b) Relative Mitotic Index (MI)

It is the calculated product from % MI of control subtracted by treatment value of vehicle control. This value gives relative change (difference i.e. increase or decrease) in MI.

4.3.5 Results of dose selection

4.3.5.1Vehicle

DMSO was selected as a suitable vehicle for the assay.

4.3.5.2 Dose

Based on the solubility, precipitation, reduction in mitotic index doses of 0.175, 0.35 and 0.525 µg/ml were selected for main study along with concurrent positive and negative controls.

4.4 MAIN STUDY CHROMOSOMAL ABERRATION

The main study for chromosomal aberration was performed as per the general procedure with little modifications.

4.4.1 SEEDING OF CELLS

Approximately 5×10^5 cells were seeded (into a medium of α-MEM with 10% serum) into 12 culture flasks (25 cm^2) each for with and without metabolic activation on day '0' (before 24 hours). This is preferable to allow time for the cells to leave the G_o stage before adding to the test agent, because of toxic concentrations of the test agent and to prevent the cell from entering the cell cycle. Thus addition of the test agent is done after 24-36 hours of culture seeding and the culture is more likely to be effective to detect aberrations.

4.4.2 Test Conditions

The test conditions used were same throughout the experiment. The incubator temperature was maintained at 37±1°C with 5% CO_2 in humidified air.

4.4.3 TREATMENT

Treatment was performed for short as well long-term exposure periods. The cells were treated with the test substance 24 hours after seeding. The cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS) medium two times to make serum free medium and treated with the test compound at the nominal concentration of 0.175, 0.35 and 0.525 µg/ml along with concurrent positive and negative controls. The positive controls used were Mitomycin-C [MMC (0.5 µg/ml)] for the absence of metabolic activation and cyclophosphmide [Cyp (25 µg/ml)] for the presence of metabolic activation.

4.4.3.1 Exposure Periods

The cells were exposed for various time intervals which are as follows:

A. Short Term Exposure

In this the cells were exposed for a short time (4.0 hour exposure) interval to expose a G_0 stage of a single cell cycle (12-18 hours) with and without metabolic activation and harvested before 18 hours along with concurrent positive (Cyclophosphamide - 25 µg/ml and Mitomycin-C - 0.5 µg/ml for with and without metabolic activation groups respectively) and negative controls.

A modification in the S9 preparation was performed as per the reference of J. Clements, 2000. The cells were exposed to above test dilutions for a short period of time (4.0 hours) with presence and absence of metabolic activation. Five ml of S9 fraction was dissolved in 45ml of media. Therefore the concentration of S9 in media is 0.01% and S9 mix is 0.1%. A modification in the concentration of colchicines (decreased) and hours of exposure (increased) to arrest more number of metaphase cells to be available for screening.

a. Composition of S9 mix

The composition of S9 mix (J. Clements, 2000) used was changed as follows:

Culture media	-	52.5.0 ml
150 mM KCl	-	01.5.0 ml
Glu-6 PO_4 (180mg/ml)	-	01.5.0 ml
β-NADP (25mg.ml)	-	01.5.0 ml
S9 fraction	-	03.0 ml

pH of the above mixture was adjusted with 1N NaOH and it is referred as the culture medium with 5% metabolic activation.

In case of without metabolic activation system 7.5 ml of 150 mM KCl was added.

Long Term Exposure (Trial-1)

In this study the cells were maintained in HAT medium (1.5 ml HAT in 60 ml of α-MEM medium without nucleotides with 10% serum) was used for more than 24 hours, to eliminate possible mutant heterozygote of CHO cells, during cell preparation, before treatment.

Cells were exposed for more than one cell cycle (1.5 cell cycle- 18 hours). This experiment was performed only in the absence of metabolic activation as S9 is toxic for long term exposures (Clements, 2000). This exposure is done with slight deviations incurrent

guidelines which asks only 24 hour exposure with test item but shows flexibility for design of exposure and sampling for recurrent exposures.

Cells were exposed for long term exposure period of 24 hrs followed by subculture and re-treatment at the interval of 24hrs. The culture was harvested on 3rd day (total 72 hour exposure) to study the accumulative effect on aberrations by repetitive treatments. This modification was made due to positive responses obtained in short term exposure period.

The cells were treated with 5% complete medium instead of 10% complete medium throughout the assay period. Therefore this assay can be named also as low serum repetitive assay.

Positive control (MMC -0.5µg/ml) was treated 18 hour before harvesting. This experiment was performed to assess the clastogenic induction potential of Endosulfan 35 EC at the lower dose level for an extended period of time of 7 days.The cells were treated with the Endosulfan 35 EC at a dose level of 0.175µg/ml on day –1 for 24 hours after seeding. On day three treatment media was removed the cells were trypsinized and again treated at 0.175µg/ml dose level as soon as the cells got settled after 3 hours of trypsinization.

The number of cells seeded = 309750/ petridish containing 5ml of culture media α-MEM with nucleotides.

Treatment concentrations used were 0.175, 0.350 and 0.525 µg/ml of culture medium. After exposure the cells were trypsinized (monolayer and supernatant were pooled and) were treated with colchicine at a concentration of 0.8µg/ml for 4.50 hrs. Cells were treated with hypotonic KCl for 30 minutes and fixed with acetomethanol. Slides were prepared after two washes with the acetomethanol fixative and the slides were prepared by hanging drop method. Slides were stained with 5% Giemsa prepared in Sorrensons buffer, pH-6.8.

Slides were observed under 40X and oil immersion (100X) for mitotic index and chromosomal aberrations respectively. Positive control treatment was performed 24 hour prior to harvesting with Ethyl methane sulphonate (EMS) at a concentration of 0.1µl/ml. Only two slides each with 50 metaphase spreads were screened as it has shown its positive response.

B. Long Term Exposure (Trial-2)

Cells were exposed for long term exposure period of 48 hrs followed by two consecutive subculture and re-treatment. The culture was harvested on 7th day to study the accumulative effect on aberrations in low dose only. The cells were treated with 5% complete medium instead of 10% complete medium. Positive control (MMC-0.5 µg/ml) was treated 18 hour before harvesting.

*Note: Both the trials of long term exposure periods are modification from the normal protocol of 24-hour exposure, in this the cells are sub-cultured as soon as they achieved confluency and retreated with the test substance with culture media containing 5% serum without metabolic activation after sub-culturing. This modification was designed to confirm

the observed positive response in a single dose level of the short term experiment for without metabolic activation.

4.4.4 Harvesting

a) Short Term Experiments

A modification in the concentration of colchicines (decreased) and hours of exposure (increased) to arrest more number of metaphase cells to be available for screening. Eighteen hours after exposure all the cells were treated with colchicine at a concentration of 0.4µg/ml, after 4.5h colchicine treatment the cells were washed with phosphate buffer and trypsinized. After trypsinization the cells (including supernatant) were collected in the centrifuge tubes and centrifuged at 1500 RPM for 10 minutes and treated with hypotonic KCl (0.56%) for about 40 minutes. The cells were fixed with 1:3 acetomethanol and two washes of fixatives were given, pellets were retained following removal of supernatant. The cells were refixed with acetomethanol suspended and stored in refrigerator.

b) Harvesting for Mid Term and Long Term Experiments

For mid term experiment the cells were harvested 24 hours after the last treatment, where colchicines (0.4µg/ml) treatment (4.5hrs) was followed by 0.56M KCl treatment for 30 minutes. Two washes of fixatives were given before dropping of the cells.

4.4.5 Slide Preparation

The slides cleaned with chromic acid were stored overnight in freezer in a solution containing methanol and distilled water in the ratio of 1:1. A few drops of the cell suspension was dropped on the slides from a distance and placed on a hot plate to dry. The slides were observed under microscope at low magnification for proper density and cell spreading by phase contrast setting.

4.4.6 Staining

Air dried slides were stained with 5% Giemsa stain for 3-5 minutes rinsed with distilled water and dried again. The slides were made permanent by mounting a cover slip with Dextran-plastisizer xylene (DPX).

4.4.7 Observation Criteria

4.4.7.1 Scoring Chromosomal Aberration

A minimum of 1000 cells were scored counted in different fields of slide per culture to determine the mitotic Index (MI). A minimum of 100 consecutive metaphases were scored under 100X oil immersion objective for structural abnormalities.

Aberrations were divided into chromatid type and chromosome type. The chromatid type aberration involves only one chromatid and the chromosome type aberration involves both chromatids at identical sites. Chromatid type aberrations like gaps, breaks, chromatid exchanges and chromosome type aberrations like chromosome gaps and breaks, rings, ploidy, dicentric etc. were scored.

4.4.7.2 Criteria of Scoring Aberrations

The criteria of scoring metaphases followed were as described by Savage (1981) with slight modifications which are as follows:

a) Chromosome type aberrations

1. Terminal and interstitial deletions

Terminal deletions were found at the distal end of the chromosomes leaving dotted chromatin and appeared single or double dotted. Similarly if these deletions were observed in the chromatid, these are referred to as interstitial deletions. Deletions were marked by presence of deleted segment near to chromatid or chromosome.

2. Gap

Chromosomal gap was defined as space between two arms of the chromatids lesser than the width of the chromosome. Similarly if the gap is present in the chromatid arm it was referred to as chromatid gap.

3. Break

Breakage of chromosome/chromatids generating space more than the widths of the chromosome/chromatids was defined as chromosome/chromatids break.

4. Fragments

The broken segments of chromatin were defined as fragment. Further a fragment may be chromatid or chromosomal. The paired fragments devoid of centromere were considered as -acentric fragments.

5. Minutes

The small interstitial deletions appearing as paired dots are classified as minutes.

6. Ring

The larger interstitial deletions in which there is a clear space in the centre of the ring are classified as acentric ring. If the centromere is present in the ring, it is known as ring chromosome. It is also defined as chromatid pair with or without centromere showing a clear space in the centre.

7. Dicentric

A metaphase showing two centromeres were classified as dicentric, the presence of three was as tricentric and multiple centromeres was as polycentric. The tricentric and polycentric were analysed as dicentric.

8. Fragment

Fragement of any chromosome showing both the chromatids but without centromeres were classified as acentric fragments.

9. Interchanges/ translocations

The orientation of the chromosome or twisting of the chromosomes in itself or generating fork like protrusion on the chromatid, giving appearance of a different chromosome not present in the normal chromosome type were considered as cases of inversion or translocation.

10. Exchanges

These were the defined as the exchange of chromatids between two or more number of chromosomes by formation of bridge between two different chromatids.All the above chromosomal and chromatid type of aberrations were analysed as percent aberrant cell and frequency for aberrant cell were obtained, an aberrant cell was defined as a cell showing one or more than one chromosome or chromatid aberration(s).

4.4.7.3 Numerical Aberrations

Any change in the number of chromosomes from 2n ± 2 (i.e. 20 ± 2) was considered as aneuploidy. The presence of aneuploids was given less importance as this may arise due to technical error during dropping of cell pellets. Polyploidy were scored when there is duplication of chromosomes in multiple of 2 and were recorded under polyploidy. Similarly existence of paired set of chromosomes in duplicate condition was defined as endoreduplication. All the above numerical aberrations were analyzed under ploidy. In present thesis a minimum of 1000 cells were analyzed for the presence of aneuploidy.

4.5 Data analysis

4.5.1 Statistical Analysis

Statisticians and toxicologists suggested various evaluation criteria of dose response analysis for chromosomal aberration. Margolin *et. al.,* (1986) suggested to use Cocharan-Armitage test. Sofuni *et. al.,* (1990) considered the dose response to be (strong) positive if it had two significant doses out of three dose groups and decided to be weakly positive if it had only one significant dose and there was a significant trend. The criteria of Galloway *et. al.* for a positive response was a clear dose related increase in the cell with structural aberrations in one experiment or a reproducible single positive dose (Galloway *et. al*., 1984).

Sofuni and Galloway didn't specify Statistical methods for their criteria. Judgement on clastogenicity can be made based on the following:

1. Dose response relationship (linear regression methods).
2. Significance of response (in comparison to the negative control).
3. Reproducibility of the results.

Data were analyzed statistically by ANOVA followed by Dunnets for mitotic indices, it could not be analyzed statistically for chromosomal aberrations as sample size was too small to analyze for ANOVA test.

METHODS FOR SYSTEMIC EFFECTS

In vivo Screening Methods

SUBCHRONIC (28 DAYS) TOXICITY TEST

As the exposure of Endosulfan is slow and long, a 28 days repetitive toxicity study was performed. This subchronic exposure type of study was performed in mice.

1. Husbandry Practices

Healthy young adult mice (*Swiss Albino*), 6-8 weeks of age, of both sexes, with nulliparous and nonpregnant females were obtained from animal breeding facility, Jai Research Foundation, Valvada-396 195, Gujarat, India, and were used for the experiment. The mice were randomly allocated to control and treatment groups of four by sex. Individual mice were identified with picric acid marking on the body coat and cage card showing experimental group, sex and mice numbers. The mice were housed in clean, sterilized, solid polypropylene cages. Clean rice (paddy) husk was used as bedding material. The cages were kept on a 5 tier rack. The mice were fed with Amrut Brand Laboratory rat pellet feed (manufactured by Pranav Agrochemicals Ltd., Pune – 411 030) and water (filtered through Aquaguard water filter system) was provided in poly propylene bottles. Both feed and water were provided *ad libitum* to all animals. Fresh feed and water was supplied on a daily basis.

2. Environmental Conditions

The animals were housed in polypropelene mice cages as 4 animals per sex per group. The mice were maintained in climatically controlled experimental room with a relative humidity range between 60 and 65%. The temperature of the experimental room was maintained between 22±3℃. The photoperiod of the experimental room was maintained manually as 12 h artificial light and 12 h darkness.

3. Acclimatization (Age, Body Weight and Sex)

Inbred *Swiss Albino* mice of 6-8 weeks of age (2.4.05, male; 2 & 4.4.05 female), males weighing about 30-40g and females about 25-35g were acclimatized for a period of 10 days. After acclimatization, the mice were divided into several groups for various treatments.

4. Number of animals

For dose selection study 16 (8 male and 8 female) animals and 32 animals were utilized for main study. Separate satellite groups of 4 male and female animals were maintained and treated during the 28 days treatment period.

5. Randomization

All the animals were weighed and allocated to individual groups of 4 animals of each sex in each group. The high dose group was supplemented with extra animals of each sex and also randomized parallel to the main study animals.

6. Dose Preparation

Endosulfan 35 EC was weighed of 40mg in a test tube dissolved with double distilled water the volume was made up to 5ml (concentration = 8mg/ml), one ml of this stock(A) was diluted with 9ml of distilled water(B). 3ml of stock (B) was again diluted with 3ml of distilled water. The animals were treated with a volume of 10ml/kg body weight/day for 28 consecutive days. All the dose dilution as were made afresh prior to treatment.

7. Dose Levels and Body Weight

Based on the results of range finding study, main study was conducted at 4.0 and 8.0mg/kg body weight/day for 28 consecutive days. Individual animal was weighed everyday before commencement of treatment and sacrifice.

8. Treatment and Route of Administration

The animals were treated with Endosulfan solutions made in sterile double distilled water and treated by oral intubations using canula as the most common form of exposure is its aqueous form. Hence, the oral route of exposure by gavage (most recommended route of oral dosing) is used in the present study. The extra animals (satellite animals) of each sex were also treated parallel to the high dose group animals.

9. Physical Examination

All the animals were examined for extrovert symptoms of toxicity before treatment once and after treatment for presence of clinical symptoms and ½, 1, 2 and 3 hours depending on severity of clinical symptoms after treatment. Symptoms observed like hyperactivity, tremor, abdominal breathing like symptoms was observed.

10. Treatment

10.1 Dose Selection
The doses tested were 10, 20, 40 and 100mg/kg body weight for range finding study.

10.2 Dose Preparation
The doses were prepared by weighing the test compound in a beaker sensitive to mg weights. Appropriate volume was made by using volumetric flasks of several capacities for different dilution ranges. All the three doses were weighed separately and volumes were adjusted accordingly.

10.3 Dosing
The animals were treated by oral tubing using mice canula. The animals were treated for control and treatment groups.

11. Sampling
After the respective treatments, haematological and biochemical analysis, histopathological evaluation and genotoxicological tests were performed.

11.1 Peripheral Blood Sampling
Peripheral blood was sampled by tail pinch through sterile needle and the blood sample was taken on the slide by touching the surface with drop of blood for peripheral blood micronucleus assay. This sampling was performed on 1st day, 1st week and 2nd week of exposure to Endosulfan before sacrificing the animals.

11.2 Blood collection
Blood was collected from orbital sinus using capillary tubes in prelabelled centrifuge tubes. A small volume of blood samples was processed immediately for RBC cholinesterase while the rest sample was allowed to clot and after that it was processed for centrifugation at 2000 rpm for serum separation to be used for analysis.

11.3 DLC Sampling
The first drop of the blood was collected on a frosted slide to a corner and a thin smear was prepared by dragging the sample behind and allowed to air dry and stained with Leishman's stain.

11.4 Sacrifice
The animals were sacrificed by CO_2 asphyxiation till the animal become morbid, soon the animals were dissected out by trained technician and subjected for observation.

11.5 Gross Observation
The gross pathological symptoms were observed immediately after sacrifice or after death of the animal at the earliest possible time.

11.6 Preparation of Plasma Samples
The plasma samples were carried out in glass vials precoated by sodium EDTA (dried overnight). The samples were hand rolled to avoid clotting and immediately processed for haematological analysis.

11.7 Collection of Epididymis

The Epididymis was collected in Dulbecco's buffer and skin was removed by making a small incision on it. The contents were chopped in to thin pieces and mixed thoroughly and processed for staining.

11.8 Staining Methods

Various staining methods were used for DLC, Peripheral Blood and Micronucleus assay and Staining for Sperm. These methods are described in respective sections.

11.9 Mounting of Slides

All the slides for DLC, peripheral Blood and Micronucleus assay were mounted after staining. All these slides were mounted by thin coverslip by using DPX after getting completely air dried. The DPX drop was dropped on to the slide and covered with coverslip.

11.10 Differential Leucocytes Counts and (Staining procedure)

- The smears were stained with Leishman's stain.
- Air dry the slide pour the stain dropwise till it cover's the whole slide.
- Keep the slide for 4-5 minutes , donot dry the slide if it dries add again stain it.
- After staining add filtered distilled water and cover the whole slide for 20 minutes.
- Rinse with distilled water wipe from the backside.

12. Observations

Various observations were made under microscope after mounting.

13. Dose Selection for Repeated Dose Toxicity Tests

Species : *Mus musculus*
Age : 6-8weeks (date of birth 2.04.05 for male and 2-4.04.05 for female)
Strain : *Swiss albino*
Body weight : Male 30-40g, Female 25-35g

13.1 Solubility and Dose Preparation

The test substance was dissolved in distilled water as it is an emulsifiable concentrate. The dilutions are as follows

Sr. No.	Weight of test substance (mg)	Volume made up to (X) ml	Dilution details	Concentration mg/ml
1	107	10	-	10.7
2	10.7	10 (A)	1ml(a)+9ml DW(B)	1.07
3	50	10(a)	1ml(a)+9ml DW(b)	0.5
4	-	-	1ml (b)+ 1ml DW	0.25

Note: Treatment volume 10ml/kg body weight.

13.2 Treatment

Animals were treated orally at 10ml per kg body weight on a single occasion. Four male and female animals were treated at 100 and 40mg/kg body weight. All the animals were died following exposure. Four and two animals were treated with 20 and 10mg/kg body weight respectively. Animals were given repeated doses of 20 and 10mg/kg body weight. Three animals out of four died after dosing while 1 animal died in the dose group of 10mg /kg body weight group.

14. MAIN STUDY

Based on the results of above dose selection assay doses 4 and 8mg/ml were selected for the main study. During main study the above doses were applied for 28 days in repetitive treatments. The main study was performed for below given assay parameters.

15. ASSAY PARAMETERS USED IN 28 DAYS TOXICITY TESTS

To assess the 28 days in vivo toxicity various parameters were tested which are as follows: Mice Weekly Body Weight, Hematology Differential Leucocyte Counts, Biochemical Test, Repeated Dose Micronucleus Assay and Sperm Morphology Test

Both Haematological and biochemical assay parameters are discussed separately in section 16. This includes 28 days repeated dose micronucleus test and described in separate section as part of genotoxicological tests in section 17 and sperm morphology test as section 18.

Analysis of Results

All the data of Clinical parameters (Glucose, Blood Urea Nitrogen, Creatinine, enzymes like Alanine aminotransferase (E.C. 2.6.1.2), Aspartate aminotransferase (E.C. 2.6.1.1) and Acetylcholinesterase (E.C. 3.1.1.7) Haematologiocal parameters were analysed using Paired Samples Test, Sig. (2-tailed) for Statistical significance at 5% α level and a 5% difference was considered as Statistically significant when compared to its concurrent control values.

Analysis of micronucleus assay and sperm morphology assay are described separately in respective sections.

16. HAEMATOLOGICAL AND BIOCHEMICAL CHANGES

16.1 Haematological Changes

Haematological and biochemical analysis were conducted on blood samples collected from all the mice at the end of the treatment during sacrifice.

The blood samples were collected by puncturing the orbital sinus plexus with the help of a fine capillary tube under ether (anesthetic) anesthesia (Riley, 1960). The orbital sinus allows the most ease in collection of many blood samples in a short time period in rodents (Annis and Darsheimer, 1975; Suber, 1994) and allows repetitive collection of blood samples with reduced variance in clinical pathology parameters (Statland and Winkel, 1984).

Around 0.6ml of blood was collected in vials containing EDTA for haematology analysis. The blood collected in a clean vial containing EDTA was directly fed into a fully automatic (Sysmex K 1000) haematological analyzer. The procedure as mentioned in Sysmex Operator Manual (1988) was followed. The results obtained were of parameters like leucocyte counts (WBC), erythrocyte counts (RBC), haemoglobin (Hb), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC).

16.2 Biochemical Changes

Biochemical changes were assayed by testing biochemical parameters on serum**.** Around 1.5 - 2.0ml of blood was collected from each mouse in clean centrifuge tubes for serum separation. The blood was allowed to clot at room temperature for 30 minutes. The serum was separated by centrifugation at 3000 rpm for 10 minutes and transferred into an

Eppendorf tube using a clean dry pasteur pipette. The serum thus separated was used for further analysis.

The serum parameters were analyzed using semi automatic serum analyzer ERBA Chem-5 plus. The analytical parameters studied were glucose, blood urea nitrogen, creatinine and total protein. The enzymes analysed were alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyltransferase (GGT).

Analytical Parameters

a) Glucose

Serum glucose was analyzed immediately after separation of the serum. Glucose was estimated by Trinder's glucose oxidase and peroxidase method (Trinder, 1969). It involves two step reactions involving glucose oxidase and hydrogen peroxidase respectively. In the first step glucose is converted to gluconic acid and hydrogen peroxide by glucose oxidase and in the second step hydrogen peroxide with the reaction of 4 aminoantipyrine is converted to a red dye by peroxidase which will give a pink colour with absorbance at 510nm.

The reagents are composed of glucose oxidase (20000 IU/L), peroxidase (3250 IU/L), 4-aminoantipyrine (0.52mM/L), 4-hydroxy benzoic acid (10nM/L) and phosphate buffer (110 mM/L) and glucose standard (100 mg/dL), 5.55mM/L.

The reagents were reconstituted at room temperature by dissolving the contents of each vial using deionised water free from contaminants. The samples were mixed well with the reagents and incubated for 15 minutes at 37 ℃. The absorbance of standards and of each sample against reagent blank were read at 510nm and expressed as mg/dL.

Calculation

$$\text{Glucose (mg/l)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \text{ x Concentration of standard (mg/dL)}$$

b) Blood Urea Nitrogen

The methodology of Talke and Schubert (1965) was followed. Urea in the presence of water is converted to ammonium and carbon dioxide by the action of urease. The ammonium reacts with alpha ketoglutarate and NADH in the presence of glutamate dehydrogenase to produce L-glutamate and NAD.

The reagents include 2-oxoglutarate (7.5mM/L), NADH (0.32mM/L), GLDH (1.000 IU/L), ADP (1.2mM/L) and tris buffer pH 7.9 ± 0.1 and 25 ℃ (100mM/L).

A volume of 10µl of the sample was mixed with 500µl of the reconstituted reagent and aspirated. The absorbance was read against blank at 340nm.

Calculation

$$\text{Urea (mg/dL)} = \frac{\Delta A \text{ of sample}}{\Delta A \text{ of standard}} \text{ X Concentrat ion of Standard (mg/dL)}$$

$\Delta A = A_1 - A_2$

c) Creatinine

The modified Jaffe reaction was followed (Jendrassik and Grof, 1938). Creatinine reacts with alkaline picrate to produce a reddish colour (the Jaffe reaction). Specificity of the assay has been improved by the introduction of an initial rate method, cephalosporin antibiotics are still major interferants. The orange-yellow colour formed is directly proportional to creatinine concentration.

The reagents comprised of picric acid (25.8mM/L), sodium hydroxide (95mM/L) and creatinine standard (2mg/dL), 0.16mM/L.

Equal volumes of picric acid and base were mixed and allowed to stand for 15 minutes before use. 50µl of the sample was taken and mixed with 500µl of the reagent and the absorbance was read against 510nm.

Calculation

$$\text{Creatinine (mg/dL)} = \frac{\Delta A \text{ of sample}}{\Delta A \text{ of standard}} \text{ X Concentration of standard (mg/dL)}$$

Δ A = Final absorbance – Initial absorbance

d) Albumin

Albumin is a carbohydrate-free protein, which constitutes 55-65% of total plasma protein. It maintains the plasma pressure, and is also involved in the transport and storage of a wide variety of ligands and is a source of endogenous amino acids. Albumin binds and solubilizes various compounds, e.g. bilirubin, calcium and long-chain fatty acids. Furthermore albumin is capable of binding with the test agent, which is the reason why lower albumin concentrations in blood have a significant effect .

Test principle

Colorimetric assay with endpoint method

• Sample and addition of R1 (buffer)

• Addition of R2 (substrate) and start of the reaction

At a pH value of 4.1 albumin displays a sufficiently cationic character to be able to bind with bromocresol green (BCG), an anionic dyestuff, to form a blue-green complex.

$$\text{Albumin + BCG} \xrightarrow{\text{pH 4.1}} \text{Albumin BCG complex}$$

The color intensity of the blue-green color is directly proportional to the albumin concentration and can be determined photometrically.

A. ENZYMES

a) Alanine aminotransferase (E.C. 2.6.1.2)

The methodology of International Federation of Clinical Chemistry (IFCC, 1956) was adopted. L-alanine combines with 2-oxoglutarate in the presence of alanine aminotransferase to produce pyruvate and L-glutamate. The pyruvate with NADH in the presence of lactate dehydrogenase gives rise to L-lactate and NAD. The reagents used were

L-alanine (400mM/L), NADH (0.18mM/L), LDH (1820 IU/L), 2-oxoglutarate (12mM/L) and tris buffer (pH 7.5 ± 0.1 at 25 ℃ – 80mM/L).

A 50µl of the sample was taken and mixed with 500µl of the reagent and the absorbance was read against blank at 340nm.

Activity of ALT (IU/L) = Absorbance/minute x 1768 (factor).

b) Aspartate aminotransferase (E.C. 2.6.1.1)

The methodology of International Federation of Clinical Chemistry (IFCC, 1980) was adopted. L-aspartate in the presence of aspartate aminotransferase combines with 2-oxoglutarate to form oxaloacetate and L-glutamate. The oxaloacetate then combines with NADH in the presence of malate dehydrogenase to produce malate and NAD. The sample pyruvate then combines with NADH in the presence of lactate dehydrogenase to form L-lactate and NAD.

The reagents used were 2-oxoglutarate (12mM/L), L-aspartate (200mM/L), MDH (≥ 545 U/L), LDH (≥ 909 U/L), NADH (≥ 0.18mM/L), tris buffer (pH 7.8 ± 0.1 at 25 ℃ – 80 mM/L) and EDTA (5.0mM/L). A 50 µl aliquot of the sample was taken and mixed with 500µl of the reagent and the absorbance was read against blank at 340nm.

Activity of AST (IU/L) = Absorbance/minute x 1768 (factor).

c) Cholinesterase Activity

Acetylcholinesterase (E.C. 3.1.1.7)

Principle

The described assay is based on the method of Voss and Sachsse (1970) for plasma and RBC and Ellman *et al.,.* (1961) for brain, in which acetylthiocholine iodide is used as substrate, which splits into acetate and thiocholine iodide. The liberated thio group of thicholine reacts with dithio bis nitrobenzoic acid (chromogen) to form a yellow coloured complex, 5-mercapto 2-nitrobenzoic acid which can be measured spectrophotometrically. The increase in colour intensity of the formed yellow complex is proportional to the cholinesterase activity and it can be measured kinetically between 400 – 420nm.

Methodology

A. Estimation of Cholinesterase Activity in Plasma and RBC

Blood samples were collected in heparinised (0.1% solution) plastic vials by puncturing the orbital sinus with the help of a fine capillary tube under ether anesthesia (Riley, 1960).

1. 20µl of blood was transferred immediately to 10ml of DTNB buffer (placed into ice bath) and mixed well. 4ml of this mixture was pipetted out for blood cholinesterase.
2. Remaining content was centrifuged for 5 minutes at 3000 rpm. 4ml of supernatant was used for plasma cholinesterase activity.
3. 4ml of DTNB without blood sample was used for blank tubes.
4. To each tube 1ml of substrate was added.
5. The tubes were placed in water bath at 30 ℃ for 10 minutes.
6. Reaction was stopped by adding 2 drops of serine salicylate and the content was mixed well.

7. The tubes were centrifuged at 3000 rpm for 5 minutes.
8. The absorbance of each tube including blank was read at 420nm.

GENOTOXICOLOGICAL TESTS

17. REPEATED DOSE (28 DAYS) MICRONUCLEUS TEST

This 28 days repeated dose micronucleus test was performed at the end of toxicity test. The bone marrow samples were taken for micronucleus analysis.

17.1 Experimental Procedure

The test method as described by Schmid (1976) and modified by Salamone and Heddle (1983) was followed. The experimental details are provided in section 1 to 10 of *in vivo* screening methods

17.2 Sacrifice

Control and treated mice were sacrificed by CO_2 asphyxiation. Both femoral bones were dissected out and cleaned from adherent tissue.

17.3 Peripheral Blood Sampling

Peripheral blood is obtained from the tail vein (refer section 10.4.1). Blood cells are immediately smeared and and then stained by conventional method of Giemsa staining.

17.4 Bone Marrow Sampling

The epicondyle tips were cut and the marrow content was flushed into a centrifuge tube, along with 3ml of fetal calf serum, using 1ml syringe and 22 gauge needles. The contents of the tubes were mixed thoroughly to dissociate cell clumps, centrifuged for 10 minutes at 2000 rpm.

17.5 Analysis

The proportion of immature among total (immature + mature) erythrocytes is determined for each animal by counting a total of at least 200 erythrocytes for bone marrow and 1000 erythrocytes for peripheral blood (Gollapudi, *et.al.*, 1995). All slides, including those of positive and negative controls, should be independently coded before microscopic analysis. At least 2000 immature erythrocytes per animal are scored for the incidence of micronucleated immature erythrocytes. Additional information may be obtained by scoring mature erythrocytes for micronuclei. When analysing slides, the proportion of immature erythrocytes among total erythrocytes should not be less than 20% of the control value. When animals are treated continuously for 4 weeks or more, at least 2000 mature erythrocytes per animal can also be scored for the incidence of micronuclei.

17.6 Slide Preparation

The supernatant was discarded and the cell pellet with 0.5ml of fetal calf serum was retained. Cells were then resuspended and a drop was placed on a clean slide using a Pasteur pipette and a smear was prepared. The prepared slides were allowed to air dry, fixed with methanol and stained the following day with 5% Giemsa stain for 10 minutes. Excess stain was removed from the slides by successive rinsing in distilled water for 1 minute. The slides were made permanent by mounting a cover slip with DPX.

