CHAPTER 2

MATERIALS AND METHODS
2.1 CHEMICALS

Mitomycin C (MMC) was purchased from Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan. Deoxyribonucleic acid (DNA), highly polymerised, yeast ribonucleic acid (RNA), Bovine serum albumin (BSA), (N-[2-Hydroxyethyl]-piperazine-N'-[2-ethanesulphonic acid]) (HEPES), 2,5-Diphenyloxazole (PPO), 1,4-bis-[5-phenyl-2-oxazolyl] benzene (POPOP), Thymidine, Trypan blue, Napthol AS - BI - glucuronide, Pararosaniline hydrochloride, Polyvinyl pyroldine were purchased from Sigma Chemical Co. (USA). Roswell Park Memorial Institute (RPMI) 1640 tissue culture medium containing L-glutamine, but not Sodium bicarbonate were purchased from GIBCO, USA. Fetal calf serum (FCS) was purchased from DIFCO, USA, heat inactivated at 56°C for 30 minutes and stored in dark at −20°C. Trichloro-acetic acid (TCA) was purchased from E. Merck, West Germany. Leishman's eosine methylene blue was purchased from BDH, U.K. Other chemicals were of 'analalr' grade.

2.1.1 CHEMICALS FOR ELECTRON MICROSCOPY

Calcium chloride and Magnesium chloride were purchased from E. Merck, West Germany. Glutaraldehyde (25%), Paraformaldehyde, Sodium veronal, Sodium phosphate (monobasic), Sodium phosphate (dibasic), Lead nitrate, Sodium citrate, Sodium chloride, Vinyl cyclohexane dioxide (ERL 4206), Nonenyl succinic anhydride (NSA), Diglycidyl ether of polypropylene glycol (DER 736), S-1 Dimethyl amino ethanol (DMAE) were purchased
from Polaron Equipment Limited, U.K. Bacto Agar and Bacto Tryptone were purchased from DIFCO, USA, Uranyl acetate was purchased from BDH, U.K., Collodian was purchased from Sigma Chemicals Co. (USA). Other chemicals were of 'analar' grade.

2.1.2 RADIO-ACTIVE CHEMICAL

$\text{H}^3\text{I}$-thymidine with specific activity 12600 mCi/m.mol and concentration 1 mCi/m.mol was obtained from Bhaba Atomic Research Centre, Trombay, India.

2.2 EXPERIMENTAL ANIMALS

Swiss albino male mice (4-6 weeks old) and weighing 20-22 gm were used in the present study. Animals were bred in our Institute's vivarium and maintained in groups of ten in metal cages with saw dust as bedding and filter tops. They were kept at uniform temperature (24°C) with alternate day and light cycle (12:12 hrs). Standard laboratory animal diet (Lipton India Limited) and water were given ad libitum.

2.3 ASCITES SARCOMA 180 TUMOUR CELL

The Sarcoma 180 tumour was obtained from the School of Tropical Medicine, Calcutta through courtesy of the Director and has been maintained by the serial transplantation in our Institute.

Originally the tumour was developed by Charlotte Friend in 1951 by intraperitoneal injection of minced Crocker Sarcoma 180
tumour tissue in Swiss albino mice. Crocker Sarcoma 180 was discovered in the Laboratory of Woglan (1914) at the Crocker Laboratory in New York (now, the Institute of Cancer Research, Columbia University) in the right maxilla of a white male mouse and was identified as carcinoma. After serial subcutaneous transplantation a sarconatous change of the tumour occurred and subsequently became a Sarcoma in 1919 and since remained stable (Stewart, H.L. et al., 1959 and Sugiuara, K., 1965).

2.3.1 MAINTENANCE OF SARCOMA 180 TUMOUR CELL LINE

Swiss albino male mice were obtained from the Animal Department of our Institute and were used for the present study. Sarcoma 180 cells were grown in ascites form by serial intraperitoneal transplantation of $10^7$ cells to 4-6 weeks old mouse, weighing 20-22 gm. Animals were kept in alternate light (12 h) and dark (12 h) conditions and were provided with normal food and water ad libitum.

