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Chemical Induced Chromosomal Aberrations In Relation To The Nuclear Cycle In Zea Mays (l)

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CHEMICAL INDUCED CHROMOSOMAL ABERRATIONS IN RELATION TO THE NUCLEAR CYCLE IN *ZEA MAYS* L.

by

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DEDICATION

With deepest gratitude, I dedicate

this thesis to my parents.

ABSTRACT

The effects of mitomycin C (MC) and hydroxylamine sulfate (HAS) on the nuclear cycle, including the production of chromosomal aberrations and mutations, have been studied. Primary root meristems of Zea mays ("Seneca 60") were given the following treatments at 24 ± 1°C; 1) a 30 min. pulse-label with ^{3}H-thymidine (1μc/ml: specific activity 6.7c/mM) immediately prior to a 2-hour treatment with MC (5μg/ml or 10μg/ml) or HAS (0.5 mg/ml), and 2) a mixture of MC or HAS and ^{3}H-thymidine for 2 hours. Frequency data from the autoradiographs were used to estimate the duration of the nuclear cycle, and to determine a stage sensitivity to chemicals.

MC appears to lengthen the duration of the total nuclear cycle in comparison to the control. The high sensitivity of the S phase and the limited sensitivity of G_2 to this drug resulted in prolonging the duration of the cycle in the S and G_2 periods.

HAS blocked the transition of late S cells to G_2 resulting in the accumulation of dividing nuclei at some point following termination of S period. The inhibition of mitosis and prolongation of the duration of nuclear cycle occurred in early G_2.

Autoradiographic experiments, in which primary roots were treated with a mixture of ^{3}H-thymidine and the chemical for 2 hours; or treated with the chemical for 2 hours followed by 2 hours in ^{3}H-thymidine, were performed in order to investigate the effects of MC

and HAS on incorporation of ^{3}H-thymidine into DNA. MC produced a strong inhibition of DNA synthesis during and after the treatment. On the contrary, HAS reduced the incorporation of ^{3}H-thymidine into DNA during the treatment, while the incorporation significantly increased after removing the roots from this drug. These observations suggest that the effect of HAS on DNA synthesis is easily reversible.

The accumulation of aberrations indicates that the cells in early S and very late G_1 are sensitive to MC, while HAS is effective in early G_2 and late S periods.

The induced aberrations are not equally distributed among the chromosomes or within a chromosome. The location of breaks was assigned to chromosome arm specific segments. The distribution patterns of chromosomal aberrations induced by these two chemicals were similar, with specific chromosome and chromosomal segments preferentially involved. The specificity of MC and HAS to induce breakage in the regions of centromeres, the secondary constriction and heterochromatin, was evident.

HAS-induced polyploidy followed from the initial induction of endoreduplication in interphase. A hypothesis for induction of endoreduplication based on a feed-back control mechanism is proposed. Experimental evidence to support the hypothesis has also been presented.

Mutagenicity studies of MC and HAS have been conducted in seed and tassel material. MC is a mutagen in Zea mays, while HAS shows little effect.

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CHAPTER 1

INTRODUCTION

Cytogenetics is a science that undertakes studies of the cytological basis of Mendelian inheritance. Cytogenetic studies also investigate chromosomal structure and behavior, and they have broadened our knowledge to include an understanding of the induction of chromosomal aberrations and mutations.

Following the rediscovery of Mendel's Laws and the discovery of X-ray induced mutagenesis by Muller (1927), Zea mays became a very important material for genetical studies. Aneuploids, euploids and mutants were accumulated, linkage groups established, many aberrations were characterized, and the meiotic chromosomes were studied intensively. However, use of the mitotic cytology of maize for studying induced chromosomal aberrations has been limited. Many workers have focused on Vicia, Allium, Hordeum or Tradescantia as material (in higher plants) for the study of induced chromosomal aberrations. Since individual chromosomes in maize somatic cells can be recognized, it is an ideal material to demonstrate the relative amounts of damage produced in specific chromosome regions.

In this study, an attempt was made to examine the effects of chemicals on mitotic inhibition and the nuclear cycle in Zea mays, and

to record the frequency of chromosomal aberrations assignable to specific chromosome regions. In addition, an interesting phenomenon, endoreduplication, has been found to be induced by hydroxylamine sulfate (HAS) in Zea mays root tips. The cells with endoreduplication undergo two or more cycles of DNA replication in interphase before entering mitosis. As they come to mitosis, the duplicated chromosomes characteristically occur in pairs. Multiple rounds of endoreduplication of the chromosome may lead to close pairing of chromatids during mitosis, yielding a structure somewhat similar to that of polytene chromosomes.

The study of HAS induced endoreduplication might well provide information on genetic control mechanisms. Since interphase has been divided into three stages on the basis of autoradiographic data, i.e., G_1, S and G_2 (Howard and Pelc, 1953), we have also been able to demonstrate that the cells in the S period could be induced by HAS to undergo the second series of DNA replication at the time of treatment. HAS also inhibited the transition of the late S cells to G_2 and the early G_2 cells, resulting in an increase in the duration of the G_2 period. Taking into account all the data, a hypothetical nuclear cycle map including the induction of endoduplication has been proposed in this thesis.

CHAPTER 2

LITERATURE REVIEW

2.1 General:

The discovery of nuclear division in the 1880's is a remarkable chapter in the history of biology. Flemming in 1882 unified the observations showing that chromosomes split longitudinally during nuclear division, and proposed the term "mitosis" for the process of cell division. The formation of daughter nuclei, and the importance of chromatin in inheritance, were fully realized during the cell division. The description of DNA, its location in chromosomes and its role in heredity did not emerge until some years later.

Following the rediscovery of Mendel's paper by three separate research workers, a relationship between heredity and cytology was established. In 1900, de Vries, Correns and Tschermak independently published accounts of their researches in hybridization in which each author referred to the experiments of Mendel which since 1865 had lain forgotten. All three had grasped the concept of the unit character in inheritance. Further, study of cell division and genetic phenomena led to the evolution of a chromosomal mechanism of sex determination (McClung, 1902). Later, in 1908 to 1916, the contributions of Boveri, Bridges, Carothers, Gulick, Merrill, Morgan, Muller and Sturtevant played an important role in the development of "Cytogenetics".

Genes in chromosomes had been visualized. Genetic transmission was parallel to chromosome behavior. It became evident that the chromosome is the vehicle of inheritance. Study of its nature and behavior has been the quest of cytogenetics for 60 years.

Study of the cell in the period between two successive mitoses (interphase) was not paid equal attention until 1941. The interphase nucleus was considered a state of inactivity and was then called the "resting stage".

In recent years, the development of cytochemistry and microspectrophotometry has provided new tools for studying the metabolic processes of the cells. Through the work of two prominent figures in these fields, Brachet (1941) and Caspersson (1941, 1950), the relationship between RNA and protein synthesis was first realized, and microspectrophotometric studies suggested that cells synthesized DNA during interphase rather than during mitosis (Ris and Mirsky, 1949: Swift, 1950).

With new techniques such as autoradiography applied to biological studies, Howard and Pelc (1951), using ^{32}P (in the form of $NaH_2P^*O_4$) labelled DNA in Vicia faba, and showed that ^{32}P was incorporated only in interphase nuclei. Thus it has become evident that during interphase metabolic activity occurs which is profoundly significant to the cell. In 1953, Howard and Pelc used a continuous labelling method and treated intact Vicia faba root meristems with ^{32}P for different intervals

after which the material was fixed, the ^{32}P in RNA was removed, and autoradiographs were prepared. On the basis of results obtained, they found the incorporation of ^{32}P into DNA during the 'middle' stage of interphase and divided the nuclear cycle (Fig. 2.1-1) into four stages:

1. Presynthetic interphase of DNA (G_1), the interval between the end of mitosis and before the onset of DNA synthesis.

2. The duration of DNA synthesis (S).

3. Postsynthetic interphase of DNA (G_2), the interval between the end of DNA synthesis and the onset of mitosis.

4. Mitosis (M).

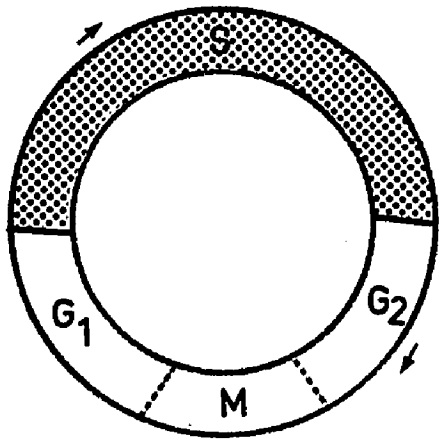


Fig. 2.1-1 Schematic representation of the nuclear cycle (after Wimber, 1960).

Since then, ^{14}C and ^{3}H have been incorporated into other nucleic acid precursors such as adenine, cytidine, uridine, deoxycytidine, thymidine, etc., which have proved to be very useful in the study of cell kinetics. So far, the most popular of these labelled substances has been ^{3}H-thymidine.

^{3}H-thymidine is specifically incorporated into DNA molecules during synthesis. Therefore, a more accurate labelling technique for the study of the duration of the nuclear cycle was developed by Quastler and Sherman (1959) in intestinal epithelium of mouse and by Wimber (1960) in Tradescantia root tips. There are recent descriptions of techniques to estimate the duration of the nuclear cycle (Thrasher, 1966; Van't Hof 1968). In essence, the material is given a short period of pulse-label with ^{3}H-thymidine. The ^{3}H-thymidine, a DNA precursor, is incorporated into DNA molecules during the DNA synthetic period. The material is sampled at intervals thereafter, and the proportions of mitoses which were labelled in autoradiographs are estimated. The change in frequency of labelled cells allows one to "read back" into interphase to ascertain the earlier events, so that the durations of each phase in the nuclear cycle can be estimated.

Many workers have reported the durations of nuclear cycles in various organisms (Table 2.1-1). It can be shown that the durations of the cycle are tissue and organism specific. Mitosis occupies a relatively small portion of the cycle and the duration of DNA synthesis, the S period, occupies about one half of the cycle in plant root meristems. In some animal tissues, about one half of the total time is spent in G_1.

Table 2.1-1

Duration of the nuclear cycle from different organisms

Organism	Temp. (°C)	Duration (hr.) G_1	S	G_2	M	Total	References
A. Animal cells in culture							
Chinese hamster fibroblast	37	2.7	5.8	2.1	0.4	11.0	Hsu et al., 1962
HeLa P_7	37	24.0	7.0	3.5	0.5	35.0	Toliver and Simon, 1967
Human amnion	37	9.8	6.8	2.2	0.6	19.4	Sisken and Morasca, 1965
Human epithelium	37	11.2	5.4	3.9	1.2	21.7	Cleaver, 1967
Mouse fibroblast	37	9.1	9.9	2.3	0.7	22.0	Dewey and Humphrey, 1962
B. Plant root meristems							
Allium cepa	24	1.5	6.5	2.4	2.3	12.8	Bryant, 1969
Tradescantia paludosa	21	1.0	10.5	2.5	3.0	17.0	Wimber and Quastler, 1963
Triticum turgidum	20	3.2	5.5	4.2	0.8	13.7	Kaltsikes, 1971
Vicia faba	21	2.5	6.2	3.3	2.0	14.0	Webster and Davidson, 1968
Zea mays	28	-1.3	6.0	3.4	2.4	10.5	Douglas, 1971
Zea mays	25	2.0	5.0	1.8	1.1	9.9	Verma and Walden, 1971

In summary, the progression of the cell in the cycle of events is usually $G_1 \rightarrow S \rightarrow G_2 \rightarrow M$ (Figure 2.1-1). It has been well documented that many metabolic activities occur during interphase. It is useful to look at some of the known metabolic events which serve as part of the prelude to mitosis. In order for a cell to divide, each of these events must have occurred at the proper rate and time. Presumably, control of any one of these event might serve as a control for the process of mitosis. In the present investigation, we have considered the following:

1. the disruption of the nuclear cycle,
2. endoreduplication, and
3. chromosomal aberrations and mutations.

2.2 The Disruption of the Nuclear Cycle:

Following eluciation of the mitotic division near the end of the nineteenth century, a number of investigators have tried to study mitosis by applying various chemicals. So far, studies on the induction of disturbances in the nuclear cycle by chemicals have formed a rather voluminous literature. It becomes evident that the question of "inhibition" requires an understanding of the mechanism of cell division and that inhibition and progress are but two sides of the same problem (Biesele, 1958).

It has been observed that some mitotic stages are especially sensitive to inhibitors while others have never been or are much

less disturbed by chemicals. According to their main effect on the nuclear cycle, inhibitors can be classified into two categories, namely those that disrupt mitosis and those that inhibit interphase.

2.21 Disruption of mitosis:

A large number of 'anti-mitotics' induce disturbances by some interference of spindle formation. The first of these agents to be discovered was colchicine. The first to recognize the colchicine effect as an inhibition of spindle function followed by an accumulation of mitotic metaphases was Ludford (1936), who worked with animal cells in vitro and in vivo. Early cytological studies on the effect of colchicine in plant material were reported by Gavaudan and Gavaudan (1937) and Dustin et al. (1937). In 1938, Levan proposed a term "C-mitosis" to describe the modification of mitotic behavior induced by colchicine. Many other agents, such as colcemide, podophyllin, vincaleukoblastine and mercaptoethanol and vinblastine have a similar effect (Gelfant, 1963; Stone, 1968).

The mechanism is by no means clear and, of course, not necessarily the same for all agents. The evidence favors the general notion that colchicine might produce its effect by interfering with the establishment of secondary bonding which is responsible for the orientation and geometry of the fully formed spindle (Mazia, 1955).

Some agents prevent the transition from late prophase to premetaphase, while there was no effect on metaphase and anaphase.

These agents, such as 3-deoxyadenosine, ethidium bromide and ribonuclease are RNA inhibitors (González-Fernández et al., 1970). The results obtained suggested that a synthesis of specific RNA during prophase was required to enable the cells to go on from the prophase to the metaphase of mitosis. The other specific effect of 3-deoxyadenosine is to block the rupture of the nuclear membrane, giving rise to a process of endomitosis in the cells treated (Giménez-Martin et al., 1971).

The formation of the cell plate can also be inhibited by the action of various chemicals, without affecting chromosomes or nuclear division. The results of the first division in the presence of these agents is a binucleate cell. Multinucleate cells are formed when repeated division occurs in the presence of an inhibitor of the cell plate formation (Giménez-Martin et al., 1965). On the other hand, González-Fernández et al. (1970) demonstrated that when karyokinesis was arrested at the prophase stage by treatment with ethidium bromide, cytokinesis followed its normal course, i.e., a prophase nucleus can be observed with a cell plate. These results suggested that cytokinesis was not dependent on the synthesis of RNA once the cell has initiated the prophase. Chemicals which suppress cell plate formation are nicotine, chloroform and caffein (Shigenaga, 1937; Kihlman and Levan, 1949), the polycyclic carcinogen; aminopyrine (Fourcade et al., 1963) and purine derivatives (Kihlman, 1949).

2.22 Inhibition of interphase:

The inhibition of the onset of mitosis does not provide much information concerning the progress of a cell towards division. Fortunately, the cycle is divided into three interphase periods plus the period of mitosis (Howard and Pelc 1953; Quastler and Sherman 1959; Wimber 1960). The concept of G_1, S and G_2 also opens a new approach to the study of interphase preparations for mitosis which may lead to a detailed "time map" of the biochemical and physiological events as they occur in relation to the time in interphase (Mazia, 1961).

I shall try to summarize the effect of chemicals on different phases of the cycle and to approach a "time map" of biochemical and physiological events. These studies should ultimately provide considerable useful information on critical processes in the cycle. Pertinent data, derived from many studies, show that the sensitivity of each phase of the interphase is drug specific (Table 2.22-1).

Furthermore, the idea of 'transition points (T Ps)' both for protein synthesis and for RNA synthesis has been suggested from studies in cultured human carcinoma strain K.B. cells with chloramphenicol and puromycin (Taylor, 1963), and in HeLa cells with actinomycin D and p-fluorophenylalanine (Mueller and Kajiwara, 1966). In 1969, Doida and Okada carefully analyzed and established the points of molecular events in the G_2 phase of mouse leukemic cells (L5178Y) by the methods of metaphase accumulation, ^{3}H-thymidine

Table 2.22-1

Representative mitotic inhibitors which affect the interphase of the nuclear cycle

Chemicals	Type of cells	Stage affected	References
A. Animal cells in culture:			
Actinomycin	Rabbit kidney cells	G_1 and S/G_2	Kishimoto and Lieberman, 1964
	Mouse leukemic cells	S/G_2	Doida and Okada, 1969
Chloramphenicol	Human K.B. cells	G_2	Taylor, 1963
Maleuric acid	Ehrlich ascites (tumor)	G_2	Okada and Roberts, 1960
Mitomycin C	Human leukocytes	S,	Nowell, 1964
Nitrogen mustard	Mouse skin epidermis	G_2	Gelfant, 1963
Puromycin	Mouse leukemic cells	G_2	Doida and Okada, 1969
	Human K.B. cells	G_2	Taylor, 1963
	Rabbit kidney cells	G2	Kishimoto and Lieberman, 1964
Thymidine	Chinese hamster (CHO)	G_1/S	Petersen and Anderson, 1964
B. Plant root meristem:			
5-amino uracil	Vicia faba	G_2,	Jakob and Trosko, 1965
	Vicia faba	S/G_2	Socher and Davidson, 1971
Chloramphenicol	Zea mays	G_2 and S	Verma, 1972
Cycloheximide	Zea mays	G_2 and S	Verma, 1972
Diaminopurine	Vicia faba	G_1 and G_2	Setterfield and Duncan, 1955
8-ethoxycaffeine	Vicia faba	G_2	Scott and Evan, 1964
Ethyl alcohol	Allium cepa	S and G_1	Arcara and Ronchi, 1967
5-fluorodeoxy-uridine	Vicia faba	G_2	Socher and Davidson, 1971
Maleic hydrazide	Vicia faba	S	Evans and Scott, 1964
2,4,5 Trichloro-phenoxy acetic acid	Vicia faba	S, and G_2	MacLeod, 1969

labelling, ^{3}H-uridine labelling, cell number counting and decline of mitotic index.

Tobey et al. (1966) and Petersen et al. (1969) studied Chinese hamster ovary (CHO) cells in which the measurement of the effects of actinomycin and cycloheximide on the G_2 of CHO cells was carried out. They found that actinomycin produced a block at 1.9 hr. before division (i.e. the end of essential RNA synthesis). Cycloheximide produced a block at 1 hr. before division (i.e. the end of essential protein synthesis). These two blocks represent TPs of the essential RNA synthesis and protein synthesis in G_2 (i.e. RNA TP and protein TP).

In microorganisms, a similar phenomenon has been described from the effect of an actinomycin pulse on Paramecium aurelia, grown in micro-cultures (Hanson and Kaneda, 1968). The TP is about three-quarters through the cycle. Cells treated after the TP were delayed to some extent (2 times longer than in the control) in the next cycle, but the first division cycle was not delayed. Cells treated before the TP were delayed in the first division which is longer or equal to a cycle time (about 5 hours at 29°C). However, the duration of the next cycle was not affected. The interpretation of Hanson and Kaneda (1968) assumes that sequential gene action through the cycle up to the TP is needed for division and that the effect of actinomycin pulse is to prevent the formation of a gene product at that time in the cycle.

The control mechanism of the cycle could be understood much better through further biochemical and physiological studies. Thus far, it has been proposed that the nuclear cycle is dependent on a sequence of transcriptions and translations. Since cells were found to be unable to continue their cycle when protein synthesis was inhibited, sequential gene action and a controlling system may be assumed.

2.3 Endoreduplication:

Since the discovery and interpretation of polytene chromosome structure by Kostoff (1930), and Heitz and Baur (1933), a number of papers have been devoted to the problems of internal chromosome reproduction. For example, the nuclei of the ilium of mosquito larvae, Culex pipiens, can reach enormous size in the resting stage prior to metamorphosis and show large numbers of chromosomes (Berger, 1937, 1938).

In plant species, Mantor (1935) found that the chimeras of Iberis root tips contained not 22 single chromosomes but 22 pairs of chromosomes. In the old part of the meristematic region of Spinacia root tip cells with the tetraploid and octaploid numbers of chromosomes occur frequently and regularly, and lie in pairs, closely attached to each other (Langlet, 1927; Lorz, 1937; Gentcheff and Gustafsson, 1939). Longlet (1927) applied the term "polysomaty" to somatic tissues of plants which contain multiples of the typical chromosome number. The configurations are best interpretated as

"diplochromosomes" (White, 1936). Ervin (1939) found similar chromosome configurations in Cucumis melo. Experiments involving the X-raying of Spinacia seeds proved that a double reproduction of chromosomes (diplochromosomes) in Spinacia occurs before the first prophase stage becomes visible (Gentcheff and Gustafsson 1939a, b). Berger (1941) confirmed this observation and stated that reproduction of the chromosomes of Spinacia and Culex occurs in the resting stage. He drew a parallelism between these two cases where the evidence points to the interpretation that the multiple chromosome complexs owe their origin to "repeated chromosome division without mitotic activity" (Berger, 1937).

White (1935) first demonstrated in Lucusta that X-ray induced "a new chromosome abnormality" which led to chromatid tetraploidy with only a diploid number. In 1936, he termed this "new chromosome abnormality" as "diplochromosomy". In Allium root meristems, Levan (1939) employing long term (3 to 5 days) treatment with growth-promoting substances such as indol-3-butyric acid (IBA) and naphthalene acetic acid (NAA) found the same chromosome configurations as described earlier by White (1936). Levan (1939) considered that the doubling of the chromosome number took place in the resting stage. Barber (1940) showed that when the potted plants of Fritillaria meleagris were placed in an incubator for two days at 30°C, the meiotic nuclear divisions were suppressed such that a diplotene nucleus lapsed directly into a pollen-grain resting nucleus. The pollen grain chromatid division took place, to give, at metaphase of the pollen grain mitosis, a diplochromosome bivalent consisting of eight chromatids. Ervin

(1941) described slight increases in the frequency of polysomatic cells in Cucumis root tips as a result of certain heat treatments and certain treatment with IBA.

Human leukocyte cultures treated with gamma rays developed polyploid cells and almost half of these cells contained diplochromosomes (Ohnuski et al., 1961). The study of X-ray induction of polyploidy showed the same effect as gamma rays (Bell and Baker, 1962).

In neoplastic tissues, polysomaty or diplochromosomes can be observed frequently. Levine (1931) described the occurrence of tetrad-like structures at metaphase in crown gall tumors of Beta vulgaris. Wipf and Cooper (1940) showed the somatic doubling of paired chromosomes in the rhizobial tubercles on the roots of certain legumes. In mouse ascites tumors, Levan and Hauschka (1953) found cells in the mitotic stages showing groups of two, four, eight or more homologous chromosomes, indicating the doubling of the chromosome number during interphases. They suggested a term "endoreduplication" to indicate these processes.

Diplochromosomes (or endoreduplication) were found in a patient with acute monocytic leukaemia (Reisman et al., 1963), and in testicular feminisation (Aspillaga et al., 1964). Biesele et al. (1962) showed endoreduplication in a leukocyte culture from a woman with a modal chromosome number of 45. She was a carrier of a 15/21 translocation and the mother of a Down's syndrome child. Diplochromosomes also were found in pernicious anaemia patients with a normal chromosome complement (Bishun et al., 1964).

In short, endoreduplication seems to occur under much the same circumstances as a number of other "non-specific" chromosome abnormalities,

but is seen in profusion only in tumors, including acute leukaemia.

In recent years, Patau and Das (1961) working with tobacco pith culture, have studied the relationship between DNA synthesis and mitosis and have concluded that DNA synthesis or replication is a prerequisite for mitosis, but both processes are essentially separate events. Data obtained from studies of human fibroblast cultures have indicated that if DNA is synthesized in excess of requirements for production of new cells, some of the existing cells become polyploid (Mittwoch et al., 1965). Bell (1964) reported endoreduplication in human leukocytes (in vitro) employing X-rays. Microspectrophotometry was used for measurements of DNA content at metaphase and showed that endoreduplicated cells in an X-irradiated culture of human leukocytes contained about twice as much DNA as diploid cells from the same culture. This is consistent with the hypothesis that a complete, second round of DNA synthesis has occurred in one nuclear cycle of these cells.

In the tobacco pith tissue cultures containing kinetin and indolyl-acetic acid (IAA) Patas and Das (1961) found that DNA synthesis can occur repeatedly in a nucleus without alternating mitosis. However, it comes to the problem of how to stop the cyclic events and repeat the DNA synthesis. Did the cell completely omit mitosis between two series of DNA doubling? Our studies concentrate on these points, and provide additional information about the phenomenon of endoreduplication.

2.4 Chromosome Aberrations and Mutations:

The word "mutation" is used to define a discontinuous and heritable change of the genetic material. There are two kinds of nuclear mutations: one is "point" mutation, which may be described as a chemical change of the gene at the molecular level, such as the incorporation of base analogues, alternation of bases of nucleic acid, transversion or exchange between bases, deletion and insertion of bases (Hayes, 1968). The other is a"chromosomal" mutation which is described as a gross morphological change in the structure of the chromosome.

Mutations arise spontaneously as well as through the action of mutagenic action. The induction of mutations was reported in 1927 by Muller. This was the first evidence that X-ray induced mutations were possible. But the first chemical mutagens were found only much later. About 32 years ago, the mutagenic action of nitrous acid was reported by Steinberg and Thom (1940) who obtained morphological mutants in Aspergillus niger and A. amstelodami by growing these species in a medium containing 0.2% $NaNO_2$. Urethane was shown to induce chromosomal breakage in Oenothera (Oehlkers, 1943). Allyl-isothiocyanate and diethy-sulphate were also found to induce gene mutations and chromosomal aberrations in Drosophila (Auerbach and Robson, 1944; 1946).

Since the mid forties, a large number of chemicals, belonging to a variety of chemical classes, have been shown to have radiomimetic

activity. Most of them produce similar cytological effects, such as stickiness, mitotic inhibition, etc., along with an important radiomimetic expression-chromosomal aberrations. The references about the chemical mutagens in microorganisms, plants and animals have been cited in review papers by Sharma and Sharma (1960), Barthelmess (1970), Fishbein et al. (1970) and Kalter (1971). An attempt to review all the available literature on the subject would be beyond the scope of this thesis. I intend to summarize only the localization of chromosomal breakage induced by chemicals. In addition, mitomycin C and hydroxylamine will be described in detail.

2.41 Location of chromosomal breakage:

In 1949, Ford proposed that nitrogen mustard did not induce chromosomal breaks randomly in Vicia faba but rather showed some specificity for heterochromatic regions. This non-randomness of chromosomal breakage was confirmed by Loveless and Revell (1949). Kihlman and Levan (1951), using 8-ethoxy caffeine (EOC) and tetramethyl uric acid, described the high frequency of breakage of the attachment thread of the satellite on the long chromosome 1 of Vicia faba. Chromatid exchanges also were localized in the nucleolar constriction of Vicia faba after treatment with EOC and deoxyadenosine (Kihlman, 1963). The distribution patterns of intra- and interchromosomal exchanges induced by ethyl alcohol and maleic hydrazide in Vicia faba are non-random and agent-specific (Michaelis and Rieger, 1968).

In Bellevalia romana, Gläss and Marquardt (1967; 1968) using nitrous acid and three nitrosamides (N-nitro-N-methylurea (NMH), N,N'-dinitroso-N,N'-dimethylterephthalamide (NMT) and 1-Nitrosoimidazolidone

(NIL)], showed that fragmentation of centromeres was more frequent than fragmentation of the chromosome arms.

A non-random distribution of chromosomal breaks has also been obtained in mammalian cells after treatments with certain chemicals. Somers and Hsu (1962) found that chromosomal breaks induced by the thymidine analogue 5-bromodeoxyuridine (BUDR) and hydroxylamine in Chinese hamster cells grown in vitro were agent-specific and location specific along chromosome 1. The aberrations induced by mitomycin C among the individual chromosomes of female and male chinese hamster cells in vitro were found to be non-randomly distribution (Natarajan and Schmid, 1971). These interesting findings included the observation that the short arm of the inactivated X chromosome (facultative heterochromatin) was seldom found to be involved in breaks. The long arm of the inactivated X was more often affected (5 to 10 times) than that of the long arm of the functional X though both are constitutive heterochromatin. Kihlman et al. (1963) have found that chromosome damage induced by deoxyadenosine and cytosine arabinoside in human leukocytes in vitro tended to be concentrated at the chromosome ends. An apparent localized distribution of chromosome damage was induced by streptonigrin among certain chromosome groups and confined to a specific segment of chromosomes, especially in the centromeric region. The secondary constrictions were most sensitive to mitomycin C (Cohen, 1969).

In Rattus natalensis cultured embryonic cells, Huang (1967) employed base analogues 5-bromodeoxy-uridine and 2-aminopurine, the viruses herpes, simplex virus and adenovirus type 12, and the carcinogens 7, 12-dimethylbenzanthracene and urethan, and showed that chromosomal

aberrations were also accumulated in heterochromatic regions. The X-chromosome and especially its long arm was more sensitive to these agents than any other chromosome.

It is clear that many different agents affect only heterochromatic regions. Therefore, one must consider the possibility that the specificity may not reside in the agent at all, but rather may reflect the sensitivity of the specific chromosome regions to non-specific environmental alteration. Natarajan and Ahnström (1969) studied the effect of bifunctional alkylating agents on two plant species - Nigella damascena and Vicia faba, the former lacking detectable heterochromatin and the latter possessing well organized heterochromatin. It has been shown that the localization of aberrations occurred in the heterochromatic regions of Vicia faba. On the contrary, Darlington and McLeish (1951) treated different species of plants with the growth regulator, maleic hydrazide (MH). They found that the frequently occurring chromosome breaks, were associated with the heterochromatic regions in Vicia faba. But in certain other species, namely, Rhoeo discolor, Muscari plumosus and Scilla sibiria, which have visible heterochromatin, MH failed to break the chromosomes. Do these species of plants contain different types of heterochromatin? If it is so, study on the location of specific chromosome breakage in relation to the heterochromatin may be useful in the determination of chromosome structure and function, particularly for those agents whose chemical action is well delineated (Somers and Hsu, 1962).

2.42 Mitomycin (MC):

MC, an antibiotic, which was isolated from Streptomyces caespitosus by Wakaki et al. (1958), demonstrated antibacterial activity as well as an antitumor effect. It causes inhibition of DNA synthesis in E. coli (Shiba et al., 1959) and degradation of DNA in E. coli as well as in tumor cells (Reich et al., 1961; Kersten, 1962; and Kersten et al., 1964), but does not interfere with RNA and protein synthesis (Reich and Franklin, 1961). Iyer and Szybalski (1964) found that MC behaved as a bifunctional alkylating agent upon chemical or enzymatic reduction.

Cytologically, MC has been shown to cause chromosomal breaks and unusual cross configurations of chromatid exchanges, especially in homologous chromosomes of human leukocytes (Nowell, 1964; Cohen and Shaw, 1964; Shaw and Cohen, 1965). The localization of chromosomal breaks has been shown to be at high frequencies at the secondary constrictions of human chromosomes and at heterochromatic regions of Chinese hamster fibroblast chromosomes (Cohen, 1969; Natarajan and Schmid, 1971). In Vicia faba chromosomal aberrations and homologous exchanges have been demonstrated by Merz (1961), Rao and Natarajan (1967) and Utsumi (1971). But in Trillium kamtschaticum MC did not cause any chromosome aberrations (Matsuura et al., 1963).

MC is a mutagen in E. coli in which it also increased the frequency of recombination (Iijima and Hagiwara, 1960). In Drosophila, it has been shown that MC increased crossing over, induced sex-linked recessive lethal mutation and X-Y chromosomal interchanges (Suzuki, 1965; Mukherjee, 1965; Schewe et al., 1971a, b). Smith (1969) has found temperature-sensitive recessive lethal mutations induced by MC in Habrobracon

sperm.

Holliday (1964) has found that MC is not mutagenic in Ustilago and Saccharomyces, but it increases mitotic crossing-over. In Glycine soja when the seeds or the stem tips of seedlings were treated with MC, it induced somatic crossing over and chromosomal aberrations leading to the different leaf spots (Vig and Paddock, 1968).

In general, MC has different effect on different organisms. In eucaryotic organisms it has induced chromosomal aberrations, mutations and somatic recombinations.

2.43 Hydroxylamine (HA):

HA is a nucleophilic and reducing agent. It was first reported as a strong mutagenic agent by Freese and his coworkers in 1961. Using bacteriophage T4, they found that the mutagenic effect was obtained only at high concentrations of HA and salt (NaCl) while low concentrations gave a rapid inactivation of the phage particles. Biochemical effects of HA at a pH of about 6 is known to react with cytosine and alter the structure of the base in such a way that it can pair with adenine instead of guanine. During DNA replication this change would result in a base-pair transition (Freese, 1963). At high concentration and high pH (pH 9), HA reacts exclusively with uracil and splits the pyrimidine ring (Schuster, 1961).

HA has been found to be an effective mutagen for animal viruses, tobacco mosaic virus, transforming DNA in B. substilis, and streptomycin resistant mutation in E. coli (Thiry, 1963; Schuster and Wittmann, 1963; Strack et al., 1964; Freese and Strack, 1962; Bresler et al., 1968 and Lie, 1964). Alikhanian et al. (1969) also found that HA induced amber mutations (UAG) in bacteriophage T4B.

Single-strand DNA viruses, phage S13 and X173, can be induced by HA in vitro as well as in vivo (Tessman et al., 1964; 1965). The effect of HA in vivo contrasts with the mutagenic effect in vitro. In vivo, HA is capable of reacting with a wide variety of molecules within the cell, not only with cytosine.

In eucaryotes, Malling (1966) and Malling and de Serres (1971) have found that genetic alterations in Neurospora induced by HA were base-pair transitions from GC to AT and not chromosome deletions. Color mutants can be induced in Schizosaccharomyces (Guglielminetti et al., 1967; Loprieno et al., 1969). Natarajan and Ramanna (1965), working with Hordeum suggested that HA is not mutagenic but that it is capable of inducing chromosome breaks. In Lycopersicum Jain et al. (1968) demonstrated that when seeds were treated with HA, M_1 plants did not possess any visible mutants. M_2 progeny, however, possessed morphological mutants.

HA induced an inactivating DNA alteration, and subsequently caused chromosomal breaks (Freese and Freese, 1966). Somers and Hsu (1962) have shown that HA induced chromosomal aberrations in in vitro cultures of Chinese hamster cells. Other effects of HA treatment were multiple constrictions, despiralization of chromosomes and

chromatid exchanges. Borenfreund et al. (1964) reported that HA and its derivatives, N-methylhydroxyl-amine and hydroxylamino-acetic acid, induced fragmentations, translocations and rearrangements of chromosomes of mouse embryo and Chinese hamster leukocyte cells in vitro. In human leukocyte cell culture, Engel et al. (1967) also found a particular sensitivity of centromeric regions under the treatment of HA.

