STUDIES ON THE REGULATIONS OF NUCLEAR AND CYTOPLASMIC ACTIVITIES IN A MUTAGEN TREATED EUKARYOTIC CELL

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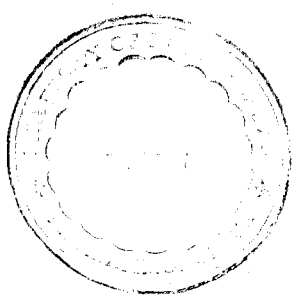

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(P.C. KESAVAN)
DEAN

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Aniruddha Gangopadhyay

(ANIRUDDHA GANGOPADHYAY)

CONTENTS

LIST OF ABBREVIATIONS

SEM	:	Scanning electron microscopy
TEM	:	Transmission electron microscopy
^{3}H Tdr	:	Tritiated thymidine
^{3}H Udr	:	Tritiated uridine
Dib^{R}	:	Dibucaine resistant
RER	:	Rough endoplasmic reticulum
HN_2	:	Nitrogen mustard

In recent time, the expression of cellular phenotypes and its regulation during the cell cycle has become an intent area of interest for the cell biologists. It is now accepted that cell differentiation and cell transformation result from some change in the information centre of the cell namely its DNA. We are still in the dark whether structural changes in the DNA molecule are always necessary for this process or changes in the controls over gene expression are equally effective, e.g., the reactivation of 'switched off' genes. Variable gene activity and its differential expression is generally being thought to be a key factor necessary for initiating cell differentiation. It is probable that cell transformation may be brought about in a number of different ways. One such way is thought to be the action of chemicals on the cell, which cause changes in the genetic material in many ways similar to that produced by radiation; the other possibility lies with viral induced cell transformation.

Mutation is a more or less permanent, uncoded relatively rare change in the kind, number, or sequence of nucleotides in the genetic material. It is not an isolated event or a simple chemical reaction, but a process in which diverse cellular functions are knitted together. It is quite likely that fundamental mutation research at the cell

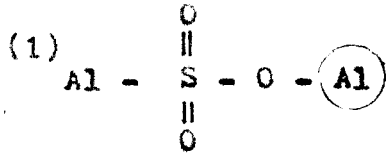
been defined by Freese (1959) and last two by Brenner et al. (1961). Another class of mutants result from deletion of segments of DNA which may be many nucleotides long. If the number of deleted nucleotides is three or a multiple of three, there is no frame-shift error beyond the site of mutation, but the protein produced may be defective because one or more amino acids are deleted from its sequence.

A particular enigma in this field arises from the wide differences in carcinogenic and mutagenic activity shown by compounds which are closely related structurally (Brookes and Lawley, 1964; Dingman and Sporn, 1967; Colburn and Boutwell, 1968). It has been shown that most, if not all, of the closely related groups of substances which are active carcinogens have the ability to interact co-valently with DNA (Sneider, 1974). The methylation of DNA produced by a number of alkylating agents, some of which are mutagenic but not carcinogenic, show that those which are powerful carcinogens may have small but possibly important differences in the sites of the DNA bases which can be alkylated (Lawley and Thatcher, 1970; Roberts et al. 1974; Maitra and Frei, 1975). Thus, while the major site of alkylation, the N-7 position on the purine base guanine is common to both carcinogens and mutagens, the minor sites the N-1 and N-3 of adenine, the N-3 of cytosine, and the

N-3 and C -6 of guanine (Craddock and Magee, 1963; Magee and Barnes, 1967; Loveless, 1969; Lawley and Thatcher, 1970; Lawley et al., 1971/2; Craddock, 1973) show some differences, particularly in the presence or absence of the O-6 methylation.

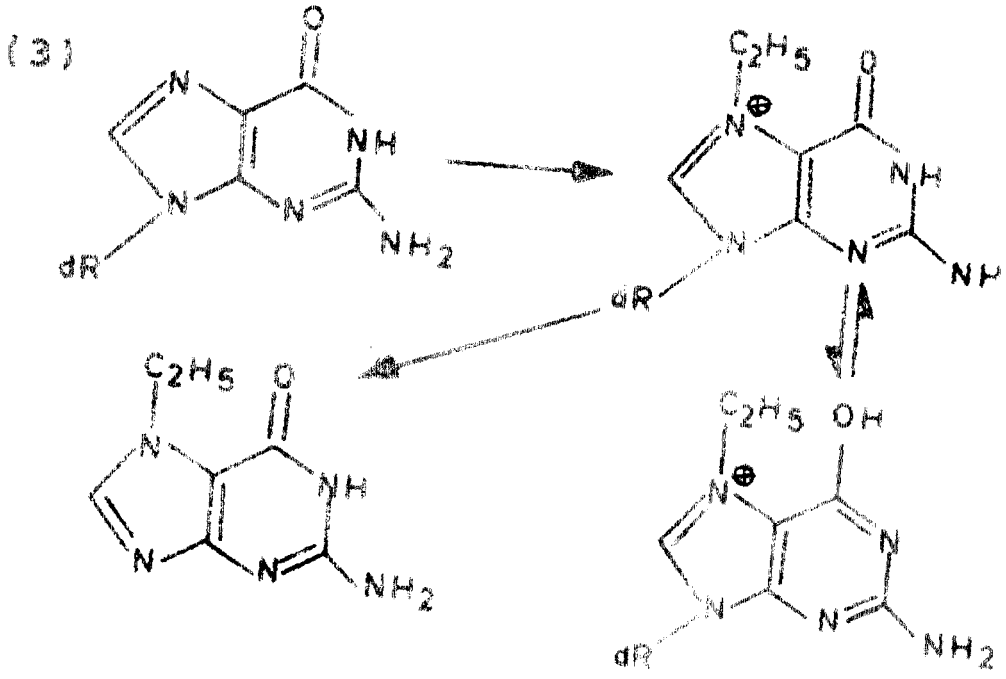
The present investigation is concerned with the action of a monofunctional alkylating agent at a single cell level. The general structure of monofunctional alkylating agent is shown below (1), which are, for e.g. Methyl methanesulfonate (MMS) and Ethyl methanesulfonate (EMS) where the alkyl group (Al) is active. The first reaction of a monofunctional alkylating agent with DNA is, simply, the addition of alkyl groups to the DNA (Strauss and Hill, 1970), although phosphates are also alkylated (Rhaese and Freese, 1969). A monofunctional agent can

(1)



occasionally cause cross-linking (either the two strands of one DNA molecule = intrastrand crosslink, or two strands of different DNA molecules = interstrand crosslink), owing to reactive DNA ends created by the alkylation-induced backbone breakage (Alexander and Stacey, 1958). One functional group is sufficient to induce mutations (at least point mutations). In polar solvents, alkylating agents give rise to positive carbonium ions, e.g., (2)

(3)


O
N
NH
N
N
NH2
dR
C2H5
O
N⊕
NH
N
N
NH
dR
C2H5
O
N
NH
N
N
NH2
C2H5
OH
N⊕
N
N
N
NH2
dR

exclusively for the present study, is considered to act as a mutagen mainly by the ethylation of purine base guanine at the 7-position and the subsequent induction of mutation either directly by hydrogen bonding with thymine instead of cytosine (GC → AT transition) or only after depurination (Orgel, 1965).

Studies with the tryptophan synthetase A protein system of Escherichia coli have shown that EMS is capable of inducing transversions as well as transitions (Yanofsky et al., 1966). Highly effective mutagenicity has been shown by using EMS in a wide variety of systems, e.g. in Drosophila (Alderson, 1964, 1965; Fahmy and Fahmy, 1957, 1961), Habrobracon (Löbbecke and von Borstel, 1962), E. coli (Loveless and Howarth, 1959; Schwartz, 1963; Verly et al., 1967), Bacillus subtilis (Corban, 1968), B. cereus (Necasek et al., 1967), bacteriophage (Loveless, 1958, 1959; Osborn et al., 1967), Neurospora (Kolmark, 1956; Malling and De Serres, 1968; Nasim and Auerbach, 1967), Schizosaccharomyces pombe (Snyder et al., 1950; Bateman et al., 1966; Nasim, 1967; Heslot, 1961), Saccharomyces cerevisiae (Lindegren et al., 1965; Lingens and Oltmans, 1964). It is also effective in inducing dominant lethal mutations in mice (Tareeva and Yakovleva, 1960) and rats (Partington and Jackson, 1963).

EMS has induced chromosome aberrations in cultured mammalian cells (Chang and Elequin, 1967) and in mice

(Cattanach et al., 1968). The carcinogenicity of EMS in neonatal mice (Walters et al., 1967) has also been investigated. Ultrastructural analysis of a chromosome segment of ethyl methanesulfonate-induced Drosophila melanogaster recessive lethals have been found to have an allelism matrix in a short chromosomal segment covered by y mal^{+} chromosome (Lifschytz and Falk, 1969). The in vivo and in vitro effects of alkylating agents, ethyl methanesulfonate, methyl methanesulfonate, hycanthone methanesulfonate and naltrexone on the amino acid composition of rat and human hemoglobin has been examined by Truong et al. (1978) who observed changes in the molar ratio of specific amino acids after mutagen exposure. Banerjee et al. (1978) have investigated EMS induced reversion in the white locus of Drosophila melanogaster to test reversion ability to wild type. Effects of EMS in mice demonstrated by the micronucleus test has been undertaken by Henry et al. (1980). Estimation of proportion of small deletions among EMS-induced point mutations at six sex linked loci of Drosophila has been undertaken (Shukla and Auerbach, 1981), where at least 60% of the point mutations are small deletions. Alkylation of rat liver DNA by EMS leads to fragmented DNA in alkaline gradients along with the accumulation of single-strand breaks (Den Engelse et al., 1981).

The frequencies of complete and mosaic mutations induced in mature Drosophila sperm by EMS at four X-chromosome

loci, y, ct, sn and f has been estimated (Brink, 1970). A comparison of ouabain thioguanine and excess thymidine resistance in EMS induced mutagenesis with L5178Y mouse lymphoma cells have been made available by Cole and Arlett (1976). Evidence of increased forward mutations in cultured Chinese hamster Don cells by EMS has been on record (Hamada et al., 1978). Sensitivity of the male germ cells of Bombyx mori and the frequency of induction of dominant lethal mutations by EMS has been determined by Datta et al. (1978). Efforts have been made (Shaw et al., 1978) to investigate conditions necessary to quantify the relationship between exposure to EMS and frequency of mutation induction at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster V79 cells. The dose-rate effects of EMS on the survival and induction of mutations in Chinese hamster Don cells were investigated by Sugiura et al. (1978). Large increase in the frequency of OUA^R chinese hamster cells mutants were found in cultures treated with various concentrations of EMS by respreading cells in 1 mM ouabain for upto 8 days after EMS treatment (Thacker et al., 1978). A series of dibucaine-resistant (Dib^R) variants of the mouse lymphoid cell line L5178Y have been induced by EMS (Vaughan et al., 1980) and isolated by selection for resistance to a short (48 h), high concentration (0.045 mM) drug pulse. Wims and Morrison (1981) have shown that EMS increased the mutation rate approximately 100 fold as compared

to spontaneous mutation rate at the immunoglobulin locus in the Y5606 cultured myeloma cell line. Evidence from the work of Generoso et al. (1981) indicates that chromosome rearrangements occur after fertilization following postmeiotic treatment of male mice germ cells with EMS. The use of mammalian cells for mutation research has been stimulated by the finding that mammalian somatic cells treated in culture with chemical mutagens, such as ethyl methanesulfonate and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), increase the occurrence of cell variants with stable, altered phenotype (Kao and Puck, 1968; Chu, 1971).

The idea to use radiation, different drugs, carcinogens, mutagens and other chemicals at different phases of the cell cycle has opened up a new field of investigation. When challenged with different kinds of hazardous physical and chemical agents at specific phases of the cell cycle, significant molecular event(s) responsible for the altered phenotypic and biochemical expressions are revealed. Since its formulation by Howard and Pelc (1953), the concept of cell cycle has had a considerable importance in cell biology. The sequence of phases of a typical cell cycle is G_1, S, G_2 and M. A growing cell undergoes a cell cycle that comprises essentially of two periods, the interphase or a period of non-apparent division and the period of division or M phase. G_1 is the period between the end of mitosis and start of

DNA synthesis, S is the phase of DNA synthesis, and G_2, the interval between the end of DNA synthesis and start of mitosis. The regulation of the duration of the cell cycle occurs primarily by arresting it at a specific point during the G_1, and the cell in the arrested condition is said to be in the G_o state (Lajtha, 1963). It has ~~been~~ often been suggested that at least the terminal part of G_1 contains immediate preparations for DNA synthesis, e.g. synthesis of such enzymes as DNA polymerase, thymidylate synthetase, kinases, concerned with production of thymidine triphosphate (Prescott, 1968). It is clear that both RNA and protein syntheses are necessary for cell progress through G_1 (Baserga et al., 1965; Kishimoto and Lieberman, 1964; Mueller et al., 1962); protein synthesis apparently is required until a very short time before DNA synthesis begins, if not at the very end of G_1 (Prescott, 1968). Broke (1977) has shown that a continuous protein synthesis is required to maintain the probability of entry into S phase. The most important point in the regulation of cell cycle occurs in the G_1 phase, during which it must decide whether the cell will start a new cell cycle or become arrested in the G_o state. Of the biochemical events that occur at defined stages of the cell cycle, the most significant one is DNA synthesis. The S phase cells contain a factor that induces DNA synthesis (Rao and Johnson, 1970). Protein synthesis is required not

only for the initiation of S in eukaryotic cells (Terasima and Yasukawa, 1966), but also for the continuation of DNA synthesis. DNA synthesis in eukaryotes may require concomitant histone synthesis which becomes associated with the newly replicated DNA (Robbins and Borun, 1967) or synthesis of other proteins not concerned directly with initiation. The G_2 period contains the events that end the chromosome replication followed by chromosome segregation. During G_2 the factors necessary for chromosome condensation are synthesized and in many cells, histone H1 becomes phosphorylated during this period (Bradbury et al., 1974). Different aspects of the cell cycle have thoroughly been reviewed by Mitchison (1971).

Some attention has recently been focussed on the socalled 'transition probability' view of the cell cycle (Burns and Tannock, 1970; Shields, 1977). It has been proposed (Burns and Tannock, 1970; Smith and Martin, 1974) that each cell is triggered into a single cycle of replication by some random events. The oscillator concept of the cell cycle suggests that regulation of replication is achieved through a switch-like process (Gilbert, 1978).

Studies on the cell cycle of amoebae indicate that the arrangement of events in the cycle may be different from other eukaryotic cells types. Nilova (1965) had reported a G_1 in A. proteus. But it was contradicted by

Ord (1968a); Prescott and Lauth (1973), where they found that G_1 period is less than one hour or may be absent. It has also been studied by Ron and Prescott (1969); Goldstein and Ron (1969) that in A. proteus a G_1 is absent, the S shows some variability in ^{3}H-thymidine incorporation from 5-9 hours after mitosis. Studies on another strain of A. proteus, Ord (1968a), found that DNA synthesis begins with the completion of cell division and the pattern displayed two peaks of DNA synthesis.

The main events of cell cycle in A. proteus has been summarised by Prescott (1973), where DNA synthesis (S period) begins at the end of mitosis. There is no G_1 period. Most of the cell cycle is occupied by G_2 period. Two hours before mitosis in G_2 the amoebae passes T-1 or transition point one, where mitosis occurs and DNA synthesis is initiated when mitosis is completed. Rao and Prescott (1970) have recorded that the synthesis of any RNA essential for mitosis and cytokinesis is completed two hours before mitosis. It indicates that mitosis and DNA synthesis in amoeba are indeed tightly coupled. Available evidence in amoeba shows that the decision to enter DNA synthesis has been made two hours before mitosis (Prescott, 1973). It has been summarised by Prescott and Goldstein (1967) that (1) the cytoplasm of G_2 phase cells lack those properties necessary for the support of DNA synthesis, and (2) the

S phase cytoplasm has properties that can induce DNA synthesis in a G_2 nucleus. The latter statement was contradicted by Ord (1969). The cytoplasmic regulation of nuclear DNA synthesis in amoeba interspecific hybrids has been reported (Rao and Chatterjee, 1974). Spear and Prescott (1980) have shown that Amoeba proteus synthesizes DNA in G_2 phase upon feeding after starvation. Makhlin et al. (1979) have shown the changes in DNA content of A. proteus nuclei during interphase. Prescott (1973) suggested that one of the few specific clues about the G_2 period in amoeba is the transition point (T_1). Once a G_2 amoeba has passed this point, it is no longer dependent upon RNA synthesis in order to proceed to mitosis, divide and initiate DNA synthesis in the new, post mitotic nucleus. The kinds of nuclear RNA synthesized by A. proteus under usual laboratory conditions have been characterized by Prescott et al. (1971) and to some extent by Goldstein and Trescott (1970). It has been on record that a significant portion of the low molecular weight nuclear RNA does not leave the nucleus during interphase (Goldstein and Trescott, 1970). The rRNA and tRNA, which comprise the bulk of cytoplasmic RNA, are not involved in the migration back into nucleus (Goldstein, 1973). Kinetic studies at various phases of the cell cycle on the relations between the nuclear activity and the variable ^{3}H-amino acid incorporation pattern in A. proteus has been examined by Chatterjee and Bell (1976). The response of amoebae to various kinds of

radiations and effect of chemicals and antibiotics on amoeba cells have been reviewed by Ord (1973b; 1979a; and Hawkins (1973). The use of chemical mutagens on amoeba has been undertaken only during the last few years. Present knowledge regarding the cellular events and cellular metabolism prior to induction of mutations is far from being complete. For example, information on the cell cycle phase specific sensitivity as well as the nucleocytoplasmic interactions at a single cell level which can provide valuable clues regarding the timing and major site(s) of action of the mutagen, remain largely obscure.

Three nitroso compounds or nitrosamides, N-methyl-N-nitrosourea (MNUrea), N-methyl-N-nitrosourethane (MNU) and N-methyl-N-nitroso-N'-nitrosoguanidine (MNNG), have been used in studies on amoeba (Ord, 1973a) with the production of similar series of effects. Ord (1968b) has shown the immediate and delayed effects of N-methyl-N-nitrosourethane on A. proteus to monitor the effects of this substance on a single cell with regard to the duration of its action, in particular, whether the action is limited to the nucleus, or to the cytoplasm. It has been observed (Ord, 1971) that unsuccessful division of an amoeba grow abnormally large after treatment with N-methyl-N-nitrosourethane. Catburn (1977) has used five mutagenic or carcinogenic chemicals on amoebae and found

a gradual movement of sensitivity from a nearly equal nuclear and cytoplasmic lethality with the chemicals methyl methanesulfonate and dimethyl sulfate to a high nuclear but low cytoplasmic lethality with MNUrea. The ICI compound 2(α-chloroisopropylamino) ethyl naphthylene hydrochloride (Tucker, 1968), which was activated by using a rat microsome preparation with the amoeba treatment (Catburn, 1977), fell between these two sensitivities. A fifth chemical, hydrazine sulfate failed to show lethal nuclear change. The site of damage in amoebae exposed to low concentrations of methyl di(β-chloroethylamine, a'nitrogen mustard') has been recorded (ord, 1956) and the effect of N-methyl-N-nitrosourethane on amoebae has been examined by Ord (1965).

Nuclear transplantation between normal and 'pale' mutant amoebae has been undertaken (Ord and Bell, 1968) to resolve the action of nucleus in controlling expression of a cytoplasmic character. Relationship between nuclear DNA and RNA synthetic activities and the changes produced by N-methyl-N-nitrosourethane in A. proteus has been examined (Ord, 1974). Ord (1976a) has recorded the changes in DNA synthesis of S phase amoeba cells exposed to N-methyl-N-nitrosourethane. An attempt has been made (Ord, 1976b) to show how the interaction of different degrees of nuclear and cytoplasmic damage may contribute to the ultimate whole

cell damage by toxic chemicals.

Chemical mutations have been induced in A. proteus by treatment with N-methyl-N-nitrosourethane (Ord, 1965, 1970). All three mutants, Mini Mutant, SpG Mutant and Pale Mutant, originated from single amoebae after treatment with N-methyl-N-nitrosourethane and have been cultured in the laboratory for more than two years, undergoing at least 100 cell cycles, without loss of mutant characteristics. Each differs from the control parent strain in a number of characteristics apparent in both nucleus and cytoplasm.

The present investigation is concerned with the study of the action of EMS- a powerful mutagenic agent on an unicellular mononucleate, eukaryotic cell namely Amoeba indica. The amoebae serve as a very useful single cell model for the study of chemical mutagenesis because of its large size, general ruggedness, long generation time etc. It offers a rare opportunity for investigating mutagen induced changes in a single cell system at morphological, biochemical and genetical level. The availability of a long cell cycle, the stages of which can be synchronised without resorting the chemical treatment or temperature shock allows the action of the mutagen to be studied fairly accurately at discrete steps of the cells' life cycle.

The main aim of the present study has been towards a better understanding of the regulation of nuclear and cytoplasmic activities and the expression of cellular phenotypes in this somatic replicating cell type when challenged with the mutagenic agent. This investigation is primarily concerned with the following aspect of the cellular responses of the mutagen exposed amoebae :-

1. To follow the nature of morphological and cytological changes induced by EMS at light and electron microscopic level at different periods of time.
2. To monitor any changes in the kinetics of nucleic acids and protein synthetic activity by tracer incorporation study after mutagen administration.
3. To investigate the cell cycle phase specific responses of the mutagen treated cells and analyse the action of the mutagen in relation to nuclear and cytoplasmic activities as a function of the age of the cell.
4. To search for any variant/cell cycle mutant which might originate after treating the cells at the defined phases of the cell cycle and characterization of the same.

This study, might hopefully provide some invaluable clues regarding the regulations of some cellular functions in general, and to understand which are especially, involved in and are operating at the different phases of the cell cycl

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.

RESULTS

Part I : Response of *Amoeba indica* to EMS treatment

Part II: Pattern of macromolecular syntheses in EMS treated cells

Part III: Isolation of mini mutant and its characteristics

PART I

Response of *Amoeba indica* to EMS treatment

A. Sensitivity to EMS exposure

B. EMS induced changes in the cell body

C. Cytoplasm and nucleus in EMS exposed cells

D. Cytochemical observations on EMS exposed cells

Amoebae were exposed to different concentrations of EMS for different time durations to investigate the response of the cells after the treatment. Healthy and attached cells were either selected from the mass culture or were selected at defined phases of the cell cycle for mutagen treatment.

A. Sensitivity to EMS exposure:

Sensitivity of the cells was considered in terms of cell survival after EMS treatment. Cell survival was checked in asynchronous populations as well as at defined phases of the cell cycle after EMS exposure. Control cells in all the cases showed more than 98% survival.

Dose response in asynchronous cells

Sensitivity of the cells exposed to the action of EMS was studied in terms of cell survival. Cells showed 98% survival while exposed to 0.1% EMS for 15 min, whereas 30% cell lethality could be detected after 15 min treatment with 0.5% EMS. However, a higher rate of cell mortality was noticed when the cells were exposed to 1.0% EMS for 15 min which was evident by only 28% cell survival after mutagen treatment (Fig. 1).

Cells were also treated with other concentrations of EMS ranging from 0.1% to 1.5% for 15 and 30 min.

However, 100% cell lethality was noted in the cells exposed to 1.5% EMS.

Cell cycle phase specific sensitivity

Sensitivity of the cells to the action of EMS at defined phases of the cell cycle was investigated in terms of cell survival. The entire cell cycle was divided into : mitotic phase (division spheres); early S phase (ES, 1 h after division); mid S phase (MS, 3½ h after division); late S phase (LS, 5½ h after division); early G_2I phase (EG_2I, 10 h after division); early G_2II phase (EG_2II. 14 h after division); mid G_2I phase (MG_2I, 20 h after division); mid G_2II phase (MG_2II, 35 h after division); MG_2III phase (MG_2III, 48 h after division); and late G_2 phase (LG_2, 66 h after division).

Differential sensitivity to the action of EMS at different phases of the cell cycle was noticed when the cells were treated with 0.1% EMS for 15 min. Maximum sensitivity, as compared to the other phases, was observed at MS phase where the cell lethality was found to be 14%. The next sensitive phase was noted to be the mitotic spheres where 12% cell mortality could be detected. However, percentage of cell survival increased from LS phase onwards and 100% survival was observed at G_2 phases. The ES phase remained insensitive (Fig. 2).

Fig. 1 : Dose-mortality curve for asynchronous cells after treatment with EMS for 15 min. At each point between 160 to 180 cells were considered. Vertical bars: ± SEM.

Fig. 2: Cell cycle phase specific sensitivity of amoebae to the action of 0.1%, 0.5% and 1.0% EMS treatment for 15 min. At each point 150 to 160 cells were counted. Vertical bars indicate ± SEM.

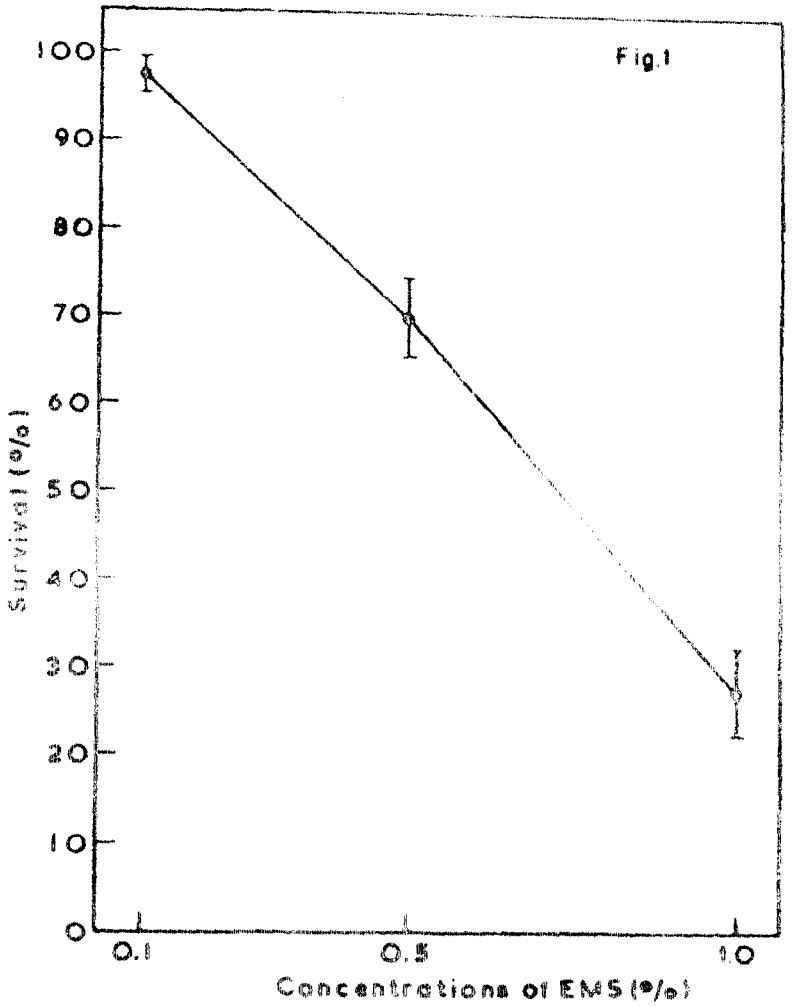


Fig.1
Survival (%)
100
90
80
70
60
50
40
30
20
10
0
0.1
0.5
1.0
Concentrations of EMS (%)

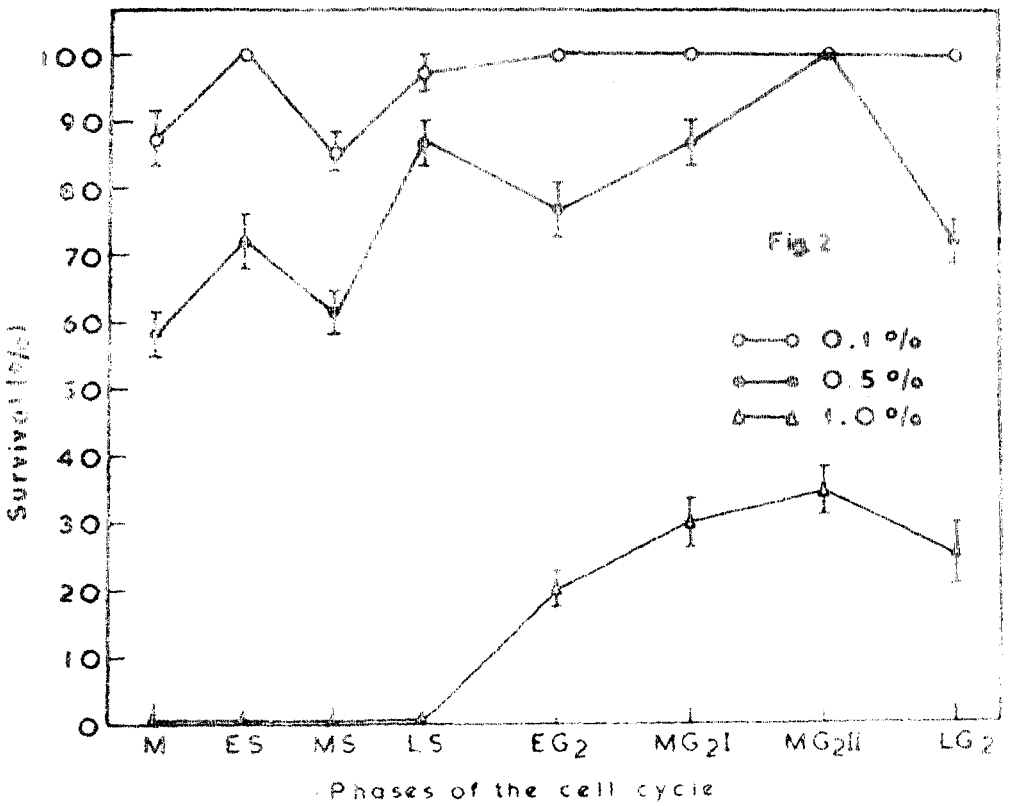


Fig.2
Survival (%)
100
90
80
70
60
50
40
30
20
10
0
0.1 %
0.5 %
1.0 %
M
ES
MS
LS
EG2
MG2I
MG2II
LG2
Phases of the cell cycle

A different pattern of cell cycle phase specific sensitivity to EMS action on the cells was observed when the cells were exposed to 0.5% EMS for 15 min. Five significant points were detected which were M, ES, MS, EG_2 and LG_2 phases (Fig. 2). Maximum cell lethality, however, was observed in M phase treated cells where 59% cell survival was noted. MS phase was found to be the second sensitive point where 38% cell lethality could be recorded. The third and fourth sensitive points were noted to be the ES and LG_2 phases where cell mortality was found to be ca. 28% in both the phases. The fifth sensitive point being the EG_2 phase where 77% cell survival was observed. However, a resistance to EMS action was found to occur from EG_2 phase onwards as evident by 100% cell survival in the cells treated at MG_2II phase (Fig. 2).

A high incidence of cell lethality was noted when the cells were exposed to 1.0% EMS for 15 min. Hundred percent cell mortality was observed in M, ES, MS and LS phases. However, resistance to the action of EMS could be detected at MG_2II phase treated cells where 65% cell mortality could be seen (Fig. 2).

However, cells were also exposed for 15 and 30 min to different concentrations of EMS ranging from 0.1% to 1.5% at specific phasesof the cell cycle, among which

Fig. 3: Spherical appearance of a cell at early hours after 15 min treatment with 0.5% EMS. Whole-mount preparation of the cell stained with Giemsa. X 720

Fig. 4: An early S phase cell after 15 min treatment with 0.5% EMS, showing elongated shape and a transparent uroid zone(u). Note the appearance of basophilic bodies. Whole mount preparation after toluidine blue staining. X 400

Fig. 5: Cell treated with 0.5% EMS for 15 min, showing formation of a distinct hyaline zone (h) at ca. 3 h after treatment. Squashed and stained with Giemsa. X 450

Fig. 6: Unstained mutagen treated living cell showing rupture of the plasma membrane before undergoing cytolysis. Arrows indicate points of rupture. X 480

3

4

5

treatment of the cells for 15 min with 0.5% EMS was found to be the most suitable dose and therefore considered as the operative dose to carry out all further experiments. This observation was also true for asynchronous cells.

B. EMS induced changes in the cell body:

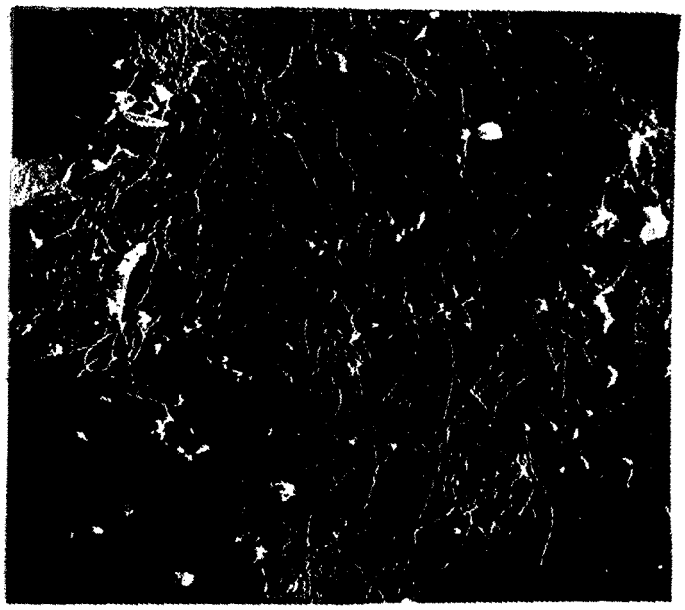
Cell shape

Majority of the cells exposed to 0.5% EMS for 15 min (operative dose) assumed spherical shape within 1/2 h after treatment (Fig. 3). Cells, however, showed normal amoeboid shape at later hours (12 h onwards). Extremely slender and elongated cell body was observed in ca. 15% cases when the ES phase cells were exposed to operative dose of EMS (Fig. 4). Stained preparations of these cells showed condensed mass of cytoplasm containing several basophilic regions and a transparent uroid zone (Fig. 4).

Hyaline zone formation and cell cytolysis

Formation of hyaline zone or separation of a transparent ectoplasm from condensed endoplasm was found to be a noticeable feature in EMS exposed amoebae. Between 30% to 40% of cells treated with operative dose of mutagen showed a distinct hyaline zone formation at early hours after treatment (Fig. 5). However, 15% to 20% of these

Fig. 7: Scanning electron micrograph showing disintegration of EMS treated cells. X 2340



cells later reverted back to normal appearance with no hyaline zone. When the cells were treated with mutagen at defined phases of the cell cycle, it was noted that the formation of hyaline zone appeared more in mitotic and S phase cells compared to G_2 phase treated cells.