2.3.2 GROWTH CHARACTERISTICS OF SARCOMA 180 CELL LINE

After 3 days of transplantation of the Sarcoma 180 tumour 0.2 ml of ascites fluid was withdrawn from the peritoneal cavity by aspiration and suspended in a fixed volume of physiological saline (2-3 ml). 0.2 ml of this diluted cell suspension was mixed with 0.15% Trypan blue in physiological saline (Wang Richard, I.H., 1963) and cell number was counted in a haemocytometer. The cells which excluded the dye were counted as 'viable'. The viable cell
number per ml of ascitic fluid was determined by multiplying this number with the appropriate dilution factor. To determine the growth characteristic of Sarcoma 180 tumour cells, the viable cell per ml of ascitic fluid were determined on different days after transplantation. The number of dead cells present, if any, was less than 5% of the total population.

On an average the number of animals used on each day was ten.

2.3.3 ADMINISTRATION OF MITOMYCIN C TO SARCOMA 180 TUMOUR

Mitomycin C received as 2 mg (potency) per vial was dissolved in 4 ml of 0.01 M phosphate buffer, pH 7.2 (concentration 0.5 mg/ml) and preserved in dark in a refrigerator. The solution was used up as soon as possible and discarded when it turned violet. Mitomycin C was administered by intraperitoneal injection in the Sarcoma 180 bearing mouse after five days of tumour transplantation. The concentration used 4 mg/kg or 7 mg/kg body weight of mouse.

2.3.4 WHOLE BODY GAMMA IRRADIATION OF SARCOMA 180 TUMOUR BEARING MOUSE

Tumour bearing mice housed in the individual spaces of a specially prepared perspex box with proper ventilation were exposed to gamma irradiation from $^{137}$Cs source (Picker, U.S.A.) with a dose rate of 0.62 Gy per minute at Hospital Wing of our Institute.
2.3.5 SCHEDULE FOR ADMINISTRATION OF MITOMYCIN C AND GAMMA IRRADIATION ON SARCOMA 180 TUMOUR BEARING MICE

After 5 day of transplantation, Sarcoma 180 tumour bearing mice were divided into four groups, animals being selected randomly.

Group 'A' : Animals were untreated and kept as control.

Group 'B' : Animals were given single injection of MMC intraperitoneally (i.p.). Dose selected were 4 mg/kg body wt., 7 mg/kg body wt. of mouse.

Group 'C' : Animals were whole body irradiated with gamma radiation. Selected doses were 4 Gy and 8 Gy.

Group 'D' : Concomitant treatment: Animal received Mitomycin C and gamma radiation concomitantly in the following sequence:

1) MMC (4 mg/kg body wt.) plus 4 Gy.
2) MMC (7 mg/kg body wt.) plus 4 Gy.
3) MMC (4 mg/kg body wt.) plus 8 Gy.
4) MMC (7 mg/kg body wt.) plus 8 Gy.

In all cases MMC was given by a single i.p. injection and animals were irradiated within 15 minutes.

The growth characteristic of Sarcoma 180 tumours in animals subjected to different treatment schedules were determined by viable cell count as done for untreated control. The inoculum size was maintained $10^7$ cells per mouse in all cases and in each group 10 animals were taken.
2.4 PER CENT GROWTH INHIBITION (PGI) OF TUMOUR CELLS

The per cent growth inhibition of tumour cells following different in vivo treatment was calculated from the relation.

\[
\text{Per cent growth inhibition (PGI) = } (1 - \frac{T}{C}) \times 100
\]

(Matsumoto, S. et al., 1986)

where \( C = \) no. of viable tumour cells per ml of ascitic fluid in untreated control animals on any day after transplantation, and
\( T = \) no. of viable cells per ml of ascitic fluid present in treated group of animals on the same day.

2.5 ESTIMATION OF DNA, RNA AND PROTEIN CONTENTS OF SARCOMA 180 CELLS

DNA, RNA and protein content of the cells were estimated by established procedure following the modified method of Schmidt, G. and Thannhuser, S.J. (1945). Tumour cells were taken out from peritoneal cavity of 2-3 untreated tumour bearing mice after different days of transplantation by cervical dislocation, washed with 10 volumes of ice-cold saline (2-3) times to remove blood cells and other materials and centrifuged at 1500g to form a pellet. 10 volume of cold saline was added to pellet and it was homogenised in a homogenizer with teflon coated pastle, this gave 10% homogenate (V/V). To one part of 10% homogenate equal volume of water and 6% perchloric acid was added and it was kept in ice for 15 minutes. After centrifugation at 1500g for 10 minutes, supernatant was collected by decantation and kept in cold. The
pellet was washed twice with 2 ml of 2.0% PCA by centrifugation and the successive supernatants added to the first supernatant.

