MATERIALS AND METHODS

A. Cell culture:

A large uninucleate protozoa, Amoeba indica, was used as an experimental model for the present investigation. Amoeba indica was collected from the local pond in Bombay, India (Rao, 1971). Cultures were maintained in the laboratory at 22 ± 1°C. Cells were cultured in glass petriplates and grown in Prescott and Carrier's (1964) amoeba medium containing NaCl 0.01 g; $CaCl_2$ 0.01 g; KCl 0.06 g; $MgSO_4$ 0.002 g; $CaHPO_4$ 0.004 g; dH_2O 1000 ml and pH was adjusted to 6.8.

Amoebae were fed with freshly harvested Tetrahymena pyriformis, a ciliated protozoa which were grown axenically in a medium containing 2 per cent proteose peptone broth in glass distilled water along with 1 g of liver extract per 1000 ml of the medium. A 250 ml ehrlenmeyer flask containing 100 ml of proteose peptone medium was then autoclaved for 20 min at 15 psi, cooled down to 22 ± 1°C and the Tetrahymenae were innoculated and grown in this broth. Three days after innoculation Tetrahymenae were harvested by centrifuging at ca. 200 rpm for 1 min and the supernatant containing proteose peptone was decanted and replaced by amoeba medium. The process was repeated four times to remove the trace of proteose peptone. Then

the pellet containing Tetrahymenae was diluted with amoeba medium.

Amoebae were fed every alternate day followed by regular replacement of fresh amoeba medium. Petriplates containing amoeba culture were changed every second day.

B. Treatment with mutagen:

A potent chemical mutagen, Methanesulfonic acid ethyl ester (Ethyl methanesulfonate, EMS) was used for the present investigation. For EMS treatment, the desired quantity was added to 0.05 ml of ethanol, then diluted with autoclaved amoeba medium (pH 6.8 ± 0.1). No significant pH change occurred during the short period of treatment. The alcohol used was equivalent to the final concentration of 0.4 per cent in the diluted EMS. In all cases freshly prepared mutagen solution was used.

Selected number of 18 h starved, healthy and attached amoebae were picked up by a braking pipette and put into syracuse watch glasses. Cells were treated with different concentrations of EMS for different periods of time. Following the treatment cells were washed thoroughly with amoeba medium to remove the mutagen. The treated amoebae were usually fed half an hour after the EMS treatment with a light suspension of Tetrahymena.

C. Cell survival and generation time in asynchronous cells:

Healthy, attached and wellfed amoebae were randomly selected from mass cultures and kept in separate watch glasses in amoeba medium. For each experiment between 30 to 60 cells were selected and the experiments were repeated 3 to 6 times. Cells were then treated with different concentrations of EMS. Amoeba medium was sucked off by braking pipette before EMS treatment. 0.4 ml of desired concentration of EMS was added by a micro syringe (Hamilton) to each syracuse watch glasses containing the amoebae.

Percentage of cell survival was determined by observing and counting the EMS exposed viable cells at different periods of time after the treatment. Control cells were kept in similar fashion in each case.

For determining generation time 30 syracuse watch glasses were taken and each filled up with 0.6 ml of amoeba medium and in all of these watch glasses one treated amoeba was transferred. This time immediately following treatment was considered to be 0 h. Every day around 24 h interval amoebae in each watch glass were counted and results were tabulated noting actual number of amoebae. Food and culture medium were replenished every alternate day. Five to ten division cycles were considered to see the

effect of EMS treatment, if any, on the generation time. Each treated lot was compared with a control set.

D. Cell cycle phase-specific sensitivity and generation time:

In A. indica the average generation time was reported to be 24 ± 2 h with a S period of 3 ± 1 h (Rao and Chatterjee, 1974). However, in our laboratory condition the generation time was found to be quite different than that was reported earlier. The entire cell cycle was found to cover a period of 72 ± 6 h, where mitotic phase took 35 to 40 min and the S phase continued upto 7 h. The S phase cells were collected immediately after the completion of mitotic division and were labelled with ^{3}H-Tdr; while the length of mitotic phase was determined by the time taken by the cells to divide into two daughter cells after the formation of a division sphere. The time covered by G_2 phase was calculated from the cell duplication time of the cells after deducting the period occupied by DNA synthesis.