17.7 Analysis of slides

The scoring of micronucleus in bone marrow cells was proposed by Boller and Schmid (1970) and Heddle (1973). Initial screening was done under low power objective in order to select area with good staining and proper morphology of erythrocytes which are generally present towards the end of the smear. A total of 2000 polychromatic erythrocytes (PCE) from each of the prepared slides were scored to calculate frequency of micronucleus. The corresponding numbers of normochromatic erythrocyte (NCE), with and without micronuclei were also recorded under 100 x oil immersion.

17.8 Evaluation and interpretation of results

There are several criteria for determining a positive result, such as a dose-related increase in the number of micronucleated cells or a clear increase in the number of micronucleated cells in a single dose group at a single sampling time. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (Richold *et.al.,* 1990 and Lowel *et al.,* 1989). Statistical significance should not be the only determining factor for a positive response. Equivocal results should be clarified by further testing, preferably using a modification of experimental conditions. A test substance for which the results do not meet the above criteria is considered non-mutagenic in this test.

18. SPERM MORPHOLOGY TEST

This particular test method has been included to find any link of Endosulfan exposure with malformations, as there are many examples of Endosulfan exposure and malformations and anomalies in human and animals. Sperms are important cells in reproductive toxicology because they can be used to asses spermatogenic damage, fertility effects and heritable genetic mutations. The mouse sperm morphology test has been a commonly used sperm test for measuring spermatogenic damage induced by physical or chemical agents. Several other mouse sperm tests have also been developed, yet their usage has been considerably more limited, these include induction of -

- Acrosome abnormalities
- In this test acrosomal morphology is observed under microscopes
- Reduction in sperm counts and
- Reduction in sperm motility

In both the above tests, number of the sperm and the swimming ability of the sperm are measured. In the mouse sperm morphology test, assessment of effects on exposed mice is based on visually scoring for the percentage of sperm with abnormal head forms in smears of sperm from the epididymis or vas deferens.

Although not widely used in mutagenicity studies, the sperm morphology test appears to be a sensitive test. Evidence that sperm-shape abnormalities were induced by selected mutagens and carcinogens has been reported (Wyrobek and Bruce, 1975; Bruce and Heddle, 1979; Soares *et al.,* 1979, Wyrobek *et al.,* 1983a, b). Although it is not clear theses changes in sperm morphology may be related to carcinogens, such a correlation may exist since 30 out of 69 carcinogens were positively identified by the sperm test and 24 out of 24 non-carcinogens were negative (Wyrobek *et al.*, 1984). In relation to mutagenicity, sperm shapes are reported to be genetically controlled by numerous autosomal and sex-linked genes (Benett, 1975; Forejt, 1976; Krzanowska, 1976).

18.1 Experimental procedure

1. The above 28 days repetitively treated mice were sacrificed by cervical dislocation and the epididymis were excised from each mouse in separate 60mm petridishes containing phosphate buffered saline.
2. The epidermal layer was removed by cutting with small scissors.
3. The epididymal contents were minced with small scissors and large tissue segments were removed.
4. The sperm suspension was transferred to testtube containing Eosin-Y (0.05%) and stained for 5 minutes.
5. The smear was prepared from suspensions and air dried.
6. Slides were mounted with coverslips.

18.2 Observation

1. The slides were observed first under 400X and then under 1000X magnification.
2. Sperms without tails or sperms that are in contact or overlaid by other sperms or debris are excluded.
3. A minimum of 1000 sperms were assessed for morphological abnormalities of the sperm head according to the criteria of Wyrobeck and Bruce (1975).
4. The slides were observed for Amorphous, banana shape, large head, small head, double head as head abnormality and sperm without hook, coiled, folded and double tailed as tail abnormality.
5. Percent Head and tail Abnormality were calculated.

18.2.1 Observation criteria

The % head abnormality was calculated based on the following formula:

$$\text{Head Abnormality} = \frac{\text{Number of abnormal cells}}{\text{Total number of cells observed}} \text{x}1100\%$$

18.3 Analysis of Results

All the data were analyzed using Paired Samples Test, Sig. (2-tailed) from the software SPSS 7.5.

18.4 Evaluation Criteria

18.4.1 Positive Response

Increase at $p \leq 0.05$ level and preferably be at least double than the negative control values.
For a test compound to be judged as a positive inducer of abnormal sperm the response should yield statistically significant increase for a minimum of two consecutive dose levels and be reproducible.

18.4.2 Negative Response

No increase in sperm abnormality to be seen up to the doses that cause whole animal lethality.

CHAPTER 3

OBSERVATION TABLES AND FIGURES

***In Vitro* Assays**

REVERSE MUTATION ASSAYS (AMES RESULTS)

TABLE: 1 (CYTOTOXICITY)

Table: 1.1 Lawn and Mean Revertants

Strain TA 98					
Without S9 mix				With S9 mix	
Sr. No.	Dose levels (µl/plate)	Mean No. of revertants per plate	Lawn	Mean No. of revertants per plate	Lawn
1	NC	18.5	Normal	20.0	Normal
2	VC	18.5	Normal	22.0	Normal
3	0.00005	16.5	Normal	31.0	Normal
4	0.0005	15	Normal	24.5	Normal
5	0.005	8	Normal	24.5	Normal
6	0.05	10	Normal	25.0	Normal
7	0.5	4.5, toxicity	MLI	16.0	Normal
8	5.0	-, Toxic	LA	20.0	Normal

Table: 1.2 Lawn and Mean Revertants

Strain TA 100					
Without S9 mix				With S9 mix	
Sr. No.	Dose levels µl/plate	Mean No. of revertants per plate	Lawn	Mean No. of revertants per plate	Lawn
1	NC	142	Normal	154.0	Normal
2	VC	147	Normal	146.5	Normal
3	0.00005	82	Normal	144.0	Normal
4	0.0005	88	Normal	135.0	Normal
5	0.005	82	Normal	197.5	Normal
6	0.05	58.5	Normal	153.5	Normal
7	0.5	44	MLI	0.000	LA
8	5.0	-	LA	-	LA

Note: NC = Negative Control, VC = Vehicle control, MLI = Moderate lawn inhibition, LA = Lawn absent, n = 2.

PLATE INCORPORATION METHOD

TABLE: 2 (REVERTANT FREQUENCY – MAIN STUDY-I)

Table: 2.1 - Strain TA 1537(-S9)								
Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**PC (SA)**
Dose (µg/plate)	0	0	0.004	0.014	0.047	0.158	0.525	0.5
Replicate1	4	7	13	11	14	16	9	688
Replicate2	4	11	15	9	27	33	14	692
Lawn	Normal	Normal	Normal	Normal	Normal	Normal	MLI	Normal
Fold Value	NA	NA	1.56	1.11	2.28	2.72	1.28	76.67
Mean	4	9	14	10	20.5	24.5	11.5	690
S.D.	0	2.8284	1.414	1.414	9.192	12.02	3.536	2.8284
S.E.	0	2.0003	1	1	6.501	8.501	2.5	2.0003
Regression Equation:y = 2.1429x + 4.7857, r = 0.13, n = 2								

REVERTANT FREQUENCY – MAIN STUDY-I

Table: 2.2 - Strain TA 98 (-S9)								
Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**2-NF**
Dose (µg/plate)	**0**	**0**	**0.004**	**0.014**	**0.047**	**0.158**	**0.525**	**50**
Replicate1	18	14	14	17	9	24	25	1336
Replicate2	14	26	12	17	15	20	28	1364
Fold Value	NA	NA	0.65	0.85	0.60	1.10	1.33	67.50
Lawn	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Mean	16	20	13	17	12	22	26.5	1350
S.D.	2.828	8.4853	1.414	0	4.243	2.828	2.121	19.799
S.E.	2	6.0009	1	0	3	2	1.5	14.002
Regression Equation:y = 1.2321x + 13.143, r =0.79, n = 2								

Note: G1- G7 are treatment groups, S.D. = Standard Deviation, S.E. = Standard Error, MLi = Mild Lawn Inhibition, SA = Sodium Azide and NF = Nitrofluorene.

REVERTANT FREQUENCY – MAIN STUDY-I

Table: 2.3 - Strain TA 1535 (-S9)								
Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**9-Aa**
Dose (µg/plate)	0	0	0.004	0.014	0.047	0.158	0.525	50
Replicate1	15	10	7	11	12	21	22	140
Replicate2	10	19	6	12	10	18	23	152
Lawn	Normal	Normal	Normal	Normal	Normal	Normal	MLI	Normal
Fold Value	NA	NA	0.45	0.79	0.76	1.34	**1.55**	10.07
Mean	12.5	14.5	6.5	11.5	11	19.5	**22.5**	146
S.D.	3.536	6.364	0.707	0.707	1.414	2.121	0.707	8.4853
S.E.	2.5	4.5007	0.5	0.5	1	1.5	0.5	6.0009
Regression Equation: y = 1.5893x + 7.6429, r =0.82, n = 2								

REVERTANT FREQUENCY – MAIN STUDY-I

Table: 2.4 - Strain TA 100 (-S9)								
Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**SA**
Dose (µg/plate)	0	0	0.004	0.014	0.047	0.158	0.525	5.0
Replicate1	154	144	98	124	86	73	37 T*	1064
Replicate2	136	158	120	130	79	88	45 T*	860
Lawn	Normal	Normal	Normal	Normal	Normal	MLi	I	Normal
Fold Value	NA	NA	0.72	0.84	0.55	0.53	0.27	6.37
Mean	145	151	109	127	82.5	80.5	41	962
S.D.	12.73	9.8995	15.56	4.243	4.95	10.61	5.657	144.25
S.E.	9.001	7.0011	11	3	3.501	7.501	4.001	102.02
Regression Equation: y = -17.125x + 173.64, r = 0.84, T*= moderately toxic, n = 2								

Note: G1- G7 are treatment groups, S.D. = Standard Deviation,
S.E. = Standard Error, 9 - Aa = 9- Aminoacridine and
SA = Sodium Azide, MLi = Moderate Lawn Inhibition, I = Lawn Inhibition.

REVERTANT FREQUENCY – MAIN STUDY-I

Table: 2.5 Strain TA 102 (-S9)								
Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	MMC
Dose (µg/plate)	0	0	0.004	0.014	0.047	0.158	0.525	0.5
Replicate1	314	328	335	359	434	422	1097	1692
Replicate2	349	296	290	445	383	434	1042	2184
Lawn	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Fold Value	NA	NA	1.00	1.29	1.31	1.37	**3.43**	6.21
Mean	331.5	312	312.5	402	408.5	428	1070	1938
S.D.	24.75	22.627	31.82	60.81	36.06	8.485	38.89	347.9
S.E.	17.5	16.002	22.5	43.01	25.5	6.001	27.5	246.04
Regression Equation: y = 90.839x + 103, r = 0.98, n = 2								

Table: 2.6 - Strain TA 1537 (+S9)								
Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**2An**
Dose (µg/plate)	0	0	0.004	0.014	0.047	0.158	0.525	50
Replicate1	3	13	14	8	13	8	16	44
Replicate2	3	7	8	15	7	11	19	49
Lawn	Normal	Normal	Normal	Normal	Normal	Normal	MLi	Normal
Fold Value	NA	NA	1.10	1.15	1.00	0.95	**1.75**	4.65
Mean	3	10	11	11.5	10	9.5	17.5	46.5
S.D.	0	4.2426	4.243	4.95	4.243	2.121	2.121	3.5355
S.E.	0	3.0005	3	3.501	3	1.5	1.5	2.5004
Regression Equation: y = 1.4821x + 4.4286, r = 0.74, n = 2								

Table: 2.7 - Strain TA 1535(+S9)								
Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**SA**
Dose (µg/plate)	0	0	0.004	0.014	0.047	0.158	0.525	0.5
Replicate1	27	27	31	40	40	28	37	2040
Replicate2	27	27	52	36	42	42	17	1886
Lawn	Normal	Normal	Normal	Normal	Normal	Normal	MLi	Normal
Fold Value	NA	NA	**1.54**	1.41	**1.52**	1.30	1.00	72.70
Mean	27	27	41.5	38	41	35	27	1963
S.D.	0	0	14.85	2.828	1.414	9.899	14.14	108.89
S.E.	0	0	10.5	2	1	7.001	10	77.012
Regression Equation: y = 0.5536x + 31.571, r = 0.38, n = 2								

Note: G1- G7 are treatment groups, S.D. = Standard Deviation, S.E. = Standard Error **,** MLi = Mild Lawn Inhibition, SA = Sodium Azide, 2-An = 2-Aminoanthracene and MMC = Mitomycin-C.

REVERTANT FREQUENCY – MAIN STUDY-I

Table: 2.8 - Strain TA 100 (+S9)								
Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**2-AF**
Dose (µg/plate)	0	0	0.004	0.014	0.047	0.158	0.525	20
Replicate1	131	187	70	179	35	182	167	2100
Replicate2	172	102	94	179	44	206	195	2026
Lawn	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Fold Value	NA	NA	0.57	1.24	0.27	1.34	1.25	14.28
Mean	151.5	144.5	82	179	39.5	194	181	2063
S.D.	28.99	60.104	16.97	0	6.364	16.97	19.8	52.326
S.E.	20.5	42.506	12	0	4.501	12	14	37.006
Regression Equation: y = 5.1786x + 118.07, r = 0.40, n = 2								

Table: 2.9 - Strain TA 98(+S9)								
Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**2-AF**
Dose (µg/plate)	0	0	0.004	0.014	0.047	0.158	0.525	20
Replicate1	14	57	36	54	42	43	46	844
Replicate2	50	44	45	46	23	62	23	1880
Lawn	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Fold Value	NA	NA	0.80	0.99	0.64	1.04	0.68	26.97
Mean	32	50.5	40.5	50	32.5	52.5	34.5	1362
S.D.	25.46	9.1924	6.364	5.657	13.44	13.44	16.26	732.56
S.E.	18	6.501	4.501	4.001	9.501	9.501	11.5	518.08
Regression Equation:y = 0.125x + 41.286, r = 0.23, n = 2								

Table: 2.10 - Strain TA 102(+S9)								
Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**2-AF**
Dose (µg/plate)	0	0	0.004	0.014	0.047	0.158	0.525	20
Replicate1	374	440	406	135 T*	38 T**	—	—	904
Replicate2	309	414	442	120 T*	20 T**	—	—	1112
Lawn	Normal	Normal	Normal	T*	I	I	I	Normal
Fold Value	NA	NA	0.99	0.30	0.07	—	—	2.36
Mean	341.5	427	424	127.5	29	—	—	1008
S.D.	45.96	18.385	25.46	10.61	12.73	—	—	147.08
S.E.	32.5	13.002	18	7.501	9.001	—	—	104.02
Regression Equation:y = -91.371x + 544.63, r = 0.75,								

Note: G1- G7 are treatment groups, S.D. = Standard Deviation, S.E. = Standard Error SA = Sodium Azide and 2-AF = 2-Aminofluorene. T*= moderately toxic, T**= Toxic, I = Inhibition, '—' = Highly toxic (No growth) , n = 2

AMES ASSAY

Revertant Frequency: Plate Incorporation Method
(In the absence of metabolic activation)

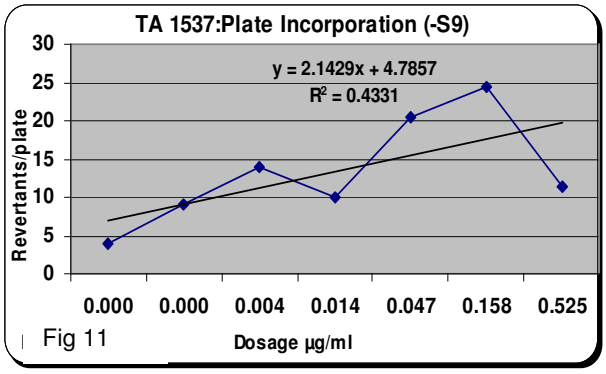


Fig 11

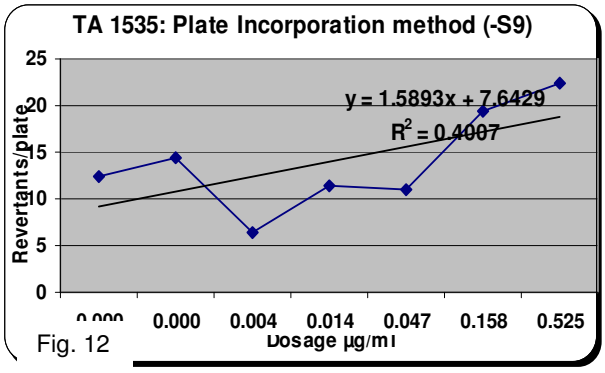


Fig. 12

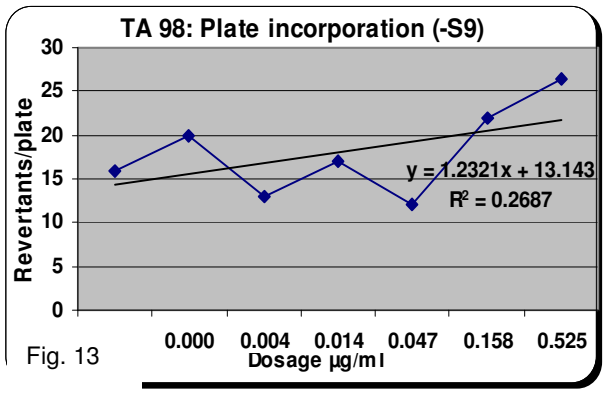


Fig. 13

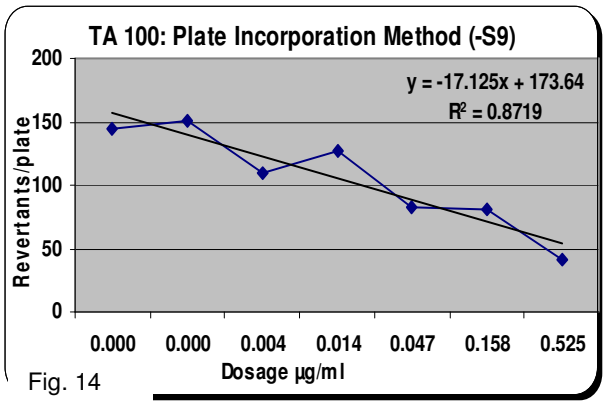


Fig. 14

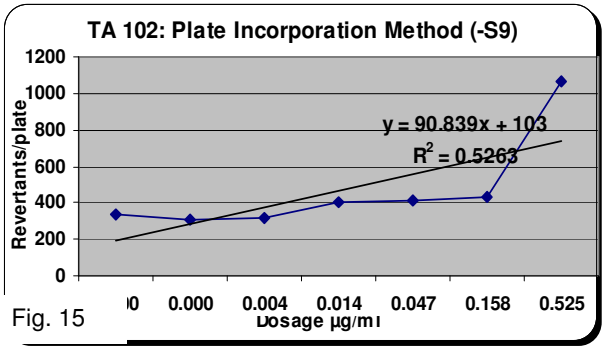


Fig. 15

Revertant Frequency: Plate Incorporation Method (In the presence of metabolic activation

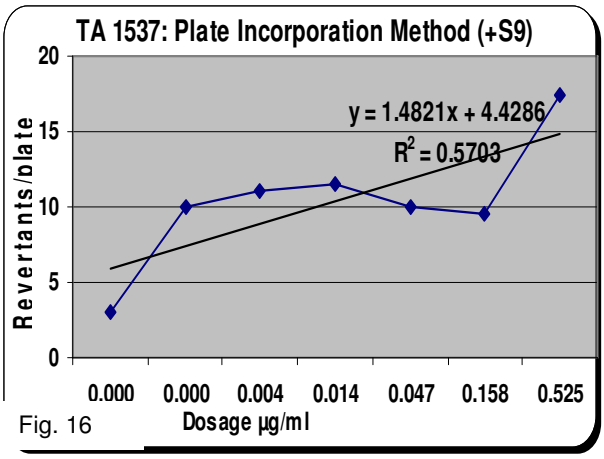


Fig. 16

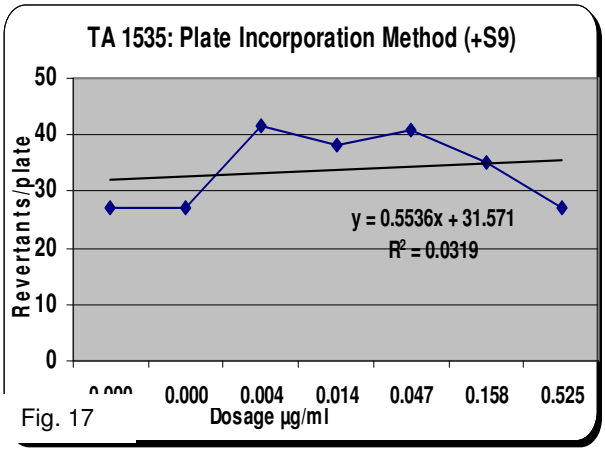


Fig. 17

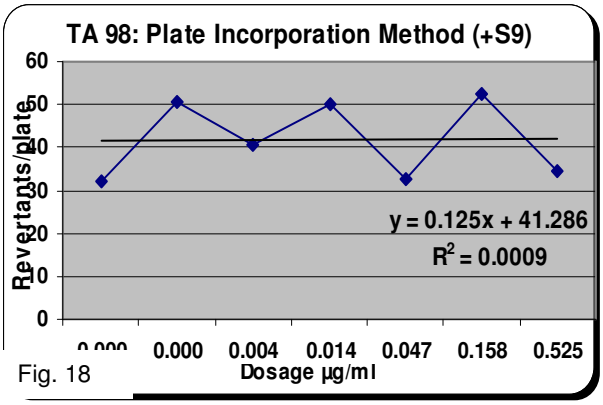


Fig. 18

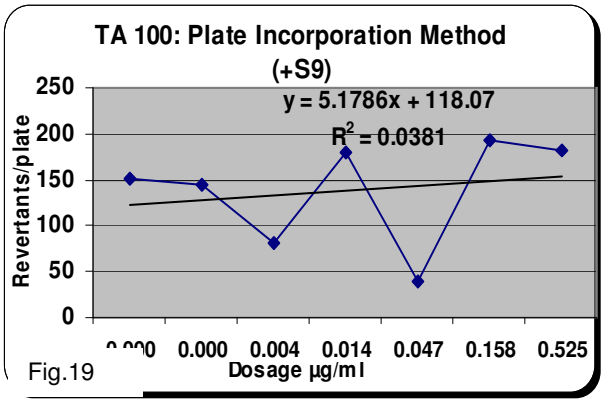


Fig.19

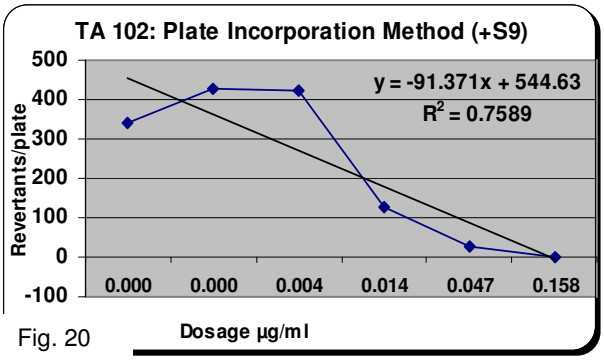


Fig. 20

TABLE: 3 - GROWTH
Optical Density of Plate Incorpotation Method and Pre Incubation Method

Plate Incorporation Method			Pre Incubation Method		
Sr. No	**Strain**	**Optical density**	**Sr. No**	**Strain**	**Optical density**
1	Blank	Adjusted to "0"	1	Blank	Adjusted to "0"
2	TA 1537	0.48	2	TA 1537	0.43
3	TA 1535	0.46	3	TA 1535	0.49
4	TA 98	0.44	4	TA 98	0.42
5	TA 100	0.44	5	TA 100	0.51
6	TA 102	0.42	6	TA 102	0.47

TABLE: 4 PRE INCUBATION METHOD
(REVERTANT FREQUENCY – MAIN STUDY-II)

Table: 4.1 - Strain :TA 1537(-S9), Pre incubation method								
Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**PC (SA)**
Dose (µg/plate)	0	0	0.004	0.014	0.047	0.158	0.525	0.5
Replicate1	8	6	15	12	13	19	8	288
Replicate2	7	10	16	14	19	28	17	384
Lawn	Normal	Normal	Normal	Normal	Normal	Normal	MLI	Normal
Fold Value	NA	NA	1.94	1.63	2.00	2.94	1.56	42.00
Mean	7.50	8.00	15.50	13.00	16.00	23.50	12.50	336.00
S.D.	4.36	5.03	8.96	7.56	9.69	14.21	8.25	199.56
S.E.	3.08	3.56	6.34	5.35	6.85	10.05	5.83	141.13
Regression Equation: y = 1.6607x + 7.0714,r = 0.66, MLI = Mild Lawn Inhibition, n = 2								

Note: G1- G7 are treatment groups, S.D. = Standard Deviation,
S.E. = Standard Error, SA = Sodium Azide,

REVERTANT FREQUENCY – MAIN STUDY-II

Table: 4.2 - Strain: TA 1535 (-S9), Pre incubation method								
Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**PC (9-Aa)**
Dose (µg/plate)	0	0	0.004	0.014	0.047	0.158	0.525	50
Replicate1	22	24	60	55	57	49	32	187
Replicate2	25	31	104	52	82	45	23	189
Lawn	Normal	Normal	Normal	Normal	Normal	Normal	MLI	Normal
Fold Value	NA	NA	2.98	1.95	2.53	1.71	1.00	6.84
Mean	23.50	27.50	82.00	53.50	69.50	47.00	27.50	188.00
S.D.	13.65	16.26	52.20	30.92	42.00	27.12	16.21	1.41
S.E.	9.65	11.50	36.92	21.86	29.70	19.18	11.46	1.00
Regression Equation: y = 10.799Ln(x) + 34.063, r = 0.33, MLI = Mild Lawn Inhibition, n = 2								

REVERTANT FREQUENCY – MAIN STUDY-II

Table: 4.3 - Strain: TA 98 (-S9), Pre incubation method								
Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**2-NF**
Dose (µg/plate)	0	0	0.004	0.014	0.047	0.158	0.525	5.0
Replicate1	32	39	26	36	23	24	36	1784
Replicate2	44	25	22	101	37	30	28	1128
Lawn	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Fold Value	NA	NA	0.75	2.14	0.94	0.84	1.00	45.50
Mean	38.00	32.00	24.00	68.50	30.00	27.00	32.00	1456.00
S.D.	22.74	19.76	14.00	51.18	18.66	15.79	18.61	463.86
S.E.	16.09	13.97	9.90	36.20	13.19	11.16	13.16	328.05
Regression Equation: y = -1.3713Ln(x) + 37.599, r = 0.06, n = 2								

Note: G1- G7 are treatment groups, S.D. = Standard Deviation,
S.E. = Standard Error, 9- Aa = 9- Aminoacridine, 2 –NF = 2-Nitroflourene.

REVERTANT FREQUENCY – MAIN STUDY-II

Table: 4.4 - Strain: TA 100 (-S9), Pre incubation method								
Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**SA**
Dose (µg/plate)	0	0	0.004	0.014	0.047	0.158	0.525	5.0
Replicate1	119	119	108	99	126	95	89	1064
Replicate2	127	121	117	160	127	106	120	860
Lawn	Normal	Normal	Normal	Normal	Normal	MLI	MLI	Normal
Fold Value	NA	NA	0.94	1.08	1.05	0.84	0.87	8.02
Mean	123.00	120.00	112.50	129.50	126.50	100.50	104.50	962.00
S.D.	71.13	69.29	65.11	80.74	73.01	58.19	62.00	144.25
S.E.	50.30	49.00	46.04	57.10	51.63	41.16	43.85	102.02
Regression Equation: y = -7.4948Ln(x) + 125.77, r = 0.46, MLI = Mild Lawn Inhibition, n = 2								

REVERTANT FREQUENCY – MAIN STUDY-II

Table: 4.5 - Strain: TA 102 (-S9), Pre incubation method								
Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	MMC
Dose (µg/plate)	0	0	0.004	0.014	0.047	0.158	0.525	0.5
Replicate1	381	466	513	438	446	319	333	904
Replicate2	502	418	380	454	395	456	336	1112
Lawn	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Fold Value	NA	NA	1.01	1.01	0.95	0.88	0.76	2.28
Mean	441.50	442.00	446.50	446.00	420.50	387.50	334.50	1008.00
S.D.	261.98	256.31	266.22	257.61	244.08	233.89	192.83	147.08
S.E.	185.28	181.27	188.28	182.19	172.62	165.41	136.37	104.02
Regression Equation: y = -41.656Ln(x) + 467.66, r = 0.68, n = 2								

Note: G1- G7 are treatment groups, S.D. = Standard Deviation, S.E. = Standard Error**,** SA = Sodium Azide, MMC = Mitomycin-C.

REVERTANT FREQUENCY – MAIN STUDY-II

Table: 4.6 - Strain: TA 1537 (+S9), Pre incubation method								
Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**2-An**
Dose (µg/plate)	0	0	0.004	0.014	0.047	0.158	0.525	5.0
Replicate1	6	10	21	19	14	7	5	189
Replicate2	9	12	18	20	12	12	3	254
Lawn	Normal	Normal	Normal	Normal	Normal	Normal	MLi	Normal
Fold Value	NA	NA	1.77	1.77	1.18	0.86	0.36	20.14
Mean	7.50	11.00	19.50	19.50	13.00	9.50	4.00	221.50
S.D.	4.58	6.43	11.36	11.26	7.54	5.95	2.24	45.96
S.E.	3.24	4.55	8.03	7.96	5.34	4.20	1.59	32.50
Regression Equation: y = -0.1754Ln(x) + 12.214, r = 0.02, MLi = Mild Lawn Inhibition, n = 2								

REVERTANT FREQUENCY – MAIN STUDY-II

Table: 4.7 - Strain: TA 1535(+S9), Pre incubation method								
Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**2-An**
Dose (µg/plate)	0	0	0.004	0.014	0.047	0.158	0.525	5.0
Replicate1	22	23	20	17	27	26	0	2680
Replicate2	27	18	15	23	30	25	0	1760
Lawn	Normal	Normal	Normal	Normal	Normal	MLi	I	Normal
Fold Value	NA	NA	0.85	0.98	1.39	1.24	0.00	108.29
Mean	24.50	20.50	17.50	20.00	28.50	25.50	0.00	2220.00
S.D.	14.36	12.10	10.41	11.92	16.50	14.64	0.30	650.54
S.E.	10.16	8.56	7.36	8.43	11.67	10.35	0.21	460.07
Regression Equation: y = -4.8051Ln(x) + 25.352, r = 0.35, MLi= Mild Lawn Inhibition, I = Inhibition, n = 2								

Note: G1- G7 are treatment groups, S. D. = Standard Deviation,
S.E. = Standard Error, 2-An = 2-Aminoanthracene.