Now to the acid soluble fraction free pellet was added 0.3 N ice cold sodium hydroxide and mixed thoroughly. Incubated the mixture at 37°C water bath for 1 hour with lid. 12% ice cold PCA was added and the mixture was kept in ice for 30 minutes. After centrifuging at 1500g for 10 minutes the supernatant was collected by decantation. The process was repeated 2 times and the supernatant of all steps were combined and used for RNA estimation.

The pellet free from RNA was dissolved in 5% PCA by vortexing and then placed in a shaking water bath at 70°C for 20 minutes. After centrifuging at 1500 g for 10 minutes the supernatant was kept. The pellet was washed twice with 5% PCA by centrifugation and successive supernatants were combined with the above supernatant. This supernatant was used for DNA estimation.

The final pellet was suspended in 0.2 N NaOH and used for protein estimation. Cells from treated group of animals were also processed in same way on same days.

DNA, RNA and protein contents were estimated according to standard procedure (Mejbaum, W., 1939; Lowry, O.H. et al., 1951; Burton, K., 1956) taking calf-thymus DNA, yeast RNA and BSA as standards for above estimation.
2.6 MEASUREMENT OF CELL AND NUCLEAR SIZES OF SARCOMA 180 TUMOUR CELL

2-3 untreated tumour bearing mice after different days of transplantation were killed by cervical dislocation and cells were aspirated from the peritoneal cavity of tumour bearing mice using 1 ml of hypodermic syringe fitted with a 24 gauze needle. Added 1 drop each on grease free clean gelatinised glass slides and thin smears were made. After semidrying the slides were fixed immediately by immersing in methyl alcohol for 3 minutes. The slides were dried in air and coverslips were mounted on the slides with polyvinyl pyrolidine. The mounted slides were observed under phase contrast microscope (Carl Zeiss, West Germany) and both cell and nuclear sizes were measured crosswise by ocular micrometer. The ocular micrometer was well calibrated with stage micrometer.

Cells from treated mice were also processed in same way on same days.

2.7 $^{3H}$-THYMIDINE UPTAKE BY WHOLE CELL AND ITS INCORPORATION INTO TOTAL DNA

Uptake of $^{3H}$-thymidine in Sarcoma 180 cells and its incorporation into TCA insoluble product was measured following the method of Alalawi, F.A. and Chapman, I.V. (1977). Tumour cells were withdrawn from peritoneal cavity of Sarcoma 180 bearing mice after 7 days of transplantation. Cells were washed three times with cold isotonic saline by low speed centrifugation at 500g for removing blood adhering materials. The final
cell pellet was suspended in a small volume of cold physiological saline (2-3 ml) and viable cell number was determined by trypan blue dye exclusion test. A part of this cell suspension was suspended in \textit{in vitro} tissue culture medium to a final concentration of $1 \times 10^6$ cells/ml. The composition of tissue culture medium was RPMI-1640 medium containing 10% heat inactivated fetal calf serum and 25 mM Hepes buffer (pH 7.4) supplemented with penicillin 100 units per ml and streptomycin 50 \mu g per ml. The cell suspension was next incubated at 37°C shaking water bath for 10 minutes. $\textit{\textsuperscript{3}}$HJ-thymidine was added to a final concentration of 4 $\mu$Ci per ml. (Stock 1 mCi/ml, Sp. activity 12600 mCi/m.mol).

For the uptake studies 1.0 ml aliquots in duplicate were removed at specific intervals. Cells were immediately chilled by adding them to 1 ml isotonic saline in tubes placed, in crushed ice to stop the reaction instantly, washing of the cells were done with cold isotonic saline by centrifugation at 1500g at 4°C for 3-4 times to remove extracellular radio-activity. A final wash was done with 50 \mu M non-radio-active thymidine in cold to remove adherent labelled thymidine. The washed pellet was suspended once more in 1 ml of isotonic saline and equal volume of 20% TCA (at 0°C) was added to precipitate the acid insoluble products. After incubation for 30 minutes at 0°C the TCA soluble pool was removed by centrifugation at 1500g at 4°C and its $\textit{\textsuperscript{3}}$HJ-thymidine content of the TCA soluble pool was measured by
transferring 0.1 ml in duplicate to the scintillation vials. 10 ml dioxan based fluor (PPO 4.0 gm, POPOP 200 mg, Napthalene 60 gm, Methanol 100 ml, Ethyleneglycol 20 ml per litre of dioxane) was added to each vial and radioactivity was determined in a liquid scintillation spectrometer (LSS-34 Electronics Corporation of India) with 45% efficiency for $^{3}H$. Total count for each time point was estimated by multiplying this count by 20 as volume of TCA soluble pool was 2 ml.