HA has been reported to be active in plant material. Chromosomal breakage and fragmentation were observed after treatments of Vicia faba and Allium cepa root tips with HA (Cohn, 1964a). The action of HA on Vicia faba chromosomes showed that the breakage points were found to be localized and these regions were found to coincide with some of known heterochromatic regions and centromeres (Natarajan and Upadhya, 1964). Cohn (1964b) studied the induction of breakage and reunion by HA and X-ray in the lateral roots of Vicia faba. He found that HA not only caused chromosome breaks but also inhibited their rejoining. On the contrary, Nicoloff et al. (1971), working with primary roots of Vicia faba, suggested that HA induced both intra- and interchanges of the chromatid type.

In short, HA is a strong mutagen in micro-organisms, while it shows little effect in higher organisms. It seems likely that the induction of mutations and chromosomal aberrations are two independent events.

2.5 Somatic Cytogenetics of Zea mays:

Meiotic chromosomes of Zea mays have been studied intensively for the last 40 years. Many cytogeneticists have concentrated on analyzing linkage groups and meiotic behavior of chromosomal aberrations (i.e. deficiencies, duplications, inversions and translocations) induced by radiation. The study of mitotic chromosomes of Zea mays has not received equal attention.

With the development of autoradiography, Clowes (1965), and Verma and Walden (1971) have demonstrated the duration of the nuclear cycle in Zea mays. Genetic control of the nuclear cycle has been suggested (Verma and Walden, 1971). On the other hand, Graf (1957) demonstrated that the number of chromosomal breaks and bridges in anaphase induced by Maleic hydrazide (MH) and N-acetyl maleic hydrazide is highly correlated with the number of heterochromatic knobs in Zea mays. However, his data on the localization of the chromosomal breaks has not been available.

Since the report by Chen (1969), a series of somatic chromosomal studies have utilized the karyotyping procedure. Filion (1970) demonstrated the amount of change required in a pachytene chromosome to detect reliably the change in a somatic karyotype. Douglas (1971) demonstrated the chromosome replication profiles in Zea mays, and suggested that the control of synchrony was at the level of the nucleus as well as within chromosome segments.

Recently, an analysis of pattern in the distribution of breakage points in the meiotic chromosomes has been reported by Jancey and Walden (1972). These authors showed that the distribution of the breakage points was not an equidistribution. In addition, mitomycin C (MC) has been shown to induce localized chromosome breaks in Zea mays (Lin and Walden, 1971).

It is clear from the literature that the study of the mitotic cytology of Zea mays has been limited. Therefore, an extensive study has been made to investigate the effects of chemicals on chromosomal aberrations and location of the chromosomal breaks in Zea mays root tip cells.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental Protocol:

An abbreviated description of the protocol is outlined in Figure 3.1-1. The flow chart lists all the major steps of this study.

3.2 Description of Material and its Preparation:

3.21 Stock information:

Two maize stocks were employed in these investigations. They are "Seneca 60" and a chromosome 9-tester stock. "Seneca 60" was obtained from Robson Seed Farms Corporation, Hall, New York. It is a single cross ydrid with homozygous, triple dominant, alleles for $\underline{Yg_2}$, $\underline{Sh_1}$ and $\underline{Wx}$ in the short arm of chromosome 9, and homozygous recessive for $\underline{su_1}$ in short arm of chromosome 4. The chromosome 9-tester stock, a homozygous recessive inbred ($\underline{yg_2}$, $\underline{sh_1}$ and $\underline{wx}$), was obtained from the Cytogenetics Laboratory, the University of Western Ontario, London,Canada. Genetic positions of the marker genes are shown in Figure 3.21-1, and the phenotypes are listed in Table 3.21-1.

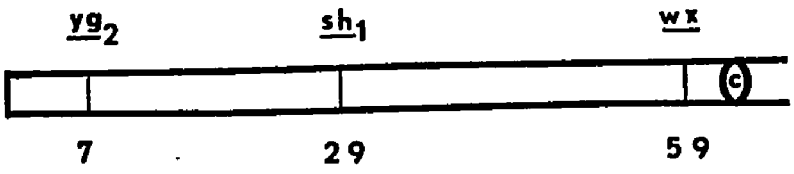


Fig. 3.21-1. Diagrammatic representation of the short arm of chromosome 9 stock showing relative positions of genes. c = centromere.

Fig. 3.1-1

Flow chart of experimental protocol.

1. ——— indicates material used, either seed or tassel.

2. —·—·— indicates a collection of data.

3. — — · — indicates chemical treatment or temperature.

4. · — — — indicates the results with which a final analysis was carried out.

5. ------ indicates interpretation and summary of data.

FIG. 3.1–1 FLOW CHART OF EXPERIMENT PROTOCOL

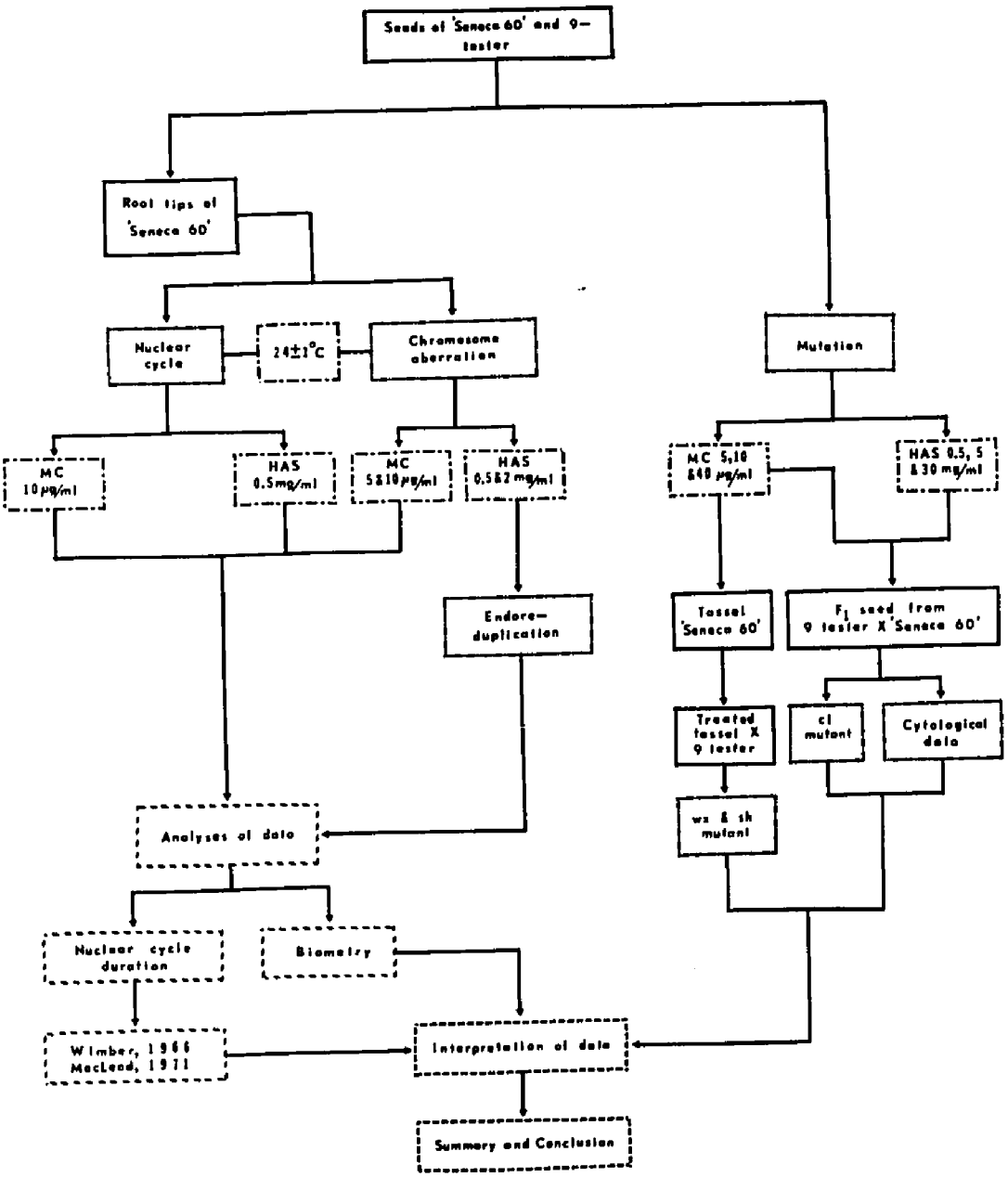


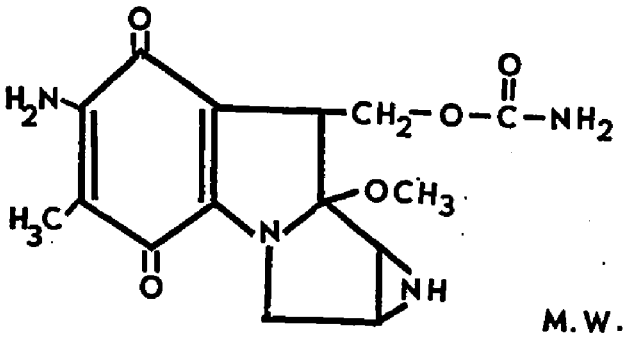
Table 3.21-1

Genetical data for chromosome stocks

Location	Linkage position	Allele symbol	Character	Phenotype
Short arm of chromosome 9:				
	7	yg_2	yellow green leaf	Persistent yellowish green plant color.
	29	sh_1	shrunken endosperm	Mature endosperm collapses to give smoothy indented crown or sides of dry kernel.
	59	wx	waxy endosperm	Endosperm produces waxy starch which reacts to I_2-KI solution with reddish brown color.
Short arm of chromosome 4:				
	71	su_1	sugary endosperm	Endosperm wrinkled, translucent, when dry: kernels distended and sweet at milk stage.

3.22 Chemical additives:

Mitomycin C (MC) and hydroxylamine sulfate (HAS) were purchased from Mann Research Laboratory, Division of Becton-Dickinson and Company, New York. Their structural formulas and molecular weights (M.W.) are shown in Figure 3.22-1.



M.W. =334.32

A; MC

$(NH_2OH)_2 \cdot H_2SO_4$ M.W. =164.14

B; HAS

Fig. 3.22-1 The structural formulas and molecular weights of MC (A) and HAS (B).

3.23 Germinating the roots, pulse labelling and their treatments:

Seeds of "Seneca 60" were washed in distilled water before germination. The seeds were germinated and grown on moist filter paper in sterile Petri dishes in a controlled germination chamber. The temperature was controlled at $24 \pm 1^{\circ}C$. Germinated kernels with primary

roots about 2 to 4 cm in length were used for these experiments.

The experimental approach consists of input in the form of cells entering the DNA synthetic period (S), pulse labelling for 30 min., removal and washing, followed by a return to the germination chamber in preparation for various collection periods. ^{3}H-thymidine, purchased from the New England Nuclear Corporation, was used for these experiments. The structural formula is shown in Figure 3.23-1. ^{3}H-thymidine, a DNA precursor, is incorporated into the DNA molecules during the DNA synthesis period. The change in frequency of labelled cells allows us to "read back" into interphase to approximate the earlier events.

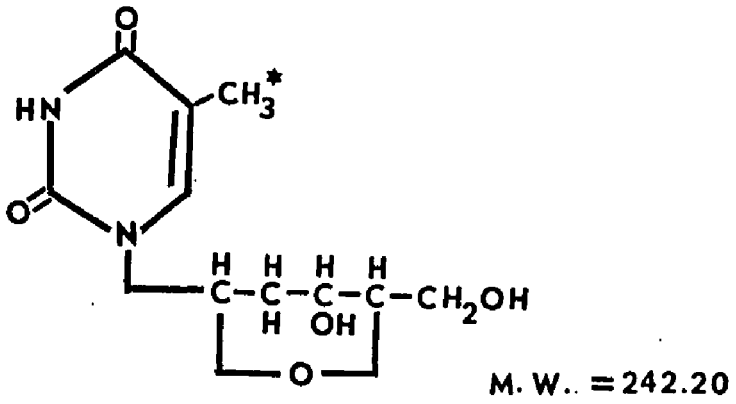


Fig. 3.23-1. The structural formula of ^{3}H-thymidine.

To study the effects of MC and HAS on the nuclear cycle, the suitable concentrations for induction of chromosomal aberrations used for these experiments included; 2 mg of MC, dissolved in 200 ml of

distilled water (= 10μg/ml), and 100 mg HAS in 200 ml (= 0.5 mg/ml). The intact roots were exposed for 30 minutes to ^{3}H-thymidine (1μc/ml; specific activity 6.7 c/mM) in Petri dishes without filter paper, followed by a MC (10μg/ml) or HAS (0.5 mg/ml) treatment for 2 hours.

After treatment, the intact roots were washed throughly in distilled water and returned to the germination chamber for further growth. The roots were collected at random from the germination chamber and fixed in freshly made glacial acetic acid: alcohol (1:3 v/v) at two hour intervals during the incubation period.

To study the effects of MC and HAS on the incorporation of ^{3}H-thymidine into nuclei, autoradiographs were prepared. The intact roots were exposed to (0.5 μc/ml) ^{3}H-thymidine for 2 hours as the control. In the treatment, two different experiments were conducted:

(a) The intact roots were treated with a mixture of the drug and ^{3}H-thymidine (0.5 μc/ml) for 2 hours. This was to study the incorporation during treatment with drugs.

(b) Roots were treated with the drug for 2 hours followed by 2 hours ^{3}H-thymidine (0.5 μc/ml), to study the post effect of the drug.

Extensive experiments on the effect of MC, HAS or colchicine on mitotic index for varying length of exposure were carried out. The intact roots were treated with MC (10 μg/ml), HAS (0.5 mg/ml) or colchicine (1 mg/ml) B-grade from Calbiochem, Los Angeles, up to 10 hours intervals during the treatment.

Pilot experiments were conducted to ascertain the suitable concentrations for chromosomal aberrations. Two concentrations of MC (5μg/ml; 10 μg/ml) and of HAS (0.5 mg/ml: 1 mg/ml) were used for these investigations. In order to make an accurate determination of stage sensitivity affected by chemicals in maize root tips, the ^{3}H-thymidine was used to label cells at a particular stage, i.e. S period of the nuclear cycle. Three types of treatments have been employed to study the effect of these drugs on the chromosomal aberrations.

Firstly, the intact roots were treated with chemicals for 2 hours without ^{3}H-thymidine involved. Secondly, the procedure followed was the same as that followed by the treatment of the nuclear cycle. Thirdly, the intact roots were treated with a mixture of ^{3}H-thymidine (1 μc/ml) and MC (or HAS) for 2 hours.

At the end of the treatment, the intact roots were given a wash and returned to the germination chamber for further growth. For the last 2.5 hours before fixation, the intact root tips were exposed to 0.3 mg/ml of 8-hydroxyquinoline (for chromosome pre-treatment). Fixations were made at two hour intervals during the incubation periods.

3.24 Tassel and seed treatments:

Two types of experiments were conducted to study the mutagenicity of MC and HAS.

3.241 Tassel treatment:

In these experiments, "Seneca 60" and a homozygous recessive chromosome 9 tester stocks were used. The experiments were carried out at the Experimental Station of Plant Sciences Department, the University

of Western Ontario, London. Before crossing, tassels of "Seneca 60" of the male parent were treated with MC (5 µg/ml) for 6 hours. The time for treatment was about 4 to 6 days before pollen shedding. The leaves surrounding the shoot were slit open with a razor blade. The tassel branches were imbedded individually and tightly in cotton. Leaves were closed and wrapped with masking tape. A solution of MC (5 µg/ml) was injected into the cotton with a hypodermic syringe until saturation was reached (about 20 to 25 ml per tassel). The cotton and the masking tape were removed after the 6 hour treatment. The pollen from treated plants was collected and used to cross onto the chromosome 9 tester plants. Mutation frequencies of the sh and wx loci were determined from the F_1 seeds. Waxy was ascertained by the staining reaction of iodine solution (1%).

3.242 Seed treatment:

F_1 seeds (9-tester X "Seneca 60") were soaked in one of three concentrations of MC (5, 10, or 40 µg/ml) or of HAS (0.5, 5 or 30 mg/ml) at 22 $\pm$ 2°C for 12 hours. In order to facilitate uniform absorption during the treatment, the flasks containing the seeds were shaken every 30 minutes. At the end of the treatment, the seeds were washed in distilled water throughly and germinated on moist filter papers in germination trays at room temperature. Three days after treatment, six roots were selected randomly from each treatment and fixed for cytological studies. The percentage germination and the root length were also scored at that time. The germinated seeds were sown in the green-house. Seedling height was determined from

14 day old seedlings grown in the greenhouse; they were transplanted to the field. Visible chlorophyll mutations of M_1 plants were scored in the field during the entire life cycle of plants.

3.25 Cytological preparation:

Root tips were fixed in freshly made solution (3 parts alcohol: 1 part acetic acid) and kept overnight at room temperature. At the end of the fixation period, the material was placed in 70% alcohol and stored in the refrigerator until needed.

Root-tip squashes were made according to Chen's (1969) schedule. Hydrolysis was accomplished in 1N HCl at 60°C for 8 min. The roots were rinsed three times in distilled water and stained in leuco-basic fuchsin for 1 to 1.5 hours. Then the roots were treated with 5% pectinase (Nutritional Biochemical Corporation) for 1.5 to 2 hours, and transfered to 45% acetic acid for 10 min. to clean the cytoplasm. The root cap was removed by touching it to the surface of the filter paper or by a microscopic spear needle. Feulgen squashes of the terminal 1.2 mm of execised root tips were prepared in 45% acetic acid. One root tip was used per slide.

For autoradiographs, slides were cleaned with Kimwipes and Mayer's adhesive was smeared directly on to a precleaned slide prior to preparing the squashes. Squash preparations were sealed with wax and kept in the slide boxes at 5°C until needed.

3.26 Autoradiography:

The technique employed in the present studies is described as follows:

The coverslips were removed by freezing the slides on a block of dry ice. When the material was throughly frozen, coverslips were popped off by lifting with a razor blade under one of the corners. The slides were rinsed immediately in distilled water and air dried.

The air-dried slides were coated with Kodak NTB2 nuclear track emulsion (diluted one to one with distilled water at 40°C) by a dipping technique. Then the slides were allowed to drain and were air dried, before storing in exposure boxes for 14 days at 4°C. All work was conducted in a dark-room equipped with a Kodak Wratten safelight filter No. 2.

After 14 days storage in light proof boxes, the coated slides were developed in D-19 at 15°C for two minutes. Subsequently, the slides were transfered to distilled water for one minute and fixed in rapid fixer for three minutes.

Following fixation, the slides were washed in running distilled water for 20 minutes and dehydrated in an alcohol series up to absolute alcohol. The autoradiographs were mounted in Euparal (Kopriva and Leblond, 1962).

3.27 Photography:

Photographs were taken on a Zeiss Photomicroscope II equipped with an automatic exposure meter. An interference band filter, green (546 mμ), was used. Pictures of cells which contained chromosomal aberrations were taken using an oil emersion lens

(Planapo 100/1.3). High contrast copy 35 mm film (DIN 13) was used. Photographic films were developed in D-19. An Agfa Varioscop (35/44) enlarger was used for enlargements. Prints of the cells were enlarged to X 4000, and processed in a Kodak Ektamatic Print Processor.

3.3 The Karyotype:

To construct a karyotype, chromosomes were cut from the enlarged photographs (X 4000). The chromosomes were identified and matched in pairs and assigned a number in the order of decreasing length (Chen, 1969).

In quantification of breaks on chromosomes, the identification and measurements of chromosomes are very important. The enlarged photographs were projected on to a white paper to yield a final magnification of approximately X 20,000. The mid-line of a chromatid was traced with a pencil and measured with a Keuffel and Esser map reader.

All chromosomes from the best 12 cells of "Seneca 60" were measured. The mean relative length, length of each arm and length of centromere were recorded and the standard error for each mean were calculated. The ratio of the long arm to short arm was calculated as an index to indicate centromeric position. For chromosome lengths, a relative value was calculated as the percentage of the total complement length (%TCL), including centromere, satellite and secondary constriction.

3.4 An Analysis of the Data

3.41 Nuclear cycle:

The durations of the nuclear cycle and of its component phases were estimated from the proportion method of Quastler and Sherman (1959) i.e., from the rhythmic appearance and disappearance of labelled mitoses.

To estimate the duration of each stage, Sparvoli et al. (1966) assumed that in the normal population of cells, the frequency of cells in a stage was directly proportional to the duration of the stage. At the same time, Wimber (1966) proposed a modified method in which the duration of G_2 and one half prophase is substituted for the duration of G_2 and prophase. In 1968, Webster and Davidson suggested that the duration of the nuclear cycle might be calculated from the percent labelled prophase curve and metaphase curve.

The frequency of labelled mitosis curve was plotted as shown in Figure 3.41-1. This is a curve of labelled mitosis as a function of time after removal from ^{3}H-thymidine. The estimate of DNA synthetic period (tS) is taken as the time between the half-maxima of the ascending and the half-maxima of the descending slopes minus labelling time. The time between the start of the experiment and the first ascending prophase curve is taken as $tG_2 + \frac{1}{2}tP$. The duration of the total cycle (tC) is the time between half maxima on the ascending slopes of the first and second labelling peaks.

Fig. 3.41-1

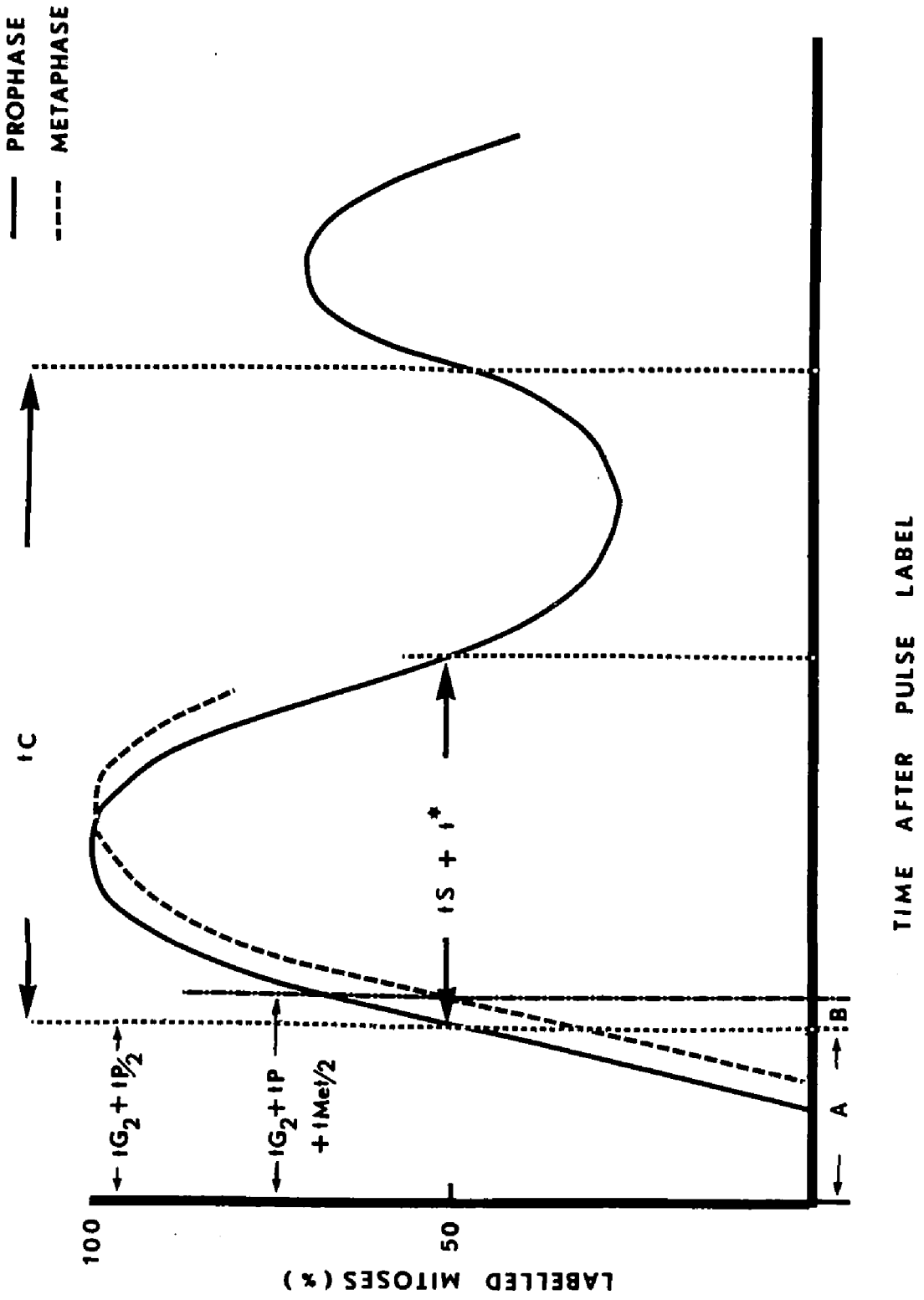
'Diagrammatic' percent labelled mitoses curve.

C = total cycle

t = time

t^* = time of pulse label (labelling time)

(See text for symbol descriptions).


PROPHASE
METAPHASE
LABELLED MITOSES (%)
100
50
TIME AFTER PULSE LABEL
A
B
tC
tS + t*
tG2 + tP/2
tG2 + tP + tMet/2

As shown in Figure 3.41-1, the duration of the nuclear cycle (tC) can be measured directly. The durations of G_2 and mitosis can be calculated as follows:

$$tG_2 + \tfrac{1}{2}tP = A \quad \text{.....(1)}$$

$$(tG_2 + tP + \tfrac{1}{2}tMet) - (tG_2 + \tfrac{1}{2}tP) = B \quad \text{.....(2)}$$

Therefore;

$$tP + tMet = 2\,B \quad \text{.....(3)}$$

The average nP/nMet ratio can be obtained from the number of prophase (nP) and the number of metaphase (nMet) in mitosis.

$$nP/nMet = K \quad \text{.....(4)}$$

Therefore;

$$nP = nMet \cdot K \quad \text{.....(5)}$$

where;

P = prophase

Met = metaphase

A = anaphase

T = telophase

t = duration

n = frequency of particular phase, e.g.,

nP = No. of prophase.

Therefore (5) can be substituted in (3) to obtain tP and tMet. tG_2 can also be derived by substituting tP in (1).

The duration of anaphase and telophase can be obtained in a similar manner;

$$\frac{tP}{nP} = \frac{tMet}{nMet} = \frac{tA}{nA} = \frac{tT}{nT}$$

Thus, the time of mitosis (tM) = tP + tMet + tA + tT. Also, tM can be calculated by the following formula (MacLeod, 1971):

$$tM = \frac{MI \times (tS + tL)}{LI}$$

where;

MI = mitotic index
LI = labelling index
tL = labelling time.

Therefore tG_1 can be calculated by substraction;

$$tG_1 = tC-(tG_2 + tS + tM).$$

In practice, slides were scored for the frequency of labelled mitotic figures and the mitotic index was calculated. For each collection period, 3 to 4 slides were examined to give a population of 6000 - 8000 cells. The control data was taken from Verma (1972).

3.42 Chromosomal aberrations:

Chromosomal aberrations were scored in metaphase or in anaphase. Metaphase scoring is preferable, since only by this method the various types of aberrations can be distinguished. The various types of frequencies of aberrations scored were chromatid breaks, isochromatid breaks, chromatid exchanges, chromosome translocation (or dicentric) and ring chromosomes. As to anaphase, the frequencies

of fragments, bridges and pseudo-chiasmata were also scored.

Both chromosomal aberration yield and presence of silver grains lying on the chromosome were calculated for determining the stage sensitivity to the chemical in maize root tips.

The relative amounts of damage produced in each chromosome and its specific chromosome region were identified and scored from the photographs. The location of each break was expressed as that percentage of the broken chromosome arm from the centromere to the break: i.e.,

$$\frac{\text{Distance from the centromere to the break}}{\text{Length of whole chromosome arm}} \times 100$$

All measurements employed the centromere as the origin and progressed along the chromosome arm in either direction. Each arm was divided into five equal segments, except that the short arms of chromosomes 6, 7, 8, 9 and 10 were divided into two equal units.

In computing the results, each chromatid break was counted as one break, and each chromosomal translocation or chromatid exchange as two breaks.

4.43 Biometry:

The following statistics were employed (Sokal and Rohlf, 1968);

4.431 Standard deviations:

For the proportions of labelled cells and mitoses, the standard deviations (S.D.) were calculated as follows;

$$S.D_{(p)} = \pm \sqrt{\frac{pq}{n}} \times 100$$

where;

p = proportion of labelled cells; or proportion mitosis;

q = (1-p), proportion of unlabelled cells; or proportion of interphase cells.

n = number of cells scored for each collection period.

For chromosomal measurements, standard errors(S.E.) were calculated as follows;

$$S.E. = \sqrt{\frac{\sum_{i=1}^{n} (X_i - \bar{X})^2}{n - 1}}$$

where;

$\bar{X}$ = mean value;

X_i = variable;

n = number of cells observed.

For chromosomal breaks, standard errors were calculated on the assumption of a Poisson distribution;

$$S.E. = \pm \sqrt{\frac{\bar{x}}{n}}$$

where;

$\bar{x}$ = mean aberration yield per cell;

n = number of cells scored.

4.432 Confidence limits for each points on percent labelled curve:

The 95% confidence limits were calculated from the formula;

$$95\% \text{ confidence limit} = \left(p \pm t_{0.05\,(\infty)} \cdot \sqrt{\frac{pq}{n}}\right) \times 100$$

where;

p = proportion of labelled cells in particular stage;

q = (1-P), the proportion of unlabelled cells;

n = number of cells scored for each collection period.

4.433 Chi-square (χ^2) test:

χ^2 was used for testing the distribution of chromosomal breakage among 10 pairs of chromosomes.

$$\chi^2 = \sum_{i=1}^{n} \left(\frac{d^2}{e}\right)$$

where;

n = classes or items;

d = deviation, i.e., observed value minus expected value;

e = expected value.

The number of labelled and unlabelled nuclei was used as the criteron and χ^2 test of a 2 by 2 contingency table was employed to determine the effects of incorporation.

$$\chi^2 = \frac{\left[(X_{11} \cdot X_{22} - X_{12} \cdot X_{21}) - \frac{N}{2}\right]^2 \cdot N}{(X_{1.})\ (X_{2.})\ (X_{.1})\ (X_{.2})}$$

4.434 t-test:

A t-statistic was calculated to test the mean number of silver grains per nucleus among different treatments. The formula used was

$$t = \frac{|\ \bar{x} - \bar{y}\ |}{\sqrt{\left(\frac{1}{N_x} + \frac{1}{N_y}\right)\left(\frac{\Sigma\ (X_i - \bar{X})^2 + \Sigma\ (Y_i - \bar{Y})^2}{N_x + N_y - 2}\right)}}$$

$$v = N_x + N_y - 2$$

where;

$\bar{x}$ = mean of the x values;

$\bar{y}$ = mean of the y values;

N_x = number of x values;

N_y = number of y values;

t = t-statistic;

v = number of degrees of freedom.

4.435 Correlation coefficient with test of significance of r;

The correlation coefficient(r)

$$r = \frac{\Sigma (X_i - \bar{X}) \cdot (Y_i - \bar{Y})}{\sqrt{\Sigma (X_i - \bar{X})^2 \cdot \Sigma (Y_i - \bar{Y})^2}}$$

was also computed.

To test the significance of r, the following test was used:

$$t = r \cdot \sqrt{n - 2/1 - r^2}$$

Degrees of freedom for this test are n - 2.

The χ^2 - test was performed on the PDP 10 computer in BASIC language while the other analyses were performed on a Programmable MONROE Calculator.

CHAPTER 4

RESULTS

Preliminary experiments were conducted to ascertain the suitable concentrations of MC and HAS, and their potentiality to induce chromosomal aberrations. The concentrations of MC and HAS found to be effective in maize for producing chromosomal aberrations were 5 - 10 µg/ml and 0.5 - 2 mg/ml for 2 hr., respectively. These concentrations did not cause any apparent lethal effect, and the root growth was healthy. Following fixation at 24 hr. after treatment, fragments and/or bridges were observed but no marked pycnotic effect was recorded. These concentrations were employed for studying the effects on nuclear cycle, mitotic index, chromosomal aberrations and mutations.

4.1. The Effect of MC on Nuclear Cycle and Mitotic Inhibition:

The frequency of labelled prophases in root tip cells of "Seneca 60" with or without a two-hour treatment with MC following 30 min. pulse-label with ^{3}H-thymidine is shown in Figure 4.1-1. The treatment with MC has lengthened the duration of the nuclear cycle. The appearance of the "first" labelling peak in the treatment curve followed a delay of 6 hr. and the peak was broader than that of the

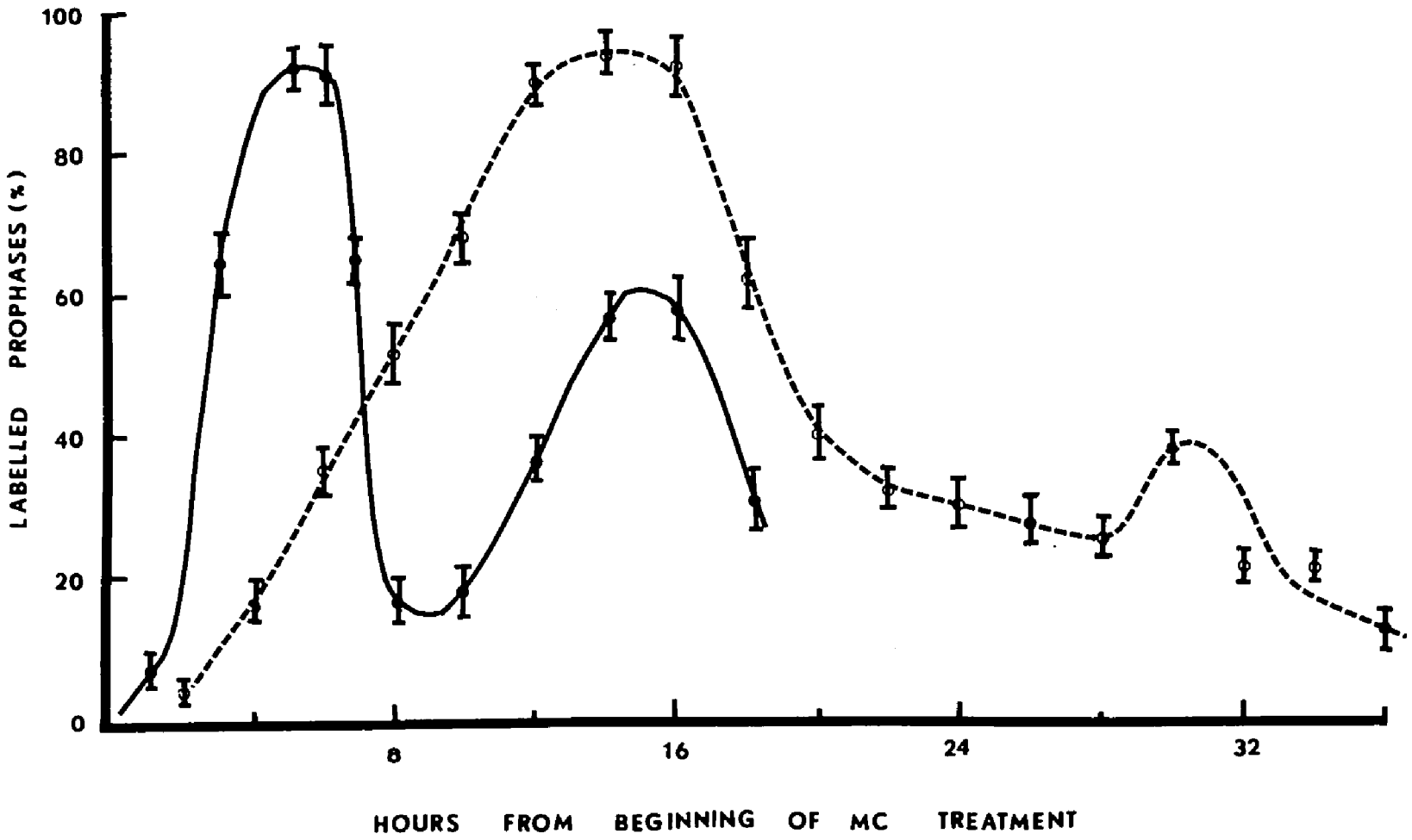
Fig. 4.1-1

A comparison of the proportions of labelled prophases at various times with or without 2 hr. treatment with (10 μg/ml) MC following the exposure of ^{3}H-thymidince for 30 min.