Cell cytolysis was noted in 5% to 8% of the cell populations exposed to operative dose of EMS. This phenomena was noticed at around 18-22 h after treatment when the cells underwent cytolysis by rupture of the plasma membrane (Fig. 6). Scanning electron microscopic observation on a cell which underwent total cytolysis has been shown in Fig. 7.

Cell fragmentation

Another notable effect of the EMS exposed cells was found to be the fragmentation of the cells as well as pinching of cytoplasm in the form of small vesicles from the cells surface. Cell fragmentation was found to be quite frequent in cells treated with 0.5% EMS for 15 min where ca. 40% cells were found to be fragmented at early hours after treatment. Sometimes the nucleus was also found to be fragmented and a portion of the nuclear fragment could also be seen to be coming out along with the cytoplasmic fragments (Fig. 8). It was observed that the process of cellular fragmentation caused the cell size to be reduced

to a considerable extent and cells of variable sizes could be observed among the treated cell populations (Fig. 9).

When the cells were treated with EMS at defined phases of the cell cycle the appearance of cytoplasmic fragmentation and pinching out the cytoplasm was noted to be proportionally more in mitotic and MS phase cells than the other stages of the cell cycle.

Effect on cell membrane

Detachment of the cells from the surface was noticed immediately after treatment with operative dose of EMS. It was noted that more than 50% cells became detached immediately after mutagen exposure, although, at later hours majority of the cells were found to be attached to the surface.

When the cells were attached to the surface the cell membrane was found to be very much sticky at later hours after EMS treatment. Sometimes the cell membrane became quite fragile immediately after mutagen exposure. Stickiness to the surface was noticed while lifting the cells by a braking pipette which in many cases led to rupture of the cell membrane due to the firm adherence to the surface. However, fragile nature of the membrane was

Fig. 8: Cytoplasmic fragmentation of the cells due to 15 min treatment with 0.5 [illegible]. f, cytoplasmic fragments; n, extruded and disorganised nucleus. Flattened cells stained with Giemsa. [illegible]

Fig. 9: Living cells showing variations in size at early hours after 15 min treatment with 0.5 [illegible]. [illegible]

Fig. 10: Scanning electron micrograph of a control ameba showing [illegible] on the cell surface. [illegible]

Fig. 11: Scanning electron micrograph of 0.5 [illegible] treated cell at 0 h. Pole caterpillar [illegible] and pore-mark appearance on the cell surface. [illegible]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u
9

not discernible at later hours, although, stickiness to the surface could be detected even at later hours after treatment specially in the ES phase cells exposed to EMS.

Scanning electron microscopic observations on cell surface:

SEM observation was made on the cell surface of the EMS exposed cells at different periods of time and was compared with that of the control cells.

In control A. indica the greater part of the cell surface showed the presence of many protrusions. Blebbing on the cell surface was also noted. Blebs were found to be scattered over the cell surface. At certain regions broad ridges were found as extensions on the cell surface to interconnect the blebs (Fig. 10). At higher magnification the protuberances on the surface membrane were found to cover almost the entire part of the cell surface (Fig. 12). Magnified view of a portion of the cell surface showed that the protuberances had smooth surfaces. Protrusions of different sizes aggregated together to make the cell surface look ununiform in nature (Figs. 14 and 19).

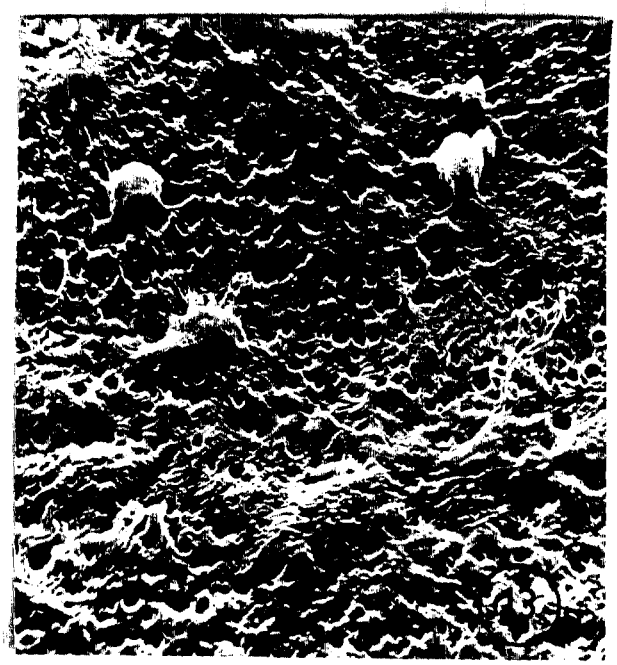
SEM observations on a magnified portion of the cell surface bleb of a control A. indica appeared with a more or less smooth surface area, although, certain mini bead like structures were observed on the blebs. Broad ridges

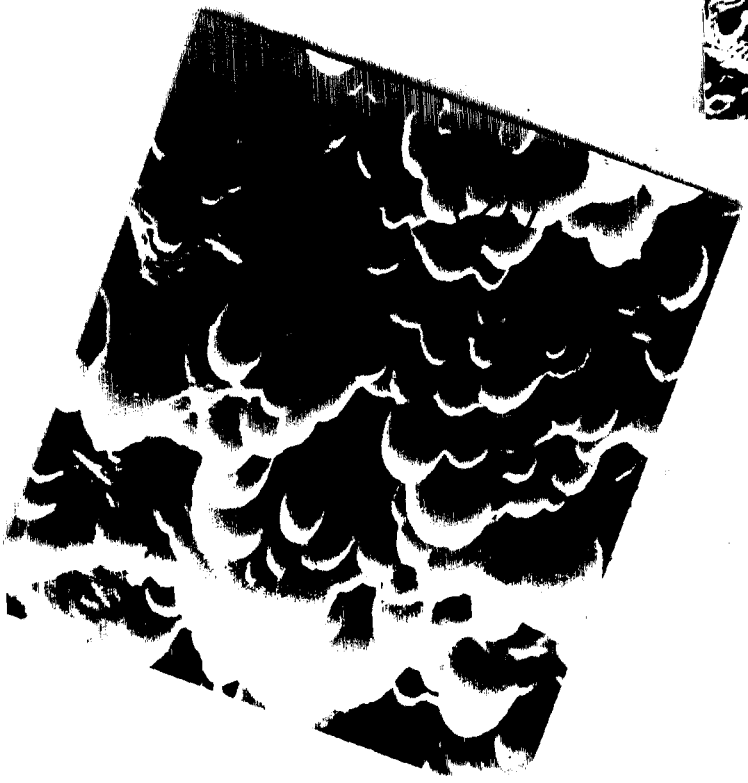
Fig. 12 : Scanning electron micrograph of a control cell with a magnified view of a randomly selected cell surface area, showing abundant protuberances. X 4500

Fig. 13 : Scanning electron micrograph showing magnified view of a randomly selected cell surface area of a treated amoeba at 0 h. Note disrupted cell surface and almost total absence of surface protuberances. X 3375

Fig. 14: Scanning electron microscopic view of a magnified portion of the surface of a normal amoeba, showing numerous protuberances. X 22500

Fig. 15: Scanning electron micrograph of a comparable magnified view of a portion of the cell surface of 0 h treated amoeba, showing wavy nature and total absence of surface protrusions. X 20250





were noted to interconnect the blebs. At certain regions the blebs coalesced to form broad ridges which extended over some area of the cell surface (Fig. 25).

Cells exposed to 0.5% EMS showed drastic change of the cell surface morphology at 0 h after treatment. The entire cell surface was found to be disrupted and ruffled. Several pock-marks and pits were found to appear on the cell surface. Protrusions from the cell surface in the form of blebs were very rarely observed (Fig. 11). Magnified view of a portion of the cell surface displayed extreme ruffling on the greater part of the surface area with prominent abundance of pock-marks and pits. The ruffling of the cell surface was due to the formation of ridges along with some mini bead like structures protruding out from the cell surface. The ridges on the cell were quite abundant and very often found to be intercepted by grooves (Figs. 13 and 15). Numerous surface protuberances, which were normal feature of the control cells, were totally absent in the treated cells.

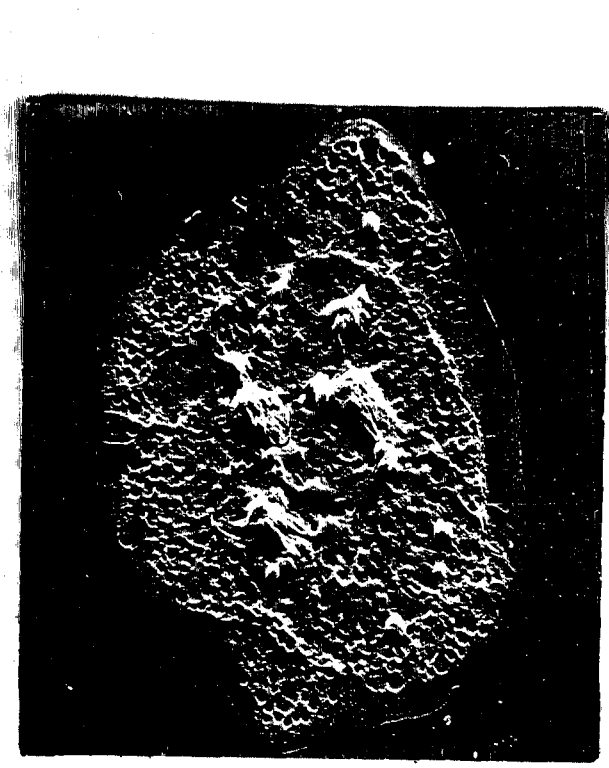
A tendency to recovery of the plasma membrane from the trauma of mutagen exposure was noted while examining the cell surface at around 12 h after treatment. A comparatively less ruffling on the surface area was noted as compared to 0 h treated cells. However, several pock-

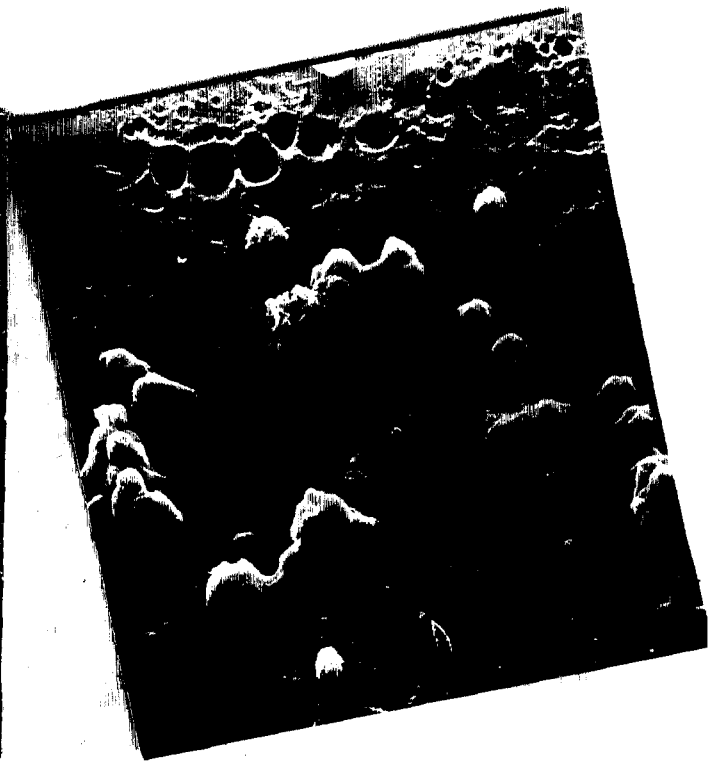
Fig. 16: Scanning electron micrograph of a EMS exposed amoeba, 12 h after treatment. Note fenestrated appearance of the cell surface as well as the appearance of some surface protuberances as found in untreated cells. X 666

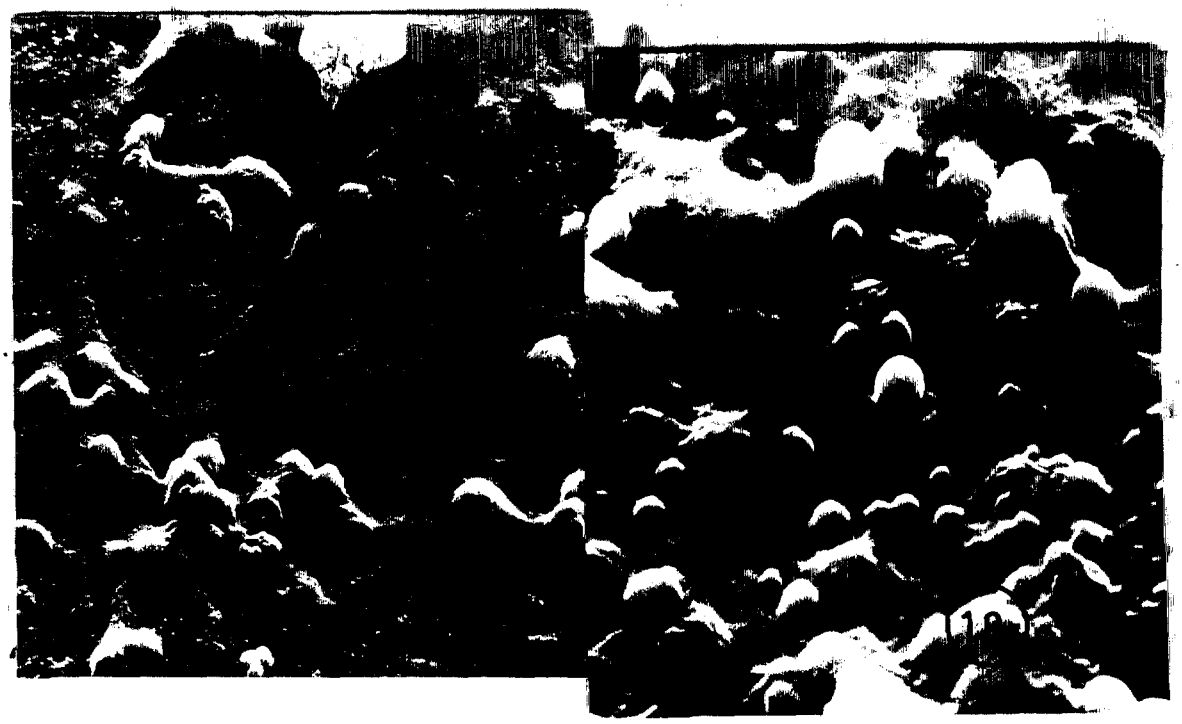
Fig. 17: A magnified scanning electron microscopic view of a portion of the EMS treated amoeba surface, 12 h after mutagen exposure. Note the crater-like structures on the cell surface and reappearance of surface blebs. X 2385

Fig. 18: A magnified scanning electron microscopic view of a portion of the cell surface of an amoeba, 12 h after EMS treatment. Note mini beads and reappearance of some cell surface blebs. X 2250

Fig. 19: Scanning electron microscopic view of a portion of the cell surface of a control amoeba. Note the cell surface protuberances which are abundant as compared to the protuberances of treated amoeba depicted in Fig. 18. X 23400







marks and crater-like holes on the cell surface was noticed along with some ridges, which were still detectable on the cell surface. Reappearance of cell surface blebs were observed around this period (Figs. 16 and 17). Magnified view of a portion of the cell surface showed a comparatively smooth surface area, although, several concavities on the surface were detected. Blebbing on the cell surface was noticed where the blebs were frequently coalesced to form ridges. Blebs contained almost a smooth surface (Figs. 17 and 18). Several mini beads appeared on the cell surface. Abundance of mini beads on the blebs were also noticed (Fig. 20). Further magnified view of a bleb showed a few pits on its surface (Fig. 21).

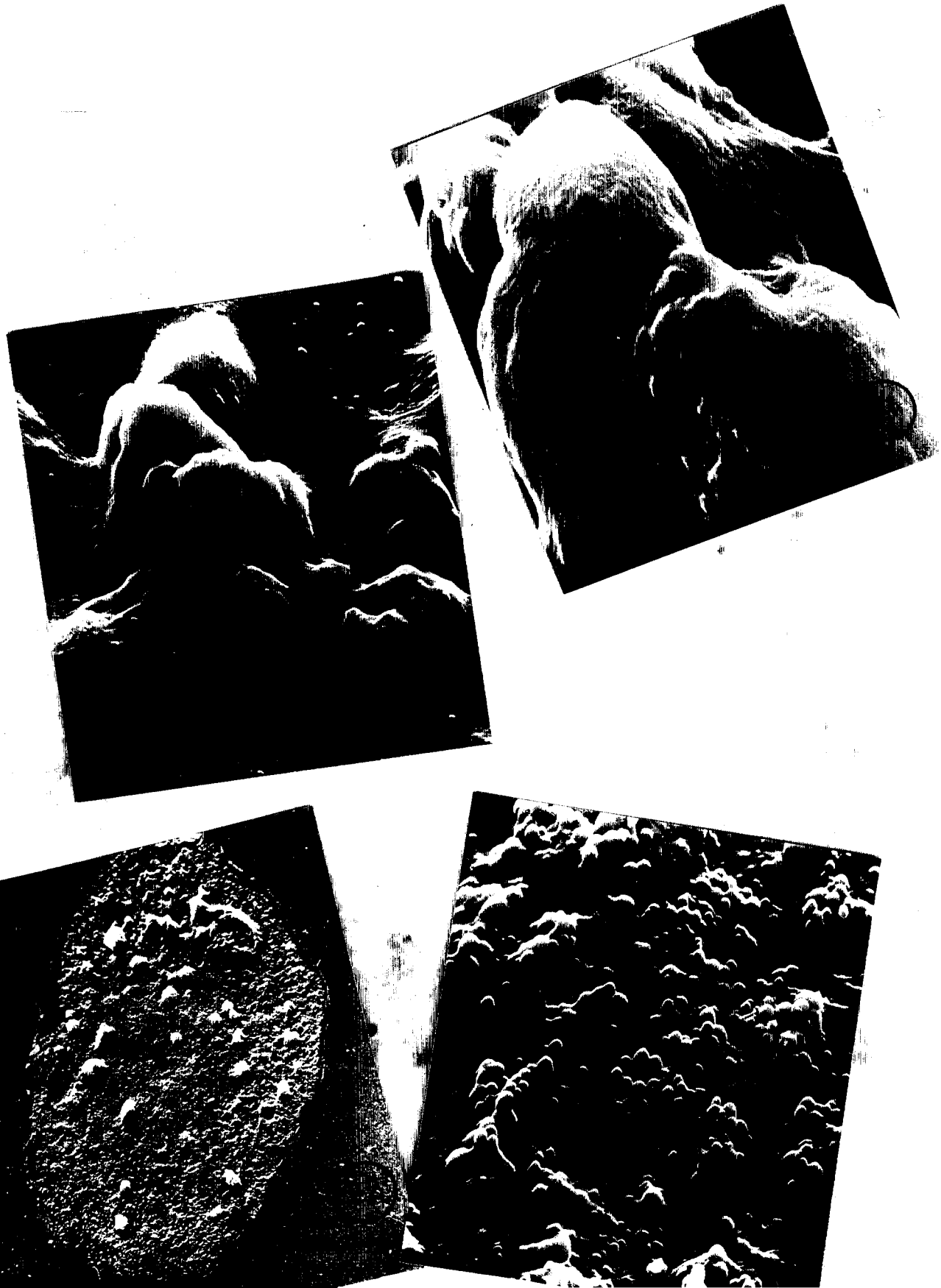
Reorganisation of the plasma membrane was observed at around 24 h after mutagen exposure where SEM observation of the treated cells surface reflected almost the same topography as displayed by the surface of control cells (Fig. 22). Observation on the greater part of the cell surface revealed the appearance of several blebs along with abundance of protuberances on the cell membrane (Fig. 23). Magnified view of a treated cell surface at this hour showed more or less a comparable surface structure as that of the control cells. However, some mini beads were also noticed on the blebs of the treated cells (Fig. 24).

Fig. 20: Scanning electron micrograph of a portion of surface of an amoeba 12 h after EMS treatment, showing cell surface blebs and mini beads. X 9000

Fig. 21: Scanning electron micrograph of an amoeba 12 h after mutagen exposure, showing magnified portion of the cell surface with bleb. X 23850

Fig. 22: Scanning electron micrograph of EMS treated amoeba 24 h after treatment. The cell surface displays almost normal morphology, indicating substantial recovery. X 990

Fig. 23: A magnified scanning electron microscopic view of a portion of the cell surface area of EMS treated cells, 24 h after the exposure. The cell surface features are more or less comparable to that of an untreated cell as depicted in Fig. 14. X 11000



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25

Cell motility and pseudopodia formation

In general, the EMS treated amoebae showed a sluggish movement as compared to normal cells. In fact, there had been an almost total cessation of motility immediately after mutagen exposure. The cells resumed their normal amoeboid locomotion at later hours after the treatment.

The nature of pseudopodia formation also underwent some changes and many cells displayed an altered pseudopodia structure after mutagen exposure. In control cells, pseudopodium was of lobose type and the lobopodium appeared as thick, roughly cylindrical with a hemispherical or conical tip. The rate of pseudopodia formation was found to be between 6 to 7 per minute in control cells.

In most of the cases cells exposed to operative dose of EMS displayed cylindrical pseudopods with blunt tips at early hours after treatment. However, cells treated at S phase, in majority of the cases, showed filamentous pseudopods with pointed tips which appeared as filopods at early hours after mutagen treatment. Pseudopodial morphology was found to resemble as that of normal type in atleast 90% cases after a few days.

Drastic reduction in the formation of pseudopodia was noticed in mutagen exposed cells at around 2-3 h after treatment. The inhibition to the formation of pseudopodia was quite apparent in cells treated with EMS at ES and MS phases (Fig. 26). However, in majority of the treated cells the frequency of pseudopodia formation appeared more or less like that of control cells around 3 to 4 days after treatment.

Phagocytosis

Considerable inhibition to the uptake of Tetrahymena was observed when the cells were exposed to EMS. Cells were fed ½ h after treatment and the experiment was continued upto 24 h. There had been an almost six fold decrease in the new phagosome formation in treated cells at initial periods after treatment as compared to the control ones (Figs. 28 and 29). This sluggish capture of the food organism was found to be continued almost upto 24 h. During that period the treated cells always showed an appreciable inhibition in their food capturing ability when compared to their normal counterparts (Fig. 27). The treated cells recovered the capacity for capturing the food organisms from 24 h onwards after mutagen treatment. Digestive function of the treated cells was also found to be impaired as evident by the presence of undigested food organisms.

Fig. 26: Formation of pseudopodia in control cells and in cells treated at defined phases of the cell cycle with 0.5% EMS for 15 min. Note a drastic inhibition in motility in MS phase treated cells.

Fig. 27: The difference in food uptake in cells treated with operative dose of EMS as compared to control cells. Phagosomes were counted on the visible part of the flattened, fixed and stained cells. Vertical bars: ± SD.

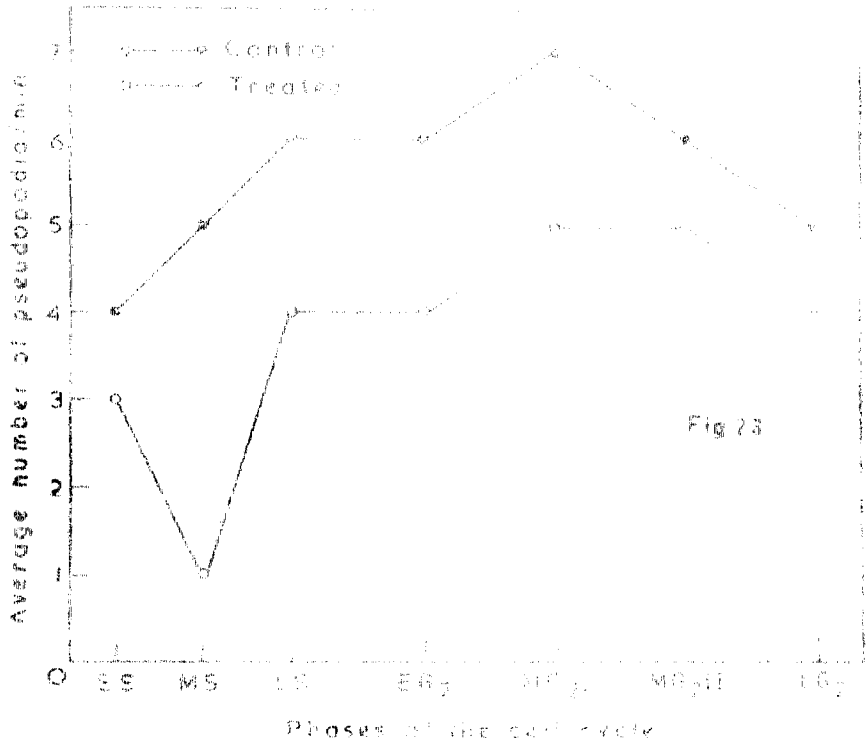

Control
Treated
Average number of pseudopodia/cell
0
1
2
3
4
5
6
Phases of the cell cycle

Fig 23

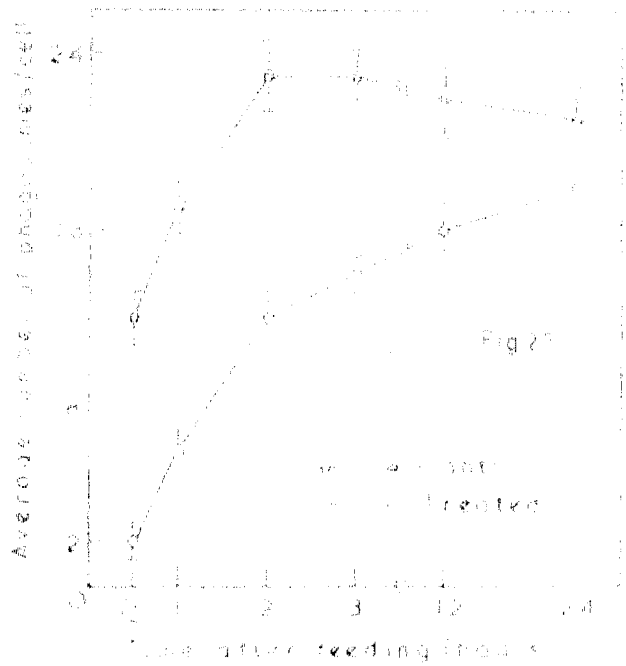

Control
Treated

Fig 24

Fig. 28: Squashed preparation of a control cell showing the formation of numerous phagosomes, ½ h after initiation of feeding with Tetrahymena. Cell stained with Giemsa. n, nucleus; p, phagosomes. X 252

Fig. 29: Cell treated with 0.5% EMS for 15 min. Note drastic inhibition in the uptake of food organism. Cell squashed and fixed ½ h after initiation of feeding. Other details as in Fig.28. X 450

28

29

Cell duplication time

The generation time of control amoebae under our laboratory condition was found to be 72 ± 6 h. Cells exposed to 0.5% EMS for 15 min showed initially, a prolonged inhibition in cell multiplication which was evident by a division delay of 48 ± 6 h before the next mitosis. However, longer generation time in successive generations was also noticed even after 3 weeks from the time of treatment (Fig. 30), and a considerable arrest in the cell multiplication process was found to be continued even for more than two months.

Differences were also noticed regarding the mitotic block in cells treated at defined phases of the cell cycle. The division delay noticed before the next divisions were initiated after the mutagen treatment has been shown in the table below:

TABLE 1

Phases of the cell cycle	M phase	ES phase	MS phase	LS phase	EG_2 phase	MG_2 phase	IG_2 phase
Generation time (h)	120±4	168±4	240±6	216±6	168±4	96±4	168±4
Division delay(h)	48±4	96±4	168±6	144±6	96±4	24±4	96±4

(± sign indicates SD of six separate experiments and the number of experimental cells at each phase varied between 120 to 130. Data represents the initial division delay after the mutagen exposure).

The generation time and division delay indicated for mitotic cells in the table 1 represent the data obtained from the first division cycle of the daughter cells, produced after treatment of a mitotic cell. However, the initial division delay of the mutagen treated mitotic cells to produce two daughter amoebae took about 40 h, while the control amoebae it was 35 to 40 minutes. The progeny of the treated mitotic cells examined for several generations also showed the mitotic arrest for a considerable period of time (Fig. 30). In all the cells treated with 0.5% EMS for 15 min at defined phases of the cell cycle a prolonged division delay was found to occur successively for several generations. However, mitotic block was much more pronounced in cells treated at S and LG_2 phases (Figs. 31, 32 and 33).

Duration of phases in the cell cycle

An alteration in the duration of S and G_2 phase of the cell cycle was monitored when the cells were treated at ES phase with 0.5% EMS for 15 min. In control cells the entire cell cycle was found to be completed by 72 $\pm$ 6 h where the mitotic phase continued for 35 to 40 min; S phase was found to continue upto 7 h while the rest of the time i.e., 64 $\pm$ 6 h was considered as G_2 phase. The length of S phase was determined by analysing the autoradiograms of

Fig. 30: Prolonged inhibition to cell multiplication process in mitotic phase cells treated with operative dose of EMS.

Fig. 31: Mitotic arrest for successive generations in ES phase cells exposed to operative dose of EMS.

Fig. 32: Mitotic delay for several generations in MS and LS phase cells treated with 0.5% EMS for 15 min.

Fig. 33: Prolonged inhibition to cell replication process in EG_2, MG_2 and LG_2 phase cells exposed to operative dose of EMS.

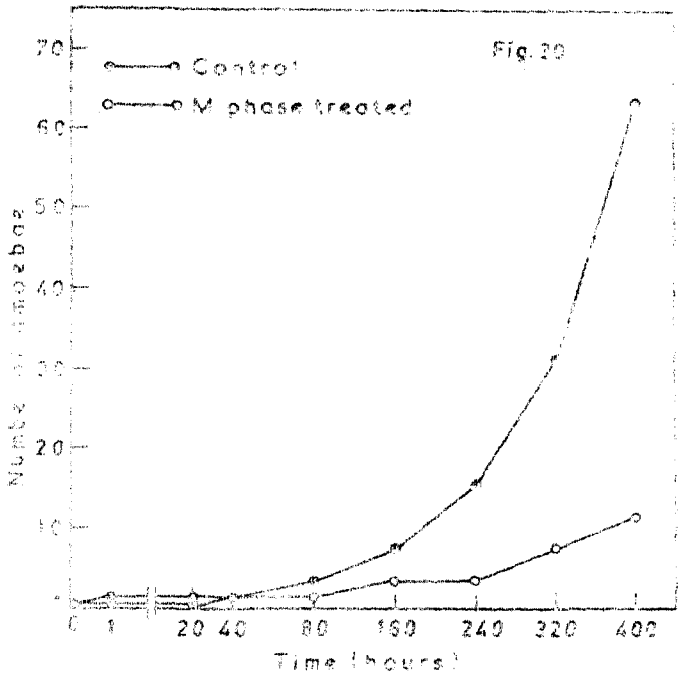


Fig. 29
Control
M phase treated
Number of amoebae
Time (hours)

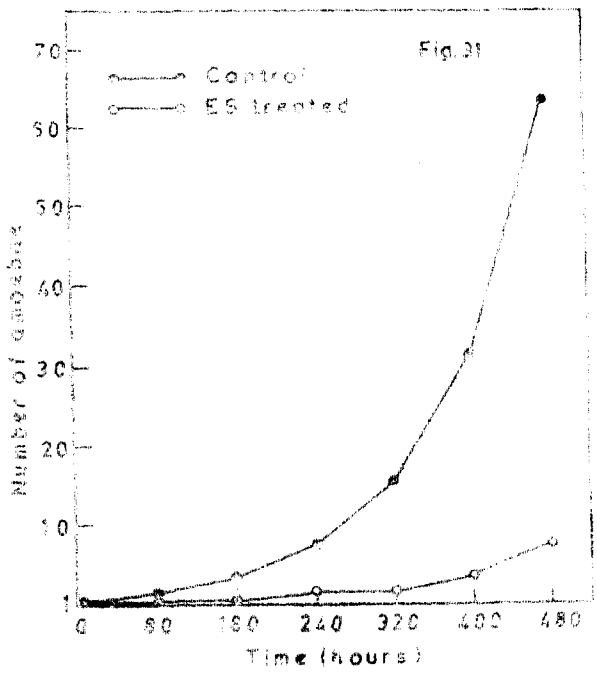


Fig. 31
Control
ES treated
Number of amoebae
Time (hours)

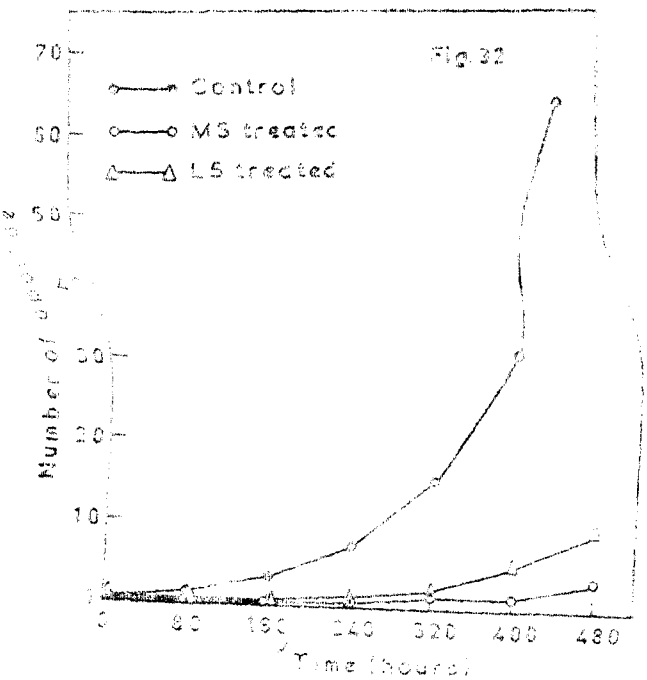


Fig. 32
Control
MS treated
LS treated
Time (hours)

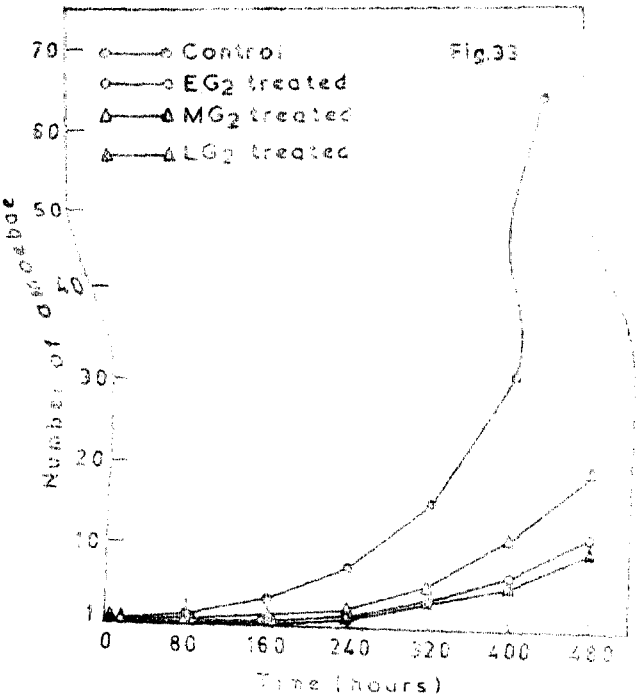


Fig. 33
Control
EG2 treated
MG2 treated
LG2 treated
Number of amoebae
Time (hours)

^{3}H-thymidine labelled cells' nuclei upto 14 h.