The determination of incorporation of labelled precursor into bulk cellular DNA was done as described below. TCA insoluble precipitates was transferred on Whatman 3 MM filter disc fitted in a 10 ml membrane filter holder and washed 5-6 times with 10 ml 5% cold TCA. The disc was further washed with cold 95% absolute alcohol, cold alcohol : ether (1:1) and finally with cold ether. All the precipitates were processed similarly. The discs were air dried and transferred to the scintillation vials. 10 ml toluene based fluor (PPO 3.92g, POPOP 80 mg per litre of toluene) was added to each vial. The total cellular uptake was estimated by adding the counts of TCA soluble pool plus insoluble pool. Cells from treated mice were also processed in same way on same day.

2.8 CYTOCHEMICAL DEMONSTRATION OF $\beta$-GLUCURONIDASE ACTIVITY IN SARCOMA 180 CELLS

The method followed was that developed by Hayashi, M. et al. (1964) which used napthol AS - BI - glucuronide in the
presence of hexazonium pararosaniline. In this simultaneous coupling reaction the coupler (hexazonium pararosaniline) is expected to trapped the liberated napthol AS - BI as it is freed from glucuronide by the enzyme at a pH 5.2. The intensity of red colour developed during the reaction depends on the enzyme activity.

Buffer and Solutions required for the method:

0.2(N) Acetate buffer (pH 5.0).
Solution A: 1.15 ml glacial acetic acid (wt. per ml 1.048 gm) in 100 ml of distilled water.
Solution B: 2.72g of sodium acetate in 100 ml of distilled water.

14.8 ml of solution A and 35.2 ml of solution B were mixed and diluted to a total volume of 100 ml with distilled water. This gave 100 ml of 0.2 N Acetate buffer (pH 5.0). Kept at room temperature.

0.05 M Sodium bicarbonate:
42 mg Sodium bicarbonate was dissolved in 10 ml distilled water. Kept at room temperature.

Substrate Stock Solution:
28 mg of napthol AS - BI - glucuronide was dissolved in 1.2 ml of 0.05 M Sodium bicarbonate solution. 0.2 N Acetate buffer (pH 5.0) was added to give a final volume of 100 ml. This stock solution could be kept at room temperature for few weeks.
Pararosaniline Stock Solution:

1g of pararosaniline hydrochloride was dissolved in acidified water (20 ml of distilled water + 5 ml conc. hydrochloric acid) with gentle warming. The solution was cooled, filtered and stored at room temperature.

Sodium nitrite, Stock Solution:

0.4g of Sodium nitrite was dissolved in 10 ml distilled water and stored in a refrigerator. Used within 3-4 days.

Working Solution:

0.3 ml of pararosaniline solution was added to 0.3 ml of sodium nitrite solution in a 50 ml beaker. After one minute, 10 ml of substrate stock solution was added. pH of the solution was adjusted to 5.2 with 1(N) NaOH. Distilled water was added to make the volume 20 ml and the solution was filtered through Whatman No. 1 filter paper.

Preparation of Smears of Sarcoma 180 cells:

Ascites Sarcoma 180 cells were aspirated from the peritoneal cavity of tumour bearing mice after different days of transplantation using hypodermic syringe with 1 ml capacity and fitted with a 24 gauze needle. Added one drop each on one end of grease free clean gelatinised (2%) glass slides and thin smears were made. After semidrying the slides were fixed immediately by immersing in ice cold ethanol for 3-5 minutes.
Staining Protocol:

Before staining all the slides were washed thoroughly with distilled water, semidried and taken in a 15 cm dia. Petridish. Smears were covered with the working solution and incubated at 37°C for 30 minutes with a glass lid. After incubation slides were washed with distilled water, dehydrated in ethanol, and cleared with xylene. Coverslips were mounted with polyvinyl pyrolidine.

Assessment of β-glucuronidase activity:

The mounted slides were observed under the optical microscope with an oil immersion objective. Subjective grading (high, moderate and low) of enzyme activity in tumour cells were carried out according to the intensity of red colour developed during the reaction. 300 cells per slide were scored. The average scoring was done by counting at least 4 slides.

Cells from treated mice were also processed in same way on same days.