One standard deviation either side of the mean is shown as a vertical bar.

Control (———).

Treated (- - - - -).


100
80
60
40
20
0
LABELLED PROPHASES (%)
8
16
24
32
HOURS FROM BEGINNING OF MC TREATMENT

control. The "second" peak was delayed until 30 hr. after the beginning of 2 hr. MC treatment. The estimates of the duration of the nuclear cycle are shown in Table 4.1-1, and were derived from Figures 4.1-1 and 4.2-1 (Tables A-2, A-3 and A-6*).

Table 4.1-1 The duration (hours) of the nuclear cycle with or without a 2 hr. treatment with MC (10 µg/ml) or HAS (0.5 mg/ml) in Zea mays root tips at 24 ± 1°C.

Treatment	Phase				
	G_1	S	G_2	M	Total
Control	1.7	5.0	2.1	1.1	9.9
MC	2.4	11.5	6.6	1.5	22.0
HAS	-	7.5	12.1	1.6	15.0

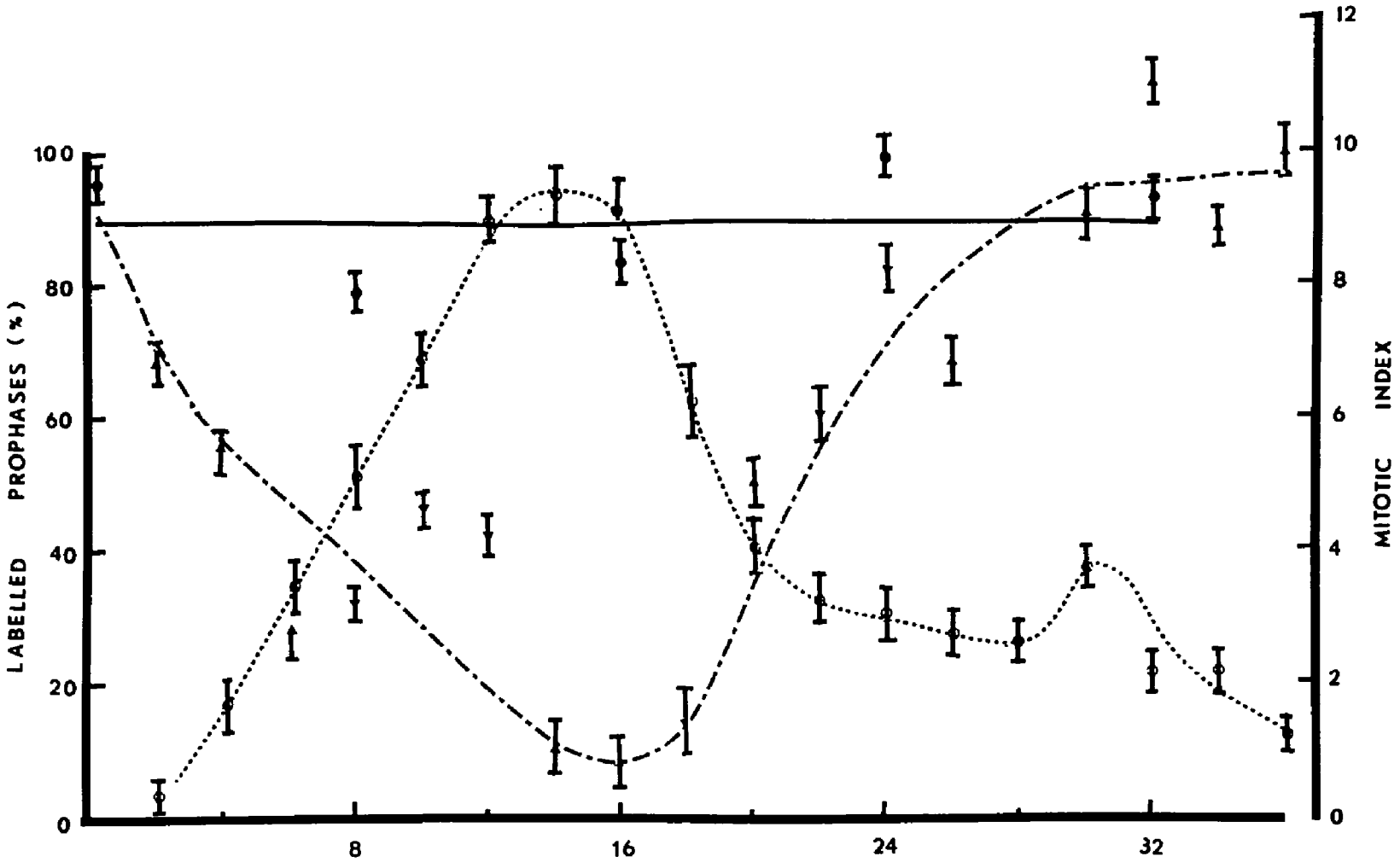
As shown in Figure 4.1-2, the relation between mitotic index and prophase labelling at various times from the beginning of a 2 hr. MC treatment indicates that the mitotic index was affected at the end of the treatment and even during the incubation period. In control roots, the mitotic index ranged from 7.9 to 9.8. Their mean is 9.0 ± 0.8 (Table A-1). A reduction in the mitotic index is evident shortly after the beginning of 2 hr. treatment, suggesting that there is

* Appendix A

Fig. 4.1-2

Relation between mitotic index (—·—·—)
and proportion of labelled prophases
(·····) at various times from the
beginning of MC (10 μg/ml) treatment for
2 hr..
Control mitotic index (———).
One standard deviation either side of the
mean is shown as a vertical bar.

N.B. Roots were given a 30 min. pulse-
label with ^{3}H-thymidine immediately
prior to a 2-hour treatment with MC.


LABELLED PROPHASES (%)
MITOTIC INDEX
100
80
60
40
20
0
12
10
8
6
4
2
0
8
16
24
32
HOURS FROM BEGINNING OF MC TREATMENT

an immediate delay in the rate of entry of cells into mitosis. The index value dropped to about 3.0 during the 6 to 8 hr. period in which the cells were in late S and very early G_2 during the treatment. At 10 to 12 hr. the observed mitotic index increased to 4.0. This showed a little effect on accumulation of the S cells toward G_2. Therefore the ascending curve of labelled prophase in the treatment provides a greater slope than that of the control. There are two factors which might lengthen the duration of G_2 plus prophase/$_2$, namely, the cells in G_2 were inhibited and/or the cells in late S period were inhibited entering into G_2 and mitosis.

During the 14 hr. to 18 hr. period after the beginning of MC treatment, the mitotic index had decreased to about 1.0, which in turn indicated that those nuclei were in a early S period and were inhibited by MC during the treatment. A sharp increase in mitotic index between 20 hr. and 24 hr. post-treatment is an indication of the entry into mitosis of cells which were in G_1 and M phase at the time of treatment. The mitotic index reached to the control value after 30 hr.. From these results, we would suggest that the site of an MC-induced block was mainly at early S phase. The high sensitivity of the S phase and the limited sensitivity of G_2 to the MC treatment result in lengthening the duration of the nuclear cycle in G_2 and S period.

Data from a continuous exposure to MC (10 μg/ml) throughout a 10 hr. period (24 ± 1°C), are presented in Figure 4.1-3 (Table A-10). Data for the control experiment are shown in Table A-9. The decline in mitotic index is proportional to increasing exposure time. The depression

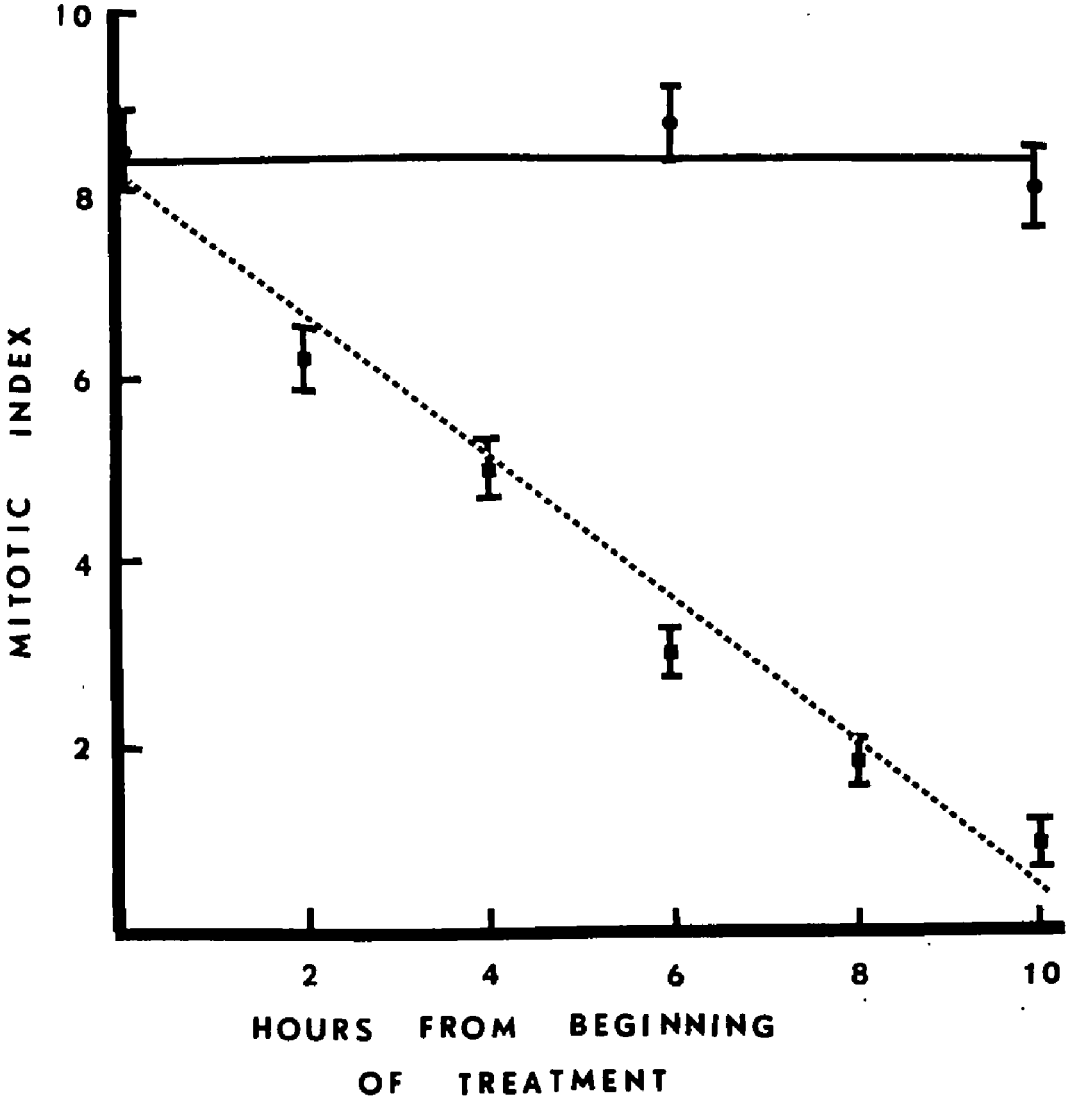
Fig. 4.1-3

The effect on mitotic index throughout a 10 hr. continuous treatment with MC (10 µg/ml).

Control (———).

Mitotic index throughout the course of MC treatment (·····).

One standard deviation either side of the mean is shown as a vertical bar.


10
8
6
4
2
MITOTIC INDEX
2
4
6
8
10
HOURS FROM BEGINNING
OF TREATMENT

of mitotic index is correlated with the length of exposure time ($r = -0.99$, $t = 13.16 > t\ (^{df = 3}_{p=.05}) = 3.18$). Analysis of the phase indices (proportion of each phase in mitosis) showed that there is a similar phase index in control and treatment. These data suggest that there is no blockage at any stage of mitosis. The decrease in mitosis appears to be due to an inhibition of interphase nuclei entering into mitosis.

4.2 The Effect of HAS on Nuclear Cycle and Mitotic Inhibition:

The results of an HAS (0.5 mg/ml) experiment are summarized in Figure 4.2-1. The data indicate that the treatment with HAS resulted in a delay in the appearance of labelled nuclei entering mitosis. There was a 10.5 hr. delay in the appearance of the "first" peak and a 15 hr. delay in the appearance of the "second" peak in the treatment curve.

The data presented in Figure 4.2-2 show that the mitotic indices declined immediately during the incubation period in comparison to control values. The mitotic indices of the control remain constant over the duration of the experiment with a mean of 9.0 ± 0.8. The relation between mitotic index and prophase labelling at various time intervals after HAS treatment indicates (the mitotic index decreased to about 0.9 - 1.8 during 6 hr. to 12 hr period from the beginning of the 2 hr. HAS treatment) that those nuclei which were in G_2 and at the end of the S period during HAS treatment. However, the mitotic index returned to the control value at 14 hr. and the curve is very steep, indicating the progress and accumulation of some S cells at the point of blockage during

Fig. 4.2-1

A comparison of the proportions of labelled prophases at various times with or without 2 hr. treatment with HAS (0.5 mg/ml) following the exposure of ^{3}H-thymidine for 30 min.

One standard deviation either side of the mean is shown as a vertical bar.

Control (——————).

Treated (- - - - - - -).

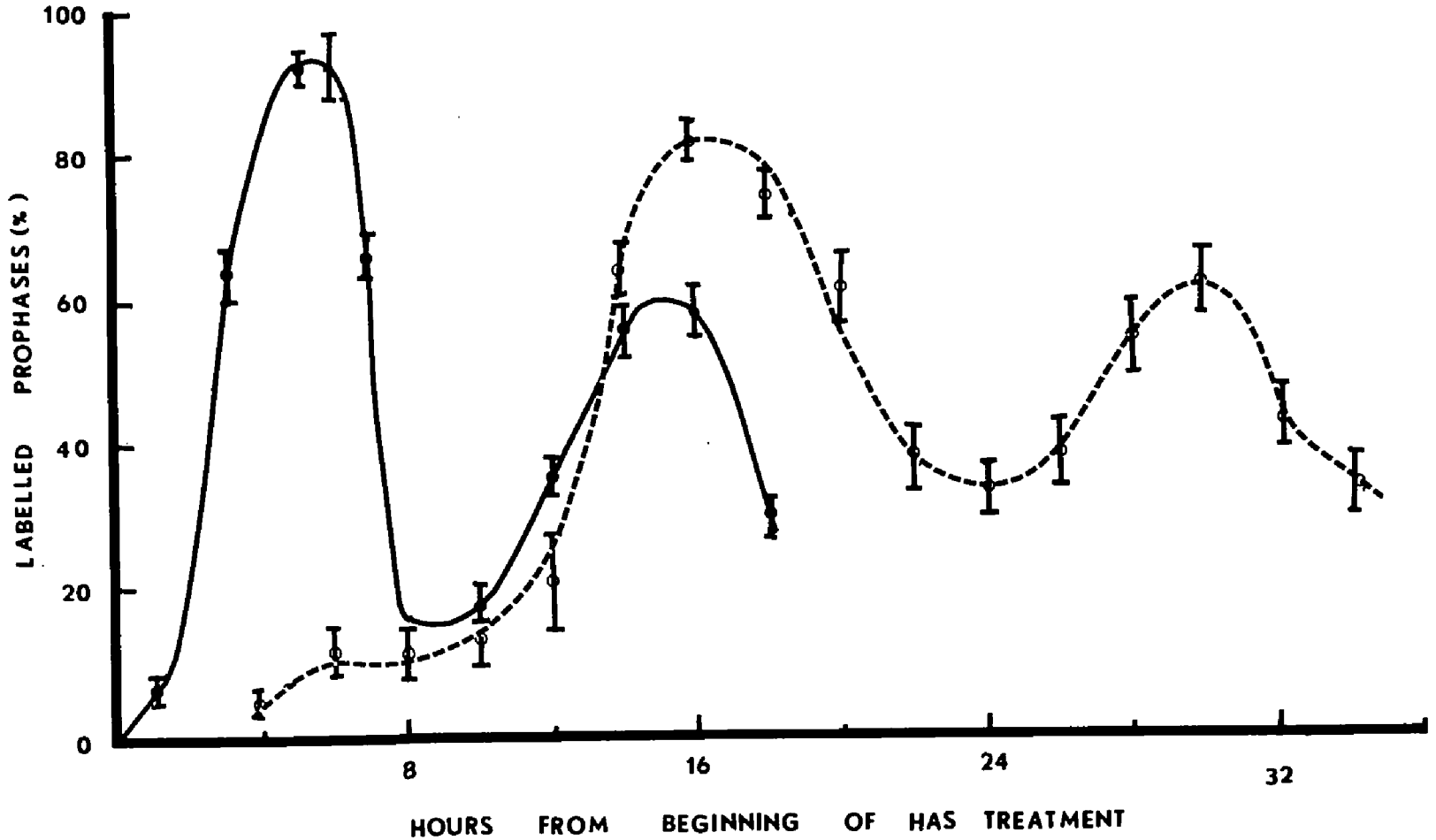
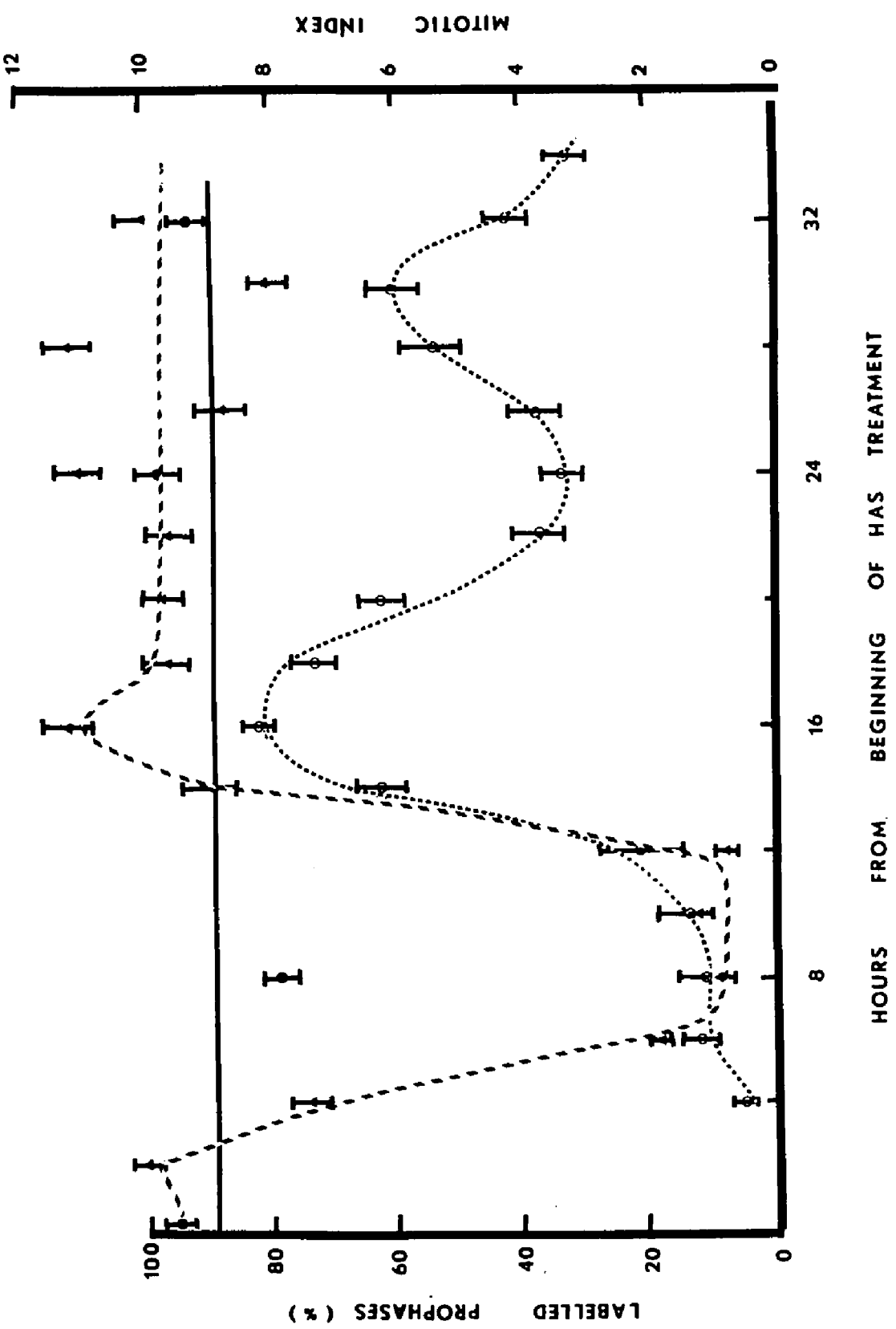

100
80
60
40
20
0
LABELLED PROPHASES (%)
8
16
24
32
HOURS FROM BEGINNING OF HAS TREATMENT

Fig. 4.2-2

Relation between mitotic index (- - - - -)
and proportion of labelled prophases
(----------) at various times from the
beginning of HAS (0.5 mg/ml) treatment
for 2 hr.
Control (———).
One standard deviation either side of the
mean is shown as a vertical bar.

N.B. Roots were given a 30 min. pulse-
label with ^{3}H-thymidine immediately
prior to a 2-hour treatment with HAS.


MITOTIC INDEX
12
10
8
6
4
2
0
LABELLED PROPHASES (%)
100
80
60
40
20
0
8
16
24
32
HOURS FROM BEGINNING OF HAS TREATMENT

inhibition. This blockage appears to be at the end of the S period. These results suggest that the effect of HAS on nuclear cycle was to inhibit and prolong the duration of G_2 cells and to inhibit the transition of late S cells to G_2. The duration of S period was also increased 2.5 hr. by this drug (Table 4.1-1).

Unlabelled dividing cells, appearing after the "first" labelling index peak, will consist of G_1 cells undergoing their first (T_1) post-treatment mitosis and also M and G_2 at their T_2 mitosis. In control roots, G_1 cells normally reach mitosis in 8 hr. (i.e., duration of G_2 + S), while after HAS treatment their arrival was delayed until about 21 hr.. We do not know the time required by M or G_2 cells to pass through the nuclear cycle following their T_1 division. Therefore, the actual duration of G_1 can not be estimated. The response of T_1 cells and T_2 cells to treatment is different. As it can be seen from the mitotic index, the mitotic index approached a normal value after 14 hr., which is at the end of S phase; the cells are blocked at this stage. Thus, the duration of G_2 at T_1 should be longer than that of G_2 at T_2. If we employ the interval between the half-maxima on the ascending slopes of the "first" and "second" labelling peaks to estimate the total duration of the nuclear cycle, and use the duration of G_2 at T_1 instead of the duration of G_2 at T_2 to obtain the duration of G_1, these estimates should underestimate the total time of the nuclear cycle and G_1 after HAS treatment (Table 4.1-1). However, G_2 phase and the transition of S to G_2 were also affected by HAS.

The results of continuous treatment with HAS (0.5 mg/ml) for periods up to 10 hr. applied to root meristematic cells are shown in

Figure 4.2-3 (Table A-11). The mitotic index index maintained the control value during the first 4 hr. of treatment. After that time, it dropped gradually in the course of the treatment. The depression of mitotic index is correlated with the length of treatment ($r = -0.89$, $t = 3.35 > t\ (^{df = 3}_{p = .05}) = 3.18$).

As shown in Figure 4.2-3, root tips treated for 8 hr. and 10 hr. yield a mitotic index which remains 5.2 and 5.6, respectively. But if root tips were left in HAS solution for 6 hr., and then transfered to the normal growth condition for 2 hr. and 4 hr., respectively, the mitotic indices dropped rapidly to 1.9 and 0.3. When the phase indices were analysed, the prophase index remained at a normal value, whereas the an increasing metaphase index and decreasing the anaphase and telophase index were observed. However, after 8 hr. continuous treatment, the metaphase index was about 43 in mitosis although the mitotic index decreased. These results indicate that HAS also had an effect on blocking the cell from metaphase to anaphase during long-term treatment. When intact roots were removed from the solution, the blocking effect disappeared and mitotic index dropped sharply.

What is the "colchicine action" on mitosis? A continuous treatment with colchicine (1 mg/ml) for 10 hr. at 24 ± 1°C yields the results shown in Figure 4.2-4 (Table A-12). An increase in mitotic index was observed. The initial increase in mitotic index at 2 hr. during the treatment results from an increased number of cells in metaphase. The highest mitotic index is 21.4 during a 6 hr. treatment. At the end of 10 hr., the mitotic index still remained at 18.4. The increase is not correlated with the duration of exposure time ($r = 0.72$,

Fig. 4.2-3

The effect on mitotic index after 6 hr. or throughout a 10 hr. continuous treatment with HAS (0.5 mg/ml).
One standard deviation either side of the mean is shown as a vertical bar.
Control (———).
Continuous treatment (·····).
6 hr. continuous treatment followed by growing in normal condition (—·—·—).

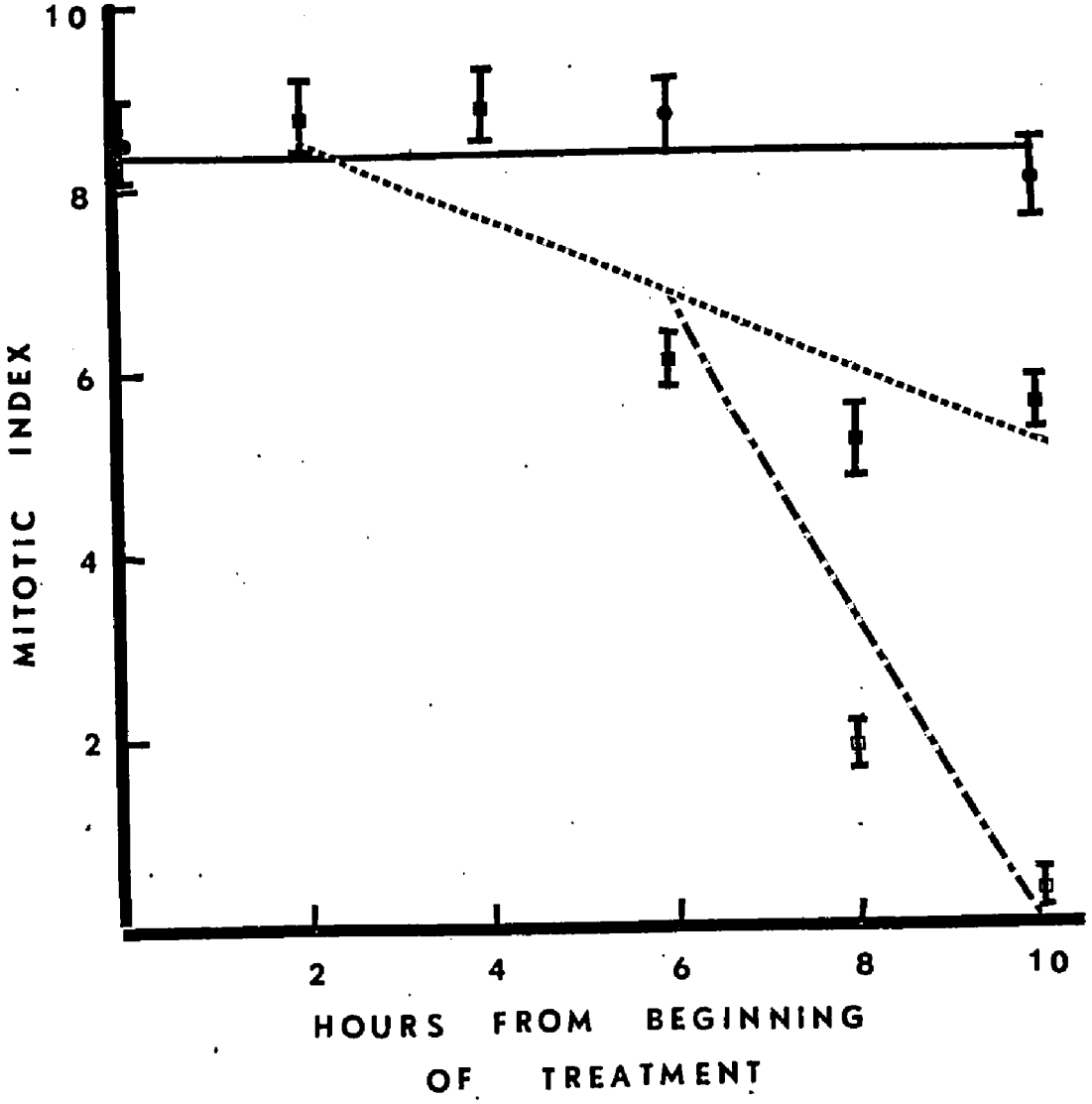

10
8
6
4
2
MITOTIC INDEX
2
4
6
8
10
HOURS FROM BEGINNING
OF TREATMENT

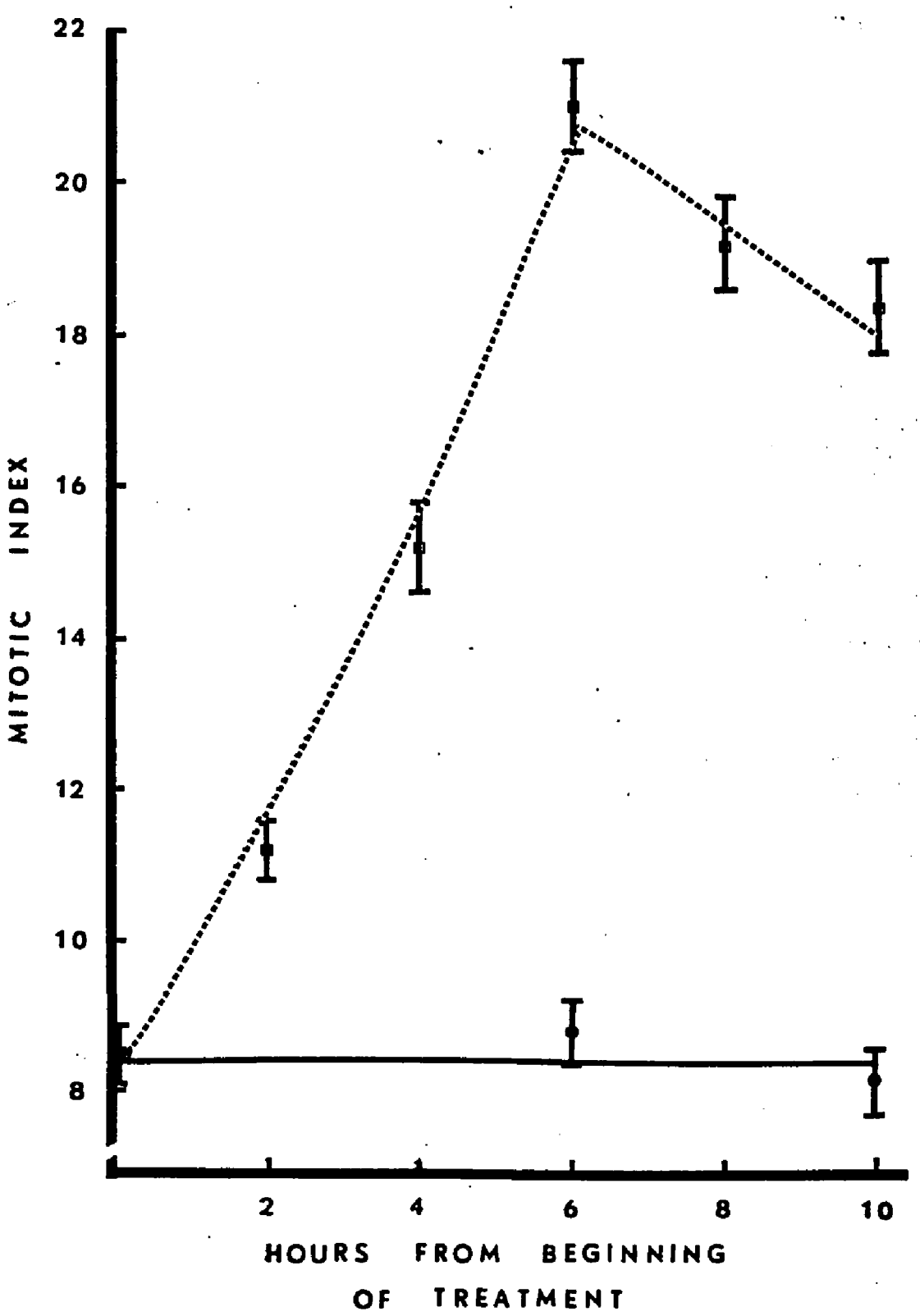
Fig. 4.2-4

The effect on mitotic index throughout a 10 hr. continuous treatment with colchicine (1 mg/ml).

One standard deviation either side of the mean is shown as a vertical bar.

Control (———).

Treated (·····).


22
20
18
16
14
12
10
8
MITOTIC INDEX
2
4
6
8
10
HOURS FROM BEGINNING
OF TREATMENT

$t = 1.82 < t\ (^{df = 3}_{p = .05}) = 3.18)$. It is evident that the colchicine treatment has stimulated an interphase population of cells to enter mitosis and accumulate at metaphase.

In summary, the action of HAS is completely different from that of colchicine. Colchicine stimulates cells in interphase to enter mitosis, suppressing cell plate formation and accumulating chromosomes at metaphase, resulting in the formation of polyploids. HAS inhibites the transition of S cells to G_2, and only temporarily affects the accumulation of metaphase figures during long-term treatment. The incidence of polyploid as induced by HAS is accounted for by endoreduplication, as we shall describe in the section 4.4.