Cell cycle phase-specific sensitivity in terms of per cent cell survival, after exposing the cells with EMS, was observed by selecting synchronized groups of amoebae from mass cultures by picking up division spheres with a braking pipette and keeping them singly in small syracuse watch glasses until they reach the desired age for EMS

treatment. Cell cycle phase-specific sensitivity was determined at different phases of the cell cycle with different concentrations of EMS, treated for 15 and 30 min. The cell cycle was divided into mitotic phase (division spheres); early-S-phase (1 h after division); mid S phase (3½ h after division); late S phase (5½ h after division); early G_2 phase (10 h after division); mid G_2I phase (20 h after division); mid G_2II phase (35 h after division); mid G_2III phase (48 h after division) and late G_2 phase (66 h after division). Selected number of cells at appropriate stages of the cell cycle were taken in syracuse watch glasses and exposed to EMS. Cell cycle phase-specific sensitivity to the action of EMS in terms of percent cell survival was plotted from the numerical data obtained by counting the numbers of viable cells at different periods after the mutagen treatment. Control cells were kept in similar fashion. Each experiment was repeated three to four times.

For determining mean generation time, cells at defined phases of the cell cycle were taken and treated with different concentrations of EMS for different time durations. The rest of the procedure was same as mentioned earlier.

Cell division delay, if any, was calculated by deducting the cell duplication time of the treated cells from the control ones.

E. Capillary cloning and culture methods for 'Mini' cells:

Some Mini cells which were obtained (vide infra) from EMS treated amoebae were cultured by adopting capillary cloning method of Ord (1979b) after minor modifications to produce a clone. The method was used to avoid other amoeba contaminations in mini cell culture and to observe the progeny obtained from a single mini cell.

A drop of modified Chalkley's medium (Ord, 1970) containing NaCl 16 g; $NaHCO_3$ 0.8 g; KCl 0.4 g; $Na_2HPO_4.12H_2O$ 0.2 g; $CaHPO_4$ 0.2 g; and $MgCl_2$ 0.2 g to 1000 ml of dH_2O diluted for use by 5 ml to 1000 ml dH_2O, was introduced into a capillary tube (of 7cm length and 5 mm diameter) which had first been fused at one end by the flame of a fish-tail gas jet. The Chalkley's medium was allowed to fill the central third of the capillary tube leaving an air gap of approximately one third of the tube length at the fused end and an equal gap at the open end. To this medium was added a small drop, approximately 5 µl, of concentrated food suspension containing ca. 200-300 Chilomonas and a single 'Mini' cell (amoeba). Capillary tubes were attached to a glass slide with plasticine and stored. The mofified Chalkley's medium was adjusted in such a way that after addition of food suspension the final pH was 5.8 to 6.0. The food suspension for capillaries was obtained from 5-10 days old wheat infusion culture. Amoebae were removed after 18-20 days by breaking the capillary tubes.

For large scale culture of the cloned 'Mini' cells, glass petriplates were used. 'Mini' cells were routinely cultured in modified Chalkley's medium using Chilomonas as food organism.

F. Squash preparation of amoeba:

Amoebae were placed on one side of a gelatenised slide and excess medium was drawn off. A drop of freshly prepared 45% acetic acid was taken on a cover slip and was inverted on the amoebae. The slide was then immediately dipped in liquid nitrogen until frozen and the cover slip was flipped off by a forceps. The slide was then post-fixed in a suitable fixative for desired time following removal of the fixative in absolute ethanol, 90% and 70% ethanol and finally air dried.

