EXPERIMENTALS

Materials and Methods

Material

1. Animals:

A Sarcoma-180 mouse ascites tumour cell line was originally obtained from Chittaranjan National Cancer Research Centre. This line is regularly maintained in our laboratory in Swiss albino male mice and was used as experimental material. This material was very suitable for experiments because of the following reasons :

(i) Microchromosomes were found in this ascites cell-line. These microchromosomes are different from the double minutes obtained in human neoplasms. The work was therefore undertaken to find out the nature of these microchromosomes by means of different banding techniques.

(ii) Additionally, this material has added advantages; the tumour cells are rapidly growing and

(iii) The processing is technically simple and presents excellent slides with an enormous number of mitotic metaphase plates.

2. Common Laboratory Chemicals :

Common laboratory chemicals used in the present experiments

were of analytical reagent grade and purchased from E. Merck (India), BDH (India) and Sarabhai Chemicals (India).

3. Fine chemicals :

Difco-trypsin was purchased from E. Merck (India) Pvt. Ltd., Deoxyribonuclease I (pancreatic 3.1.4.5) was purchased from Sigma Chemical Company, U.S.A.

4. Stains :

Giemsa powder was purchased from E. Merck (India) Pvt. Ltd.. Basic Fuchsin was purchased from BDH (India).

5. Fluorescent compounds :

Quinacrine dihydrochloride, sold under the commercial name 'Atebrin' was purchased from George T. Gurr (Div. of Baird and Tatlock, Romford RMI IHA).

Hoechst was obtained as a gift from Dr. H. Loewe, Frankfurt.

Acridine orange was purchased from B.D.H. (India).

6. Radioactive Precursor :

The radioactive precursor ^{3}H-thymidine (specific activity - 12,600 mci/m.mole) used in the present investigation was obtained from Bhaba Atomic Research Centre, Trombay, India.

7. Autoradiographic film :

Kodak fine-grain autoradiographic stripping plates AR 10 CAT 300 3522 was obtained from Kodak Limited (England).

8. Developer - D19b

To prepare the developer the following compounds were dissolved in 100 ml distilled water one after another and then the developer was stored in a brown-coloured glass-bottle in the freeze.

Elon	..	0.2 gm
Sodium sulphite	..	7.2 gm
Hydroquinone	..	0.8 gm
Sodium carbonate	..	4.8 gm
Potassium bromide	..	0.4 gm

9. Fixer :

High speed fixer (May and Baker Ltd., India) was diluted three times with distilled water.

10. Giemsa stain :

Giemsa stain was prepared by dissolving 1 gm of powdered Giemsa in 54 ml glycerol; this was kept overnight at 60°C. Next day 84 ml methanol was mixed after cooling and filtered. The resulting solution is stored as 'Stock Giemsa' in the freeze.

11. Feulgen stain :

Feulgen stain was prepared by dissolving 0.5 gm of basic fuchsin in 100 ml boiling distilled water. It was cooled at 58°C and filtered. The filtrate was cooled down to 26°C. To it 10 ml N.HCl and 0.5 gm potassium metabisulphite was added. The mouth of the container was closed with a stopper; container was wrapped in a black paper and stored in a cool dark chamber.

After 24 hours, 0.5 gm of charcoal powder was added, shook thoroughly and filtered. The final solution was stored in a coloured glass bottle, wrapped with a black paper and stored in the freeze.

12. Colloidal developer :

2 gms of powdered gelatin was dissolved in 100 ml of deionized water and 1 ml pure formic acid. Controlled stirring for 10 minutes is required to dissolve the gelatin. The colloidal developer thus prepared was stored in brown coloured glass bottle.

13. Diphenyl amine reagent :

1.5 gm of diphenylamine was dissolved in 100 ml of glacial acetic acid and 1.5 ml concentrated sulphuric acid was added. The solution was stored in amber-coloured bottle in the dark.

Methods

A. Transplantation of mice :

Ascites fluid was drawn out from the peritoneal cavity of a tumour-bearing mouse with a sterile syringe. The fluid was diluted 5 times with sterile normal (0.9%) saline. From this diluted fluid, 0.5 ml was injected into the peritoneal cavity of each Swiss albino male mice. The tumour was grown for 5-6 days. These tumour-bearing mice was used for different experiments.