4.3 Effects of MC and HAS on Incorporation of ^{3}H-thymidine:

The results of the MC (5 µg/ml) experiments are presented in Table 4.3-1. There is a highly significant difference between treated and control roots in the frequency of labelled nuclei and in grain counts (Tables B-1 and B-2*). It has been shown that there is a 13% reduction in the frequency of labelled interphase, and a 40% inhibition of incorporation of ^{3}H-thymidine into nucleus as compared with the control. The results of the MC treatments in both MC I. and MC II. (Table 4.3-1) are not significantly different from each other in grain counts per nucleus (Table B-2) which suggest that the inhibitory action of MC lasts for some time after the roots have been removed from MC. The grain count distributions are shown in Figure 4.3-1. It is clear that MC strongly reduces the incorporation of the radioactive precursor into

* Appendix B

Table 4.3-1

Effect of MC (5 µg/ml) or HAS (0.5 mg/ml) on mitotic index, autoradiographic labelling index and labelling of nuclear DNA with ^{3}H-thymidine (0.5 µc/ml) at 24 ± 1°C.

Treatment*	Mitotic index	% labelled interphase nuclei	No. of silver grains per nucleus	Labelling as % reduction of control
Control	9.3 ± 0.44	63.5 ± 0.65	50.1 ± 2.98	-
MC I.	7.1 ± 0.44	49.5 ± 0.72	31.8 ± 2.37	36.5
MC II.	6.8 ± 0.46	49.2 ± 0.81	28.8 ± 3.31	42.4
HAS I.	10.3 ± 0.62	51.1 ± 0.80	28.8 ± 1.23	42.6
HAS II.	8.2 ± 0.48	56.7 ± 0.77	37.3 ± 4.60	25.5

* Control = 2 hrs. in water followed by exposure for 2 hrs. to ^{3}H-thymidine.

MC = mitomycin C, HAS = hydroxylamine sulfate.

I. = 2 hrs. in water followed by exposure for 2 hrs. to a mixture of MC or HAS and ^{3}H-thymidine.

II. = 2 hrs. in MC or HAS followed by exposure for 2 hrs. to ^{3}H-thymidine.

The material was fixed immediately after the treatment.

Fig. 4.3-1

Frequency of grain counts per nucleus following ^{3}H-thymidine treatment.

Control (1*).

MC I. (2).

MC II. (3).

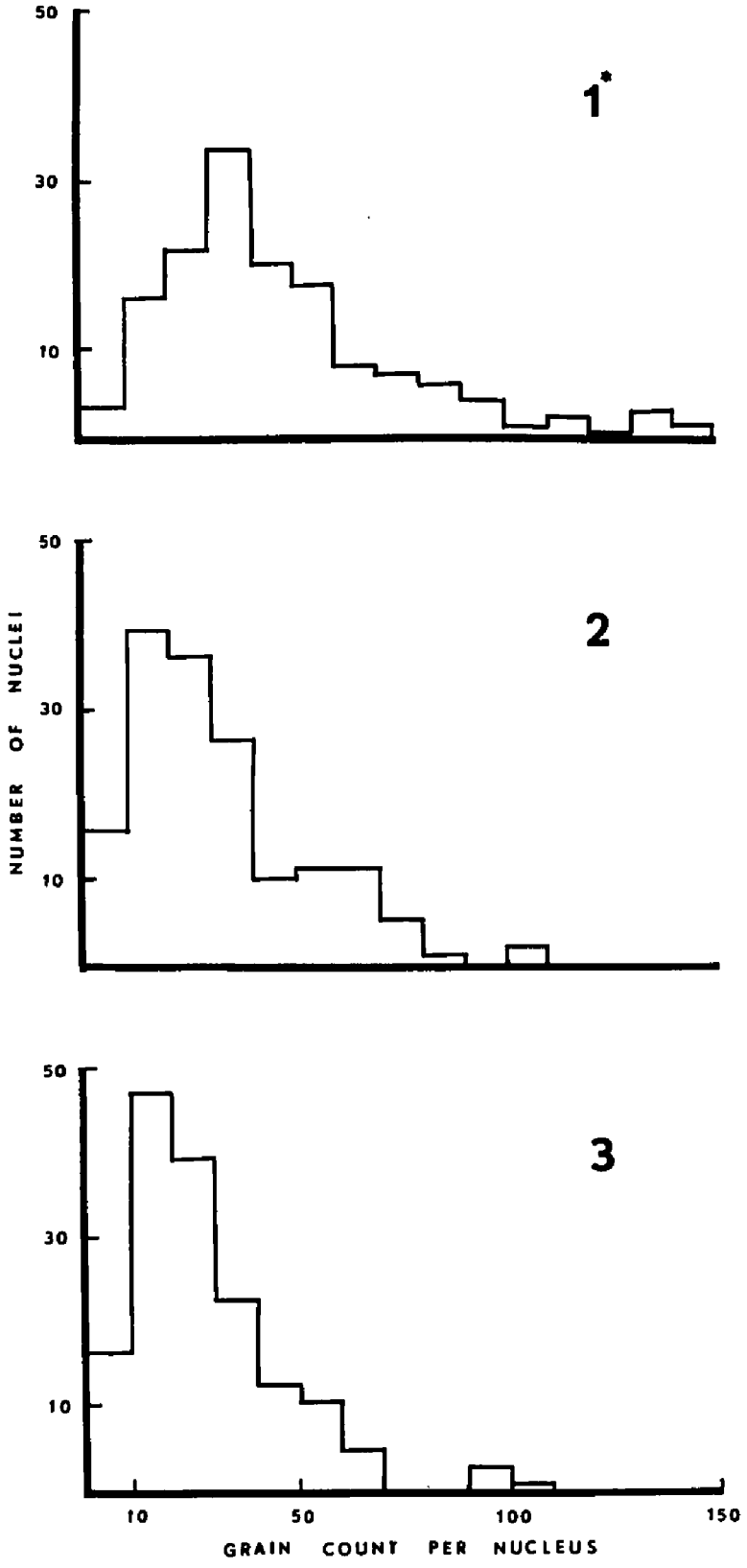
Data are based on 4 samples for each treatment, 40 nuclei per sample.

Control = 2 hrs. in water followed by exposure for 2 hrs. to ^{3}H-thymidine.

MC = mitomycin C

MC I. = 2 hrs. in water followed by 2 hrs. to a mixture of MC and ^{3}H-thymidine.

MC II. = 2 hrs. in MC followed by 2 hrs. to ^{3}H-thymidine.


50
30
10
1*
50
30
10
NUMBER OF NUCLEI
2
50
30
10
3
10
50
100
150
GRAIN COUNT PER NUCLEUS

DNA (or the rate of DNA synthesis) and reduces the percentage of labelled interphases, not only during the time of treatment but also after the treatment.

The data from the HAS treatment experiments on incorporation of ^{3}H-thymidine into the nucleus are shown in Table 4.3-1. The frequency of labelled interphase nuclei and the average grain counts per nucleus are significantly lower than in the control (Tables B-1 and B-2). The results between HAS I. and HAS II. experiments are significantly different in the mean grain counts per nucleus (Table B-2). The grain count distributions after treatment with HAS are shown in Figure 4.3-2. The results indicate that HAS inhibits the incorporation of ^{3}H-thymidine into DNA (or reduces the rate of DNA synthesis) during the treatment. The incorporation increased soon after the removal from HAS.

It is clear that the action of HAS on inhibition of DNA synthesis is different from that of MC. HAS has a temporary effect on the inhibition whereas in the presence of MC, the inhibitory effect lasts for some time after treatment. These results support our data that MC lengthens the duration of the S period and reduces the mitotic index in the S period at the time of treatment.

4.4 Endoreduplication:

As a by-product of the present research, an interesting phenomenon "endoreduplication" has been found to be induced by HAS in Zea mays root meristems. Endoreduplication has been defined as a process of polyploidization occurring during interphase, and as the reduplicated nucleus

Fig. 4.3-2

Frequency of grain counts per nucleus following ^{3}H-thymidine treatment.

Control (1*).

HAS I. (2).

HAS II. (3).

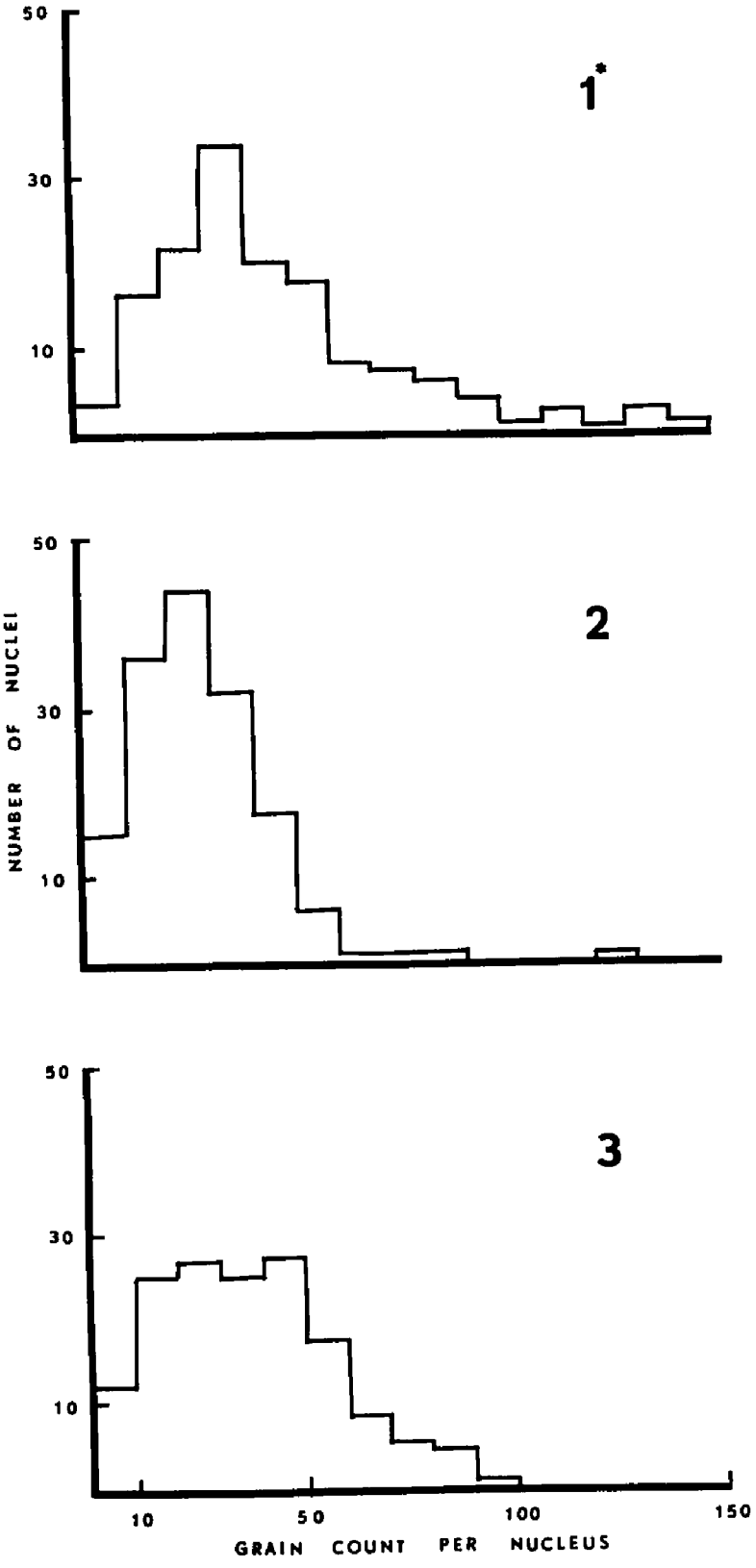
Data are based on 4 samples for each treatment, 40 nuclei per sample.

Control = 2 hrs. in water followed by exposure for 2 hrs. to ^{3}H-thymidine.

HAS = hydroxylamine sulfate.

HAS I. = 2 hrs. in water followed by exposure for 2 hrs. to a mixture of HAS and ^{3}H-thymidine.

HAS II. = 2 hrs. in HAS followed by exposure for 2 hrs. to ^{3}H-thymidine.


50
30
10
1*
50
30
10
NUMBER OF NUCLEI
2
50
30
10
3
10
50
100
150
GRAIN COUNT PER NUCLEUS

enters mitosis, diplochromosomes are seen. Diplochromosomes were not observed in untreated root tips. The origin of the chromosome doubling is quite different from that of the colchicine-induced doubling. Colchicine prevents spindle organization and cell plate formation during mitosis. On the other hand, endoreduplication takes place during interphase.

A cytological feature of endoreduplicated cells can be seen in prophase and metaphase of mitosis following the endoreduplication. At prophase, chromosomes show a quadripartite structure instead of the usual bipartite structure (Figure 4.4-1A, 1B). The four threads of chromatids coil two by two forming two major strands (each strand with two chromatids). Each centromere joins four chromatids (two major strands). The centromere divides into two during late prophase or metaphase. The chromosomes reach their greatest contraction and the four chromatids lie in parallel (Figure 4.4-1C, 1D). The number of chromosomes is double and they remain arranged in pairs. These are called "diplochromosomes". A comparison between normal metaphase and diplochromosomes is shown in Figure 4.4-1E. It is clear that diplochromosomes are two times larger than normal metaphase chromosomes. Immediately after metaphase the centromeres divide once more and then the anaphase movement begins. The cytological behavior at anaphase and telophase is similar in a nucleus possessing diplochromosomes and in a tetraploid nucleus (Figure 4.4-1F). The next nuclear cycle would be the same as in a tetraploid.

Fig. 4.4-1

A comparison between normal chromosomes and diplochromosomes. The diplochromosomes were induced by HAS.

A. Normal prophase

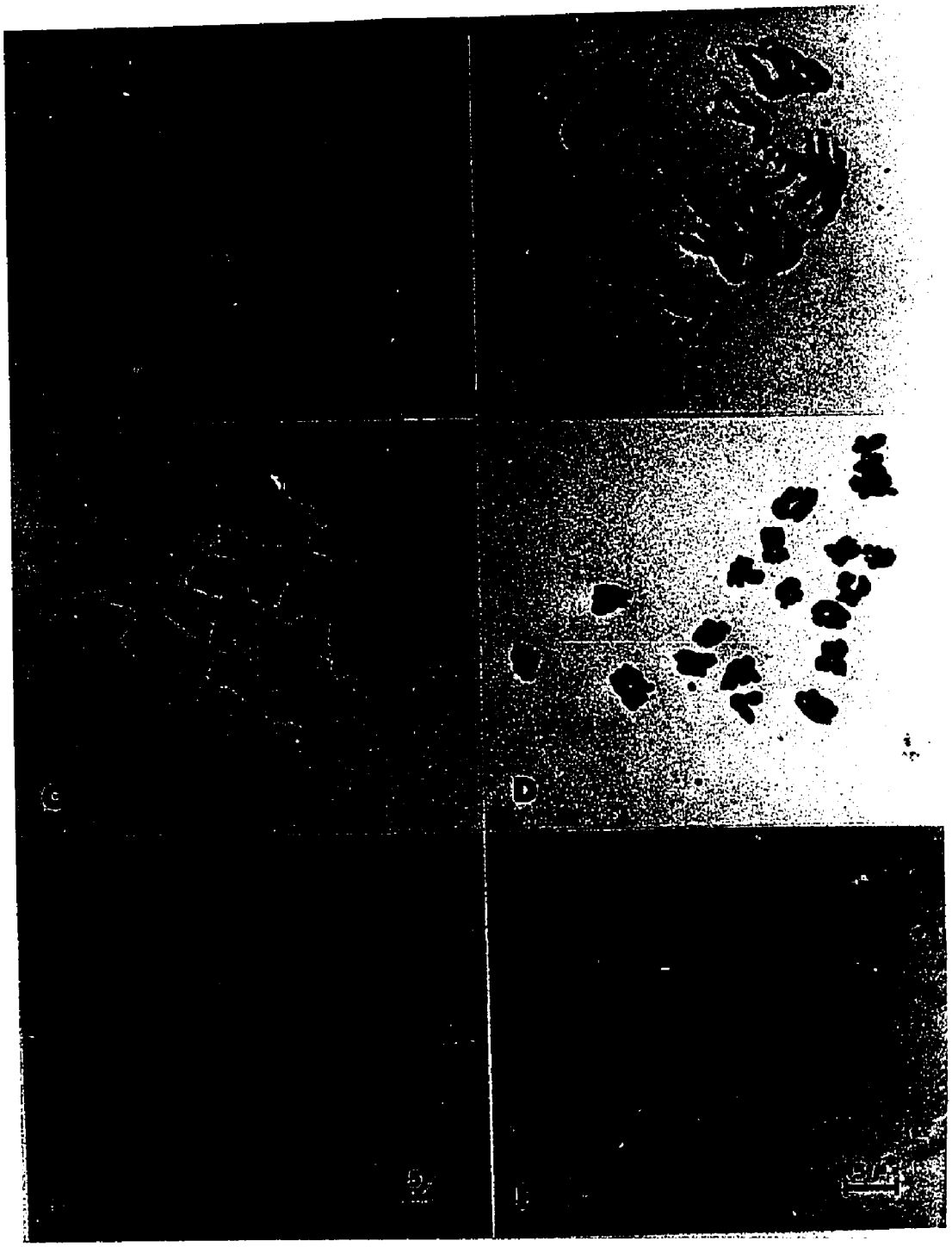
B. Diplochromosomes in prophase.

C and D. Diplochromosomes in metaphase.

E. Normal metaphase and diplochromosomy.

F. Diplochromosomy at anaphase is similar to tetraploid nucleus.

* A, B, C, D and F are same scale.


C
D


A
B
C
D
E
F
5μ
5 μ

The frequencies of endoreduplicated cells at metaphase (diplochromosomes) are presented in Table 4.4-1. The data indicate that the cells with diplochromosomes were seen first in the 20 hr. after treatment collection period. The frequency of diplochromosomes increased to 5.3% and 7.1% at 24 hr. and 28 hr. respectively. At 32 hr. after 2 hr. treatment, no cells with diplochromosomes were observed. Apparently, the induction of endoreduplication by this drug occurs at particular stage(s) in the nuclear cycle.

It has been well documented that cells undergoing endoreduplication require two or more series of DNA replication in interphase before entering mitosis. Therefore, the following question arose; during which stage(s) of the nuclear cycle can cells be induced by HAS to undergo the second series of DNA replication? There appear to be at least 6 possibilities, namely;

1. G_1
2. S $\Big\rangle$ because of successive DNA doublings.
3. G_2 - because of a complete omission of mitosis.
4. G_1 and S.
5. S and G_2.
6. G_1, S and G_2.

With a view to answering this question, the cells should be labelled with ^{3}H-thymidine during S period. Therefore, intact roots were treated with a mixture of HAS and ^{3}H-thymidine for 2 hr.. The roots were fixed at two-hour intervals during 20 to 32 hr. period after treatment. Analyses of the time of appearance of diplochromosomes, and their labelling events,

Table 4.4-1

Frequencies of endoreduplicated cells (at metaphase) at various times after the intact roots were treated with HAS for 2 hrs. at 24 ± 1°C.

Treatment (conc.) mg/ml	Hours after treatment	No. of metaphases scored	No. of metaphase cells with diplochromosomes	% cells with diplochromosomes
0.5	20	71	2	2.8
0.5	24	227	12	5.3
0.5	28	266	19	7.1
0.5	32	225	0	-
2.0	28	353	36	10.2

enable us to ascertain at which stages of interphase the cells can be induced to undergo endoreduplication. For example, if diplochromosomes were labelled, the cells were in S period at the time of treatment. If the diplochromosomes were labelled and unlabelled during their early appearance but not in late appearance then the cells were in S and G_2 at the time of treatment, and so on. The data are presented in Table 4.4-2. The results show that over 90% of cells with diplochromosomes were labelled (Figure 4.4-2). If we compared the duration of the nuclear cycle with the time of diplochromosome appearance after HAS treatment, as shown in Figure 4.4-3, the appearance of diplochromosomes followed the descending curve of the "first" labelling peak. As we have mentioned before, the transition of S cells to G_2 was also blocked by this drug. From the above information, we would suggest that the cells in S period, at the time of treatment, were induced to undergo a second round of DNA replication. The endoreduplicated cells did not pass through G_2 before undergoing the second round of DNA replication. A hypothetical nuclear-cycle map to explain the induction of endoreduplication will be described in the discussion.

4.5 Somatic Cytology of Zea mays ("Seneca 60"):

There are 10 pairs of chromosomes in diploid cells of Zea mays ("Seneca 60") with a total metaphase length of 40.8 ± 3.6 microns. The karyotype is shown in Figure 4.5-1. The arm ratios and their total relative lengths are also indicated in Figure 4.5-2 (Table C-1*). Similar chromosomal measurements of "Seneca 60" have been reported by Chen (1969). However, his measurements did not include the lengths of

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* Appendix C

Table 4.4-2

Frequencies of endoreduplicated cells (at metaphase) at various times after the intact roots were treated with a mixture of HAS (0.5 mg/ml) and ^{3}H-thymidine (1 µc/ml) for 2 hrs. at 24 ± 1°C.

Hours after treatment	No. of metaphases scored	No. of metaphase cells with diplochromosomes				% cells with diplochromosomes
		L*	U*	Total	(%) labelled cells	
20	215	2	0	2	100	0.9
22	289	10	0	10	100	3.5
24	750	36	2	38	94.7	5.1
26	406	24	0	24	100	5.9
28	612	38	3	41	92.7	6.7
30	892	29	1	30	96.7	3.4
32	559	-	-	0	-	-

* L = Labelled, U = Unlabelled.

Fig. 4.4-2

Autoradiograph of diplochromosomes:

A. Late prophase.

B. Metaphase.


A
B
5 μ


A
B
5 μ


A
B
5 μ

Fig. 4.4-3

A comparison between nuclear cycle and time of diplochromosome appearance after the beginning of 2 hr. treatment with HAS (0.5 mg/ml).

Labelled prophase (———).

Diplochromosomes (- - - - - -).

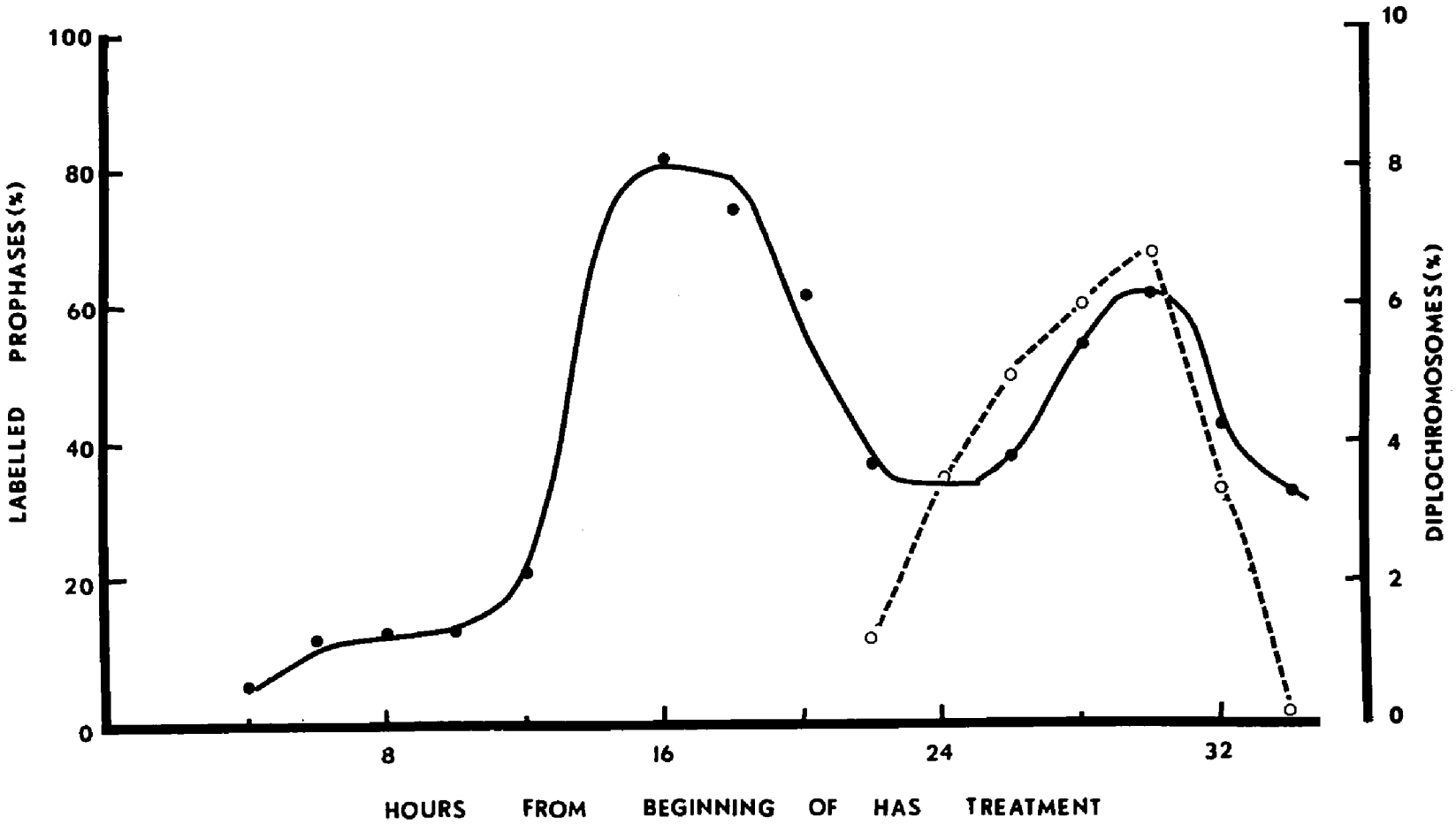

100
80
60
40
20
0
LABELLED PROPHASES(%)
10
8
6
4
2
0
DIPLOCHROMOSOMES(%)
8
16
24
32
HOURS FROM BEGINNING OF HAS TREATMENT

Fig. 4.5-1

A. A normal cell in mitotic metaphase of Zea mays.

B. The somatic karyotype from A.

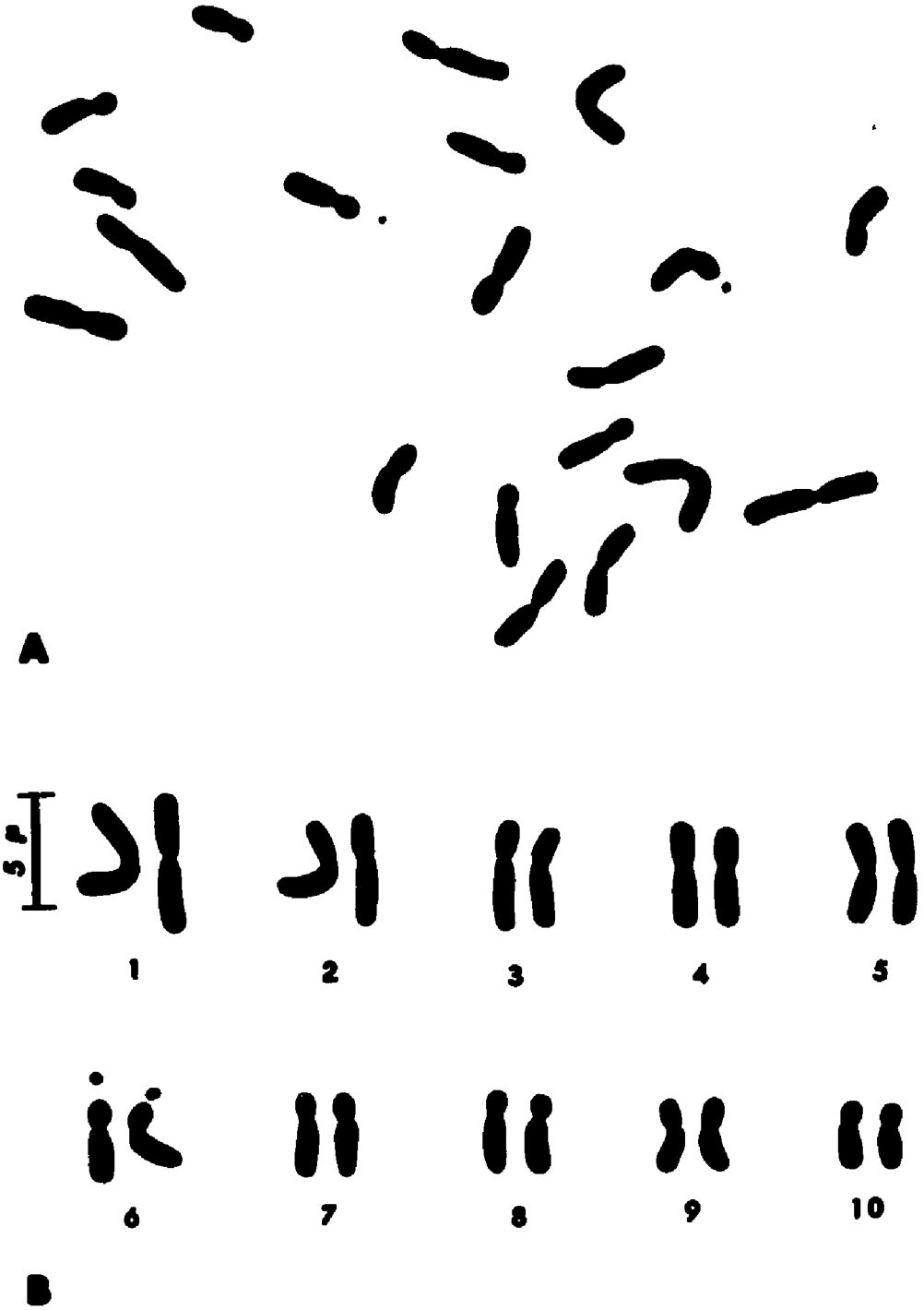
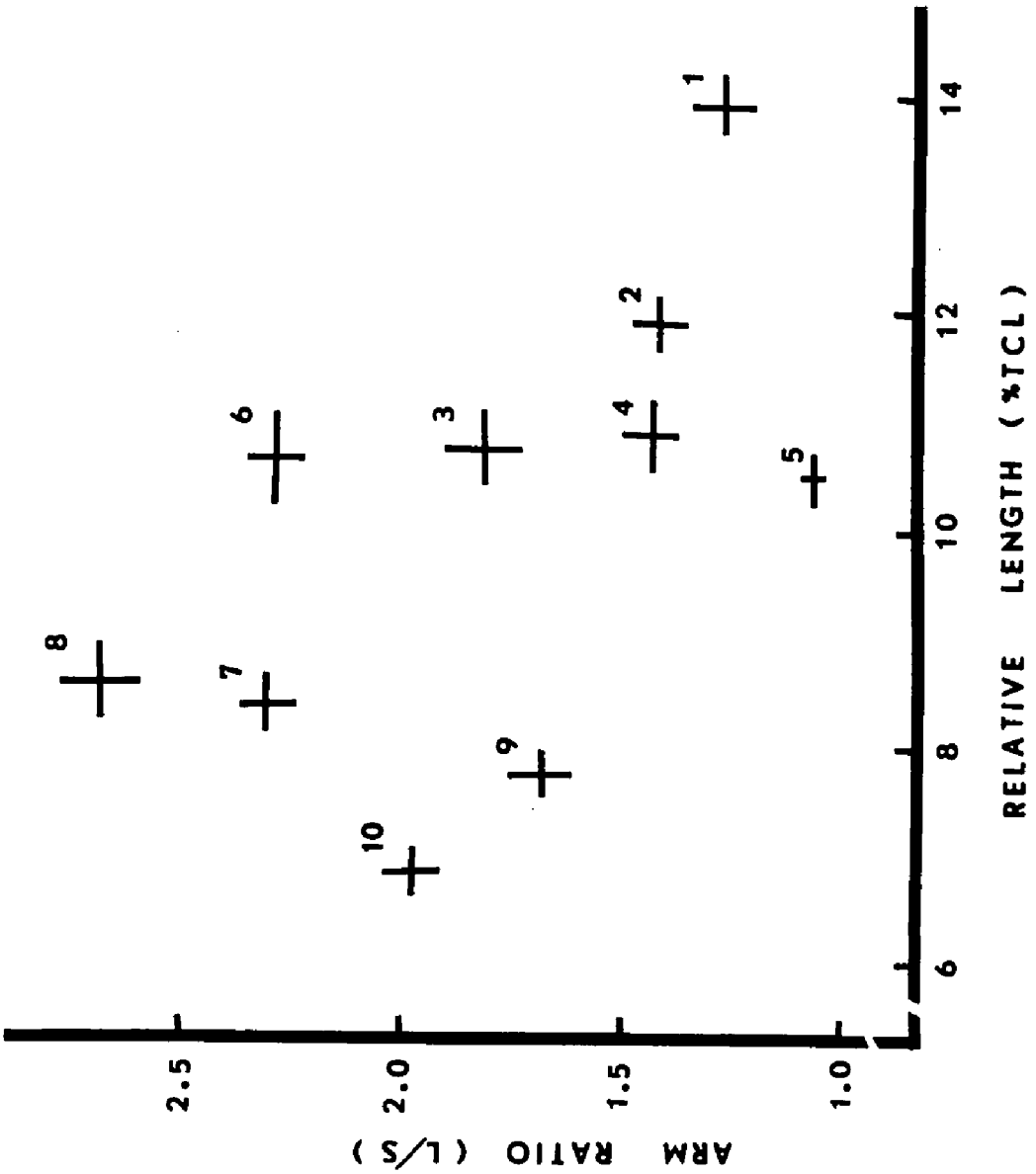

A
5μ
1
2
3
4
5
6
7
8
9
10
B

Fig. 4.5-2

Diagrammatic representation of the means and standard errors of the 10 pairs of chromosomes of "Seneca 60". One standard error either side of the mean is shown as a vertical and horizontal bars.


ARM RATIO (L/S)
2.5
2.0
1.5
1.0
RELATIVE LENGTH (%TCL)
6
8
10
12
14
1
2
3
4
5
6
7
8
9
10

centromeres, nucleolar constrictions, and satellites as the total chromosome length.

The 10 pairs of chromosomes can be distinguished from each other by the combination of their relative lengths, arm ratios and cytological markers (e.g., short arm of chromosome 6 with nucleolar constriction). Therefore, the following questions on the induction of chromosomal aberrations have been investigated:

1. Is there an equal distribution of chromosomal breakage induced by chemicals among the 10 pairs of chromosomes?

2. What are the relative amounts of damage produced in a specific chromosomal region of the chromosome by a chemical?

4.6 Inductions of Chromosomal Aberrations and Mutations with MC:

4.61 General observations:

MC proved to be an effective chromosome-breaking agent in Zea mays root meristematic cells. When the duration of treatment was 2 hr., a reasonably high proportion of aberrations was obtained with 5 µg/ml and 10 µg/ml. No aberrations appeared immediately after fixation of MC treated roots. The aberrations were observed when intact roots were incubated for a period after the treatment. If root tips were fixed at 24 hr. and 44.5 hr. after treatment, metaphase fragments, anaphase fragments and bridges, and micronuclei were observed (Figure 4.61-1).