G. Method for counting pseudopodia:

Formation of pseudopodia per minute was counted by a stop watch half an hour after feeding the cells with Tetrahymena or Chilomonas (for 'Mini' cells) and checked at different time intervals. Fifteen cells were considered per point and an arithmetic mean value was plotted.

H. Quantitation of phagocytosis:

A drop of Tetrahymena suspension was added into a syracuse watch glass containing the EMS treated amoebae

in 0.6 ml of amoeba medium. Amoebae were allowed to feed and cell samples were collected on gelatenised slides at different time intervals after EMS treatment and squashed. Cells were post-fixed in 1:3 acetic-ethanol followed by processing in down grades of ethanol and finally air dried. A control set was run at the same time. Cells were stained with Giemsa and mounted in DPX. Examination of the cells for counting the number of phagosomes on the visible part of the cells was carried out under a Carl Zeiss Jena (GDR) microscope with X200 magnification. Fifteen to twenty cells were examined at each point.

I. Measurement of cell size and nuclear diameter:

For measuring cell size and nuclear diameter, more than hundred cells were randomly selected from the mass culture and the measurement was done by placing an ocular meter in the eyepiece of a Carl Zeiss Jena 'Amplival' microscope under X100 magnification.

J. Staining procedures:

Giemsa stain : For general staining purposes cells were fixed in 1:3 acetic-ethanol, stained in buffered 0.2% Giemsa and made permanent.

Toluidine blue : Basophilic reactions was noted by staining Zenker-fixed squashed cells with 0.1% toluidine blue in 1% ethanol. Cells were stained for 15-20 seconds followed by washing in dH_2O, finally air dried and mounted in DPX.

Periodic acid-Schiff (PAS) reaction (adopted from Gurr, 1956) : The method was used to locate glycogen, mucopolysaccharides and mucoproteins. Squashed cells, fixed in Bouin's fixative were oxidised for 10 minutes in 1% aqueous periodic acid, rinsed in dH_2O and transferred to Schiff's reagent for 45 min, washed in freshly prepared sulphite wash water (3 changes, 1 min each) and then kept in running tap water for 10 min, dehydrated and mounted in DPX.

Sudan black B : Sudan black B staining technique was used for locating bound lipids in amoeba. Squashed cells were fixed in 10% formalin, washed in 50% ethanol and air dried. Cells were stained in saturated Sudan black B in 70% ethanol for 30 min at room temperature followed by quick rinse in 70% ethanol for differentiation, finally washed in running tap water, blot dried and mounted in glycerine jelly.

Mercury-Bromophenol blue : Mercury-Bromophenol blue method of Bonhag (adopted from Pearse, 1968) was used for

locating the protein moiety in amoebae. Cells were fixed in freshly prepared Carnoy's fixative, washed thoroughly in 90% ethanol brought down to water and stained in mercuric bromophenol blue (1% $HgCl_2$ and 0.05% bromophenol blue in 2% aqueous acetic acid) for two hours at room temperature. Cells were then washed in 0.5% acetic acid solution for 3-5 min, dehydrated in two changes of tertiary butyl alcohol (5 min each) transferred to xylene and finally mounted in euparal.

Alkaline phosphatase : Modified Gomori method (adopted from Pearse, 1968) was followed for locating the activity of alkaline phosphatase.

Cells fixed in chilled acetone, were incubated for 45 min in incubating mixture at 37°C, passed through 2% Cobalt nitrate and diluted yellow Ammonium sulphide in 40% acetone (0.1 ml of yellow Ammonium sulphide dissolved in 50 ml of 40% acetone), dehydrated and made permanent.

Acid phosphatase : Lead nitrate method for acid phosphatase of Gomori (adopted from Pearse, 1968) was found suitable for locating the activity of acid phosphatase in amoebae.