2.9 BIOCHEMICAL ESTIMATION OF ACID DNASE ACTIVITY IN SARCOMA 180 CELLS


Preparation of Tumour Cells Homogenates:

Cells collected from 2-3 tumour bearing mice sacrificed by cervical dislocation after different days of transplantation,
were washed 2-3 times with cold physiological saline to remove adhering blood cells and other materials etc. by centrifugation at 1500g for 5 minutes. Final cell pellet was suspended in 2-3 ml cold saline and viable number of cells were determined by trypan blue dye exclusion test. The cell suspension was further diluted to 1x10^6 cells/ml in cold saline and sonicated in a sonifier (Labsonic 2000, Switzerland) at 0°C. The sonified suspension of the total homogenate was used as source of enzyme.

The activity of this enzyme was determined by measuring the amount of acid soluble mononucleotide released from DNA per 20 minute at 37°C. Unit of enzyme activity was defined as the amount of enzyme that catalyses the formation of n mole of mononucleotide per minute. Specific activity refers to units of activity of this enzyme per mg of protein. Free acid DNase activity was measured as follows: the reaction mixture (total volume of 1 ml) contained 0.5 ml of homogenate, 1 mg C.T. DNA, and was 0.2 M KCl, 0.05 M in sodium acetate buffer (pH 5.0) and 0.25 M in sucrose. After a 20 minute incubation at 37°C, the reaction was stopped by adding 1.5 ml of a chilled 1(N) solution of perchloric acid. After 20 minute standing in ice the precipitated proteins and potassium perchlorate were removed by centrifugation at 1500g for 30 minutes at 4°C and the clear supernatant was carefully separated by use of Pasteur pipette.

The absorbance of all the supernatant were measured at 260 nm in Pye Unicam Sp8-100 (U.K.) UV-VIS Spectrophotometer
using cuvettes of 1 cm path length. Reference cuvette contained mixture of 1 ml of saline and 1.5 ml of 1(N) perchloric acid. The blank tube contained 1 mg C.T. DNA and 0.5 ml saline and was in 0.2 M KCl, 0.05 M Na-acetate buffer (pH 5.0) and in 0.25 M Sucrose and processed in the same way as the experimental tubes. Reading of blank was subtracted from that of experimental tubes and multiplied by proper dilution factor to get the absorbance of the acid soluble mononucleotide released from DNA. Average extinction co-efficient of mononucleotides at 260 nm was assumed to be 8.5x10^3 M^{-1} \text{cm}^{-1} (Abe, I. and Sato, S., 1976). The protein was determined according to method of Lowrey, O.H. et al. (1951) taking BSA as standard. Cells from treated mice were also processed in same way on same days.

2.10 TRANSMISSION ELECTRON MICROSCOPY IN SARCOMA 180 TUMOUR CELLS


Buffer, fixatives and solutions required for the method:

Phosphate buffer (PBS-Mg) 0.03 M, pH 7.2 containing 0.155 M Sodium chloride and 0.001 M Magnesium chloride. 316 moSms.

Solution A : 0.2 M NaH_2P_4O_7 \cdot 2H_2O (3.12g/100 ml)

Solution B : 0.2 M Na_2HPO_4 \cdot 2H_2O (3.56g/100 ml)

28 ml of solution A was mixed to 72 ml of solution B to get 100 ml of 0.2M phosphate buffer pH 7.2.
Added 0.9g Sodium chloride (NaCl) and 20 mg of Magnesium chloride (MgCl₂), 2H₂O to 15 ml of 0.2 M phosphate buffer and diluted with distilled water to a total volume of 100 ml. This gave 0.03 M phosphate buffer pH 7.2 with 0.155 M Sodium chloride and 0.001 M Magnesium chloride.

**Veronal acetate (VAC) base solution:**

This solution was prepared by taking Sodium acetate 9.7g; Sodium veronal 14.7g; Sodium chloride 17.0g and distilled water to 500 ml. This solution could be stored at 4°C indefinitely.

Veronal acetate (VAC) buffer - 412 mOsm.

To 40 ml of VAC base added 100 ml of distilled water; the pH of this solution was adjusted to 7.2 with 1(N) HCl and the volume was brought to 200 ml with distilled water. 222 mg Calcium chloride was added to this buffer.

**Glutaraldehyde:**

Mixed 4 ml of 25% glutaraldehyde with 6 ml of 0.03 M phosphate buffer (PBS - Mg) pH 7.2 to make 10 ml of 10% glutaraldehyde solution. This solution was stored in cold (4°C). Immediately before use, 10% glutaraldehyde was diluted to working concentration i.e. 2% with the same buffer.