As shown in Table 4.61-1, types and frequencies of chromosome aberrations were scored in roots treated with MC for 2 hr. at 44.5 hr.. The most frequent aberrations were centromere breaks, chromatid

Fig. 4.61-1

Chromosomal aberrations induced by MC:

A. Fragment at metaphase.

B. Bridge and fragment at anaphase.

C. Bridge at telophase.

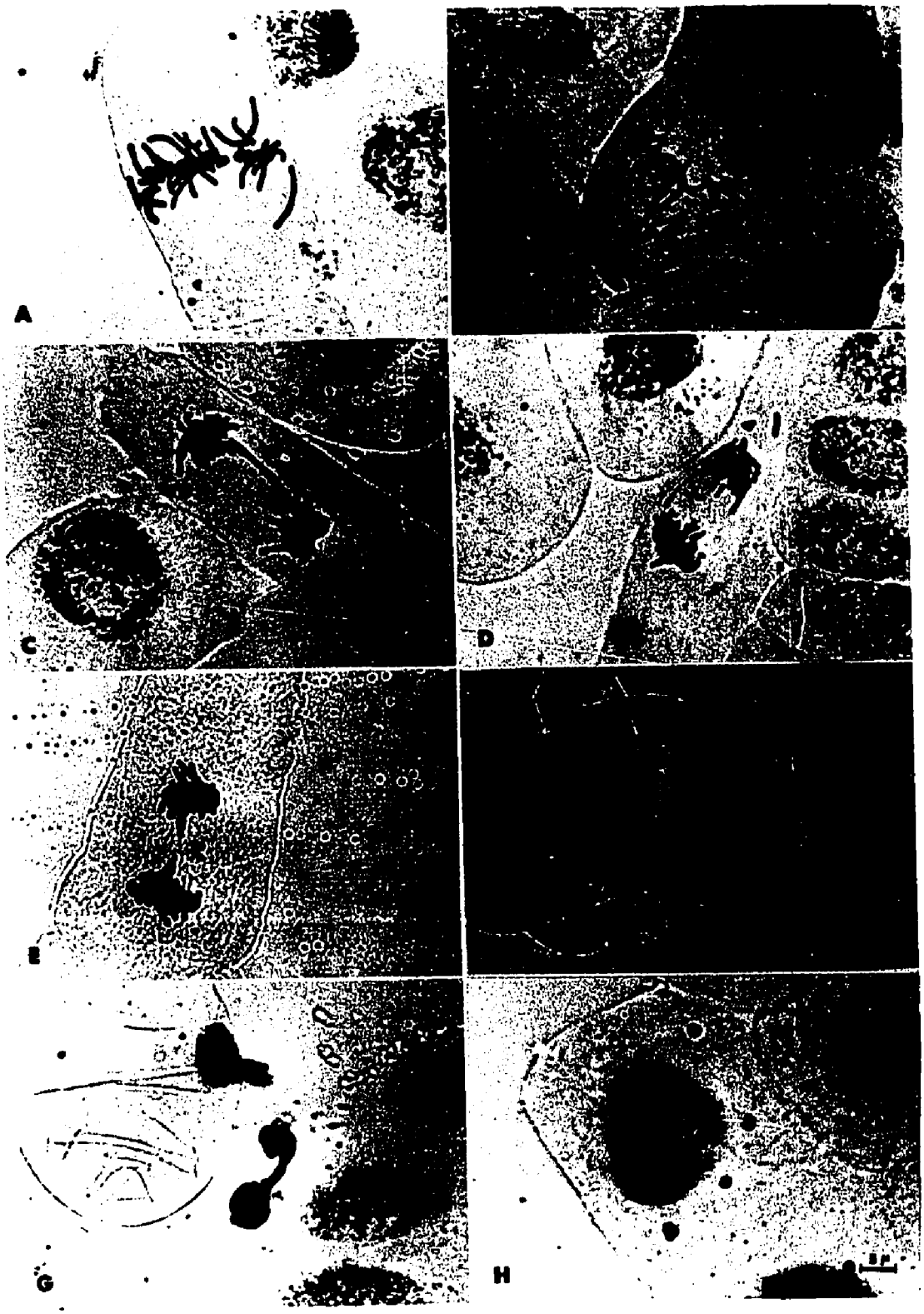
D. Bridge and fragment at telophase.

E. Broken bridge and fragment at telophase.

F. Multiple fragments at telophase.

G. Sticky bridge.

H. Micronuclei at interphase.


A
C
D
E
G
H

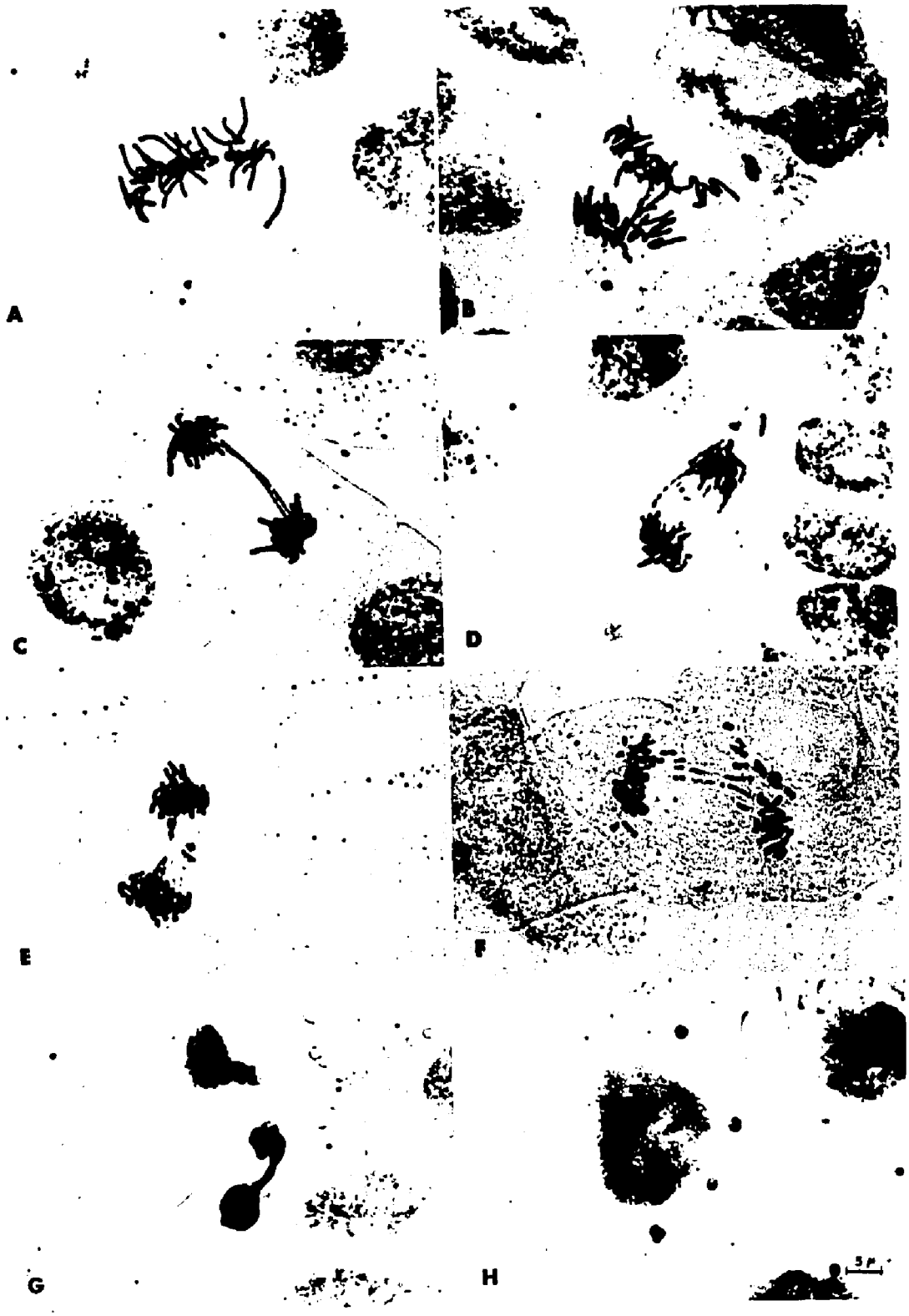

A
B
C
D
E
F
G
H
5 μ

Table 4.61-1

Types and frequencies of chromosome aberrations induced by MC at 24 ± 1°C.

Treatment (2 hr.)	Fixation time (hr.) after treatment	No. of Metaphases analysed	No. (or %) of abnormal metaphases	Aberrations of each type (per 100 cells)					
				Chromatid breaks	Isochromatid breaks	Centromere breaks	Chromatid exchanges	Chromosome translocations	Ring chromosomes
Control	-	100	0	-	-	-	-	-	-
5 µg/ml	44.5	226	85 (37.6%)	16.4	19.5	31.0	9.7	4.4	0.9
10 µg/ml	44.5	206	128 (62.1%)	31.6	54.4	77.2	19.4	3.9	2.4

breaks, isochromatid breaks and chromatid exchanges. Chromosomal translocations (dicentric chromosomes) and ring chromosomes also occurred (Figure 4.61-2). Both the mean number of breaks per 100 cells and the percentage of cells with breaks increased with the concentration of the agent. The percentage of abnormal metaphase cells was 37.6% and 62.1% for concentrations of 5 µg/ml and 10 µg/ml of MC, respectively.

4.62 Distribution of chromosomal breaks:

The distribution of chromosome breaks among the 10 pairs of chromosomes observed is presented in Table C-4. If MC were acting 'equally' per unit of chromatin then the distribution of breaks should be correlated with the lengths of the chromosomes. The relative lengths in percent of the complement of metaphase chromosomes are also shown. The expected numbers of breaks for individual chromosomes were calculated from the relative lengths. The data were subjected to Chi-square analysis and a highly significant value was obtained $\left(\chi^2 = 107.6 > \chi^2_{\substack{df = 9 \\ p = 0.01}} = 21.7\right)$. It is clear that the breaks were not equally distributed among each pair of chromosomes or even among long arms, short arms or centromeric regions of different pairs of chromosomes (Tables C-5, C-6, and C-7).

An attempt has been made to assign the location of each break not only to a specific arm, but also in most cases to a segment within arms. Pictorial examples of some regions of chromosome No. 1, 3, 5 and 8 are shown in Figure 4.62-1. As shown in Figure 4.62-2, the array of breaks among the 10 pairs of chromosomes indicates a localized

Fig. 4.61-2

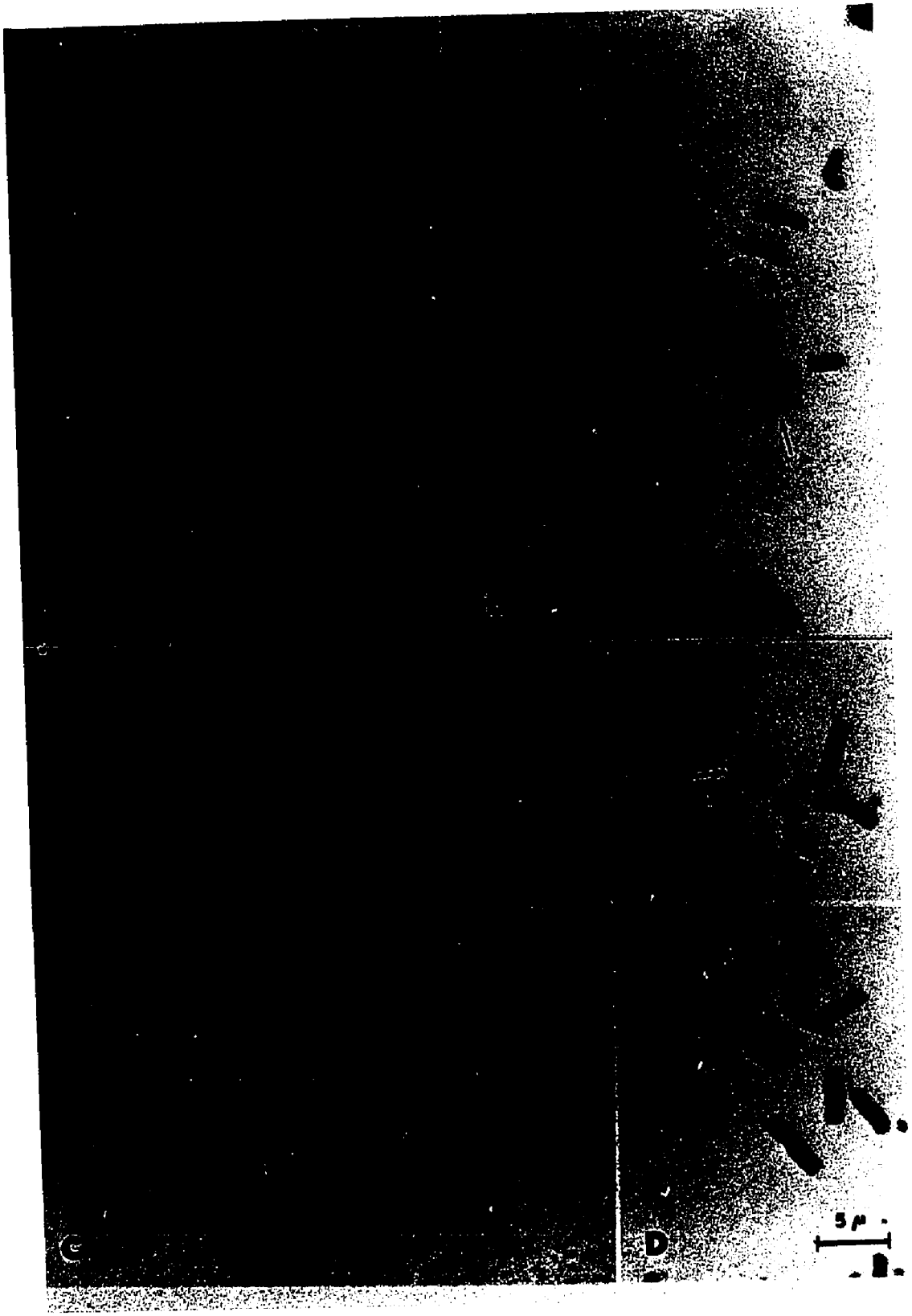
Metaphase cells illustrate types of chromosomal breaks induced by MC. Arrows indicate types of chromosomal aberrations:

A. Chromatid break and centromeric break.

B. Multiple chromatid exchanges and isochromatid break.

C. Isochromosomal break and chromosomal translocation.

D. Ring chromosome.


C
D
5 μ

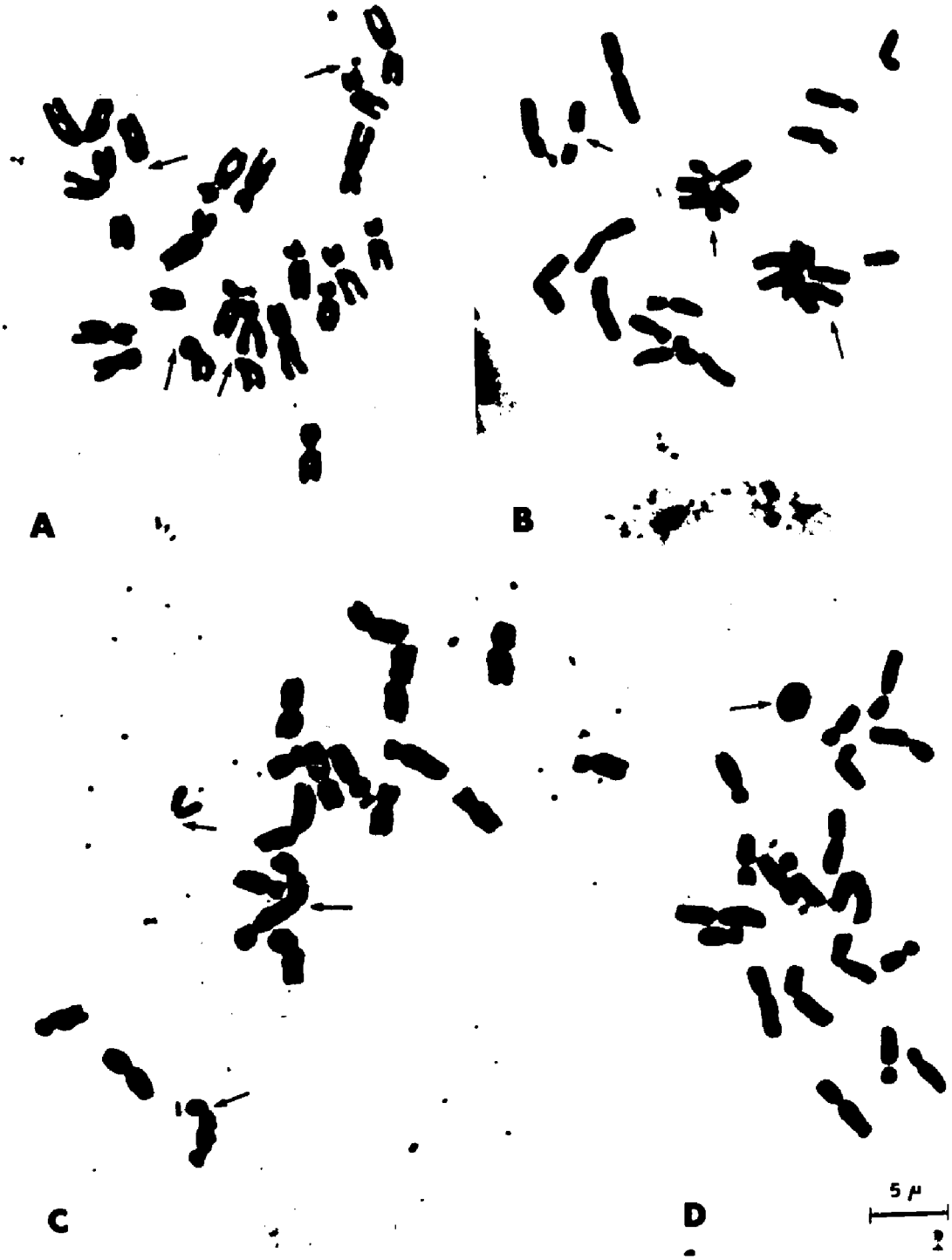
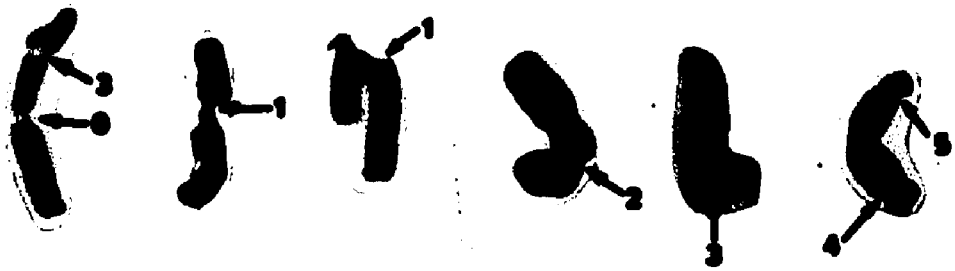

A
B
C
D
5 μ

Fig. 4.62-1

Pictorial example of some regions of chromosome No. 1, 3, 5 and 8 which were damaged by MC.
The numerals represent the regions of the breaks (Table C-2 foot-note explanation).



CHROMOSOME 1

CHROMOSOME 3

CHROMOSOME 5

CHROMOSOME 8

CHROMOSOME 1

CHROMOSOME 3

CHROMOSOME 5

CHROMOSOME 8

Fig. 4.62-2

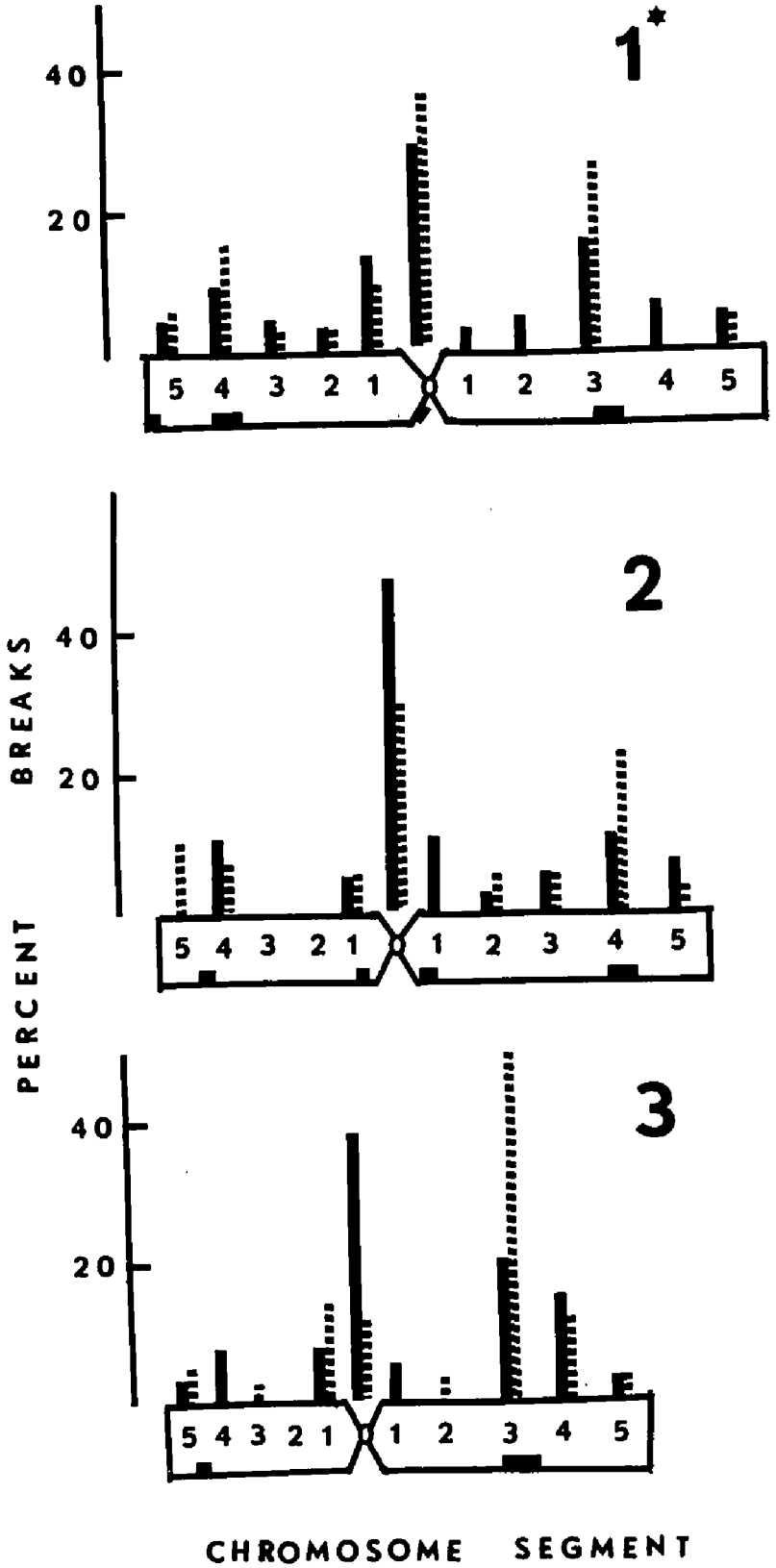
The distribution of chromosomal breaks. Diagram of relative frequencies of damage for each chromosome region in percent of total recorded from a chromosome, from chromosome No. 1 to chromosome No. 10.

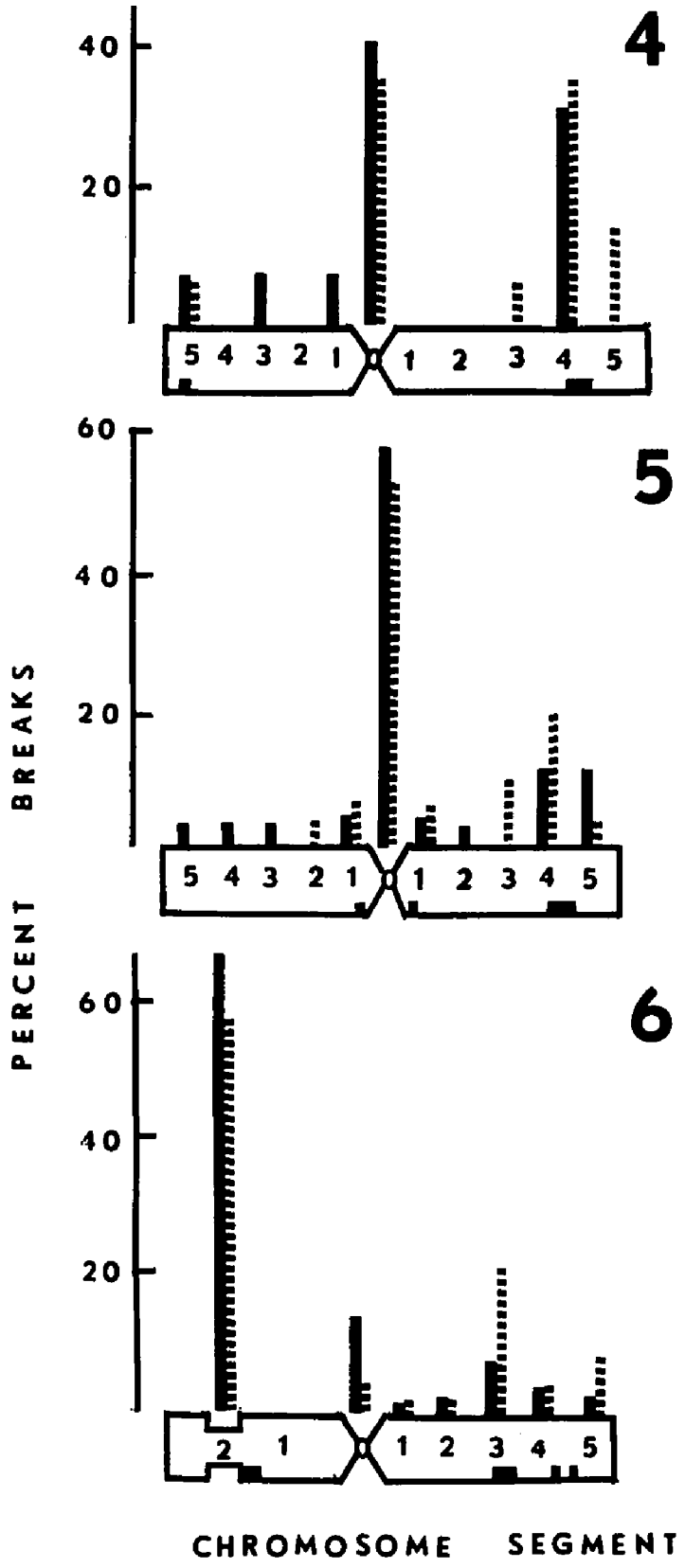
MC (———).

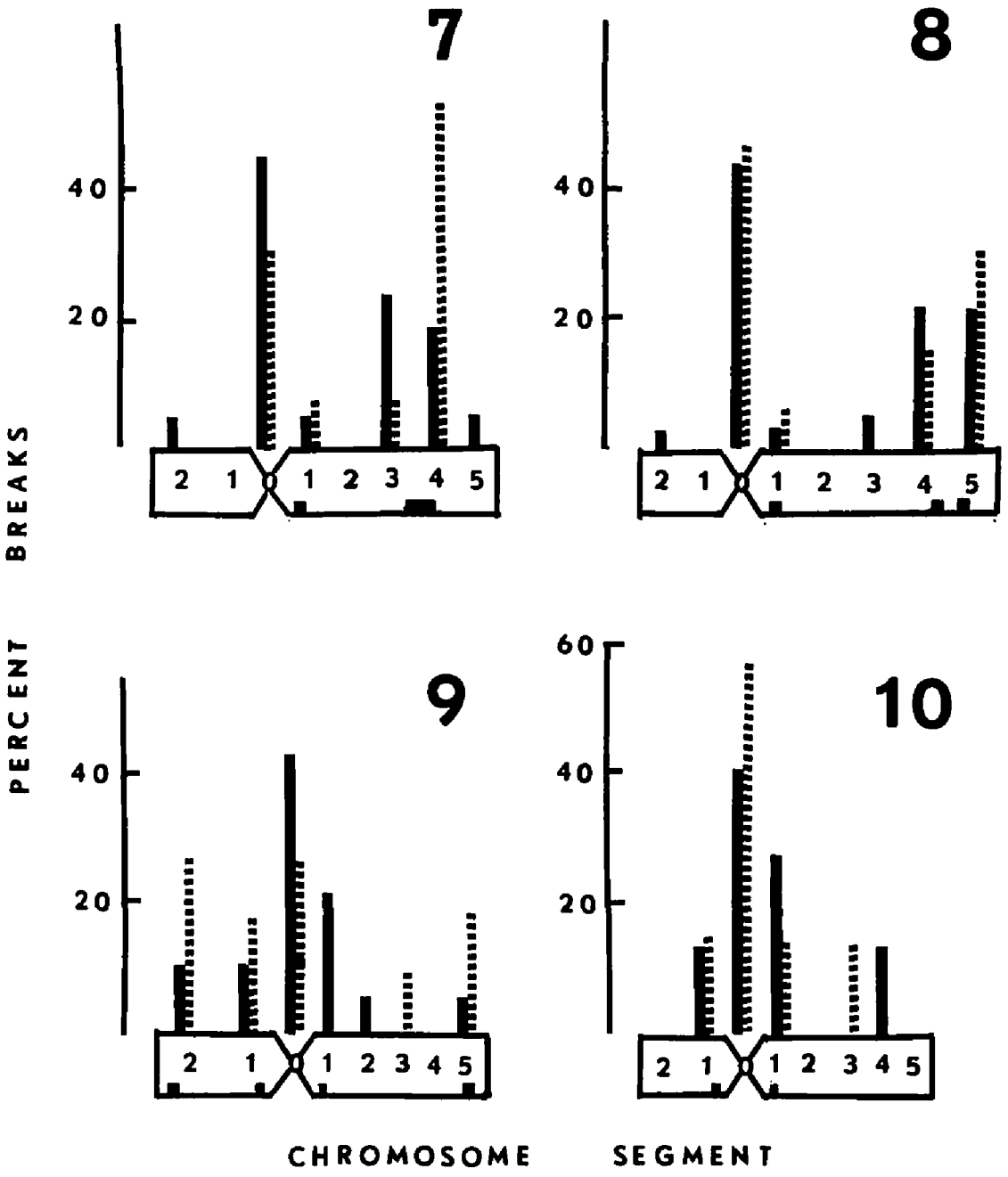
HAS (.....).

*1, 2, 3 ..... to 10; represent chromosome No. 1, chromosome No. 2, and so on.

N.B. Black square indicates the location of heterochromatin region in the diagrammatic representation of the chromosome. The secondary constriction is shown in the short arm of chromosome 6.


1*
40
20
5 4 3 2 1 1 2 3 4 5
2
40
20
5 4 3 2 1 1 2 3 4 5
3
40
20
5 4 3 2 1 1 2 3 4 5
PERCENT BREAKS
CHROMOSOME SEGMENT


4
40
20
5 4 3 2 1 1 2 3 4 5
5
60
40
20
5 4 3 2 1 1 2 3 4 5
6
60
40
20
2 1 1 2 3 4 5
PERCENT BREAKS
CHROMOSOME SEGMENT


7
8
9
10
40
20
60
PERCENT BREAKS
2 1 1 2 3 4 5
CHROMOSOME SEGMENT

distribution. The greatest concentration of breaks appears at the centromere. When one examines regional sensitivity of the intra-chromosomal segment, the breakage frequency is relatively higher in heterochromatic regions and the secondary contriction in comparison to other regions of the chromosome.

4.63 Chromosomal aberrations in relation to the nuclear cycle:

The results for both aberration yield and labelling index obtained are presented in Figure 4.63-1 (Table C-12) from an experiment which included the exposure of roots for 30 min. to ^{3}H-thymidine and then for 2 hr. to MC (5 µg/ml). The data on aberration yield show clearly that very few aberrations were observed from the first post-treatment mitosis of these cells (in G_2 at the time of treatment). The aberrations which were observed occurred in labelled cells. These data indicate that there is an increase in sensitivity with increasing recovery time, i.e., the cells in early S showing a higher aberration yield than the cells in late S at the time of treatment. The aberration frequency during the period 16 hr. to 22 hr. from the beginning of MC treatment is higher than other periods; these cells would have been in late G_1 and early S period at the time of treatment.

The results of the second experiment with a mixture of ^{3}H-thymidine and MC are summarized in Figure 4.63-2 (Table C-13). The highest frequency of aberrations was 0.97 and 1.14 per cell during the 18 hr. and 22 hr. periods, respectively. At 22 hr., labelled metaphases had decreased to 50%, while about one half of the cells with aberrations were labelled. These results suggest that those cells had been in early S and late G_1 during the treatment. The aberration yield data confirm

Fig. 4.63-1

Proportion of labelled metaphases (———)
and frequency of aberrations (▪▪▪▪▪▪▪▪▪▪▪▪) after
exposure for 30 min. to ^{3}H-thymidine followed
by 2 hr. MC (5 µg/ml) treatment.
One standard deviation either side of the
mean is shown as a vertical bar.