Squashed cells fixed in chilled acetone were incubated at 37°C for 45 min in freshly prepared 0.01 M Sodium-β-glycerophosphate in 0.05 M Sodium acetate buffer (pH 5.0), containing

0.004 M lead nitrate, passed through diluted yellow Ammonium sulphide (0.15 ml in 50 ml dH_2O), washed, dehydrated and made permanent.

K. Liquid scintillation spectrometry :

Incorporation study

Syntheses of DNA, RNA and protein were measured by the incorporation of ^3H-thymidine (Methyl T) (sp. act. 17.8 Ci/mM); ^3H-uridine-T (G) (sp. act. 10.9 Ci/mM and ^3H-I-leucine T(G) (Sp. act. 7.6 Ci/mM) into EMS exposed cells. Amoebae were incubated for one hour at each point with 50 µl of labelled precursor, washed with amoeba medium atleast 4 times and transferred to unlabelled precursor for 15 min (1 h in case of thymidine label) with 3 changes. Cold thymidine (5×10^{-2}M), uridine (5×10^{-2}M), leucine (2×10^{-3}M) were used to wash and chase the labelled cells. Unlabelled precursor solutions were prepared in sterile amoeba medium and the pH was adjusted to 6.8. Desired number of cells were then transferred into test tubes containing 0.5 ml of EDTA buffer (pH 7.5) containing 0.15 M KCl and 2% SDS as cell lysing medium. For investigating protein, DNA and RNA syntheses, cells were similarly lysed and 300 µg of BSA (dissolved in sterile dH_2O, pH 6.8) as carrier protein; 400 µg of DNA (dissolved in SSC buffer, containing 0.15 M Tri-sodium citrate dihydrate and 0.015 M NaCl, pH adjusted to 7.0 with 0.1 M NaOH) and 400 µg of RNA

(dissolved in 50 mM Tris-HCl buffer, pH 7.5) were added as carrier followed by precipitation with a final concentration of 10% (v/v) (for protein) and 20% (v/v) (for DNA and RNA) chilled TCA.

After addition of TCA, sample solutions were allowed to settle the precipitate at 4°C for 4 to 12 h. The precipitates were collected on Millipore membrane filters (Pore size 0.45 um, 25 mm dia., Millipore Intertech Inc., Mass., USA) with the help of a Maxflow filter holder (Maxflow, Bombay, India) and washed several times with 5% (v/v) chilled TCA under water suction pump, dried under an infrared lamp and the incorporated radioactivity was counted by Packard Tri Carb Liquid Scintillation Spectrometer (Model 3380) in scintillation vials containing 10 ml of toluene based liquid scintillant (PPO 4 g; POPOP 100 mg; in 1000 ml of sulphur free toluene). Procedure was slightly modified for samples to be assayed for protein synthesis. 1 ml of 0.5 N NaOH was added to the sample after TCA precipitation (for 12 h at 4°C) and kept for 12 h at 37°C, added 50 µl of 6N HCl to the sample for neutralization, allowed to stand for 1 h at 0°C and then collected on membrane filter. Thus an alkaline hydrolysis of RNAs (especially t-RNAs) was carried out which might have had taken some labelled amino acid during synthesis. Control cells were also processed in the same way.

Counting was done through ^{3}H channel. Counting efficiency of the Scintillation counter was found to be 60% for ^{3}H. The radioactivity was expressed in terms of counts per minute (cpm) after background deduction and quenching correction. Channel ratio and autostandard was checked.

L. Enzyme extraction:

The specificity of tracer thymidine was checked by DNase digestion. DNase solution of 0.3mg/ml concentration (pH 7.0) was prepared and the digestion was carried out for 6 h at 37°C by dipping the labelled squashed cells in the DNase solution. After enzymatic digestion the slides were washed thoroughly in dH_2O, air dried and processed for autoradiography.

M. Autoradiography:

For autoradiography, the labelled and squashed amoebae were fixed in 1:3 acetic-ethanol, washed with 90% ethanol and treated with 5% (v/v) TCA for 5 min at 5°C to remove unincorporated labelled precursors followed by washing thoroughly in absolute ethanol and rectified spirit and ultimately air dried. The cells were then processed for autoradiography.