REVERTANT FREQUENCY – MAIN STUDY-II

Table: 4.8 - Strain: TA 98(+S9), Pre incubation method								
Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**2-AF**
Dose (µg/plate)	0	0	0.004	0.014	0.047	0.158	0.525	20
Replicate1	25	24	34	39	42	40	16	3880
Replicate2	32	52	38	41	29	44	13	4376
Lawn	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Fold Value	NA	NA	0.95	1.05	0.93	1.11	0.38	108.63
Mean	28.50	38.00	36.00	40.00	35.50	42.00	14.50	4128.00
S.D.	16.82	26.03	20.88	23.11	21.48	24.24	8.21	350.72
S.E.	11.90	18.41	14.77	16.34	15.19	17.14	5.80	248.04
Regression Equation: y = -1.296Ln(x) + 35.078, r = 0.09, n = 2								

REVERTANT FREQUENCY – MAIN STUDY-II

Table: 4.9 - Strain: TA 100(+S9), Pre incubation method								
Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**2-AF**
Dose (µg/plate)	0	0	0.004	0.014	0.047	0.158	0.525	20
Replicate1	107	109	133	144	181	175	167	608
Replicate2	118	122	174	140	164	179	195	702
Lawn	Normal	Normal	Normal	Normal	Normal	Normal	MLI	Normal
Fold Value	NA	NA	1.33	1.23	1.49	1.53	1.57	5.67
Mean	112.50	115.50	153.50	142.00	172.50	177.00	181.00	655.00
S.D.	65.18	67.00	90.96	82.00	99.93	102.12	105.13	66.47
S.E.	46.10	47.38	64.33	57.99	70.67	72.22	74.35	47.01
Regression Equation: y = 38.695Ln(x) + 103.45, r = 0.93, MLI = Mild Lawn Inhibition, n = 2								

Note: G1- G7 are treatment groups, S.D. = Standard Deviation,
S.E. = Standard Error, 2-AF = 2-Aminofluorene

REVERTANT FREQUENCY – MAIN STUDY-II

Table: 4.10 - Strain: TA 102(+S9), Pre incubation method								
Group	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**2-AF**
Dose (µg/plate)	0	0	0.004	0.014	0.047	0.158	0.525	20
Replicate1	366	466	506	471	390	337	300	896
Replicate2	448	408	445	447	394	249	203	912
Lawn	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Fold Value	NA	NA	1.09	1.05	0.90	0.67	0.58	2.07
Mean	407.00	437.00	475.50	459.00	392.00	293.00	251.50	904.00
S.D.	238.53	253.96	276.22	265.27	226.30	174.70	152.80	11.31
S.E.	168.69	179.61	195.34	187.60	160.04	123.55	108.06	8.00
Regression Equation: y = -70.956Ln(x) + 474.27, r = 0.57, n = 2								

Note: G1- G7 are treatment groups, S.D. = Standard Deviation,
S.E. = Standard Error, 2-AF = 2-Aminofluorene.

AMES TEST

Revertant Frequency: Pre Incubation Method (In the absence of metabolic activation

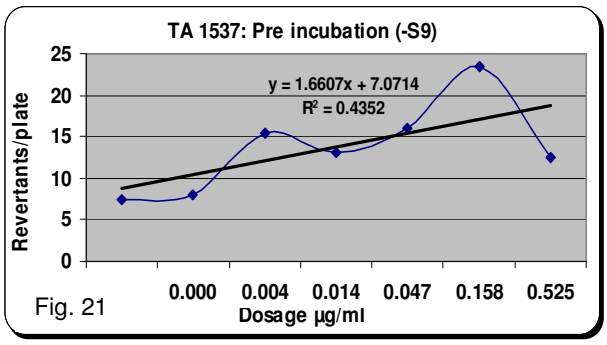


Fig. 21

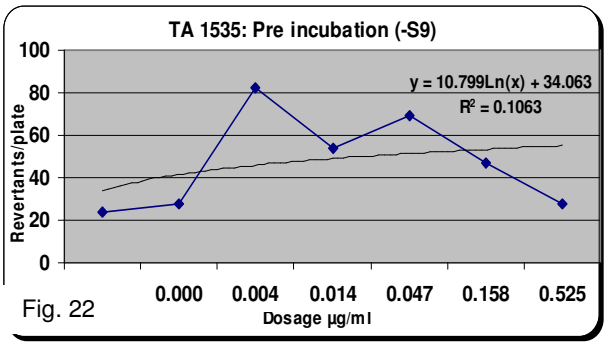


Fig. 22

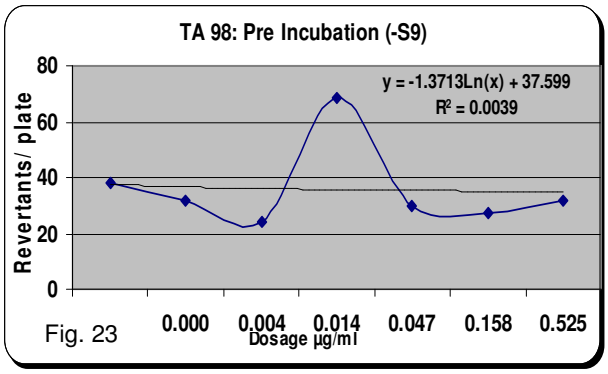


Fig. 23

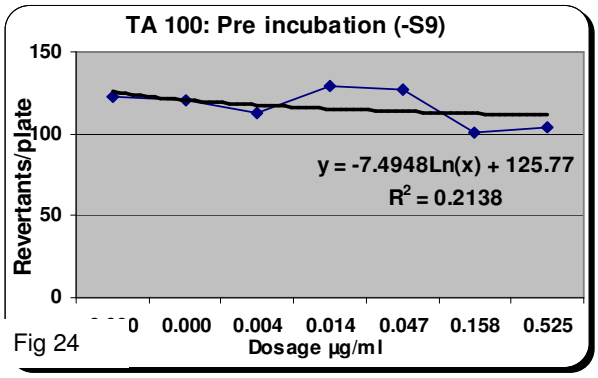


Fig 24

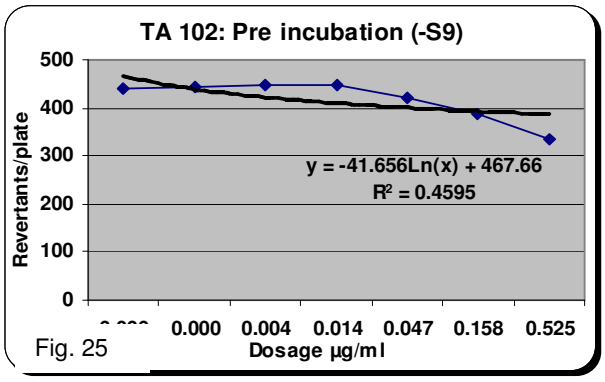


Fig. 25

AMES TEST

Revertant Frequency: Pre Incubation Method (In the presence of metabolic activation

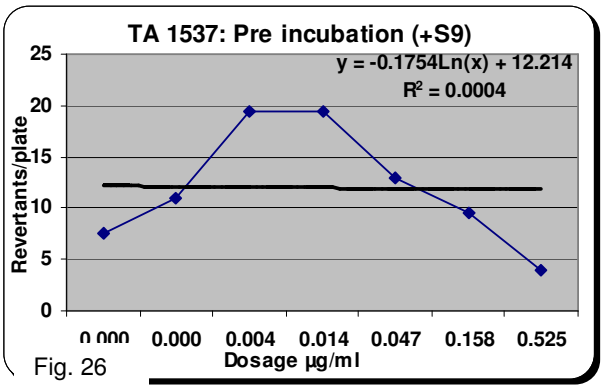


Fig. 26

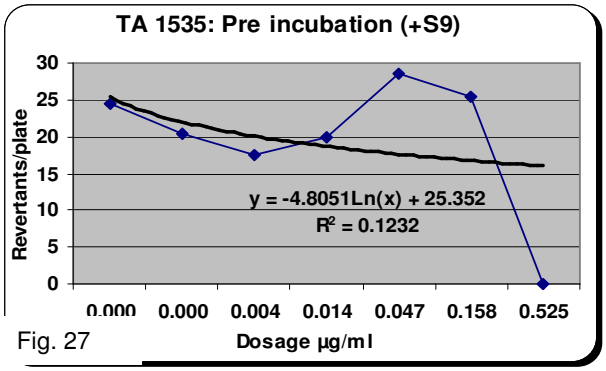


Fig. 27

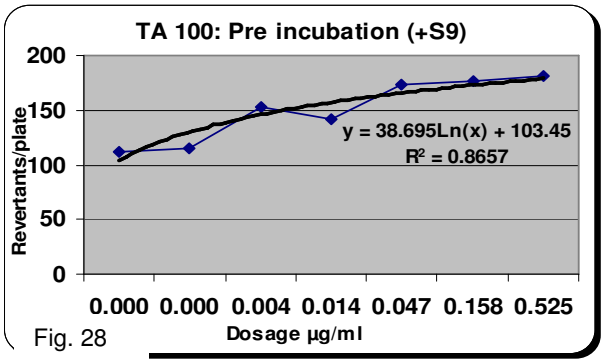


Fig. 28

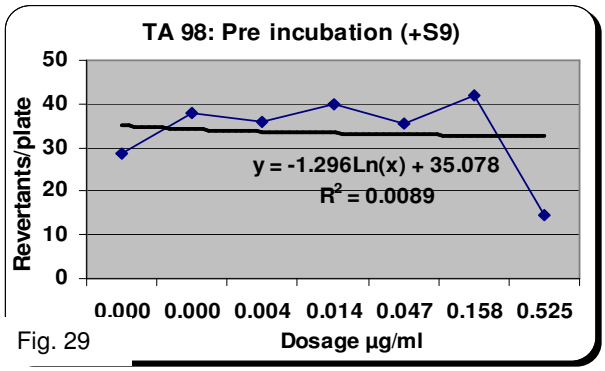


Fig. 29

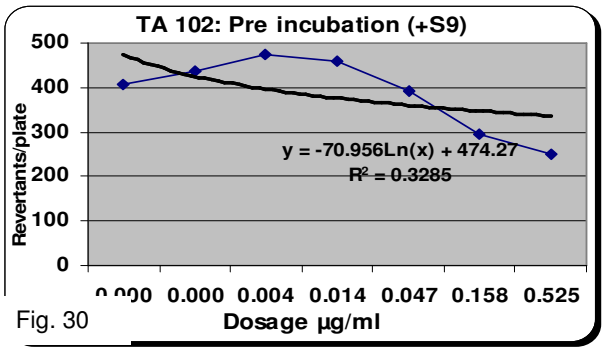


Fig. 30

FORWARD MUTATION ASSAYS: DOSE SELECTION STUDY

TABLE: 5 SURVIVAL FREQUENCIES (Cytotoxicity)
Table: 5.1 (Without Metabolic Activation)

Survival frequency : Dose Selection (without metabolic activation, -S9)									
Dose levels (µg/ml)	No of cells seeded	No of colonies observed				ACE	%ACE	RCE	%RCE
		R1	R2	Mean	SD				
VC(0)	100	115	94	104.5	14.849	1.045	104.5	1	100
0.175	100	94	105	99.5	7.7782	0.995	99.5	0.952	95.2
0.35	200	151	122	136.5	20.506	0.682	68.2	0.653	65.3
0.525	205	50	92	71.0	29.698	0.346	34.6	0.331	33.1

Note: VC = Vehicle Control, N = Number of Cells seeded, ACE = Absolute Clonning Efficiency, RCE = RelativeClonning Efficiency

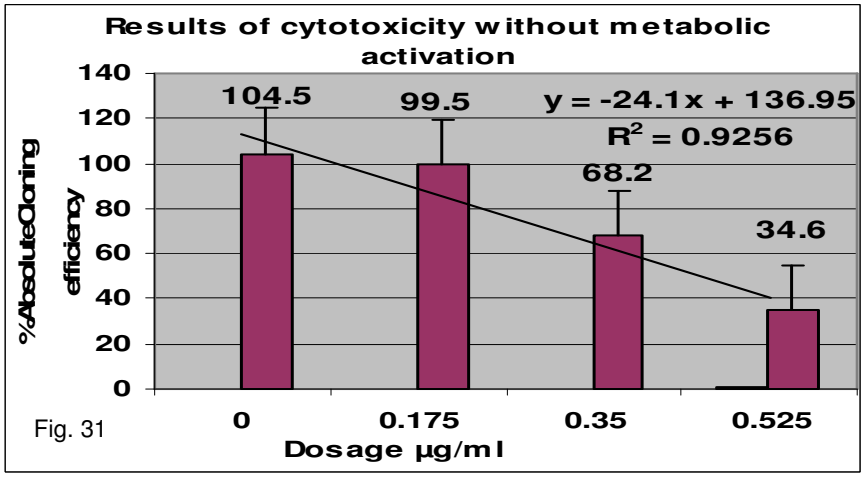


Fig. 31

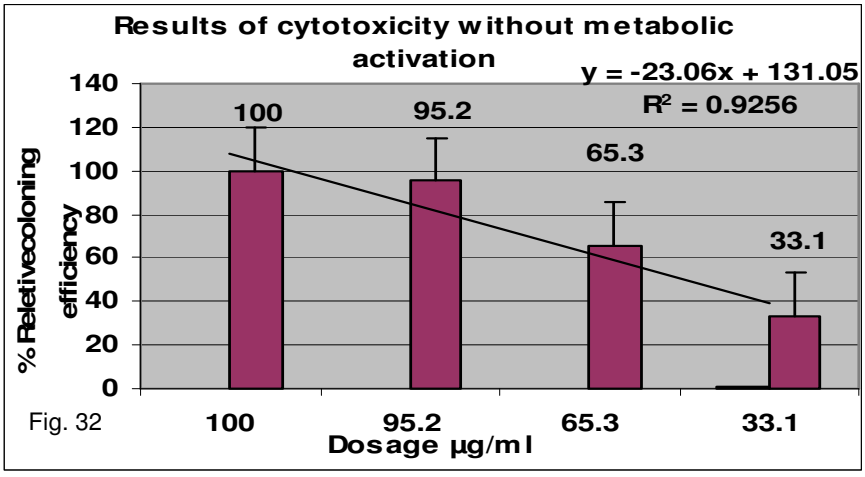


Fig. 32

FORWARD MUTATION ASSAYS: MAIN STUDY

TABLE: 5 SURVIVAL FREQUENCIES (BEFORE MUTANT SELECTION)

Table: 5.2 (Without Metabolic Activation)

Survival frequency of the cells before selection without metabolic activation (-S9)									
Dose levels (µg/ml)	No of cells seeded	No of colonies observed				ACE	%ACE	RCE	%RCE
		R1	R2	Mean	SD				
NC(0)	200	207	215	211	5.66	1.055	105.50	1.27	127.11
VC(0)	200	193	139	166	38.18	0.830	83.00	1.00	100.00
0.175	200	119	105	112	9.90	0.560	56.00	0.67	67.47
0.35	300	262	232	247	21.21	0.823	82.33	0.99	99.20
0.525	400	28	32	30	2.83	0.075	7.50	0.09	9.04
PC(10 µl EMS)	200	124	134	129	7.07	0.645	64.50	0.78	77.71

Table: 5.3 (With Metabolic Activation)

Survival frequency of the cells before selection with metabolic activation (+S9)									
Dose levels (µg/ml)	No of cells seeded	No of colonies observed				ACE	%ACE	RCE	%RCE
		R1	R2	Mean	SD				
NC(0)	200	162	137	149.5	17.68	0.748	74.75	1.00	99.67
VC(0)	200	157	143	150	9.90	0.750	75.00	1.00	100.00
0.175	200	173	185	179	8.49	0.895	89.50	1.19	119.33
0.35	300	143	126	134.5	12.02	0.448	44.83	0.60	59.78
0.525	200	112	101	106.5	7.78	0.533	53.25	0.71	71.00
PC (12 µg B(a)P	200	168	103	135.5	45.96	0.678	67.75	0.90	90.33

Note: NC = Negative Control, VC = Vehicle Control, PC = Positive Control, ACE = Absolute Clonning Efficiency, RCE = Relative Clonning Efficiency, EMS = Ethy Methane Sulphonate and B (a) P = Benzo-a -pyrene

TABLE 6: MUTATION FREQUENCY WITH SURVIVAL FREQUENCY (Without Metabolic Activation)

Table: 6.1 (Without Metabolic Activation)

Mutation frequency and survival frequency of the cells without metabolic activation (-S9)												
Dose levels (µg/ml)	N	No of colonies observed				ACE	Cells/ml counted	Volume of cells added (ml)	No. of Cells Plated	No. of Clonable Cells	0	MF
		R1	R2	Mean	SD							
NC(0)	200	183	165	174	12.73	0.870	285000	0.5	142500	123975	3	4.84
VC(0)	200	121	137	129	11.31	0.645	227500	0.5	113750	73369	1	2.73
0.175	200	122	106	114	11.31	0.570	250000	0.5	125000	71250	2	5.61
0.35	200	109	120	114.5	7.78	0.573	368750	0.4	147500	84444	1	2.37
0.525	400	222	224	223	1.41	0.558	153750	2.0	307500	171431	0	0.00
PC (10µl EMS)	403	260	244	252	11.31	0.625	65000	2.9	188500	117871	38	64.48

Table: 6.2 (With Metabolic Activation)

Mutation frequency and survival frequency of the cells with metabolic activation (+S9)												
Dose levels (µg/ml)	N	No of colonies observed				ACE	Cells/ml counted	Volume of cells added (ml)	No. of Cells Plated	No. of Clonable Cells	O	MF
		R1	R2	Mean	SD	NA						
NC(0)	200	211	221	216	7.07	1.080	277500	1.0	277500	299700	4.00	2.67
VC(0)	200	142	113	127.5	20.51	0.638	193750	1.0	193750	123516	6.00	9.72
0.175	200	113	125	119	8.49	0.595	363750	0.5	181875	108216	0.00	0.00
0.35	200	129	116	122.5	9.19	0.613	557500	0.8	446000	273175	2.00	1.46
0.525	207	255	238	246.5	12.02	1.191	103750	2.0	207500	247095	3.00	2.43
PC (12µg BaP)	300	236	343	289.5	75.66	0.965	358750	0.5	179375	173097	29.00	33.51

Note: NC = Negative Control, VC = Vehicle Control, PC = Positive Control, N = Number of Cells seeded, O = No. of Colonies observed on selective plates, ACE = Absolute Clonning Efficiency, EMS = Ethy Methane Sulphonate, B(a)P = Benzo-a –pyrene, MF = Mutation Frequency /10^6 cells, n = 5.

Forward Mutation Assay – Data Graphs-I

Gene Mutation Assays

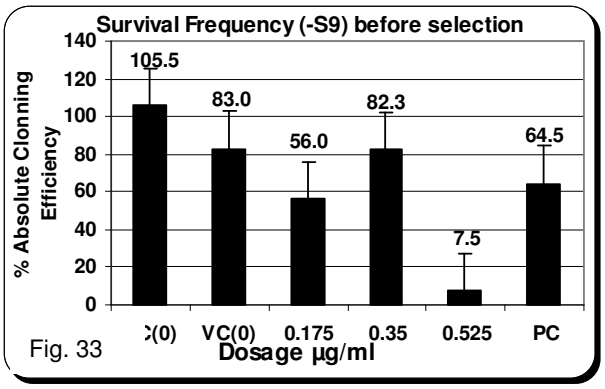


Fig. 33

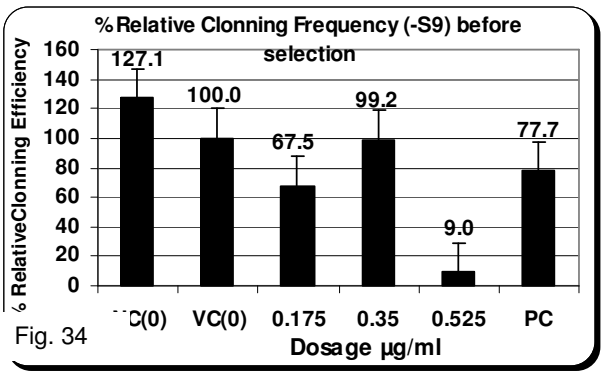


Fig. 34

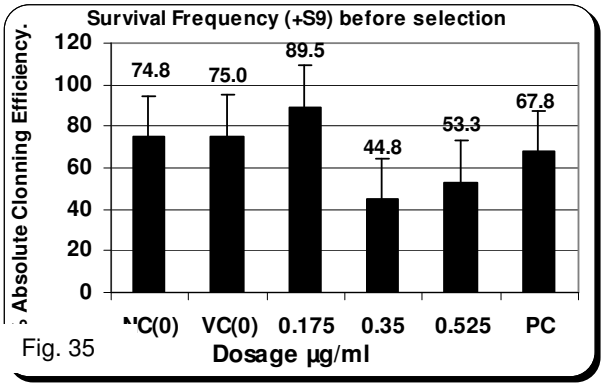


Fig. 35

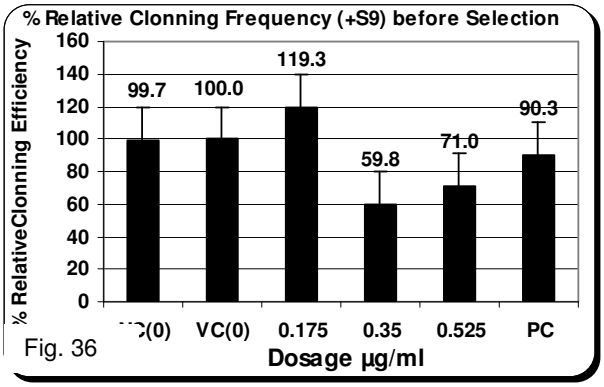


Fig. 36

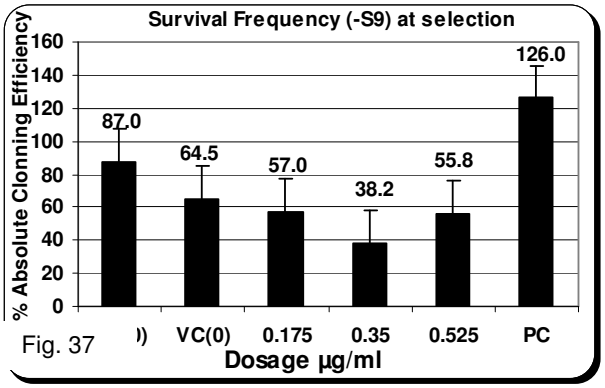


Fig. 37

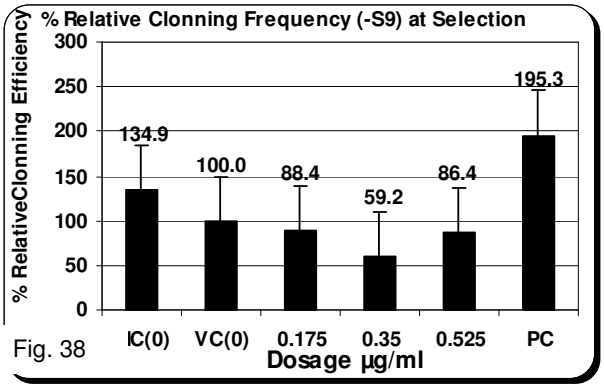


Fig. 38

Forward Mutation Assay – Data Graphs-II

Forward Mutation Assays

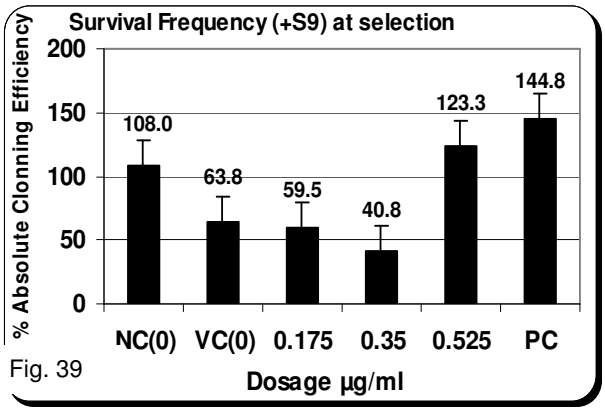


Fig. 39

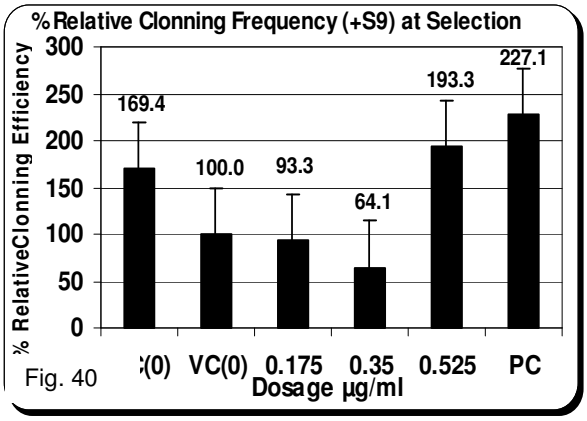


Fig. 40

Mutation Frequencies

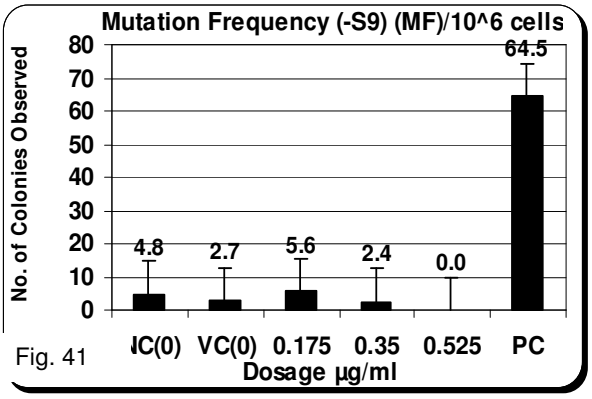


Fig. 41

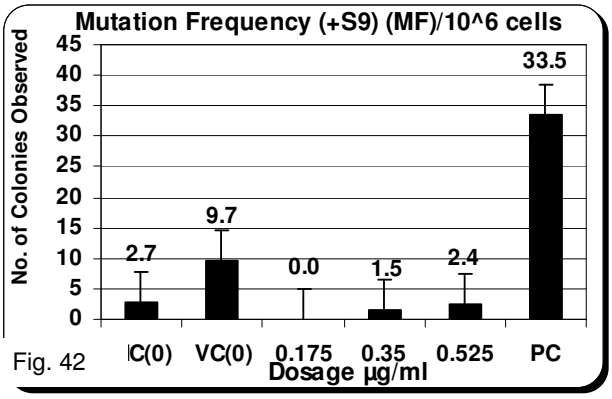


Fig. 42

CHROMOSOMAL ABERRATION TESTS

TABLE- 7: DOSE SELECTION ASSAYS CHROMOSOMAL ABERRATION

Table -7.1 Mitotic Index

Without Metabolic activation (4.0 hour Exposure, 2 hour colchicine 0.4µg/ml)						
Dose group	**Dose Levels µg/ml**	**Mitotic Index**		**Mean MI**	**Relative MI**	**% Reduction**
		Replicate 1	**Replicate 2**			
NC	0.00	4.3912	3.920	4.1556**	NA	NA
VC	0.00	1.998	2.4496	2.5691	100	NA
T1	0.045	1.959	2.076	2.0175	78.53	21.47
T2	0.178	1.764	1.3623	1.56315	60.84	39.16
T3	0.712	1.1522	1.1596	1.1559*	44.99	55.01
$y = -18.271x + 116.77$, $R^2 = 0.9952$, $r = 0.997597$						

Table -7.2 Mitotic Index

With Metabolic activation (4.0 hour Exposure, 2 hour colchicine 0.4µg/ml)						
Dose group	**Dose Levels µg/ml**	**Mitotic Index**		**Mean MI**	**%Relative MI**	**% Reduction**
		Replicate 1	**Replicate 2**			
NC	0.00	1.6553	2.453	2.05415	NA	NA
VC	0.00	2.2705	2.606	2.4382	100	NA
T1	0.045	1.994	2.078	2.036	79.25	20.75
T2	0.178	2.645	2.249	2.447	95.25	4.75
T3	0.712	1.8578	1.4559	1.65685	64.49	35.51
$y = -9.0528x + 107.38$, $R^2 = 0.523$, $r = 0.7231$						

Note: NC = Negative Control, VC = Vehicle Control, T1 – T3 are treatment concentrations, MI = Mitotic Index, n = 2, * = $p \leq 0.05$ and ** $p \leq 0.01$

Data Graphs
Chrosomal Aberration Assays (Dose Selection)

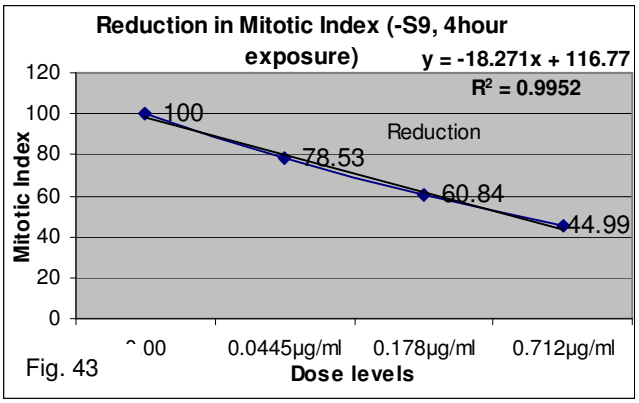


Fig. 43

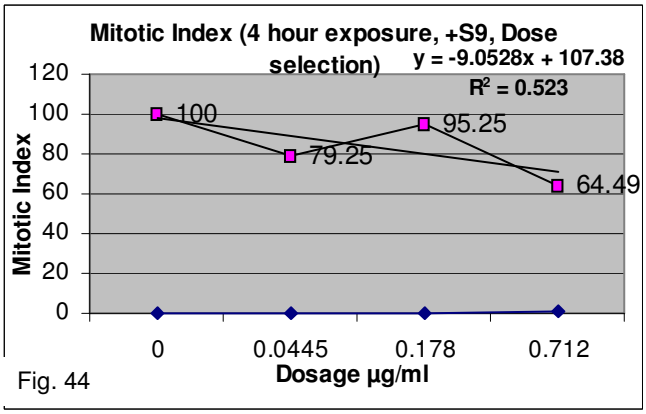


Fig. 44

CHROMOSOMAL ABERRATION

TABLE -8 MAIN STUDIES (MITOTIC INDEX AND CHROMOSOMAL ABERRATIONS)

Table - 8.1: Short-Term Exposure Mitotic Indexes (-S9)

Mitotic Index: Main Study Without metabolic activation (4h exposure)								
Dose		M	No. of cells		% Mitotic index	mean %mi	% Relative MI	Increase
Group	levels µg/ml		observed	Total				
NCR1	0	59	1050	1109	5.3201	4.6729	92.85	NA
NCR2	0	44	1049	1093	4.0256			
VCR1	0	56	1000	1056	5.3030	5.0325	NA	NA
VCR2	0	51	1020	1071	4.7619			
T1R1	0.175	96	1016	1112	8.6331	8.1487	161.92	61.92
T1R2	0.175	84	1012	1096	7.6642			
T2R1	0.350	46	1021	1067	4.3112	6.7447	134.02	34.02
T2R2	0.350	105	1039	1144	9.1783			
T3R1	0.525	83	1084	1167	7.1123	5.2835	104.99	4.99
T3R2	0.525	37	1034	1071	3.4547			
PCR1	MMC (0.5)	52	1046	1098	4.7359	4.5820	91.05	-8.95
PCR2	MMC (0.5)	48	1036	1084	4.4280			

Note: NC = Negative Control, VC = Vehicle Control, PC = Positive Control, M = Metaphases, MMC = Mitomycin-C, R = Replicate and n = 2, Values are not significant at any of the tested concentrations.