A change in the length of the cell cycle was noted when the amoebae were treated with 0.5% EMS for 15 min at ES phase. The DNA synthesis was prolonged upto 14 h instead of 7 h as in control cells. The entire G_2 phase was also found to be prolonged and it covered a period of 153 ± 4 h. The cell duplication time taken by the treated cells was found to be 168 ± 4 h.

Variant cell production

EMS treatment brought about some remarkable changes in the size of amoebae. The cells treated with 0.5% EMS for 15 min displayed a variable size range from 'Giant' to 'Mini', although, in general, EMS treatment resulted into the reduction of cell size and many of these cells resumed 'apparent normal' form after 5 to 6 generations.

Three types of vaiant cells could be detected which were: (i) Mini cells; (2) Intermediate cells and (3) Giant cells. The 'Mini' cells were found to be smallest in size; the 'Intermediate' cells types had a bigger size than 'Mini' cells and smaller than normal amoebae whereas the 'Giant' cells were found to be considerably bigger as compared to control amoebae and could be detected in the culture around 10 to 12 days after treatment (Fig. 34).

However, 'Mini' and 'Intermediate' cells appeared in the culture approximately between 22 to 24 h after mutagen exposure.

Many of the giant cells were usually lethal, in few cases, they assumed 'apparent normal' size after several mitotic divisions and became viable. Most of the giant cells which were examined showed a multinucleate condition (Fig. 35). Mini sized cells were also of frequent occurrence after mutagen administration to the amoebae. Broadly, these mini cells could be categorised into two major groups: (i) the cells which after a period of time regained their normal size and (ii) the group of cells which permanently remained smaller in size, compared to controls. Besides these cells, an array of intermediate sized cells were also observed which mostly were regulated back to 'apparent normal' size after 8 to 9 division cycles. The size variations of different cell types obtained from the measurements of more than sixty living and fixed cells of the different categories are as follows:

TABLE 2

Cell types	Average cell size (µm ± SD)	Nuclear diameter (µm ± SD)
A. indica	397 ± 11	33 ± 2.5
Giant cell	594 ± 27	52 ± 3
Intermediate cell	295 ± 15	28 ± 1.5

However, between 40% to 50% of the variant cells were found to be lethal within 15-20 days following mutagen treatment. Only few mini cells were found to form a stable clone out of the other variant cells which appeared after the treatment. A detailed characterization of the mini cells has been considered in a separate chapter.

The table below shows the number of variants produced after exposing known number of cells with the operative dose of EMS.

TABLE 3

Total No. of cells treated	No.of variants produced	Types of Variants		
		No.of Mini cells	No. of Intermediate cells	No.of Giant cells
2018	935	238 x 96 *142 @ 84 **12	632 x 565 *67 @ 565	65 x 7 *58 @ 7

(X, viable cells; *, non-viable cells; @, cells reverted back to normal forms; **, formed stable clone of mini cells).

C. Cytoplasm and nucleus in EMS exposed cells:

(I) The cytoplasm

As seen under light microscope

A recognisable difference in the cell body of amoebae could be observed after exposing the cells to

Fig. 34: Living cells showing size variations. Size variants produced after exposing the cells with EMS. Normal A. indica, right side on the top; intermediate sized cell, second cell at the top from the right side; giant cells and mini cells. X 322

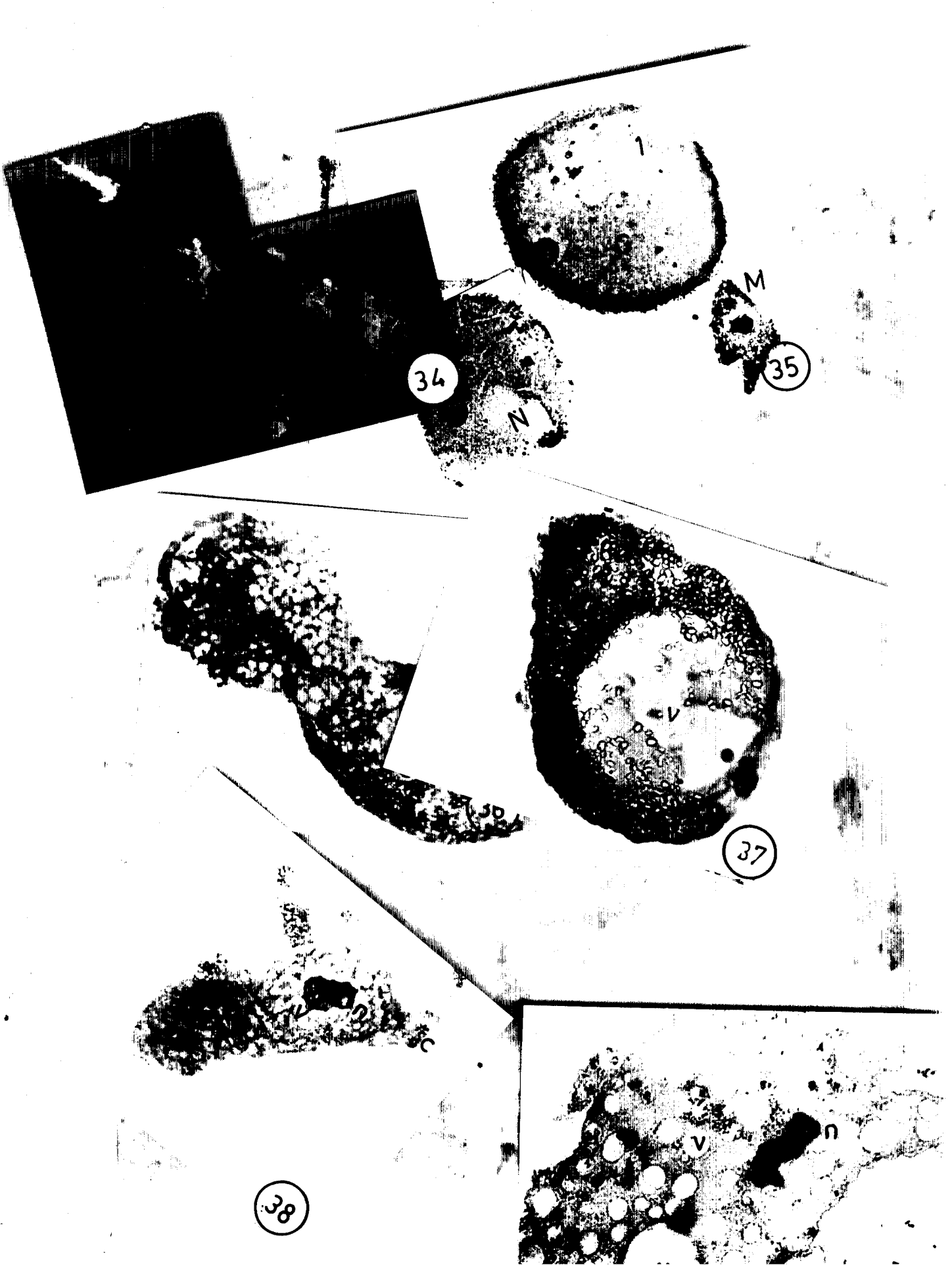
Fig. 35: Squashed and Giemsa stained cells, showing size variants produced after treating the cells for 15 min with 0.5% EMS. Cells were fixed 10 days after treatment. Note the binucleate (1 and 2) 'Giant' cell (G) with a 'Mini' cell (M) and a normal cell (N). X 144

Fig. 36: Wholemount preparation of an anucleate cell body around 3 h after treating the ES phase cells with operative dose of EMS. Note hypervacuolated cytoplasm and cluster of crystals (C). Cell stained with Giemsa. X 450

Fig. 37: Wholemount preparation of a cell at around 1 h after EMS exposure. Note spherical shape with a big vacuole (v); eccentric nucleus (n) and cluster of cytoplasmic crystals (c) around the vacuole. Cell stained with Giemsa. X 720

Fig. 38: Squashed EMS exposed cell at around 12 h after mutagen treatment, showing extensive vacuolation (v) in the cytoplasm. Note pycnotic nucleus (n) and crystals (c). Stained with Giemsa. X 320

Fig. 39: Toluidine blue stained thin section of a cell treated with operative dose of EMS and fixed around 12 h after treatment, showing hypervacuolated cytoplasm (v). n, nucleus. X 900

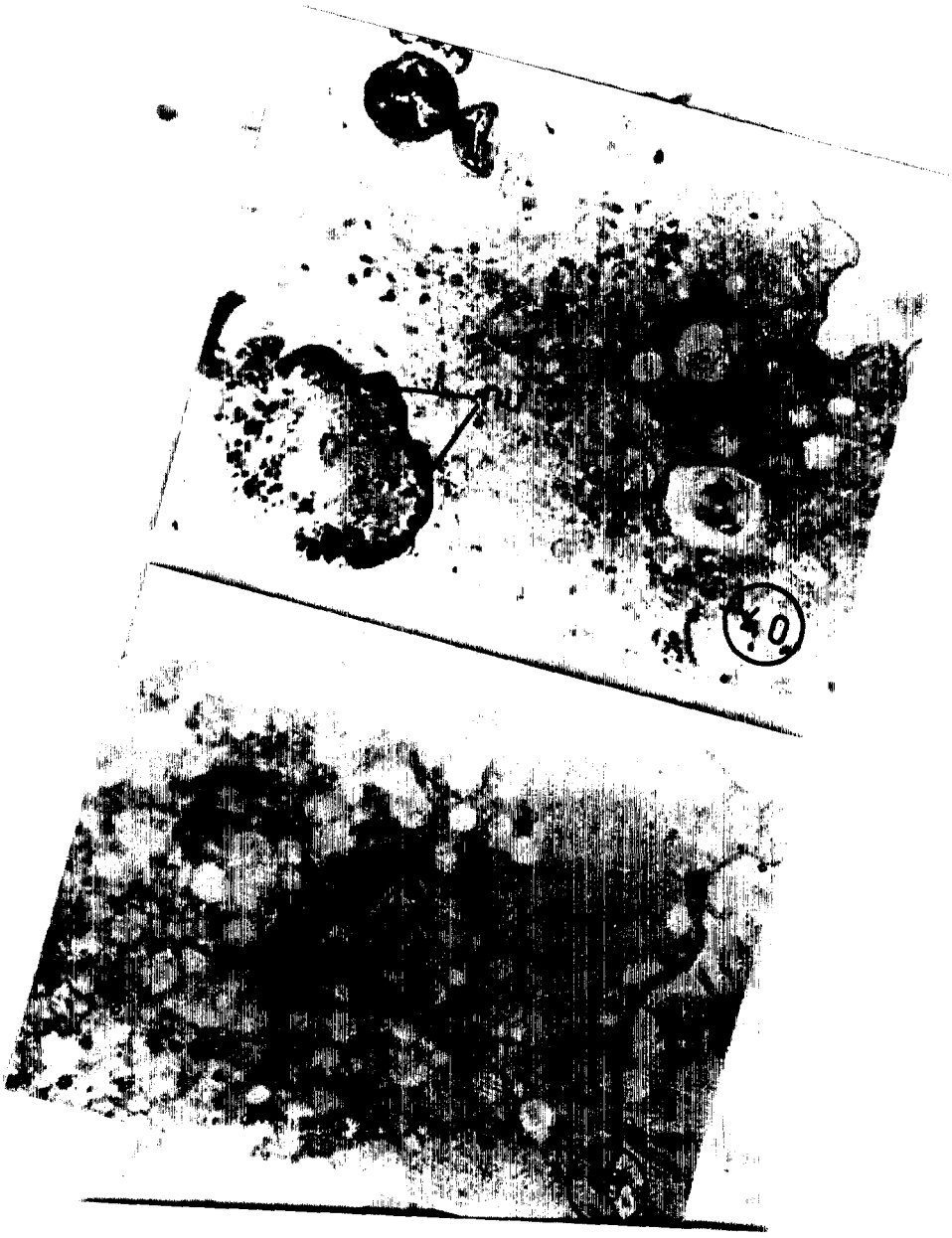

1
M
34
35
N
3D
V
37
V
n
38

operative dose of EMS. Anucleated amoebae were often observed among the treated cells within 3-4 h following mutagen exposure. A higher frequency of nuclear ejection was noted within 2-3 h in cells exposed at S phase with 0.5% EMS for 15 min. In stained preparations, an extremely hypervacuolated condition of the cytoplasm could be detected in the anucleate cell bodies where the cytoplasmic crystals were found in clusters, more towards the cell periphery (Fig. 36). In some cases a big vacuole was noticed within an hour after mutagen administration. The vacuoles were so big in size that the entire cell cytoplasm was pushed apart towards the periphery and the cell appeared spherical with no pseudopodia. The nucleus was also found to be eccentric and a cluster of crystals was observed along the periphery of the vacuole (Fig.37). These cells underwent cytolysis within 36 h after treatment. Amoebae exposed to operative dose of EMS demonstrated extensive vacuolation in the cytoplasm in atleast 40% of the cells even around 12 h after mutagen exposure, where the cytoplasmic crystals could be traced as clusters around some of the vacuoles, sometimes also at the tips of the advancing pseudopods. A typical pycnotic nucleus was also observed in some

Fig. 40: Thin section of a portion of control cell stained with toluidine blue. Cell fixed at around 24 h along with the treated cell after the initiation of the experiment. n, nucleus; nu, nucleoli. X 1600

Fig. 41: Thin section of a portion of toluidine blue stained cell treated with 0.5% EMS for 15 min and fixed at around 24 h after treatment. Note fenestrated endoplasm. v, vacuoles; pm, plasma membrane; n, nucleus. X 1600

Fig. 42: MS phase cell treated with operative dose of EMS and fixed five days after treatment. Note crystals (c) of different shapes. Squashed cell stained with Giemsa. X 320




C

cells at this stage (Fig. 38). The hypervacuolated nature of the treated cells' cytoplasm was further witnessed by the observations on thin sections of treated amoebae at ca. 12 h where the entire cytoplasm was found to be crowded with vacuoles (Fig. 39). However, at later hours after treatment the hypervacuolated nature of the cell cytoplasm was almost indistinct and appeared more or less comparable to control amoebae.

In some cases cells exposed to 0.5% EMS for 15 min showed a fenestrated cytoplasm at ca. 24 h after treatment. This nature of cytoplasm could also be noticed in treated cells examined at around 36 h. The most noticeable feature was found to be the appearance of some vacuoles just below the plasma membrane and a continuation of these vacuoles could be traced from the endoplasm towards the plasma membrane. However, the control cells did not show such a situation (Figs. 40 and 41). More or less the same feature was reflected in MS phase cells treated with operative dose of EMS, in addition to that appearance of several large sized crystals of different shapes were quite apparent in the cytoplasm, few days after treatment (Fig. 42). Cytoplasmic damage was less evident in G_2 phase treated cells as compared to S phase ones.

As observed under electron microscope

The observation on the hypervacuolated nature of the mutagen exposed cell was further strengthened by fine structural analysis of treated cells at ca. 12 hr after treatment. The vacuoles were found to be surrounded by a membrane and therefore delimited from the cytoplasm. The cytoplasm of the treated cells displayed several electron dense particles (Fig. 44), whereas, the control cell cytoplasm displayed almost no such particles (Fig. 43).

Several cytoplasmic organelles showed a great deal of disorganisation immediately after EMS treatment. The cisternae of the golgi bodies became disorganised and were found to be scattered. Usual convex and concave faces of the golgi cisternae was absent in most of the cases. Unusual swelling at certain regions of the golgi cisternae was quite apparent immediately after treatment. The vesicular elements of the golgi bodies were in most cases found to be dispersed. Electron dense inclusions within the vesicular elements of the disorganised golgi bodies were noticed (Fig. 45). An almost normal morphology of the golgi bodies was found to be present around 24 h after treatment where more or less a regular stacking of the golgi bodies, like that of the control cells could be discernible (Fig. 46).

Fig. 43: Electron micrograph of a normal A. indica showing a portion of cytoplasm with mitochondrion, vesicles and plasma membrane. M, mitochondrion; p, polysomes, v, vesicles; f, microfibres; rer, rough endoplasmic reticulum; pm, plasma membrane. X 20000

Fig. 44: Electron micrograph of EMS treated amoeba about 12 h after treatment, showing hypervacuolated condition of the cytoplasm. Note also the presence of electron dense particles. va, vacuole, ep, electron dense particles; ci, cytoplasmic inclusion; vm, vacuolar membrane. X 13500

Fig. 45: Electron micrograph of a portion of mutagen exposed amoeba at 0 h showing the disorganised nature of the golgi bodies. g, golgi body; ig, inclusions within the vesicular element of golgi body; pm, plasma membrane; ep, electron dense particles; rer, rough endoplasmic reticulum; r, free ribosomes; s, swelling of golgi cisterna; M, mitochondrion. X 22500

Fig. 46: Electron micrograph of a mutagen treated amoeba 24 h after the treatment. Golgi body shows almost normal structural reconstitution. g, golgi body; rer, rough endoplasmic reticulum; nu, nuclear honeycomb layer; M, mitochondria; vc, vesiculated cristae. X 22500


P
Pm
Mt
43
Pm
P
Vc
R

Rough endoplasmic reticulum was often found in association with the golgi cisternae in EMS exposed cells, almost immediately after treatment. Free ribosomal particles in the form of monomers were quite abundant in the cytoplasmic matrix around the rough endoplasmic reticulum (Fig. 45). However, at later hours after treatment, in most of the cases, the ribosomes were found either to be arranged on the cisternae of rough endoplasmic reticulum or in the form of polysomes (Fig. 46) like that of normal cells.

Electron microscopic studies also revealed striking changes in the mitochondrial structure in EMS exposed amoebae. The mitochondria became greatly enlarged immediately after treatment in majority of the cases. The tubular cristae often occupied a narrow peripheral region and patches of electron dense material appeared in most of the mitochondria examined. Sometimes, the limiting membrane and the inner membranes of the mitochondria 'crossed-over' where the broken limiting membrane ended abruptly as either a limiting membrane or as an inner membrane (Fig. 48). Electron-lucent zone in the mitochondrial matrix was quite apparent in several cases. Bursting of mitochondria was also noted which led to the release of mitochondrial content through the cytoplasm (Fig. 49).

Fig. 47: Electron micrograph of a portion of the cytoplasm of control amoeba, showing a mitochondrion (M). X 45000

Fig. 48: Electron micrograph of a mutagen administered cells at 0 h, showing the mitochondrial enlargement. ep, electron dense patches; el, electron-lucent area; e, electron dense particles; arrows, broken membrane showing 'cross-over' between outer and inner mitochondrial membranes. X 45000

Fig. 49: Electron micrograph of a portion of an amoeba at early hours after EMS treatment. el, electron-lucent zones; bm, bursting mitochondrion; M, mitochondria; v, vesicles; g, disorganised golgi body; arrow, release of vesicles from the membrane surface. X 30000


M
ep
ep
e
48
bm
49

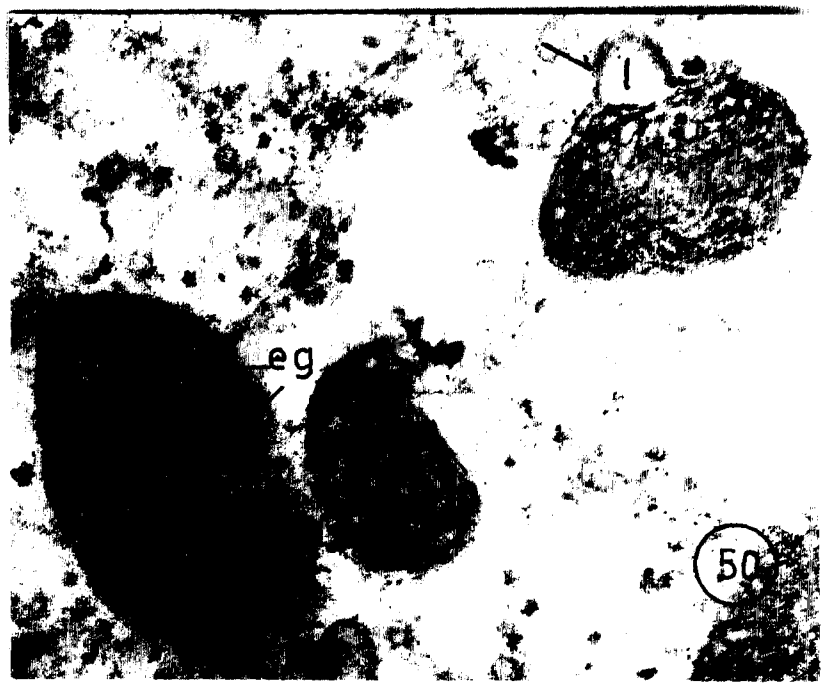
However, at later hours after treatment the mitochondrial size was found to be almost regulated back to normal form and the tubular cristae reappeared in most of the mitochondrion like that of control mitochondria (compare Figs. 49 and 50). Several other striking differences were encountered at around 24 h after treatment which were: (a) some mitochondria were found with vesiculated cristae (Fig. 46), (b) appearance of numerous electron dense dots as inclusions in the mitochondrial matrix as well as on the tubular cristae, although these particles were also quite abundant in the cytoplasm (Fig. 50), (c) sometimes, extension of the limiting membrane of the mitochondria in the form of a protrusion which encircled an electron-leucent zone was noted (Fig. 50), (d) appearance of some mitochondrion which showed greater electron-opacity (Fig. 51) as compared to control mitochondria.

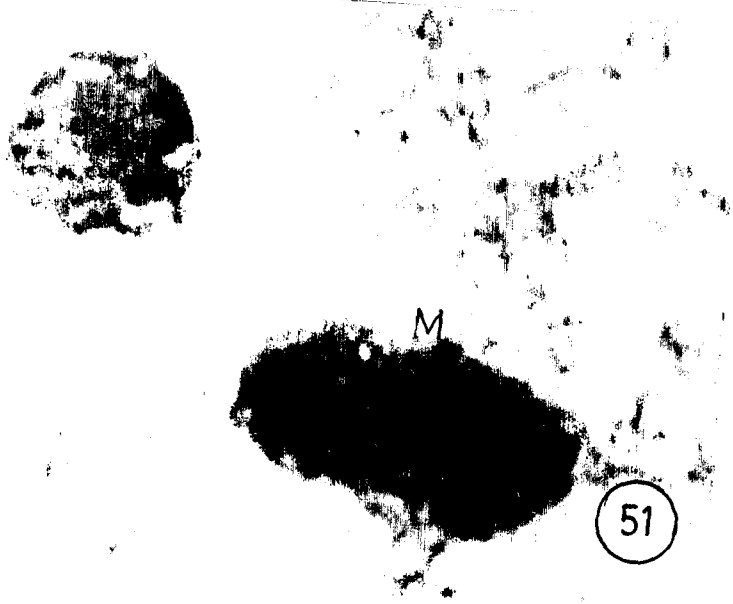
One noticeable feature of the EMS exposed cells was the presence of many electron dense particles in the cytoplasm which were almost totally absent in the untreated amoebae (Figs. 45, 47 and 48). The nature of these particles is unknown.

In some mutagen treated cells several vesicular bodies containing electron dense granular inclusions

Fig. 50: Electron micrograph showing the mitochondria of a treated amoeba 24 h after the mutagen treatment. Note the size of the mitochondria have become comparable to the control cells (Fig. 47), eg, electron dense granular inclusions; l, electron-lucent area; arrow, extension of limiting membrane as protrusion. X 45000

Fig. 51: Electron micrograph of a portion of the treated cells 24 h after treatment, showing electron-opacity of mitochondria (M). X 42500


l
eg
50


M
51

were found to be pinched out from the surface of the cells (Fig. 49). This phenomena could not be observed in control cells (Fig. 47).

(II) The nucleus

As seen under light microscope

Some interesting changes in the nucleus were encountered after treating the cells with EMS in a mass culture and also at defined phases of the cell cycle.

An increase in the size of the nucleus was found to be a general occurrence in cells exposed to 0.5% EMS. Measurement of the nuclear diameter within 8 to 10 h after treatment showed that in treated amoebae the mean diameter varied between 45 ± 4 µm while in control cells it was 33 ± 2.5 µm (Figs. 52 and 53).

Treatment of 0.5% EMS for 15 min was found to cause a marked difference in the nuclear structure. It was noted that within 3 to 4 h after treatment the nucleus became disorganised and in most of the cases nuclear elements appeared in the form of small dots, condensed more towards the nuclear periphery leaving more or less a lighter zone at the centre of the nucleus. Sometimes the dark dots of nuclear materials were placed close to each other and interconnected to form a coiled

thread-like appearance (Fig. 54). Reorganisation of the nucleus was, however, observed at around 12 h onwards after EMS treatment with the appearance of nuclear envelope and conspicuous nucleoli (Fig. 55).

Cells treated with EMS at ES phase showed sprouting out of the nuclear materials around 12 to 14 h after treatment. Detailed description of this observation has been included elsewhere (Chapter III). When the cells were exposed to EMS for 15 min at MS phase with the same dose, a completely different picture was recorded. Atleast in 20% of the treated cells the nuclear membrane was not at all discernible within 3 to 4 h after treatment. The nucleus was found to be totally disorganised where the nuclear elements appeared in the form of beads of different sizes ranging from 1 to 4 µm in diameter. However, these beads were more or less arranged in such a fashion so as to form a spherical mass which could be recognised as the nuclear mass (Fig. 56). These cells were generally non-viable. No significant variation could be observed in cells treated with the same dose at late S phase.

Another interesting feature was noted in EMS treated ES phase cells where around 16 to 18 h after

treatment a multinucleate condition was observed in ca. 20% cases. The diameter of these nuclei was found to vary between 22 to 28 µm (Fig. 57).

As seen under electron microscope

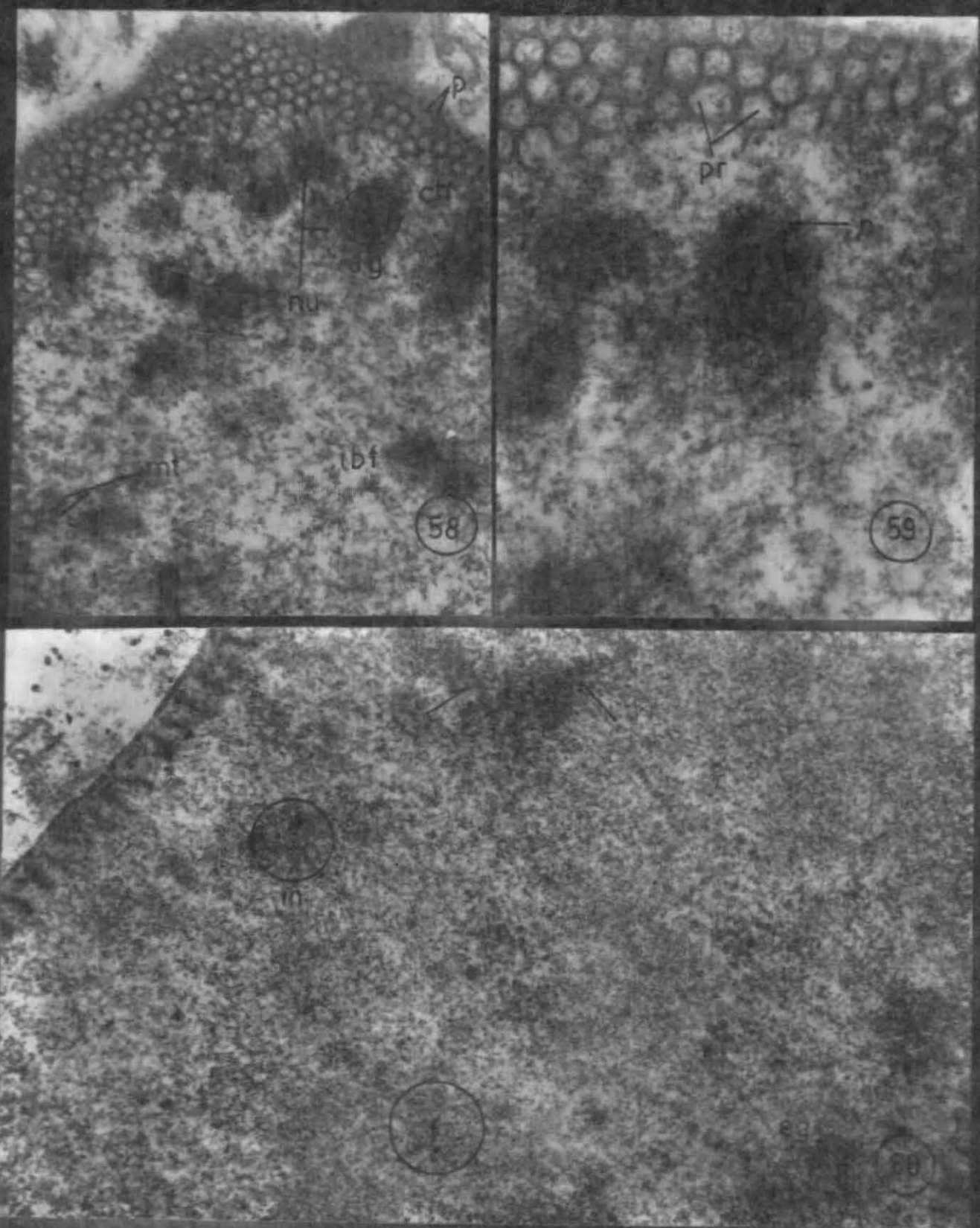
In control *A. indica* the nucleus was bounded by a nuclear envelope. It consisted of two membranes which enclosed a perinuclear cisterna and were fused at intervals to form pores. A honeycomb layer (fibrous lamina) was found in continuation with the inner membrane of the nuclear envelope. The fibrous lamina consisted of a series of adjascent hexagonal prisms with their long axes perpendicular to the surface of the nucleus. One end of the fibrous lamina abuts the inner surface of the inner membrane of the nuclear envelope, while the other end opened to the nucleoplasm (Fig. 58). Several small beaded thread-like materials were noticed within the honeycomb packets, which were probably the ribonucleoprotein helices (Fig. 59). These helices were also abundant in the nucleoplasma (Fig. 58).

The nucleus contained multiple nucleoli which were normally arranged in a mew just beneath the honeycomb layer of the nuclear envelope. The substructure of

Fig. 58: Electron micrograph of a portion of control cell nucleus. p, pores, nu, nucleoli; mt, microtubules (in T.S.); dg, dense granular inclusions; ibf, electron dense body with fibrous material; ch, chromatin. X 17000

Fig. 59: Electron micrograph of a portion of control *A. indica* cell nucleus magnified. n, nucleolus; dg, dense granular inclusions; pr, almost hexagonal prisms. X 45000

Fig. 60: Electron micrograph of a portion of EMS exposed cell nucleus at 0 h after treatment. Note disorganised and inconspicuous nature of the nucleoli as compared to control cell (Fig. 59). in, inconspicuous nucleoli; eg, electron dense granules within the nucleolus; f, condensed fibrous elements; arrows, branching of a nucleolus. X 275000

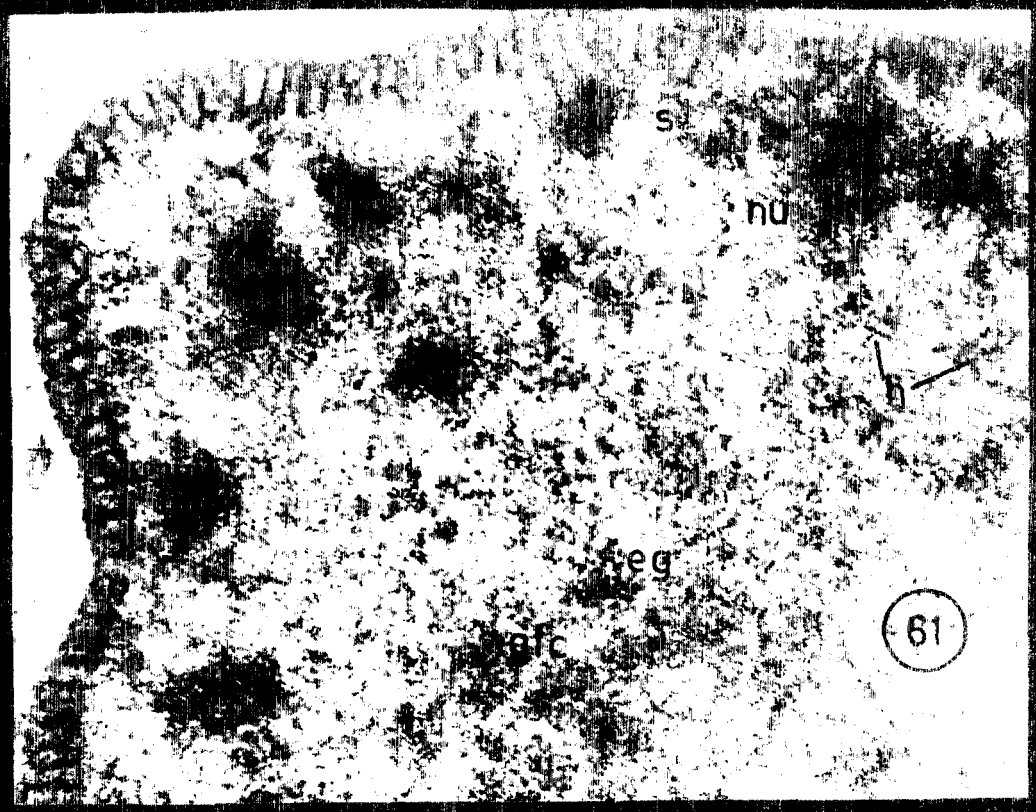

p
chr
g
nu
mt
lbf
58
pr
p
59
m
60

the nucleoli showed granular and fibrillar components, intermixed and packed together. The lighter masses were designated as the chromatin material. However, some small circular annuli-like structures were noticed just below the nuclear honeycomb layer as microtubules. The nucleoplasm was found to be largely occupied by fibrous material which were found to be organised more compactly in certain regions. In some places electron dense bodies were observed in association with the fibrous material (Fig. 58).