(a) Conventional autoradiography : This procedure was followed for only one experiment where 18 h starved healthy cells were randomly picked from the mass culture and exposed to 0.5% EMS for 15 min. Cells were pulse labelled for 1 hour at each point with 200 mCi/ml of 50 µl ^{3}H-L-leucine (sp. act. 3.3 Ci/mM). ^{3}H-L-leucine was mixed in sterile amoeba medium and the pH was adjusted to 6.8. After incubation, cells were washed thoroughly with amoeba medium and cold L-lecuine (2×10^{-3} M, prepared in amoeba medium, pH 6.8) for 15 min and squashed.

Kodak fine grain autoradiographic stripping film AR 10 (Kodak Ltd., London) was cut into smaller rectangular pieces and allowed to float in dH_2O (pH adjusted to 7.0) at 23°C by facing the emulsion side down. Labelled and squashed cells were coated with the film strips (emulsion side facing the specimen), dried by using a air blower and transferred the slides in a light proof bakelite box containing silica gel. The entire operation was carried out in a dark room by using a 25 Watt lamp fitted with a Kodak Safelight filter, No. 1. The bakelite boxes were kept in darkness at 4°C for 16 days for radioactive exposure, finally developed in Kodak D19b developer for 9 min at 10°C and fixed in acid fixer (May and Baker, India) for 5 min. Cells were stained with Giemsa, air dried and mounted in euparal. Control cells were processed in the same manner.

(b) Liquid scintillation autoradiography : A rapid liquid scintillation method for autoradiography (Gaddipati and Sen, 1978) was adopted to carry out the entire experiment on cell cycle of A. indica and 'Mini' cells. This technique was followed to minimise the time of exposure and to get higher resolution of the autoradiograms. It was also found to reduce background grain development markedly on the films.

DNA and protein synthesis was investigated throughout the cell cycle of control, treated A. indica and 'Mini' cells. For DNA synthesis 50 µl of ^{3}H-thymidine (sp.act. 17.8 Ci/mM) was used at each point. For protein synthesis 50 µl of ^{3}H-L-leucine (sp. act. 3.3 Ci/mM) of the strength 200 µCi/ml of sterile amoeba medium (pH 6.8) was used. Cells were incubated for 1 hour with labelled precursors through each point unless and otherwise mentioned.

Labelled and squashed cells were coated with Kodak AR 10 stripping film, dried and stored at 4°C in a light-proof bakelite box, containing silica gel, for 24 h, immersed in scintillation fluid (containing PPO 6 g and dimethyl POPOP 0.1 g in 1000 ml sulphur free toluene) in a Coplin jar, tightly sealed and kept in the dark at 20°C for 72 h and finally brought to 25 ± 1°C. In complete darkness they were successively transferred to

xylene and passed through down grades of ethanol. The preparations were developed for 7 min in Kodak D19b developer at 20°C, briefly rinsed in dH_2O and fixed in acid fixer for 5 min, washed, stained with Giemsa and mounted in euparal.

N. Grain counting procedure:

For quantitative analysis of protein synthesis grains were counted by placing an eye-piece graticule on the ocular. 2700 μm^2 area was counted from three randomly selected cytoplasmic zones in each cell. For DNA synthesis, grain counts were done per nucleus under an eye-piece graticule examining several labelled nuclei per point. Grains were always counted under X100 oil immersion objective and viewed through a Carl Zeiss-Jena, Amplival microscope (GDR). Background on the slides and cytoplasmic grain incorporations (in case of DNA synthesis) were deducted to get the actual count.