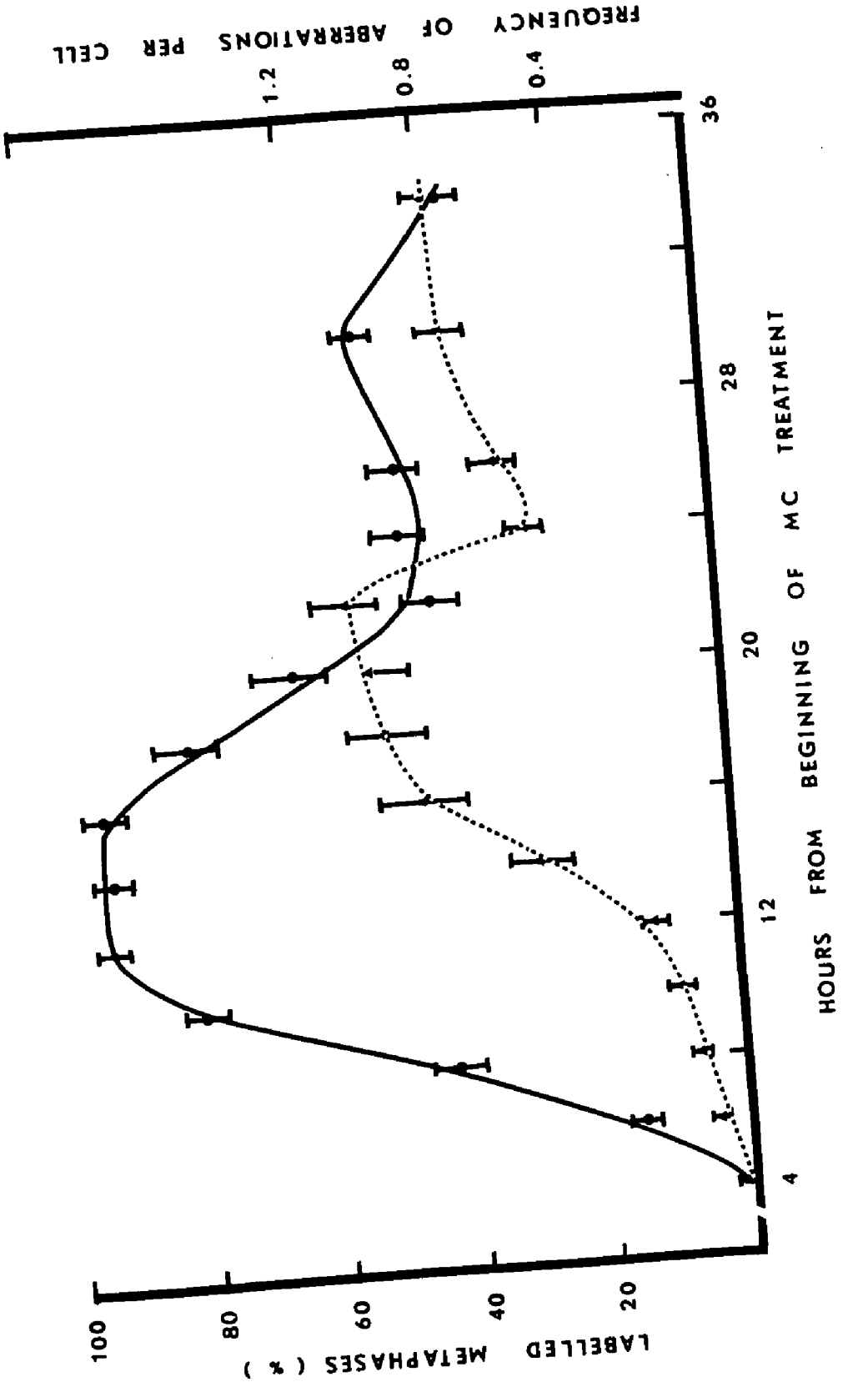
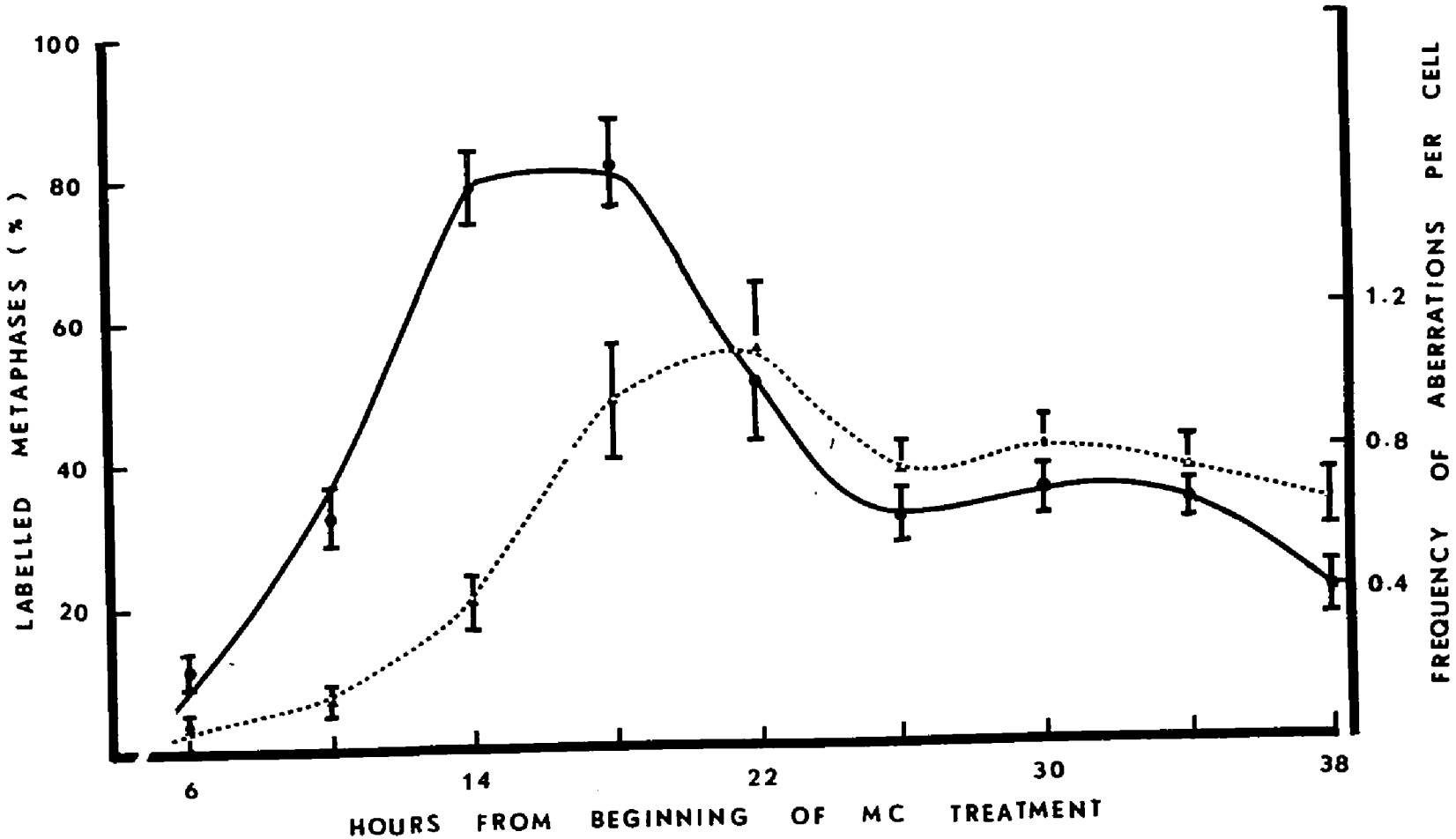

FREQUENCY OF ABERRATIONS PER CELL
1.2
0.8
0.4
36
28
20
12
4
HOURS FROM BEGINNING OF MC TREATMENT
LABELLED METAPHASES (%)
100
80
60
40
20

Fig. 4.63-2

Proportion of labelled metaphases (———) and frequency of aberrations (·····) after exposure for 2 hr. to a mixture of ^{3}H-thymidine and MC (5 µg/ml). One standard deviation either side of the mean is shown as a vertical bar.


100
80
60
40
20
LABELLED METAPHASES (%)
1.2
0.8
0.4
FREQUENCY OF ABERRATIONS PER CELL
6
14
22
30
38
HOURS FROM BEGINNING OF MC TREATMENT

the results obtained from the first experiment. It is clear that chromosomes, as measured by breakage, were most sensitive to MC in the early S period and late G_1.

4.64 Induction of mutation:

In the first experiment, pollens of triple dominant stock of "Seneca 60" $\underline{Yg_2}$, $\underline{Sh}$ and $\underline{Wx}$ were treated with MC for 6 hr. as described earlier. As shown in Table 4.64-1, the total mutation frequency induced by MC was 0.12% for the $\underline{sh}$-locus and 0.09% for $\underline{wx}$-locus, as compared to 0.01% for each of the shrunken and waxy loci in the control, an increase of almost 10 fold .

In a second experiment, the effects of MC were studied on seed germination, seedling growth, root growth, mutation rate and chromosomal breakage. The results are presented in Table 4.64-2. Seeds treated with one of the three concentrations employed showed that there was no effect on germination as compared to the control, but survival decreased as the concentration was increased. The reduction of root length and plant height was proportional to the concentration of MC employed. The highest concentration (40 µg/ml) produced a 59.1% reduction in root length over the control value.

The various types of cytological abnormalities of root tip mitosis and their frequencies are shown in Table 4.64-3. Anaphase bridges and fragments occurred frequently in the treated materials, although very few aberrations were found in the control material. However, the reduction of root length$\left[r = -0.99,\ t = 14.6 > t\ (^{df\ =\ 1}_{p\ =\ 0.05}) = 12.7\right]$

Table 4.64-1

Frequency of mutants obtained in a cross of
$\underline{yg_2}$ $\underline{sh_1}$ $\underline{wx}$ X $\underline{Yg}$ $\underline{Sh}$ $\underline{Wx}$ following MC treatment.

Treatment	Population (No. of seeds)	No. of mutants			Total mutants			
		Single		Double				
		$\underline{sh_1}$	$\underline{wx}$	$\underline{sh_1}$ & $\underline{wx}$	$\underline{sh_1}$	frequency %	$\underline{wx}$	frequency %
Control	7877	1	1	0	1	0.01	1	0.01
5 µg/ml	8915	4	1	7	11	0.12	8	0.09

Table 4.64-2

Effect on germination, seedling growth and viable chlorophyll mutation rate following the treatment of F_1 maize seeds (9 tester X "Seneca 60") with MC for 12 hrs. at room temperature.

Concentration (µg/ml)	No. of seeds	% germination	Root length*		Plant height**		Survival after 30 days (%)	Viable chlorophyll mutation (M_1 plant basis) % (no.)
			Mean length (cm)	% reduction of control	Mean height (cm)	% reduction of control		
0	100	94.0	6.6 ± 1.6	-	24.7 ± 5.0	-	100	- (0)
5	150	88.7	4.6 ± 2.3	30.3	20.7 ± 8.0	16.2	90.2	10.5 (14)
10	150	92.0	4.2 ± 1.9	36.4	21.2 ± 6.9	14.2	87.7	13.7 (19)
40	200	91.0	2.7 ± 1.7	59.1	17.9 ± 6.0	27.5	85.7	12.6 (23)

* 3 days after treatment.

** 14 days after treatment.

Table 4.64-3

Effect of the treatment of F_1 maize seeds (9 tester X "Seneca 60") with MC for 12 hrs. at room temperature on chromosome breakage, as studied in first root tip mitosis at 3 days after treatment.

Concentration (µg/ml)	No. of anaphase cells scored	No. of abnormal anaphases	% abnormal anaphases	No. of bridges and pseudo-chiasmata	No. of fragments	No. of lagging chromosomes
0	413	3	0.7	2	1	2
5	233	20	8.6	15	14	1
10	242	31	12.8	14	26	5
40	195	49	25.1	20	43	15

and frequency of chromosome aberrations $\left(r = 0.99,\ t = 8.6 > t\ (^{df = 1}_{p = 0.1}) = 6.3\right)$ show a correlation with the concentration of chemical.

The most striking phenotypic change in the MC-treated M_1 plants were yellow, albino and pale-green longitudinal stripes of various sizes in leaves (Figure 4.64-1). The frequency of viable chlorophyll 'mutations' is shown in Table 4.64-2. No chlorophyll mutants were found in the control. These experiments suggest that MC is also a mutagen in Zea mays.

4.7 Induction of Chromosomal Aberrations and Mutations with HAS:

4.71 General observations:

The results presented in Table 4.71-1 show the types and frequencies of chromosome aberrations resulting from a 2 hr. HAS treatment. The general effects of HAS on Zea mays chromosomes are observed as chromatid breaks, isochromatid breaks and centromere breaks (Figure 4.71-1). Multiple chromosomal aberrations or shattering of chromosomes also occurred in about 1% of the nuclei. Occasionally, the chromosomes became despiralized (Figure 4.71-2A, 2B). These phenomena were observed during the 14 hr. to 30 hr. recovery period. Chromatid exchanges were rare after 2 hr. HAS treatment. However, subchromatid exchanges still could be observed (Table 4.71-1). The latter resulted in the formation of bridges at anaphase and telophase. Additionally, lagging chromosomes and tripolar nuclei also appeared at anaphase and telophase. Interphase showed micronucleus (Figure 4.71-3).

Fig. 4.64-1

Chlorophyll mutants induced by MC.

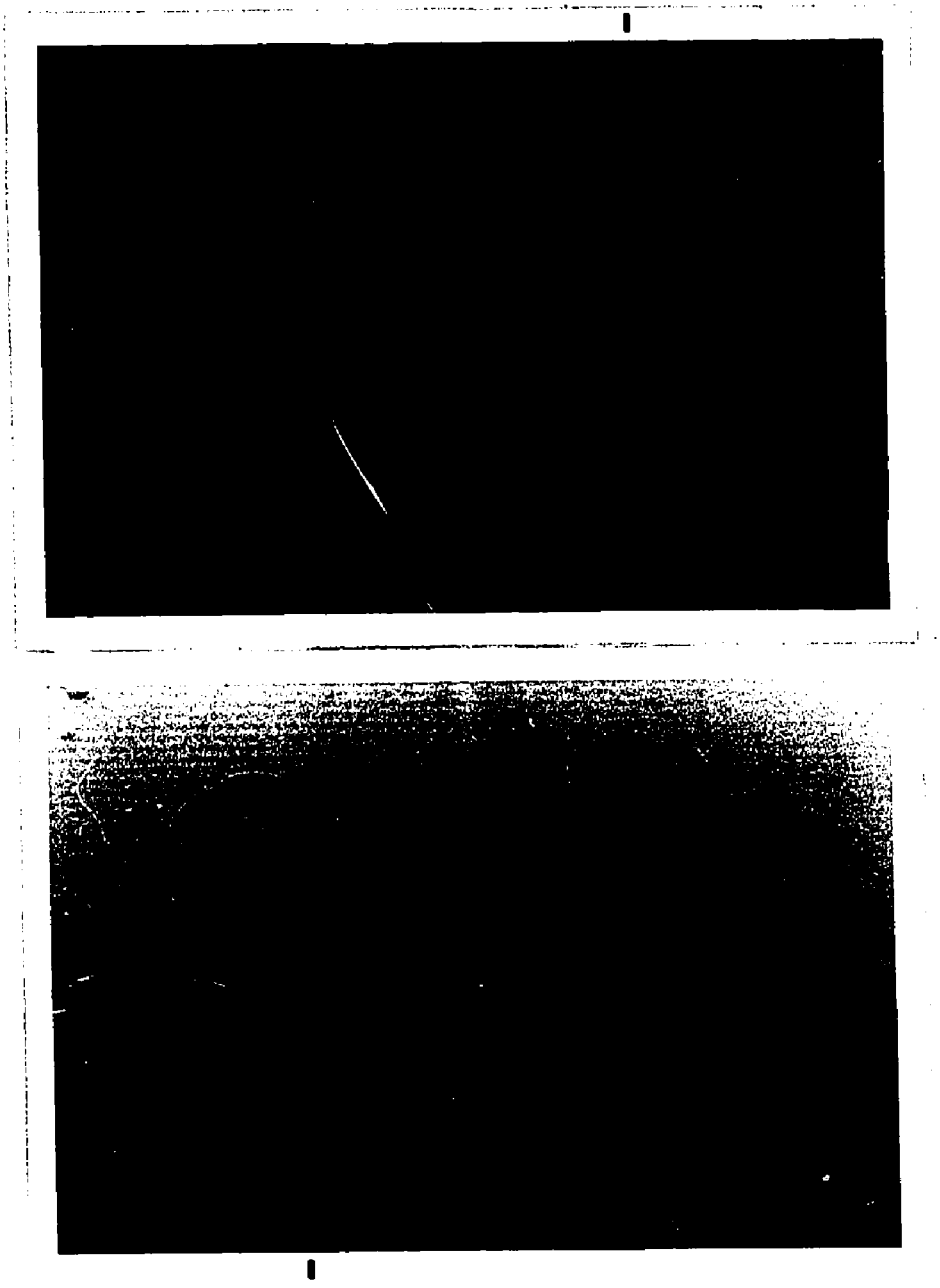


Table 4.71-1

Types and frequencies of chromosome aberrations resulting from treatment with 2 hr. HAS, at 24 ± 1°C.

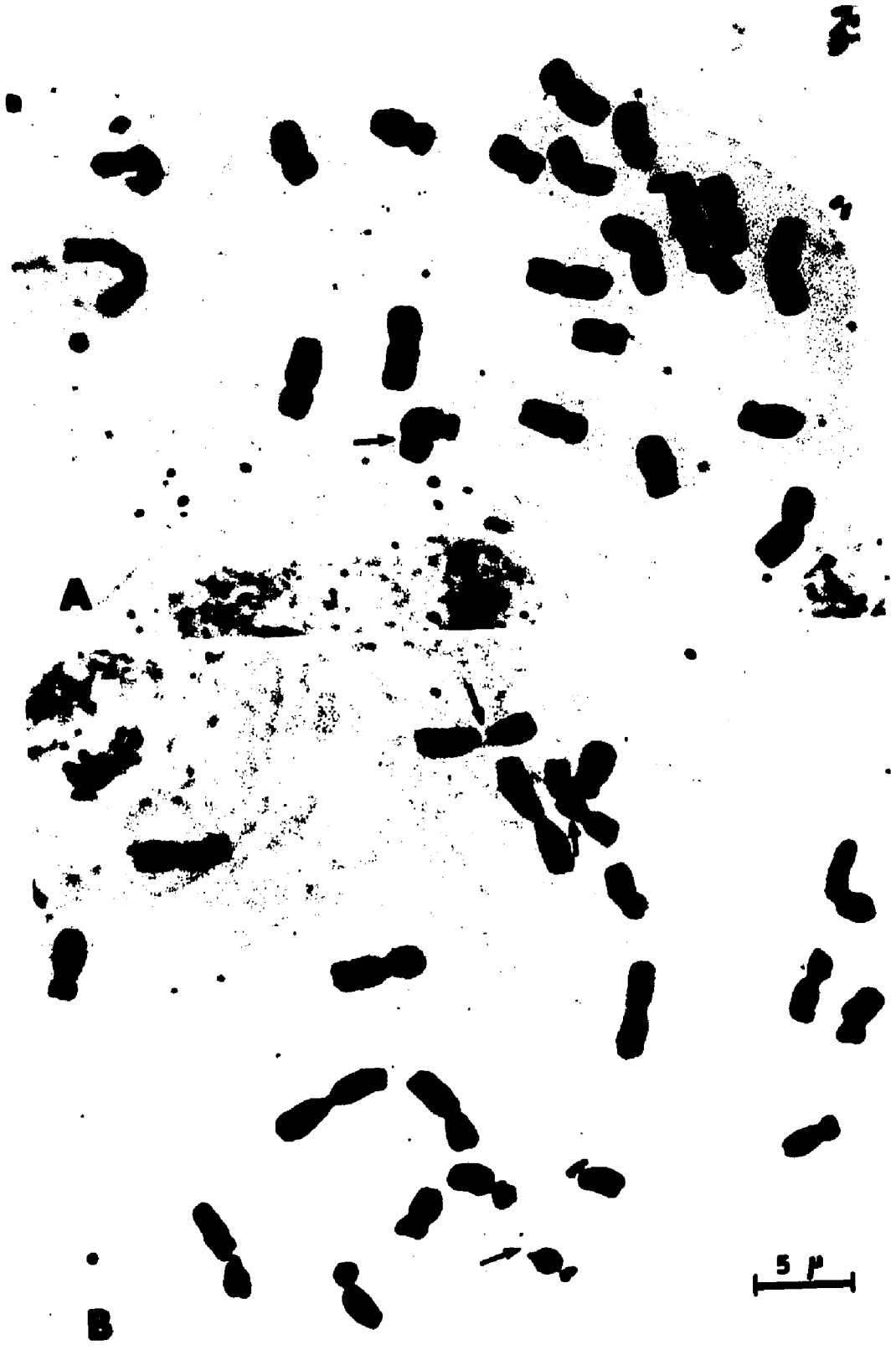
Treatment Concentration (mg/ml)	Fixation hrs. after treatment	No. of metaphases scored	% abnormal metaphases	Aberrations (per 100 cells)						
				Chromatid breaks	Isochromatid breaks	Centromere breaks	Chromatid exchanges	Subchromatid exchanges	Chromosome translocation.	Total breaks per cell
Control	-	100	0	-	-	-	-	-	-	-
0.5	3	168	8.3	7.14	0	1.2	0	2.4	0	0.13±0.02
0.5	5	50	20.0	10.00	4.0	12.0	0	0	0	0.26±0.07
0.5	16	123	25.2	12.20	0.8	14.6	1.6	6.5	0.8	0.45±0.06
0.5	20	71	11.3	9.86	1.4	4.2	0	1.4	0	0.18±0.05
0.5	24	227	12.3	7.49	3.1	7.9	0	0.9	0	0.20±0.03
0.5	28	266	14.3	7.14	3.0	8.9	0	1.5	0	0.22±0.03
2.0	3	201	10.5	7.46	3.0	1.0	0	1.0	0	0.13±0.02
2.0	28	353	19.6	7.65	3.4	7.7	0.3	2.8	1.1	0.27±0.02

Fig. 4.71-1

Metaphase cells illustrate types of chromosomal breaks induced by HAS.
Arrows indicate types of chromosomal aberrations:

A. Isochromatid break.

B. Centromeric break and chromatid break.


A
B
5 μ

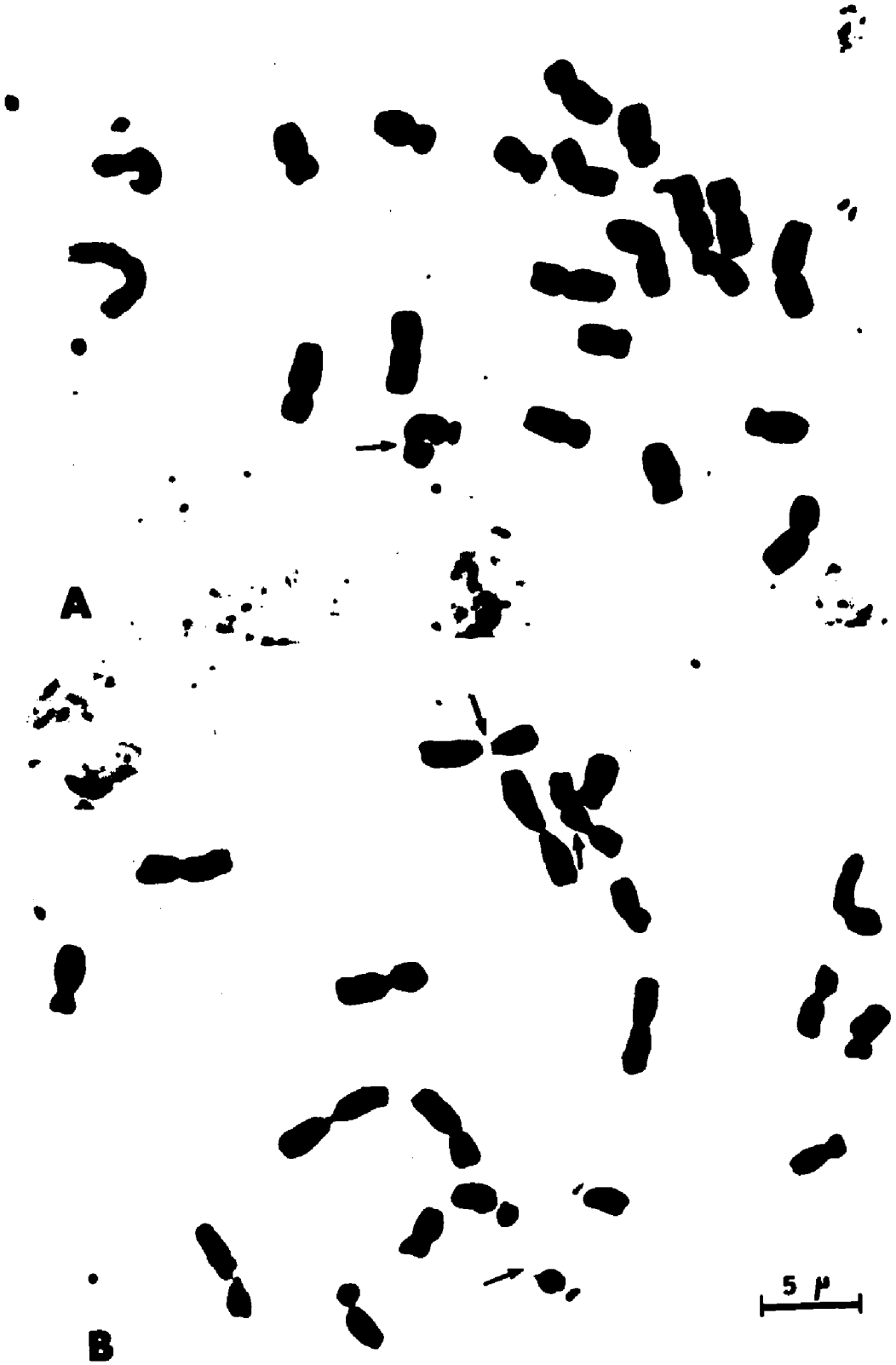

A
B
5 μ

Fig. 4.71-2

Metaphase cells illustrating chromosomal aberrations:

A. Chromosomal despiralization induced by HAS.

B. Multiple chromosome breaks induced by HAS.

C. Multiple chromosome breaks and exchanges induced by MC.


A
B
C
5 μ


A
B
C
5 μ

Fig. 4.71-3

Chromosomal aberrations induced by HAS:

A. Pseudochiasma and fragment at anaphase.

B. Fragment at telophase.

C. Bridge at telophase.

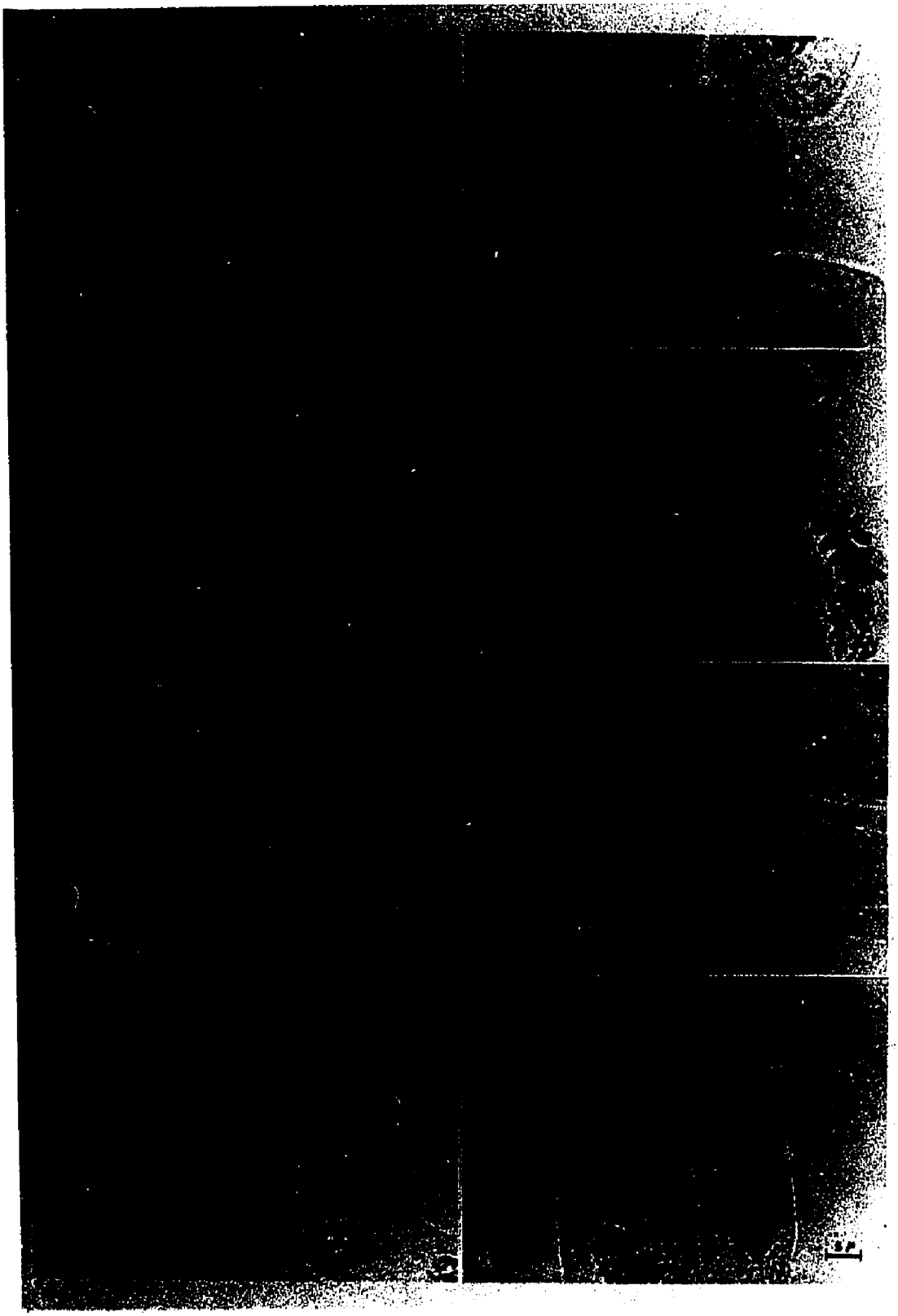
D. Bridge and fragment at telophase.

E. Tripolar nuclei at telophase.

F. Lagging chromosome at anaphase.

G. Lagging chromosome and fragment at telophase.

H. Micronucleus at interphase.




A
C
D
F
G
H
5 μ

4.72 Distribution of chromosomal breaks:

The data describing the distribution of chromosomal breaks over the 10 pair of chromosomes after fixation at 24 hr. and 28 hr. are shown in Table C-3. If one considered the length of chromosomes proportional to number of breaks, an equal distribution of the chromosomal breaks would be expected. Tables C-8, C-9, C-10 and C-11 indicate that χ^2-test for the distribution of breaks over total chromosomes, chromosomal arms and centromeres reveals a highly significant deviation from an equal distribution.

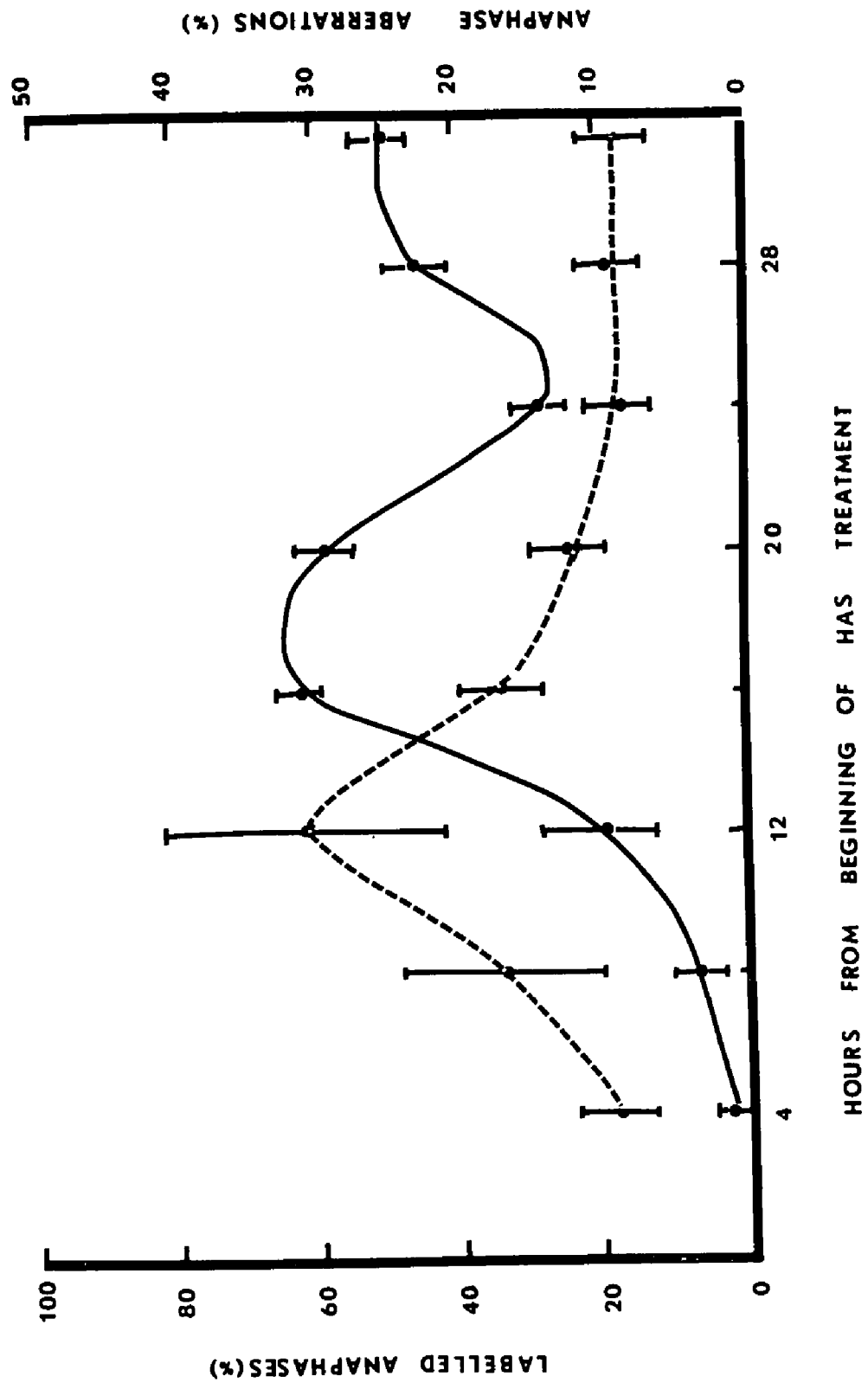
The results summarized in Figure 4.62-2 show that the distribution pattern of breaks was highly agent-specific, with specific chromosomes and chromosome segments involved. The specificity of HAS and MC to induce breakage in the centromere, nucleolar constriction and heterochromatic region was evident.

4.73 Chromosomal aberrations in relation to the nuclear cycle:

A relationship between nuclei at different stages in the nuclear cycle at the time of treatment and their sensitivity to the induction of chromosomal breakage is shown in Figure 4.73-1. These results indicate that the highest frequency of chromosomal aberrations occurred during the 8 to 16 hr. period involving those nuclei which had been in early G_2 and late S at the time of treatment. The frequency of aberrations induced in nuclei which were in G_1 and early S phases is low.

Fig. 4.73-1

Proportion of labelled anaphases (———)
and frequency of anaphase aberrations
(- - - - - - -) after exposure for 30 min.
to ^{3}H-thymidine followed by 2 hr. treatment
with HAS (0.5 mg/ml).
One standard deviation either side of the mean
is shown as a vertical bar.


ANAPHASE ABERRATIONS (%)
50
40
30
20
10
0
LABELLED ANAPHASES (%)
100
80
60
40
20
0
4
12
20
28
HOURS FROM BEGINNING OF HAS TREATMENT

4.74 Induction of mutations:

F_1 seeds (9 tester X "Seneca 60") were treated with HAS for studying its effects on germination, root growth, seedling growth, chromosomal breakage and chlorophyll mutations. The data are summarized in Table 4.74-1. The concentrations used proved to have no effect on germination, although they did show some influence on survival. The reduction of root length and plant height is proportional to the concentrations used.

The types and frequency of aberrations are given in Table 4.74-2. Very few aberrations (0.7%) were observed in the control. The frequency of chromosomal aberrations is correlated with the concentrations $\left[r = 1,\ t = 159.3 > t\ (^{df\ =\ 1}_{p\ =\ 0.01}) = 63.7\right]$. The reduction of root length is also correlated with the chromosomal aberrations $\left[r = -0.97,\ t = 5.4 > t\ (^{df\ =\ 2}_{p\ =\ 0.05}) = 4.3\right]$. However, chlorophyll mutations are very low 0 - 2%. These suggest that HAS has little effect as a mutagen in Zea mays.