**Paraformaldehyde:**

300 mg paraformaldehyde was suspended in a small volume of distilled water (1 ml) and dissolved by heating with addition of one or two drop of 1(N) Sodium hydroxide (NaOH). The pH of
the clear solution was adjusted to 7.0 with 1(N) hydrochloric acid, and the volume was brought to 10 ml with PBS-Mg buffer. Double aldehyde fixative (Glutaraldehyde: Paraformaldehyde :: 1% : 1.5%, 110 moSm.

Equal parts of 2% glutaraldehyde was mixed with 3% Paraformaldehyde to obtain a final concentration of 1% glutaraldehyde and 1.5% paraformaldehyde.

Osmium tetroxide fixative 1%,446 moSm :

An ampule containing 0.1g solid Osmium tetroxide was cleaned with detergent and washed with tap water thoroughly. Distilled water (30 ml) containing in a 50 ml beaker was heated to about 60°C and the ampule was dipped into warm water to brought out all the content in one end. The ampules was opened and the contents dropped into 10 ml of VAC buffer in a amber coloured bottle under a hood. The solution was kept at 4°C in dark to protect from light. The solution could be stored at 4°C till it becomes coloured.

Agar in VAC :

0.2g of bacto-agar was suspended in 10 ml of VAC buffer and the solution was kept in a water bath at 45°C till the agar melted down to a clear solution. The solution could be stored at 4°C.

Tryptone :

0.1g of Bactro-Tryptone was dissolved in 10 ml distilled water and 0.05g of Sodium chloride was added to this solution.
The solution was divided into 1 ml aliquot and sterilized by autoclave and stored in cold.

**Uranyl acetate (UAC) in VAC:**

0.05g of Uranyl acetate was dissolved in 10 ml of veronal acetate buffer by slightly warming; used freshly prepared solution.

**Dehydrating agent:**

100% absolute alcohol was diluted to graded series of 30%, 50%, 70% and 95% with distilled water.

**Embedding Medium:**

A low viscosity epoxy resin was prepared according to following formula (Spurr, A.R., 1969):

- ERL-4206 (Vinylcyclohexane dioxide) - 9.2 ml
- D.E.R. 736 (Diglycidyl ether of polypropylene glycol) - 8.25 ml
- NSA (nonenyl succinic anhydride) - 25.5 ml

The first three were taken in a 50 ml beaker and mixed thoroughly by vortexing. The beaker with the solution was weighed in a balance and accelerator DMAE (dimethyl amino ethanol) of 0.4g was added by weight and immediate stirred with a glass rod, care was taken to avoid bubbles during mixing.

**Staining solution:**

**Lead citrate stain:**

Distilled water was boiled in a beaker for 30 minutes to remove dissolved carbon dioxide and kept under cover. 15 ml of
Carbon dioxide free water was taken in a 25 ml volumetric flask and 0.88g of Sodium citrate and 0.66g of Lead nitrate were added to it. The mixture was shaken with stopper intermittently for 30 minutes till milky white colour appeared. Added 4 ml of 1(N) Sodium hydroxide (NaOH) slowly to this mixture till it became colourless and volume was brought to 25 ml with CO₂ free distilled water.

**Uranyl acetate stain:**

25 ml of absolute alcohol was taken in a 50 ml volumetric flask and 0.5g of uranyl acetate was added gently to alcohol till saturated solution of uranyl acetate forms. The saturated solution of uranyl acetate was kept in dark at room temperature. At the time of staining (1 ml) of saturated uranyl acetate was mixed with equal volume of absolute alcohol (1 ml).

**Collodion film preparation:**

200-300 mesh copper grids were placed in a filter paper with mat side upwards and again placed it on a small piece of wire gauze. This wire gauze was placed in a 8 cm diameter Petri-dish which contains an outlet fitted with rubber and pinch cork. Distilled water was added to it. The grids were washed 3-4 times to make them dust free. The Petri-dish was once more filled with distilled water, one or two drop of 0.25% collodion solution was added gently on the water surface when a thin film formed. Water was drained slowly and when the
film settled down over the grids, grids were transferred carefully on a piece of filter paper in a separate Petridish and finally stored in a dust free cabinet.