The electron microscopic observations on the nucleus of the treated cell showed that the nuclear structure became greatly affected after EMS administration. Immediately after treatment the nucleoli became inconspicuous and lost their discrete entity. Nucleolar elements from the peripheral region of the nucleoli were found to be dispersed in the nucleoplasm. Occasionally, several electron dense granules were noted within the discrete nucleolus. Sometimes branching of the nucleoli was also observed. Disorganisation of chromatin material was quite apparent. The fibrillar components of the nuclear sap became condensed at certain regions as compared to control cells. The helices became very scanty and indistinct as compared

Fig. 61: Electron micrograph of a portion of the nucleus of a EMS treated cell around 12 h after treatment, showing conspicuous nucleoli like that of control, indicating their reconstitution. nu, nucleoli; gfc, granulofibrillar components; eg, aggregation of electron dense granules; h, helices; s, shedding out of honeycomb layer. X 20800

Fig. 62: Electron micrograph of a portion of the nucleus of EMS treated cells around 24 h after treatment, showing sharp contour of the nucleoli. Note almost comparable nucleoli as that of control (Fig. 59). b, branching of nucleoli; arrow, fussion of two nucleoli; ep, electron dense particles; nu, nucleoli. X 18000


s
nu
b
eg
61


nu
b
62

to control cells (Figs. 58 and 60). At around 12 h after treatment, the nucleoli tended to be reconstituted beneath the nuclear envelope. The granulofibrillar nature of the nucleoli could be discernible at this stage, although, a tendency of the nucleolus to anastomose with each other was noticed. The nucleoplasm was found to be stubbed with highly dense granular and fibrillar components as compared to controls. At certain regions aggregation of electron dense granules was observed, which were found to be interdispersed with the fibrillar components of the nucleoplasm. Prominent helices were quite abundant in the interior of the nucleus (Fig. 61). However, at later hours after treatment well defined nucleolar structure was noted which was found to be almost comparable to that of normal amoeba (Fig. 62). In treated cell nuclei, in general, certain electron dense particles were noted. The nature of these particles are not known (Fig. 62).

D. Cytochemical observations :

Several cytochemical techniques were employed to detect a few cell substances to investigate the effect of EMS on localisation of these substances at definite intervals of time, after treatment with 0.5% EMS for 15 min. In each case 18 to 20 cells were observed

to get an average picture about the intensity of of staining of the specific cytochemical reactions.

Basophilia

Treatment with EMS resulted in the ununiform localisation of basophilia. Such effect was found to be dominant in toluidine blue stained cells at early hours after treatment. Although, this ununiformity could rarely be observed in cells at 24 h after treatment (Table 4). It was noted that immediately after treatment the basophilic reaction at certain regions of the cytoplasm was more intense than the rest of the area of the cell. However, at later hours after treatment the basophilic positive areas were found to be aggregated together to give an extensive cytoplasmic areas with a high positive reaction as compared to control cells (Figs. 63 and 64).

Mercuric bromophenol blue

Mercuric bromophenol blue staining was used to examine the intensity of reaction of the protein moiety in treated cells as compared to control.

Immediately after treatment the intensity of staining was found to be appreciably less as compared to

control cells (Figs. 65 and 66). An uneven distribution of reaction intensity was observed all over the cytoplasm of the treated cells. However, a fairly higher intensity of staining could be recorded on the nucleus as indicated by dark blue colour of the dye. At later hours after treatment the cells appeared to have much less differentiation in the staining intensity as compared to control cells (Table 4). It was observed that the nucleus was darkly stained along with an appreciably dense and uniform staining throughout the cytoplasm.

Sudan black B

Sudan black B staining technique was used to monitor the site of reaction and intensity of staining of bound lipids in EMS treated cells, compared to controls.

In control cells bound lipids were observed in the form of bluish-black droplets scattered all over the cytoplasm. The intensity of staining was noted to be uniform throughout the cytoplasm. Immediately after EMS treatment a diffused lipid staining reaction was noted all over the cytpplasm of the cell. Stained bound lipids appeared in the form of small dots scattered evenly over the cytoplasm except at the region of the

Fig. 63: Squashed control cell showing poor basophilic reaction after staining with toluidine blue. X 283

Fig. 64: Cell treated with 0.5% EMS for 15 min, squashed and stained in toluidine blue at ca. 12 h after treatment. Note intense basophilia at certain places. X 283

Fig. 65: Squashed preparation of a control cell showing mercuric bromophenol blue reaction. Note high staining intensity all over the cell cytoplasm and nucleus. X 283

Fig. 66: A cell treated with 0.5% EMS for 15 min squashed and fixed 0 h after treatment and stained with mercuric bromophenol blue. Note less staining intensity of the cells as compared to control (Fig. 65). X 283

Fig. 67: Squashed control cell showing intensity of bound lipid reaction after staining with Sudan black B. X 450

Fig. 68: Cell treated with 0.5% EMS for 15 min, squashed and stained with Sudan black B at 0 h after treatment, showing reduction in the intensity of staining as compared to control cells. Note Sudanophobic nucleus (n) scanty sudanophilic bound lipids. X 450

68
67
66
65
64
63

advancing pseudopods where very few sudanophilic granules could be detected. The nucleus remained sudanophobic both in treated and control cells (Figs. 67 and 68). At later hours after treatment the staining intensity appeared to be higher as compared to the staining intensity of treated cells at early hours (Table 4).

Periodic acid Schiff

Periodic acid Schiff (PAS) staining technique was used to study the intensity of polysaccharides (mucopolysaccharides and muceproteins) staining in EMS treated cells as compared to the controls.

In control cells polysaccharides and mucoproteins had a purple colour with PAS. The reaction was found to occur evenly all over the cytoplasm. An appreciably higher staining intensity was noted in the cytoplasm of the cell. Very less PAS positive substances were noted on the nucleus (Fig. 69). At 0 h after treatment, a totally diffused nature of PAS reaction could be noted throughout the cytoplasm and the localisation of PAS positive substances were detected mostly at the tip of the advancing pseudopods (Fig. 70). The nucleus remained PAS negative. However, at ca. 24 h after treatment a recovery was noted in the treated cells as revealed by the

Fig. 69: A squashed control cell stained by PAS technique, showing intense PAS reaction all over the cell. Note poor PAS positive substances on the nucleus (n). X 283

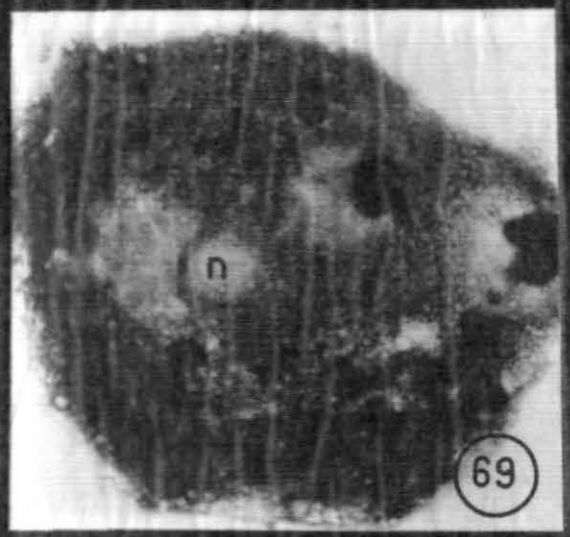
Fig. 70: Cell treated with 0.5% EMS for 15 min, squashed and stained through PAS reaction, showing less staining intensity of PAS positive substances at 0 h after treatment. Note detectable localization of PAS positive substances at the tips of the advancing pseudopods (arrows), n, nucleus. X 283

Fig. 71: A squashed control cell prepared for APase activity showing a high intensity of staining. X 283

Fig. 72: Cell treated for 15 min with 0.5% EMS, showing diffused reaction at 0 h after treatment. Note fairly positive APase activity on the nucleus (n). X 283

Fig. 73: A control cell squashed and stained for AcPase activity, showing a homogeneous intensity of reaction in the cytoplasm and a fairly higher activity on the nucleus (n). Arrows, sites of activity. X 283

Fig. 74: EMS treated squashed cell showing diffused AcPase reaction in the cytoplasm, 12 h after treatment. Note a binucleate cell. X 283


n
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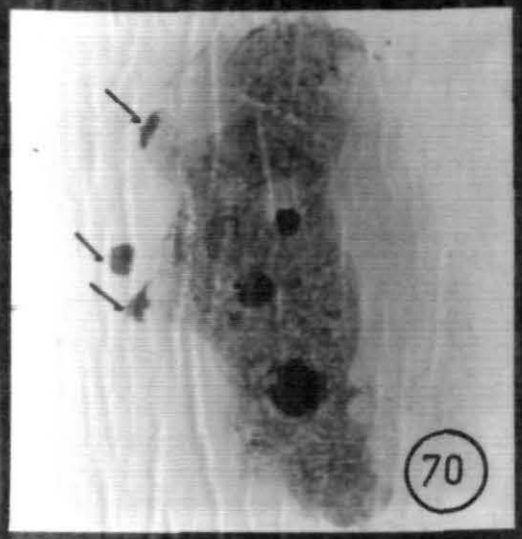
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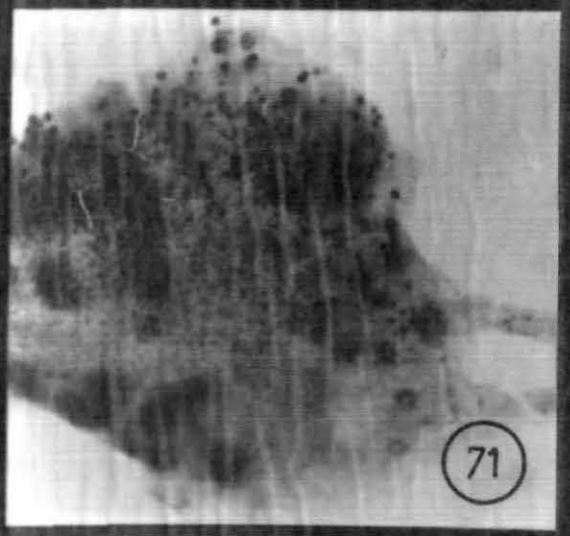
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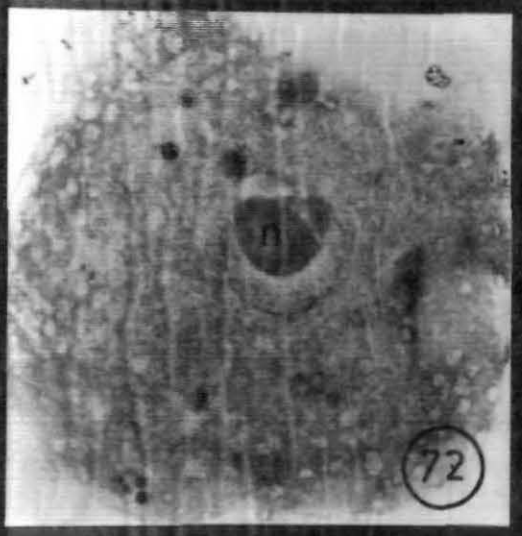
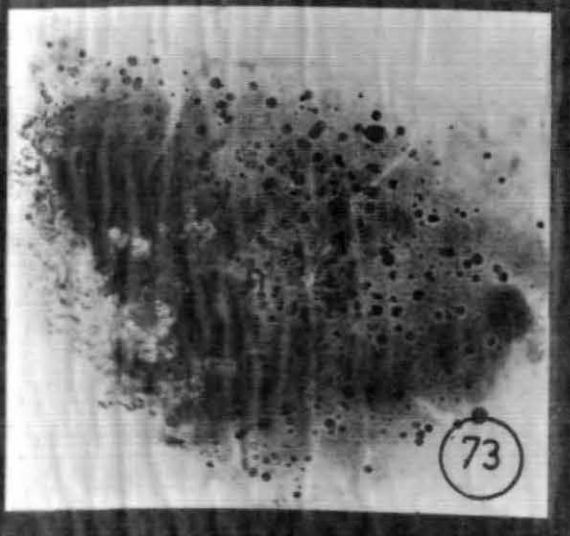

n
72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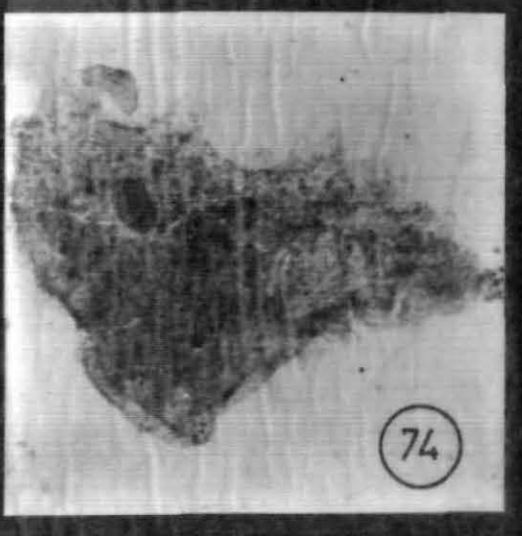
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appearance of more PAS positive substances in the cells (Table 4).

Alkaline phosphatase

Presence of nonspecific alkaline phosphatase (E.C. 3.1.3.1; APase) and its intensity of staining was detected by the appearance of black deposits of cobalt sulphide in the cell.

In control cells an appreciably higher intensity of APase was noted tobe distributed evenly throughout the visible part of the cell. However, a similar kind of staining could be detected on the nucleus (Fig. 71). Immediately after treatment with EMS a diffused reaction was noted as compared to control cells (Fig. 72). However, the intensity of staining reaction was found to be much higher at later hours after treatment, where the APase activity could be detected by the appearance of a fairly higher accumulation of reaction products (Table 4).

Acid phosphatase

The non-specific acid phosphatase (E.C. 3.1.3.2; AcPase) was detected by the appearance of black dots of lead sulphide in the cells.

In control cells a homogeneous intensity of staining reaction was observed in the cytoplasm which was evident by the appearance of black dots scattered evenly throughout the cytoplasm. A fair intensity of reaction could also be noted on the nucleus (Fig. 73). Immediately and even at early hours after treatment a considerably less intensity of AcPase was noted, which was apparent by the diffused nature of AcPase reaction in the cytoplasm as compared to control cells (Fig. 74). However, somewhat higher intensity of reaction was recorded on the nucleus as compared to treated cell's cytoplasm. Concentration of AcPase was found to be quite high at later hours after treatment as compared to immediately treated cells (Table 4).

TABLE 4

The intensity of cytochemical staining in control cells and in cells exposed to 0.5% EMS for 15 min. Intensity has been expressed in the form of +.

Staining reaction	Type of cells C=control T=treated	Overall staining intensity 0 h	24 h	Remark
Basophilia	C	++	++	Intense basophilia in treated cells at later hours.
	T	+	++++	
Mercuric bromophenol blue	C	++++	++++	Staining intensity in treated cells appeare more or less comparabl to control at later hours.
	T	++	+++	
Sudan black B	C	++++	++++	Considerably less staining intensity even at later hours in treated cells as compared to control.
	T	+	++	
Periodic acid Schiff	C	++++	++++	Very weak PAS reaction at early hours, follow ed by substantial recovery at later hour in treated cells.
	T	+	+++	
Alkaline phosphatase	C	++++	++++	Staining intensity in treated cells at later hours, comparable to that of control.
	T	++	++++	
Acid phosphatase	C	++++	++++	Recovery of AcPase staining intensity at later hours in treated cells, compar- able to control.
	T	+	+++	

PART II

Pattern of macromolecular syntheses in EMS exposed cells

A. Profile of DNA synthesis

B. Spectrum of RNA synthesis

C. Pattern of protein synthesis

To study the pattern of some macromolecular syntheses in EMS exposed amoebae, cells were either selected randomly from the mass culture or were picked up at defined phases of the cell cycle. Pattern of macromolecular syntheses was determined by incubating the cells with radioactive precursors followed by processing of the cell samples either for autoradiography or for Liquid Scintillation Spectrometry.

A. Profile of DNA synthesis:

Two types of experiments were carried out to investigate the pattern of DNA synthesis: (1) cells treated only once with the operative dose of EMS at ES phase followed by ^{3}H-Tdr incorporation at different periods of time upto 14 h; (2) cells treated with EMS at ES phase, MS phase and LS phase followed by immediate labelling with ^{3}H-Tdr.

The profile of DNA synthesis was investigated by labelling the control and the treated cells after mitosis with tritiated thymidine for 1 h through each point. Amoebae of desired age were selected, incubated with labelled precursor, and autoradiograms were prepared to analyse DNA synthesis in terms of counting the silver grains incorporated in the nuclei. In ^{3}H-thymidine incorporation study, sometimes a little higher cytoplasmic incorporation of the radioactivity was noted in the autoradiographic preparations of both control and treated cells. However, it never exceeded more than 13% of the total nuclear radioactivity. In one case cells

of desired age were labelled with ^{3}H-thymidine and processed for counting the radioactivity through Liquid Scintillation Spectrometer.

1. DNA synthesis in cells treated only at ES phase

^{3}H-thymidine incorporation study by autoradiography showed that the DNA synthesis in control A. indica was initiated immediately after mitosis as evident by the incorporation of labelled thymidine in the amoeba nuclei. The peak DNA synthesis was noted at MS phase i.e., 3½ h after division (Fig. 77) which was followed by a decline in the incorporation of tritiated thymidine into the nuclei at LS phase (5½ h after division). The pattern of DNA synthesis was, however, traced upto 14 h (EG_2II phase) after mitosis, and there had been a continual decrease in the incorporation of label upto 14 h. All the nuclei after 10 h continued to be labelled at a lower rate and accounted for ca. 10 to 13% of the activity when compared to the peak incorporation period (Fig. 75). DNase treatment removed ca. 98% of the nuclear grains. From the above observations, it was evident that S phase of A. indica in our laboratory condition occupied approximately first 7 h of the cell cycle, the rest of the period being considered as G_2 phase.

A drastic change in the pattern of DNA synthesis was noticed when the ES phase cells were treated with 0.5% EMS

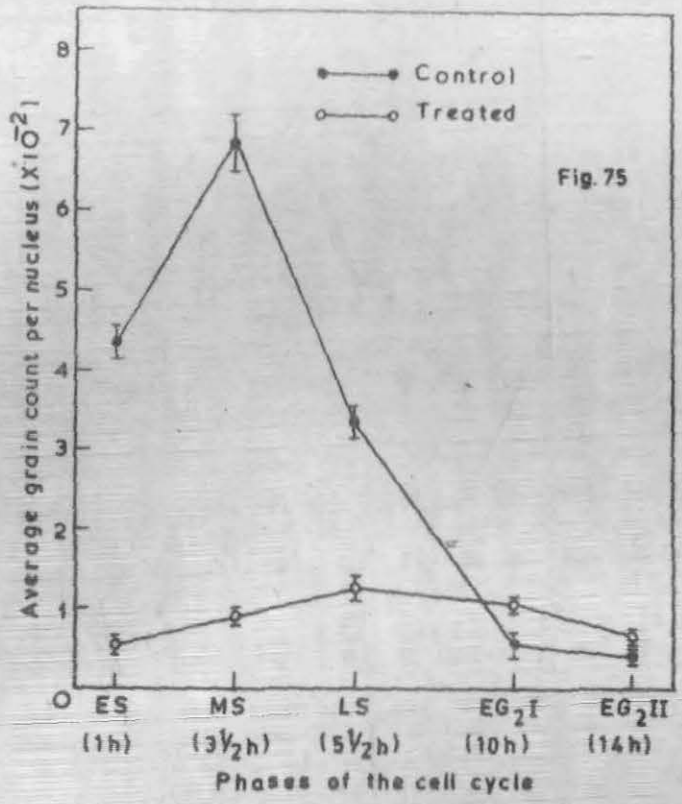
for 15 min followed by labelling the cells for 1 h with ^{3}H-thymidine through each point upto 14 h (EG_2II phase). A decline in the DNA synthesis was observed in these cells as compared to controls. Maximum depression in the incorporation of tritiated thymidine into the treated nuclei was noted immediately after treatment at ES phase, where the inhibition was found to be 88% when compared to its normal counterparts. However, ^{3}H-thymidine incorporation was noted to be somewhat increased from MS phase onwards when 87% depression could be detected at MS phase (Figs. 77 and 78), and 64% depression at LS phase nuclei as compared to the nuclei of control cells of the corresponding age. The peak of DNA synthesis was noted to be eliminated in treated cells where it showed maximum ^{3}H-thymidine incorporation at LS phase while in control cells the peak synthesis was observed at MS phase. A gradual depression in DNA synthesis was observed after LS phase, although, no significant change in the pattern of DNA synthesis could be detected between LS and EG_2I phase cells (Fig. 75). It was interesting to note that DNA synthesis was substantially prolonged upto EG_2I and EG_2II phases of the treated cells as evident by a low ^{3}H-thymidine incorporation into the nuclei of the treated amoebae between 10 to 14 h ages where DNA synthesis was found to be higher as compared to control cells of those age groups (Fig. 75). However, the cytoplasmic

grain incorporation was also noted to be relatively higher as compared to control cells of 10 to 14 h old age groups.

The pattern of ^{3}H-thymidine incorporation in the nuclei noted from autoradiographic preparations of the cells was also confirmed by the data obtained from counting the radioactivity in Liquid Scintillation Spectrometer. In control cells, DNA synthesis was found to be initiated immediately after mitosis and maximum incorporation of the labelled precursor was observed at around 3 h (MS phase) after cell division as displayed by a peak of incorporated tritiated thymidine. A decline in DNA synthesis was noticed between 4 to 6 h after division. Synthesis of DNA was found to be accelerated once again at ca. 7 h after division which had been shown by the appearance of a second peak of incorporated ^{3}H-thymidine at that hour, although, the amount of radioactivity counted at this point was found to be more or less similar as compared to 5 h old cells and no significant difference could be detected between the 5 h and 7 h old cells. However, no comparable autoradiographic preparation was made at that hour. From 7 h onwards there had been a decline in the synthesis of DNA and the synthetic pattern was followed upto 14 h where a much lower rate of tritiated thymidine incorporation was detected after 7 h (Fig. 76).

Fig. 75: The DNA synthetic patterns as revealed by autoradiograms of the control amoebae and of amoebae exposed to EMS at ES phase. Each point represents the average grain count of 20 to 25 nuclei from two separate experiments. Vertical bars indicate ± SEM.

Fig. 76: Pattern of ^{3}H-thymidine incorporation in control cells and in cells treated with EMS at ES phase. Cells labelled for 1 h through each point. Radioactivity was expressed as cpm/10 cells. Each point represents an average radioactivity of three separate experiments, containing 20 cells at each point. Vertical bars: ± SEM.


Control
Treated
Fig. 75
Average grain count per nucleus (X10⁻²)
0
1
2
3
4
5
6
7
8
ES
(1h)
MS
(3½h)
LS
(5½h)
EG2 I
(10h)
EG2 II
(14h)
Phases of the cell cycle

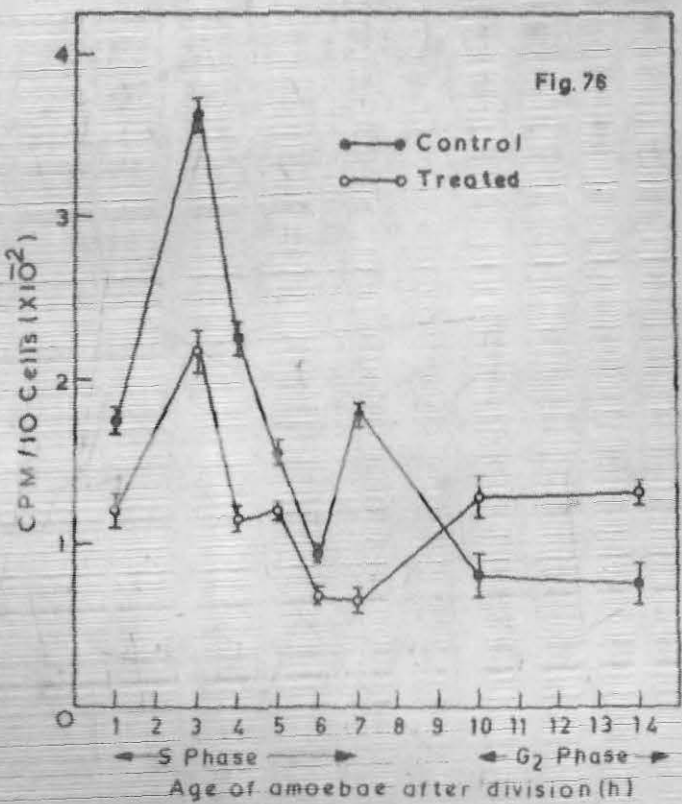


Fig. 76
Control
Treated
CPM/10 Cells (X10⁻²)
0
1
2
3
4
1 2 3 4 5 6 7 8 9 10 11 12 13 14
S Phase
G2 Phase
Age of amoebae after division (h)

Fig. 77: Autoradiograph of a control cell nucleus showing DNA synthesis at MS phase. Cell labelled with ^{3}H-thymidine, squashed and stained with Giemsa. X 2000

Fig. 78: Autoradiograph showing nucleus of a cell treated with EMS at ES phase and labelled with ^{3}H-thymidine through MS phase for 1 h, squashed and stained with Giemsa. X 2000

Fig. 79: Autoradiograph of the nucleus of a cell exposed to EMS at MS phase followed by incubation for 1 h with tritiated thymidine at MS phase, squashed and stained with Giemsa. X 2000

77
78
79

When the cells were exposed at ES phase with operative dose of EMS, a depression in the DNA synthetic activity could be noticed in these cells. In treated cells a differential pattern of DNA synthesis was observed as compared to control cells where one major peak of DNA synthesis could be found at around 3 h as revealed by counting the incorporated radioactivity by Liquid Scintillation Spectrometer. Incorporation of labelled precursor was found to be decreased from 3 h onwards while between 4 h to 5 h it showed more or less same pattern of incorporation. Decline in DNA synthesis was, however, found to be continued after 5 h and could be traced upto 7 h. An increase in the ^{3}H-thymidine incorporation was, however, noted between 10 to 14 h after treatment (Fig. 76).

2. Spectrum of DNA synthesis in cells treated separately at ES, MS and LS phases

When the cells were exposed to 0.5% EMS for 15 min at ES, MS and LS phases separately, an altered pattern of DNA synthesis was observed when the cells were labelled with ^{3}H-thymidine at those different phases immediately after the treatment (Fig. 80).

Cells treated at ES phase with EMS displayed a significant reduction in DNA synthetic activity as

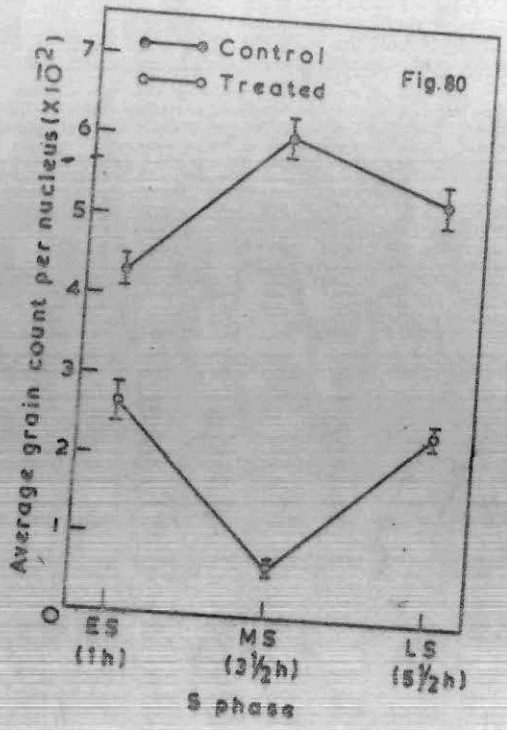
revealed by the presence of reduced number of silver grains in the nuclei where the depression was found to be 63% as compared to ES phase control cells. However, a drastic reduction in the pattern of DNA synthesis could be detected when the cells were exposed to EMS at MS phase where the depression in ^{3}H-thymidine incorporation into the nuclei was found to be 89% compared to MS phase control cells (Figs. 77 and 79). Cells treated at LS phase with EMS displayed appreciably less inhibition to DNA synthesis as compared to MS phase treated cells and the decline in ^{3}H-Tdr incorporation was noted to be 45% as compared to LS phase control cells (Fig. 80).

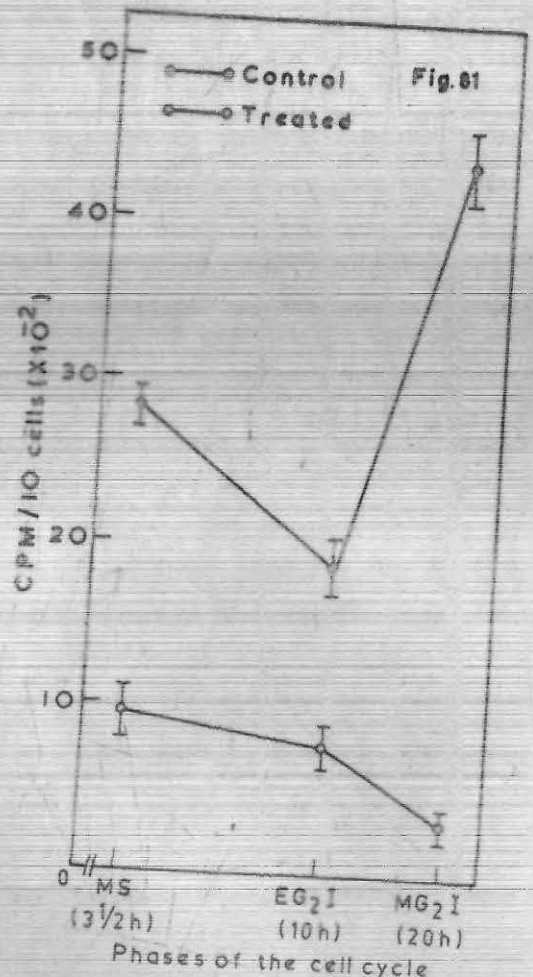
B. Spectrum of RNA Synthesis:

It was clear from earlier observations that the MS phase had been most sensitive to the action of EMS as regard to cell survival as well as DNA synthesis. It was decided to check the pattern of RNA synthesis in these MS phase treated cells. Consequently, RNA synthetic activity was assayed by investigating ^{3}H-uridine incorporation pattern of these cells at MS, EG_2I and MG_2I phases. Cells were labelled with ^{3}H-uridine for 1 h through each point and the amount of incorporated radioactivity was checked by Liquid Scintillation counting (Fig. 81).

Fig. 80: Autoradiographic grain count/data of the DNA synthesis in control cells and in cells treated with EMS separately at ES, MS and LS phases followed by labelled thymidine incorporation for 1 h through each phase. Each point represents an average grain count per 20 to 25 nuclei from two separate experiments. Vertical bars indicate ± SEM.

Fig. 81: Liquid Scintillation counting of ^{3}H-uridine incorporation in control cells and in cells treated with EMS at MS phase. The average count obtained from three separate experiments containing 20 cells at each point has been shown. Vertical bars: ± SEM.


Control
Treated
Fig.80
Average grain count per nucleus (X10⁻²)
0
1
2
3
4
5
6
7
ES
(1h)
MS
(3½h)
LS
(5½h)
S phase


Control
Treated
Fig.81
CPM/10 cells (X10⁻²)
0
10
20
30
40
50
MS
(3½h)
EG₂I
(10h)
MG₂I
(20h)
Phases of the cell cycle

In control cells, tritiated uridine incorporation was found to be higher at MG_2I phase as compared to MS and EG_2I phases. RNA synthesis was found to be lower at EG_2I phase than that of MS phase cells.

The profile of RNA synthesis was found to differ in cells exposed to EMS at MS phase followed by labelling the cells with ^3H-uridine at MS, EG_2I and MG_2I phases. A depression in the incorporation of labelled uridine into the total cellular RNAs of the cell was noted at all the above mentioned phases. Depression in the RNA synthetic activity was found tobe maximum at MG_2I phase as indicated by 92% decline in the incorporation of tritiated uridine compared to its normal counterpart. It was also noticed that immediately after EMS treatment at MS phase the RNA synthetic activity was substantially lowered, compared to control cells, as indicated by 66% depression in the incorporation of tritiated uridine (Fig. 81).

C. Pattern of Protein Synthesis:

The tritiated leucine incorporation pattern was investigated in the EMS exposed amoebae from asynchronous cell populations as well as in the cells from specific phases of the cell cycle. The labelled amino acid incorporation pattern of the mutagen exposed asynchronous

amoebae at various hours after treatment has been described first. The next section includes the cell cycle phase specific incorporation pattern of radioactive precursor by the treated cells as compared to control amoebae.

Protein synthesis in EMS exposed asynchronous cells

The pattern of protein synthesis in EMS treated asynchronous cells was investigated by autoradiographic analysis of labelled cells.

Autoradiographs of ^{3}H-leucine labelled cells displayed a fairly homogeneous distribution of silver grains throughout the cell. Autoradiograms of the tritiated leucine labelled control cells showed more or less linear increase in the incorporation of the labelled precursor into amoeba proteins between 0 to 24 h after the initiation of the experiment (Fig. 82).

When the cells were exposed to EMS an initial depression in the incorporated ^{3}H-leucine was noted which continued upto 12 h after treatment. However, an increase in the incorporation of labelled leucine could be detected at ca. 24 h after treatment where the protein synthetic ability of the cells has been found to be accelerated several folds and the synthetic activity was almost similar

Fig. 82 : Pattern of ^{3}H-leucine incorporation in control amoebae and in cells treated with EMS. Each point represents the mean of 25 to 30 cells. Vertical bars indicate ± SEM.