Table 4.74-1

Effect on germination, seedling growth and viable chlorophyll mutation rate following the treatment of F_1 maize seeds (9 tester X "Seneca 60") with HAS for 12 hrs. at room temperature

Concentration (mg/ml)	No. of seeds	% germination	Root length*		Plant height**		Survival after 30 days (%)	Viable chlorophyll mutation (M_1 plant basis) % (no.)
			Mean length (cm)	% reduction of control	Mean height (cm)	% reduction of control		
0	100	94.0	6.6 ± 1.6	-	24.7 ± 5.0	-	100	- (0)
0.5	100	95.0	6.0 ± 2.4	9.1	22.9 ± 3.9	7.7	98.9	2.0 (2)
5.0	100	92.0	3.7 ± 2.4	43.9	20.8 ± 4.8	15.8	95.7	- (0)
30.0	150	92.0	0.8 ± 0.9	87.9	18.2 ± 7.0	26.3	93.4	1.3 (2)

* 3 days after treatment.

** 14 days after treatment.

Table 4.74-2

Effect of the treatment of F_1 maize seeds (9 tester X "Seneca 60") with HAS for 12 hrs. at room temperature on chromosome breakage, as studied in first root tip mitosis at 3 days after treatment.

Concentration (mg/ml)	No. of anaphase cells scored	No. of abnormal anaphases	% abnormal anaphases	No. of bridges and pseudo-chiasmata	No. of fragments	No. of lagging chromosomes
0	413	3	0.7	2	1	2
0.5	169	5	3.0	2	0	6
5.0	228	11	4.8	4	5	3
30.0	147	22	14.7	5	10	8

CHAPTER 5

DISCUSSION

The purpose of the present study was to examine the effects of mitomycin C (MC) and hydroxylamine sulfate (HAS) on the nuclear cycle, the induction of chromosomal aberrations and mutations in Zea mays. The main features of my thesis are outlined as follows:

1. Alterations in the nuclear cycle components and inhibition of mitosis by the application of MC and HAS.

2. The induction of endoreduplication and the provision of a model for its explanation.

3. The frequency of chromosomal aberrations assignable to specific chromosomes.

4. An examination of the stage sensitivity of nuclei regarding the production of chromosomal aberrations.

5. An investigation of the induction of chlorophyll mutants, and mutants at the waxy and shrunken loci.

5.1 Alteration of the Nuclear Cycle and Mitotic Inhibition:

The effects of MC (10 µg/ml) and HAS (0.5 mg/ml) on the disruption of the nuclear cycle and mitotic inhibition are reported.

5.11 Mitomycin C (MC):

The main effect of MC on the duration of the nuclear cycle in Zea mays root meristems was a marked increase in the duration of S and G_2, as compared with the control value (Table 4.1-1). When roots were exposed to ^{3}H-thymidine followed by treatment with MC, S lasted for 5.5 hr. longer than the control. Figure 4.1-2 indicates that mitotic indices were reduced during the recovery time. Mitotic division was inhibited in those cells which were in the S period during the time of treatment. These results are in agreement with those of Nowell (1964), who offered a similar conclusion from his study of MC in human leukocyte cultures.

The autoradiographic data (Table 4.3-1) on DNA synthesis from the present study reveal that DNA synthesis was inhibited during the treatment and also after treatment with MC. It has been known for some time that MC produced an inhibition of DNA synthesis and degradation of DNA in bacterial and mammalian cells (Reich et al., 1961; Shatkin et al., 1962). Iyer and Szybalski (1964) have shown in Sarcina lutea (DNA with 71% G + C) and in Cytophase johnsonii (33% G + C) that MC cross-links complementary DNA strands; a high content of G and C favors this crossing linking reaction. The lethal effect (in vivo) of MC is proportional to the G and C contents in DNA (Iyer and Szybalski, 1964). The inhibition of DNA synthesis was due to the cross-linking of DNA (Matsumoto and Lark, 1964). The data obtained from our autoradiographs support this interpretation of the

biochemical effect of MC on the inhibition of DNA synthesis.

A comparison of the labelling indices from the control roots and roots exposed to ^{3}H-thymidine following MC treatment or to a mixture of ^{3}H-thymidine and MC (Table 4.3-1) shows that MC not only reduces the rate of DNA synthesis (40%), but also decreases the number of cells synthesizing DNA. MC appears to be an inhibitor of the onset of DNA synthesis, i.e., it inhibits the transition of G_1 cells to S, resulting in a lower labelling index and the accumulation of the cells in G_1. The proposal of the ability to inhibit the onset of DNA synthesis is supported in the literature: 2,4,5-trichlorophenoxy-acetic acid was reported to inhibit the $G_1 \rightarrow S$ transition in Vicia faba (Macleod, 1969).

The duration of G_2 is 4.5 hr. longer following MC treatment than in the untreated roots (Table 4.1-1). The mitotic index curve (Figure 4.1-2) shows that there is a considerable decline in the mitotic index during and immediately after the treatment. These results suggest that G_2 cells may be prevented from dividing or slowed in their progress toward division. On the contrary, Natarajan and Schmid (1971) have indicated, in Chinese hamster cell cultures treated with a lower concentration (0.6 μg/ml) of MC for 45 min., the appearance of labelled cells in mitosis was not delayed significantly. However, a continuous fall in the mitotic index was obtained after treatment.

Chemical determinations of protein and nucleic acid contents have shown that MC had a relatively severe inhibitory action on DNA synthesis, and a moderate action on protein and RNA syntheses (Kurodo and Furuyama, 1963). Both RNA and protein syntheses occurred throughout interphase,

but there was evidence that specific proteins were synthesized during a specific period (G_2) of interphase (Tobey et al., 1966). Petersen et al. (1969) found that actinomycin (an RNA synthesis inhibitor) and cycloheximide (a protein synthesis inhibitor) produced different blocks at the G_2 period in Chinese hamster ovary (CHO) cells. Verma and Walden (1971) have shown that cycloheximide and chloramphenicol (protein synthesis inhibitors) resulted in an extension of the duration of G_2 in Zea mays root tips.

G_1 and mitosis were not significantly affected by MC (Table 4.1-1). Data from the continuous treatment experiments (Tables A-9 and A-10) also indicate that phase indices were similar to those of the control. These observations permit the suggestion that each stage of mitosis was not sensitive to MC. The decrease in the mitotic index was due rather to inhibition or blockage in certain stage(s) of interphase.

These results demonstrate that MC inhibition of mitosis in Zea mays root tip cells is actually due to slowing down DNA synthesis. MC also partially inhibits the transition of G_1 to S. However, it should be noted that the higher concentrations of MC show an effect on G_2 period.

5.12 Hydroxylamine sulfate (HAS):

The results from the nuclear cycle experiments show that following HAS treatment there was a block at the end of S resulting in the accumulation of the dividing nuclei at some point following termin-

ation of S period (Figure 4.2-2). Therefore, it may be presumed that the transition of S cells to G_2 was inhibited. Consequently, lengthening the duration of G_2 (10 hr.) was obtained (Table 4.1-1).

In human carcinoma cells (HeLa S3), Puck and Steffen (1963) showed that mitotic cells continued to accumulate after a radiation-induced block had been established. Doida and Okada (1969) demonstrated that radiation produced a block somewhere in the middle of the G_2 stage of mouse leukemic cells, and inhibited protein synthesis causing mitotic delay. By using the biochemical specificity of actinomycin D (an m-RNA inhibitor) and puromycin (a protein inhibitor), Tobey et al. (1966) have provided evidence for the presence of two temporal markers of biological significance which subdivide the G_2 phase of the nuclear cycle and which are related to mitosis. These two markers represent the end of essential m-RNA synthesis in early G_2, and the end of essential protein synthesis in the middle of G_2 for mitosis in Chinese hamster ovary (CHO) cells.

The biological effects of hydroxylamine (HA) are complicated. HA splits amino-acyl ester bonds and inhibits Ac-phe-tRNA binding to ribosomes (Zachau et al., 1958; Jimenez et al., 1970). Clark (1963) has reported that HA inhibited amino acid decarboxylases in plant, insect and mammalian tissues. HA is also a potent inhibitor of the enzyme transaminase whose activity is vital in the general metabolic machinery of the cells (Grossman and Brown, 1969). The results from Zea mays (Figure 4.2-2, explanation in text) show that some point following termination of S period was blocked and that the duration of G_2 was

lengthened by HAS. The time map of the HAS blocking position resembles more closely the blocking position of actinomycin D than that of puromycin (Tobey et al., 1966). The basis of the response of the transition of S to G_2 is unknown. It seems reasonable to predict that some specific proteins which are important for the progression of the cells to mitosis may be affected by HAS. The cells were blocked at the S/G_2 transition resulting in accumulation and delay before entering mitosis. These results are in agreement with hypothesis of blockage of the cell progression from interphase to mitosis which has been proposed by many workers (Lea, 1962; Tobey et al., 1966; Doida and Okada, 1969). The results also suggest that the proposed proteins for the transition of S to G_2 are very important for mitosis, in addition to the synthesis of m-RNA in early G_2 (Tobey et al., 1966).

It should be stressed that in the present experiments the decrease in grain counts and labelling indices during HAS treatment may be caused by a direct effect on nucleotide metabolism which, in turn, would reduce the rate of exogenous thymidine incorporated into DNA. Freese and Freese (1966) have demonstrated that HA with an NOH group, produced hydrogen peroxide and nitroso compounds in the presence of oxygen, and resulted in inactivating effect on DNA. Furthermore, it is well known that HA is highly toxic at low concentrations in bacteria (Gray and Lambert, 1948). Kihlman et al. (1966) have shown that hydroxylamine hydrochloride inhibited the incorporation of ^{32}P into DNA and RNA in root tips of Vicia fabia. However, Young and Hodas (1964) indicated that HA inhibited DNA, RNA and protein syntheses in HeLa cells.

Among other possibilities, it could be that HAS affected RNA and protein synthesis resulting in the lengthening of the duration and inhibition of DNA synthesis. It has been demonstrated that mammalian cells in S which were treated with an inhibitor of protein synthesis showed a rapid, marked decline in DNA synthesis (Littlefield and Jacob, 1965; Young, 1966).

As shown in Table B-1, the interphase labelling index also decreased during the treatment with HAS. Therefore, HAS seems to be involved in the control of the onset of DNA synthesis, i.e., the transition of G_1 to S. However, a significant increase in grain count value and labelling index was obtained following the treatment. The results suggest that the effect of HAS on inhibition of DNA synthesis is easily reversible.

The mitotic index decreased significantly in early G_2 at the time of treatment (Figure 4.2-2). The inhibition of mitotic activity by HA has been described in Vicia faba (Natarajan and Upadhya, 1964; Cohn 1964). In the continuous treatment, mitotic indices dropped slowly during the course of treatment. When intact roots were removed from the solution and grown for 2 hr. or 4 hr. in the absence of HAS on moist filter paper, the mitotic indices decreased rapidly (Figure 4.2-3). Analyzing the phase indices during the continuous treatment, the accumulation of metaphases was also obtained. Therefore, it may be presumed that HAS produced a blocking effect on the cell from metaphase to anaphase during long-term treatment. This effect disappeared when intact roots were removed from HAS treatment.

In conclusion, it is indicated from our work that cells were blocked at some point following termination of DNA replication and were also inhibited in early G_2 resulting in lengthening the duration of G_2 and causing a mitotic delay. The duration of S period was only slightlyaffected. In the long-term treatment, this drug also has some effect on blocking the metaphase cell proceeding to anaphase.

5.2 Endoreduplication

The biochemical events occurring in the nuclear cycle of the cell can be divided into two classes: 1) "cyclic events" - those that appear only at certain definite times in the nuclear cycle, such as the replication of DNA during the S period and some specific enzymes, and; 2) "continual events" - those that are more or less persistent, e.g., energy sources (ATP), carbohydrate and protein syntheses. The former appear to be under genetic control and to be sequentially ordered. Prescott and Goldstein (1967) studied nuclear transplantation in Amoeba proteus and suggested that the presence of an initiator of DNA synthesis in the cytoplasm of cells in the S period could stimulate new DNA synthesis in a G_2 nucleus. Transfer of a nucleus from an S phase cell into a G_2 cell resulted in a sharp reduction of DNA synthesis (Prescott, 1968). It is evident that the control of the cyclic events is achieved through a sequence of transcriptions and translations. The continual reactions may be largely regulated by biochemical kinetics and equilibria.

Practical questions confront us: What events necessarily go in sequences? What are the "transitions" or "points of no return" in the nuclear cycle resulting in the regulation of chromosome replication and segregation? In the normal condition, the nuclear cycle is governed by sequential gene activation. Masters and Pardee (1965) have found that the syntheses of four enzymes of Bacillus subtilis, - histidase, aspartate transcarbamylase, ornithine transcarbamylase and dehydraquinase - are in linear sequence both as a function of the nuclear cycle and in relation to the genetic map. In Schizosaccharomyces pombe, Bostock et al. (1966) reported that both the periodic and continuous synthesis of enzymes occurred during the cycle. Furthermore, it was found that basal synthesis of sucrase and maltase was continuous, while there was periodic synthesis of aspartate transcarbamylase and ornithine transcarbamylase which may be controlled autogenously. In Physarum polycephalum, Cummins (1969) suggested that the sequence of DNA replication was controlled by specific initiator proteins. These findings therefore extend our knowledge of the nuclear cycle to include the view that the modes of enzyme syntheses are found in both prokaryotic and eukaryotic cells. The biochemical events are step by step. There is a direct causal connection between each marker and the one that follows it.

Endoreduplication may permit us to order these events somewhat more accurately. In nuclei which have undergone two series of DNA replications before entering mitosis, the duplicated chromosomes characteristically occur in pairs at metaphase. Why can the cells be induced to undergo a second series of DNA synthesis and then enter mitosis?

A hypothetical nuclear cycle map must be consistent with the following observations:

1. HAS treated cells were inhibited at the termination of DNA replication (presumably, a transition factor between the two phases was affected), and lengthened the duration of the G_2 period.

2. DNA synthesis was inhibited up to 43% of the control value during the 2 hr. treatment of HAS. When the intact roots were removed from HAS, DNA synthesis inhibition within 2 hr. was reduced to 26%. The duration of DNA synthesis was prolonged 2.5 hr. after the 2 hr. treatment.

3. The intact roots were treated with a mixture of HAS and ^{3}H-thymidine for 2 hr.. The roots were fixed for a two-hour interval during 20 to 32 hr. period after treatment. Our results show that over 90% of cells with diplochromosomes were labelled (Table 4.4-2 and Figure 4.4-2). Therefore, the cells in S period could be induced to undergo a second series of DNA replication by HAS at the time of treatment. The cells in G_1 and G_2 could not be induced. Therefore, each step of the nuclear cycle has its own control system.

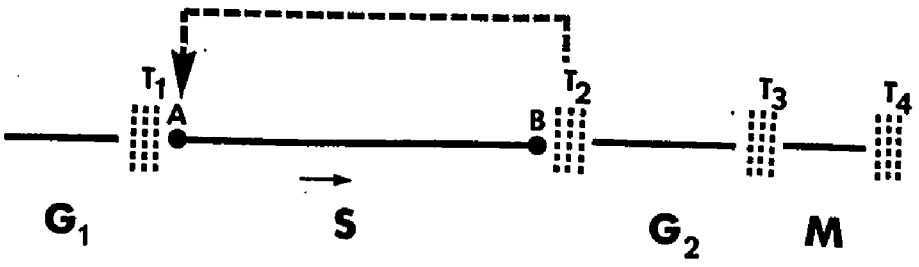


Fig. 5.2-1 Diagrammatic representation of a hypothetical control mechanism for nuclear cycle including the induction of endoreduplication.

In Figure 5.2-1, "A" represents the initiator for each DNA replicon to start the synthesis of DNA, i.e., bringing about the interaction between DNA and DNA polymerase necessary for DNA synthesis. "B" is a terminator which determines the end of DNA synthesis for each replicon. T_1, T_2, T_3 and T_4 represent the transition factors. There are two functions for each transition factor, e.g., T_2;

1. It controls the transfer of cells from S to G_2.
2. It inhibits the initiator "A" (to initiate the next cycle of DNA synthesis after finishing DNA synthesis).

The T_2 factor functions only at the end of S after the terminator "B" is activated. The nucleus can pass through S to G_2, if the circuit is open". The signal reverts to "A" and the nucleus stays in the S period if "T_2" is "closed".

When the T_2 transition factor was affected by HAS, the nucleus remained in the S period. If the T_2 factor could not inhibit initiator "A" activity at the end of the first DNA synthesis, initiator "A" would bring about the start of the next cycle of DNA synthesis (i.e., a second series of DNA synthesis). Once the effect of HAS was removed from the T_2 factor, the nucleus continued on into the G_2 stage and to mitosis, permitting the observation of the diplochromosomes.

Our results and hypothesis are in agreement with the findings of Johnson and Rao (1971), who also demonstrated that DNA synthesis was not generally begun in nuclei going through the G_2 period, even in the presence of the inducer (initiator of DNA synthesis). Van't Hof and Kovacs (1971), working on Vicia and Pisum excised root tissue culture, suggested that two principal controlling points, G_1/S and G_2/M, are energy dependent and sensitive to radiations. Recently, Maugh II (1972) has described a molecular control mechanism that normally holds cell growth in check. The rationale for this phenomenon is the existence of chalones (a type of glycoproteins), endogenous mitotic inhibitors that control cell proliferation by negative feedback inhibition. That is, mature cells might synthesize and release chalone. High levels of chalone within the tissue system would then inhibit further replication of immature cells.

In summary, the hypothesis for induction of endoreduplication presented above is based on the idea negative feedback control. The cell moving from one phase to another phase is controlled by a negative

feedback system of transition factor. We presume that the transition factor might serve as a controlling element to govern the cell traversing through the cycle.

5.3 Chromosomal Aberrations and Mutations with MC and HAS.

5.31 Mechanism of action of MC and HAS on chromosomes.

MC is a derivative of urethan and of ethylenimine. It is biologically inactive in its natural state, but it becomes a bifunctional alkylating agent upon chemical or enzymatic reduction *in vivo* (Iyer and Szybalski, 1964). Iyer and Szybalski (1963; 1964) have shown that MC extensively cross-links complementary DNA strands. Such cross-links may inhibit DNA replication leading to deletions or to breakages in the double helix of DNA (Laurence, 1963).

Fishbein *et al.* (1970) described mutations induced by alkylating agents in which the altered base, usually a guanine quatermized at the N-7 position, was assumed to ionize differently, leading to occasional pairing errors. The other possibility was that the alkyl group on the N-7 of guanine labilizes the β-glycosidic bond, resulting in depurination. The loss of this base would leave a gap in the DNA template, such that during subsequent replication, a deletion or an insertion of a base into the new strand opposite the deletion may occur. Brookes *et al.* (1969) suggested that the depurination site will subsequently yield cleavage of the polynucleotide chain by β-elimination process. It could be an important mechanism for chromosomal breakage.

Hydroxylamine sulfate (HAS) is a salt of hydroxylamine (HA). HA is a nucleophilic and reducing agent. The reaction of HA with DNA is pH dependent (Phillips and Brown, 1967). At high concentrations, (0.1 to 1.0M) and high pH (pH 9), HA reacts exclusively with the uracil moieties of nucleic acids. At low pH (pH 6) and high concentration, HA reacts exclusively with the cytosine moieties of DNA, aminating only at the C-4 atom (Freese et al., 1961). But at low concentrations (< 0.1M), the pH is not a critical condition; the reaction involves all four bases (Freese et al., 1961; Freese and Freese, 1965). Both chemical and genetic results suggest that the strong mutagenic effect of HA observed under all these conditions induces only transitions from G-C to A-T (Freese and Freese, 1966).

HA with free NOH group produces, in the presence of oxygen, hydrogen peroxide and a nitroso compound. The free radicals or hydrogen peroxide (via radicals) directly inactivate DNA. If the inactivating DNA alteration is not repaired, it may lead to chromosomal breaks (Freese and Freese, 1966). However, HA has been found to induce a mainchain cleavage in isolated DNA at sites which were believed to be peptide esters holding the polynucleotide units together (Bendich et al., 1963; Bendich and Rosenkranz, 1963). These amino acid ester linkages would be equivalent to the R-linkers which, according to the chromosome model of Taylor (1963), link the replicating units of the chromosomal DNA in a tandem-like fashion. This may constitute a more realistic explanation of the chromosomal cleavages.

In short, it may be noted that MC and HAS differ with respect to chemical structure and mode of action. Since both of them induce

chromosome breakage, it may be assumed that widely different pathways induced by each chemical leads to more or less the same biological effect, i.e., chromosome breakage. However, the difference between MC and HAS cytologically appears to be that MC induces a higher frequency of chromatid exchanges, translocations and ring chromosomes. These types of aberrations are induced by HAS only rarely. Multiple fragmentations and exchanges were found after MC treatment (Figure 4.71-2C). HAS induced fragmentations and despiralizations (Figure 4.71-2A, 2B), which is in agreement with the findings of Somers and Hsu (1962) in Chinese hamster cells. Furthermore, MC is highly active as a chromosome-breaking agent in Zea mays, but HAS induced a lower frequency of chromosome breaks.

5.32 Localization of chromosomal breakage.

The ten pairs of somatic chromosomes of Zea mays can be recognized from each other (Figure 4.5-1). It is an excellent material to study the relative amounts of damage produced in specific chromosome regions by different chemicals. Random occurrence of chromosomal breaks would tend toward an equal distribution. Chromosome breaks induced by MC and HAS are not equally distributed among or within chromosomes (Tables C-4, C-5, C-6, C-8, C-9 and C-10). Even among the centromeres the distribution of the breakage is localized (Tables C-7 and C-11). A similar observation on meiotic chromosomes of Zea mays induced by radiations has been reported by Jancey and Walden (1972). The incidence of somatic chromosomal breaks induced by MC or HAS in the centromeres, secondary constriction and heterochromatin were higher than expected (Lin and Walden, 1971; 1972).

According to the morphological differentiation of chromosomes, two major kinds of chromatin have been recognized, namely, eu- and heterochromatin. A possible role of heterochromatin in the production of aberrations has been reported by Natarajan and Ahnström (1969):

1. The induced lesions remain open for a long time due to the late replication of the DNA in these regions.

2. The lesions remain in close contact in view of the formation of chromocenters.

Natarajan and Ahnström (1969) supported the reasons outlined above and showed that very few aberrations were induced by MC in Nigella damascena, a species which lacks heterochromatin chromocenters and late replicating regions of heterochromatin DNA.

Evans (1964), based on his experiments with Vicia faba using ^{3}H-thymidine and on the work of other authors, suggested that there is a general rule governing the time of DNA synthesis in the eu- and heterochromatin regions of autosomes. Chromosome regions showing positive heteropycnosis in the interphase and early stages of mitosis appear to replicate late in S phase, usually after completion of replication in the euchromatin. Douglas (1971) has reported similar results in Zea mays. It is possible that there is a certain relationship between the late replication of heterochromatin and localization of chromosome breakage in the heterochromatic regions. Further, the chromosomes possessing constitutive heterochromatin were more often involved in aberrations than those of functional heterochromatin in Chinese hamster (Natarajan and Schmid, 1971). Although both types of heterochromatin replicate late in the S period, they probably possess different sequences

or different types of DNA in their regions. However, it is of interest to note that quinacrine mustard (QM) and related compounds most probably bind to the constitutive heterochromatin and not to the functional heterochromatin (Caspersson et al., 1969; George, 1970). Horn and Walden (1971) showed that some of the heterochromatin in Zea mays was not fluorescent after staining with QM, especially around the centromeric regions of the chromosomes. Our data also show that the higher frequencies of chromosomal breaks occurred in the heterochromatic regions apart from the centromeres rather than in those adjacent to the centromeric region (Figure 4.62-1).

McLeish (1953) explained the correlation between the breakage points and the heterochromatin distribution by assuming that the final process of breakage took place either within, or on the surface of the chromocenters. However, as postulated by Kihlman (1961), the chromocenter hypothesis does not account satisfactorily for the differing degree and types of localization found after treatment with different agents. The reaction mechanism of the agent and the chemical composition of the affected regions of the chromosomes may be also involved in the localized chromosome breakage.

Natarajan and Upadhya (1964), and Kumar and Natarajan (1965) suggested that certain regions of chromosomes could be defined in their longitudinal differentiation as being rich in A-T or G-C pairs, responding selectively to the action of specific mutagens. In Vicia faba the breaks produced by ethyl methane sulfonate (EMS), which is preferential to guanine, overlapped those induced by HA, which is specific for cytosine. Somers and Hsu (1962) have shown that 5-bromodexyuridine

(5-BdUR) and HAS induced localized breaks in mammalian chromosomes and the breakage points for these two agents are non-overlapping.

However, Michaelis and Rieger (1958) showed that chromosome aberrations induced by automutagenic agents as well as anaerobiosis were mostly localized in heterochromatic regions. Rather than support the above suggestions, this latter observation points towards the general tendency of these regions to be involved in aberrations.

Very little is known about the chemical composition of the centromeres. Our data indicate a preferential effect of MC and HAS on centromeric regions in Zea mays chromosomes. These results are in agreement with the findings of Somers and Hsu (1962), who employed HA in Chinese hamster cell culture. These authors suggested the possible composition of centromeres as being rich in G-C contents, since these regions were not preferentially damaged by 5-BdUR. However, Swaminathan et al. (1962) have indicated that a high frequency of aberrations was induced by EMS in the centromeric regions of the chromosomes of Hordeum vulgare and Triticum vulgare. Natarajan and Upadhya (1964) showed all possible types of breaks and reunions involving the centromeric regions of all the chromosomes of Vicia faba, indicating that EMS and HA preferentially broke these regions. Thus, Natarajan and Upadhya (1964) suggested that any mutagen which acted on either guanine or cytosine induced breaks in the centromeric region in plant chromosomes. Our results obtained from HAS and MC support this conclusion.

Following the treatment with MC and HAS in Zea mays root tips, a high frequency of chromosomal aberrations resulted in the regions of the secondary constriction. Similar results have been reported with

MC in Vicia faba and in human leukocytes by Merz (1961) and Cohen (1969) respectively. Natarajan and Ramanna (1965) have also indicated that HA induced the localization of breaks in secondary constrictions in Hordeum vulgare.

In addition, Kihlman (1966) has shown that the accumulation of 8-ethoxycaffeine and deoxyadenosine in the nucleus specially concentrated in the nucleolar region of chromosome 1 of Vicia faba. 8-ethoxycaffeine and deoxyadenosine have been reported to inhibit DNA synthesis and to accumulate dATP in the cells (Odmark and Kihlman, 1965). Natarajan and Upadhya (1964) suggested that 8-ethyoxycaffeine and deoxyadenosine interfered with thymidine synthesis and induced breaks at the secondary constriction regions. These authors suggested that the secondary constriction regions might be rich in A-T pairs of DNA. If the nucleolar region is rich in A-T pairs, which is the main factor to cause breaks at this region. Our results also indicate that MC and HAS induced a high frequency of breaks at secondary constriction (Figure 4.62-2). MC attacks guanine, while HA attacks cytosine. However, Kihlman (1966) suggested that the localization of breaks in the nucleolar regions of Vicia faba resulted from the accumulation of 8-ethoxycaffeine and deoxyadenosine on the surface of nucleolus and therefore suppressed the synthesis of deoxyribonucleotides. By appropriate autoradiographic studies, Collaghan and Grun (1961) have shown that maleic hydrazide accumulated preferentially at the nucleolar regions before getting distributed to other parts of the cells. It could be possible that a higher concentration of a chemical distributed at the nucleolar region caused the localization of breaks in this region.

In conclusion, many factors such as the reaction mechanism of the agent and its distribution in the nucleus, as well as chemical composition and physiological activity of the affected regions of chromosomes, may be involved in the production of localized chromosome breakage. Furthermore, a study on the chemical composition of chromosomal DNA by molecular hybridization of cytological preparations would be helpful to understand the molecular structure of specific regions of Zea mays chromosomes, and might provide more information on the action of chemicals on chromosomal aberrations on the molecular basis.

5.33 Chromosomal aberrations in relation to the nuclear cycle.

It has been shown that most of the chromosome-breaking agents not only produced chromosome aberrations but also caused mitotic delay (Hsu et al., 1962: Kihlman, 1966; Lin, 1971; Lin and Walden, 1971). The combination of labelling with ^{3}H-thymidine and applying a chromosome breaking agent enables one to compare with a high degree of accuracy of the radiomimetric sensitivity among cells in various stages of the nuclear cycle. It is possible to detect not only S-cells but G_2-cells as the unlabelled cells which reached division before the labelled (S) cells.

The results presented in Figure 4.63-1, 4.63-2 and 4.73-1 show that stage sensitivity induced by MC and HAS is very different from each other. As shown in Figures 4.63-1 and 4.63-2, very few aberrations were observed in the cells which were in G_2 at

the time of the MC treatment. A similar observation has been reported for other alkylating agents, such as ethyl methane sulfonate and nitrogen mustard (Kihlman, 1966). The aberration yield was highest in those cells that had been in early S and/or late G_1 at the time of treatment. Similar results induced by MC have been reported by Nowell (1964) in human chromosomes from leukocyte cultures.

As for the HAS treatment, the results of aberrations indicate that the treatment of cells in early G_2 and late S periods was effective in inducing chromosome aberrations (Figure 4.73-1). The aberrations produced were chromatid and subchromatid breaks. The yield of aberrations in early S and G_1 cells was low. These results are in agreement with the proposition of Cohn (1964), who suggested that breakage might be induced during or after the period of DNA synthesis. However, HAS is of low efficiency in the induction of chromosomal aberrations in Zea mays as well as other organisms, such as Vicia faba and Allium cepa (Cohn, 1964; Kihlman et al., 1966; Nicoloff et al., 1971).

Hydroxyurea, a derivative of HA, produced chromosomal breaks in G_2 (Kihlman et al., 1966). Young and Hodas (1964) showed that hydroxyurea inhibited only DNA synthesis, while HA inhibited RNA and protein syntheses as well. However, HA induced chromosomal damage by direct action on the cytosine moiety of DNA and main-chain breaks in DNA (Freese et al., 1961; Freese and Freese, 1966; Bendich et al., 1963).

Another possibility may be that HA cleaves acetyl coenzyme A, thereby disrupting oxidative phosphorylation and reducing the concentrations of cellular and mitochrondrial ATP (Fishbein and Carbone, 1963).

Ahnström and Natarajan (1966) suggested a hypothesis that the deficiency of deoxyribonucleoside triphosphates could cause chromosomal aberrations which were due to a reversal of the DNA polymerase reaction. These authors also indicated that 5-fluoro-2-deoxyuridine and 2-deoxyadenosine induced chromosomal breaks (mainly in G_2) are the result of a reversal of DNA polymerase reaction.

On the basis of the cytological data at hand, a stage sensitivity to HAS has been indicated. The action of HAS on different stages apparently involves direct and indirect actions on DNA and chromosomes.

5.34 Induction of mutations with MC and HAS.

The results presented in Tables 4.46-1 and 4.46-2 show that MC increased the frequency of mutations almost 10 times higher than that of the control value. This is in constrast to the findings of Holliday (1964) in Ustilago and Saccharomyces. However, the high mutagenicity of MC has been reported in Escherichia, Drosophila and Habrobracon sperm (Iijima and Hagiwara, 1960; Suzuki, 1965; Schewe et al., 1971; Smith, 1969).

The possible mechanism of mutation induced by MC has been described in Section 5.31. There is a general increase in the yield of cytological aberrations and the lethal effect corresponding to an increase in the concentration. This may be because higher concentrations caused a higher frequency of chromosomal aberrations and disturbed the physiological balances.

The results of HAS treatment with seeds show that chlorophyll mutations were very low, about 0-2%. These suggest that HAS has

a low effect on induction of mutations in Zea mays. Similar results from the M_1 generation of treated plants have been reported by Jain et al. (1968) in Lycopersicon. Natarajan and Ramanna (1965) indicated HA is non-mutagenic in Hordeum. The authors also suggested two processes - chromosome breakage and mutagenesis - were independent of each other in Hordeum. However, HA has reported to be a strong mutagen in microorganisms (Freese et al., 1961; Freese and Strack, 1962; Malling and de Serres, 1971). The mutagenic action of HA has been mentioned in Section 5.31.

The frequency of chromosomal aberrations is correlated with the exogenous concentrations of mutagen. However, the questions of diffusion into the nuclei and inactivation of the chemical during the treatment can not be approached using the methods of the present study. The sample size in our study on chlorophyll mutations was small, and we did not observe M_2 plants. Some recessive mutations could not be observed from M_1 treated plants. Consequently, our observations must be considered with these constraints in mind.

CHAPTER 6

SUMMARY

The purpose of the study reported in this thesis was to consider the effects of chemical mutagens on chromosome structure and gene mutations. This thesis considers the following facets: 1) the effects of mitomycin C (MC; 10 µg/ml) and hydroxylamine sulfate (HAS; 0.5 mg/ml) on the nuclear cycle and mitotic inhibition; 2) the induction of endoreduplication and a hypothetical approach for its explanation; 3) from metaphase chromosomes, the frequency of chromosomal aberrations assignable to specific chromosomes; 4) the stage sensitivity of chromosomal aberrations; 5) the induction by MC and HAS of chlorophyll mutations, and waxy and shrunken loci; and 6) an analytical approach to experimental data. The findings are outlined as follows:

6.1 Mitomycin C (MC)

1. The effect of MC (10 µg/ml) on the duration of nuclear cycle in Zea mays root meristem cells, was studied with autoradiographic methods and it was shown that the total nuclear cycle was prolonged for an additional 12 hrs., as compared with the control value (10 hrs.) at $24 \pm 1^{o}C$.

2. The high sensitivity of the S phase and some affectivity of G_2 to the MC treatment resulted in lengthening the duration of the nuclear cycle in the S and G_2 periods.

3. There was a significant reduction in mitotic indices after treatment in comparison with the control values. The results indicate that the mitotic inhibition was mainly in the S period.

4. The results from autoradiographic studies show that MC (5 µg/ml) reduced the incorporation of the radioactive precursor into DNA and decreased the percentage of labelled interphase nuclei, not only during the time of treatment but also after the treatment.

5. MC produced a high frequency of chromosomal aberrations, mostly isochromatid breaks, chromatid breaks and exchanges, and it was concluded that this drug is very active as a chromosome-breaking agent in Zea mays.

6. The induced aberrations are not equally distributed among the chromosomes or within a chromosome. The high frequency of damage occurred in the regions of the nucleolar constriction, centromeres and heterochromatin.

7. The cells which are preparing for DNA synthesis and chromosome duplication (cells in early S and very late G_1) are sensitive to MC.

8. Mutagenicity of MC was studied and the results indicated that it induced chlorophyll, waxy and shrunken mutations in Zea mays.