B. Preparation of chromosomes from mouse - ascites tumour cells :

5-6 days after transplantation a tumour-bearing mouse was injected interperitoneally with 0.2 ml of 0.05% colchicine in order to arrest the metaphase cells. 2 hours after injection the fluid was collected by sacrifycing the mouse. The fluid was washed with 0.9% saline (pre-warmed at 37°C) and by centrifugation at 800-900 rpm. The cells were then collected and one part of fluid was mixed with 4 parts of a hypotonic saline solution (containing 1 part of normal saline and 3 parts of distilled water) previously warmed at 37°C. This was incubated at 37°C for 45 minutes with occasional stirring to avoid clumping of the cells. It was then centrifuged in a clinical centrifuge at 800-900 rpm for 5 minutes and the supernatant was discarded. Then fixative (acetic alcohol (1:3) kept previously at 4°C) was added drop by drop with constant squeezing until

sufficient amount of fixative was added and left for one hour at 4°C. The fixative was changed by centrifuging at 2000 rpm for 5 minutes, the fluid was discarded and new fixative was added. The fixative was changed three times at an interval of half an hour.

For slide preparation, one drop of fluid was placed at one end of a grease-free slide and was blown in one direction (blowing method). The slides were then stained with 5% phosphate-buffered(0.06M, pH 6.8) Giemsa for 15 minutes; the slides were then rinsed with distilled water, left to dry overnight and mounted in euparal. The slides were observed under a Zeiss photomicroscope and photographs from well-scattered metaphase plates were taken.

C. Staining of chromosomes with Feulgen reagent :

The air-dried chromosome preparations were hydrolyzed with N.HCl (prewarmed at 60°C) for 8-10 minutes at 60°C. They were then dipped into Feulgen solution for one hour. Then the slides were rinsed thrice in SO_2 - water (200 ml of water, 10 ml of 1(N) HCl and 10% sodium bisulphite) each time for 2 minutes. The preparations were washed in running water, passed through alcohol grades and mounted.

D. Staining of chromosomes with acridine orange :

125 mg acridine orange was dissolved in 100 ml distilled water. The air-dried chromosome preparations were stained with

this acridine orange solution for 15 minutes. The slides were then rinsed in distilled water, blotted dry and mounted in euparal.

E. Study of Anaphase :

0.2 ml of fluid was drawn, diluted 4 times with normal saline and smear was drawn. After thorough drying the slides were fixed in acetic alcohol (1:3) and stained with Giemsa. Rinsed with distilled water and mounted in euparal.

F. Localization of centromeric heterochromatin by alkali C-banding technique :

For alkali C-banding the method of Polani (237) was followed.

The air-dried chromosome preparations were immersed in sodium phosphate buffer (0.06M, pH 6.8) for one hour at room temperature. They were then incubated in 0.07M NaOH in water (previously warmed at 37°C) for 45-50 seconds at 37°C, quickly dipped into ice-cold 2XSSC (0.3M sodium chloride plus 0.03M trisodium citrate) for a few seconds and rapidly dehydrated through 50%, 70%, 90% and absolute alcohol giving two changes in each grade. The preparations were then incubated in 2XSSC at 60°C for one hour. Washed in distilled water, stained in 2% phosphate-buffered (0.06M, pH 6.8) Giemsa for 15 minutes. Slides were rinsed in distilled water, blotted dry, mounted in DPX and examined.

G. Localization of centromeric heterochromatin by $Ba(OH)_2$ method:

The air-dried chromosome preparations were immersed in

sodium phosphate buffer (0.06M, pH 6.8) for one hour at room temperature. They were then incubated in a saturated solution. Of $Ba(OH)_2$ (previously warmed at 60°C) for 5-8 minutes at 60°C. They were then dipped in 0.2N HCl for a few seconds in order to clean the scum deposited from $Ba(OH)_2$. The slides were then incubated in 2XSSC at 60°C for one hour. Washed in distilled water, stained in 2% phosphate buffered (0.06M, pH 6.8) Giemsa for 15 minutes. Slides were rinsed in distilled water, blotted dry, mounted in DPX and examined.