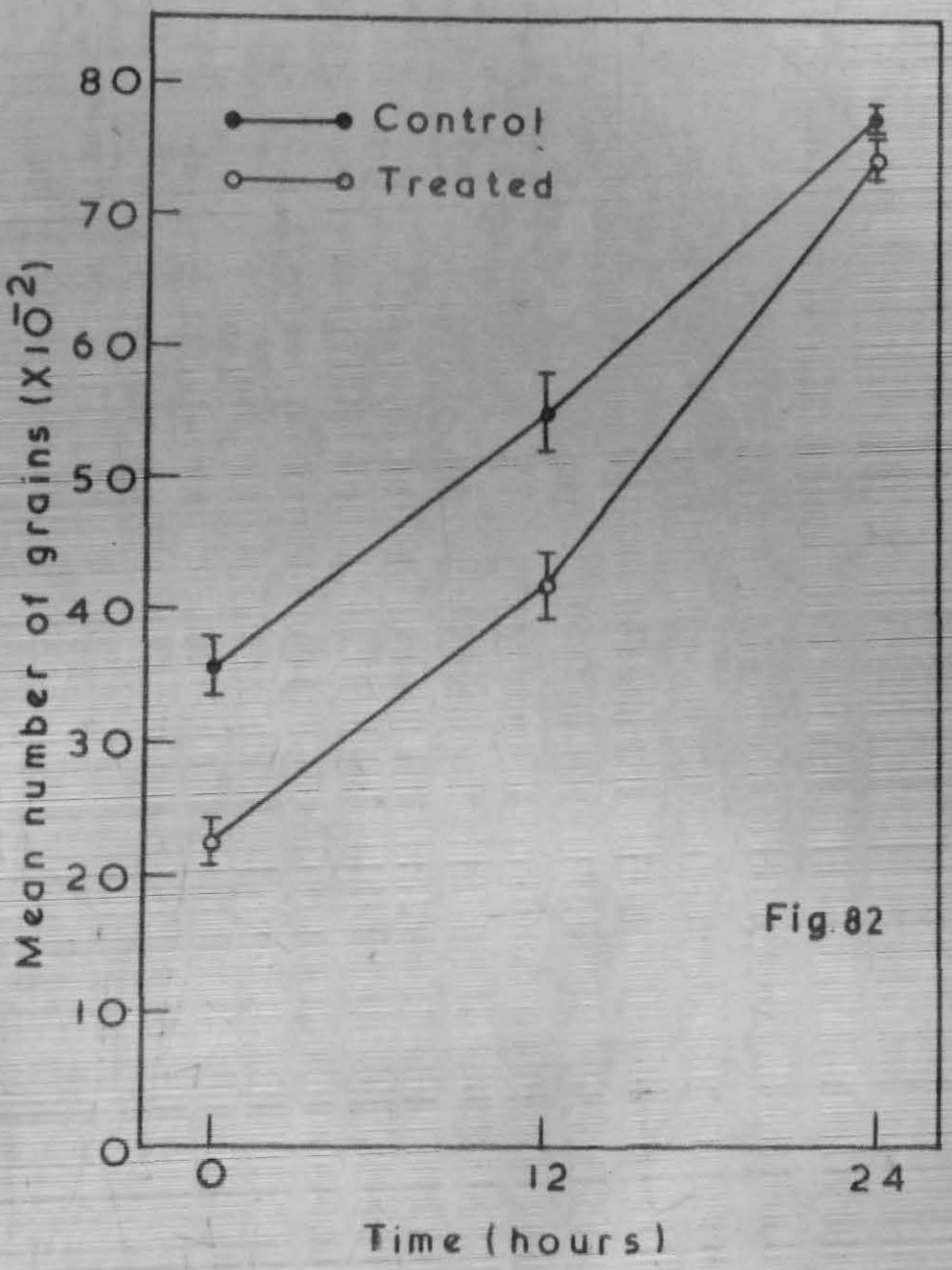

Control
Treated
Mean number of grains (X10⁻²)
80
70
60
50
40
30
20
10
0
0
12
24
Time (hours)

Fig. 82

to that of the control cells. Maximum depression to labelled leucine incorporation was monitored at 0 h after treatment where 36% depression could be detected as compared to control cells (Fig. 82).

Pattern of protein synthesis at defined phases of the cell cycle

Pattern of protein synthesis in control and treated cells was investigated by undertaking two separate experiments as follows: (1) cells once treated at ES phase with EMS and the pattern of incorporation of ^{3}H-leucine was followed through different phases of the cell cycle; (2) cells treated once at MS phase and incorporation of tritiated leucine was checked at different phases of the cell cycle thereafter.

Cells were labelled with ^{3}H-leucine and processed either for autoradiography or for Liquid Scintillation counting. Autoradiographic preparations of ^{3}H-leucine incorporated cells displayed a homogenous distribution of silver grains in the cytoplasm as well as on the nucleus.

(1) Protein synthesis in cells treated at ES phase

Autoradiographs of the tritiated leucine labelled control cells displayed a variable pattern of incorporation of the labelled precursor at different phases of the cell cycle. At ES phase a higher incorporation of tritiated

leucine into amoeba proteins was observed which was followed by a decline in the labelled precursor incorporation at MS and LS phases. The LS phase cells, however, showed less protein synthetic activity as measured by ^{3}H-leucine incorporation compared to other phases of the cell cycle. An enhanced incorporation of ^{3}H-leucine could be observed at EG_2 phase, which was followed by a decline in protein synthesis at MG_2I and MG_2II phases. Acceleration in the incorporation of the labelled leucine was noticed at MG_2III phase (Fig. 87) and the maximum protein synthetic activity could be detected at LG_2 phase which displayed the highest grain density.

When the ES phase cells were exposed to EMS the pattern of protein synthesis showed a significant difference from the control cells (Fig. 87). In ES phase treated cells the main peak of labelled precursor incorporation was observed at MS phase where 29% inhibition in the incorporation could be detected as compared to MS phase control cells. However, decline in the tritiated leucine incorporation was noticed immediately after treatment at ES phase which was found to be 65%. A gradual decline in the incorporation of ^{3}H-leucine was noticed at LS and EG_2 phases where the depression was found to be 62% and 89% respectively as compared to the control cells of same phases (Figs. 83 and 84). From the MG_2I phase onwards upto LG_2 phase the depression of protein synthesis

was noted to be between 53-63% as compared to control cells. In all the phases of the cell cycle there had been a significant depression in the labelled leucine incorporation in EMS treated cells as compared to controls (Fig. 87).

The pattern of ^{3}H-leucine incorporation throughout the cell cycle in control and in ES phase treated cells was further checked by counting the radioactivity in Liquid Scintillation Counter. In control cells, a higher incorporation of labelled leucine was detected at ES phase which was followed by a decline in ^{3}H-leucine incorporation at MS and LS phases. An acceleration to the incorporation of tritiated leucine into amoeba proteins was noticed from EG_2 phase onwards and a peak of protein synthetic activity was observed at MG_2I phase which once again followed a decline in the protein synthetic activity at MG_2II phase. However, ^{3}H-leucine incorporation was found to be accelerated from MG_2III phase onwards (Fig. 88).

Cells treated at ES phase showed a continuous depression in ^{3}H-leucine incorporation throughout the cell cycle. In treated cells a small peak of tritiated leucine incorporation was detected at MS phase where the depression was found to be only 23% when compared to its normal sequence. Maximum depression in protein synthesis was, however, observed

at EG_2 phase as evident by 92% decline in ^3H-leucine incorporation, compared to control cells. A slow rate of increase in labelled amino acid incorporation was noted from MG_2I phase onwards and could be traced upto LG_2 phase. Immediately after treatment at ES phase, cells showed a substantial inhibition in tritiated leucine incorporation where the decline was found to be 83% in comparison to control cells (Fig. 88).

2. Protein synthesis in cells treated at MS phase

When the MS phase cells were treated with EMS, a completely different pattern of protein synthesis was observed in them. In control cells more or less the same pattern of ^3H-leucine incorporation was observed as mentioned earlier.

Autoradiographs of the cells exposed to EMS at MS phase displayed a depression to protein synthesis immediately after treatment where decline in the amount of incorporated labelled leucine was found to be 64% as compared to MS phase control cells (Figs. 85 and 86). However, an increase in the amount of tritiated leucine incorporation in the treated amoeba proteins could be noticed at LS and EG_2 phases where no significant difference in the profile of protein synthesis was noted when compared to controls. A decline in ^3H-leucine incorporation in treated

Fig. 83: Autoradiograph of a EG_2 phase control cell labelled with ^3H-leucine for 1 h at EG_2 phase, squashed and stained with Giemsa. X 640

Fig. 84: Autoradiograph of a EG_2 phase cell treated at ES phase with EMS, incorporated with ^3H-leucine for 1 h at EG_2 phase and squashed, showing appreciably less grain density as compared to control (Fig. 83). Cell stained with Giemsa. X 640

Fig. 85: Autoradiograph of a MS phase control cell labelled with ^3H-leucine for 1 h at MS phase, squashed and stained with Giemsa. X 400

Fig. 86: Autoradiograph of a cell treated with EMS at MS phase and labelled immediately after treatment for 1 h with ^3H-leucine, squashed and stained with Giemsa. Note less grain density as compared to control (Fig. 85). X 640

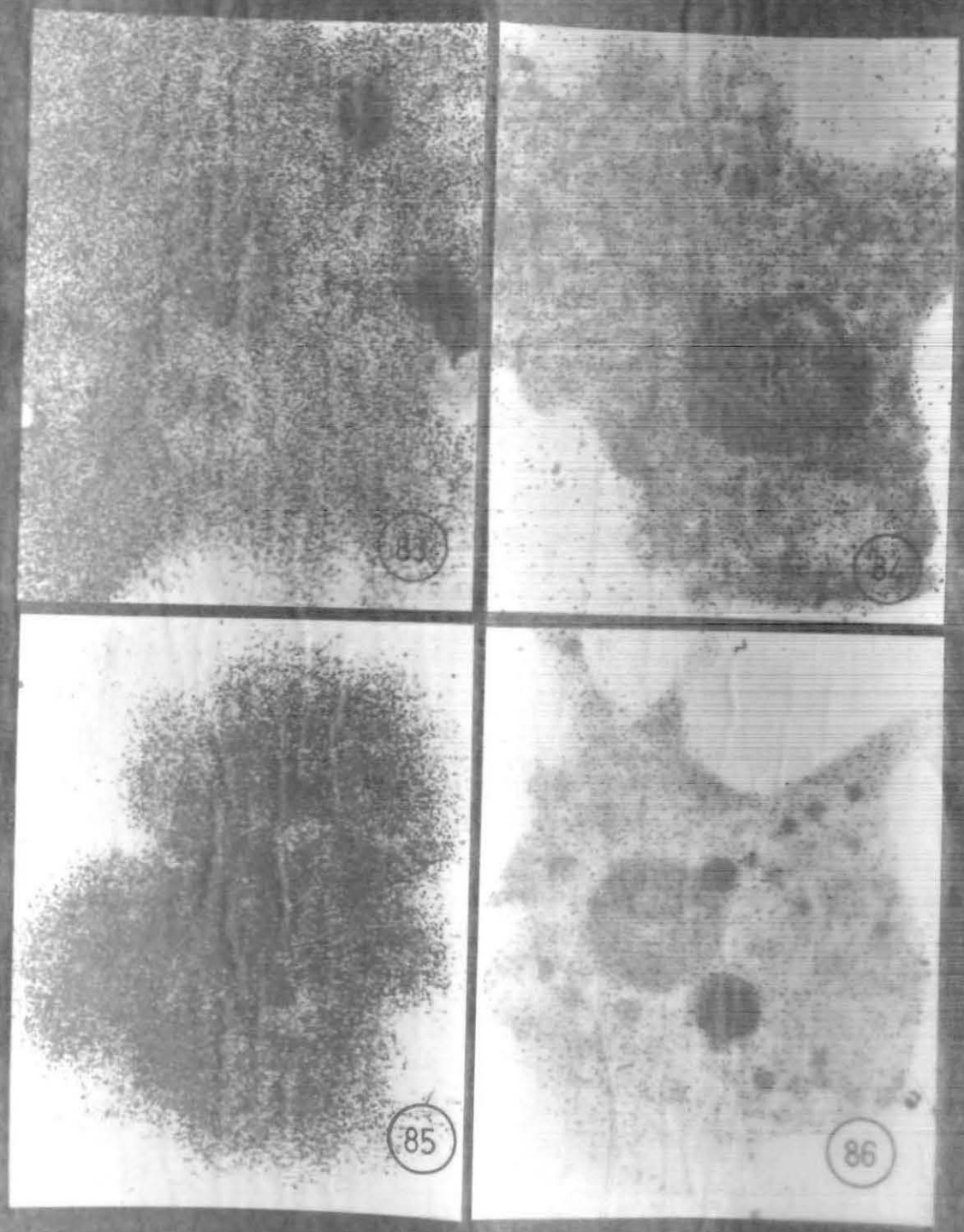

83
84
85
86

Fig. 87: Pattern of ^{3}H-leucine incorporation in control cells and in cells treated with EMS at ES phase. Each point represents the mean grain count of 20 to 25 cells. Vertical bars indicate ± SEM.

Fig. 88: Liquid Scintillation counting pattern of ^{3}H-leucine incorporation in control cells and in cells treated with EMS at ES phase. Radioactivity counted from different age groups of cells shows an average count obtained from 3 separate experiments containing 20 cells at each point. Vertical bars: ± SEM.

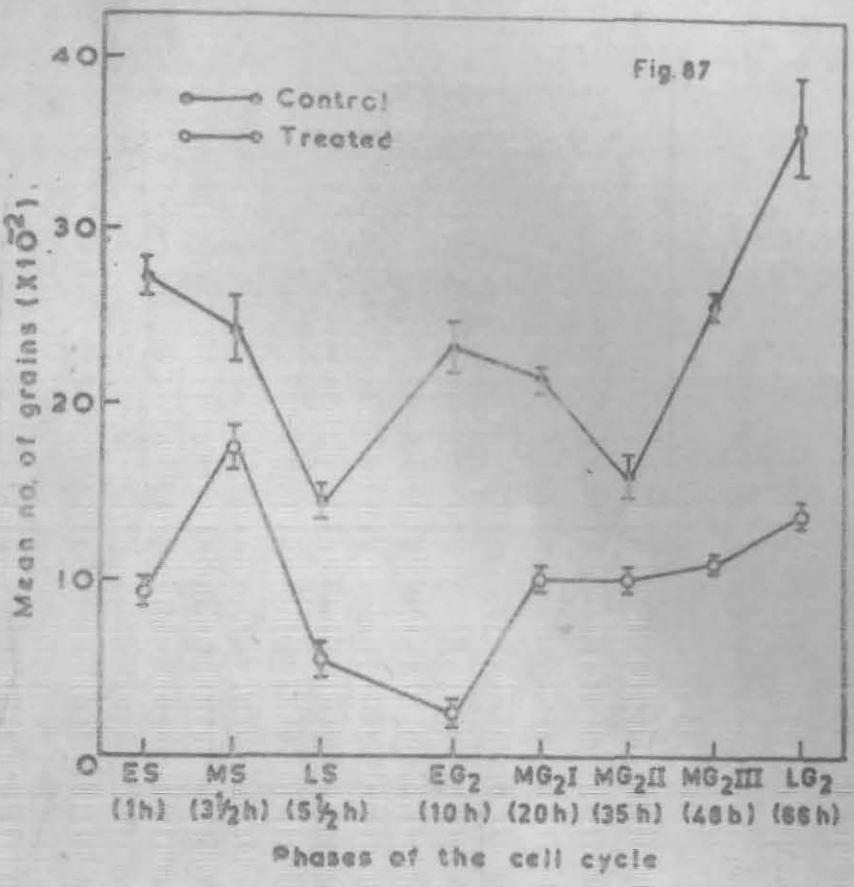


Fig. 87
Control
Treated
Mean no. of grains (X10^{-2})
40
30
20
10
0
ES (1h)
MS (3½h)
LS (5½h)
EG$_2$ (10h)
MG$_2$I (20h)
MG$_2$II (35h)
MG$_2$III (48h)
LG$_2$ (66h)
Phases of the cell cycle

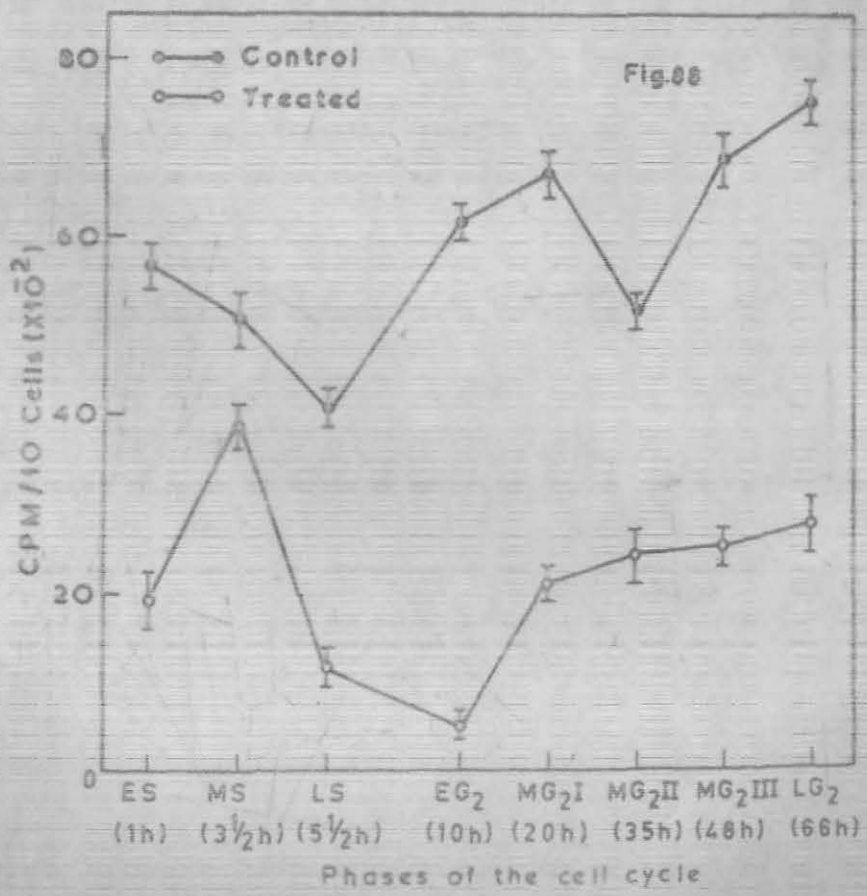


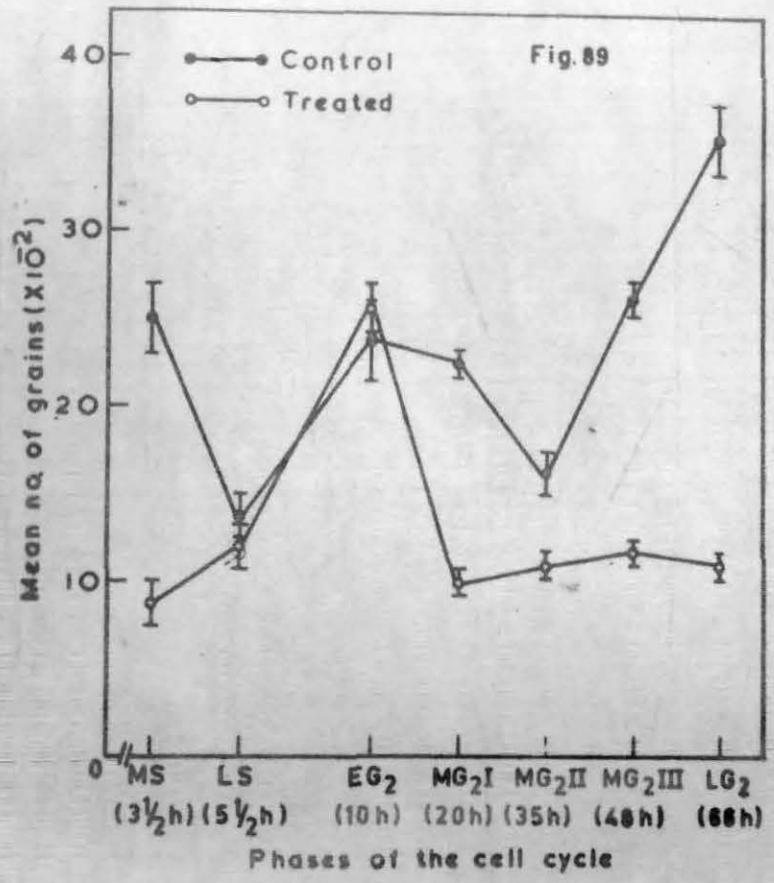
Fig. 88
Control
Treated
CPM/10 Cells (X10^{-2})
80
60
40
20
0
ES (1h)
MS (3½h)
LS (5½h)
EG$_2$ (10h)
MG$_2$I (20h)
MG$_2$II (35h)
MG$_2$III (48h)
LG$_2$ (66h)
Phases of the cell cycle

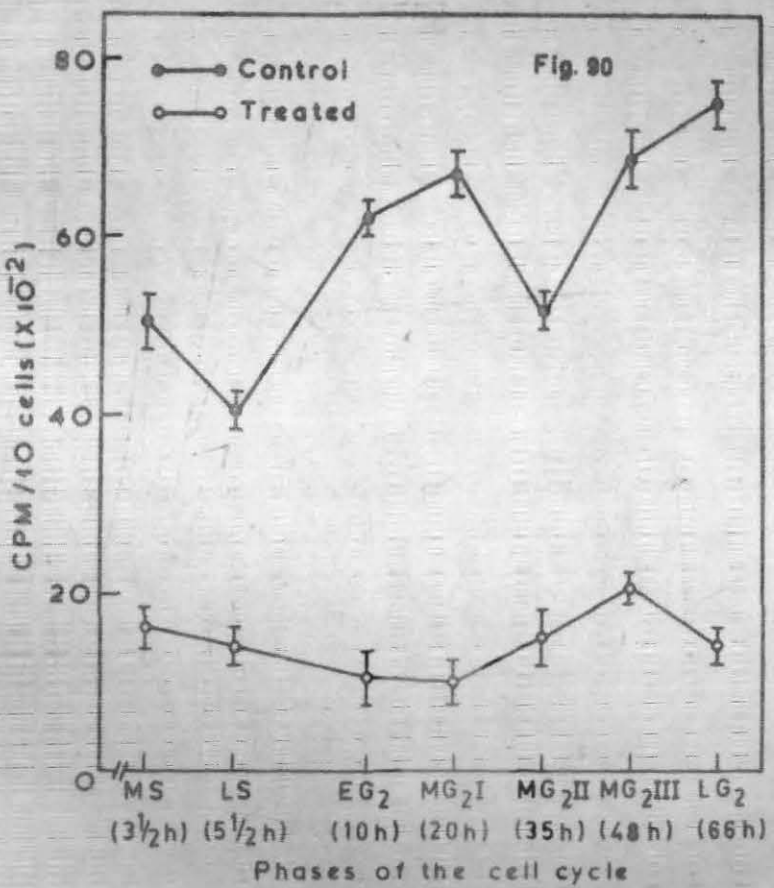
cells was observed from MG_2I phase onwards and could be followed upto LG_2 phase where a substantially lower level of synthetic activity could be detected throughout (Fig. 89)

The patterns of ^3H-leucine incorporation of control and MS phase treated cells, as revealed by autoradiographic data, were also checked by counting the radioactivity of incorporated labelled leucine by Liquid Scintillation Spectrometer. The control cells showed more or less the same profile of labelled leucine incorporation as described earlier. A completely different pattern of tritiated leucine incorporation was noted in MS phase treated cells. There had been a continuous depression in the protein synthetic activity throughout the cell cycle in treated cells. Maximum depression in the incorporation of ^3H-leucine was observed at MG_2I phase which was evident by 85% decline in the labelled precursor incorporation as compared to control cells. Immediately after EMS treatment at MS phase the depression in ^3H-leucine incorporation was found to be 68% and thereafter there had been a gradual decline in tritiated leucine incorporation upto MG_2I phase. However, a slow rate of increase in the incorporation of ^3H-leucine was noted from MG_2II phase and a small increase in the protein synthetic activity was noticed at MG_2III phase (Fig. 90).

Fig. 89: Profile of ^{3}H-leucine incorporation in control cells and in cells exposed to EMS at MS phase. Each point represents the mean grain count of 22-28 cells. Vertical bars: ± SEM.

Fig. 90: Liquid Scintillation counting pattern of ^{3}H-leucine incorporation in control cells and in cells treated with EMS at MS phase. Radioactivity counted from different age groups of cells shows an average count obtained from 3 separate experiments, involving 20 cells at each ppoint. Vertical bars: ± SEM.


Control
Treated
Fig. 89
Mean no. of grains (X10⁻²)
40
30
20
10
0
MS
LS
EG2
MG2I
MG2II
MG2III
LG2
(3½h)
(5½h)
(10h)
(20h)
(35h)
(48h)
(66h)
Phases of the cell cycle


Control
Treated
Fig. 90
CPM/10 cells (X10⁻²)
80
60
40
20
0
MS
LS
EG2
MG2I
MG2II
MG2III
LG2
(3½h)
(5½h)
(10h)
(20h)
(35h)
(48h)
(66h)
Phases of the cell cycle

PART III

Isolation of mini mutant and its characteristics

A. Origin of mini amoeba

B. Frequency of mini mutant production

C. Clone formation of mini mutant

D. Structural analysis of mini amoeba

E. Cytochemical studies

F. Pattern of macromolecular syntheses

G. Duration of different phases of the cell cycle

It was possible to induce mutation in A. indica by treatment with the chemical mutagen, EMS. A 'Mini' amoeba mutant was found which differed visibly from the parental strain in such a way that the individual amoeba could be recognized and change in its transformation from the normal cell types could be traced.

A. Origin of 'mini' amoebae:

Healthy and attached amoebae starved for 18 h were randomly selected from the culture dishes and exposed to 0.5% EMS for 15 min. Treated cells were washed several times with amoeba medium to remove the trace of mutagen and fed with freshly harvested Tetrahymenae half an hour after the treatment. The treated cells were examined at 1 h interval to monitor any detectable morphological alteration. It was noticed that between 30-34 h after treatment, some of the treated cells were considerably of smaller size than the other treated cells and they were designated as 'Mini' cells hereafter. These cells were collected and kept separately in syracuse watch glasses for further studies. The cell division in 'Mini' cells was found to be delayed by about six days after its origin. Once the mini amoebae began to divide it was observed that between 5-6 division cycles a clone was formed of mini amoebae of approximately same sizes

which were smaller than normal A. indica. However, ca. 40% mini cells were found to be lethal within 3-5 days after their origin and among the viable mini cells ca. 88% were found to revert back to normal sized cells after about two months from the date of its origin. Only ca. 12% of the surviving mini cells were found to form a clone of approximately equal sized cells and ultimately emerged as a stable clone of mini cells. Some 'Intermediate' sized cells also appeared in the treated cells' culture but none of these cells were able to maintain their size. These cells were found to be smaller than normal cells and larger than mini cells and were found to revert back to the normal size within 6-8 generations.

A more critical study was undertaken to find out the particular stage(s) of the cell cycle responsible for the production of mini mutant. Synchronised cells were exposed at 1 h intervals with 0.5% EMS for 15 min throughout the cell cycle including the mitotic cells. It was noticed that when the ES phase (1 h after division) cells were treated with operative dose of EMS these 'Mini' cells appeared at around 36 ± 3 h after the treatment (Fig. 91).

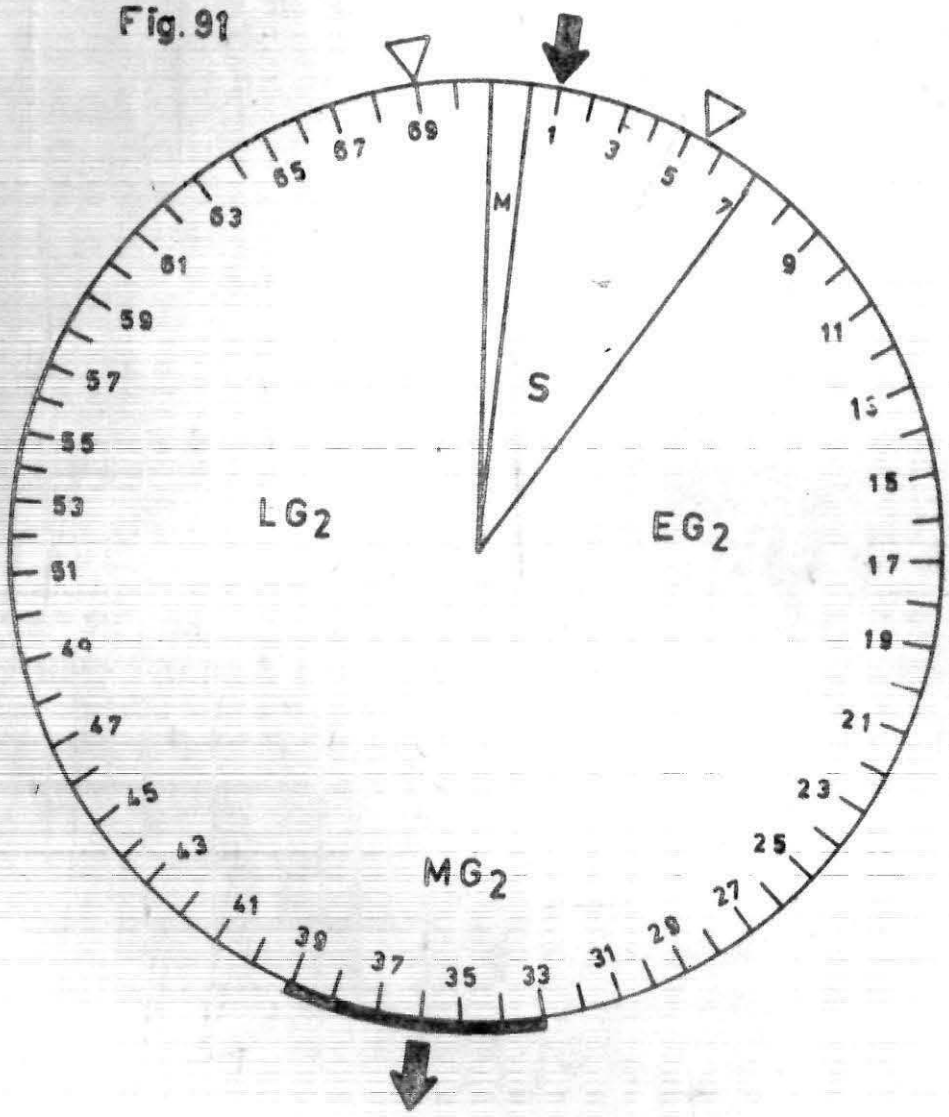
Earlier to this origin of 'Mini' cells which was encountered around 36 ± 3 h after the treatment of ES phase

cells with EMS, a striking change in the treated cells' nuclei could be observed in several cases. When the periodic samples of these cells were prepared for light and electron microscopy, it was found that in several cases there had been the occurrence of sprouting out of the nuclear material around 12 to 14 h after the treatment. More than one such budding off of the nuclear material could be seen in some cases. Presumably, a portion(s) of the nuclear substance which had come out became reorganised to form small and discrete nuclear body with prominent nucleoli (Figs. 92, 93 and 94).

In several cases the emergence of 'Mini' cells could be noticed from the treated cells ca. around 36 h after treatment of the cells at ES phase. In cytological preparations it was observed that these 'Mini' cells emerged out from the treated cells containing a comparatively smaller nucleus along with some part of cytoplasm (Fig. 95). These 'Mini' cells were ultimately found to be detached from the treated cells by cytoplasmic fission and later by division multiplied to form a clone of 'Mini' cells. A division delay of 5-6 days was noticed after its origin from the ES phase treated cells, although, once the division was initiated the mini cells produced the cells of almost equal sizes and formed a stable clone. However, upto three months

Fig. 91: A time model showing the appearance of mini amoeba mutant after treating the ES phase cells with 0.5% EMS for 15 min. Experiments involved the treatment of amoebae at 1 h intervals through the cell cycle. Black arrow on the top indicates point of mutagen exposure which leads to the appearance of stable mini cells at around 36 ± 3 h after treatment (black arrow at the bottom). Arrow heads indicate the points of mutagen treatment which led to the production of non-viable mini cells. Numbers within the circle denote hours.

Fig. 91


69
1
3
5
7
9
11
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39
41
43
45
47
49
51
53
55
57
59
61
63
65
67
M
S
LG2
EG2
MG2

Fig. 92: Cell treated with 0.5% EMS for 15 min at ES phase, showing sprouting out of nuclear material (arrows) at around 14 h after treatment. Squashed cell stained with Giemsa. X 1600

Fig. 93: Thin section of a cell treated with EMS at ES phase. Cell fixed at ca. 14 h after the treatment, stained with toluidine blue. Note three nuclei (arrows) with distinct nucleoli. Fv, food vacuole. X 1600

Fig. 94: Electron micrograph of a portion of ES phase treated cell fixed around 14 h after treatment, showing the cell nucleus (n1), a mini nucleus (n2) as well as a portion of another nuclear element (n3). X 4500

Fig. 95: Possible origin of a mini amoeba cell from an A. indica treated at ES phase. Cell fixed at ca. 36 h after treatment. Note binucleate parental treated cell and the mini cell with considerably smaller nucleus. Arrow indicates point of cell detachment and an almost completed process of cytoplasmic fission. X 252

18
13
n1
n2

after its production the cell cycle time was found to vary considerably. Mini cells were also found to originate when the cells were treated with EMS at LS (5½ h after mitosis) and LG_2 (69 h after division) phases and were noticed to appear in the culture at 69 ± 2 h and 72 ± 3 h respectively of the cell cycle after treatment. However, the mini cells originated from these phases of the cell cycle were found to be non-viable as they died within 15 to 20 days after their origin.