**Specimen preparation:**

10 day old 1-2 tumour bearing mice were killed by cervical dislocation and cells were collected from peritoneal cavity. They were washed with saline to remove any blood or adhering materials by low centrifugation. Cell pellet was fixed in double aldehyde for 30 minutes at room temperature, after 30 minutes washed the loose cell pellet 2 times with PBS-Mg buffer and 4 times with VAC buffer by low centrifugation at 500g for 1-2 minutes. Small volume (1 ml) of melted agar in a VAC was mixed thoroughly with cell pellet and transferred quickly on a cleaned glass slide to form a 1 mm thick film. The slides were kept at 4°C for 10-15 minutes for Agar to solidify. 1 mm\(^2\) were cut from their agar film and 5-6 pieces were transferred into each vial containing 1 ml of 1% Osmium tetroxide in VAC, 0.1 ml of tryptone solution was added to them. Vials with stoppered were kept in dark for 15-18 hours at room temperature. Fixative was discarded using a Pasteur pipette and washed 2 times with VAC buffer. The sample was next treated with 0.5% uranyl acetate for 90 minutes at R.T. (Watson, M.L., 1958). Solution was discarded by decantation and specimen was washed 2 times with VAC buffer. The specimen was dehydrated with different graded of alcohol as follows (Trump, B.F. and Harris, C.C., 1979):
30% Alcohol in 0.1% Sodium chloride for 5 minutes,
50% Alcohol in 0.1% Sodium chloride for 5 minutes,
70% Alcohol for 5 minutes,
95% Alcohol for 10 minutes,
and finally 3 times with absolute alcohol for 10 minutes each.

After dehydration the sample was infiltrated with 1:1 mixture of embedding medium (Spurr, A.R., 1969): Alcohol for 1 hour in a stoppered vial, 2:1 mixture of embedding medium: Alcohol for 1 hour in a stoppered vial, and finally with undiluted embedding medium for 1 hour without stopper. The sample was embedded in Beam capsules and incubated at 60°C for 24 hours. The Block was kept one day at R.T. for hardening.

Staining Protocol:

The sections were cut into thin (400-600 Å) gray colour by 'NOVA' ultramicrotome (LKB, Sweden) and thin sections were mounted on matside of 200-300 mesh copper grids precoated with collodian film. The grids were dried.

Uranyl acetate diluted with alcohol was added dropwise by a Pasteur pipette to 5 or 6 places on a wax bed in a 6 cm dia. Petridish. Thin sections on collodian coated grids were immersed in Uranyl acetate solution (Cole, K.E. et al., 1986) one in each drop and kept for 1 hour with cover. After staining the grids were washed with a jet of distilled water for few seconds and soaked in filter paper for drying. They were next immersed in lead-
citrate solution (Reynolds, E.S., 1963) added dropwise on a separate wax bed in a Petridish which also contained few pellets of sodium hydroxide to avoid lead carbonate deposition. After 10 minute of staining each grids were washed by dipping them in distilled water for few seconds with three consecutive changes and dried in filter paper by touching the grids cornerwise. All the grids were preserved in a grid box before viewing under electron microscope.

**Examination of Sections:**

The sections were examined in Model H-600 Hitachi (Japan) transmission electron microscope operated at an accelerating voltage of 75 KV. Photographs were taken on Fuji orthochromatic film at an electronic magnification ranging from 4000 to 10,000 and positive prints were made in Agfa paper with photographic enlargement.

Cells from treated mice were also processed in same way on same day.

**2.11 EVALUATION OF HOST'S LIFE SPAN**

Sarcoma 180 ascites tumour cells were transplanted into Swiss male mice of approximately equal age and weight. After 5 day of transplantation a group of mice were kept as control and different doses of mitomycin C and whole body gamma radiation were administered to the rest of animals as described in Sec. 2.35. The animals were observed daily till death. The
survival of the tumour bearing mice after being subjected to
different treatment schedule of MMC and radiation was deter­
mined by taking twenty animals in each group.

The median survival time was determined according to the
protocol of National Cancer Institute, USA (Wick, M.M. and Mui,
A., 1981). It is expressed as the day when the number of survi­
viving animals was \((N-1/2)\) where 'N' denotes the total number of
animals used at the beginning of the experiment. Each set of
experiments was repeated five times. The per cent increase in
life span of Sarcoma 180 tumour bearing mice following mitomycin
C and radiation treatment was calculated from the relation
\%
\quad \text{ILS} = (\frac{T}{T'} - 1) \times 100
\]
where \(T\) and \(T'\) are the median survival
time in days of treated and untreated group respectively (Wick,

**Toxic death:**

Toxic death was considered if the treated animals died
before the matched untreated control.

