CHAPTER 2

1. MATERIALS AND METHODS

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°C 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°C 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°C 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
$C_{13}H_{10}N_2 \cdot HCl$, 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], $Na\ NH_4HPO_4 \cdot 4H_2O$, 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°C 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°C
Procedure: Add 25g to 1l of distilled water & sterilize by autoclaving at 121°C 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°C 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°C 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°C.

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°C for approx 5 days, for long storage aliquot and freeze at -20°C.

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°C 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°C). 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°C 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°C). 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°C.

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 0°C 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±3°C 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, - 80°C) 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 37°C. 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

Volume of standard (µl)	Actual weight (µg)	Volume of distilled Water (µl)	Final volume (µl)
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°C 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 45°C 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°C 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 45°C 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 37°C 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

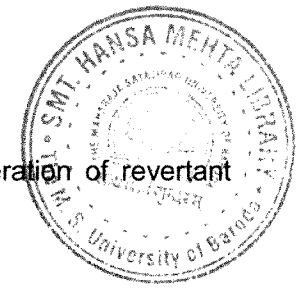
With few exceptions it is believed that this assay is more sensitive than the plate incorporation assay, because short-lived mutagenic metabolites may have a better chance reacting with the tester strains in the small volume of preincubation mixture, and the effective concentration of S9 mix in the preincubation 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 45°C 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 37°C 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:

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



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

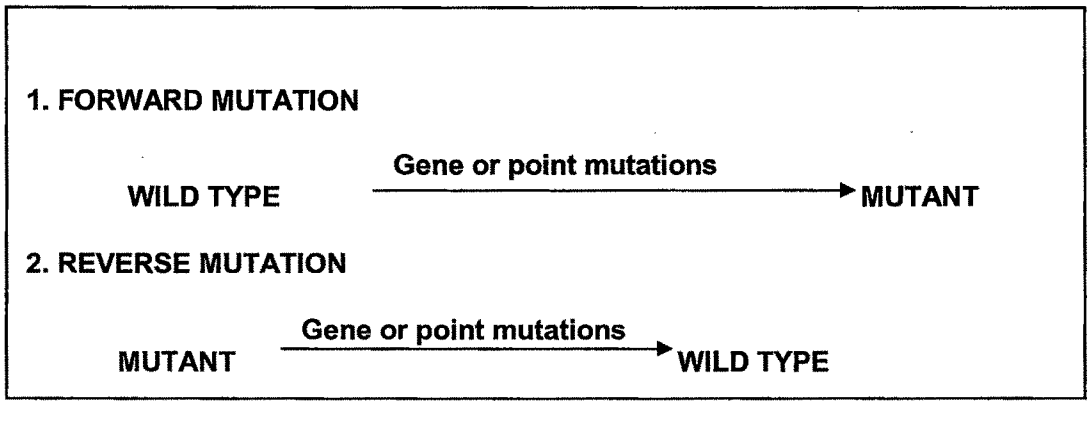
The test article can be considered positive if it produces at least 2- fold increase in the mean revertants/plate of at least one of these tester strains over the mean revertants/plate of the appropriate vehicle control.

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°C. 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°C 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°C 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 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

[C] = 0.525 µg/ml α_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 α_1

2) 100 µl was added to 4.90 ml medium without serum

[C] = 0.35µg/ml α_2

3) 150 µl was added to 4.85 ml medium without serum

[C] = 0.525µg/ml α_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.

Chapter 2

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 45°. 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_0 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.

B. 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.

C. 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 7^{th} 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°C. 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 °C. The absorbance of standards and of each sample against reagent blank were read at 510nm and expressed as mg/dL.

Calculation

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

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 °C (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

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

$$\Delta A = \text{Final absorbance} - \text{Initial absorbance}$$

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 °C – 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 °C – 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 °C 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:

$$\text{Head Abnormality} = \frac{\text{Number of abnormal cells}}{\text{Total number of cells observed}} \text{x1100\%}$$

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 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.