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

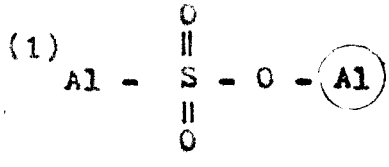
been defined by Freese (1959) and last two by Brenner et al. (1961). Another class of mutants result from deletion of segments of DNA which may be many nucleotides long. If the number of deleted nucleotides is three or a multiple of three, there is no frame-shift error beyond the site of mutation, but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

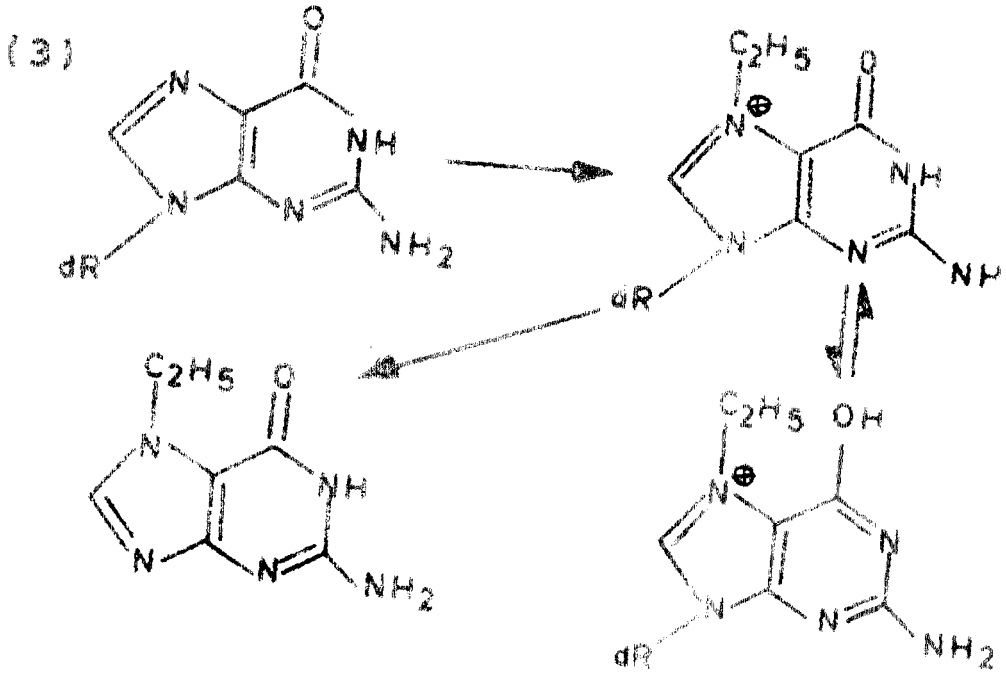
The present investigation is concerned with the action of a monofunctional alkylating agent at a single cell level. The general structure of monofunctional alkylating agent is shown below (1), which are, for e.g. Methyl methanesulfonate (MMS) and Ethyl methanesulfonate (EMS) where the alkyl group (Al) 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