B. Frequency of mini mutant production :

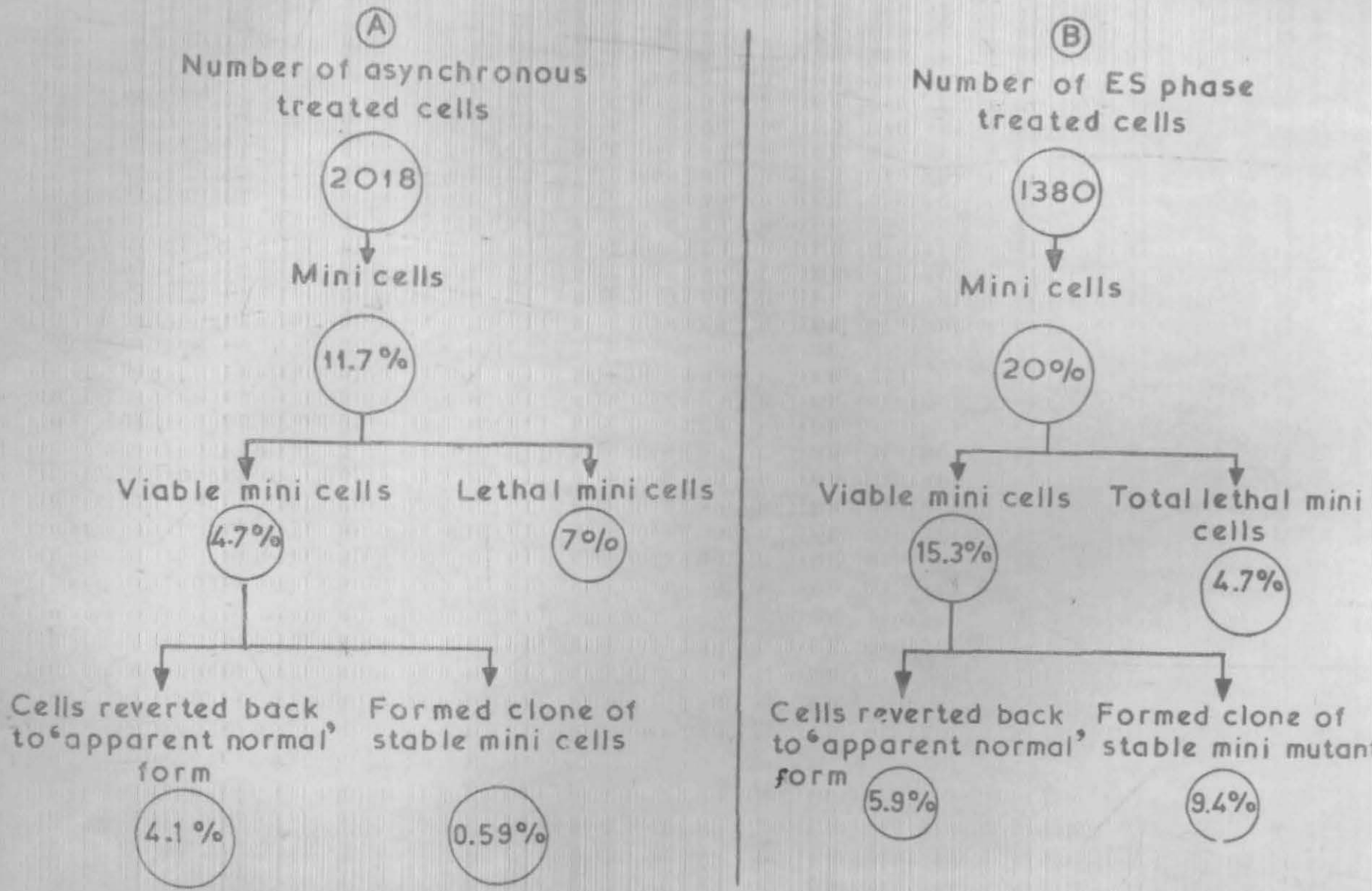
Frequency of mini mutant production varied between the mutants obtained from asynchronous cells and the mutants which emerged after treating the ES phase cells with operative dose of EMS. When 2018 asynchronous cells from 36 separate experiments, were treated with 0.5% EMS, approximately 12% mini cells were obtained, out of which some mini cells were found to be non-viable while among the viable ones a large number of cells reverted and appeared to be 'apparent normal' in size and the rest of the mini amoeba cells formed a stable clone. The frequency of the production of mini mutant amoeba was in the order of ca. 0.6% (Fig. 96A). These mini cells were found to be quite stable and had already crossed more than 600 generations in our laboratory

without any loss of mutant characters (see also Part I, "Variant cell production").

The frequency in the production of mini amoeba mutant varied when they originated from ES phase treated cells. When 1380 number of ES phase cells from 39 separate experiments, were treated with operative dose of EMS only 20% mini cells were found to originate from those treated cells among which ca. 2% cells were found to be lethal after 15-20 days from the time of their origin and ca. 6% cells reverted back to the 'apparent normal' form after about two months from the time of its origin. However, ca. 2.6% cell death occurred within five months after the origin of these mini cells. The rest of the mini cells were found to form a clone of mini amoeba mutants which had already crossed more than 650 generations ~~with~~ maintaining the mutant characteristics intact. The frequency of mini mutant production was in the order of 9.4% (Fig. 96B), which was found to be significantly higher as compared to the frequency of mutants obtained from asynchronous treated cells. No discernible difference could be noticed among the mini cells originated from asynchronous cells and ES phase treated cells. So, hereafter description will be restricted to the mini amoebae originated from ES phase treated cells.

Fig. 96: Frequency of stable mini mutant production in asynchronous cells treated with 0.5% EMS for 15 min.(A) and in cells treated at ES phase with EMS(B).

Fig. 96


A
Number of asynchronous treated cells
2018
Mini cells
11.7%
Viable mini cells
Lethal mini cells
4.7%
7%
Cells reverted back to 'apparent normal' form
Formed clone of stable mini cells
4.1%
0.59%
B
Number of ES phase treated cells
1380
Mini cells
20%
Viable mini cells
Total lethal mini cells
15.3%
4.7%
Cells reverted back to 'apparent normal' form
Formed clone of stable mini mutant
5.9%
9.4%

C. Clone formation of Mini mutant:

Stable 'Mini' mutants originated from ES phase treated cells were cloned by a capillary cloning technique of Ord (1979) after minor modifications. The detailed technique followed here has already been discussed elsewhere (vide page no. 23). A clone was formed from a single mini mutant which was introduced into a capillary tube along with modified Chalkley's medium containing Chilomonas as food and allowed to divide. From this first clonal culture 30-35 cells were obtained which were once again subjected to capillary cloning and ultimately 500-600 cells were obtained from the clone formed from a single cell. These cells were then allowed to grow in petriplates with modified Chalkley's medium and Chilomonas was used as food organism. Modified Chalkley's medium was found to be more suitable than Prescott and Carrier's amoeba medium for two reasons: (1) the generation time of mini cells cultured in modified Chalkley's medium was found to be 20 ± 2 h whereas the cells cultured in Prescott and Carrier's medium showed a cell duplication time of 29 ± 4 h; (2) mini cells appeared quite healthy when cultured in modified Chalkley's medium as compared to the amoeba medium of Prescott and Carrier.

Chilomonas feeding was found to be most suitable as the cells did not survive after they were fed with Tetrahymena.

D. Structural changes in mini amoebae:

Cell size and nuclear diameter

The average cell size of mini cells differed greatly when compared to control A. indica. The cell size was measured in living cells as well as in flattened and fixed cell preparations (Figs. 97 and 98). Amoeba proteus was also measured for comparison as it represents a widely known species of amoeba.

The nuclear diameter was found to be quite smaller in size when compared to control A. indica and A. proteus Fig. 98).

The measurements on the random sample of all three types of cells are given in the table below:

TABLE 5

Cell Types	Average cell size (μm ± SD)	Nuclear diameter (μm ± SD)
A. proteus	536 ± 38	49 ± 3.2
A. indica	397 ± 11	33 ± 2.5
Mini mutant	224 ± 7	22 ± 1.8

Pseudopodia and feeding activity

The average number of pseudopodia formation per minute at different phases of the cell cycle of mini cells

Fig. 97: Living cell photomicrograph shows size difference among A. proteus (top), mini mutant (middle) and A. indica (lower). X 256

Fig. 98: Squashed and Giemsa stained preparations of A. proteus, mini cells and A. indica showing nuclear size difference. P, A. proteus; M, mini cells; I, A. indica; n, nucleus. X 92

Fig. 99: Squashed and Giemsa stained mini cells' preparation showing only one ingested Tetrahymena (t) in each cell. X 92

Fig.100: A squashed mini cell showing formation of more than 20 phagosomes after Chilomonas feeding. Note cell sample prepared for autoradiography. C, Chilomonas. X 400

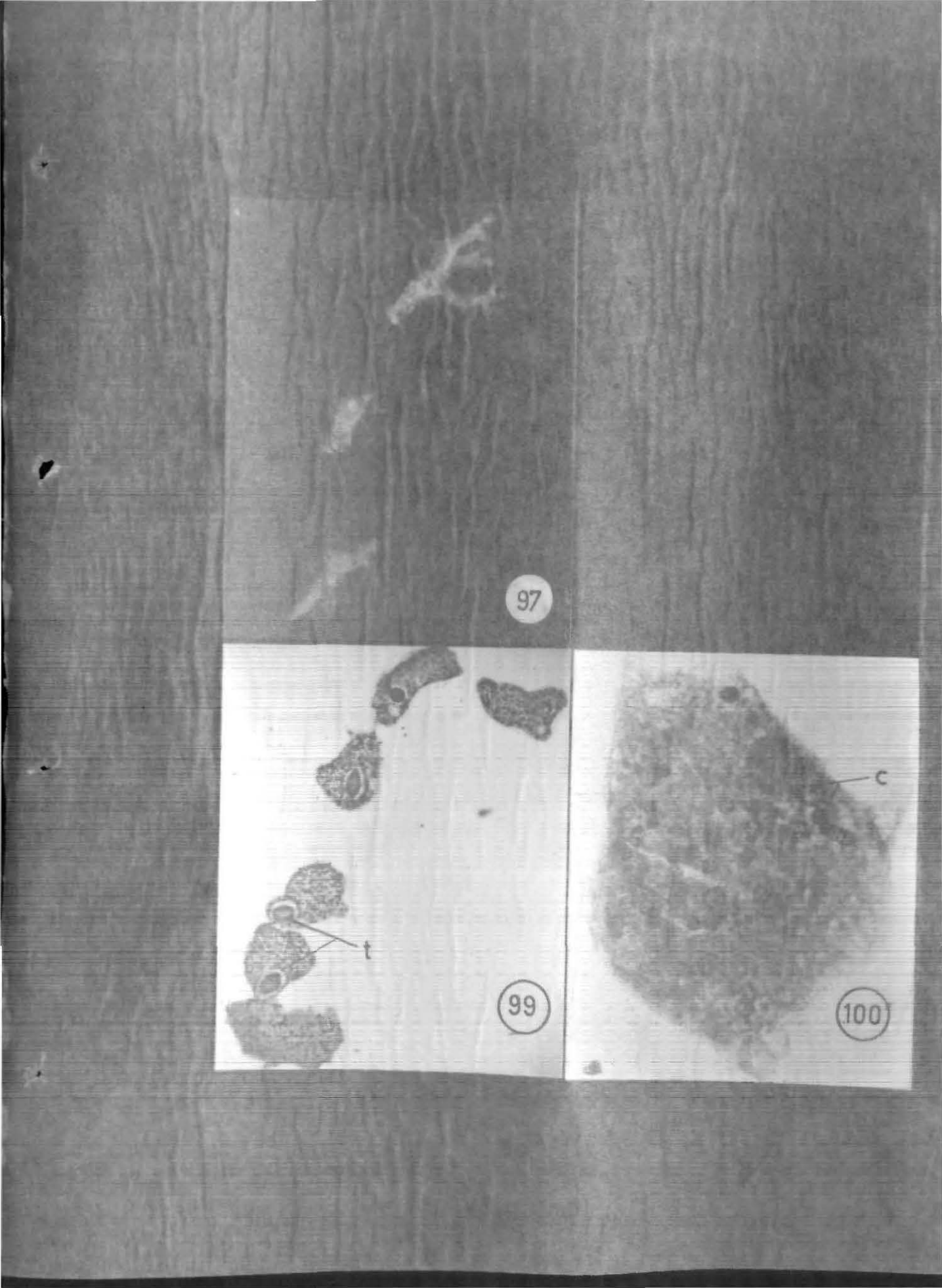

97
t
99
c
100

Fig. 101: Pseudopodial throw per minute at different phases of the cell cycle of mini cells.

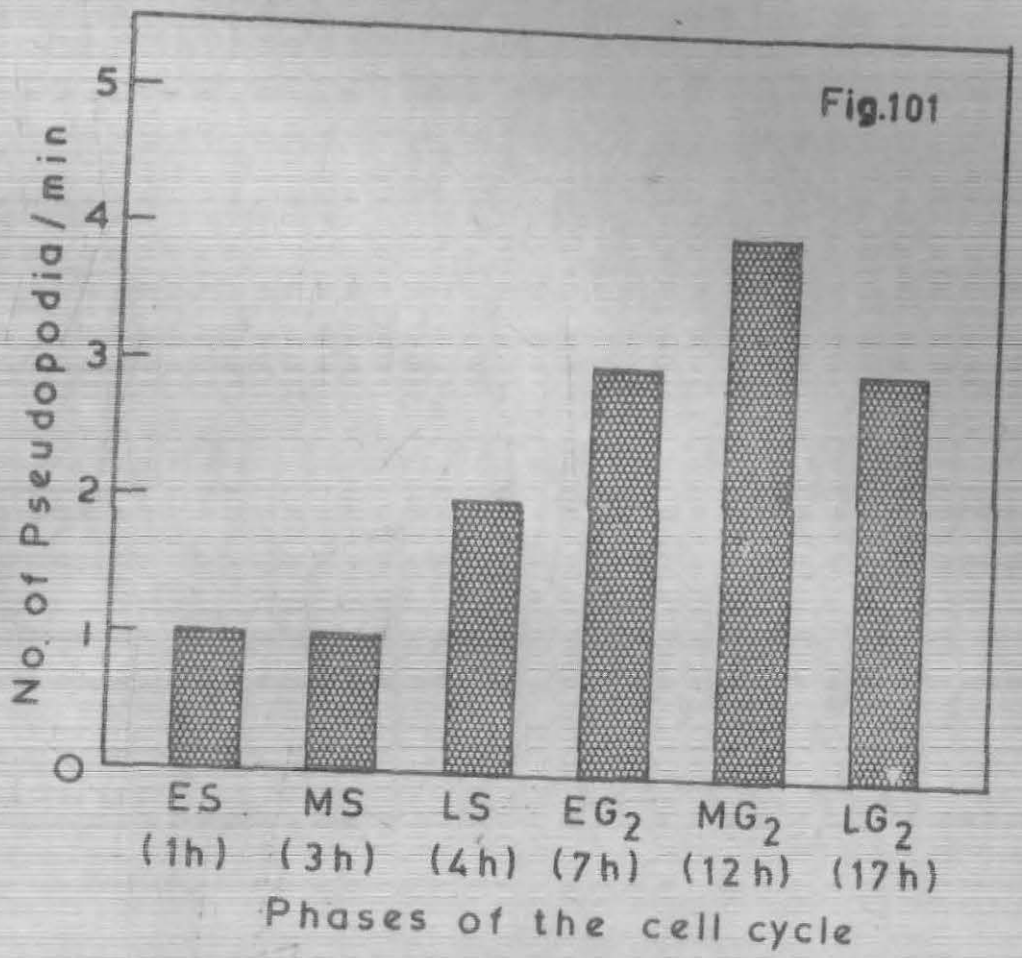


Fig.101
No. of Pseudopodia/min
0
1
2
3
4
5
ES (1h)
MS (3h)
LS (4h)
EG2 (7h)
MG2 (12h)
LG2 (17h)
Phases of the cell cycle

was found to be less when compared to control A. indica (Figs. 26 and 101). The pseudopodia of the majority of mini cells appeared fine and long with a more or less pointed tip, whereas in control A. indica it was roughly cylindrical with a hemispherical tip.

The cloned amoeba mutants were supplied with Tetrahymena as their food. It was noticed that the mini cells could capture only one Tetrahymena at a time, although, the food was available to them ad lib: (Fig. 99). Moreover, the mini cells became lethal after being fed with Tetrahymena for more than three days. It was noted that the mini cells could capture more than 20 Chilomonas at a time (Fig. 100). No lethality was observed in cells fed with Chilomonas and they were found to remain quite healthy after feeding. From the above observations, it was found suitable to feed the mini cells with Chilomonas. The control. A. indica, however, could feed both on Tetrahymena and Chilomonas equally well.

Cell membrane

The cell membrane of the mini amoeba was found to have developed a great deal of stickiness to the surface as compared to control A. indica. The increased adherence to to the surface was evident while picking up the cells from

the surface by the braking pipette, which often resulted in rupture of the cell membrane. This increased stickiness has been found to be a constant feature of mini cells.

Scanning electron microscopic view of the surface area of the plasma membrane of mini amoeba mutant showed distinct differences in surface structure as compared to A. indica control cells. The cell surface of mini cells displayed a completely uneven surface. Several ridges were found on the cell surface which were interconnected with each other. Several pits or concavities were noticed on the greater parts of the cell surface. Blebs were found to be scattered on the cell surface (Fig. 102). Magnified SEM view of the greater area of the plasma membrane showed the formation of several mini beads on the cell surface which appeared as very small projections on the cell surface. Several blebbed areas on the cell surface were also investigated on the cell membrane (Fig. 103). Magnified SEM view of the blebbed zones of the mini cells showed some striations in these areas. The blebs were found to be interconnected by ridges and the blebs appeared to contain several smooth protuberances on its surface. The cell surface around the blebs contained several mini beads of unequal sizes (Fig. 105). A highly magnified view of a cell surface bleb showed several larger beads on its surface. These beads contained a conical projection

Fig. 102: Scanning electron micrograph of the surface of a mini mutant showing appearance of blebs, ridges and concavities. X 900

Fig. 103: Scanning electron micrograph of a portion of mini cell surface with blebs. X 3307

Fig. 104: Scanning electron micrograph of a portion of A. indica control cell surface showing several protuberances. X 5775

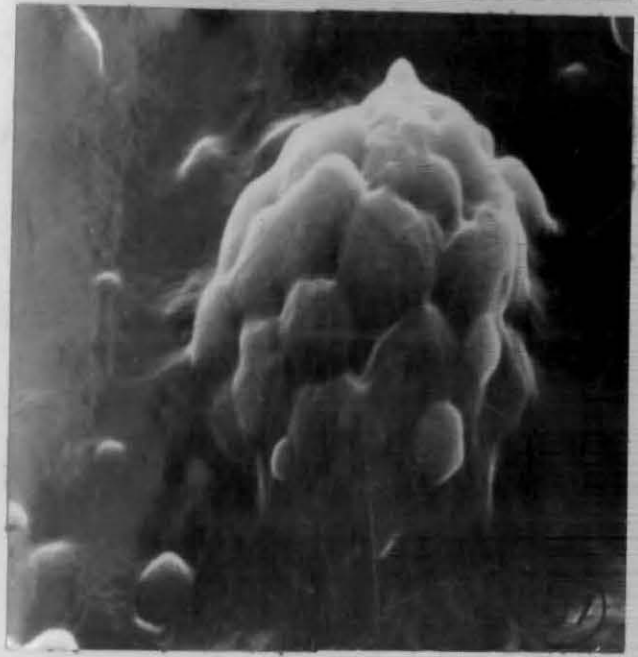
Fig. 105: Scanning electron micrograph of a magnified portion of the cell surface of mini amoeba mutant. Note blebs. X 9450

102

103

Fig. 106: Scanning electron micrograph of a highly magnified portion of a control *A. indica* cell surface showing almost smooth out-line of the bleb. X 20250

Fig. 107: Scanning electron micrograph of a highly magnified portion of the cell surface of mini cell. Note a bleb with several protuberances on its surface. X 22500



leading away from the cell surface. Formation of several large beads of the blebs led to the appearance of angular depressions in between the beads (Fig. 107).

In control A. indica, as described earlier (See Part I, "SEM observations on cell surface") the surface was mostly covered by protuberances which were absent in mini cells' surface. The ridges and mini beads which were abundant in mini cells were very rarely visible in control A. indica. Pits or concavities which were observed on mini cells' surface were absent in control A. indica. The structure of the blebs also differed greatly from as mini cells (Figs. 10, 102, 104, 105, 106 and 107)/they did not have the beaded appearance like that of mini cells.

Light microscopic observations

The nucleus of mini cells appeared almost spherical in shape with dense nuclear material (Fig. 108). Thin section of the nucleus showed that the nucleoli were arranged mostly in a single row just beneath the nuclear envelope (Fig. 111). Approximately 4% binucleate mini cells were also noticed when these cells were randomly observed in cytological preparation (Fig. 109).

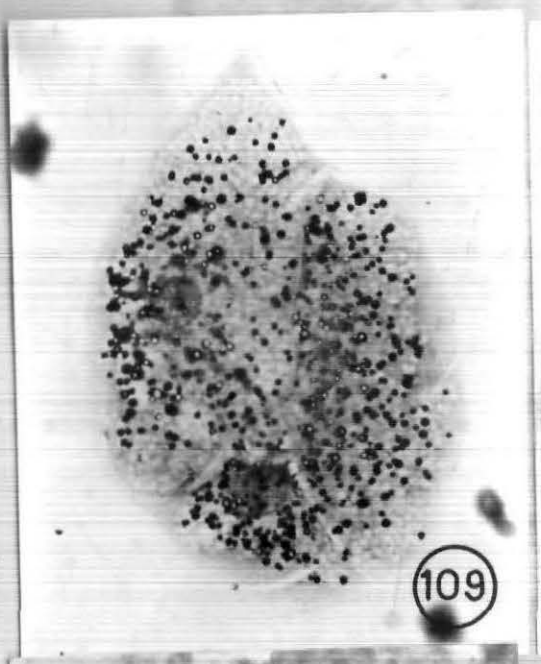
Mini cell's cytoplasm was found to be nonhomogeneous in nature (Fig. 108). Vacuolated condition of the endoplasm

Fig. 108: Squashed and Giemsa stained preparation of a mini amoeba showing nucleus (n) and crystals(c) of round shape. X 360

Fig. 109: Squashed and Giemsa stained preparation of a binucleate mini cells. X 640

Fig. 110: Thin section of a mini amoeba mutant showing vacuolated endoplasm and homogeneous ectoplasm. Section stained with toluidine blue. X 2000

Fig. 111: Toluidine blue stained thin section of a mini cell showing nuclear structure. n, nucleoli. X 2000



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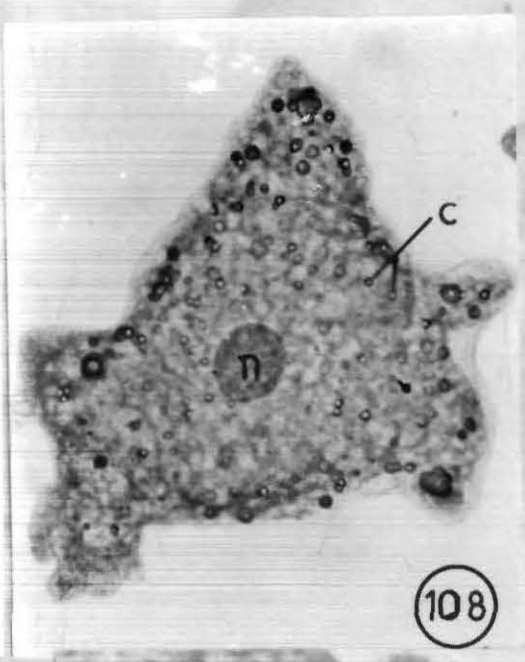

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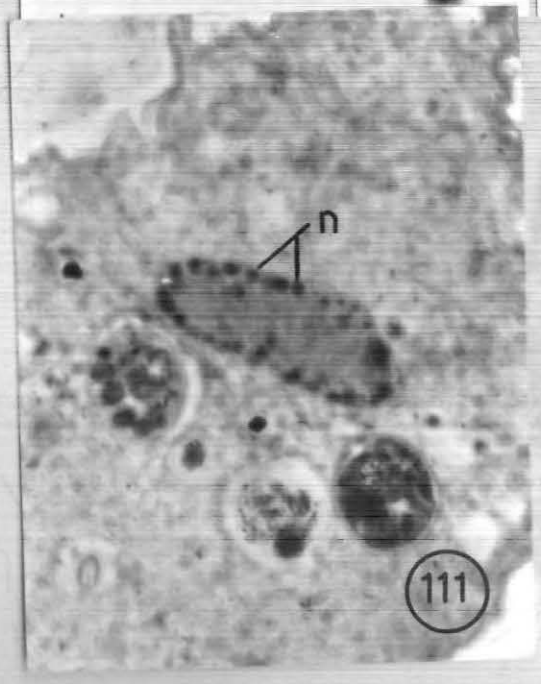
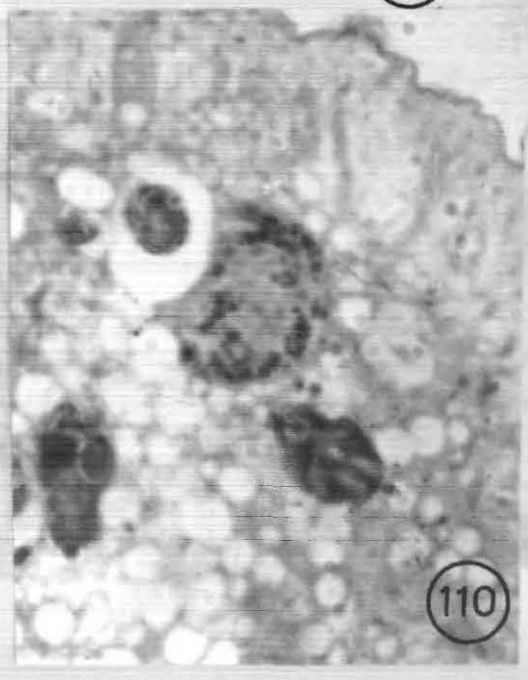


111



110

was noticed in the thin sections (Fig. 110). The cytoplasmic crystals were found to be very scanty and were round in shape (Fig. 108), whereas, in control A. indica, cytoplasmic crystals showed varied structural configurations. In binucleate mini cells, however, a greater number of round shaped crystals were observed as compared to mononucleate mini cells (Figs. 108 and 109).

Fine structural analysis

The nucleus of mini amoeba mutant was surrounded by a nuclear envelope, consisting of two membranes which enclosed a perinuclear cisterna and were fused at intervals to form pores. Nuclear honeycomb layer was also noticed like that of the control, A. indica. A striking feature of mini cells was found to be the appearance of a layer of microfibrils around the outer membrane of the nuclear envelope which was not present in control A. indica (Figs. 112, 113 and 115). The nucleus contained multiple nucleoli which had sharp contours as compared to the control A. indica. Nucleoli were found to be arranged mostly at the periphery just beneath the nuclear envelope. The infra structure of the nucleoli displayed a highly condensed and granulated appearance, as compared to control A. indica, with some intranucleolar fibrils (Figs. 114 and 115). The nucleoli were occasionally intercepted from one

Fig. 112: Electron micrograph of a greater part of A. indica control nucleus. h, honeycomb layer; nu, nucleoli; p, pores. X 9000

Fig. 113: Electron micrograph of the entire mini amoeba nucleus. Note nucleus with highly packed chromatin. nu, nucleoli; h, honeycomb layer; ch, chromatin. X 9000

Fig. 114: Electron micrograph of a portion of A. indica control nucleus. p, pores; nu, nucleoli; dg, dense granular inclusions; ibf, electron dense body with fibrous material. X 18000

Fig. 115: Electron micrograph of a portion of nucleus of mini mutant. Note formation of a layer of microfibrils (mf) around the nuclear envelope. ep, electron dense particle; p, nuclear pores; if, intranucleolar fibrils. X 20000

115
ep
if
d
mi

h
p
nu
112

113
h
nu
mi

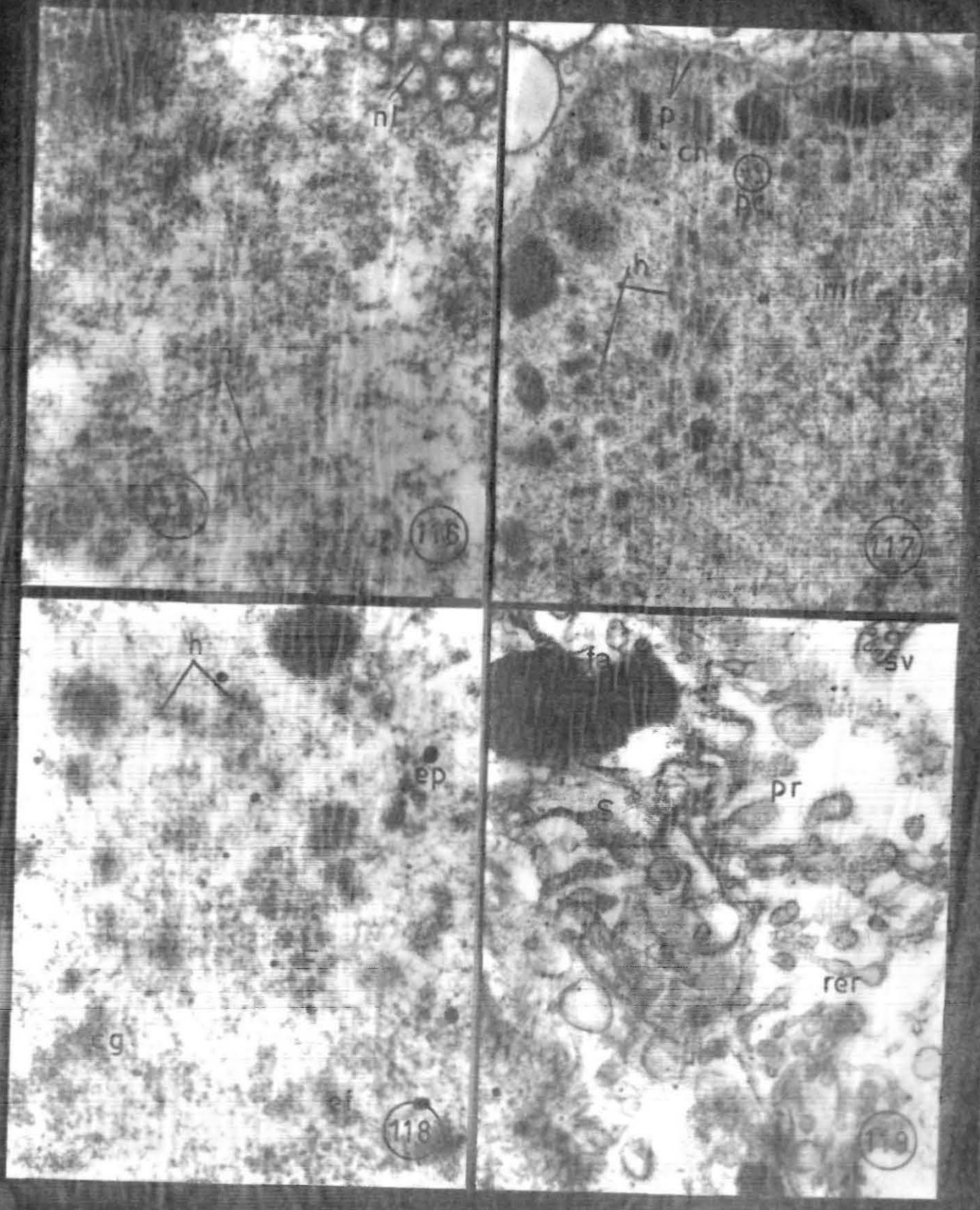
p
nu
lg
114

Fig. 116: Electron micrograph of a portion of nucleus along with a part of honeycomb layer of control *A. indica* cell. cg, chromatin granules; h, helices; hl, honeycomb layer (in part). X 45000

Fig. 117: Electron micrograph of a greater part of mini cell nucleus. Note nucleolar perichromatin granules (pc); imf, interchromatin microfibrils; h, ribonucleoprotein helices; p, nuclear pores; ch, chromatin. X 16000

Fig. 118: Electron micrograph of the core of mini cell nucleus, showing electron dense bodies interdispersed with fibrous material (F). ch, granulated chromatin; ep, electron dense particle; h, ribonucleoprotein helices; ef, fibrous material with electron dense bodies associated with some annular structure. X 24000

Fig. 119: Electron micrograph of an area around the mini cell nucleus with a portion of nucleus. S, ribosomes arranged in spirals on the surface of a cisterna of rough endoplasmic reticulum; pr, polyribosomes; rer, rough endoplasmic reticulum; r, ribosomes; fa, lipid droplet; sv, small vesicles within a comparatively large vesicle. X 22500

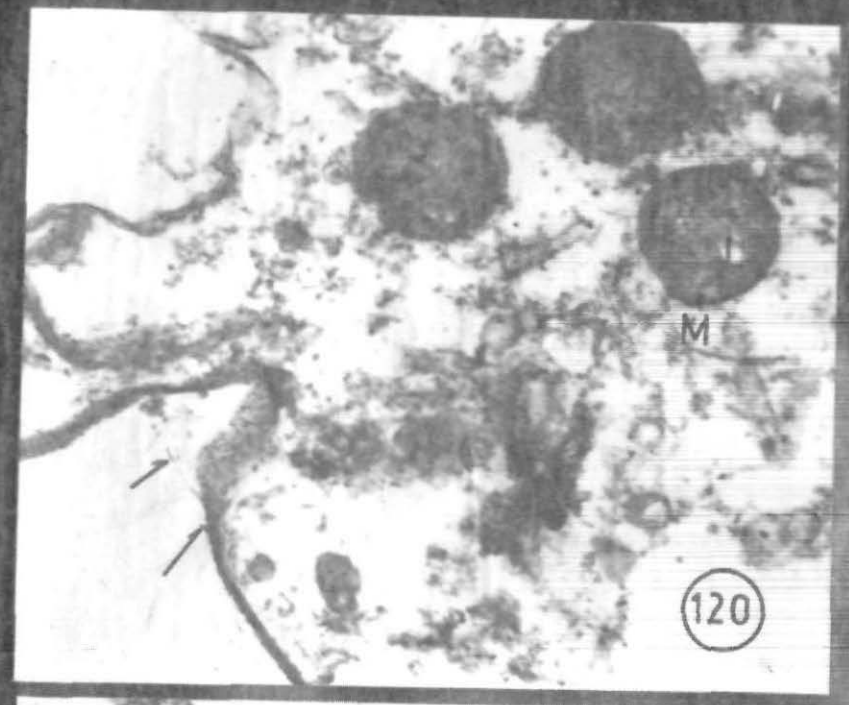

nf
116
p
ch
pc
h
imf
117
h
ep
g
ef
118
fe
sv
pr
s
rer
119

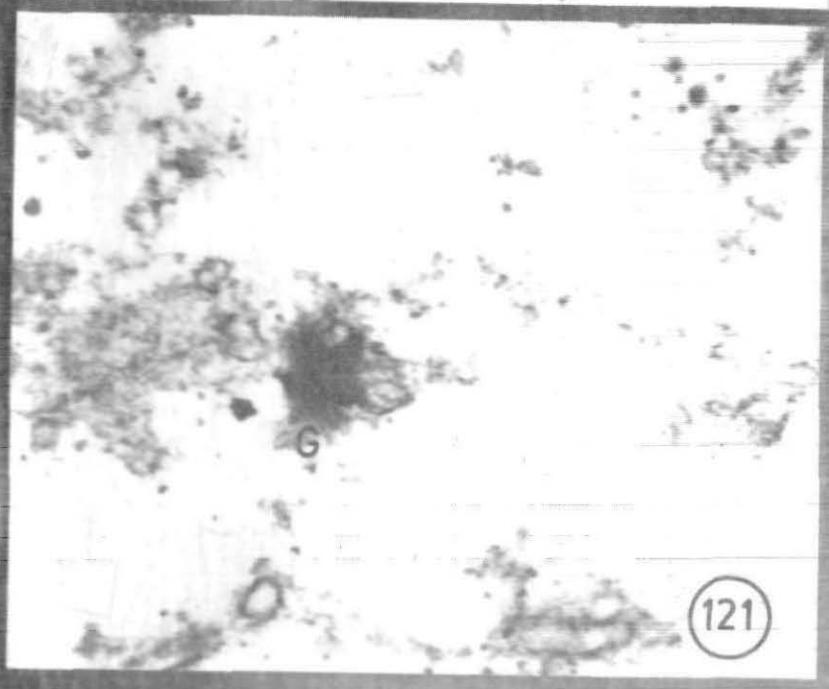
another by a mass of chromatin. Highly dense and granulated chromatin materials, unlike the control, A. indica, were noticed in the nucleoplasm along with the appearance of nucleolar perichromatin granules (Figs. 113 and 117). Sometimes the interchromatin spaces were found to be occupied by abundant interchromatin microfibrils. Certain regions at the core of the nucleus were greatly occupied by masses of fibrous materials, which were associated with electron dense bodies. These electron dense bodies were interdispersed with the fibres, unlike that of the control A. indica. Several helices were also observed in the nucleoplasm (Figs. 116 and 118). The most noticeable feature of the mini cell nucleus was the appearance of some electron dense particles within the nucleus (Figs. 115 and 118). These particles were of unknown origin.