TABLE -8 MAIN STUDIES (MITOTIC INDEX AND CHROMOSOMAL ABERRATIONS)

Table - 8.2: Short-Term Exposure Mitotic Indexes (+S9)

Mitotic Index : Main Study With metabolic activation (4h exposure)								
Dose		M	No. of cells		%Mitotic Index	Mean %MI	% Relative MI	Increase
Group	Levels µg/ml		Observed	Total				
NCR1	0	112	1006	1118	10.0179	10.7850	115.84	NA
NCR2	0	131	1003	1134	11.5520			
VCR1	0	109	1071	1180	9.2373	9.3101	100.00	NA
VCR2	0	102	1041	1143	8.9239			
VCR3	0	110	1016	1126	9.7691			
T1R1	0.175	196	1174	1370	14.3066	14.8305**	156.06	56.06
T1R2	0.175	195	1075	1270	15.3543			
T2R1	0.350	115	1002	1117	10.2954	9.8161	103.29	3.29
T2R2	0.350	107	1039	1146	9.3368			
T3R1	0.525	106	1088	1194	8.8777	9.0589	95.32	4.68
T3R2	0.525	107	1051	1158	9.2401			
PCR1	Cyp(25)	85	1012	1097	7.7484	6.6962*	70.46	29.54
PCR2	Cyp(25)	78	1304	1382	5.6440			

Note: NC = Negative Control, VC = Vehicle Control, PC = Positive Control,
M = Metaphases, Cyp = Cyclophosphamide, R = Replicate n = 2,
* = $p \leq 0.05$ and ** $p \leq 0.01$

Table - 8.5: Short-Term Exposure Numerical Chromosomal Aberrations

Numerical Aberrations (-S9) 4 hour exposure						
Dose levels	**Ploidy**					
µg/ml	**Aneuploidy**	**Polyploidy**	**ER**	**Total Ploidy**	**Mean**	**SD**
0	0	3	0	3	2.00	1.41
0	0	1	0	1		
0	0	2	0	2	1.50	0.71
0	0	1	0	1		
0.175	0	1	0	1	1.00	0.00
0.175	0	1	0	1		
0.350	0	2	0	2	2.00	0.00
0.350	0	2	0	2		
0.525	0	1	0	1	1.00	0.00
0.525	0	1	0	1		
MMC (0.5)	0	4	0	4	5.50	2.12
	0	7	0	7		

Note: E.R = Endoreduplication, S.D. = Standard Dviations, MMC = Mitomycin-C.

Table - 8.6: Short-Term Exposure Numerical Chromosomal Aberrations

Numerical Aberration (+S9) 4 hour exposure						
Dose levels	**Ploidy**					
µg/ml	**Aneuploidy**	**Polyploidy**	**ER**	**Total Ploidy**	**Mean**	**SD**
NC	0	6	0	6	4.50	2.12
NC	0	3	0	3		
VC	0	14	0	14	11.00	3.61
VC	0	12	0	12		
VC	0	7	0	7		
0.175	0	4	0	4	5.50	2.12
0.175	0	7	0	7		
0.350	0	5	0	5	8.00	4.24
0.350	0	11	0	11		
0.525	0	14	0	14	10.00	5.66
0.525	0	6	0	6		
Cyp(25)	6	4	1	11	10.5	0.71
Cyp(25)	4	6	0	10		

Note: NC = Note Negative Control, VC = Vehicle Control, PC = Positive Control, ER = Endoreduplication, Cyp = Cyclophosphamide.

TABLE -9:LONG-TERM EXPOSURE (TRIAL-I) Table- 9.1: Chromosomal Aberrations

Chromosomal Aberration without metabolic activation (Long Term) Trial-I																		
Dose levels	M	Gap		Break		Dl.	Fragment		R	Ex.	Int.	Dic.	Trs. /inv.	Total Aberration		Aberrant Metaphases		% Ab Cell WT gap	Mean % aberrant Cell ± S.D
µg/ml		C	Cl	C	Cl		ACF	F						with gaps	WT gaps	with gap	WT gap		
NC	100	0	0	1	0	0	0	1	0	0	0	0	0	2	2	1	2	2	2.40 ± 1.14
NC	100	2	0	1	1	1	0	1	0	0	0	1	1	8	6	5	4	4	
NC	100	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	3	3	
NC	100	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	1	1	
NC	100	0	0	0	0	0	0	2	0	0	0	0	0	2	2	2	2	2	
VC	100	1	2	2	3	0	0	0	0	0	0	1	0	9	6	8	5	5	5.80 ± 2.77
VC	100	0	4	0	0	1	3	8	0	0	0	1	0	17	13	12	9	9	
VC	100	0	1	0	0	0	2	0	0	0	0	0	0	3	2	3	2	2	
VC	100	0	0	0	1	1	1	4	0	0	0	0	1	8	8	8	8	8	
VC	100	0	7	0	4	1	0	1	0	0	0	1	0	14	7	8	5	5	
0.175	100	0	0	1	0	2	2	9	0	0	0	1	1	16	16	12	12	12	8.80 ± 2.77
0.175	100	1	2	1	2	0	3	2	0	1	0	4	0	16	13	13	11	11	
0.175	100	0	2	1	0	0	2	1	0	0	1	0	0	7	5	7	5	5	
0.175	100	0	1	1	1	0	0	6	0	1	0	1	0	11	10	9	8	8	
0.175	100	0	0	0	2	0	3	2	1	0	0	2	0	10	10	8	8	8	
0.350	100	0	2	0	0	3	1	3	0	0	0	0	0	9	7	7	6	6	9.40 ± 4.39
0.350	100	5	0	0	5	2	2	6	0	0	0	0	0	20	15	17	16	16	
0.350	100	0	6	0	1	4	3	2	0	0	1	0	0	17	11	15	11	11	
0.350	100	0	4	0	2	0	1	1	0	0	1	0	0	9	5	8	5	5	
0.350	100	0	1	2	0	1	3	3	0	0	1	1	0	12	11	11	9	9	
0.525	100	0	4	0	0	5	2	5	0	0	0	3	0	19	15	15	14	14	11.47 ± 13.36
0.525	100	1	2	1	2	3	5	0	0	0	0	4	1	19	16	19	17	17	
0.525	84	0	2	0	1	0	4	0	0	0	0	1	1	9	7	9	7	8.3	
0.525	100	1	6	2	7	0	2	6	0	2	0	1	2	21	22	19	18	18	
EMS (0.1µl/ml)	100	0	3	10	14	5	12	9	1	9	6	7	6	82	79	43	43	43	37.00 ± 8.49
EMS (0.1µl/ml)	100	0	6	7	5	7	0	2	0	1	0	2	6	36	30	33	31	31	

Note: NC = Negative Control, VC = Vehicle Control, Dl. = Deletion, Int. = Interchange, WT = Without, Ab, = Aberrant and EMS = Ethyl Methyl Sulphonate.

Table- 9.2: Numerical Aberrations

Numerical Aberrations : (-S9) Trial-I (Long Term Exposure)						
Dose levels	**Aneuploidy**	**Polyploidy**	**ER**	**Total Ploidy**	**Mean**	**SD**
NC	0	7	0	7	6.20	2.95
NC	0	8	0	8		
NC	0	7	0	7		
NC	0	1	0	1		
NC	0	8	0	8		
VC	0	3	0	3	5.00	1.87
VC	0	6	0	6		
VC	0	3	0	3		
VC	1	6	0	7		
VC	1	5	0	6		
0.175	0	8	0	8	5.40	2.41
0.175	1	5	0	6		
0.175	0	7	0	7		
0.175	1	3	0	4		
0.175	0	2	0	2		
0.350	0	17	0	17	9.40	4.34
0.350	0	6	0	6		
0.350	0	8	0	8		
0.350	0	8	0	8		
0.350	0	8	0	8		
0.525	0	6	2	8	7.00	1.41
0.525	0	7	0	7		
0.525	0	5	0	5		
0.525	0	8	0	8		
EMS (0.1µl/ml)	0	13	0	13	12.50	0.71
	0	12	0	12		

TABLE -10:LONG-TERM EXPOSURE (TRIAL-II) With Excess colchicines

Trial-II (Chromosomal Aberration without metabolic activation), with excess colchicine																			
Dose levels	**M**	**Gap**		**Break**		**Dl.**	**Fragment**		**R**	**Ex.**	**Int.**	**Dic.**	**Trs. /inv.**	**Total Aberration**		**Aberrant Metaphases**		**Aberrant cell WT gap**	**Mean % aberrant Cell**	**S.D.**
□g/ml		**C**	**Cl**	**C**	**Cl**		**ACF**	**F**						**with gaps**	**without gaps**	**with gap**	**WT gap**			
NC (0.00)	100	1	0	1	0	0	0	1	1	0	0	1	0	5	4	5	4	4	4.50	0.71
NC (0.00)	100	0	0	1	0	1	0	2	0	1	0	1	0	6	6	5	5	5		
VC (0.00)	100	1	1	1	1	0	1	0	0	1	0	0	0	6	4	6	4	4	3.67	2.12
VC (0.00)	100	1	0	1	4	1	1	1	0	0	0	0	1	10	9	8	7	7		
0.175	100	1	1	0	1	0	5	2	0	0	0	0	0	10	8	8	7	7	9.00	5.16
0.175	100	0	0	0	0	0	7	1	0	0	0	3	1	12	12	11	11	11		
0.175	100	0	0	0	0	0	0	1	0	0	0	0	2	3	3	3	3	3.0		
0.175	100	0	7	3	6	1	4	1	1	0	0	1	0	19	17	18	15	15		
MMC 0.5)	50	3	0	4	4	2	1	7	1	0	0	3	0	25	22	14	13	26	31.52	2.12
MMC (0.5)	27	0	2	1	1	5	5	0	1	0	0	3	0	18	16	10	10	37.04		

Note: NC = Negative Control, VC = Vehicle Control, MMC = Mitomycin-C, Dl. = Deletion, Int. = Interchange, WT = Without

Table- 10.1: Numerical Aberrations

Numerical Aberration (-S9) with excess colchicine						
Dose levels	**Ploidy**					
µg/ml	**Aneuploidy**	**Polyploidy**	**ER**	**Total Ploidy**	**Mean**	**SD**
NC (0.00)	4	6	0	10	7.00	4.24
NC (0.00)	3	1	0	4		
VC (0.00)	1	4	0	5	5.00	0.00
VC (0.00)	0	5	0	5		
0.175	2	2	3	7	13.75	6.70
0.175	10	5	4	19		
0.175	8	12	0	20		
0.175	0	6	3	9		
MMC(0.5)	2	6	3	11	7	5.66
MMC(0.5)	3	0	0	3		

Note: NC = Negative Control, VC = Vehicle Control, MMC = Mitomycin-C.

Data Graphs
Chrosomal Aberration Assays

Chromosomal Aberration

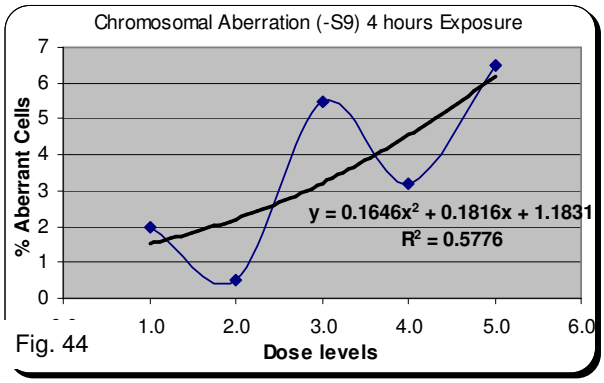


Fig. 44

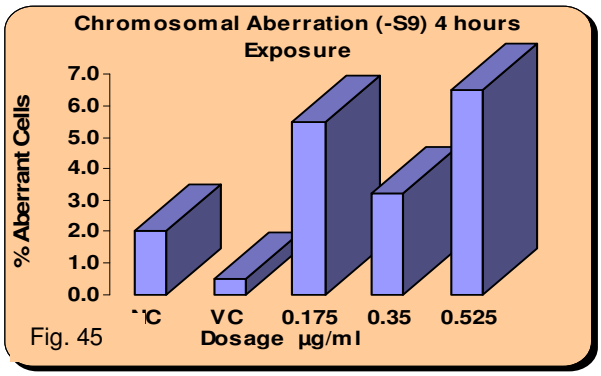


Fig. 45

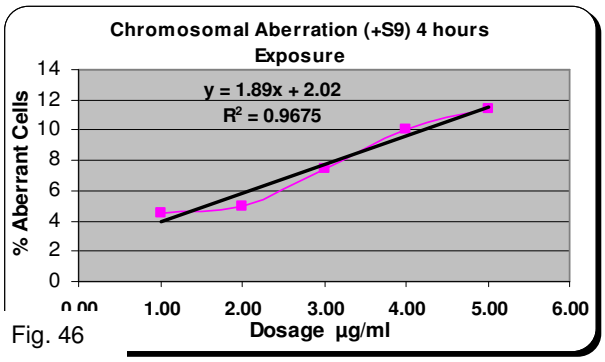


Fig. 46

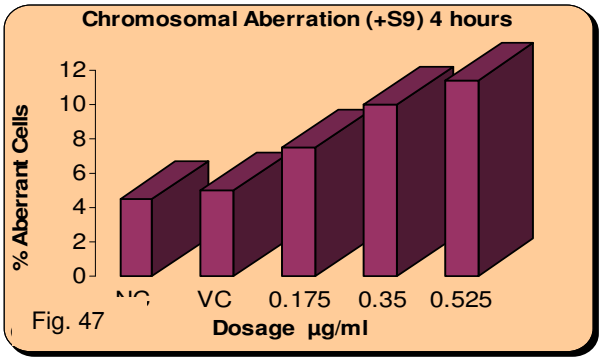


Fig. 47

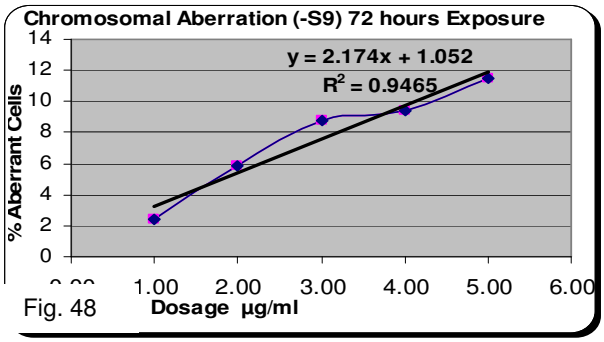


Fig. 48

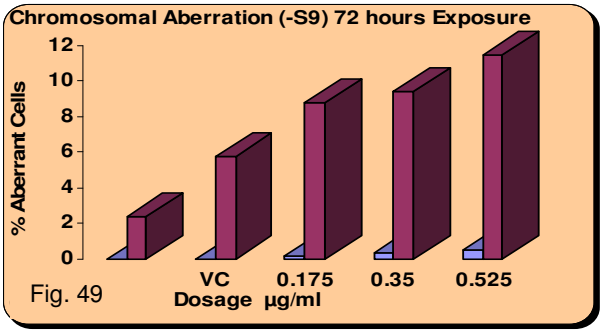


Fig. 49

IN VIVO ASSAYS

Result Summary Tables

Table: 11 Symptoms of toxicity (Range Finding Study)

Dose (mg/kg body weight)	Mortality		Symptoms Morphological	Gross pathology
	Male	Female		
100	Died (1/1)	Died (1/1)	Both male and female Abdominal breathing and convulsions, catalapsy and death	Male and female Liver: mottling (+++) Lung : congestion (++) Kidney : mottling (+)
40	Died (1/1)	Died (1/1)	Both male and female Abdominal breathing and convulsion, catalapsy and death	Male and female Liver: mottling (+) Lung : congestion (+) Kidney : mottling (+) only male
20 (day-1)	Died (0/1)	Died (0/1)	Male Abdominal breathing was observed only in male animal, which got recovered after 4 hrs. Female The animal was devoid of symptoms.	-
20 (day-2)	Died (2/3)	Died (0/1)	Male (before death) Exaggerated response to sound stimuli, Clonic tonic convulsion of fore and hind limbs, Catalapsy, Salivatioin. Lacrimation, Convulsion, Writhing. Dilation of pupil. Male Moderate abdominal breathing was observed Female Normal.	male Liver: mottling (++) Lung : congestion (+) Kidney : mottling (+)
10 (day-1)	Died (1/1)	Died (0/1)	Male Salivation and death Female Normal.	male Liver: mottling (+) Lung : congestion (+) Kidney: -

TABLE: 12: MAIN STUDY (GROSS PATHOLOGICAL OBSERVATION AT NECROPSY)

Animal No.	Dose mg/Kg body weight	sex	External observation	organ	Gross pathological observation
1-8	0.0	Male and female	-	-	-
9-16	4.0	Male and female	-	-	-
17-20	8.0	male	Abdominal breathing, Tremors, Salivation	Lung(Right cranial lobe) Liver Kidney	Mild congestion - -
			Abdominal breathing, Tremors, Salivationand nasal discharge	Lung (all the lobes) Liver Kidney	Congestion Mottling (mild) -
			Hyper activity Abdominal breathing	Lung Liver Kidney	- mottling(mild) -
21-24		female	Tremor, writhing,salivation finally bleeding from mouth	Lung Liver Kidney	- - -

TABLE: 13 – WEEKLY BODY WEIGHT

Table: 13.1 Mice Weekly Body Weight (Male)

WEEK	GROUP	MEAN	SD	SE	p-value significance
WEEK-1	G1	40.39	3.49	1.75	-
	G2	39.71	3.13	1.56	0.767
	G3	40.77	1.20	0.60	0.847
WEEK –2	G1	40.30	3.26	1.63	-
	G2	41.84	1.88	0.94	0.468
	G3	42.12	1.87	0.93	0.430
WEEK-3	G1	42.59	2.34	1.17	-
	G2	42.64	1.14	0.57	0.971
	G3	42.16	1.89	0.95	0.829
WEEK-4	G1	43.00	3.91	1.95	-
	G2	42.57	0.82	0.41	0.840
	G3	40.27	3.39	1.69	0.418

Table: 13.2 Mice Weekly Body Weight (Female)

WEEK	GROUP	MEAN	SD	SE	p-value significance
WEEK-1	G1	35.66	4.42	2.21	-
	G2	36.46	2.76	1.38	0.797
	G3	32.82	1.40	0.70	0.380
WEEK –2	G1	35.23	4.11	2.05	-
	G2	36.02	2.86	1.43	0.802
	G3	34.97	0.94	0.47	0.901
WEEK-3	G1	38.22	4.49	2.25	-
	G2	38.77	1.86	0.93	0.859
	G3	36.45	1.12	0.56	0.554
WEEK-4	G1	36.90	4.17	2.08	-
	G2	37.11	1.93	0.97	0.936
	G3	36.02	1.54	0.77	0.734

Note: G1 – G3 are Control (G1) and treatment groups (G2, G3), SD = Standard deviation and SE = standard error.

ESULTS OF HEMATOLOGY (FEMALE)

	GROUP	MEAN	SD	SE	P-VALUE SIGNIFICANCE
WBC	G1	5.20	1.49	0.74	-
	G2	4.88	1.44	0.72	0.352
	G3	4.85	1.00	0.50	0.438
RBC	G1	8.47	0.35	0.17	-
	G2	8.55	0.58	0.29	0.827
	G3	8.02	0.42	0.21	0.024*
Hb	G1	14.25	0.62	0.31	-
	G2	14.38	1.24	0.62	0.874
	G3	14.23	0.98	0.49	0.961
HCT	G1	42.13	0.78	0.39	-
	G2	42.33	3.17	1.59	0.905
	G3	42.15	1.00	0.50	0.952
MCV	G1	-49.78	1.13	0.56	-
	G2	-49.50	0.59	0.30	0.754
	G3	-51.63	0.83	0.42	0.015*
MCH	G1	-8.38	16.85	8.43	-
	G2	-16.85	0.40	0.20	0.383
	G3	-9.13	17.15	8.58	0.060
MCHC	G1	33.83	0.91	0.46	-
	G2	33.95	0.51	0.25	0.855
	G3	34.00	1.23	0.62	0.689
PLT	G1	842.75	225.61	112.80	-
	G2	1109.00	76.83	38.42	0.161
	G3	1125.50	81.62	40.81	0.136

Note: WBC = White blood cells, RBC = red blood cells, Hb = Haemoglobin, HCT = hemetocrit,
MCV = mean corpuscular volume, SD = Standard Deviation, SE = Standard Error, MCH = Mean Corpuscular Haemoglobin, MCHC = Mean Corpuscular Haemoglobin Concentration, PLT = platelets, * $p \leq 0.05$.

TABLE: 14.4 INDIVIDUAL ANIMAL VALUES (FEMALE ANIMALS)

HAEMATOLOGICAL ANALYSIS OF WHOLE BLOOD IN FEMALE ANIMALS								
ANIMAL NO.	WBC (103/MM3)	RBC (106/MM3)	Hb (G/DL)	HCT (%)	MCV (MM3)	MCH (PG)	MCHC (G/DL)	PLT (103/MM)
5	6.8	8.15	13.5	41.4	-50.8	-16.6	32.6	882
6	5.4	8.94	15	43.1	-48.2	-16.8	34.8	1121
7	3.2	8.51	14.4	42.4	-49.8	16.9	34	792
8	5.4	8.27	14.1	41.6	-50.3	-17	33.9	576
MEAN	5.2	8.4675	14.25	42.125	-49.775	-8.375	33.825	842.75
SD	1.49	0.35	0.62	0.78	1.13	16.85	0.91	225.61
REF. RANGE-4TH WK.	2.47± 0.80	9.34 ± 0.52	12.99 ± 0.57	37.96 ± 2.00	40.66 ± 0.70	13.92 ± 0.40	34.24 ± 0.54	260.40 ± 2718
REF.RANGE-12TH WK	5.26-6.92	10.35-11.05	14.82-15.89	44.38-47.32	42.17-43.39	14.13-14.69	33.57-34.03	10.82-14.21
13	6	9.09	15.6	45.1	-49.6	-17.2	34.6	1090
14	5.9	8.68	14.9	43.7	-50.3	-17.2	34.1	1071
15	2.9	8.69	14.3	42.7	-49.1	-16.5	33.5	1053
16	4.7	7.72	12.7	37.8	-49	-16.5	33.6	1222
MEAN	4.875	8.545	14.375	42.325	-49.5	-16.85	33.95	1109
SD	1.44	0.58	1.24	3.17	0.59	0.40	0.51	76.83
REF. RANGE-4TH WK.	2.47± 0.80	9.34 ± 0.52	12.99 ± 0.57	37.96 ± 2.00	40.66 ± 0.70	13.92 ± 0.40	34.24 ± 0.54	260.40 ± 2718
REF.RANGE-12TH WK	5.26-6.92	10.35-11.05	14.82-15.89	44.38-47.32	42.17-43.39	14.13-14.69	33.57-34.03	10.82-14.21
21	6.3	7.94	13.9	41.9	-52.8	-17.5	33.2	1210
22	4.5	8.57	15.6	43.6	-50.9	-18.2	35.8	1014
23	4	8.01	13.3	41.3	-51.6	16.6	33.2	1144
24	4.6	7.56	14.12	41.8	-51.2	-17.4	33.8	1134
MEAN	4.85	8.02	14.23	42.15	-51.625	-9.125	34	1125.5
SD	1.00	0.42	0.98	1.00	0.83	17.15	1.23	81.62
REF. RANGE-4TH WK.	2.47± 0.80	9.34 ± 0.52	12.99 ± 0.57	37.96 ± 2.00	40.66 ± 0.70	13.92 ± 0.40	34.24 ± 0.54	260.40 ± 2718
REF.RANGE-12TH WK	5.26-6.92	10.35-11.05	14.82-15.89	44.38-47.32	42.17-43.39	14.13-14.69	33.57-34.03	10.82-14.21

Note: WBC = White blood cells, RBC = red blood cells, Hb = Haemoglobin, HCT = hemetocrit, MCV = mean corpuscular volume, SD = Standard Deviation, SE = Standard Error, MCH = Mean Corpuscular Haemoglobin, * $p \leq 0.05$.

TABLE: 15 ANALYSIS RESULTS OF DIFFERENTIAL LEUCOCYTE COUNTS

TABLE: 15.1 (MALE ANIMALS) 1ST DAY AFTER EXPOSURE

PARAMETER	GROUP	MEAN	SD	SE	p - value of significance
LYMPHOCYTES	G1	79.00	6.98	3.49	-
	G2	74.25	11.95	5.98	0.514
	G3	75.00	5.29	0.65	0.671
NEUTROPHILS	G1	16.75	4.99	2.50	-
	G2	23.00	11.97	5.99	0.501
	G3	22.75	5.97	2.98	0.308
MONOCYTES	G1	1.50	1.29	0.65	-
	G2	1.75	2.36	1.18	0.886
	G3	1.25	0.96	0.48	0.761
EOSINOPHILS	G1	2.75	1.50	0.75	-
	G2	1.00	0.82	0.41	0.133
	G3	1.00	1.15	0.58	0.133

TABLE: 15.2 INDIVIDUAL ANIMAL VALUES DLC COUNTS (MALE ANIMALS)

1ST DAY AFTER EXPOSURE DIFFERENTIAL LEUCOCYTE COUNT (MALE ANIMALS)						
MICE NO	LYMPHOCYTE COUNT	NEUTROPHIL	MONOCYTE	EOSINOPHIL	BASOPHIL	TOTAL CELLS
1	73	22	1	4	0	100
2	84	13	2	1	0	100
3	73	20	3	4	0	100
4	86	12	0	2	0	100
MEAN	79	16.75	1.5	2.75	0	100
SD	6.98	4.99	1.29	1.50	0.00	0.00
REF.RANGE	78.01-84.71	13.13-19.67	1.0-2.0	1.0-2.0	0-1	—
9	86	12	0	2	0	100
10	60	39	0	1	0	100
11	82	16	2	0	0	100
12	69	25	5	1	0	100
MEAN	74.25	21	1.75	1	0	100
SD	11.95	11.97	2.36	0.82	0.00	0.00
17	76	24	0	0	0	100
18	72	27	1	0	0	100
19	82	14	2	2	0	100
20	70	26	2	2	0	100
MEAN	75	22.75	1.25	1	0	100
SD	5.29	5.97	0.96	1.15	0.00	0.00

Note: G1 – G3 are Control (G1) and treatment groups (G2, G3), SD = Standard Deviation,
SE = Standard Error.

TABLE: 15 ANALYSIS RESULTS OF DIFFERENTIAL LEUCOCYTE COUNTS
TABLE: 15.3 (FEMALE ANIMALS) 1ST DAY AFTER EXPOSURE

PARAMETER	GROUP	MEAN	SD	SE	p-value significance
LYMPHOCYTES	G1	82.50	1.29	0.65	-
	G2	79.75	9.11	4.55	0.591
	G3	75.50	6.35	3.18	0.155
NEUTROPHILS	G1	14.25	2.75	1.38	-
	G2	17.25	8.42	4.21	0.536
	G3	20.50	3.70	1.85	0.127
MONOCYTES	G1	1.25	0.96	0.48	-
	G2	2.00	1.63	0.82	0.486
	G3	2.00	1.41	0.71	0.319
EOSINOPHILS	G1	2.00	0.82	0.41	-
	G2	1.00	0.82	0.41	0.182
	G3	2.00	1.63	0.82	1.000

TABLE: 15.4 INDIVIDUAL ANIMAL VALUES DLC COUNTS (FEMALE ANIMALS)

1ST DAY AFTER EXPOSURE DIFFERENTIAL LEUCOCYTE COUNT (FEMALE ANIMALS)						
MICE NO	LYMPHOCYTE COUNT	NEUTROPHIL	MONOCYTE	EOSINOPHIL	BASOPHIL	TOTAL CELLS
5	82	16	0	2	0	100
6	81	17	1	1	0	100
7	84	11	2	3	0	100
8	83	13	2	2	0	100
MEAN	82.5	14.25	1.25	2	0	100
SD	1.29	2.75	0.96	0.82	0.00	0.00
REF.RANGE	83.5-92.32	6.6-14.25	1.0-4.0	0-1	0	—
13	67	29	2	2	0	100
14	88	9	2	1	0	100
15	84	15	0	1	0	100
16	80	16	4	0	0	100
MEAN	79.75	17.25	2	1	0	100
SD	9.11	8.42	1.63	0.82	0.00	0.00
21	78	19	1	2	0	100
22	79	19	2	0	0	100
23	66	26	4	4	0	100
24	79	18	1	2	0	100
MEAN	75.5	20.5	2	2	0	100
SD	6.35	3.70	1.41	1.63	0.00	0.00

Note: G1 – G3 are Control (G1) and treatment groups (G2, G3), SD = Standard Deviation,
SE = Standard Error.

TABLE: 16 ANALYSIS RESULTS OF DIFFERENTIAL LEUCOCYTE COUNTS

Table: 16.1 (Male animals) 1st week after exposure

Parameter	GROUP	MEAN	SD	SE	p-value significance
Lymphocytes	G1	77.50	6.61	3.30	-
	G2	70.75	6.13	3.07	0.279
	G3	50.00	11.60	5.80	0.031*
Neutrophils	G1	21.00	6.58	3.29	-
	G2	26.00	6.32	3.16	0.369
	G3	48.25	12.31	6.16	0.037*
Monocytes	G1	1.00	0.82	0.41	-
	G2	0.25	0.50	0.25	0.215
	G3	1.00	0.82	0.41	1.000
Eosinophils	G1	0.50	0.58	0.29	-
	G2	3.00	1.83	0.91	0.063
	G3	0.75	0.96	0.48	0.638

Table: 16.2 Individual Animal Values DLC counts (Male Animals)

1st week after repetative exposure DLC counts (Male animals)						
Mice No	**Lymphocyte count**	**Neutrophil**	**Monocyte**	**Eosinophil**	**Basophil**	**Total cells**
1	69	29	2	0	0	100
2	77	22	0	1	0	100
3	79	20	1	0	0	100
4	85	13	1	1	0	100
mean	77.5	21	1	0.5	0	100
SD	6.61	6.58	0.82	0.58	0.00	0.00
9	69	30	0	1	0	100
10	74	24	0	2	0	100
11	77	18	1	4	0	100
12	63	32	0	5	0	100
mean	70.75	26	0.25	3	0	100
SD	6.13	6.32	0.50	1.83	0.00	0.00
17	45	53	1	1	0	100
18	67	30	1	2	0	100
19	47	53	0	0	0	100
20	41	57	2	0	0	100
mean	50*	48.25*	1	0.75	0	100
SD	11.60	12.31	0.82	0.96	0.00	0.00

Note: G1 – G3 are Control (G1) and treatment groups (G2, G3), SD = Standard Deviation,
SE = Standard Error, DLC = differential Leucocyte Count, * $p \leq 0.05$.

TABLE: 17-REPEATED TREATMENT BIOCHEMICAL TEST (Male)

Table: 17.1- Biochemical Test (Male)

	GROUP	MEAN	SD	SE	p-value significance
GLUCOSE	G1	145.85	15.38	8.88	-
	G2	152.95	13.69	7.91	0.651
	G3	149.65	8.03	4.64	0.756
ALT	G1	53.52	21.11	14.22	-
	G2	43.91	19.19	11.08	0.473
	G3	47.16	19.76	11.41	0.721
AST	G1	109.36	57.89	8.49	-
	G2	59.76	18.95	10.94	0.965
	G3	61.17	13.62	7.87	0.917
CRE	G1	0.45	0.41	0.23	-
	G2	0.20	0.03	1.93	0.371
	G3	0.19	0.03	1.60	0.369
BUN	G1	18.77	1.15	0.66	-
	G2	17.64	2.49	1.44	0.275
	G3	16.67	2.31	1.34	0.270
ALB	G1	2.61	0.28	0.16	-
	G2	2.96	0.15	0.01	0.057
	G3	2.62	0.30	0.18	0.919
CHE	G1	3111	195.61	-	-
	G2	3554	741.68	-	0.465
	G3	3616.25	633.04	-	0.314

Note: G1 – G3 are Control (G1) and treatment groups (G2, G3), ALT = alanine aminotransferase, AST = aspartate aminotransferase, CRE = creatinine, BUN = blood urea nitrogen, ALB = albumin, CHE = choline esterase, SD = Standard Deviation, SE = Standard Error.