H. Localization of centromeric heterochromatin by hot SSC method :

For C-banding with hot-SSC the method of Pera (238) was followed.

The air-dried chromosome preparations were immersed in sodium phosphate buffer (0.06M, pH 6.8) for one hour at room temperature. The slides were then boiled in 0.1 XSSC for 2 minutes and subsequently renaturated in 2XSSC at 60°C for one hour. They were then washed in distilled water, stained in 2% phosphate buffered (0.06M, pH 6.8) Giemsa for 15 minutes. Slides were rinsed in distilled water, blotted dry, mounted in DPX and examined.

I. Localization of intercalary heterochromatin by G-banding technique:

For G-banding the method of Seabright (239) was followed.

The proteolytic enzyme trypsin was prepared as 0.2% in 0.06M sodium phosphate buffer (pH 6.8) and stored in freezing condition. The air-dried chromosome preparations were incubated in 0.06M sodium phosphate buffer (pH 6.8) at 37°C for one hour. For the induction of G-banding, the slides were flooded with the above-mentioned trypsin solution for 10-15 seconds (217) and then rinsed twice with distilled water. Stained in 2% phosphate buffered (0.06M, pH 6.8) Giemsa for 15 minutes. Slides were rinsed in distilled water, blotted dry, mounted in DPX and examined.

J. Localization of centromeric heterochromatin by banding with Deoxyribonuclease I :

The air-dried chromosome preparations were incubated in 0.06M sodium phosphate buffer (pH 6.8) at 37°C for one hour. The slides were treated with a DNase solution (500 µg/ml in glass distilled water. containing 2.5×10^{-3}M $MgSO_4$) for 3 minutes at 37°C. After the treatment, the slides were rinsed in running water, stained with 5% phosphate buffered Giemsa for 15 minutes. Washed in distilled water, blotted dry, mounted in DPX and examined.

K. Localization of some specific regions by N-banding technique:

For N-banding the method of Bobrow et al. (218) was followed.

The pH of a 2% Giemsa in distilled water was adjusted to 11 by means of a suitable alkali. The air-dried chromosome prepara-

tions were immersed in this Giemsa solution for 20 minutes at 37°C. Slides were rinsed in distilled water, blotted dry, mounted in DPX and examined.

L. Localization of nucleolus organizing regions by silver-staining method :

For the selective staining of Ag-NORs the method of Howell et al. (219) was followed. The method requires the use of two solutions : A colloidal developer and a 50% $AgNO_3$ solution. The latter solution was prepared by dissolving 4 gms of silver nitrate into 8 ml of deionized water and the solution is stored in brown glass bottle.

For staining the NORs, 2 drops of the colloidal developer and 4 drops of aqueous silver nitrate solution are pipetted onto the surface of a slide containing chromosome preparations. The solutions were mixed and covered with a cover-glass. The slide was then placed inside an incubator maintained at 70°C. Within one minute, the silver-staining mixture turns yellow and within 4 minutes it becomes golden brown. The slide was then removed and both the cover-glass and the slide were rinsed off in deionized water. The slide was blotted dry, mounted in euparal and examined.

M. Determination of late-replicating chromosomal regions by means of autoradiography :

Five ascites tumour-bearing mice were taken 5-6 days after transplantation and 20 μCi of ^{3}H-thymidine was injected

(vi) The slides were hanged to dry for 2-3 hours.

(vii) When dried, the completed preparations were kept in light-tight boxes containing dry silica gels for exposure for 8 days.

After 8 days, the slides were processed in a dark room using red safe-light as follows :

(i) The slides were developed in D19b developer for 10 minutes.

(ii) The slides were then rinsed with distilled water for few seconds.

(iii) The slides were then fixed in high speed fixer for 10 minutes.

(iv) They were then washed in distilled water, passed through alcohol grades and finally mounted in euparal.

The slides were examined and metaphase plates showing grains over the chromosomes were photographed.

N. Fluorescence band through Quinacrine dihydrochloride staining:

The air-dried chromosome preparations were rinsed in Mc Ilvaine's citric acid - Na_2HPO_4 buffer (0.2M Na_2HPO_4 adjusted with 0.1M citric acid to pH 5.5). The slides were stained for 5 minutes in a 0.5% aqueous solution of quinacrine dihydrochloride, washed for 3 minutes in running water, rinsed in McIlvaine's buffer (pH 5.5) and mounted in the same buffer. For the microscopic analysis, a Zeiss fluorescence microscope was used equi-

pped with an HBO 200W/4 lamp, BG12 exciting filter and a 53/44 barrier filter.