Vacuoles and vesicles were quite abundant in the cytoplasm. The membrane bound spaces stubbed with ribosomes were identified as granular endoplasmic reticulum. The configuration of granular endoplasmic reticulum was found to be vesicular. The smooth endoplasmic reticulum appeared in the form of cisterae. Ribosomal particles and polyribosomes were also noted to be free in the cytoplasmic matrix. Ribosomes were occasionally found to be arranged in spirals or in circular pattern on the cisternal surface of the granular endoplasmic reticulum as seen in tangential sections

Fig. 120: Electron micrograph of a portion of cytoplasm and cell membrane of mini cell showing some spiny projections and filamentous structures on the cell membrane (arrows). Note electron dense particles and an electron-lucent area (L) in the mitochondrion. M, mitochondrion. X 22500

Fig. 121: Electron micrograph of a cytoplasmic area of mini cell, showing golgi body (G). X 33000


M
120


G
121

(Figs. 113 and 119). Microfibres in the cytoplasm was also noted (Fig. 120).

Spiny projections and filamentous structures were observed on the plasma membrane of mini cells. Mitochondria were found to be more or less spherical with irregular and indistinct cristae which showed a tendency to anastomose with each other, whereas in control A. indica distinct tubular cristae were noted. Several electron dense particles were detected in the mitochondrial matrix and on the mitochondrial membranes as well as on the golgi stacks which were of unknown origin and could only be observed in mini cells. Occasionally, electron-lucent region in the mitochondrial matrix was also observed (Figs. 120 and 121).

E. Cytochemical studies

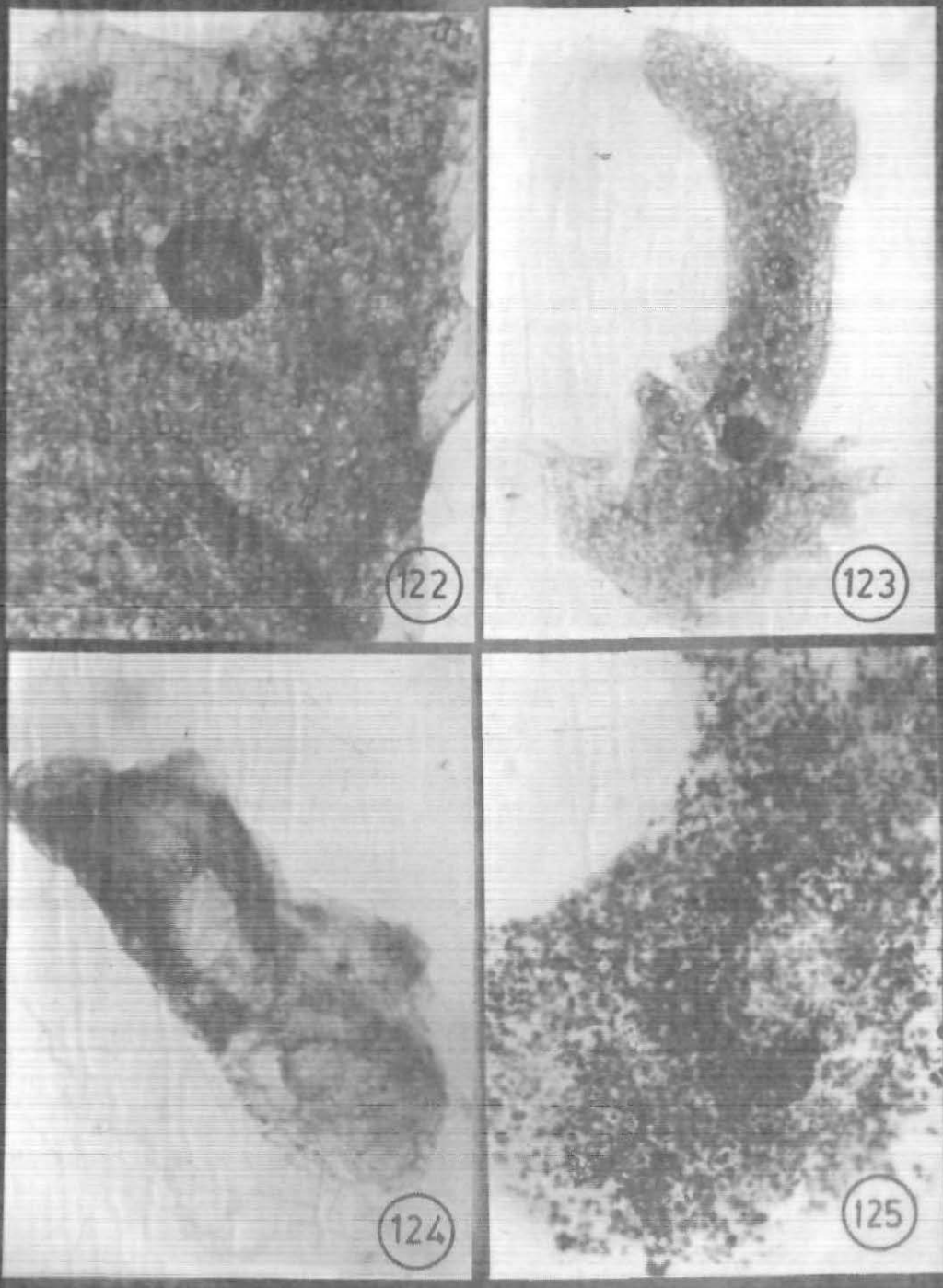
In mini amoeba mutants highly positive basophilic reaction was observed when the cells were stained with toluidine blue. Basophilic reaction was found to be uniform all over the cytoplasm (Fig. 122). Staining reaction of mercuric bromophenol blue with the protein moiety was also checked. A fairly positive and moderate staining reaction was observed in the cytoplasm, although, comparatively less intensity of staining was observed towards the cell periphery. The nucleus also found to be highly stained (Fig. 123). A greater staining intensity

Fig. 122: Portion of a squashed cell with nucleus of mini mutant amoeba, showing intense basophilic reaction after toluidine blue staining. X 800

Fig. 123: Squashed mini cell stained with mercuric bromophenol blue. Note staining intensity on the nucleus. X 315

Fig. 124: Squashed mini cell showing PAS reaction. Note staining intensity at the advancing zone. X 315

Fig. 125: Portion of a squashed mini cell showing highly sudanophilic nature of the cytoplasm after sudan black B staining. X 640


122
123
124
125

was noticed in the advancing zone of the cell as compared to the uroid when the cells were subjected to PAS reaction. In control A. indica reaction occured almost uniformly on the cell cytoplasm. However, a very faint staining reaction was noted on the nucleus (Fig. 124). The mini cells were also stained with Sudan black B to detect bound lipids. The intensity of reaction was found to be quite high as demonstrated by black dots scattered evenly over the cell cytoplasm. The mini cell nucleus was also found to be fairly sudanophilic (Fig. 125).

F. Pattern of macromolecular syntheses:

Pattern and duration of DNA synthesis

The profile of nuclear DNA synthesis in mini cells was investigated by labelling the cells with ^{3}H-thymidine immediately after mitosis and was followed upto 7 h. Cells were labelled for 30 min after mitosis and at 1 h interval thereafter to follow the pattern of incorporation of tritiated thymidine into the nuclear DNA. The labelled cells were squashed and processed for autoradiography. Cytoplasmic grain incorporation was found to be less than 8% of the total count on the nucleus.

The pattern of DNA synthesis obtained from the autoradiographic preparations revealed that very less incorporation of tritiated thymidine occurred upto ½ h after mitosis.

However, an acceleration of DNA synthesis was noticed from 1 h onwards and the peak synthesis was detected at 3 h after division (Fig. 128). A gradual decline in the incorporation of ^{3}H-thymidine was found to occur from 4 h and onwards. Very less DNA synthetic activity was observed between 5 to 7h(Fig. 126). The incorporation of ^{3}H-thymidine at ½ h after division was found to be 3% while compared to peak DNA synthesis displayed at 3 h and 10 to 16% of the DNA synthesis was noticed between 5 to 7 h when compared to the maximum DNA synthesis of the mini cells at 3 h. The duration of DNA synthesis period was found to be shorter in mini cells as compared to control A. indica.

Profile of protein synthesis:

The pattern of protein synthesis was investigated by labelling mini cells with ^{3}H-leucine throughout the cell cycle. Cell samples were processed for autoradiography and the pattern of protein synthetic activity was analysed by counting the silver grain over the cytoplasm. A homogeneous distribution of silver grains was noticed in the cells. Cells were labelled for 1 h with ^{3}H-leucine at each point.

^{3}H-leucine incorporation was found to be decreased between 2 to 4 h after mitosis. The increased incorporation of labelled leucine into mini amoeba proteins was noticed

Fig. 126: Pattern of DNA synthesis in mini amoeba. Cells labelled with ^{3}H-Tdr for ½ h (at one point) and 1 h at other points after division and processed for autoradiography. Each point represents average grain count of 18 to 22 nuclei of two separate experiments. Vertical bars: ± SEM.

Fig. 127: Pattern of ^{3}H-leucine incorporation in mini amoeba cells incorporated for 1 h at each point and processed for autoradiography. Each point represents mean grain count of 30 to 35 amoebae of 2 separate experiments. Vertical bars: ± SEM.

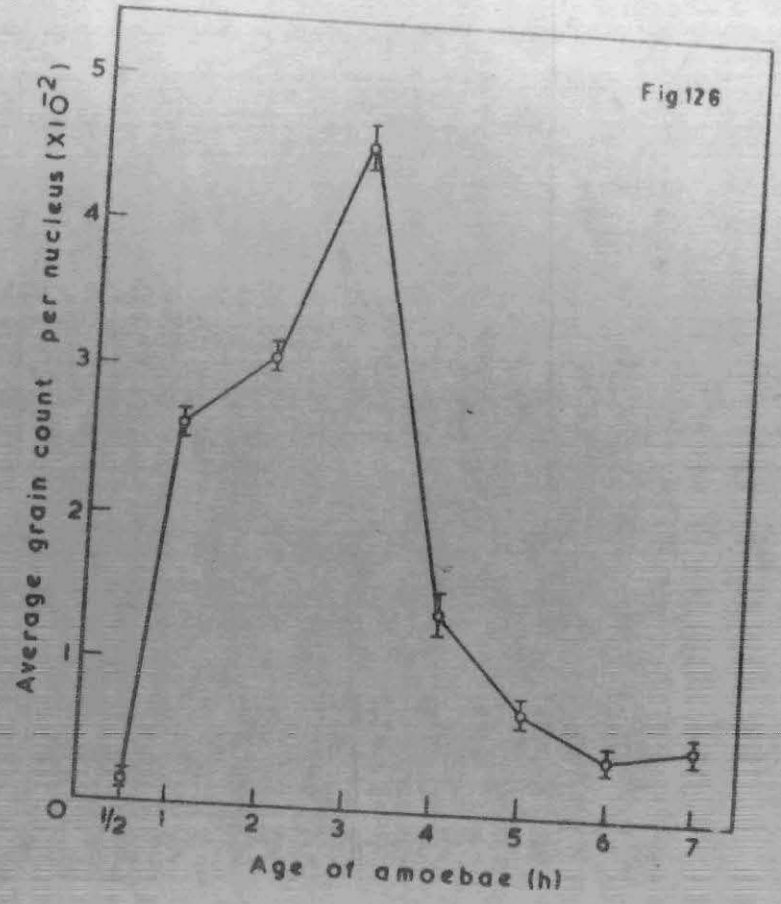


Fig 126
Average grain count per nucleus (X10⁻²)
0
1
2
3
4
5
1/2
1
2
3
4
5
6
7
Age of amoebae (h)

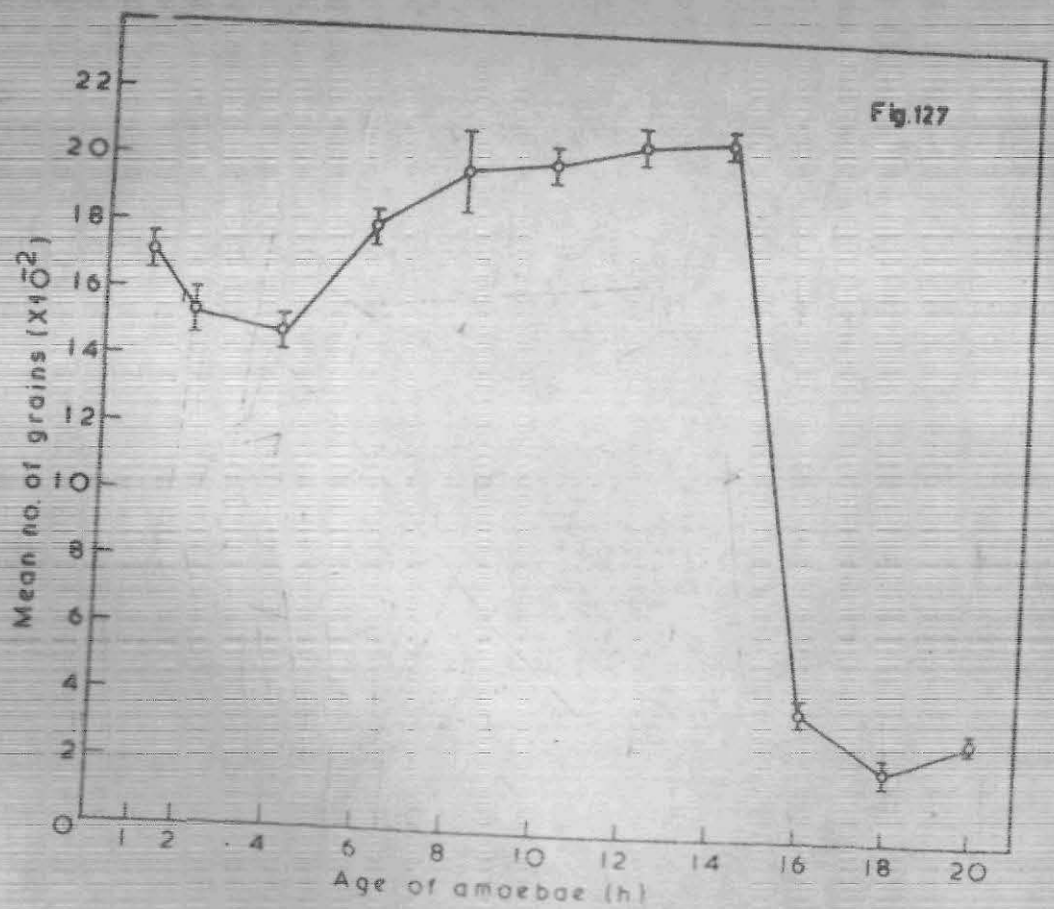
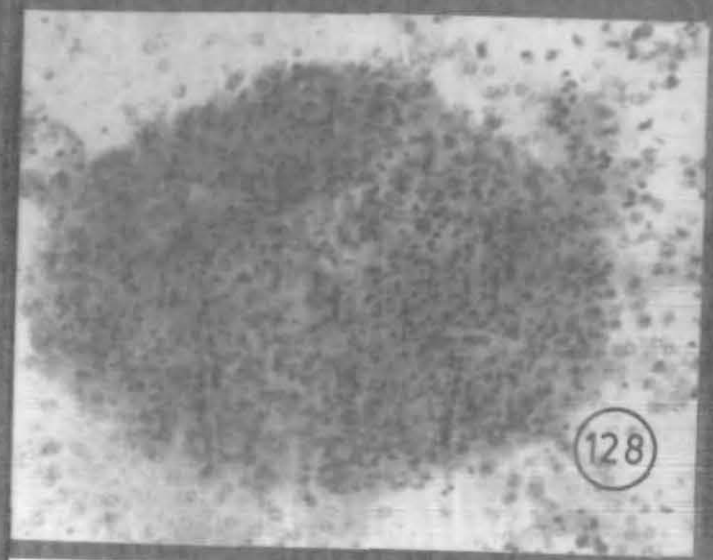


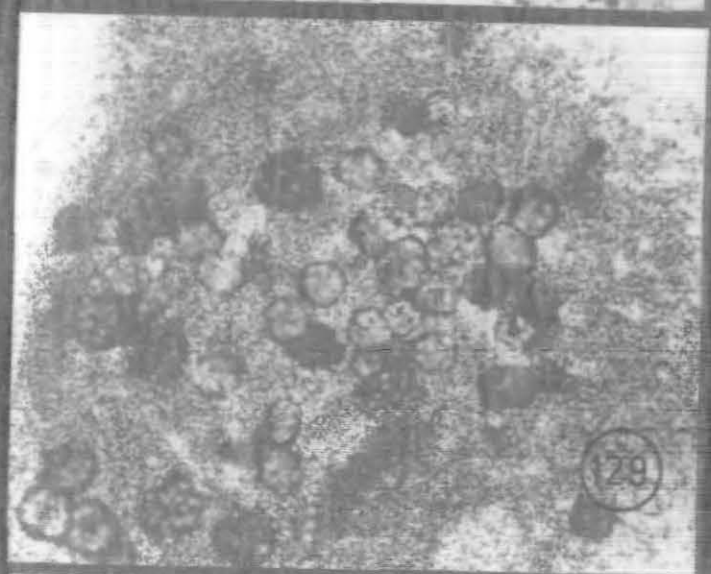
Fig.127
Mean no. of grains (X10⁻²)
0
2
4
6
8
10
12
14
16
18
20
22
1
2
4
6
8
10
12
14
16
18
20
Age of amoebae (h)

Fig. 128: Autoradiograph of a mini cell nucleus showing DNA synthesis at 3 h after division. Cell labelled with ^{3}H-Tdr, squashed and stained with Giemsa. X 2520

Fig. 129: Autoradiograph of a ^{3}H-leucine labelled mini cell at 14 h after division, showing high grain density. Cell squashed and stained with Giemsa. X 640

Fig. 130: Autoradiograph of a ^{3}H-leucine labelled mini mutant at 16 h after division, showing very less incorporation. Other details as in Fig. 129. X 640


128


129


130

from 6 h onwards and it was found to follow similar pattern of incorporation of labelled precursor upto 14 h, where it showed the maximum incorporation of radioactive amino acid in the cell (Fig. 129). The most striking change in the pattern of ^{3}H-leucine incorporation was noted between 16 to 20 h old cells where a sharp decline in the incorporation of the labelled amino acid was observed (Fig. 127). The decrease in the incorporation of ^{3}H-leucine into mini cell proteins between 16 to 20 h was found to be 80% to 82%, as compared to the maximum incorporation of label by 14 h old cells (Fig. 130).

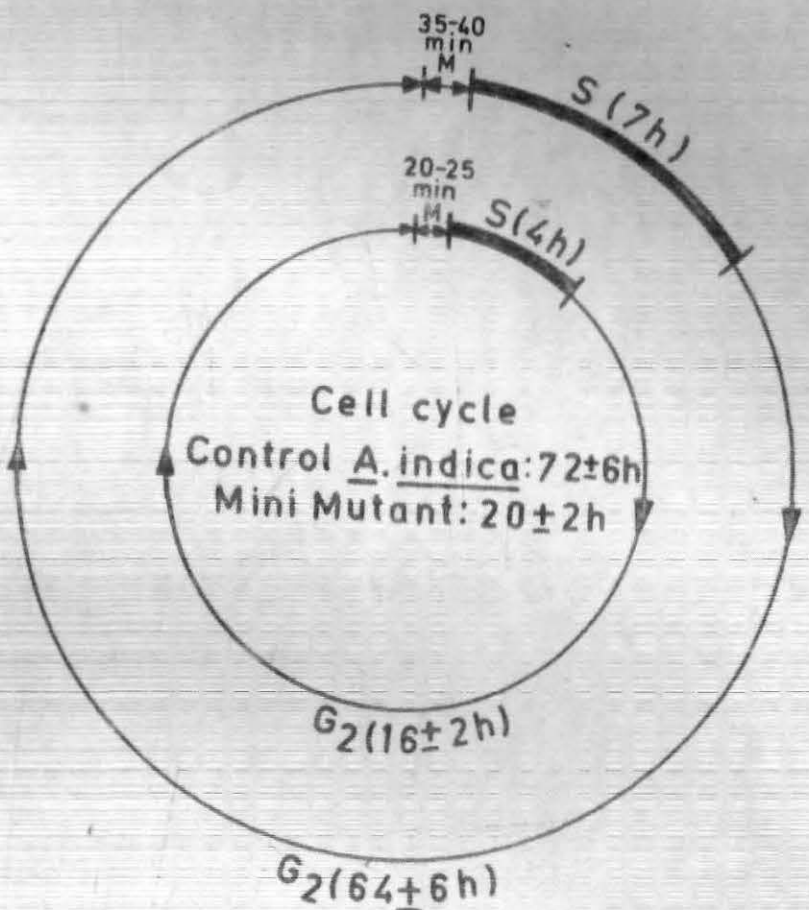
G. Duration of different phases of the cell cycle:

In control A. indica the cell cycle covered a period of 72 ± 6 h where M phase had a duration of 35 to 40 min, the S phase covered upto 7 h and the rest of the time was occupied by G_2 phase of 64 ± 6 h.

In 'Mini' amoeba mutant the entire cell cycle was found to be completed within 20 ± 2 h where the mitotic phase was restricted upto 20 to 25 min. The S phase occupied 4 h while the G_2 became 16 ± 2 h (Fig. 131). In mini cells the cell cycle was found to be very short as compared to the cell cycle of control A. indica.

Fig. 131: Model showing the duration of different phases of the cell cycle of Mini cell (inner circle) as compared to the cell cycle of control *A. indica* (outer circle). S phase was determined by ^{3}H-Tdr incorporation. ± SD.

Fig.131


35-40 min
M
S (7h)
20-25 min
M
S(4h)
Cell cycle
Control A. indica: 72±6h
Mini Mutant: 20±2h
G2(16±2h)
G2(64±6h)

DISCUSSION

The present study tries to make a critical attempt to investigate the actions of ethyl methanesulfonate, a potent mutagenic agent, on amoeba cells both at structural and functional level. The emphasis of the present study has been directed mainly towards the induction of mutation and investigation of the action(s) of EMS on amoebae, mostly at defined phases of the cell cycle.

The effects of EMS on amoebae are both immediate, apparent following treatment, and delayed, appearing at later hours after mutagen exposure. EMS has been found to affect amoeba's cell membrane severely following treatment as has been evident from the changes in cell shape, pseudopodial structure and inhibition in the formation of pseudopodia. Similar effects have also been recorded by Ord (1976a) after treating the amoebae with another mutagen, N-methyl-N-nitrosourethane. Detachment of the cells immediately after treatment and inhibition in the formation of pseudopodia along with drastic inhibition in food uptake, i.e. impairment in the process of phagocytosis, which really amounts to the alteration of membrane functions due to the damage of either the cell surface mucopolysaccharides and membranee incorporated proteins or lipid bilayer of the amoeba cell

membrane by the alkylating action of EMS (Ross, 1962). The other possibility lies with the indirect damage to the structures of the microfilaments which are involved in the process of cell movement (Jeon and Jeon, 1975). It has become evident that amoeba acts as an organised unit where initiation of new pseudopodia and the moments of inhibition or reversal of movement of older fronts are distinctly correlated (Mast, 1932; Grebecki and Klopocka, 1981). It is quite likely that sluggish movement and inhibition in pseudopodia formation may be due to damage of both nucleus and cytosol of the cell caused by EMS. It has been observed (Goldstein and Jelinek, 1966; Jahn, 1967) that movement in amoeba stops following enucleation and motility is restored instantly on insertion of a nucleus from another amoeba. Jeon (1968) has reported lack of adhesion to the substratum and rounding up of amoebae after enucleation. In agreement with these results, it can be suggested that the lack of attachment and sluggish movement of amoebae at intial hours after EMS treatment may be due to impairment of nuclear activity by the action of EMS. Extreme stickiness of the amoebae to the surface, after they have become attached at later hours following treatment, indicates profound alteration, conformational and/or biochemical,

of the cell membrane. It has been recorded (Hoover et al., 1980) that divalent ions like Mg^{2+}, Ba^{2+}, La^{3+}, Ni^{2+}, Zn^{2+} and Mn^{2+} are involved in promoting adhesion of the cells to the surface. They are of the opinion that (1) if for some reason one divalent cation binds more readily to the cell surface due to physical properties of that ion, it might reduce surface charge more effectively; thus, electrostatic repulsion will be lowered, allowing more attachment; (2) there can be enzymes on the cell which require specific ions for activation; (3) the divalent cations may bind to specific proteins of the membrane, and in doing so cause conformational changes which contribute to a more adhesive surface. It can be suggested that the action of EMS might reduce surface charge and thus electrostatic repulsion will be lowered, allowing firm attachment.

In some cases, fragile nature of amoeba cell membrane has been noted immediately after EMS treatment, which often leads to total cytolysis, indicating membrane damage due to EMS treatment. The same phenomeana has been reported in irradiated amoebae (Ord and Daniellí, 1956). Fragility of the amoeba cell membrane has been found to be associated with the damage of golgi apparatus after MNU treatment (Ord, 1979a).

Scanning electron microscopic analyses of the cell surface of EMS treated cells support the statement that

there has been a remarkable alteration in the membrane surface structure immediately following the treatment. It shows disorganization of the surface structure as evident by obliteration of surface protuberances and blebs which are characteristic features of normal *A. indica*. Immediately after treatment, cell surface becomes disrupted as has been seen by the appearance of ruffles, pock-marks, pits and several mini bead-like structures. A change in the surface structure probably indicates damage to the mucopolysaccharide coat, the existence of which has been on record (Pappas, 1959; Nachmias and Marshall, 1961; Chapman-Andresen, 1962; Marshall and Nachmias, 1965), the lipid bilayer and membrane protein complexes due to intervention of EMS.

Membrane damage can be correlated with the damage of golgi apparatus which has been observed immediately after EMS treatment. Autoradiographic studies (Flickinger, 1975; Read and Flickinger, 1980) have shown that glycoprotein which occurs in the golgi apparatus is a precursor to the cell membrane components. This finding, together with morphological and cytochemical similarities (Revel and Ito, 1967; Stockem, 1969; Wise and Flickinger, 1970a) suggests that much of the material in the golgi complex represents cell-surface components either in the process

of assembly or completed and awaiting transport to cell surface (Flickinger, 1981). Hence, damage to the golgi apparatus by EMS can produce lesion in the biosynthesis of membrane components leading to alteration in the membrane surface structure, which is quite evident from the SEM analyses of the cell membrane. Appearance of pock-marks, cavities and pits on the EMS treated cell surface thus reveal some characteristic nature of the interaction of EMS with amoeba cell membrane components. At later hours after treatment, membrane recovery has been noted along with the reconstitution of golgi apparatus, indicating functional correlations with structural recovery of the membrane, reconstitution and functioning of golgi apparatus and resumption of cell motility. It seems reasonable to speculate that action of EMS not only leads to ethylation of DNA (Lawley and Brookes, 1963b; Lawley and Shah, 1972; Sun and Singer, 1975; Fujiwara, 1975; Singer, 1976) but also has a remarkable membrane damaging action leading to alteration of cell surface structures by acting directly on the cell membrane and indirectly on the membrane replacement machinery. The structural changes on the cell membrane can also be linked with the alteration of nuclear activity due to EMS exposure. Detachment of the cells from the surface, as mentioned earlier, may also be due to the disruption of normal surface structure. However, the functional

significance of the protrusions, blebs, mini beads and ruffles on the membrane surface is not entirely understood at present.

EMS induced diverse cellular damage has been rendered apparent both from light and electron microscopic observations of the cytoplasm, nucleus and cell organelles. Pinching out of the cytoplasm in the form of membrane bound vesicles from the cell surface following EMS treatment appears to be a consequence of defensive mechanism operating to eject cytotoxic substances. EMS induced cytotoxicity has been observed (Röhrborn, 1970; Peterson et al., 1979) in cultured mammalian cells. Cell fragmentation due to EMS treatment leads to the appearances of cells of variable sizes. Similar type of fragmentation has been noted after X-irradiation in Chaos chaos (Schaeffer, 1946), C. diffuens (Wichterman and Honegger, 1958), Acanthamoeba (Mookerjee et al., 1962) and in other species of amoebae following treatment with nitrogen mustard or X-rays (Daniels, 1951, 1952; Ord and Danielli, 1956; Ord, 1956, 1968b), suggesting failure of surface layer to congeal at the places of vacular eruption which leads to fragmentation and even bursting of the cell body.

Hypervacuolated and fenestrated nature of the EMS treated amoeba cytoplasm reflect a massive structural disorganization of the cell. Hypervacuolization has also been observed in X-irradiated Acanthamoeba (Chatterjee, 1968) and nitrogen mustard treated chick cells in culture (Fell and Allsopp, 1949). Hypervacuolation is essentially a kind of surface precipitation reaction (Heilburnn, 1956), which shows that extensive stimulation or injury release calcium inside the cell augmenting surface precipitation reaction that causes hypervacuolization of the cell cytoplasm. Formation of hyaline zone immediately after EMS treatment is probably indicating cellular damage. Nuclear ejection leading to the formation of anucleate cell body after EMS treatment can be explained as a phenomenon of EMS action. Similar type of effect has also been recorded in Entamoeba histolytica (Nasset and Kofoid, 1928) subjected to radium rays and in irradiated Acanthamoeba (Chatterjee, 1968). It has been observed that S phase cells are more vulnerable to cytoplasmic damage when exposed to EMS as compared to G_2 phase, possibly indicating nucleus mediated cytoplasmic damage. It has been recorded (Ord, 1968b) that the transfer of nuclei between N-methyl-N-nitrosourethane (MNU) treated and control amoebae showing majority of the effects after MNU treatment are mainly due to nuclear damage, although,

cytoplasmic damage is also evident. Alteration in the form of cytoplasmic crystals few days after treatment of MS phase cells with EMS has been noted. Altered forms of crystals can also be produced in amoebae by nuclear DNA lesions after MNU treatment (Ord, 1970, 1973a).

The golgi bodies have been found to be greatly affected as evidenced by the disorganization of golgi cisternae and dispersion of its vesicular elements at early hours after EMS treatment. Studies on amoeba golgi bodies indicate the presence of glycoprotein, acid mucopolysaccharides, acid phosphatase and thymidine pyrophosphatase in the golgi stacks which are associated with the packaging of hydrolases to form primary lysosomes, membrane turnover as well as other enzymatic processes such as those related to digestion (Stockem, 1969; Wise and Flickinger, 1970a,b; 1971; Flickinger, 1975; Stockem and Korohoda, 1975). It has been noted that immediately after EMS treatment, along with the disorganization of golgi the intensity of PAS positive substances decreases considerably as observed from cytochemical studies, suggesting changes in golgi function. It can be ascertained that EMS does act both on membrane and golgi bodies leading to lesion in golgi function such as membrane turnover and the enzymatic processes relating to digestion. Disorganization of the golgi bodies has been found to be

associated with the impairment of nuclear activity in amoeba (Flickinger, 1969) which suggests that EMS probably affects golgi bodies through the impairment of nuclear activity. At later hours after treatment structural recovery of the golgi bodies has been noted along with the recovery of PAS positive substances, golgi related membrane and digestive functions which suggest that EMS is responsible for structural disorganization as well as functional lesion of golgi bodies in amoebae.

A change in the pattern of ribosomes immediately following EMS treatment has become evident by the appearance of monomers of ribosomal particles in the cytoplasm. It may be due to less of their attachment to mRNA and endoplasmic reticulum due to intervening action of EMS, resulting to failure in the process of initiation of polypeptide chain which may cause increase in the number of free ribosomes as has been recorded in amoebae after MNU (Ord, 1976b), N-methyl-N-nitrosourea treatments and by UV-radiation (Ord, 1979a). At later hours after treatment the ribosomal pattern appears almost like the control cells, indicative of recovery in the process of ribosomal arrangement, from the trauma of EMS exposure.

Remarkable change in the mitochondrial form and structure is quite evident immediately following EMS

treatment. Extreme swelling of the mitochondria, reduction of tubular cristae to a narrow peripheral zone and in some cases rupture of the mitochondrial membrane have been observed at early hours after EMS treatment which are also on record after administration of MNU (Ord, 1976b) N-methyl-N-nitrosoguanidine (MNNG) (Ord, 1979a) and cadmium (Ord and Al-Atia, 1979) to the amoebae. It can be suggested that swelling of the mitochondria may occur by relative sliding of the two membranes and unfolding of the cristae which leads to the alteration of membrane permeability and ion transport due to EMS action. Bursting of the mitochondria may either be due to excessive swelling caused by EMS toxicity within the cell or it may be related to the damaging action of EMS as such on the membrane bound organelles. Appearance of electron-dense spot, inclusions within the matrix of cristae, in few cases electron-opacity of the mitochondrial matrix and development of vesicular cristae have been noted after EMS exposure. Similar findings have been reported after MNU treatment to amoeabe (Ord, 1976b). Electron-lucent zone which has been found to develop after EMS treatment is of similar type as has been shown by Flickinger (1973) after ethidium bromide treatment to amoebae. Abnormalities in the mitochondrial form with the presence of material inclusions are often associated with an arrest of organelle activity or actual degradation associated with metabolic changes (Fujita and

Machino, 1964; Ollerich, 1968; Ueda and Tsuji, 1971; Morisset, 1974; Oliveira, 1977) as has become evident from the drastic reduction in the intensity of cytochemically detectable boundlipids in the cell immediately following EMS treatment. It suggests a change in the glycolysis and/or oxidative phosphorylation pathways of the cell (Ord, 1979a). However, at later hours after EMS exposure the mitochondrial size has been found to be regulated back to almost normal form, but the cytochemically detectable lipid intensity in the cell still does show considerably less intensity suggesting an attempt to structural recovery and lesion in complete functional recovery.