CHOLINESTRASE ASSAYS

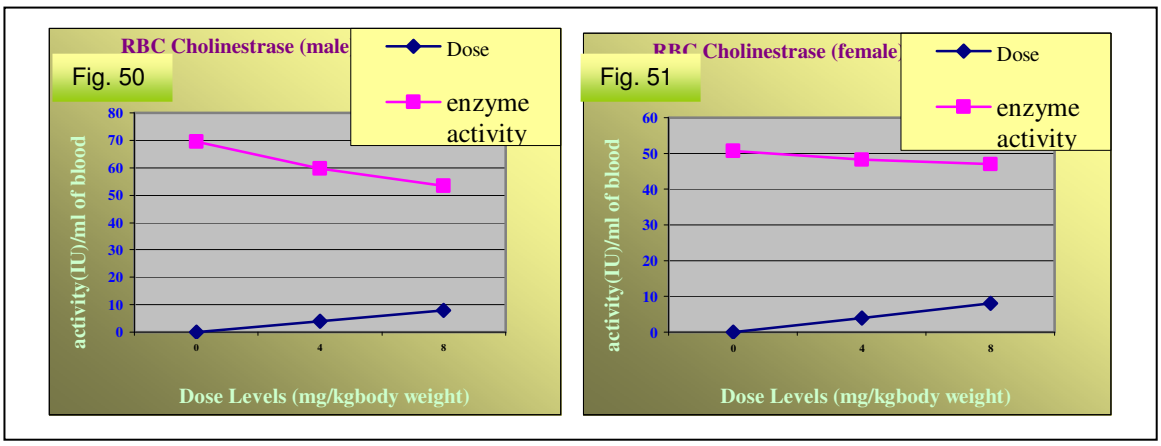


TABLE: 17- REPEATED TREATMENT BIOCHEMICAL TEST (MALE)

Table: 17.2 BIOCHEMICAL TEST (MALE)

REPEATED TREATMENT BIOCHEMICAL TEST (MALE)							
Animal No.	**Glucose**	**ALT**	**AST**	**CRE**	**BUN**	**ALB**	**CHE**
1	136	28.3	40.63	0.31	19.67	2.62	3332
2	161.1	75.65	151.63	0.18	19.32	2.83	2816
3	160.5	36.99	65.39	0.15	16.8	2.84	3063
4	125.8	73.12	179.79	1.14	19.3	2.16	3233
MEAN	145.85	53.515	109.36	0.445	18.7725	2.6125	3111
SD	15.38	21.11	57.89	0.41	1.15	0.28	195.61
Ref. Range- 4th Wk.	132.7± 20.7	89.9±34.1	92.1±36.7	0.2±0.0	19.7±2.8	2.4±0.1	—
Ref. Range- 12th wk	106.94-161.06	32.86-42.24	43.78-106.22	0.12-0.30	18.43-23.37	—	—
9	149.9	24.13	44.12	0.18	18.32	3.15	3104
10	164.5	25.37	40.89	0.16	20.07	2.99	4482
11	131.8	61.73	87.98	0.25	13.47	2.98	4030
12	165.6	64.4	66.04	0.2	18.69	2.73	2600
MEAN	152.95	43.9075	59.7575	0.1975	17.6375	2.9625	3554
SD	13.69	19.19	18.95	0.03	2.49	0.15	741.68
Ref. Range- 4th Wk.	132.7± 20.7	89.9±34.1	92.1±36.7	0.2±0.0	19.7±2.8	2.4±0.1	—
Ref. Range- 12th wk	106.94-161.06	32.86-42.24	43.78-106.22	0.12-0.30	18.43-23.37	—	—
17	162	80.09	78.36	0.18	19.73	2.83	3494
18	140.6	34.82	60.53	0.24	15.85	2.91	3617
19	151	44.36	65.32	0.17	17.64	2.62	4566
20	145	29.36	40.45	0.18	13.44	2.13	2788
MEAN	149.65	47.1575	61.165	0.1925	16.665	2.6225	3616.25
SD	8.03	19.76	13.62	0.03	2.31	0.30	633.04
Ref. Range- 4th Wk.	132.7± 20.7	89.9±34.1	92.1±36.7	0.2±0.0	19.7±2.8	2.4±0.1	—
Ref. Range- 12th wk	106.94-161.06	32.86-42.24	43.78-106.22	0.12-0.30	18.43-23.37	—	—

Note: ALT = alanine aminotransferase, AST = aspartate aminotransferase, CRE = creatinine, BUN = blood urea nitrogen, ALB = albumin, CHE = choline esterase, SD = Standard Deviation.

Table: 17 REPEATED TREATMENT BIOCHEMICAL TEST
Table: 17.3 BIOCHEMICAL TEST (FEMALE)

PARAMETERS	GROUP	MEAN	SD	SE	p-value significance
GLUCOSE	G1	123.73	25.64	14.81	-
	G2	140.63	12.65	7.31	0.41
	G3	178.63	12.32	7.11	0.06
ALT	G1	40.42	6.07	3.51	-
	G2	27.44	5.68	3.28	0.026
	G3	28.81	4.74	2.73	0.07*
AST	G1	70.57	9.15	5.28	-
	G2	54.47	5.7	3.29	0.09
	G3	52.83	4.19	2.42	0.10
CRE	G1	0.28	0.07	4.31	-
	G2	0.21	0.05	2.78	0.37
	G3	0.15	0.03	1.47	0.039*
BUN	G1	19.66	4.43	2.56	-
	G2	14.98	2.15	1.24	0.18
	G3	16.88	1.35	0.78	0.40
ALB	G1	3.31	0.12	6.868E	-
	G2	3.16	0.39	0.23	0.58
	G3	3.11	0.32	0.19	0.36
CHE	G1	5439.75	998.07	576.23	-
	G2	5525.00	670.24	386.96	0.89
	G3	5748.75	446.16	257.59	0.54

Note: G1 – G3 are Control (G1) and treatment groups (G2, G3), ALT = alanine aminotransferase, AST = aspartate aminotransferase, CRE = creatinine, BUN = blood urea nitrogen, ALB = albumin, SD = Standard Deviation, SE = Standard Error, CHE = choline esterase, *$p \leq 0.05$.

Table: 17 REPEATED TREATMENT BIOCHEMICAL TEST

Table: 17.4 BIOCHEMICAL TEST (FEMALE)

Biochemical Analysis Individual Animals Data (Female)							
Animal No.	**Glucose**	**ALT**	**AST**	**CRE**	**BUN**	**ALB**	**CHE**
5	135.8	47.02	58.86	0.34	24.8	3.13	5649
6	84.9	45.86	65.14	0.15	19.24	3.3	6944
7	119.5	33.51	75.84	0.31	21.82	3.46	4907
8	154.7	35.27	82.42	0.31	12.78	3.35	4259
MEAN	123.725	40.415	70.565	0.2775	19.66	3.31	5439.75
SD	25.64	6.07	9.15	0.07	4.43	0.12	998.07
Ref. Range-4th Wk.	80.9±14.92	85.3±7.9	88.2±20.32	0.23±0.08	20.8±3.7	2.85±0.27	—
Ref. Range-12th wk	133.44-177.56	34.11-98.99	68.98-103.02	0.1-0.23	15.45-18.35	—	—
13	153.4	37.05	58.63	0.16	12.64	3.32	6303
14	135.1	23.49	48.05	0.25	17.29	3.45	5458
15	151.5	23.01	61.47	0.27	16.94	3.38	5853
16	122.5	26.2	49.74	0.17	13.04	2.48	4486
MEAN	140.625	27.4375	54.4725	0.2125	14.9775	3.1575	5525
SD	12.65	5.68	5.70	0.05	2.15	0.39	670.24
Ref. Range-4th Wk.	80.9±14.92	85.3±7.9	88.2±20.32	0.23±0.08	20.8±3.7	2.85±0.27	—
Ref. Range-12th wk	133.44-177.56	34.11-98.99	68.98-103.02	0.1-0.23	15.45-18.35	—	—
21	159.5	23.64	59.87	0.14	15.28	3.13	5280
22	182.8	35.75	51.61	0.12	18.95	3.27	6480
23	193.6	30.51	50.95	0.19	17.06	3.45	5557
24	178.6	25.35	48.88	0.15	16.24	2.58	5678
MEAN	178.625	28.8125*	52.8275	0.15*	16.8825	3.1075	5748.75
SD	12.32	4.74	4.19	0.03	1.35	0.32	446.16
Ref. Range-4th Wk.	80.9±14.92	85.3±7.9	88.2±20.32	0.23±0.08	20.8±3.7	2.85±0.27	—
Ref. Range-12th wk	133.44-177.56	34.11-98.99	68.98-103.02	0.1-0.23	15.45-18.35	—	—

Note: ALT = alanine aminotransferase, AST = aspartate aminotransferase, CRE = creatinine, BUN = blood urea nitrogen, SD = Standard Deviation, ALB = albumin, CHE = choline esterase, * $p \leq 0.05$.

REPEATED DOSE MICRONUCLEUS ASSAY

TABLE 18: PCE TO TOTAL ERYTHROCYTES RATIO

Table: 18. PCE/TOTAL ERYTHROCUTES MALE AND FEMALE ANIMALS

Sex	GROUP	MEAN	SD	SE	p-value significance	Regration
Male	G1	0.60	3.14	1.57	-	Y = 0.0024x + 0.6128 r = 0.49
	G2	0.64	3.90	1.95	0.318	
	G3	0.62	1.50	7.52	0.438	
Female	G1	0.66	5.75	2.87	-	Y = -0.0163x + 0.6779 r = 0.87
	G2	0.65	4.83	2.41	0.960	
	G3	0.53	5.69	2.84	0.085	

Note: G1 – G3 are Control (G1) and treatment groups (G2, G3), PCE = Polychromatic Eruthrocytes, SD = Standard Deviations, SE = Standard Error, n = 4.

TABLE: 19. BONE MARROW MICRONUCLEUS FREQUENCY

Table: 19.1 Micronucleus frequency mice bone marrow (Male Animals)

Micronucleus frequency mice bone marrow (Male Animals)							
Animal Number, Sex & Dose group	**PCE**	**MN PCE**	**NCE**	**MN NCE**	**Total Erythrocytes (E)**	**% MN PCE among total**	**% MN NCE among total**
A no 1-4, Male (0.0) mg/Kg Body weight	2621	3	1888	1	4513	0.066	0.022
	2581	3	1827	1	4412	0.068	0.023
	2666	3	1814	2	4485	0.067	0.045
	2527	1	1369	0	3897	0.026	0
Mean	2598.8	2.5	1724.5	1	4326.8	0.057	0.022
SD	59.11	1	239.19	0.82	289.65	0.021	0.018
A no 9-12, Male (4.0) mg/Kg Body weight	2740	12	1232	1	3985	0.301	0.025
	2542	4	1332	2	3880	0.103	0.052
	2507	7	1547	0	4061	0.172	0
	2528	6	1661	3	4198	0.143	0.071
Mean	2579.3	7.25	1443	1.5	4031	0.18	0.037
SD	108.13	3.4	195.94	1.29	133.8	0.086	0.031
A no 17-20, Male (8.0) mg/Kg Body weight	2548	9	1562	4	4123	0.218	0.097
	3210	16	1919	3	5148	0.311	0.058
	2615	15	1483	5	4118	0.364	0.121
	2940	12	1941	4	4897	0.245	0.082
Mean	2828.3	13	1726.3	4	4571.5	0.285**	0.09**
SD	306.72	3.16	237.64	0.82	530.76	0.066	0.027

Note: PCE = Polychromatic Erythrocytes, NCE = Normochromatic Erythrocytes
MN PCE = Micrnocleated Polychromatic Erythrocytes, % = Percent,
MN NCE = Micrnocleated Normochromatic Erythrocytes, SD = Standard Deviations,
SE = Standard Error, n = 4, ** = Values are significant at 0.001.

Table 19.2 Micronucleus frequency mice bone marrow (Female Animals)

Micronucleus frequency mice bone marrow (Female Animals)							
Animal Number, Sex & Dose group	**PCE**	**MN PCE**	**NCE**	**MN NCE**	**Total Erythrocytes (E)**	**% MN PCE among total**	**% MN NCE among total**
A no 5-8, Female (4.0) mg/Kg Body weight	2780	2	1123	0	3905	0.051	0
	2505	2	1150	1	3658	0.055	0.027
	2658	2	1930	3	4593	0.044	0.065
	2721	6	1449		4176	0.144	0
Mean	2666	3	1413	1.33	4083	0.073	0.023
SD	118.33	2	374.99	1.53	400.44	0.047	0.031
A no 13-16, Female (4.0) mg/Kg Body weight	2581	12	1387	1	3981	0.301	0.025
	2565	10	1295	1	3871	0.258	0.026
	2805	11	1149	3	3968	0.277	0.076
	2511	3	1728	1	4243	0.071	0.024
Mean	2615.5	9	1389.75	1.5	4015.75	0.227	0.038
SD	129.83	4.08	245.87	1	159.25	0.106	0.025
A no 21-24, Female (8.0) mg/Kg Body weight	2823	11	3462	6	6302	0.175	0.095
	3423	14	3108	7	6552	0.214	0.107
	2706	10	2248	3	4967	0.201	0.06
	2620	11	1860	3	4494	0.245	0.067
Mean	2893	11.5	2669.50	4.75	5578.75	0.209**	0.082
SD	363	1.73	742.36	2.06	1003.53	0.029	0.022

Note: PCE = Polychromatic Erythrocytes, NCE = Normochromatic Erythrocytes
MN PCE = Micrnocleated Polychromatic Erythrocytes, % = Percent
MN NCE = Micrnocleated Normochromatic Erythrocytes, SD = Standard Deviations,
SE = Standard Error, n = 4, ** = Values are significant at 0.001.

Data Graphs

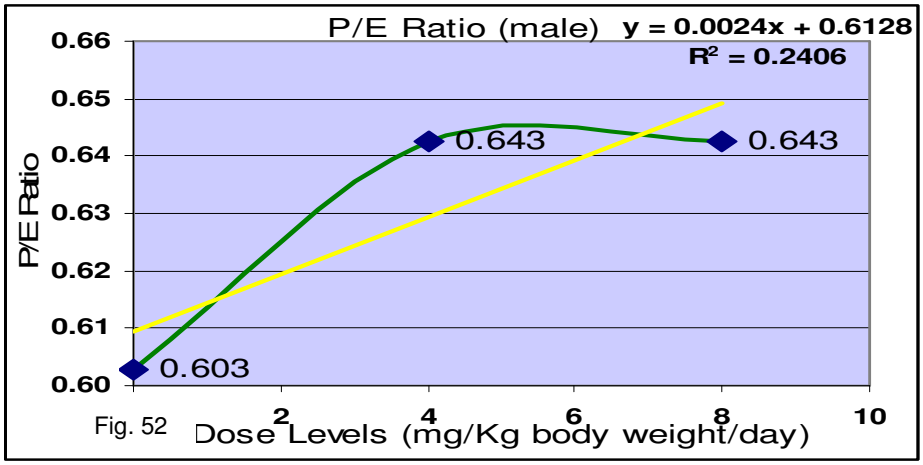


Fig. 52

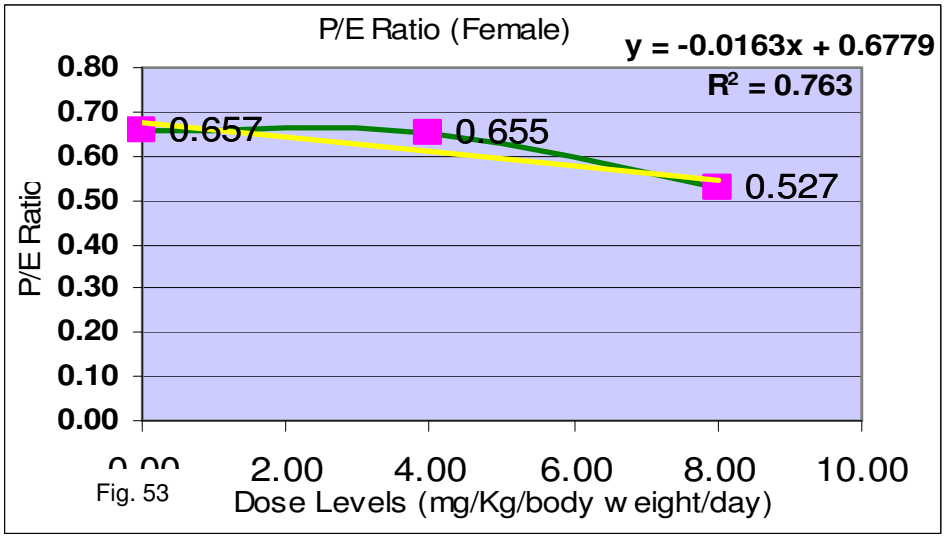


Fig. 53

Table: 20 Sperm Morphology Test

Sperm Morphology												
Dose mg/Kg body weight	Head - Abnormality									Tail - Abnormality		
	N	Total Abnormal Sperm	% Head Ab.	Am.	banana shape	large head	small head	double head	without hook	Coiled	Folded	DT
0	1005	8	0.796	1	0	0	5	0	2	0	0	0
0	1253	5	0.399	1	1	0	0	0	3	1	0	0
0	1003	9	0.897	4	0	2	1	0	2	0	0	0
0	1061	3	0.283	1	0	0	0	0	2	0	0	0
4	1004	16	1.594	3	1	1	5	0	6	3	10	0
4	1046	16	1.530	5	0	2	3	0	6	3	17	0
4	1007	7	0.695	3	0	1	0	0	3	0	12	1
4	1002	16	1.597	6	1	0	1	1	7	1	16	1
8	1003	49	4.885	29	3	4	10	0	3	3	277	0
8	1009	39	3.865	19	4	6	7	0	3	3	176	0
8	1000	36	3.600	16	2	7	6	0	5	62	200	0
8	1001	67	6.693	27	8	9	12	0	11	50	95	0

Note: N = Number of Sperm Examined, % Head Ab. = **%** Head Abnormality Am. =Amorphous, DT = Double tailed

Table: 20.1

Dose mg/Kg body weight	Total No. of Sperm Examined	Total Abnormal Sperm	Relative fold values	%Abnormality	p- value
0	1080.5	6.25	NA	0.58	NA
4	1014.75	13.75	2.2*	1.36	0.03
8	1006	47.75	7.64**	4.75	0.0011

Note: n = 4, * p≤0.05 and ** = p≤0.01

Table: 21. MICRONUCLEI IN PERIPHERAL BLOOD

Table 21.1: (1ST DAY OF EXPOSURE)

Dose Group	Mice Number & Sex	Im. RBC	Matured RBC	MN RBC	Total RBCs	Ratio of mature RBC	MN RBC		% MN RBC
							Mean	SD	
0.0	1 M	21	1002	1	1024	0.979	1.25	0.50	0.122
	2 M	12	1022	2	1036	0.986			
	3 M	12	1047	1	1060	0.988			
	4 M	13	1017	1	1031	0.986			
	5 F	17	1029	1	1047	0.983	1.25	0.50	0.116
	6 F	8	1019	2	1029	0.99			
	7 F	7	1064	1	1072	0.993			
	8 F	13	1215	1	1229	0.989			
4.0	9 M	5	1020	2	1027	0.993	1.25	0.96	0.123
	10 M	5	1014	0	1019	0.995			
	11 M	10	1026	1	1037	0.989			
	12 M	19	1009	2	1030	0.98			
	13 F	6	1013	1	1020	0.993	1	0.00	0.098
	14 F	10	1024	1	1035	0.989			
	15 F	8	1021	1	1030	0.991			
	16 F	15	1026	1	1042	0.985			
8.0	17 M	7	1004	1	1012	0.992	1.25	1.26	0.117
	18 M	9	1019	3	1031	0.988			
	19 M	15	1223	0	1238	0.988			
	20 M	11	1025	1	1037	0.988			
	21F	12	1058	2	1072	0.987	2.25	0.50	0.213*
	22F	13	1087	2	1102	0.986			
	23F	11	1051	2	1064	0.988			
	24F	15	1038	3	1056	0.983			

Note: Im. RBC = Immature Red Blood Cells, MN RBC **=** Micronucleated Red Blood Cells,

SD = Standard Deviations, n = 4, $p \leq 0.05$**.**

Table: 21. MICRONUCLEI IN PERIPHERAL BLOOD

Table – 21.2 : (1ST Week after EXPOSURE)

Dose Group	Mice Number & Sex	Immature RBC	Matured RBC	MN RBC	Total RBCs	Ratio of mature RBC	MN RBC		% MN RBC
							Mean	SD	
0.00	1 M	3	1013	0	1016	0.997	0.5	0.58	0.049
	2 M	2	1003	0	1005	0.998			
	3 M	3	1008	1	1012	0.996			
	4 M	6	1021	1	1028	0.993			
	5 F	9	1011	0	1020	0.991	1.25	1.26	0.118
	6 F	1	1186	3	1190	0.997			
	7 F	6	1002	1	1009	0.993			
	8 F	12	1041	1	1054	0.988			
4.0	9 M	6	1019	3	1028	0.991	2	0.82	0.191
	10 M	2	1043	2	1047	0.996			
	11 M	6	1104	1	1111	0.994			
	12 M	4	1033	2	1039	0.994			
	13 F	5	1035	1	1041	0.994	1.5	1.29	0.145
	14 F	5	1015	0	1020	0.995			
	15 F	2	1022	2	1026	0.996			
	16 F	7	1070	3	1080	0.991			
8.0	17 M	2	1182	1	1185	0.997	2	2.16	0.180
	18 M	23	1076	5	1104	0.975			
	19 M	3	1138	0	1141	0.997			
	20 M	10	1043	2	1055	0.989			
	21F	4	1007	4	1015	0.992	3.25	0.96	0.313
	22F	6	1060	2	1068	0.993			
	23F	9	1065	4	1078	0.988			
	24F	12	1019	3	1034	0.985			

Note: Im. RBC = Immature Red Blood Cells, MN RBC **=** Micronucleated Red Blood Cells,
SD = Standard Deviations, n = 4.

Table: 21. MICRONUCLEI IN PERIPHERAL BLOOD

Table – 21.3: (2nd Week after EXPOSURE)

Dose Group	Mice Number & Sex	Immature RBC	Matured RBC	MN RBC	Total RBCs	MN RBC		MN RBC
						Mean	SD	
0.00	1 M	4	1030	1	1035	1.25	0.96	0.117
	2 M	5	1154	0	1159			
	3 M	5	1012	2	1019			
	4 M	4	1065	2	1071			
	5 F	8	1047	4	1059	2.5	1.29	0.230
	6 F	4	1068	3	1075			
	7 F	11	1105	1	1117			
	8 F	3	1120	2	1125			
4.0	9 M	10	1035	3	1048	1	1.41	0.095
	10 M	8	1040	0	1048			
	11 M	4	1149	1	1154			
	12 M	7	1001	0	1008			
	13 F	11	1043	3	1057	1.75	1.50	0.169
	14 F	9	1071	0	1080			
	15 F	26	1006	3	1035			
	16 F	20	1018	1	1039			
8.0	17 M	17	1020	2	1039	4	1.83	0.394*
	18 M	18	1046	5	1069			
	19 M	46	995	6	1047			
	20 M	23	1003	3	1029			
	21F	10	1005	2	1017	2.25	1.89	0.216
	22F	13	1036	5	1054			
	23F	18	1007	1	1026			
	24F	24	1115	1	1140			

Note: Im. RBC = Immature Red Blood Cells, MN RBC **=** Micronucleated Red Blood Cells,

SD = Standard Deviations, n = 4, $p \leq 0.05$**.**

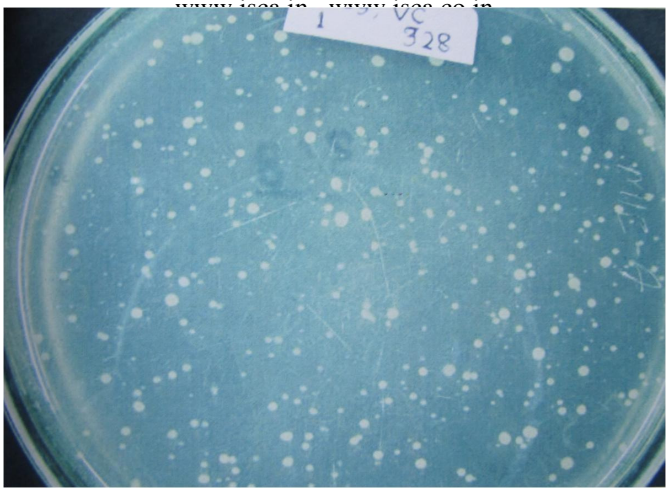
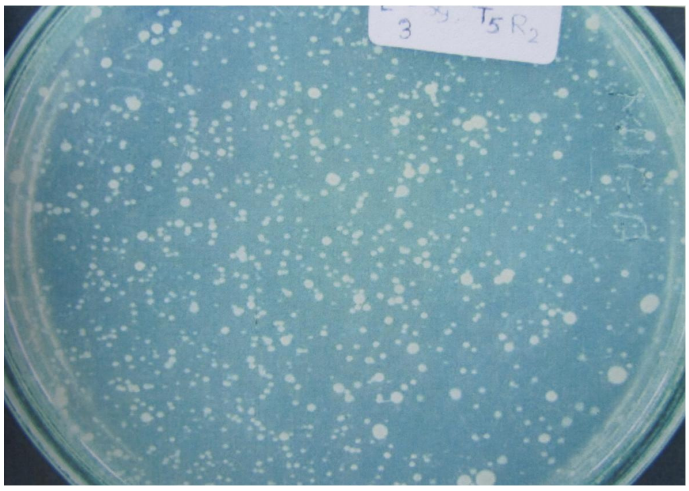
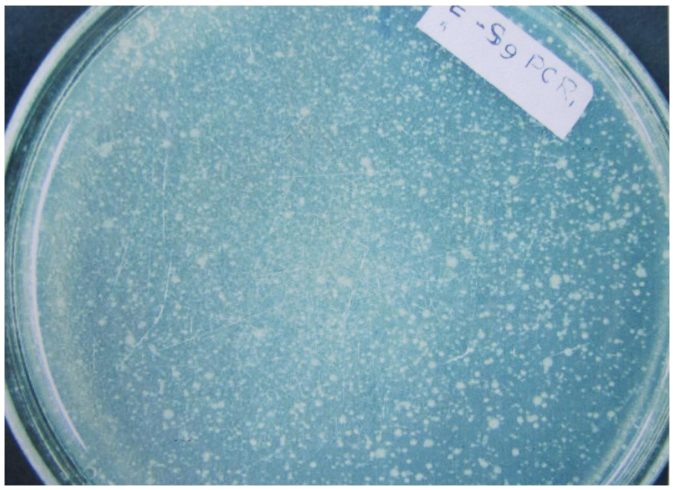
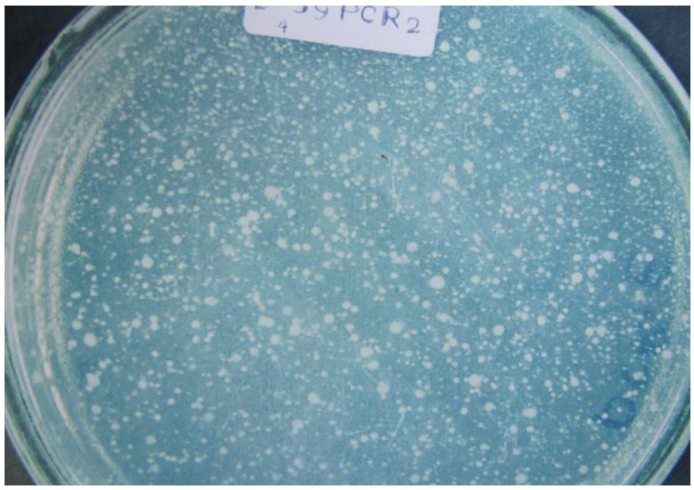


Figure 54: Ames colonies; Vehicle Control group showing less number of colonies in the absence of metabolic activation.

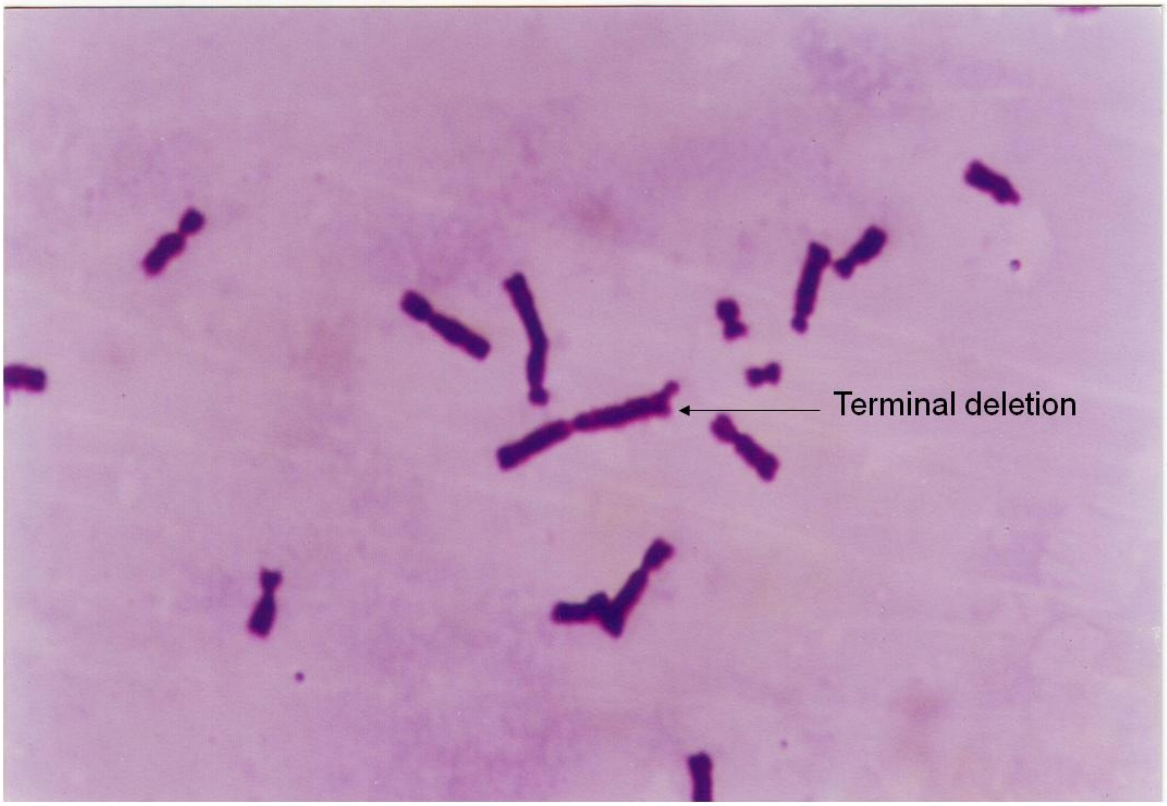


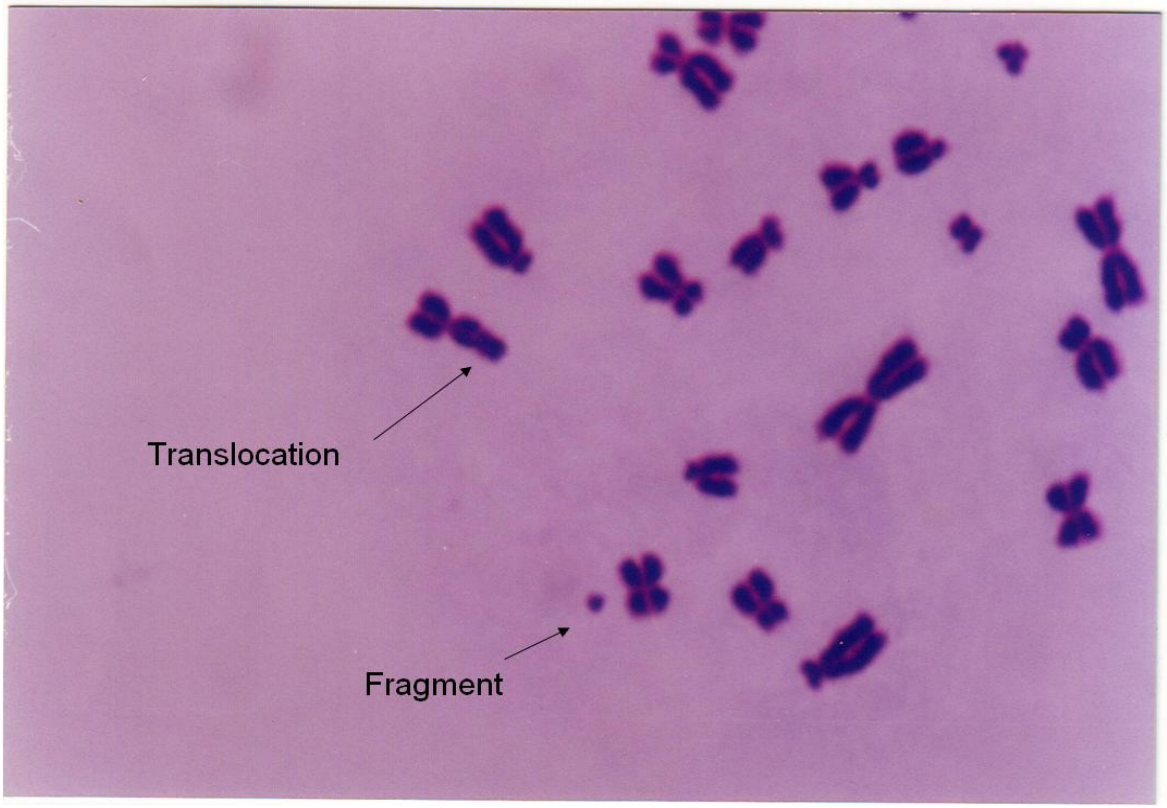
Figures 55 and 56: Ames colonies; Strain TA 102 showing a positive response in Endosulfan treated plates in the absence of metabolic activation.