O. Localization of AT-rich heterochromatin by Hoechst 33258 banding method:

Hoechst staining was done as described by Latt (240). A stock stain solution 50 µg/ml in distilled water was stored at 4°C in a light-tight bottle. Slides with air-dried chromosome preparations were rinsed 10 minutes in PBS (0.15M NaCl, 0.03M KCl, 0.01M sodium phosphate pH 7.0); stained 10 minutes in a 1/100 dilution of Stock Hoechst in PBS, rinsed 5 minutes in PBS, rinsed 5 minutes in distilled water and finally mounted in distilled water. Slides were viewed through a Zeiss fluorescence microscope (excitation filter BG12, barrier filter 53/44).

P. Localization of centromeric heterochromatin by acridine orange banding technique :

The air-dried chromosome preparations were immersed in sodium phosphate buffer (0.06M, pH 6.8) for one hour. They were then denatured by 0.07M NaOH and reassociated by 2XSSC as described earlier. The slides were then stained in 10^{-4}M acridine orange for 15 minutes, washed in distilled water and mounted in distilled water. Slides were viewed through a Zeiss fluorescence microscope (excitation filter BG12, barrier filter 58/44).

Q. Method for cell counting :

The cell counting is done with the help of the homocytometer (1/400 mm^2). The homocytometer consists of a slide with a groove in the middle; there are 25 big squares on each side of the groove.

For cell counting, 0.1 ml of ascites fluid was drawn and diluted 10 or 20 times as it is required. One drop of this diluted fluid was placed on the groove and covered with a coverglass. The total number of cells on four squares situated at the corners of the 25 squares were counted. The value was then multiplied by the dilution factor and subsequently by 10^5. The resulting value gives the number of cells present per millilitre of fluid.

R. Estimation of DNA per nucleus :

1 ml of ascites fluid was drawn and hypotonic treatment was performed with saline (diluted as 1:3) at 37°C for 45 min. The cells were then ruptured by homogenization in a Sorvall Omni-mixer at 5K for 3 minutes. The nuclei were then precipitated by treatment with 10% chilled trichloro-acetic acid for half an hour. It was then centrifuged. The residue was treated with 5% perchloric acid at 85-90°C for 15 min. It was then centrifuged; the supernatant was preserved and the residue was again treated with 5% perchloric acid at 85-90°C for 15 min. It was centrifuged. The two supernatants were mixed and diphenylamine colour

reaction was performed as 1 ml of PCA extract + 2 ml of diphenylamine reagent; incubated at room temperature for 48 hours. The intensity of blue colour developed was then measured at 660 nm in colorimeter. The amount of DNA per ml of fluid was calculated from the standard curve. Cell counting per ml of fluid was done by the method described above. Amount of DNA per nucleus was obtained by the following formula :

$$\frac{\text{Amount of DNA per ml of fluid}}{\text{Total number of nuclei per ml of fluid}} = \text{Amount of DNA per nucleus}$$

S. Measurement of extraction of DNA through DNase I extraction:

0.6 ml of ascites fluid was drawn and hypotonic treatment was performed with saline (diluted as 1:3) at 37°C for 45 min. The cells were then ruptured by homogenization in a Sorvall Omni-mixer at 5K for 3 min. Three equal portions were made from this fluid. The first was taken as control. It was treated with distilled water containing 0.0025M $MgSO_4$ at 37°C for 2 hours. The second was treated with DNase solution (500/ug/ml containing 0.0025M $MgSO_4$) for 3 min at 37°C and the third was treated with the same concentration of DNase for 2 hours at same temperature. It was then precipitated with 5% PCA uranyl acetate reagent for half an hour in cold. After half an hour, the supernatant was taken and diphenylamine colour reaction was performed as 1 ml of PCA extract + 2 ml diphenylamine reagent; incubated at room temperature for 48 hours. The intensity of the blue colour developed was then measured at 660 nm in colorimeter.