The cell nucleus has been found to be the seat of effects produced by EMS treatment. Enlargement of the nucleus has been observed in EMS treated cells at early hours after treatment which has also been reported in hydroxyurea treated HeLa cells (Grant and Grasso, 1978). Nuclear enlargement may be due to impairment in cytoplasmic cleavage (Chatterjee, 1968) or is associated with growth inhibition (Grant and Grasso, 1978). Division delay and giant cell production have been noted after EMS treatment, which suggest that nuclear enlargement and mitotic arrest are presumably different manifestations of common EMS toxicity. Disorganization of the nuclei, appearance of pycnotic and bizarre shaped nuclei after

EMS treatment have been noticed. Similar types of nuclear disorganization has been observed in amoeba cells after treatment with nitrogen mustard (Daniels, 1952; Ord, 1956), X-rays (Ord and Danielli, 1956) and alkylating agents (Ord, 1968b, 1976b).

EMS induced nuclear alteration is indicative of nuclear damage. The pycnotic nucleus which has been observed is also reported in irradiated amoebae (Chatterjee, 1968), indicating a lethal nuclear damage. Cell treated only at ES phase with EMS shows multipucleate condition around 18 h after treatment which may be due to nuclear fragmentation caused by EMS treatment. It can be suggested from the present finding that S phase cells are more vulnerable to nuclear fragmentation as compared to G_2 phase ones after EMS treatment.

Electron microscopic analysis of the EMS treated cells show remarkable changes in the nucleoli immediately following EMS exposure. Disorganization of the nucleoli has been noted along with the loss of their peripheral region. Branching of the nucleoli is often observed. The ribonucleoprotein helices, which are generally abundant in the control nucleus, are found to be almost absent. Similar results are obtained after treatment of amoebae with MNU (ord, 1976b) and MNUrea (Ord, 1979a) showing separation of nuclear helices from their attachment

and a mass exodus of the same from the nucleus. Studies with EM autoradiography of amoebae whow that the peripheral region contains nucleolar DNA (Minassian and Bell, 1976a,b) and the mRNA and/or rRNA are packed in a helical form in groups near the nucleoli (Stevens, 1967; Wise et al., 1972). Present investigation indicates that EMS induced nucleolar damage which is associated with disappearance of RNP helices from the nucleus, suggesting lesion in the RNA synthetic activity as has also been recorded by Ord (1976b) after MNU and MNUrea treatment. Nucleolar reorganization has been noted accompanied by the reappearance of RNP helices at later hours along with occasional fusion of the nucleoli, suggesting structural recovery. Budding off of the nuclear honeycomb layer is evident in almost all the cells following EMS treatment, indicating EMS induced damage to the nuclear membrane.

Sensitivity of the amoebae to the action of EMS has been observed in terms of cell lethality and division delay which shows that lethality and division delay are dose as well as cell cycle phase dependent. With increasing dose more lethality of the asynchronous cells has been noted which also correspondingly relates to the prolonged delay in division. It suggests that cell sensitivity is dependent upon dosages of EMS which shows that a linear dose-effect relationship is dose rate

dependent, as gradual sensitivity of the cells is achieved at progressively higher dosages. Cell cycle experiments on amoeba cells with EMS show a substantial difference in the sensitivity at different phases of the cell cycle. It has been noted that the mitotic and S phase, especially MS phase, cells are more vulnerable to EMS action, compared to G_2 phase ones. It has been observed that cells treated with EMS at MS phase, the period corresponds to the peak DNA synthesis, show greater sensitivity along with prolonged delay in division for successive generations. Similar type of S phase sensitivity affects have been observed in MNU and MNUrea (Ord 1971, 1976a,b) treated amoebae where nuclear transfer studies show that the nucleus is more sensitive than cytoplasm which has also been documented by abnormalities in division. It implicates replication as being responsible for the greater sensitivity of the S phase nucleus.

A direct correlation between DNA replication and sensitivity to nitrosamides has been reported by a number of workers. The replicating fork of E. coli has been found to be 220 times more sensitive to base change than other regions of the DNA (Guerola et al., 1971); the DNA which is neither replicating nor transcribing, is less affected as shown by transformation studies, than active DNA

(Kimball and Setlow, 1972). Greater sensitivity of the cells during DNA synthesis, particularly at periods of peak DNA synthesis has been on record (Kelly and Legator, 1970; Barranco and Humphrey, 1971). It has been accepted that DNA, rather than the DNA precursors, is more sensitive material to alkylation within the cell and is probably also the primary site of alkylation (Wheeler, 1962; Kihlman, 1966). Maximum sensitivity of the cells to the action of EMS is restricted to mitotic and S phase, especially MS phase, which corresponds to the peak of DNA synthesis, implicating phase-dependent sensitivity of the amoebae to the ethylating action of EMS. Loss of chromatin associated protein during mitosis may be the cause of increased sensitivity of the mitotic cells to EMS action. Cell mortality is indicative of severe ethylating and cytotoxic effects of EMS leading to extensive disruption and irreversible block of synthetic process. The possibility of cytoplasmic sensitivity can not be ruled out as the LG_2 phase cells are also found to be quite sensitive at higher dosages where negligible amount of DNA synthesis occurs. It suggests the effect of cytoplasmic volume which may act as an amplifier of damage by increasing the number of active alkylating species of EMS within the cell as has also been shown by Ord (1974, 1976b) in amoebae cells after MNU and MNUrea treatment.

Prolonged division delay for successive generations has been the general effect observed in EMS treated cells. Division delay is more pronounced in S phase specially MS phase, treated cells as compared to cells treated at other phases of the cell cycle. DNA synthesis has also been found to be depressed and continues for a longer period in ES phase treated cells. An almost similar situation has been recorded in HN_2 treated mouse fibroblast cells (Caspersson et al., 1963; Gelfant, 1963) and in HeLa cells (Plant and Roberts, 1971; Roberts et al., 1971a,b) after treatment with several alkylating agents, suggesting that these chemicals block cell division in G_2. In contrast to this (Evans and Scott, 1964), using root tips of Vicia faba, have found that G_2 cells are not delayed whereas S phase cells are considerably retarded after HN_2 treatment. Since DNA synthesis and oxidative phosphorylation are, as a rule, prerequisite for cell division, inhibition to these processes generally results in inhibition to mitosis (Edmunds, 1964; Kihlman, 1966). Present findings suggest a correlation between mitotic arrest and extension of different phases of the cell cycle. It indicates that EMS acts as a mitotic inhibitor probably by bringing out alkylation (Wheeler, 1962) of the replicating DNA. It has been observed that some binucleate giant cells have been found to appear in the culture, several

days following EMS exposure which has also been recorded by Ord (1968b, 1973a) after MNU and MNUrea treatment to amoebae, indicating growth without cytokinesis due to damage of both nucleus and cytoplasm.

DNA synthesis has been found to be greatly inhibited in EMS treated ES phase amoebae where the depression in DNA synthesis continues throughout the S period. Alkylating agents can inhibit DNA synthesis by alkylation of DNA which can be the result of both the alkylated DNA having a reduced primer activity of a competition for sites on the DNA polymerase between normal and alkylated deoxyribonucleotide triphosphates, if the latter are unable to function as substrates in polymerase reaction (Wheeler, 1962). EMS can ethylate several sites of DNA. The site to be most frequently ethylated is the N-7 position of guanine (Brookes and Lawley, 1961[a,b]; Loveless, 1969; Sun and Singer, 1975).

EMS has been found to inhibit DNA synthesis and cause DNA damage in germ cells of male mice (Sega, 1974) and in Paramecium (Cohen, 1980) by ethylating several sites of DNA. It has been shown that nuclear DNA synthesis in amoebae continues at a reduced rate in presence of, and immediately after exposure to MNU (Ord, 1974, 1976a). DNA synthesis has been found to be inhibited in HeLa cells

immediately after MMS treatment (Painter, 1977) which is responsible for blocking the initiation of replicons but the replicons which have already been initiated after MMS treatment remain unaffected. A severe inhibition to protein synthesis also follows immediately after MMS treatment which probably is partially responsible for reducing the rate of DNA replication (Painter, 1977). It has been also suggested (Brookes and Lawley, 1961b; Lawley and Brookes, 1963a) that the characteristic cytotoxic action of difunctional alkylating agents is due to their ability to cross-link the twin strands of the DNA molecules, thus preventing its replication. Lawley and Brookes (1965); Loveless, 1966) have shown that the sensitivity of the alkylating agents, including EMS, depends on the activity of enzymes which repair damaged DNA. Many enzymes used to repair the process of DNA synthesis have particular requirements for efficient functioning, e.g. Mg^{++} by DNA polymerase, and have energy requirements from ATP molecules (Eskin and Linn, 1972; Kornberg, 1974). If the energy systems are damaged and synthesis of new enzymes are inhibited in treated cells then the degree of such damage can affect the repair process.

Present findings, along with the supporting informations indicate that EMS induced inhibition and continued

depression of DNA synthesis are due to lesion in the process of replication by ethylating damage to all the available sites of replicating DNA. It is also suggestive of ethylating lesion in the synthesis or activation of the enzymes (Goldstein *et al.*, 1962; Rutman, 1964) involved both in replication as well as repair of damage DNA. It is suggested that either the DNA template or enzymes required for replication may be affected by EMS action. Anderson and Burdon (1970) have shown that MNNG affect the activity of DNA polymerase in hamster fibroblast cells. It has been suggested (Clarkson and Michell, 1979) that the initial severe reduction in the rate of DNA replication caused by MMS is mediated through an inhibition of protein synthesis. Mitochondria have been found to be damaged by EMS action, indicating a possibility of correlation between the prolonged impairement of replication and lesion in the process of oxidative phosphorylation.

Another experiment on DNA synthesis, which involves treatment of amoebae with EMS at ES, MS and LS phases shows a proportional inhibition in the synthesis of DNA among ES, MS and LS phases treated cells. Severe depression to DNA synthesis has been noted in MS phase treated cells,

which coresponds to the peak period of DNA synthesis of control cells. A relatively less inhibition of DNA synthesis has been noted in ES and LS phases treated cells. It has been shown (Ludlum, 1965) that DNA may be more easily attacked by alkylating agents when undergoing maximum replication. MMS is known to reduce the production of new DNA in human cells (Buhl and Regan, 1973) by interruption in DNA chain elongation as a result of either block in the parental DNA (Fox and Fox, 1969), or a block in the initiation of adjacent replicating units (Roberts *et al.*, 1971a,b). The proportional inhibition of DNA synthesis in EMS treated amoebae indicates that DNA is more vulnerable to EMS action at MS phase where more replicating sites are available for ethylation, whereas at ES and LS phases the inhibition of DNA synthesis is substantially less as these periods correspond to the periods of less replication activity.

^{3}H-Uridine incorporation study of control amoebae shows an alternate peak-and-dip pattern of RNA synthesis throughout the S and G_2 phases, suggesting continuous changes in the rate of RNA synthesis or the size of the uridine precursor pools (Ord, 1973c) and permeability of uridine molecules (Hawkins, 1975). Some of the points of the cell cycle are clearly defined and thus only these points are considered for the present study. Treatment by

EMS at MS phase following incorporation of ^{3}H-uridine shows immediate inhibitionn to total RNA synthesis and continuation of the labelling experiment with MS phase treated cells reveals a continued depression in the process at least upto MG_2I phase. Lesion in the process of RNA synthesis has been recorded after HN_2 treatment to guinea-pig cells in culture (Levis and Nadai, 1964). Brookes et al. (1969) suggested transcriptional damage after some monofunctional alkylating agent treatment in E. coli. EMS studies of amoebae show a disorganization of nucleoli and chromatin following EMS treatment. EM autoradiographic studies on amoebae have shown (Minassian and Bell, 1976b) that peripheral regions of nucleoli contain nucleolar DNA. The RNP helices of nucleus and cytoplasm of amoebae contain packed mRNA protein and/or rRNA of nucleus of A. proteus (Stevens, 1967; Flickinger, 1968) and might contain transcriptional products (Wise et al., 1972). The RNP helices are found to disappear from the nucleus immediately after EMS exposure. It is on record (Karasaki, 1968; Fakan and Puvion, 1980) that transcription sites are associated with nucleolar chromatin areas. Present investigation on the inhibition of RNA synthesis after EMS treatment is indicative of severe transcriptional lesion, which may be due to ethylation of RNA bases (Singer and Fraenkel-Conrat, 1969). Continued

inhibition to RNA synthesis may be related to the inactivation of DNA as a template for mRNA synthesis (Kiho and Rich, 1964) or lesion in the repair enzyme system and enzymes required for transcription (Lawley and Brookes, 1965; Brookes *et al.*, 1969).

Protein synthesis, as assayed by ^{3}H-leucine incorporation, has been found to be inhibited immediately after EMS exposure. Cytochemical study with mercuric bromophenol blue stain also shows appreciably less staining intensity following treatment. EMS administration also inhibits alkaline phsophatase and acid phosphatase activities immediately after treatment as revealed by differences in the intensity of cytochemical staining between normal and EMS exposed amoebae. It has been shown that alkylating mutagenic chemicals alkylate proteins (Ross, 1962) resulting in the depression of protein synthesis (Fox and Fox, 1967a,b; Painter, 1977). Depression in the enzyme activity or enzyme inhibition in *E. coli* has been recorded (Lawley and Brookes, 1965) after treatment with alkylating chemical mutagens. Present data is in agreement with the above findings and suggests that inhibition in protein synthesis as well as reduced cytochemical reactions of alkaline and acid phosphatases are due to ethylating action of EMS. The synthetic activity along with the intensity of cytochemically detectable proteins including the enzymes like alkaline and acid phosphatases, however, have been found

to be almost regulated back to normal level at later hours, indicating a recovery of the treated cells.

Different patterns of inhibition of protein synthesis have been noted when the amoeba are exposed to EMS at ES and MS phases followed by labelling the cells with ^{3}H-leucine at different periods of the cell cycle. In both the eES and MS phases treated cells a marked depression in protein synthesis has been noted throughout the cell cycle. However, in ES phase treated cells the protein synthetic activity has been found to be gradually increased at later periods of the cell cycle. On the other hand, in MS phase treated cells the inhibition has been more pronounced and a continuous decline in the pattern of protein synthesis has been observed. It is worth while to note that both at ES and MS phases inhibition of DNA synthesis occurs following EMS treatment but the inhibition is more severe when the cells are treated at MS phase. Moreover, a considerable inhibition to RNA synthesis has also been observed at MS phase after EMS exposure.

An inhibition of protein synthesis in human cells (Buhl and Regan, 1973) and in Chinese hamster ovary cells (Clarkson and Michell, 1979) in culture after MMS treatment has been demonstrated, suggesting translational lesion. Allan and McCalla (1967) have noted inhibition of DNA, RNA and protein synthesis after treatment with radiometic

agents and have suggested that delayed inhibition of protein synthesis is due to slow inactivation of some compounds related to protein synthesizing system. In E. coli, Brookes et al. (1969) have shown that the action of monofunctional alkylating agents cause modification of DNA, trancription and mRNA translation that results in inhibition of synthetic activity, suggesting inactivation due to alkylation of cellular proteins. The repair enzyme system is a possible target for inactivation (Lawley and Brookes, 1965; Loveless, 1966; Brookes et al., 1969). Findings of other workers are consistent with the present report and indicative of severe lesion in the translational machinery due to ethylating action of EMS on repair enzyme system as well as alkylation of cellular proteins. The prolonged and severe inhibition to protein synthesis of MS phase treated cells suggest inactivation of DNA as a template for mRNA synthesis and hence cause translational lesion.

Comparative studies on the ethylating action of EMS on replication, transcription and translation in amoebae indicate that sub-lethal dose of EMS affects replication, transcription and translation.

The most significant finding of the present investigationn has been the production of a 'mini' amoeba mutant

emerged as a result of treating the cells with EMS, a powerful chemical mutagen. The 'mini' mutant differs visibly from the parental strain and bears definite morphological, physiological, biochemical and ultrastructural characteristics. This 'mini' mutant strain has been cultured in the laboratory for more than two years, undergoing over 650 generations maintaining the unaltered mutant characteristics. Some intermediate and giant sized cells are also encountered following EMS treatment which are either lethal or are found to regulate back to normal forms.

It has been observed that mini mutant can be produced both from asynchronous population as well as from a defined phase of the cell cycle following EMS treatment. It has become evident from the present experiment that after treatment of amoebae at 1 h intervals through the cell cycle the stable mini mutant originates only from ES phase treated cells, although, appearance of mini cells has also been recorded after treatment of the cells at LS and LG_2 phases, but these mini cells are nonviable. The frequency of mutant production is much less in asynchronous cells compared to ES phase treated population. It suggests that the ES phase cells available in mass culture at the time of treatment are a few in number which are only capable to produce viable mini cells. These mini cells are quite stable

in their size and are capable of producing only mini cells by the process of the cell division and maintain unaltered mutant characteristics.

EMS has been proved to be an effective mutagen for prokaryotes and several eukaryotes (Schalet, 1978) and is able to produce mutations in Tetrahymena (Carlson, 1971), Paramecium (Cohen, 1980) and mammalian cells in culture (Vaughan and Stadler, 1980). EMS acts as a mutagen mainly by the ethylation of guanine at 7-position, i.e. 7-ethylguanine formation (Swann and Magee, 1971) and subsequent induction of mutation either directly by hydrogen bonding with thymine instead of cytosine (GC → AT transition) or only after depurination (Orgel, 1965). The alkylation of 0-6 position of guanine is also thought to be a possible source of mutations following EMS exposure (Loveless, 1969; Loveless and Hampton, 1969; Kimball et al., 1971). 0-6-Alkylguanine is known to be miscoding and, therefore, mutagenic (Gerchman and Ludlum, 1973; Singer, 1979). The genetic alteration in EMS induced ad-3B mutations of Neurospora crassa have been identified (Malling and De Serres, 1968) and indicating a case of base pair substitution. EMS treatment may cause error in repair replication (Anderson and Fox, 1974) by ethylation of guanine at N-7 position or 0-6 ethylguanine formation or both following subsequent inductionn of mutation either

directly by hydrogen bonding with thymine instead of cytosine (Swann and Magee, 1971) or formation of apurinic acid (Strauss, 1961) due to breakdown of alkylated guanine resulting in error of pairing and thus leading to mutation. The possibility of extensive intragenic deletions (Freese and Cashel, 1964; Strauss and Wahl, 1964) due to ethylating action of EMS cannot be ruled out.

The nucleus of amoeba has long been considered polyploid, though the existence of polyploidy is based on circumstantial evidence (Ord, 1973a). A mutation in amoeba cell which has many representatives of each gene will require a change in all, or at least many, of them. Where EMS is present for only a short period it is necessary for all the copies of the genes to be vulnerable at the same time, while the majority of the other genes are protected. Two activities of the chromatin are expected to leave genes vulnerable to EMS, DNA replication and DNA transcription. Provided that during replication all the equivalent loci replicate their DNA at the same time, or that during transcription all the copies of a given gene 'switch on' simultaneously, these periods are likely to give vulnerability to particular genes while other periods are expected to be less affected. Expression of inheritable change in a polyploid system e.g., A. indica, might indicate

nonrandom change of specific cistrons, such as indicated in amoeba cells (Ord, 1976b) and also in other systems (Botstein and Jones, 1969; Guerola et al., 1971; Guerola and Creda-Olmedo, 1975) after treatment with MNU.

The ES phase dependent EMS induced mini mutant production in amoebae suggests that mutagenic action of EMS is primarily confined to the period of DNA replication where maximum DNA ethylation is possible. The ES phase specific origin of stable mini mutant indicates that the particular genetic point(s) appear to be affected, and thus responsible for the production of mutation, is restricted to a short period of DNA replication. Findings of Kee and Haber (1975) with nitrosoguanidine treatment and cell cycle-dependent induction of mutation along a yeast chromosome and mutations during the cell cycle phases of hamster tissue culture cells (Orkin and Littlefield, 1971) as well as induction of mutation in Paramecium (Cohen, 1980) by EMS at DNA replicating phase, strongly support the present findings.

No detectable induction of mutation by EMS in MS phase treated cells, suggests that exposure to EMS at that particular phase has a preferential lethal action on the cells involving mini mutant production which is also evident from the sensitivity test. It indicates a

close correlation between lethal and mutational damage.

The probable mode and time of mini cell origin after EMS treatment to ES phase cells have been critically investigated. It has become evident that around 12 to 14 h following treatment of ES phase cells, sprouting out of the nuclear material occurs which are found to form discrete and smaller nuclei. At around 36 h after treatment a portion of the parental treated cell cytoplasm is found to be separated by cytoplasmic fission containing a nucleus which is quite smaller in diameter as compared to the nucleus of parental cells. These cells are found to form a stable clone of mini mutants. It is quite likely that the mini cell containing the nucleus, which probably has originated from the budding off process from the parental nucleus, is carrying altered genetic informations, thus leading to a stable clone of mini mutants with distinct mutant characteristics. It can be suggested that the smaller size of the mini mutant nucleus may be due to balanced elimination and subsequent redistributionn of nuclear material during the process of nuclear budding. The viability of mini mutant suggests that the nucleus is capable of performing all essential functions required for its survival and multiplication.

The mini cells are quite smaller in size, measuring about about half as compared to A. indica and approximately one third that of A. proteus. The nuclear diameter is also noted to be considerably smaller than A. indica and A. proteus. The cell size and nuclear diameter of the mini cells are consistent and hence it can be considered as a size mutant.

Mutations have been produced in A. proteus by treatment with MNU (Ord, 1970). Three mutants have been obtained which are: mini, pale and SpG mutants after treatment of the amoebae at mid G_2, end G_2 and end S phases respectively of the cell cycle. These are found to be stable and having specific mutant characteristics, which are mainly changes in the cell size, nuclear diameter and the cytoplasmic crystals. Nuclear transfers between control and MNU induced mutant amoebae suggest that phenotypic changes are dependent on nuclear changes (Ord, 1970, 1976b) which is also probably true for the present study. In giant amoeba, Chaos chaos X-irradiation during division (Schaeffer, 1946) results in the production of stable clone of smaller amoebae. However, no information is available to consider this cell type as a mutant.

The cytoplasmic triuret crystals of amoebae (Granbaum et al., 1959; Griffin, 1961) are found to change their form after MNU treatment (Ord, 1970), suggesting alteration

of nuclear DNA or its closely associated chromatin protein. In EMS induced mini mutants, the crystalline form has also been found to be changed and only one type of crystals, which are round in shape, are observed and are less in number. So far, no attempt has been made to investigate the use and fate of the triuret crystals in amoebae. However, the use of ammonia and the common carbamyl phosphate step as the starting point in both pyrimidine base and urea biosyntheses, as well as the replication and division lesions which always accompany the crystal change (Ord, 1979a) suggest a possible correlation between the two.

Drastic changes in the membrane of mini amoebae have been noted. Scanning electron microscopic observations of the mini mutant cell membrane show profound alteration in the membrane surface structure, compared to parental cells as evident by disappearance of cell surface protuberances and appearance of ridges, several concavities and mini beads on the cell surface. The structure of the blebs has been found to be altered drastically by the appearance of larger beads having conical projections. All these features strongly suggest drastic alteration in the membrane characteristics and surface architecture of the mini mutant. The pseudopodial form in mini cells is distinctly different from the parental cells. The mini

cell membrane has developed a great deal of stickiness to the surface and this has remained a constant feature of mini cells. Increased stickiness of the mini cells to the substratum definitely indicates change(s), structural and probably conformational, of the cell membrane. It has been observed (Hoover et al., 1980) that divalent cations have profound influence in promoting cell adhesion. Affinity of one divalent cation to bind more readily to the cell surface might reduce surface charge more effectively, thus lowering the electrostatic repulsion and hence allowing more attachment. The other possibility lies with preferential binding of divalent cations to specific proteins, leading to conformational changes which may cause more adhesiveness to the substratum. It has been pointed out (Curtis, 1967; Weiss, 1967) that physical nature of cell adhesion depends upon the radius of curvature of 'probes' the cell is able to produce. Small radii of curvature are particularly favourable for electrostatic interactions so that tips of membranous projections should be the reasonable places to seek divalent cationic bridges (Pethica, 1961) in cell to substratum adhesion.

One of the most important features of the mini cell has been the change in its food habit. The control A. indica

can feed on both Tetrahymena and Chilomonas equally well whereas the mini mutants are found to die if they are fed with Tetrahymena. It has been observed that mini cells can capture only one Tetrahymena although the food is available to them ad lib. It is also noted that even after ingesting only one Tetrahymena the mini cells die within a very short period of time. On the other hand, they can survive quite well with Chilomonas feeding and are able to capture approximately twenty Chilomonas at any given time without any deleterious effects. It suggests that Tetrahymena as a food organism for mini cells is probably too large which the mini cells are unable to ingest and hence die. It is likely that the total membrane area required to capture and for the formation of more than one phagosome for Tetrahymenae at a time is inadequate in mini mutant cells. The observations indicate that Chilomonas, being of smaller size than Tetrahymena, is the most suitable food organism for mini mutants. Formation of several phagosomes at a given time is suggestive of comfortable physiological state of the mini cells in relation to Chilomonas capture, phagosome formation, ingestion and assimilation of nutrients.

TEM analysis of mini cell shows that the nucleus is surrounded by a nuclear honeycomb layer, which generally acts as a reinforcement for nuclear membranes (Fawcett, 1966).

It seems likely that the presence of fibrous lamina in the mini mutant's nucleus is indicative of toughness of its nuclear envelope as in A. proteus (Flickinger, 1974). The most striking feature of mini mutants is the appearance of a layer of microfibrils around the outer membrane of the nuclear envelope, suggesting involvement of new structural protein(s) and is a characteristic feature of mini mutants. The nucleoli of mini cells are found to be arranged just beneath the nuclear envelope as in other species of amoebae (Flickinger, 1968, 1974). The highly condensed and sharp contour of nucleoli probably suggest a higher degree of intermixing and close packing of granular and fibrillar elements unlike the parental cells, thus indicating a distinct characteristic feature of mini mutant nucleoli. The mini cell nucleus is packed with highly dense and granular chromatin materials which are not observed in parental cells. The electron-dense bodies interdispersed with fibrous material are noticed in the mini cell nuclei, unlike the parental amoebae. All these features of the mini mutant nuclei strongly suggest its specific characteristics and uniqueness, thus leading to the conclusion that the mini cell nucleus is definitely different in structure from the parental cell.

The cytoplasmic matrix of the mini cell has been found to be different when compared to its parental stock. Vacuolization of the endoplasm has been a distinctive feature,

indicating altered nature of cytoplasm. There has been a mixture of small and large free polysomes in addition to the regions of RER where attached polysomes form a variety of spiral and/or circular pattern(s) and are found to be distributed mainly near the peripheral nuclear zone. It indicates that mini cells have an active cell cycle passing through all cyclic phases and probably are involved in making a large variety of proteins. The appearance of vesicular RER stubbed with ribosomes, suggest a characteristic structural pattern. The mitochondria of mini mutants are found to be almost spherical in shape containing irregular and indistinct cristae which often show a tendency to anastomose unlike the parental cells, indicating structural difference.

The mini cells are found to be highly basophilic and sudanophilic. PAS positive substances have also been detected histochemically, indicating presence of these substances in appropriate amounts.

The pattern of DNA synthesis in mini cells differs from parental cells. In mini mutants, upto $\frac{1}{2}$ h following mitosis, DNA synthetic activity has been found to be quite negligible while the peak synthesis is noted at around 3 h after mitosis, and thereafter a decline in ^{3}H-Tdr incorporation follows indicating termination of S period. It has been noted that the S period has become shorter

compared to parental cells. The pattern of protein synthesis in mini cells, in terms of ^{3}H-leucine incorporation reveals an almost steady state of synthesis upto 14 h following mitosis, where it shows the maximum incorporation of ^{3}H-leucine. A sudden drop in ^{3}H-leucine incorporation occurs between 16 to 20 h after division.

Analysis of the cell cycle of mini cells reveal a shorter duration of different phases compared to parental cells leading to a overall shorter cell cycle. It takes approximately one fourth the time to complete a cell cycle compared to parental cells. The mitotic phase covers 20-25 min; the S period takes 4 h; while 16 $\pm$ 2 h is required to cover G_2 phase. Thus the whole cell cycle has been found to be completed within 20 $\pm$ 2 h instead of 72 $\pm$ 6 h as found in parental cells, suggesting change in the cell cycle timing and length of different phases. Alteration in the cell cycle timings has also been recorded in MNU induced amoeba mutants (Ord, 1970, 1973a) where in all the cases a longer cell cycle has been noted.

It has been suggested that there is a size control over the DNA division cycle (Killander and Zetterberg, 1965; Hartwell et al., 1974; Prescott, 1976). It is likely (Carter and Jagadish, 1978) that attainment of a critical cell size itself is not the trigger but perhaps some property

which changes with size such as accumulation of a specific cell component, leads to faster cell division.

The cell cycle progression depends on consecutive and/or simultaneous action of a series of genes. Malfunctioning or quantitative change in the action of any of these genes owing to mutation might lead to alteration in the cell cycle progression. A mutation that under permissive conditions alters the cell cycle in a unique way is a cell cycle mutant (Simchen, 1978) as has been induced in cultured mammalian cells (Roscoe et al., 1973; Smith and Wigglesworth, 1972, 1974). A detailed review of the cell cycle mutants (Simchen, 1978) indicates that the cell cycle mutants bring about arrest of the cell at specific stages and each mutant shows a characteristic morphology of arrest at the nonpermissive condition at different stages.

The foregoing discussion and experimental evidences strongly suggest that EMS induced mini amoeba cell is a size mutant which has a cell cycle phase specific origin, bears a totally altered pattern of cell cycle and has specific structural, physiological and biochemical identity. Present study, however, does not satisfy all the characteristics to designate it as a cell cycle mutant and hence further characterization is required before considering the mini cells as a cell cycle mutant.

SUMMARY

The effect of ethyl methanesulfonate (EMS) - a mutagenic chemical on amoebae especially at defined phases of the cell cycle had been investigated.

1. Sensitivity of the cells to the action of EMS was found to be dose and cell cycle phase dependent where maximum sensitivity, in terms of cell lethality, was noted in mitotic and S phase cells.

2. Treatment of 0.5% EMS for 15 min was found to be the most suitable dose to carry out all the experiments.

3. Cell membrane was found to be greatly affected as was evident by the disorganisation of cell surface structure, revealed by SEM analysis. Cell detachment at early hours following EMS exposure and development of increased surface stickiness at later hours were also noted.

4. A change in the cell shape, cessation of cell motility, inhibition in the formation of pseudopodia along with the impairment in the process of phagocytosis were observed in the treated cells.

5. Delay in cell division was found to be a general effect after EMS treatment.

6. EMS induced cellular damage was noted as evident by cell cytolysis, cell fragmention, formation of hyaline zone and appearance of hypervacuolization.

7. Three types of variant cells were produced after EMS treatment namely, mini cells; intermediate-sized-cells and giant cells among which only few mini cells were found to retain smaller size. The other cell types were either lethal or were regulated back to normal form. The giant cells were generally multinucleate.

8. Formation of altered form of cytoplasmic crystals was noted at later hours following treatment of the cells at MS phase.

9. Several cell organelles, like golgi bodies and mitochondria, were found to be disorganised after EMS exposure. Ribosomal particles were often observed as monomers through the cytoplasm.

10. The cell nucleus was found to be affected much after the treatment which showed nuclear swelling, fragmentation of the nucleus, formation of bizarre nucleus, shedding off the nuclear honeycomb layer, disorganisation of the nucleoli and chromatin materials, branching and fusion of the nucleoli. RNP helices were almost absent at early hours in the treated cell nucleus.

11. Cytochemical studies of the treated cells showed ununiform distribution of basophilia and distinctly less intensity of staining reaction of total protein moiety, bound lipids, PAS positive substances and alkaline and acid phosphatases.

12. ^{3}H-thymidine incorporation study showed depression in DNA synthesis throughout the S period in ES phase treated cells. Proportionally more inhibition to DNA synthesis was noted in MS phase treated cells, compared to ES and LS phase treated cells.

13. Total cellular RNA synthesis, as assayed by ^{3}H-uridine incorporation, was found to be inhibited to a great extent in MS phase treated cells and the depression in the synthetic activity continued for quite long thereafter.

14. Protein synthesis as measured by ^{3}H-leucine incorporation, was inhibited immediately following EMS treatment. Cell cycle experiments showed a continued inhibition of protein synthesis throughout the cell cycle in both ES and MS phase treated cells.

15. The most important finding of the present investigation had been the production of a mini amoeba mutant which appeared after treating the cells at ES phase. This mutant strain had been cultured in the laboratory

for more than two years and has undergone over 650 generations todate with unaltered mutant characteristics. The following features were encountered in mini mutants:

(a) It has a cell cycle-phase-specific origin. The mini cells were emerged out from the parental cells by containing a comparatively smaller nucleus, which appeared after its fragmentation, along with some part of the cytoplasm.

(b) The frequency of mini cell production was in the order of 9.4%.

(c) These cells were considerably smaller in size and also contained a nucleus of smaller diameter, compared to parental cells.

(d) Chilomonas were found to be the suitable food organism for mini cells as they could not survive after Tetrahymena feeding.

(e) A great deal of alteration of the cell surface structure was evident from SEM analysis. These cells also developed a great deal of stickiness to the surface.

(f) A change in the form of cytoplasmic crystals was evident.

(g) The nucleus contained extensively dense nucleoli and had a highly dense and granulated nature of chromatin. Appearance of a layer of microfibrils around the nuclear boundary was observed.

(h) The mitochondria had an anastomosing nature of cristae. The endoplasmic reticulum had vesiculated nature and were stubbed with ribosomes.

(i) Duration of S phase and the pattern of DNA synthesis differed from the parental cells.

(j) All the phases of the cell cycle were reduced and thus the entire cell cycle was shortened to a considerable extent as compared to control as well as the treated parental cells.

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