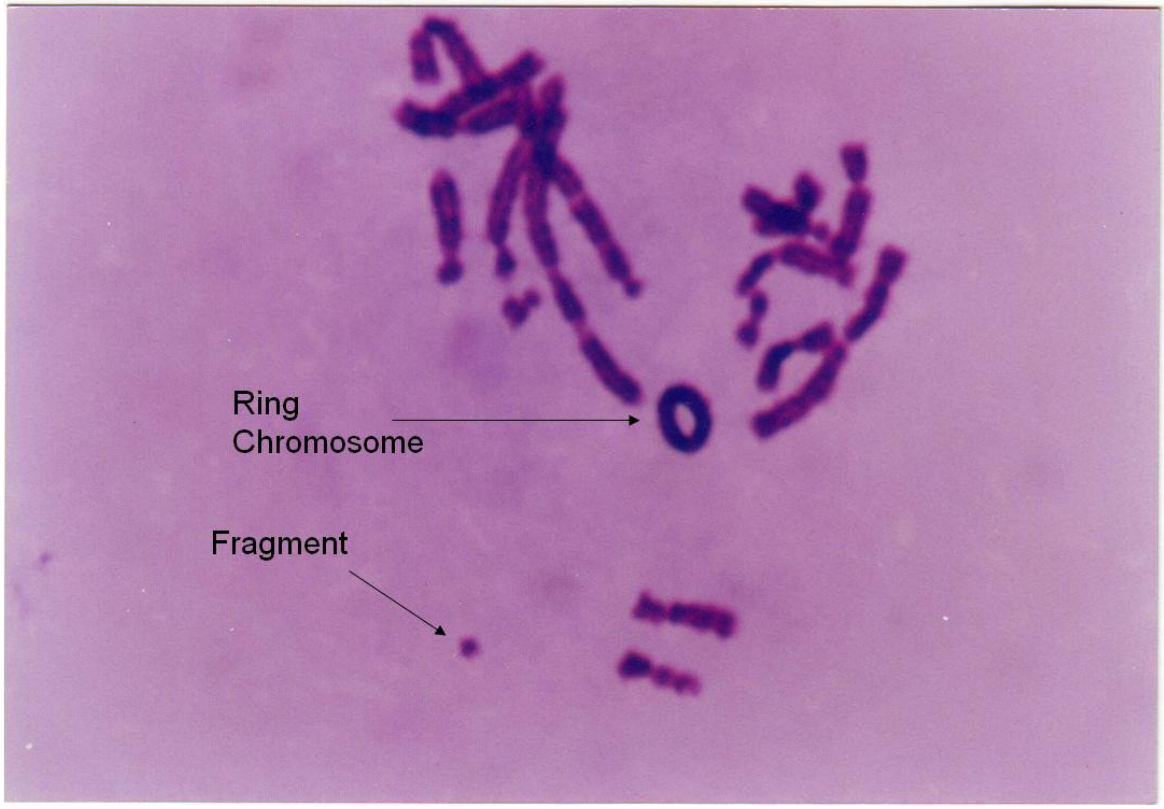


Figures 57 and 58: Ames colonies; Strain TA 102 showing a positive response in plates treated with Mitomycin C (0.5µg/plate).





Figures 59 and 60: Chromosomal aberrations in Endosulfan treated groups



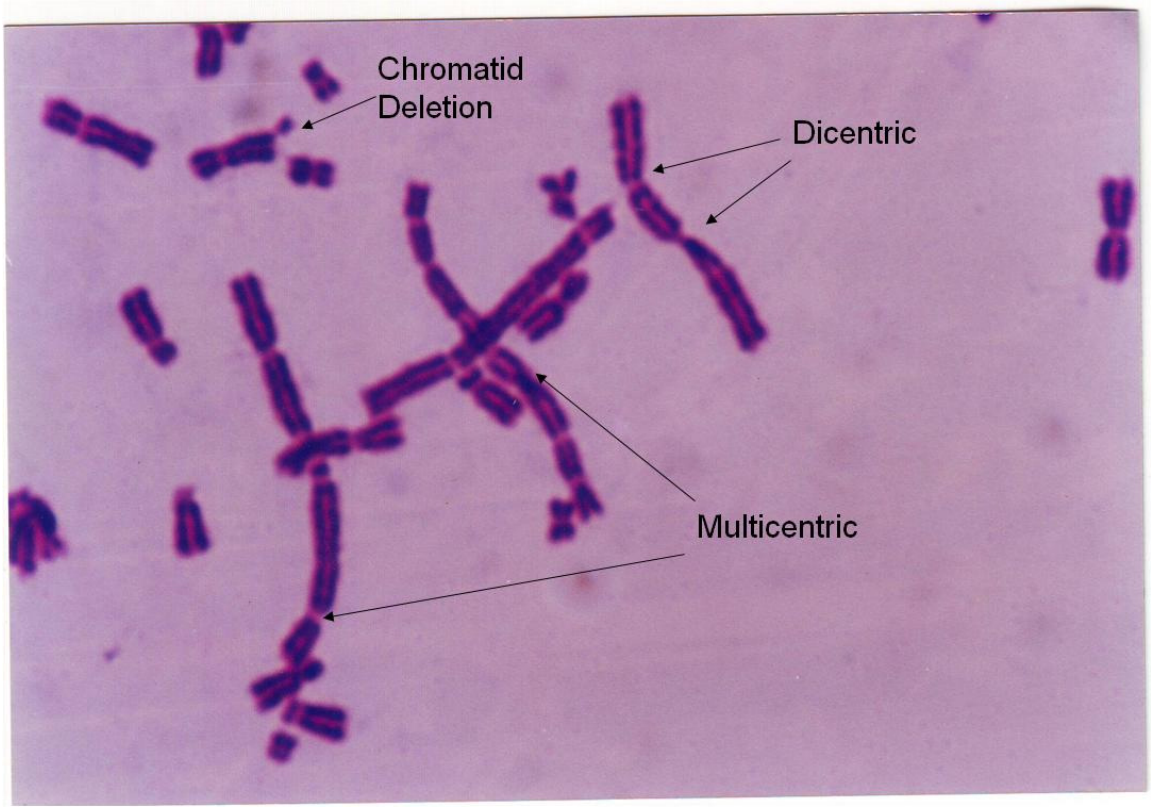
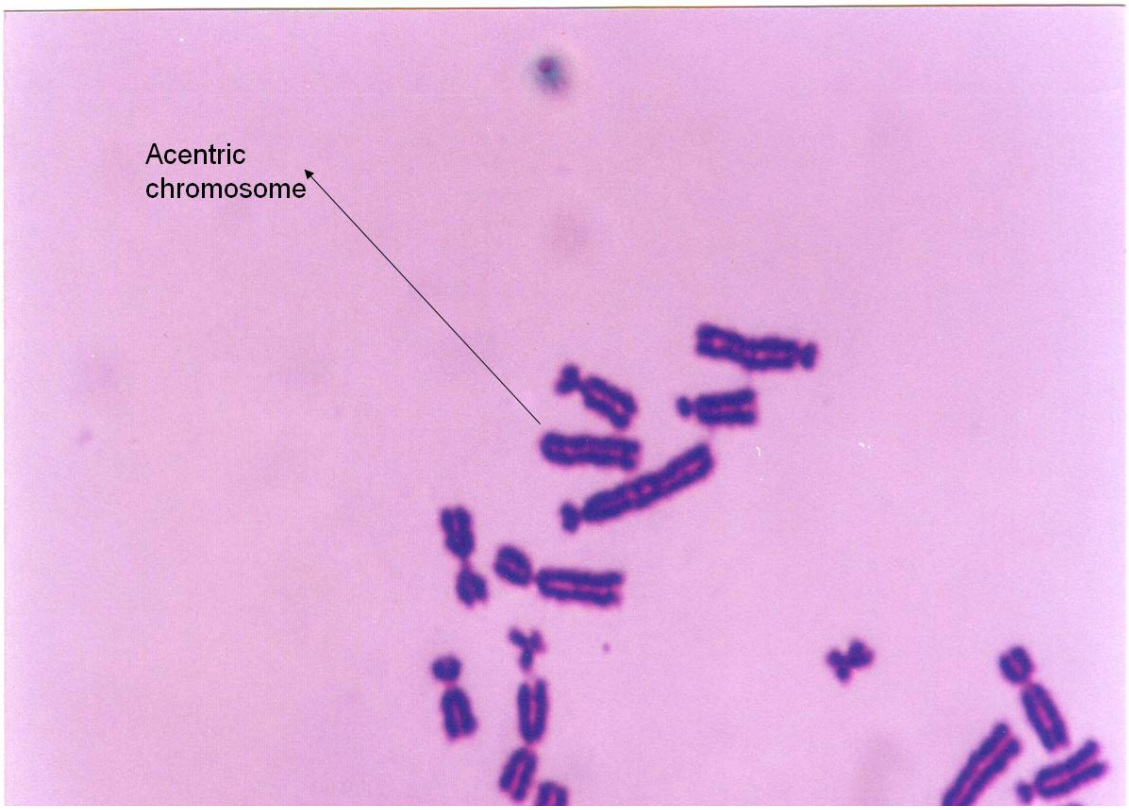


Figure 61 and 62: Chromosomal aberrations in Endosufan treated groups



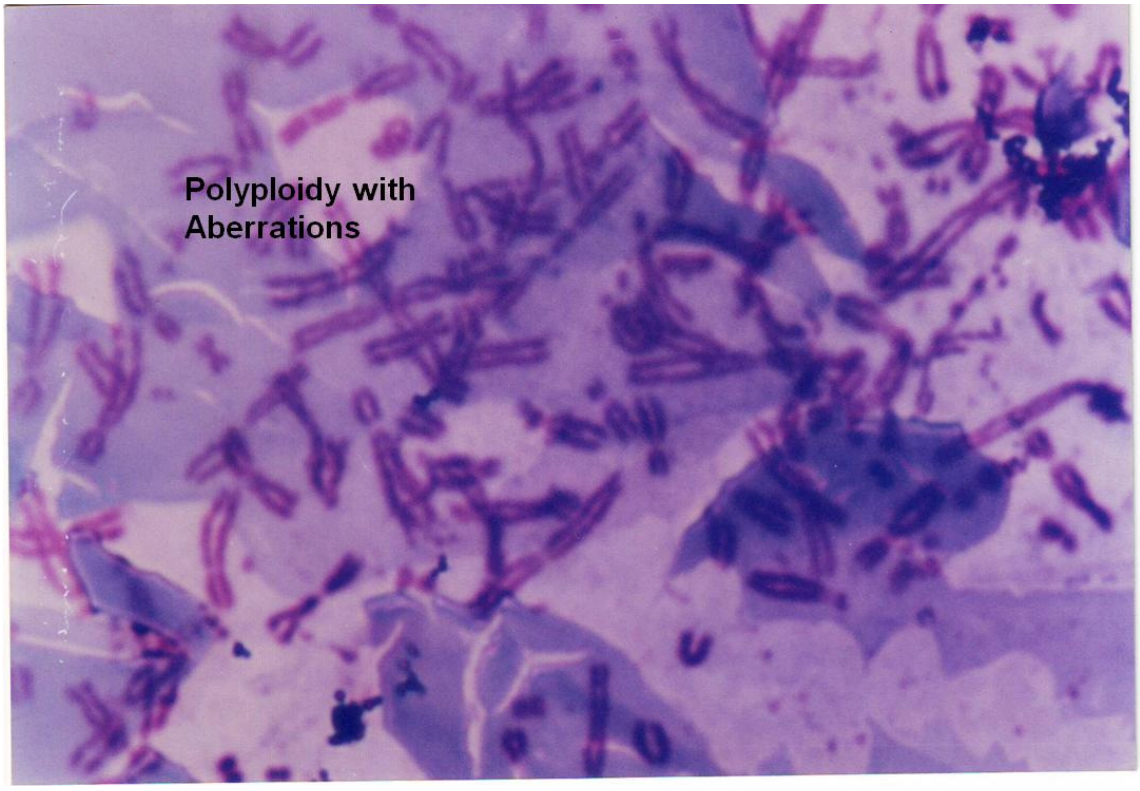
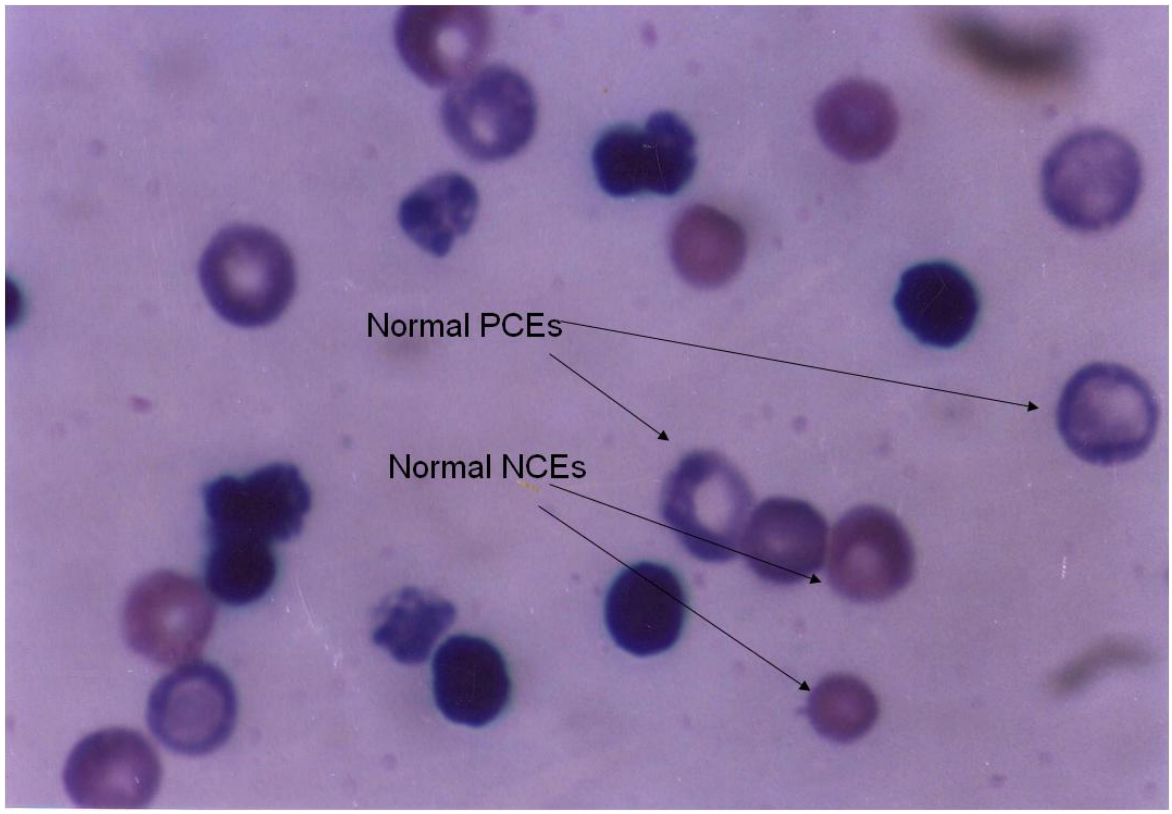


Figure 63 and 64: Chromosomal aberration in Endosulfan treated groups.



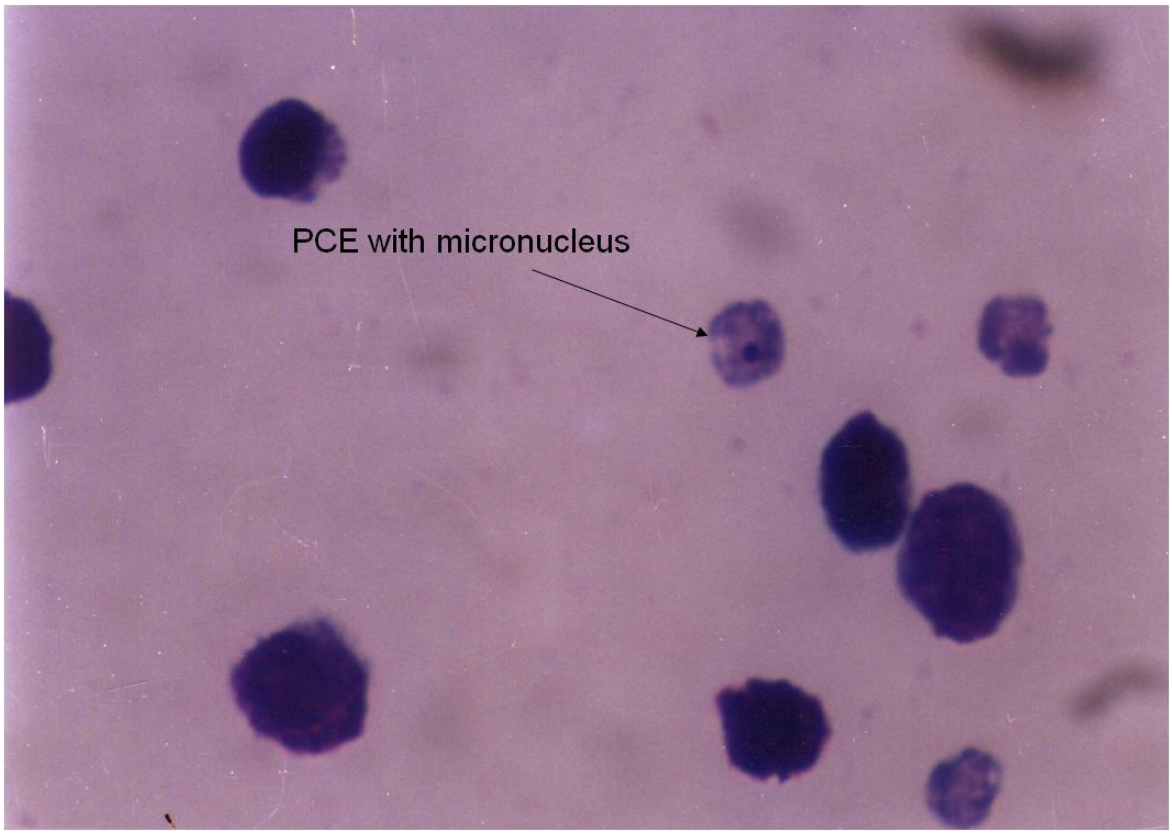
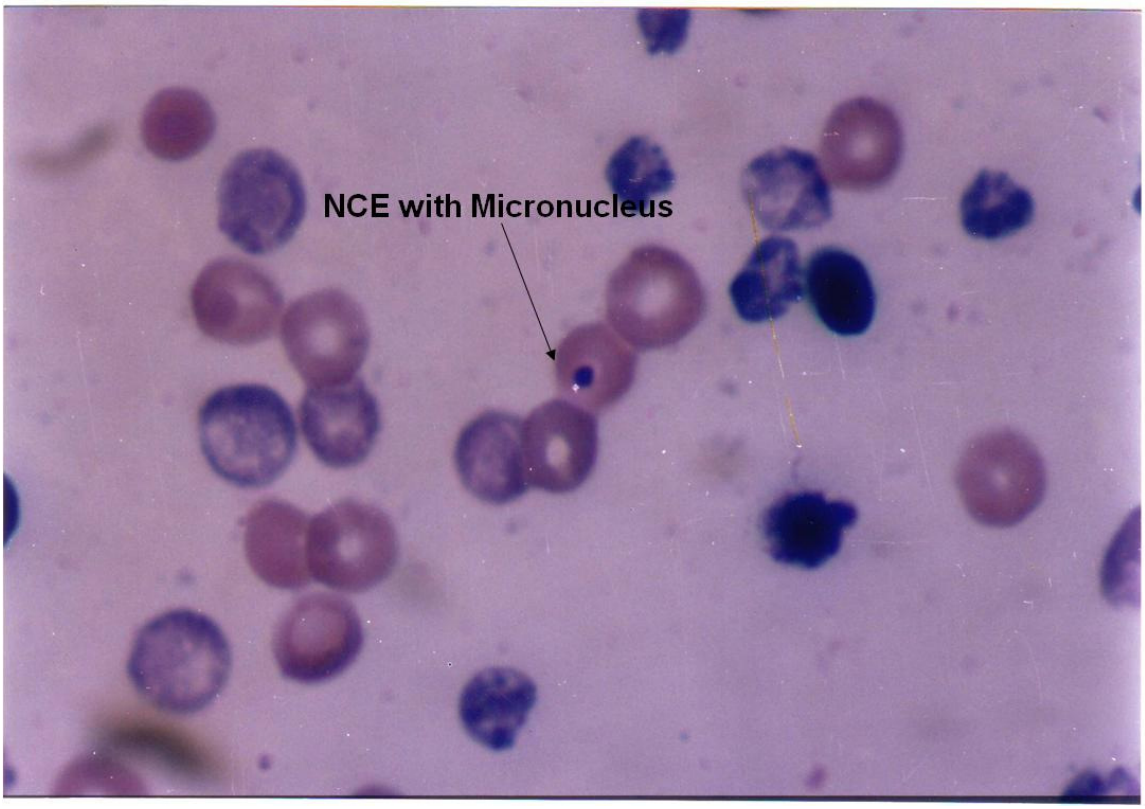


Figure 65 and 66: Polychromatic and Normochromatic Erythrocytes (PCE and NCE) in Bone Marrow.



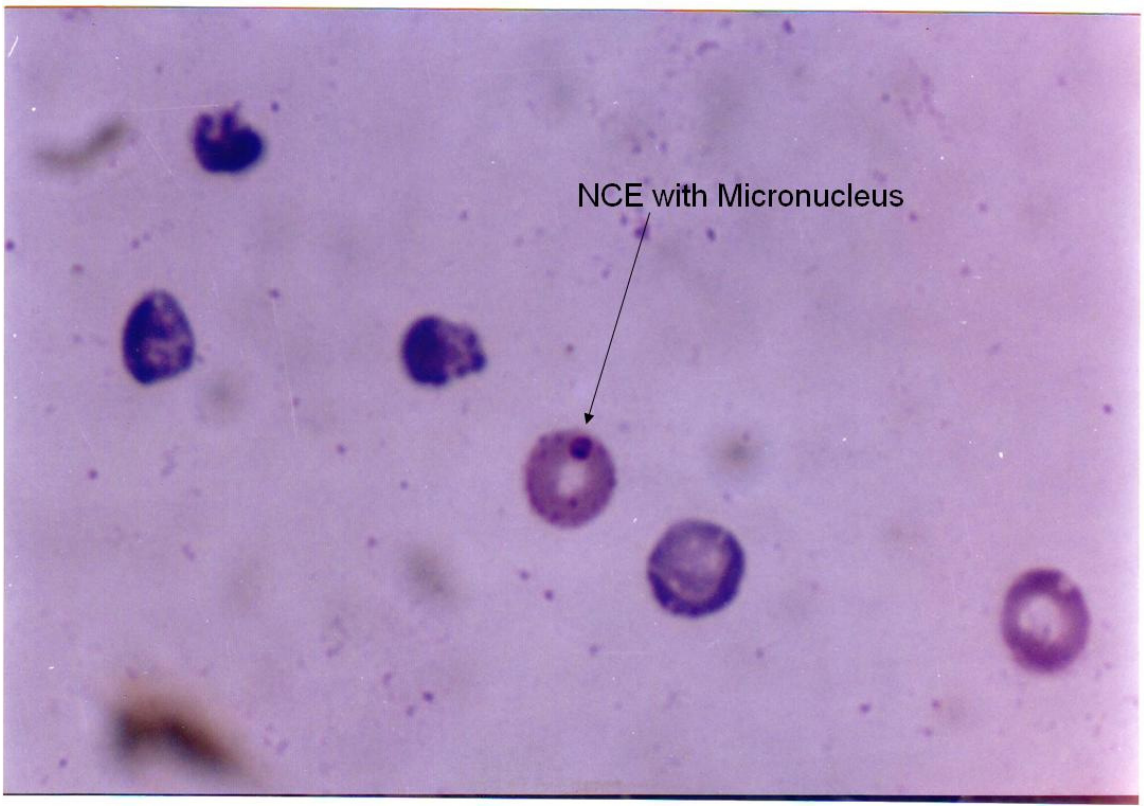
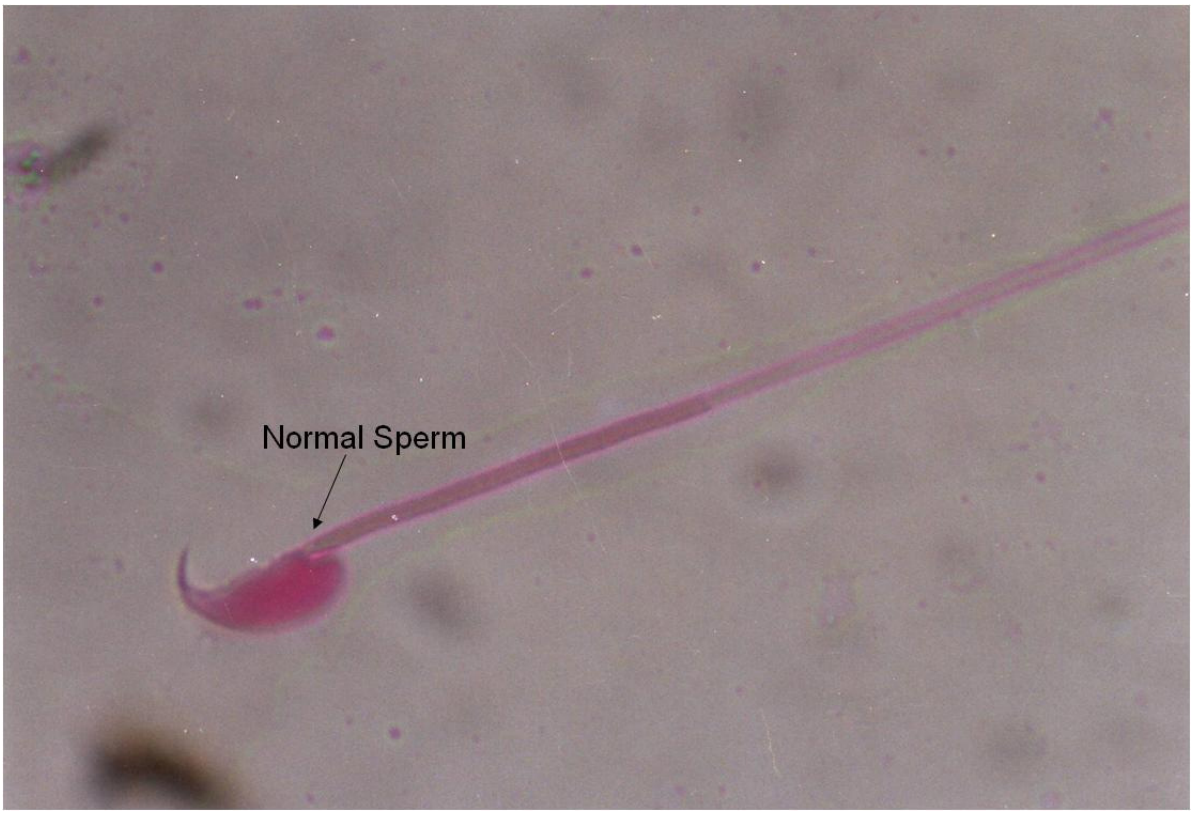


Figure 67 and 68: Normochromatic Erythrocytes (NCEs) with micronucleus.