2.12 HAEMATOLOGICAL RESPONSES IN NORMAL AND TUMOUR BEARING MICE

At different days interval after tumour transplantation
various haematological parameters namely peripheral blood cell
counts RBC, WBC (Total and Differential), haemoglobin concen­
tration and bone marrow cellularity were studied along with
normal mice (Wintrobe, M.M. et al., 1974).

**Blood collection :**

Blood was drawn from tail vein, disodium salt (EDTA) in a
concentration of 1.5 mg/ml of blood was used as the anticoagulant. For the differential counts of leucocytes, freshly drawn blood from tail vein (without anticoagulant) was used.

**Haemoglobin concentration and erythrocyte count:**

The concentration of haemoglobin was measured by the usual procedure using Shali's haemoglobinometer. For the erythrocyte counts, blood samples were diluted 1:200 with a diluting fluid containing neutral glycerine 20 ml; Sodium sulphate 8.0g, Sodium chloride 1.0g, Methyl violet 25.0g and distilled water. Thoma pipette was horizontally shaken and a drop of resultant mixture was discharged under the cover glass of an improved Neubauer hemocytometer and the corpuscles were allowed to settle. The number of erythrocytes in 80 small squares were counted under light microscope. The number of cells in 1/ul of undiluted blood was calculated following the standard formula (Dacie, J.V. and Lewis, S.M., 1975):

\[
\text{Erythrocyte count} = N \times \frac{1}{0.02} \times 200 \text{ (dilution)}
\]

\[
= N \times 10,000
\]

**Total count of leucocytes:**

Blood was diluted (1:20) with a diluting fluid containing 3 ml of glacial acetic acid, 97 ml of distilled water and a pinch of methylene blue powder, in a WBC pipette. The resultant mixture was charged in the Neubauer haemocytometer and the number of
cells in four corner blocks (each block is subdivided into 16 squares) was determined. The total leucocyte counts/μl of blood was calculated by multiplying the average number of cells in four blocks by 200 (Wintrobe, M.M. et al., 1974).

Differential count of leucocytes:

The differential distribution of leucocytes was calculated by microscopical examination of Leishman stained blood smears.

Leishman stain preparation:

Leishman's eosine methylene blue 1 g; acetone free methyl alcohol 500 ml. The mixture was stirred on a magnetic stirrer for 30 minutes at room temperature in a dark glass container. The stain was filtered and stored in a dark place for 1-2 weeks with occasional shaking.

Phosphate buffer (pH 6.4-6.8):

Phosphate (monobasic) 6.63g; Sodium phosphate (dibasic) 3.2g and distilled water 1 litre.

Preparation of blood smear, staining and counting:

(a) Blood smears prepared from freshly drawn blood was made on clean glass slides. The smear was covered with undiluted stain and kept for one minute.

(b) The stain was diluted two volume of buffer solution.

(c) The slides remained in this condition for 10-12 minutes and then flooded with tap water. The slides were air-dried and observed under oil immersion objective of a light microscope.
(d) Different types of white blood cells were scored on the basis of their nuclear and cytoplasmic characteristics. The per cent distribution of each type of cell was calculated after scoring at least 500 leucocytes per slide.

**Total count of femoral marrow cells:**

Animals were killed by cervical dislocation. The femoral bone of mouse were washed with cold saline and ends of the femur bone were snipped open with scissors and the marrow plug was flushed out by forcefully injecting saline through the bone cavity by inserting a 26 gauze needle. The marrow plug, collected in 2-3 ml of saline was dissociated into single cell suspension by repeatedly passing this suspension through 22 and 23 gauze needles. 0.1 ml of cell suspension were diluted with saline and used for total counts.

Total number of nucleated cells per femur was determined by multiplying this number with dilution factor. Haematological parameters of treated tumour bearing mice were also processed in the same way on same days.

2.13 **STATISTICAL ANALYSIS**

Results from different experiments were statistically evaluated by student's 't' test. Statistical significance was assigned when \( P < 0.05 \). Between group, comparison on any day was made by analysis of variance (cell number per ml of ascitic fluid due to radiation, mitomycin C and mitomycin C plus radiation), using the
F test to establish significant differences between the groups. Confidence limit at level 95% were determined among the ratio of protein to DNA (P/D) for untreated and treated cells (Zar, J.H., 1984).