O. Scanning electron microscopy:

Amoebae were placed on a coverslip and fixed for 1 h at 4°C in Karnovsky's fixative (Karnovsky, 1965), containing 5% glutaraldehyde and 4% formaldehyde in 0.1 M cacodylate buffer at pH 7.3. Cells were rinsed in 0.05 M cacodylate buffer (pH 7.3) and post-fixed for 1 h at 4°C in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.3),

washed thoroughly in dH_2O, dehydrated in a series of ethanol and acetone and dried in a Sorvall Critical Point Drier with liquid CO_2. The specimens were coated with Gold in vacuum and examined in a Cambridge Stereoscan, model S4-10 operated at 30 kv.

P. Transmission electron microscopy:

Cells were fixed for 1 h at 4°C in Karnovsky's fixative (Karnovsky, 1965), containing 5% glutaraldehyde and 4% formaldehyde in 0.1 M cacodylate buffer at pH 7.3, rinsed in 0.05 M cacodylate buffer (pH 7.3) and post-fixed at 4°C for 1 h in 1% osmium tetroxide in 0.1 M cacodylate buffer at pH 7.3, rinsed in cacodylate buffer (pH 7.3) and in dH_2O. Amoebae were than embedded in a 2 mm cube of 2% agar (Flickinger, 1969). Agar cubes containing cells were dehydrated in graded series of ethanol followed by propylene oxide, then infiltrated and embedded in Araldite. Embedding was done in beam capsules and polymerization of the resin solution was carried out at 60°C for 16 h.

Sections were cut in an LKB Ultratome IV using glass knives prepared with LKB Knifemaker 7800B. 0.5 µm thin sections were cut and stained with 1.0% toluidine blue in 1.0% borax for light microscppic observations. Ultrathin

sections ranging from 700-800 Å were cut and mounted on uncoated copper grids. Grids were then stained with uranyl acetate and lead citrate (Reynolds, 1963). Electron micrographs were obtained with a Siemens 1A electron microscope operated at 80 kv.

Q. Photomicrography :

Photomicrographs were taken through Carl Zeiss Photomicroscope-II using ORWO (35 mm) 125 ASA panchromatic films. Neopan (FUJI) 400 ASA films were used in Carl Zeiss 'Tessovar' Photomacrographic Zoom system for taking photographs of living cells.

R. Radioisotopes and chemicals:

All radioactive precursors were purchased from Isotope Division, Bhabha Atomic Research Centre, Bombay, India. Liver extract, Ethyl methanesulfonate, Thymidine, Uridine, Leucine, Lysine, Ethylenediamine tetraacetic acid (disodium salt, dihydrate), SDS (Sodium laurylsulfate), BSA (Bovine serum albumin), Deoxyribonucleic acid, Ribonucleic acid, Trizma base, PPO (2,5, diphenyloxazole), POPOP (1,4 bis, [2-(5-phenyloxazolyl)]benzene, phenyl-oxazolylphenyl-oxazolyl-phenyl), Dimethyl POPOP (1,4-bis [2-(4-Methyl-5 phenyloxazolyl)] benzene), DNase (Deoxyribo-nuclease), Cacodylic acid (sodium salt) and Osmium tetroxide

were purchased from Sigma Chemical Company, St. Louis, MO, USA. Proteose peptone was obtained from DIFCO Laboratories, USA. Giemsa's stain and sodium-β-glycerophosphate were purchased from BDH Poole, England. Toluidine blue was purchased from Allied Chemicals, New York, USA. From E. Merck, W. Germany, Bromophenol blue, Sodium veronal (Barbital), and TCA (Trichloroacetic acid) were obtained. Euparal was purchased from Merck, Germany. Yellow Ammonium sulphide was purchased from Riedel-de Haena, Germany. Napthalene and Propylene oxide were obtained from Koch-Light Laboratories Ltd., England. Glutaraldehyde, DDSA (Dodecenyl succinine anhydride), DMP 30 (2,4,6 dimethyl aminomethyl phenol), Araldite 502, Uranyl acetate, Lead citrate and copper grids were purchased from Electron Microscopy Sciences, Washington, USA.

All other chemicals used were of highest purity grade commercially available in India.