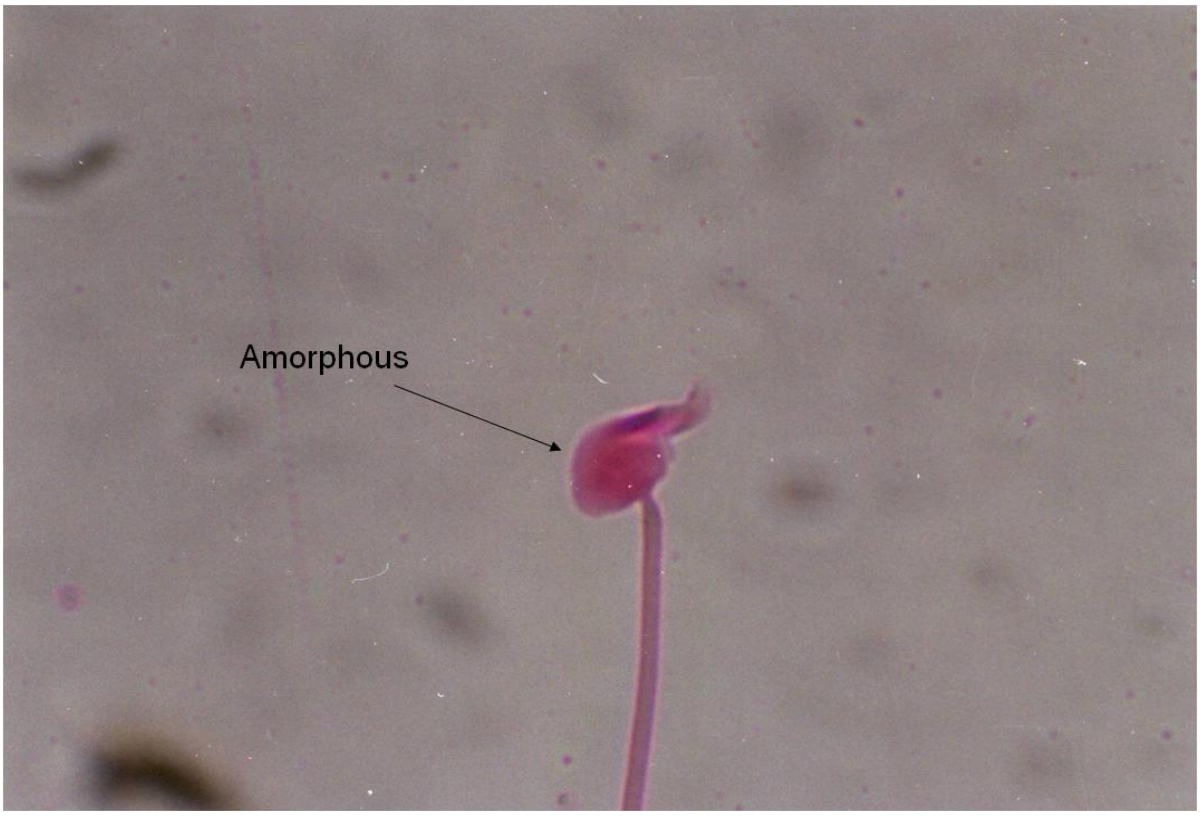
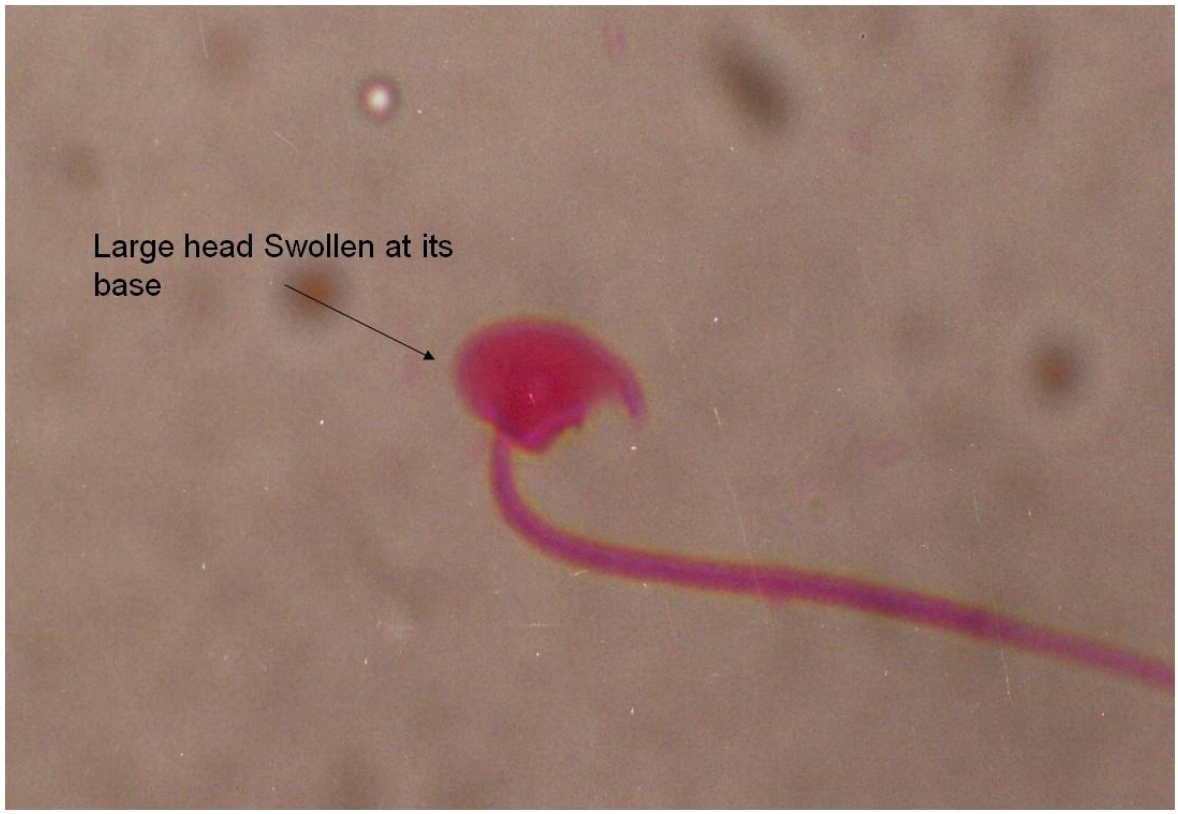


Figure 69 and 70: Sperm Morphology



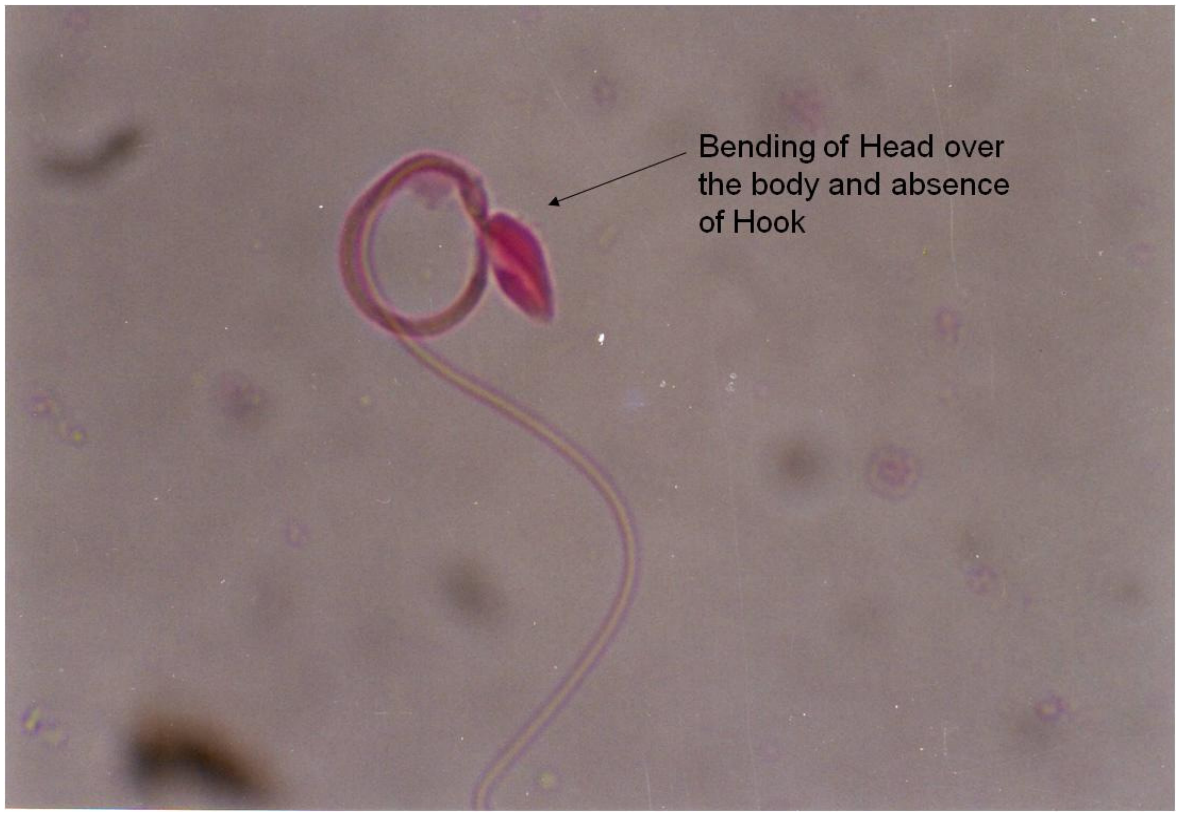


Figure 71 and 72: Abnormal Sperms

Figure 73 and 74: Tail abnormalities in spems

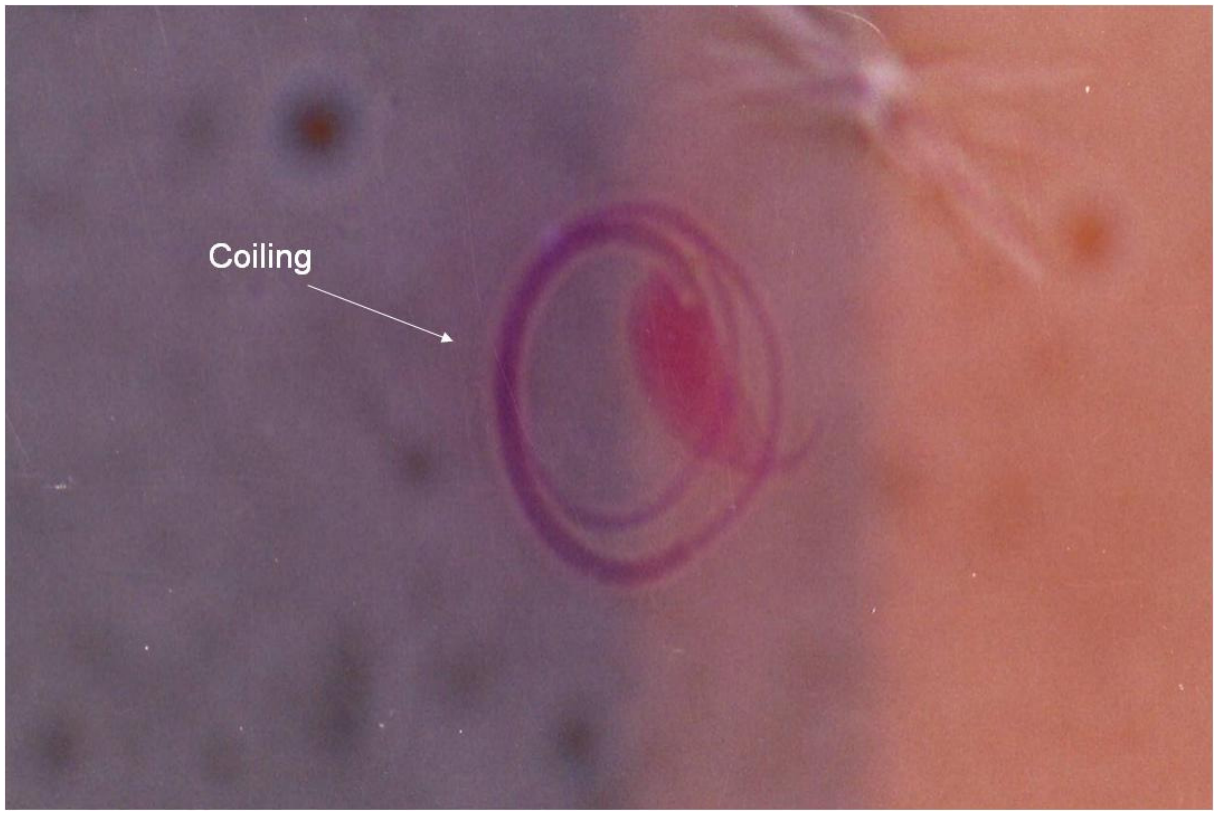


Figure 75: Abnormal coiling in sperms.

CHAPTER 4

EXPERIMENTAL EVIDENCES OF TOXICITY

4.1 *IN VITRO* DOSE SELECTION TESTS

4.1.1 Ames Tests

The doses for the current study were selected based on the comprehension of a dose range study using the test substance (Endosulfan) alone as well as supplementing it with metabolic activator - S9 mix (table:1,1.1 and1.2). The essentials of the same are outlined as follows:

- There was complete lawn inhibition (absence of lawn) at the dose of 5µl/plate (i.e. 1750µg/plate). Therefore, the dose 5µl/plate (i.e. 1750µg/plate) was not considered for main study.
- Only moderate lawn inhibition could be observed in the plates which were not supplemented with metabolic activator. However, lawn was absent at the highest applicable dose for both the strains (TA 98 and TA 100) tested.
- Nevertheless the plates supplemented with metabolic activator showed a strain specific response wherein the lawn was normal in TA 98 while completely absent in TA 100 at the dose of 0.5µl/plate.
- Dose dependent reductions in number of revertant colonies were apparent in both the strains challenged with or without metabolic activator.

Based on the above results, for the presence and absence of metabolic activation groups, doses of 4, 14, 47, 158 and 525 µg/plate were selected for the main experiment.

4.1.2 Cell Gene Mutation Test

Results of precipitation

No visible sign of precipitation was observed after treatment. However, an observation under inverted microscope revealed the presence of globules in the groups treated with highest concentrations.

Results of confluency

There was no noticeable difference in confluency of the cell in any of the groups studied except for the highest dose group, where a patchy distribution of monolayer was observed.

pH

Change in pH was not recorded at any of the doses since all the measured pH values were within the biological pH range of 7.4 ± 0.2.

Cytotoxicity

Dose dependent decrease in percentage (%) Relative Cloning Efficiency (%RCE) was observed which was 100, 95.2, 65.3 and 33.1 in control (0.0µg/ml), low (0.175µg/ml), mid (0.35µg/ml) and high (0.525 µg/ml) doses respectively for the absence of metabolic activation (table: 5, 5.1, fig: 31 & 32). The above results of % RCE, are in agreement with the reported requirement of survival frequency (70 - 90% for low dose, 40-65% for mid dose and

10-35% for high dose) for low, mid and high doses (Gupta, 1984). However, percentage Relative Cloning Efficiency was not observed for the presence of metabolic activation during dose selection assay due to atypical response.

4.1.3 Chromosomal Aberration Test

Dose selection study

A short-term exposure test for cytotoxicity was performed for dose selection wherein morphological (confluency) and mitotic index studies were performed.

Morphological observations under inverted microscope

All the cells were dead at 45.57 and 11.39µg/ml and more than 1/4th of the cell population was observed detached (died) in 2.848µg/ml treatment group. Slides could not be prepared at these dose levels due to observed high toxicity in the groups subjected to test substance with or without metabolic activator.

Mitotic Index

In the absence of metabolic activation

Mean mitotic indices of 4.1556, 2.5691, 2.0175, 1.56315 and 1.1559 were observed for the doses of distilled water, DMSO and 0.045, 0.178 and 0.712µg/ml of Endosulfan respectively. Moreover, a dose dependent reduction (21.47%, 39.16% and 55.01%) was noticed in the test substance treated groups. The results were significant for the highest ($p \leq 0.05$) and control values (table: 7.1). A regression equation of $y = -18.271x + 116.77$, $R^2 = 0.9952$, $r = 0.99$, is obtained which shows that a 50% reduction in mitotic index can be expected at a dose of 0.572µg/ml. The above regression also points to a significant dose dependent decrease in mitotic index (fig: 43).

In the presence of metabolic activation

The observed mean mitotic indices were 2.05415, 2.5691, 2.036, 2.447 and 1.65685 for the same dose levels (of distilled water, DMSO and 0.045, 0.178 and 0.712µg/ml of Endosulfan) mentioned above for the absence of metabolic activation. This finding was contradictory to the mitotic index observed in the absence of metabolic activation and shows no significant inhibition of mitotic index in the groups supplemented with mitotic activator. The percent reductions were 20.75%, 4.75%, 35.51% respectively for low, mid and high doses (table: 7.2). A regression equation ($y = -9.0528x + 107.38$, $R^2 = 0.523$, $r = 0.0.7231$) is derived which shows that a 50% reduction can be obtained at 1.109µg/ml concentration (fig: 44).

Three doses *viz.*, 0.175, 0.350 and 0.525 were selected based on the results of confluency and mitotic index of both the presence and absence of metabolic activation groups for main study (refer table: 7.1 and 7.2). A higher dose for the S9 mix group was not selected to maintain uniform dose levels in both the cases. Therefore, the cells in the main study were not exposed to a near cytotoxic dose. Moreover, the selection of these doses was also based on the results of gene mutation experiments to make homogeneity in the selected doses.

In the present study however, the Mitotic Index (MI) was not much useful in dose selection. The mitotic index is 'the ratio' of the number of cells seen in mitosis to the total number of cells present. Only in an ideal 'steady-state' population, there is a simple relationship between MI and mitotic duration. Such populations are seldom realized, especially when the cell population is disturbed by clastogenic treatments. Simple

interpretation of MI is therefore not always possible and MI is not therefore a useful measure of 'mitotic rate'. Treatment, for instance, which affect the differential length of interphase and mitosis can result in either an increase or decrease in MI (Lovell, *et al*., 1989).

4.2 IN VITRO MAIN STUDY

4.2.1 Ames test

4.2.1.1 Growth

All the Salmonella tester strains (TA 1537, TA 1535, TA 98, TA 100 and TA 102) showed optimum growth before treatment in both the methods of treatment. The optical density was in the range of 0.42-0.51 (table: 3)

4.2.1.2 Lawn Inhibition Test and Reduction in Number of Revertant Colonies

Strain specific and treatment specific toxicities were observed which are as follows:

Plate incorporation method (in the absence of metabolic activation)

Mild lawn inhibitions were observed in the strain TA 1537 and strain TA 1535 at the highest concentration *viz*., 525µg/plate. However, in strain TA 100, moderate to complete lawn inhibition were observed at the concentrations of 158 and 525µg/plate (fig: 55 to 58). All the other strains showed normal lawn at all the tested dose levels (table: 2, 2.1, 2.2, 2.3, 2.4, and 2.5).

Reduction in number of revertant colonies, were observed only in strain TA 100, rest all the strains exhibited normal colony counts (fig: 54).

Plate incorporation method (in the presence of metabolic activation)

Mild lawn inhibitions were observed in the strains TA 1537 and Strain TA 1535 at the highest concentration of 525µg/plate. While the strain TA 102 has shown excessive toxicity and lawn inhibition has started from 0.014 to 525µg/plate in dose dependent manner. The dose 0.014 has given moderate and complete lawn inhibition. All other strains showed normal lawn at all the tested dose levels (table: 2.6, 2.7 and 2.10).

Reduction in number of revertant colonies was observed only in strain TA 102 subjected to doses 0.047 and 158. However, 525µg/plate dose group showed zero colony counts. The results of both lawn inhibition and reduction in colony counts point to severe toxicity in strain TA 102 and moderate toxicity in the strain TA 100 (table: 2.8 and 2.10). Strain TA 98 has shown normal lawn in all the dose groups (table: 2.9).

4.2.1.2 Lawn Inhibition Test and Reduction in Number of Revertant Colonies

Strain specific and treatment specific toxicities were observed which are as follows:

Pre incubation method (in the absence of metabolic activation)

Strain TA 1537 and strain TA 1535 treated with the highest concentration exhibited mild lawn inhibition (table: 2.6). However, moderate lawn inhibitions were observed in TA 100 at the concentrations of 158 and 525µg/plate. All other strains exhibited normal lawn at all the tested dose levels (table: 4.4).

Further, no reduction in the number of revertant colonies was observed in any of the strains studied (table: 4.3 and 4.5).

Pre incubation method (in the presence of metabolic activation)
Mild lawn inhibitions were observed in the strain TA 1537 and strain TA 100 at the highest concentration of 525µg/plate while the strain TA 1535 showed a dose dependent toxicity from 158 to 525µg/plate. The dose 158 and 525µg/plate evoked moderate and complete lawn inhibition. Rest all the strains showed normal lawn at all the tested dose levels (table: 4.6, 4.7 and 4.9).

An obvious reduction in number of revertant colonies was observed only in strain TA 1535 at the dose of 158 and 525µg/plate. The dose 525µg/plate resulted in zero colony counts (table: 4.7).

Both lawn inhibition and reduction in colony counts in strains TA 1535 and TA 100 indicate toxicity to test substance (table: 4.7 and 4.9).

From the above results it could be inferred that Endosulfan induces strain dependent toxicity. The toxicity is also found to be depended on metabolic activation. The metabolic end product generated during oxidative transformation seems to be toxic to the bacterial strain TA 102 which is sensitive to oxidative mutations. However, in the absence of metabolic activation, toxicity gets reduced and mutagenicity gets triggered and shows positive response in strain TA 102 indicated by 3 fold increase in revertant frequency.

4.2.1.3 Revertant Frequency
Plate incorporation method
TA 1537 (-S9)
An increase in revertant colonies per plate was observed in the strain TA 1537. The doses of 4, 14, 47, 158 and 525 µg/plate evoked 1.56, 1.11, 2.28, 2.72, and 1.28 fold increase in revertant colonies over the concurrent control (fig: 11). The result indicates moderate increase in mutation frequency (revertant frequency) and also absence of mutagenicity for the group (table: 2.1). However, the increase in mutant frequency was not consistent as indicated by the low r value ($y = 2.1429x + 4.7857$, $r = 0.13$).

TA 1535 (-S9)
A definite increase ($y = 1.5893x + 7.6429$, $r = 0.82$) in revertant colonies per plate was observed in the strain TA 1535 since 0.45, 0.79, 0.76, 1.34, 1.55 time increase in revertant frequency was noticed in the dose groups over the concurrent control (table: 2.3, fig: 12). The current observation indicates moderate increase in mutation frequency (revertant frequency) and absence of mutagenicity for the test substance in the absence of metabolic activation.

TA 98 (-S9)
A slight increase ($y = 1.2321x + 13.143$, $r = 0.79$) in revertant colonies per plate was observed in the strain TA 98. The observed levels of increase were 0.65, 0.85, 0.60, 1.10 and 1.13 times for the chosen doses over the respective control (table: 2.2, fig: 13). The current observation indicates absence of mutagenicity for the test group.

TA 100 (-S9)
This strain showed decrease ($y = -17.125x + 173.64$, $r = 0.84$) in revertant frequency in all the tested dose groups and became toxic to the highest dose (525 µg/plate) tested (table:

2.4). The strain subjected to treatment exhibited 0.72, 0.84, 0.55, 0.53 and 0.27 time increase in revertant frequency compared to that of control (fig: 14). The observed nonspecific response points to absence of mutagenicity for the group.

TA 102 (-S9)
A statistically significant increase (y = 90.839x + 103, r = 0.98) in revertant colonies per plate was observed in the strain TA 102. A dose dependent increase of 1.00, 1.29, 1.31, 1.37 and 3.43 time revertant frequency was apparent for studied strain (table:2.5, fig: 15). The higher r value indicates significant increase in mutation frequency (revertant frequency) and mutagenicity of Endosulfan in the absence of metabolic activation for TA 102.

TA 1537 (+S9)
A nonspecific increase in revertant colonies per plate was observed in the strain TA 1537. The recorded increase in revertant frequency in the treatment groups compared to that of control was as follows 1.10, 1.15, 1.00, 0.95 and 1.75 (table: 2.6, fig: 16). The values indicate aberrant increase in mutation frequency (revertant frequency) and absence of mutagenicity for TA 1537 subjected to various doses of Endosulfan together with S9.

TA 1535 (+S9)
The strain TA 1535 when treated with serial concentration of test substance showed an increase in the frequency of revertant colonies compared to that of control (table: 2.7, fig: 17). However, a negative correlation between the frequencies of colony reversion to increase in concentration of Endosulfan was quite apparent in this group. The result therefore, indicates absence of mutagenicity in the test system using TA 1535.

TA 98 (+S9)
The doses of 4, 14, 47, 158 and 525 µg/plate evoked 0.80, 0.99, 0.64, 1.04 and 0.68 fold increase in revertant frequency compared to that of concurrent control (table: 2.9, fig: 18). The comparable mutation frequencies (revertant frequency) for varied doses indicate absence of mutagenicity in this group.

TA 100 (+S9)
The revertant frequency of 0.57, 1.24, 0.27, 1.34 and 1.25 fold over the respective control observed in the test substance dosed group indicates non-significant increase in mutation frequency (revertant frequency) and hence, absence of mutagenicity for TA 100 treated with Endosulfan and S9 supplement (table:2.8, fig: 19).

TA 102 (+S9)
The dose groups 4, 14, and 47µg/plate exhibited 0.99, 0.30 and 0.07 fold increase in the frequency of revertant colony in TA 102 (table: 2.10, fig: 20). The decrease in revertant frequencies with increase in dose could be due to excessive toxicity in the presence of metabolic activation.

Positive Control
The *Salmonella* tester strains TA 1537, TA 1535, TA 98, TA 100 and TA 102 showed 76.67, 10.07, 67.5 and 6.21 times increase in revertant frequency when challenged with known mutagen in the absence of metabolic activation. However, in the presence of metabolic activator (S9) the frequency of revertant colony got changed to 4.65, 72.7, 26.97, 14.28 and

2.36 respectively for the *Salmonella* tester strains TA 1537, TA 1535, TA 98, TA 100 and TA 102 (table: 2.1, 2.2, 2.3, 2.4 and 2.5).

The results of positive control show sensitivity of tester strains towards its specific mutagen. The increase in frequency of revertant mutation in the positive control groups compared to vehicle control also signifies that the strains used in the assay are capable to detect mutations and proves efficacy and validity of the test performed.

Pre incubation method

TA 1537 (-S9)
In this strain an increase in the frequency of revertant colony to the tune of 1.94, 1.63, 2.00, 2.94 and 1.56 fold was observed when treated with test substance at a dose level of 4, 14, 47, 158 and 525µg/plate (table:4.1). However, regression analysis (y = 1.6607x + 7.0714, r = 0.66), shows that there is no difference in colony counts when compared to control (fig: 21).
TA 1535 (-S9)
The observed increase in values of revertant colonies per plate was 2.98, 1.96, 2.53, 1.71 and 1.00 times in the respective treatment groups compared to control group (table: 4.2). However, no statistically significant difference in revertant colonies per plate (y = 10.799Ln(x) + 34.063, r = 0.33) was observed in any of the treatment group compared to the concurrent control (fig: 22).

TA 98 (-S9)
Compared to respective control an increase of 0.75, 2.14, 0.94, 0.84 and 1.00 fold, was observed in TA 98 when treated with sublethal doses of Endosulfan (table:4.3). There is however, no significant increase in revertant colonies per plate for the selected doses in TA 98 as evident from the regression equation y = -1.3713Ln (x) + 37.599, r = 0.06 (fig: 23).

TA 100 (-S9)
TA 100 when subjected to the test substance in the range of 4, 14, 47, 158 and 525 µg/plate induced 0.94, 1.08, 1.05, 0.84 and 0.87 fold increase in the frequency of revertant colony (table: 4.4). Though, this strain shows slight decrease (y = -7.4948Ln(x) + 125.77, r = 04) in the frequency of revertant colony with increase in dose it is not significant statistically (fig: 24).

TA 102 (-S9)
A non-significant decrease (y = -41.656Ln(x) + 467.66, r = 0.68) in revertant colonies with increase in dose was observed in the strain TA 102 (fig: 25). The strain showed 1.01, 1.01, 0.95, 0.88 and 0.76 times increase in the frequency of revertant colony in the dosed groups compared to concurrent control (table: 4.5).

TA 1537 (+S9)
The strain TA 1537 treated with selected doses of Endosulfan registered 1.77, 1.18, 0.86, 0.36 and 0.42 increase in frequency of revertant mutation over the respective control (table:

4.6,). Further analysis however, revealed that there exist no significant correlation between the dose and the frequency of revertant mutation (fig: 26).

TA 1535 (+S9)
The increase in revertant colonies per plate observed in the strain TA 1535 was in the tune of 0.85, 0.98, 1.39, 1.24 and 0.00 times compared to that of control (table: 4.7, fig: 27). Regression analysis showed a dose dependent decrease in frequency of revertant mutation ($y = -4.8051\text{Ln}(x) + 25.352$, $r = 0.35$) possibly due to toxicity in the high doses groups.

TA 98 (+S9)
The observed increase in the frequency of revertant mutation in the treated groups was 0.95, 1.05, 0.93, 1.11 and 0.38 when compared to that of control (table: 4.8, fig: 29). The result indicates aberrant response to increase in doses of test substance by the strain TA 98 in the presence of metabolic activator ($y = -1.296\text{Ln}(x) + 35.078$, $r = 0.09$).

TA 100 (+S9)
The strain subjected to increase in concentration of test substance showed 1.33, 1.23, 1.49, 1.53 and 1.57 fold increase in revertant mutation compared to that of control (table: 4.9, fig: 28). The current result indicates mild increase ($y = 38.695\text{Ln}(x) + 103.45$, $r = 0.93$) in mutation frequency (revertant frequency) and absence of mutagenicity for the test substance in the presence of S9.

TA 102 (+S9)
The strain TA 102 when treated with Endosulfan together with metabolic activator registered 1.09, 1.09, 0.90, 0.67 and 0.58 fold increase in revertant colony compared to that of control (table: 4.10, fig: 30). The observed dose dependent decrease in revertant frequencies ($y = -70.956\text{Ln}(x) + 474.27$, $r = 0.57$), could be due to excessive toxicity in the presence of metabolic activation at the highest given doses.

A careful scan through the available literature revealed that Endosulfan has been reported toxic to yeast and is also designated a potent mutagen without metabolic activation (Yadav *et. al*., 1982). Moreover, Endosulfan is known to induce reverse mutations and mitotic gene conversion and also increase the percentage of aberrant colonies in *Saccharomyces cerevisiae.* But it did not induce mitotic cross-overs (Yadav *et. al.,* 1982). The above observations indicate that Endosulfan is capable of inducing chromosome breakage and loss. Endosulfan also induced cytotoxic activity (significant increase in the number of crossover colonies) in the yeast strain *Saccharomyces cerevisiae* T2 (deficient in repair system) but not in *Saccharomyces cerevisiae* T1 (L'Vova, 1984).

However, mutagenic activity was not reported for Endosulfan when applied to various strains of the *Salmonella typhimurium viz.,* TA97a, TA98, TA1535, TA1537, TA1538 without metabolic activation (Moriya *et.al*.,1983, Pedenekar *et.al*.,1987) or for *Escherichia coli w*ithout metabolic activation (Moriya *et.al*.,1983). Endosulfan also tested negative in the *Salmonella* mutagenicity test with or without metabolic activation (Dorough *et.al.,*1978).

The present experimental results are in agreement with the above findings wherein Endosulfan has been tested non mutagenic in the strains of *Salmonella typhimurium* TA98, TA1535, TA1537 except for TA102 when applied without S9. Further, the current study indicates that in the presence of metabolic activation the compound (Endosulfan 35% EC)

get metabolized and becomes non mutagenic to the strain TA102. Dubois and coworkers (1996) opined that Endosulfan could induce the formation of DNA adducts in both fetal rat hepatocytes and Hep G2 (human liver hepatoblastoma) cells this activity strongly correlated with high induction of CYP3A gene expression and hence, metabolic activation.

4.2.2 Cell Gene Mutation Test

4.2.2.1 Cloning Efficiency

In the current study the % absolute cloning efficiency observed were 105.50%, 83.00%, 56.00%, 82.33%, 7.50% and 64.50 % for the dosage of NC (0.00) VC (0.00), 0.175, 0.35, 0.525µg/ml and PC (10 µl EMS) respectively in the absence of metabolic activation system (table:5.2).

Percent relative cloning efficiency were 127.11%, 100.00%, 67.47%, 99.20%, 9.04% and 77.71 for the dosage of NC (0.00), VC (0.00), 0.175, 0.35, 0.525µg/ml and PC (12µg B(a)P /ml of culture) respectively in the absence of metabolic activation system (table: 5.2). The absolute cloning efficiency was significantly decreased in 0.525µg/ml of culture, indicates possible cytotoxicity at the high dose level.

The absolute cloning efficiency observed in the present study were 74.75%, 75.00%, 89.50%, 44.83%, 53.25% and 67.75% for the dosage of NC (0.00), VC (0.00), 0.175, 0.35, 0.525µg/ml and PC (10µl of EMS/ ml of culture) respectively in the presence of metabolic activation system (table: 5.3).

The currently observed % relative cloning efficiency were 99.67%, 100.00%-del, 119.33%, 59.78%, 71.00% and 90.33% for doses of 0.175, 0.35 and 0.525 µg/ml, respectively in the presence of metabolic activation system (table: 5.3). However, no decrease in relative cloning efficiency was recorded in the presence of metabolic activation. The result indicates absence of cytotoxicity for the dosage of NC (0.00), VC (0.00), 0.175, 0.35, 0.525 and PC (12µg of B (a) P) /ml of culture) µg/ml respectively in the presence of metabolic activation system (table: 5.3).

CHO cells have been previously reported to be particularly sensitive to the toxic effects of an S9 mix (Kirkland *et al*., 1989). Clastogenic effects of an S9 mix have been detected in CHO cells, but not in human lymphocytes. This may be due to an S9 mediated generation of reactive oxygen species in the cells, which might be inactivated by blood components in the lymphocyte cultures (Kirkland *et al*., 1989). Clastogenic activity of S9 has also been reported in mouse lymphoma cells (Cifone *et al*., 1987).

4.2.2.2 Mutation Frequency

A comparison with the concurrent control value revealed no dose related increase in mutation frequency in the treatment groups of the present study (table: 6.1 and 6.2, Fig. 33 to 42). However, Endosulfan was reported positive in forward mutation assay of mouse lymphoma cell line (MLTK thymidine kinase assay) by McGregor *et.al*, (1988) in the absence of metabolic activation.

The observed negative response in mutation frequency might be due to ineffectiveness of CHO-HGPRT assay for mutation. There are cases where the cell lines are sensitive for detecting clastogenicity but are poor to detect mutagenicity of the compound (Moore *et al*., 1989). The CHO HGPRT assay system gives little or no evidence of genotoxicity (Moore *et al*., 1989).

The inability of CHO HGPRT assay to detect increased mutation frequency as observed by aberrant cell frequency (chromosomal aberration) obtained in same CHO cell line (refer section 4.2.1.3 and 4.2.3.2) and a positive response obtained in Ames test in strain TA 102 is hard to comprehend. However, it may be explained by the prevailing hypothesis that "CHO *hgprt* locus is unable to evaluate the clastogenic component of a genotoxic compound due to hemizygous nature of the *hgprt* locus which permits recovery primarily of mutations of a single gene" (Evans *et. al.,* 1986; Hsie *et al.,* 1986, Stankowaski and Hise, 1986; Yandell *et al*., 1986, Little *et al*., 1987; Moore *et al*., 1987).

A test of long term exposure (i.e. 24 or 48 hour exposure) was not performed in the present experimental protocol and hence, it could be possible that one might have missed out mutant cells in the above mentioned experiment. Therefore, a long term assay needs to be performed in order to make any affirmative conclusion. Further, it is also necessary to perform the assay with more sensitive methods such as mouse lymphoma thymidine kinase ($TK^{-/+}$). However, these were beyond the scope of the current protocol and will be incorporated in the future plan of work.

An increase in cloning efficiency observed in the mid dose group indicates mitogenic nature of the test compound or cellular disintegration or loss of cell-cell contacts at that dose level. Further, in the present study a different result was obtained in the presence of metabolic activation group as compared to that of test substance alone treated group. A mild increase in cloning (both absolute and relative) efficiency in low dose group indicates growth promoting (mitogenic) nature of the metabolites of Endosulfan at low dose level.

4.2.3 Chromosomal Aberration Test
Short Term Exposure
4.2.3.1 Mitotic Index

The mitotic index observed were 4.6729%, 5.0325%, 8.1487%, 6.7447%, 5.2835% and 4.5820% respectively for the dosage of NC (0.00), VC (0.00), 0.175, 0.35, 0.525 µg/ml and PC (0.5 µg of Mytomycin-C / ml of culture) in the absence of metabolic activation system (table: 8.1). A dose related reduction in mitotic index was not observed which indicates that the compound tested is not affecting the cells at their G0, S, G2 and M phases of cell division but affects the viability by some other mechanism.

The mean mitotic index in the presence of metabolic activation were 10.7850%, 9.3101%, 14.8305%, 9.8161%, 9.0589% and 6.6962% respectively for the dosage of NC (0.00), VC (0.00), 0.175, 0.35, 0.525µg/ml and PC (25µg of Cyclophosphamide/ml of culture). A dose dependent increase ($p \leq 0.01$) was observed in 0.175µg of endosulphan/ml of culture (table: 8.2). However, the mild increase in mitotic index in low dose group indicates to a mild mitogenic nature of the test compound at the given dose level. This could be because the compound or its metabolic end product might be acting as stimulating factor for cells under exposure. There is an isolated report that Endosulfan is inducing cell cycle

kinetics in two different mammalian cells in culture (Sebastien *et al.,* 2007). An increase in mitotic index predicts aneugenic response of the test agent under investigation.

4.2.3.2 Percent Aberrant Cells
Structural Chromosomal Aberration
The structural chromosomal aberrations observed in the present study include acentric chromosome, translocation, fragment, deletions, dicentrics, multicentrics ring chromosomes (fig: 59 to 63).

4 -hour -S9 CAM
A comparable result was observed between S9 and vehicle control indicating that DMSO itself at 1% exposure level did not induce any chromosomal aberration. A comparable result was also observed between vehicle control and low dose group, indicates low level of aberration at this (0.175µg/ml) dose level (table: 8.3 fig: 44 & 45). However, test chemical induces cytotoxicity at the higher dose level and hence, reduces the population of cells in metaphase.

Dose dependent increase in % aberrant cells was observed in the present study. A polynomial trend was observed between the groups with a regression formula of y = 0.1646x2 + 0.1816x + 1.1831 and r value of 0.76. This shows a strong relationship of a dose response with a polynomial trend. However, a test for significance was not performed as standard deviation was found more between the groups.

A two-fold increase in the aberrant cell frequency indicates clastogenic nature of the chemical in question at the doses of 0.35µg/ml and higher. The above results indicate a biologically significant response as the aberrant cell frequency showed two-fold increase in mid and high dose groups. This result is considered positive as per the criteria mentioned by Sofuni *et. al.,* (1990) and Galloway *et. al*., (1984).

Numerical Aberration
An increase in occurrence of polyploidy was observed in all the tested dose levels, indicates that colchicine at the extended exposure (> 2 hours) duration affects mitosis and increases occurrence in polyploidy (table: 8.5, 8.6; fig: 64).

4 - hour +S9 CAM
A linear trend in dose dependency was apparent between the groups as evidenced by the regression formula y = 1.89x + 2.02 and r value of 0.98 (table: 8.4; fig: 46 & 47). The results however, were further not analyzed statistically for a test of hypothesis as standard deviation was more between the groups. The present finding gain credence from the observations of Sobti *et al.,* (1983) who reported chromosomal anomalies in cultured human lymphoid cells exposed to Endosulfan with and without metabolic activation.

In continuation to the above-mentioned study for short-term exposure, a long-term study was planned to assess the clastogenic nature of the compound for longer exposure in the following experiment.

Long Term Exposure (Trial –I)
Without metabolic activation (72 hour)

A dose dependent increase in aberration was observed between the groups with a regression formula of y = 2.174x + 1.052 and r value of 0.97 (table: 9.1 & 9.2, fig: 48 & 49). The equation shows a strong relationship of a dose response with a linear trend. A significant ($p \leq 0.05$) increase in % aberrant cells was observed in all the groups except 0.350µg/ml.

Long Term Exposure (Trial –II)
Without metabolic activation (7 days)
Dose induced chromosomal aberrations was confirmed by this set of experiment (table:10, 10.1). In summary, Genotoxicity studies of Endosulfan have provided evidence that the test compound is mutagenic and clastogenic.

4.3 IN VIVO DOSE SELECTION STUDY
4.3.1 Mortality
Mortality was found to be 100% for doses 100mg/kg body weight and 40 mg/kg body weight while 75% and 25% mortality were observed at the doses of 20 and 10 mg/kg body weight respectively.

The detail of endpoint (mortality) observed in each group was as follows:

100 mg/kg body weight
All the animals were found dead after treatment in this dose group.

40 mg/kg body weight
Complete mortality was observed both in male and female mice in this group.

20 mg/kg body weight
Mortalities were observed in male (2 out of 3) mice only.

10 mg/kg body weight
One animal died at 10mg/kg body weight.

From the above dose range study a safer dose of 8 mg/kg body weight was selected as high dose for the main studies.

4.3.2 Clinical Symptoms
The symptoms of toxicity were more severe within one hour (40-60 minutes) of treatment. The animal died after one hour, if there is severe toxicity.

The observed morphological symptoms of toxicity were: Exaggerated response to sound stimuli, clonic tonic convulsion of fore and hind limbs, Catalapsy, Salivation, Lacrimation, Convulsion, Writhing and Dilation of pupil.

The gross pathological symptoms of toxicity observed were mottling in liver, lung and kidney of dead animals in both the sexes. These symptoms were co-examined by a trained veterinary pathologist for confirmation.

Further, based on the reference of Khan and Sinha (1993) a dose of 3 mg/Kg body weight Endosulfan (35% EC) was provided for 7 days using oral intubation in *Mus musculus*.

4.4 IN VIVO MAIN STUDY

4.4.1 Clinical Symptoms

4.3.2.1 Morphological

The morphological symptoms observed include abdominal breathing and convulsions, catalapsy and death (table: 11).

The most prominent signs of acute overexposure to Endosulfan in both humans and animals are hyperactivity, tremors, decreased respiration, dyspnea, salivation, and tonic-clonic convulsions. Five cases of acute lethal poisoning in humans resulting from accidental or intentional ingestion of Thiodan® were reported in an earlier study by Terziev *et al*. (1974).

Control group, low dose group

Control (0.0 mg/kg b.wt) and Low dose (4.0 mg/kg b.wt.) group animals showed no adverse clinical symptoms of toxicity.

High dose group

The animals exhibited overt signs of toxicity within 30-120 minutes of the treatment, which disappeared gradually after 2 hours of treatment. Symptoms of toxicity were first observed only in the high dose (8.0mg/kg b. wt.) group male animals. These symptoms include hyperactivity, tremor and abdominal breathing.

Three male animals died due to toxicity and showed severe tremors, writhing and salivation. The animals died at different intervals of the treatment *viz.,* 19th, 20th and 21st day of the treatment. However, even though few female animals exhibited hyperactivity and mild tremors they survived course of experiment.

On day 26th post-treatment, female animals exhibited hyperactivity, salivation and severe tremor. However, on 27th day of treatment the animals exhibited severe tremor, salivation, and lethargy. Out of the total one female animal died within two hours of treatment. This animal showed tremor, writhing, excessive salivation and finally vomited blood before it died. All other animals got recovered after treatment and were found to be normal at the time of sacrifice.

The above results indicate that:

1. Male animals are more sensitive (sex related toxicity) to the test substance in question.
2. Endosulfan at the given dose evoked acute and accumulative toxic effects (which was much prominent in female animals though) and
3. Hence, justifies 8.0 mg/kg b. wt as the highest applicable dose for a 28 day repeated dose study.

Signs of acute lethal Endosulfan poisoning in animals are similar to those observed in humans and include hyper excitability, dyspnea, decreased respiration, and fine tremors followed by tonic-clonic convulsions. Oral LD_{50} (median lethal dose) values for technical-grade Endosulfan vary depending on species, sex, formulation tested, and nutritional status of the animal being exposed (Gupta and Gupta 1979; WHO 1984). With regard to species sensitivity, mice appear to be quite sensitive to Endosulfan lethal effects, with a reported LD_{50} value of 7.36 mg/kg in males (Gupta *et al.* 1981) and 2 out of 10 male mice dying after

administration of 7.5 mg technical Endosulfan/kg in the diet for 7 days (Wilson and LeBlanc 1998). The sensitivity to mortality in male mice compared to female is also supported by NCI (1978).

The two isomers of Endosulfan (α and β) also reported to have different LD_{50} values in rats. The α-isomer is more toxic than the β-isomer in female rats, with an oral LD_{50} value of 76 mg/kg b. wt. versus an LD_{50} value of 240 mg/kg b. wt. for β-Endosulfan (Maier-Bode 1968; Hoechst 1975, 1990). The same difference was reported in female albino mice, the lethal dose for α-Endosulfan being 11 mg/kg b. wt. versus 36 mg/kg b. wt. for β-Endosulfan (Dorough *et al.* 1978). The lethal dose for Endosulfan sulfate in mice was comparable to that of the α-isomer, 8 mg/kg b. wt. (Dorough *et al.* 1978). Also, Hoechst (1966a, 1966b) had reported an LD_{50} of 14 mg/kg b. wt. for α-Endosulfan and 17 mg/kg b. wt. for β-Endosulfan in female mice.

Further, increased mortality was observed in both male rats (at doses of 20.4 mg/kg/day and above) and male mice (at doses of 0.46 mg/kg/day and above) in a 2-year bioassay conducted by the National Cancer Institute (NCI 1978). The authors attributed the excessive mortality in the male rats to treatment-related toxic nephropathy. The high mortality in male mice was possibly due to fighting since no other treatment related cause for the deaths could be determined. But in present case mortality is treatment related and manifested by clinical symptoms. Survival in females of both species was unaffected by Endosulfan (NCI 1978). However, survival was significantly decreased in female rats that consumed 5 mg/kg/day for 2 years (FMC 1959b), and in female mice that consumed approximately 2.9 mg technical grade Endosulfan/kg/day for 2 years (Hoechst 1988b; Hack *et al.* 1995). In these studies, survival in male rats was not affected at 5 mg/kg/day for 2 years (FMC 1959b) and survival in male mice was not affected at 2.51 mg/kg/day for 2 years (Hoechst 1988b).

4.3.2.2 Gross pathological observation

Immediately after death the mice, were dissected out and examined by specialized pathologists at and during different phases of experiments. The organs examined were liver, kidney, lungs, testes, gastrointestinal tract (oesophagus, stomach, ileum etc.) as presumed target organs.

The overall gross symptoms, observed include mottlings in liver, lung and kidney as overt symptoms of toxicity in both the sexes (table:12).

Case reports of human poisonings and studies in animals indicate that during acute oral exposure to lethal or near-lethal amounts of Endosulfan, involvement of a large number of organ systems (respiratory, cardiovascular, gastrointestinal, hematological, hepatic and renal) is observed. However, during long-term exposure, the liver and kidney appear to be the primary systemic target organs (Hack *et al.* 1995).

4.3.2.3 Histopathological observation

In the absence of any prominent symptoms in the target organs, detailed histopathological examination of these tissues were not carried out. The present notion gains credence from the observations of Dikshith and coworkers (1988). They in a 30 repeated dermal dose study of Endosulfan up to 62.5mg/kg/day (males) and 32 mg/kg/day (females) in rat reported no effect on organ weight or histoarchitecture.

4.4.2 Body weight

Dose related changes in body weight were not observed both in male and female animals (table: 13.1 and 13.2). This indicates Endosulfan toxicity can not be judged by change in animal body weight. The present findings also corroborates with the findings of Wilson and LeBlanc (1988) wherein no significant change in mice body weight was observed for food treated with technical grade Endosulfan at 15mg/kg in the food for 7 days.

In another finding, body weight gain was significantly reduced in male but not in female mice in the diet supplemented with 2.5mg Endosulfan/kg/day for 24 months (Hoechst 1988b; Hack *et al*. 1995). Neither food nor water consumption was significantly altered in mice or rats administered with technical grade Endosulfan in the diet for 24 months (Hack *et al*. 1995). The present finding for 28 days also supports indirectly the above report as there is no significant difference in body weight.

4.4.3 HAEMATOLOGY

Male Animals

WHITE BLOOD CELLS (WBC)

A slight increase in WBC count was obtained in all the groups as compared with the reference ranges (table: 14.1, 14.2). However, the data was statistically not significant when compared to that of concurrent control values, except an increase in HCT and MCHC at p≤ 0.05 which is of no any biological significance.

RED BLOOD CELLS (RBC)

There observed no differences in the RBC count in all the treatment and control groups. Further, the data is also comparable to reference range (table: 14.1 and 14.2).

HAEMOGLOBIN (Hb)

There were no differences in the haemoglobin count in all the groups (table: 14.1 and 14.2). This result is also comparable to its reference range and it is not significant.

HEMATOCRIT (HCT)

Here too the values were comparable to reference ranges and though a statistically significant increase (p≤0.05) was observed in high dose group, the result obtained is biologically insignificant (table: 14.1 and 14.2).

MEAN CORPUSCULAR VOLUME (MCV)

The Mean Corpuscular Volume (MCV) values were comparable to reference ranges, and among themselves. The results obtained are biologically and statistically insignificant (table: 14.1 and 14.2). A negative value was observed in the print generated by instrument being used. This is a calculation based result which should be considered positive instead negative values, it has been reported as per the instrumental print.

MEAN CORPUSCULAR HAEMOGLOBIN (MCH)

MCH is also a calculation type of result which is negative in the instrumental print; its values are also comparable to reference ranges and among themselves (table: 14.1 and 14.2). Hence, it is not significant.

MEAN CORPUSCULAR HAEMOGLOBIN CONCENTRATION (MCHC)

The values obtained were comparable to reference ranges and among themselves (table:14.1 and 14.2). Though, a statistically significant result is obtained, the data is biologically not significant.

PLATELETS (PLT)

An increased average platelet count was observed compared to the reference ranges (table: 14.1 and 14.2). However, there was no difference in the value among all the groups studied including the control and therefore, this could not be considered as increased platelet count.

Female Animals

WHITE BLOOD CELLS (WBC)

A slight increase in mean WBC count was observed in all the groups as compared to the references values (table: 14.3 and 14.4). However, the data is statistically and biologically not significant though a mild decrease was noticed in the treated groups compared to control.

RED BLOOD CELLS (RBC)

There observed no difference in the mean RBC count among the groups (table: 14.3 and 14.4). Also, the data is comparable to the reference range though it is statistically significant ($p \leq 0.05$).

HAEMOGLOBIN (Hb)

There are no differences in the haemoglobin count in any of the group (table: 14.3 and 14.4). The observed values are also comparable to its reference range and it is not significant.

HEMATOCRIT (HCT)

Here again the values are comparable to reference ranges (table: 14.3 and 14.4) and the result obtained is biologically and statistically insignificant.

MEAN CORPUSCULAR VOLUME (MCV)

The Mean Corpuscular Volume (MCV) values were akin to its reference ranges, and among themselves (table: 14.3 and 14.4). The results obtained are statistically significant ($p \leq 0.05$) and biologically insignificant. A negative value was observed in the print generated by instrument being used; this is an algorithm based result and should be considered positive instead of negative readings.

MEAN CORPUSCULAR HAEMOGLOBIN (MCH)
MCH is also a calculation based result which is negative in the instrumental print; its values are also comparable to the reference ranges and among themselves (table: 14.3 and 14.4). Hence, it is not significant.

MEAN CORPUSCULAR HAEMOGLOBIN CONCENTRATION (MCHC)
The values obtained are comparable to its reference ranges and among themselves (table14.3 and 14.4). The data is biologically and statistically not significant.

PLATELETS (PLT)
An increased mean platelet count was observed in the present study compared to the reference ranges (table: 14.3 and 14.4). However, there is not much difference in the values of PLT amongst the groups studied including control and hence, can not be considered as increased platelet counts.

There is no historical data available on Endosulfan induced haematological changes in mice. Mixed results have been reported from studies examining haematological effects of dermal exposure to Endosulfan in rats. Although decreased haemoglobin was observed in male rats following daily application of doses of Endosulfan of 18.75 mg/kg b. wt. for 30 days (Diikshith *et al.,* 1988), similar results have not been observed in female rats or in male rats at similar doses in other studies. For example, no haematological parameters were adversely affected following exposure of females to doses of 32 mg/kg/day for 30 days (Diikshith *et al.,* 1988). In addition, no adverse effects on routine haematological parameters were observed following exposure of rats for 30 days for 6 hours per day, 5 days per week to doses of Endosulfan ranging from 12 to 192 mg/kg/day (males) and from 3 to 48 mg/kg/day for females (Hoechst 1985d). Similarly negative results were obtained in a comparable 30 day rat study using slightly lower Endosulfan doses (Hoechst 1985c). The above difference in different results of these studies is unclear but may have been related to differences in the age of the rats or the application protocol.

4.4.4.1 Differential Leucocyte Count
One day after exposure
Comparable results were obtained in animals of both the sexes (table: 15.1, 15.2,15.3 and 15.4).

One week after exposure
A dose dependent decrease in lymphocytes was observed in male animals (table: 16.1 and 16.2). The decrease was statistically significant ($p \leq 0.05$) in the high dose group whereas, a dose dependent increase was observed in neutrophils. This increase was statistically significant ($p \leq 0.05$) in the high dose group and may be due to the relative decrease of lymphocytes. This difference may be related to exposure with Endosulfan but are very low and cannot be interpreted as biologically significant increase. No other changes were observed in male animals after one week of repetitive exposure.

The observed changes were subtle in case of female animals and hence, cannot be considered as biologically significant. Therefore, it can be inferred from the present study that there is no difference, in differential leucocyte counts in both the sexes after one week of exposure.

The results of the earlier studies also support the current results. Sub-acute and chronic-duration studies using Endosulfan revealed no effects on hematological parameters or on routine gross and histopathological examination of bone marrow and the spleen in mice or dogs (FMC 1959a, 1967; Hoechst 1984b, 1988b, 1989c).

4.4.5 BIOCHEMICAL OBSERVATIONS

Biochemical tests were performed to find changes in glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CRE), blood urea nitrogen (BUN) and Cholinesterase (CHE) both in male and female animals.

Male Animals

A slight increase in cholinesterase was found in the treated animals which however, was statistically and biologically not significant, this might be due to Endosulfan induced neurogenic response (table: 17.1 and 17.2, fig: 50). All the other parameters mentioned in section 4.4.5 were within normal range and hence not significant.

Female Animals

A statistically significant decrease ($p \leq 0.05$) in alanine aminotransferase (ALT) and creatinine (CRE) was observed which is well within the biological range hence, changes are computed insignificant. All other parameters were normal (table 17.3 and 17.4, fig: 51).

Repeated dose studies have generally not shown adaptive or adverse effects on the liver. Routine gross and microscopic pathology has not revealed adverse hepatic effects in mice exposed to 2.51 mg/kg/day (males) or 2.86 mg/kg/day (females) for 2 years (Hoechst 1988b; Hack *et al.* 1995), in rats exposed to 5 mg/kg/day (females) or 2.9 mg/kg/day (males) for 2 years (Hack *et al.* 1995; FMC 1959a; Hoechst 1989a), or in dogs exposed to 1 mg/kg/day for 2 years (FMC 1967). Serum alkaline phosphatase was, however, elevated in dogs exposed to 0.67 mg/kg/day (males) or 0.6 mg/kg/day (females) for 1 year, suggesting adverse effects on the liver; however, no effects on liver weight, liver function, or microscopic pathology were observed (Hoechst 1989c).

4.4.6 BONE MARROW MICRONUCLEUS TEST

4.4.6.1 Ratio of Polychromatic to total Erythrocytes (P/E ratio) male and female

A mild suppression in P/E ratio was observed (table: 18). However, this suppression was statistically not significant. Moreover, this suppression indicates toxicity to bone marrow and was more prominent in female animals.

4.4.6.2 % MN PCE and % MN NCE

Male Animals

A dose dependent increase ($p \leq 0.01$) in %micronucleus frequency was observed both in polychromatic (%MN PCE) and normochromatic erythrocytes (%MN NCE) in male animals after 28 days repeated administration at the dose of 8 mg/Kg body per day (table,19 and 19.1, fig: 52). An increase in %micronucleus frequency in polychromatic (%MN PCE) was statistically not significant but biologically significant.

Female Animals

Female animals also showed a dose dependent increase (p≤ 0.001) in % micronucleus frequency both in polychromatic (%MN PCE) and normochromatic erythrocytes (%MN NCE) after 28 days repeated dosing at the dose of 8 mg/Kg body weight (table: 19.2; fig: 53; fig: 65).

PERIPHERAL BLOOD MICRONUCLEUS TEST

Peripheral blood was examined to evaluate bone marrow toxicity by comparing mature and immature red blood cell ratio in peripheral blood. For this, ratio of mature RBC among total was examined. The result reveled no much difference in the ratio of mature RBC among total in all the treated groups of both male and female animals (table: 21,21.1, 21.2 and 21.3).

Peripheral blood Micronucleus frequency

Micronuclei can also be scored in the polychromatic erythrocytes (PCE) in mouse peripheral blood (fig: 66). The significant advantage of the peripheral blood assay is less invasive blood sampling, which allows for repetitive sampling from the same animal over time for kinetic studies of micronucleus (MN) induction (MacGregor, 1980 and 1983). The peripheral blood micronucleus test was originally thought to be only applicable to the mouse because the mouse spleen is known to be incapable of removing micronucleated erythrocytes in the blood. However, recent studies in the rats also showed that newly formed micronucleated PCE (also referred to as reticulocytes) are also detectable in peripheral blood before they are removed from the spleen (Holden, 1997). Furthermore, the ability to score micronuclei in normochromatic erythrocyte (NCE), which accumulate in the peripheral blood, permits the evaluation of clastogenicity of a test article in the standard multiple dose toxicological studies. Indeed, retrospective evaluation of micronuclei in NCE of peripheral blood (fig: 67 & 68), have been performed in several mouse cancer bioassays in the National Toxicology programme (NTP) (Choy, 1997; MacGregor, 1990). Such studies provide examples that the micronucleus test can be incorporated into routine bioassays for the assessment of Genotoxicity under the same exposure conditions as in the animal bioassays.

1St Day of Exposure

The mean % MN frequency in RBC observed were 0.122, 0.123 and 0.117 in male and 0.116, 0.098 and 0.213 in female animals (table: 21.1). The female animals were shown statistically increase (p≤ 0.05) in percent micronucleated red blood cells.

1St Week of Exposure

The mean % MN frequency in RBC observed were 0.049, 0.191and 0.180 in male and 0.118, 0.145 and 0.313 in female animals (table: 21.2). Though the incidence of micronucleus was high in female animals of high dose group there were no difference in the 1st day and 1st week response.

2nd Week of Exposure

The mean % MN frequency in RBC observed were 0.117, 0.094, and 0.394 in male and 0.230, 0.169 and 0.216 in female animals (table: 21.3). An increase in peripheral blood micronucleus assay was observed in 2nd week of exposure in high dose male animals (p≤ 0.05) was observed. The above finding confirms the occurrence of increased micronucleus frequency in bone marrow cells followed by increase in peripheral blood.

4.4.7 SPERM MORPHOLOGY TEST

A dose dependent statistically significant ($p \leq 0.05$ for 4.0 mg/Kg body weight and $p \leq 0.01$ for 8.0 mg/Kg body weight, respectively) increase in percent abnormality of the sperm head was observed in the current study (table: 20). The observed abnormality include large head, small head, amorphous head, bending of head with no hook, double tail and coiling of tail (fig: 69 to 75). Further, the relative fold values were 2.2 and 7.64 to the doses of 4.0 and 8.0mg/Kg body weight respectively when compared to that of concurrent control group (table: 20.1). Both the above results indicate that the Endosulfan after 28 days consecutive exposure causes sperm abnormality.

Khan and Sinha (1996) observed significant reduction in sperm count of 35% (w/w) Endosulfan technical as active principle, and the remaining 65% (w/w) being solvents, emulsifiers and stabilizers. An increase in abnormal sperm count was observed by 14% at a dose of 3 mg/kg bodyweight/day for 35 consecutive days in Swiss albino mice.

The gross abnormalities in sperm head shape for Endosulfan were also observed and described as pin shaped, bottom-heavy (Soars *et. al.,* 1979), triangular, amorphous, rudimentary (Rastogi and Levin, 1987; Bhunya and Behera, 1987), hammer shaped, sickle shaped and flat based (Sinha and Prasad, 1990). Some sperms were characterized as having distinctly larger (twice the area) or smaller (half the area) head size as compared to normal, as documented by Seuanez *et.al.,*1977. Sperm with abnormal head number (twin-headed) were also recorded (Soars *et. al.,*1979; Sinha and Prasad, 1990).

Sinha and Saxena (1997) also found sperm abnormality in Druckery rats (3 weeks old) at the doses of 2.5, 5.0 and 10 mg/kg/day for 90 days with technical grade Endosulfan. A significant increase was observed in sperm head *viz.,* no hook, excessive hook, amorphous, pin and short head; and tail abnormalities were also observed as coiled flagellum, bent flagellum and bent flagellum tip. These findings are in agreement with the currently observed sperm abnormality in Endosulfan treated mice.

Lastly, the present study using a combination of *in vitro* and *invivo* test system to evaluate the toxicity, revealed beyond doubt that the test substance Endosulfan is a mutagen to the bacterial system however, even though it is found clastogenic, its mutagenic potency in mammalian system was equivocal. This warrant further confirmatory test like mouse lymphoma assay (MLA). Nevertheless, the results of the present study confirmed beyond doubt the aneugenic, clastogenic, and spermatotoxic potency of the test substance in question and hence, it is logical to surmise that Endosulfan might impart developmental anomalies in non target organisms including human. It is therefore, suggested that this pesticide needs to be recommended to be listed in Appendix III of Rotterdam Convention and ultimately convince the agrochemical industry to rapidly phase out the production of Endosulfan by suggesting appropriate alternate safe synthetic and/or biological pest control measures.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

Conclusion

Endosulfan is mutagenic to bacterial test systems only in the absence of exogenous metabolic activation system. The mutagenicity was strong in case of *Salmonella* strain TA 102. Nonetheless, mutagenicity should be tested in sensitive strain like *Eschrichia coli* WP_2 uvrA with or without plasmids, since these strains are similar to *Salmonella* strains TA 102 in terms of sensitivity to mutate, for further confirmation.

Endosulfan was found to induce toxicity and clastogenicity in mammalian cells. The mutagenicity of Endosulfan is equivocal for mammalian cells. On one side it gives clear increase in clastogenic response and induces various chromosomal aberrations while on the other side somatic cell mutagenicity was not found in single trial of short term exposure. An experimental design including long term exposure with repeat experiments is required to detect mammalian cell mutagenicity as short term exposure may lead to death of the mutant cell (during 7 days expression period, CHO HGPRT assay) or expression period should be reduced to select mutant cells for expression. Therefore, confirmation on mutagenicity should be taken only after performing more sensitive test like mouse lymphoma assay (MLA).

Endosulfan is extremely toxic which leads to mortality on acute dosing and becomes clastogenic when applied repetitively both *in vitro* and *in vivo* tests.

Systemic exposure is all neurogenic type and clearly detected by increase in cholinesterase levels in serum. The hazards of Endosulfan is clearly due to targeting to the nervous system as is detected by the symptoms observed after exposure (all pertaining to nervous system) therefore, it is extremely toxic to the living system.

Endosulfan induces abnormality in sperm head and tail morphology. The anueniginecity of Endosulfan is very clearly detected by repetitive 28 days micronucleus test. This may be because Endosulfan takes time to get absorbed in to the mammalian system. The menace of Endosulfan induced malformations in human being could be related to its anuegenicity and gamete abnormality However, further carefully designed study on reproductive and developmental toxicity needs to be undertaken in order to consolidate the current notion.

Recommendations

The indications are that Endosulfan poses serious risks to human health, especially under conditions of use in developing countries. Indeed, the chemical has been implicated in scores of cases of accidental death across the globe and long-term exposure has been linked to a range of serious disorders among villagers of southern India.

This pesticide kills indiscriminately, affecting not only pests, but also a range of other harmless or beneficial insects, with similar ramifications for species further up the food chain. Endosulfan's ability to harm is reflected in its mutagenic, aneugenic and systemic toxicity. This document presents ample evidence that Endosulfan might pose considerable risk to humans and the environment. In light of this the following recommendations are made:

Endosulfan is a highly dangerous, outdated chemical, the safe use of which cannot be guaranteed by many poor countries where it is still used. Governments should ban Endosulfan use, and Designated National Authorities in countries that are signatories of the Rotterdam Convention (India is party since 2005) should propose the chemical for inclusion in the Convention's Prior Informed Consent procedure. Endosulfan is already referred Chemical Review Committee (CRC 2) to be included in Annexure III. However, the decision is still pending.

Endosulfan is a persistent chemical that has been demonstrated to bioaccumulate in exposed organisms. As such, it should be included on the list of Persistent Organic Pollutants targeted for global elimination by the Stockholm Convention to further promote better practice. The World Health Organization should upgrade Endosulfan from Class II (Moderately Hazardous) to Class Ib (Highly Hazardous), in line with the USA's EPA classification. Such a move would assist many countries, which has banned all Class Ia and Ib chemicals, to promote safer agrochemical practices.

Safe alternatives to Endosulfan must be researched, identified and widely promoted. Pesticides Action Network Asia-Pacific lists a number of alternatives to Endosulfan use in different agricultural contexts. These include use of botanical pesticides (neem extracts) and parasitic wasps in rice production, and the use of baculoviruses, natural enemies and pheromone traps to control cotton pests (Source: Environmental Justice Foundation Ltd. www.ejfoundation.org).

Ultimately, the action most ably protecting human and environmental health would be the withdrawal from sale of Endosulfan. This requires the agrochemical industry to rapidly phase out production of Endosulfan and to dispose of all stockpiles safely.

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