6.2 Hydroxylamine Sulfate (HAS):

1. The data from autoradiographic experiments show that HAS (0.5 mg/ml) appears to block some point following termination of S to G_2 resulting in accumulating the dividing nuclei at the end of S period and to inhibit in early G_2. Therefore, lengthening the duration of G_2 (10 hrs.) was obtained. The duration of S period was prolonged for an additional 2.5 hrs., as compared with the control (5 hrs.) at 24 ± 1°C.

2. Furthermore, it was also concluded that HAS reduced the incorporation of ^{3}H-thymidine into DNA during the treatment. The incorporation of ^{3}H-thymidine showed a significant increase following the removal from the drug, suggesting that the effect of HAS on DNA synthesis is easily reversible.

3. The incidence of chromosomal breaks was localized as follows: The regions in the centromeres, heterochromatin and nucleolar constriction were more sensitive to HAS.

4. The results of aberrations indicate that the treatment of cells in early G_2 and late S periods was effective in inducing chromosomal aberrations.

5. The other cytological effect of HAS is the induction of endoreduplication. A hypothesis for induction of endoreduplication based on a feedback control mechanism is proposed.

6. The preliminary experiment on chlorophyll mutations of M_1 plants suggests that HAS has little effect on induction of mutations in Zea mays, although it is a strong mutagen in microorganisms.

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APPENDIX A

Frequency Data on Mitosis and Nuclear Cycle

Table A-1

Mitotic index and phase index of mitosis in root tips at 24 ± 1°C (control)

Hours*	Mitotic index	Phase index				Total cells scored
		Prophase	Metaphase	Anaphase	Telophase	
0	9.5 ± 0.22	50.7	17.6	5.7	25.9	16956
8	7.9 ± 0.27	51.4	14.3	5.2	29.0	10138
16	8.3 ± 0.31	49.5	14.9	5.4	30.1	7821
24	9.8 ± 0.35	54.2	13.4	5.0	27.4	7162
32	9.3 ± 0.31	51.7	16.0	4.7	27.7	8724

* Hours from beginning of treatment.

Table A-2

Percentage of labelled nuclei in root tips of "Seneca 60" at 25°C*

Hours after pulse	Percent labelled mitoses			
	Prophase	Metaphase	Anaphase	Telophase
1	6.0 ± 1.0	2.5 ± 1.4	0	0.9 ± 0
3	63.4 ± 3.4	39.7 ± 6.0	19.0 ± 8.5	32.2 ± 6.0
5	92.5 ± 1.0	74.8 ± 3.2	80.4 ± 5.8	74.7 ± 3.3
6	92.2 ± 2.1	95.5 ± 2.3	90.0 ± 6.7	91.0 ± 3.2
7	66.2 ± 2.3	52.4 ± 3.3	61.0 ± 6.5	52.2 ± 4.3
8	16.9 ± 2.6	19.6 ± 4.0	11.4 ± 5.2	23.4 ± 4.0
10	17.4 ± 1.4	12.3 ± 2.3	16.6 ± 7.4	13.0 ± 2.4
12	35.9 ± 2.2	14.2 ± 2.5	18.5 ± 7.4	9.9 ± 1.7
14	56.0 ± 2.0	45.1 ± 3.4	48.5 ± 6.0	40.7 ± 3.2
16	58.6 ± 2.5	51.2 ± 4.6	42.3 ± 9.6	55.0 ± 4.0
18	29.9 ± 2.5	35.6 ± 4.3	34.0 ± 8.8	37.9 ± 4.5

* From Verma (1972; Table A-4).

Table A-3

Percentage of labelled nuclei in root tips of "Seneca 60" at various times after roots exposed for 30 min. to ^{3}H-TdR followed by 2 hr. in MC (10 µg/ml) at 24 ± 1°C.

Hours*	Mitotic index	Percent labelled mitoses				Total cells scored
		Prophase	Metaphase	Anaphase	Telophase	
2	6.9 ± 0.27	4.4 ± 1.11	0	0	0	8645
4	5.7 ± 0.25	16.7 ± 2.48	1.2 ± 1.22	0	3.4 ± 1.53	8230
6	2.9 ± 0.18	33.1 ± 4.28	19.0 ± 5.15	33.3 ± 27.22	9.6 ± 3.45	8923
8	3.0 ± 0.20	51.1 ± 4.30	41.0 ± 7.88	30.0 ± 14.49	28.6 ± 6.45	7658
10	4.6 ± 0.21	68.6 ± 2.72	51.5 ± 6.15	23.8 ± 9.29	33.3 ± 5.14	9962
12	4.2 ± 0.22	90.5 ± 2.42	74.6 ± 5.49	52.9 ± 12.11	67.3 ± 4.47	7988
14	1.0 ± 0.12	93.3 ± 4.56	84.6 ± 10.00	66.7 ± 27.27	76.2 ± 9.29	6629
16	0.8 ± 0.10	92.9 ± 4.87	88.9 ± 10.48	71.4 ± 17.07	66.7 ± 9.62	8176
18	1.5 ± 0.14	62.1 ± 6.37	85.0 ± 7.98	53.6 ± 13.53	85.7 ± 7.64	7616
20	5.0 ± 0.33	40.8 ± 4.49	24.2 ± 7.46	41.7 ± 14.23	37.2 ± 7.37	4198
22	6.0 ± 0.38	33.8 ± 3.85	20.0 ± 6.76	27.3 ± 13.33	26.7 ± 6.59	4005
24	8.3 ± 0.44	32.5 ± 3.41	24.1 ± 5.82	17.7 ± 9.25	17.9 ± 4.18	4015
26	5.0 ± 0.20	27.6 ± 3.96	19.4 ± 7.10	16.7 ± 15.21	20.4 ± 5.76	7049
28	4.3 ± 0.28	25.0 ± 3.71	14.4 ± 5.78	16.7 ± 15.21	14.5 ± 4.24	5784
30	9.3 ± 0.32	39.0 ± 2.34	30.9 ± 4.17	45.8 ± 10.17	27.3 ± 3.36	8186
32	11.7 ± 0.39	21.6 ± 1.89	15.7 ± 3.39	4.2 ± 4.08	14.8 ± 2.48	6854
34	9.0 ± 0.41	21.3 ± 2.22	18.0 ± 4.92	6.7 ± 6.44	13.2 ± 3.29	4960
36	10.4 ± 0.38	16.9 ± 1.87	14.6 ± 3.54	8.3 ± 5.64	12.2 ± 2.56	6554

* Hours after the beginning of MC treatment

N.B. Labelling index = $\frac{10806}{30000}$ = 36.0%

Table A-4

The 95% confidence limits on the labelled mitoses treated with MC (10 µg/ml) for 2 hr. at 24 ± 1°C

Hours*	Prophase	Metaphase	Anaphase	Telophase
2	6.6 - 2.2	-	-	-
4	21.6 - 11.9	3.6 - 0	-	6.5 - 0
6	41.5 - 24.0	29.1 - 8.9	86.7 - 0	18.4 - 0.7
8	59.5 - 42.7	56.5 - 25.6	58.4 - 1.6	41.2 - 15.9
10	74.0 - 63.3	63.5 - 39.5	42.0 - 5.6	43.4 - 23.2
12	95.2 - 85.7	85.3 - 63.9	76.7 - 29.2	76.0 - 58.5
14	102.3 - 84.4	104.2 - 65.0	120.0 - 13.3	94.4 - 58.0
16	102.4 - 83.3	109.4 - 68.3	104.9 - 38.0	85.5 - 47.8
18	74.6 - 49.6	100.6 - 69.4	80.1 - 27.0	100.7 - 70.7
20	49.6 - 32.0	38.9 - 9.6	69.6 - 13.8	51.7 - 22.8
22	41.3 - 26.2	33.3 - 6.8	53.4 - 1.1	39.6 - 13.8
24	39.1 - 25.8	35.5 - 12.7	35.8 - 0	18.7 - 9.7
26	35.3 - 19.8	33.2 - 5.4	46.5 - 0	31.7 - 9.1
28	32.3 - 17.7	26.7 - 4.1	46.5 - 0	22.8 - 6.2
30	43.6 - 34.4	39.1 - 22.7	65.8 - 25.9	33.9 - 20.7
32	25.3 - 17.9	22.3 - 9.0	13.6 - 0	19.7 - 10.0
34	25.6 - 16.9	27.7 - 8.4	19.3 - 0	19.7 - 6.7
36	20.6 - 13.2	21.6 - 7.7	19.4 - 0	17.3 - 7.2

* Hours after the beginning of MC treatment.

Table A-5

Mitotic index and phase index of mitosis obtained at various times after 2 hr. treatment with MC (10 µg/ml), at 24 ± 1°C.

Hours*	Mitotic index	Phase index				Total cells scored
		Prophase	Metaphase	Anaphase	Telophase	
2	6.9 ± 0.27	57.6	18.4	2.9	21.2	8645
4	5.7 ± 0.25	48.7	17.4	3.0	30.9	8230
6	2.9 ± 0.18	47.5	22.7	1.1	28.6	8923
8	3.0 ± 0.20	57.9	16.7	4.3	21.0	7658
10	4.6 ± 0.21	62.9	14.3	4.5	18.2	9962
12	4.2 ± 0.22	43.6	18.7	5.0	32.6	7988
14	1.0 ± 0.12	44.8	19.4	4.5	31.3	6629
16	0.8 ± 0.10	41.1	13.2	10.3	35.3	8176
18	1.5 ± 0.14	51.8	17.9	11.6	18.8	7616
20	5.0 ± 0.33	57.7	15.9	5.8	20.7	4198
22	6.0 ± 0.38	62.4	14.5	4.5	18.6	4005
24	8.3 ± 0.44	53.5	16.2	5.1	25.2	4015
26	3.0 ± 0.20	59.6	14.6	2.8	23.0	7049
28	4.3 ± 0.28	54.4	15.6	2.4	27.6	5784
30	9.3 ± 0.32	57.4	16.2	3.2	23.1	8186
32	11.7 ± 0.39	59.2	14.3	3.0	23.5	6854
34	9.0 ± 0.41	59.1	13.7	3.4	23.8	4960
36	10.4 ± 0.38	59.2	13.1	3.5	24.1	6554

* Hours from beginning of MC treatment.

Table A-6

Percentage of labelled nuclei in root tips of "Seneca 60" at various times after roots exposed for 30 min. to ^{3}H-TdR followed by 2 hr. in HAS (0.5 mg/ml) at 24 ± 1°C

Hours*	Mitotic index	Percent labelled mitoses				Total cells scored
		Prophase	Metaphase	Anaphase	Telophase	
2	10.0 ± 0.22	0	0	0	0	7321
4	7.4 ± 0.32	5.4 ± 1.36	2.1 ± 1.49	0	2.0 ± 1.39	6660
6	1.8 ± 0.15	12.2 ± 3.45	6.7 ± 6.44	0	9.7 ± 5.31	7512
8	0.9 ± 0.10	11.4 ± 4.78	12.4 ±11.65	0	6.7 ± 6.44	7883
10	1.2 ± 0.11	13.6 ± 4.22	7.1 ± 6.88	0	0	9326
12	0.8 ± 0.10	21.2 ± 7.12	9.1 ± 8.67	0	5.6 ± 5.40	8097
14	9.0 ± 0.36	64.0 ± 2.94	44.7 ± 5.39	37.5 ± 12.00	37.9 ± 3.45	6259
16	11.3 ± 0.37	82.4 ± 1.84	68.5 ± 4.12	61.1 ± 11.49	66.6 ± 2.83	7516
18	9.7 ± 0.36	73.9 ± 2.58	60.8 ± 4.96	63.2 ± 11.07	66.1 ± 2.89	6818
20	9.8 ± 0.32	62.2 ± 2.30	57.0 ± 4.26	59.1 ± 7.41	62.2 ± 2.84	8789
22	9.6 ± 0.33	37.5 ± 2.49	36.1 ± 4.34	40.0 ± 6.32	30.8 ± 3.18	7765
24	11.1 ± 0.38	34.7 ± 2.26	36.1 ± 4.88	29.8 ± 6.67	30.2 ± 3.01	6776
26	8.7 ± 0.32	38.9 ± 2.67	41.0 ± 4.80	33.1 ± 9.06	40.1 ± 3.58	7529
28	11.3 ± 0.37	53.9 ± 2.37	55.8 ± 4.47	50.0 ± 8.57	49.6 ± 3.27	7157
30	8.0 ± 0.31	61.2 ± 2.83	45.1 ± 4.72	45.2 ± 8.94	44.9 ± 3.47	7651
32	10.2 ± 0.35	42.1 ± 2.29	33.1 ± 4.13	39.5 ± 7.45	35.7 ± 3.20	7615
34	10.0 ± 0.35	33.2 ± 2.44	38.1 ± 4.57	22.1 ± 6.92	32.0 ± 3.15	7147

* Hours after the beginning of HAS treatment.

N.B. Labelling index = $\frac{11274}{30000}$ = 37.6%

Table A-7

The 95% confidence limits on the labelled mitoses treated with HAS (0.5 mg/ml) for 2 hr. at 24 ± 1°C.

Hours*	Prophase	Metaphase	Anaphase	Telophase
2			-	4.7 - 0
4	8.1 - 2.7	5.1 - 0	-	20.1 - 0
6	19.0 - 5.5	19.3 - 0	-	19.3 - 0
8	20.7 - 2.0	35.2 - 0	-	-
10	21.9 - 5.4	20.6 - 0	-	16.1 - 0
12	35.2 - 7.3	26.1 - 0	61.0 - 14.0	44.6 - 31.1
14	69.8 - 58.3	55.3 - 34.2	83.6 - 38.6	72.1 - 61.0
16	86.0 - 78.8	76.6 - 60.4	84.9 - 41.5	71.8 - 60.5
18	78.9 - 68.8	70.5 - 51.1	73.6 - 44.6	67.7 - 56.6
20	66.7 - 57.6	65.4 - 48.7	52.4 - 27.6	37.0 - 24.6
22	42.4 - 32.7	44.6 - 27.6	42.9 - 16.7	36.1 - 24.3
24	39.1 - 30.2	45.6 - 26.5	50.9 - 15.4	47.1 - 33.1
26	44.1 - 33.7	50.4 - 31.5	66.8 - 33.2	56.0 - 43.2
28	58.6 - 49.2	64.6 - 47.1	62.7 - 27.6	51.7 - 38.1
30	66.8 - 55.7	54.3 - 35.8	54.1 - 24.9	42.0 - 29.4
32	46.6 - 37.7	41.2 - 25.0	35.7 - 8.6	38.1 - 25.8
34	38.0 - 28.5	47.0 - 29.1		

*Hours from beginning of HAS treatment.

Table A-8

Mitotic index and phase index of mitosis obtained at various times after 2 hr. treatment with HAS (0.5 mg/ml) at 24 ± 1°C

Hours*	Mitotic index	Phase index				Total cells scored
		Prophase	Metaphase	Anaphase	Telophase	
2	10.0 ± 0.35	67.1	11.5	2.3	19.0	7321
4	7.4 ± 0.32	56.2	19.1	4.1	20.6	6660
6	1.8 ± 0.15	65.2	10.9	1.5	22.5	7512
8	0.9 ± 0.10	65.7	11.9	0	22.4	7883
10	1.2 ± 0.24	56.9	12.1	0	31.0	9326
12	0.8 ± 0.10	53.2	17.7	0	29.0	8097
14	9.0 ± 0.36	47.1	15.0	2.8	35.0	6259
16	11.3 ± 0.37	50.2	14.9	2.1	32.7	7516
18	9.7 ± 0.36	44.2	14.7	2.9	38.1	6818
20	9.8 ± 0.32	47.4	15.7	5.1	31.7	8789
22	9.5 ± 0.33	47.0	16.4	8.1	28.4	7765
24	11.1 ± 0.38	49.9	12.9	6.3	30.9	6776
26	8.7 ± 0.32	51.1	16.1	4.1	28.6	7529
28	11.3 ± 0.37	50.7	16.0	4.2	29.1	7157
30	8.0 ± 0.31	45.1	18.1	5.1	31.7	7651
32	10.2 ± 0.35	50.7	16.8	5.5	27.1	7615
34	10.0 ± 0.35	48.2	15.9	5.1	30.8	7147

* Hours from beginning of HAS treatment.

Table A-9

Mitotic and phase indices throughout a 10 hr. in distilled water as a control at 24 ± 1°C

Hours of treatment	Mitotic index	Phase index				Total cells scored
		Prophase	Metaphase	Anaphase	Telophase	
0	8.5 ± 0.38	49.2	15.6	5.2	29.9	5436
6	8.8 ± 0.38	51.0	15.2	3.5	30.3	5537
10	8.1 ± 0.40	55.1	12.1	4.0	28.8	4621

Table A-10

Mitotic and phase indices throughout a 10 hr. continuous treatment with MC (10 µg/ml) at 24 ± 1°C.

Hours of Treatment	Mitotic index	Phase index				Total cells scored
		Prophase	Metaphase	Anaphase	Telophase	
2	6.2 ± 0.27	58.8	14.9	3.3	23.0	7784
4	5.0 ± 0.24	52.8	11.3	4.2	31.6	9305
6	3.0 ± 0.20	58.3	15.6	4.2	21.8	7142
8	1.8 ± 0.15	62.0	12.4	1.4	23.4	7868
10	0.9 ± 0.12	51.9	20.4	0	27.8	6292

Table A-11

Mitotic and phase indices throughout a 10 hr. continuous treatment with HAS (0.5 mg/ml) at 24 ± 1°C.

Hours of treatment	Mitotic index	Phase index				Total cells scored
		Prophase	Metaphase	Anaphase	Telophase	
2	8.7 ± 0.37	65.8	22.6	1.2	12.5	5850
4	8.9 ± 0.39	61.0	36.7	0	2.3	5341
6	6.1 ± 0.33	50.6	40.1	0.9	8.4	5308
8	5.2 ± 0.37	53.1	43.6	0	3.2	3585
10	5.6 ± 0.32	53.5	34.7	0.7	11.1	5110
6 + 2*	1.9 ± 0.21	36.8	39.5	5.3	18.4	4041
6 + 4**	0.3 ± 0.07	73.7	15.8	0	10.5	6126

*6 + 2 = 6 hr. in HAS and then 2 hr. in normal growth condition.

**6 + 4 = 6 hr. in HAS and then 4 hr. in normal growth condition.

Table A-12

Mitotic and phase indices throughout a 10 hr. continuous treatment with colchicine (1 mg/ml) at 24 ± 1°C.

Hours of treatment	Mitotic index	Phase index				Total cells scored
		Prophase	Metaphase	Anaphase	Telophase	
2	11.9 ± 0.39	35.1	64.2	0.1	0.5	6766
4	15.8 ± 0.55	24.7	65.6	1.0	8.7	4369
6	21.4 ± 0.54	27.7	64.7	0.6	7.0	5715
8	19.9 ± 0.60	20.8	69.0	0.6	9.7	4364
10	18.4 ± 0.58	24.3	63.4	1.2	11.1	4467

APPENDIX B

χ^2 and t Test on Incorporation of ^{3}H-thymidine

Table B-1

2 X 2 contingency χ^2 test for interphase labelling index between different treatments.

Treatment[+]	No. of labelled interphase nuclei	No. of unlabelled interphase nuclei	Total	χ^2 value [++]
Control	3477	1999	5476	205.3 **
MC I.	2373	2426	4799	
Control	3477	1999	5476	189.3*
MC II.	1884	1949	3833	
Control	3477	1999	5476	143.0**
HAS I.	2014	1923	3937	
Control	3477	1999	5476	44.7**
HAS II.	2338	1783	4121	
MC I.	2373	2426	4799	6.3 *
MC II.	1884	1949	3833	
HAS I.	2014	1923	3937	25.0**
HAS II.	2338	1783	4121	

+ See Table 4.3-1 footnote explanation.
++ Based on df = 1.
* Significant; P< 0.05
** Highly significant; P< 0.01.

Table B-2

t-test for the mean value of the grain counts between different treatments.

Treatment[+]	Grain No. per nucleus[++]	t-value
Control MC I.	50.1 ± 2.98 31.8 ± 2.37	9.6**
Control MC II.	50.1 ± 2.98 28.8 ± 3.31	9.5**
Control HAS I.	50.1 ± 2.98 28.8 ± 1.23	13.2**
Control HAS II.	50.1 ± 2.98 37.3 ± 4.60	4.7**
MC I. MC II.	31.8 ± 2.37 28.8 ± 3.31	1.5 NS
HAS I. HAS II.	28.8 ± 1.23 37.3 ± 4.60	3.5*

+ See Table 4.3-1 footnote explanation. ++ Data are based on 160 nuclei, i.e.: 4 samples for each treatment, 40 nuclei were scored from each sample. NS = Not significant: $P > 0.05$.
* = Significant: $P < 0.05$. ** = Highly significant: $P < 0.01$.

APPENDIX C

Data on Chromosomal Measurements and Aberration Frequencies

Table C-1

Chromosome length measurements of "Seneca 60"*

	Chromosome number									
	1	2	3	4	5	6	7	8	9	10
A. Total relative length (% TCL)										
Mean	13.91	11.88	10.72	10.91	10.38	10.70	8.37	8.56	7.71	6.86
S.E.**	0.28	0.26	0.30	0.37	0.35	0.42	0.29	0.41	0.18	0.25
B. Long arm										
Mean	7.31	6.53	6.39	5.94	4.93	5.31	5.32	5.71	4.35	4.03
S.E.	0.27	0.26	0.23	0.23	0.18	0.24	0.23	0.31	0.18	0.22
C. Short arm										
Mean	5.85	4.60	3.57	4.21	4.69	2.34	2.30	2.09	2.61	2.08
S.E.	0.20	0.12	0.17	0.22	0.18	0.12	0.07	0.14	0.12	0.08
D. Arm ratio										
Mean	1.25	1.42	1.79	1.42	1.05	2.27	2.31	2.73	1.67	1.93
S.E.	0.07	0.07	0.11	0.07	0.02	0.06	0.07	0.11	0.10	0.10

N.B. * 12 cells were used.
** Standard error.

Relative length of centromere: Mean = 0.75
S.E. = 0.08

Short arm of chromosome 6; Nucleolar constriction; Mean = 1.15
S.E. = 0.25

Satellite; Mean = 1.16
S.E. = 0.32

Table C-2

The distribution of chromosomal breaks caused by MC

Chromosome		Location: Short arm 5*	4	3	2	1	0	Long arm 1	2	3	4	5	Total breaks
1	%	4.5	9.1	4.5	3.0	13.6	28.8	3.0	4.5	16.7	7.6	4.5	66
	No.	3	6	3	2	9	19	2	3	11	5	3	
2	%	–	10.5	–	–	5.3	47.4	10.5	2.6	5.3	10.5	7.9	38
	No.	0	4	0	0	2	18	4	1	2	4	3	
3	%	2.6	7.7	–	–	7.7	38.5	5.1	–	20.5	15.4	2.6	39
	No.	1	3	0	0	3	15	2	0	8	6	1	
4	%	8.33	–	8.3	–	8.3	41.7	–	–	–	33.3	–	12
	No.	1	0	1	0	1	5	0	0	0	4	0	
5	%	2.4	2.4	2.4	–	4.8	57.1	4.8	2.4	–	11.9	11.9	42
	No.	1	1	1	0	2	24	2	1	0	5	5	
		Distal			Proximal								
6	%	67.4			–		14.6	1.1	2.2	7.9	4.5	2.2	89
	No.	60			0		13	1	2	7	4	2	
7	%	4.5			–		45.5	4.5	–	22.7	18.2	4.5	22
	No.	1			0		10	1	0	5	4	1	
8	%	2.8			–		44.4	2.8	–	5.6	22.2	22.2	36
	No.	1			0		16	1	0	2	8	8	
9	%	11.1			11.1		44.4	22.2	5.6	–	–	5.6	18
	No.	2			2		8	4	1	0	0	1	
10	%	–			14.3		42.9	28.6	–	–	14.3	–	7
	No.	0			1		3	2	0	0	1	0	

* The location of each break was expressed as that % of the broken chromosome arm from the centromere to the break. 0 = centromere, 1=1-20%, 2=21-40%, 3=41-60%, 4=61-80%, 5=81-100%.

Table C-3

The distribution of chromosomal breaks caused by HAS

Chromosome		Location: Short arm 5*	4	3	2	1	0	Long arm 1	2	3	4	5	Total breaks
1	%	6.4	14.9	2.1	2.1	8.5	36.1	–	–	25.5	–	4.3	47
	No.	3	7	1	1	4	17	0	0	12	0	2	
2	%	10.5	7.9	–	–	5.3	29.0	10.5	5.3	5.3	23.7	2.6	38
	No.	4	3	0	0	2	11	4	2	2	9	1	
3	%	5.6	–	2.8	–	13.9	11.1	–	2.8	50.0	11.1	2.8	36
	No.	2	0	1	0	5	4	0	1	18	4	1	
4	%	7.1	–	–	–	–	35.7	–	–	7.1	35.7	14.3	14
	No.	1	0	0	0	0	5	0	0	1	5	2	
5	%	–	–	–	3.1	6.2	53.1	6.3	–	9.4	18.8	3.1	32
	No.	0	0	0	1	2	17	2	0	3	6	1	
		Distal			Proximal		4.1	2.0	2.0	22.5	4.1	8.2	49
6	%	57.1			–								
	No.	28			0		2	1	1	11	2	4	
7	%	–			–		30.8	7.7	–	7.7	53.9	–	13
	No.	0			0		4	1	0	1	7	0	
8	%	–			–		46.5	7.7	–	–	15.4	30.8	13
	No.	0			0		6	1	0	0	2	4	
9	%	27.3			18.2		27.3	–	–	9.1	–	18.2	11
	No.	3			2		3	0	0	1	0	2	
10	%	–			14.3		57.1	14.3	–	14.3	–	–	7
	No.	0			1		4	1	0	1	0	0	

* The location of each break was expressed as that % of the broken chromosome arm from the centromere to the break. 0 = centromere, 1=1-20%, 2=21-40%, 3=41-60%, 4=61-80%, 5=81-100%.

Table C-4

"Goodness of fit" test for equal distribution of chromosome breaks in various chromosomes induced by MC, based on percentage of unit chromosome lengths.

	Chromosome number										
	1	2	3	4	5	6	7	8	9	10	Total
% of unit chromosome length	13.91	11.88	10.72	10.91	10.38	10.70	8.37	8.56	7.71	6.86	100
Observed	66	38	39	12	42	89	22	36	18	7	369
Expected	51.33	43.84	39.56	40.26	38.30	39.48	30.88	31.59	28.45	25.31	369
X^2	4.19	0.78	0.01	19.84	0.36	62.11	2.55	0.62	3.84	13.25	107.6**

** P < 0.01

Table C-5

"Goodness of fit" test for equal distribution of chromosome breaks in various long arm of chromosomal segments induced by MC, based on percentage of unit chromosome lengths.

	Long arm of chromosome										
	1	2	3	4	5	6	7	8	9	10	Total
% of unit chromosome length	7.31	6.53	6.39	5.94	4.93	5.31	5.32	5.71	4.35	4.03	55.8
Observed	24	14	17	4	13	16	11	19	6	3	127
Expected	16.63	14.86	14.54	13.51	11.22	12.08	12.10	12.99	9.90	9.17	127
X^2	3.27	0.05	0.42	6.69	0.28	1.27	0.10	2.78	1.54	4.15	20.6**

** P < 0.01.

Table C-6

"Goodness of fit" test for equal distribution of chromosome breaks in various short arm of chromosomal segments induced by MC, based on percentage of unit chromosome lengths.

	Short arm of chromosome										Total
	1	2	3	4	5	6	7	8	9	10	
% of unit chromosome length	5.85	4.60	3.57	4.21	4.69	4.65	2.30	2.09	2.61	2.08	36.7
Observed	23	6	7	3	5	60	1	1	4	1	111
Expected	17.72	13.93	10.81	12.75	14.20	14.08	6.97	6.33	7.91	6.30	111
X^2	1.57	4.51	1.34	7.46	5.96	149.76	5.11	4.49	1.93	4.46	186.6**

** P < 0.01

Table C-7

"Goodness of fit" test for equal distribution of centromeric breaks in various chromosomes induced by MC, based on percentage of unit chromosome lengths.

	Centromere										Total
	1	2	3	4	5	6	7	8	9	10	
% of unit centromere	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	7.5
Observed	19	18	15	5	24	13	10	16	8	3	131
Expected	13.10	13.10	13.10	13.10	13.10	13.10	13.10	13.10	13.10	13.10	
X^2	2.66	1.83	0.28	5.01	9.07	0	0.73	0.73	1.99	7.79	30.1**

** P < 0.01.

Table C-8

"Goodness of fit" test for equal distribution of chromosome breaks in various chromosomes induced by HAS, based on percentage of unit chromosome lengths.

	Chromosome number										
	1	2	3	4	5	6	7	8	9	10	Total
% of unit chromosome length	13.91	11.88	10.72	10.91	10.38	10.70	8.37	8.56	7.71	6.86	100
Observed	47	38	36	14	32	49	13	13	11	7	260
Expected	36.17	30.89	27.87	28.37	26.99	27.82	21.76	22.25	20.05	17.83	260
X^2	3.24	1.64	2.37	7.28	0.93	16.12	3.53	3.85	4.08	6.58	49.6**

** $P < 0.01$.

Table C-9

"Goodness of fit" test for equal distribution of chromosome breaks in various long arm of chromosomal segments induced by HAS, based on percentage of unit chromosome lengths.

	Long arm of chromosome										
	1	2	3	4	5	6	7	8	9	10	Total
% of unit chromosome length	7.31	6.53	6.39	5.94	4.93	5.31	5.32	5.71	4.35	4.03	55.8
Observed	14	18	24	8	12	19	9	7	3	2	116
Expected	15.19	13.57	13.28	12.34	10.25	11.03	11.06	11.87	9.04	8.37	
X^2	0.09	1.45	8.65	1.53	0.30	5.76	0.38	2.00	4.04	4.85	29.1**

** $P < 0.01$.

Table C-10

"Goodness of fit" test for equal distribution of chromosome breaks in various short arm of chromosomal segments induced by HAS, based on percentage of unit chromosome lengths.

	Short arm of chromosome										Total
	1	2	3	4	5	6	7	8	9	10	
% of unit chromosome length	5.85	4.60	3.57	4.21	4.69	4.65	2.30	2.09	2.61	2.08	36.7
Observed	16	9	8	1	3	28	0	0	5	1	71
Expected	11.33	8.91	6.92	8.16	9.08	9.01	4.45	4.05	5.06	4.03	71
X^2	1.92	0	0.17	6.28	4.07	40.02	4.45	4.05	0	2.28	63.2**

** P < 0.01

Table C-11

"Goodness of fit" test for equal distribution of centromeric breaks in various chromosomes induced by HAS, based on percentage of unit chromosome lengths.

	Centromere										Total
	1	2	3	4	5	6	7	8	9	10	
% of unit centromere	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	7.5
Observed	17	11	4	5	17	2	4	6	3	4	73
Expected	7.30	7.30	7.30	7.30	7.30	7.30	7.30	7.30	7.30	7.30	73
X^2	12.89	1.88	1.49	0.72	12.89	3.85	1.49	0.23	2.53	1.49	39.5**

** P < 0.01.

Table C-12

Yields of the various aberration types obtained at various times after roots exposed for 30 min. to ^{3}H-thymidine followed by 2 hr. MC (5 µg/ml) at 24±1°C

Hours from beginning of MC treatment	Total metaphases scored	% metaphase aberrations	% labelled metaphases	No. of metaphases nuclei		No. of chromatid breaks		No. of isochromatid breaks		No. of chromosome translocations & rings		Aberration frequencies per cell
				L*	U*	L	U	L	U	L	U	
4	202	4.5	1.0 ± 0.01	2	200	0	1	0	8	0	0	0.04 ± 0.01
6	212	8.5	15.6 ± 2.44	33	179	0	3	2	14	0	1	0.09 ± 0.02
8	188	12.8	42.6 ± 3.60	80	108	4	3	7	11	0	0	0.13 ± 0.02
10	203	15.8	80.3 ± 2.54	163	40	1	1	30	2	2	0	0.18 ± 0.03
12	221	24.4	94.6 ± 1.41	209	12	2	0	44	2	4	0	0.24 ± 0.03
14	54	38.9	94.4 ± 3.00	51	3	0	0	25	0	3	0	0.57 ± 0.10
16	45	64.4	95.5 ± 3.00	43	2	0	0	38	0	3	0	0.91 ± 0.14
18	59	62.7	79.7 ± 5.19	47	12	0	1	41	9	2	2	1.00 ± 0.13
20	55	65.5	63.6 ± 6.48	35	20	0	0	31	24	0	1	1.03 ± 0.13
22	124	75.0	41.9 ± 4.35	52	72	5	6	42	76	3	6	1.11 ± 0.09
24	151	40.4	46.4 ± 4.00	70	81	0	7	21	39	0	10	0.56 ± 0.06
26	201	42.8	45.3 ± 3.36	91	110	1	4	32	77	0	8	0.64 ± 0.06
30	222	55.9	55.4 ± 3.31	123	99	8	12	75	60	6	4	0.78 ± 0.06
34	148	52.7	37.8 ± 3.87	56	92	2	6	29	67	2	4	0.78 ± 0.07

* L = Labelled; U = Unlabelled.

Table C-13

Yields of various aberration types obtained at various times after 2 hr. treatment with a mixture of MC (5 µg/ml) and (1 µg/ml) ^{3}H-thymidine at 24±1°C.

Hours from beginning of treatment	Total metaphases scored	% metaphase aberrations	No. of metaphase nuclei		% labelled metaphase cells	No. of chromatid breaks		No. of isochromatid breaks		No. of chromosome interchanges & rings		Aberration frequencies per cell
			L*	U*		L	U	L	U	L	U	
6	181	7.7	21	161	11.6 ± 2.23	2	5	1	4	0	2	0.07 ± 0.02
10	121	11.6	39	82	32.2 ± 4.24	3	4	4	6	0	0	0.14 ± 0.03
14	76	32.9	60	16	78.9 ± 4.69	5	0	20	4	1	0	0.40 ± 0.07
18	33	66.7	27	6	81.8 ± 6.70	2	0	22	8	0	0	0.97 ± 0.17
22	38	71.1	19	19	50.0 ± 8.12	1	2	18	20	0	1	1.14 ± 0.17
26	148	43.9	47	101	31.8 ± 3.74	5	4	32	56	4	5	0.77 ± 0.07
30	213	57.2	75	138	35.2 ± 3.16	10	17	64	75	1	4	0.82 ± 0.06
34	176	48.3	57	117	33.5 ± 3.26	5	7	40	73	4	2	0.77 ± 0.06
38	101	38.6	20	81	20.0 ± 3.87	0	5	41	42	0	3	0.66 ± 0.08

* L = Labelled nuclei.
U = Unlabelled nuclei.

Table C-14

Types and frequencies of chromosomal aberrations observed in anaphases at various times after roots exposed for 30 min. to ^{3}H-thymidine followed 2 hr. treatment with HAS (0.5 mg/ml) at 24 ± 1°C.

Hours from beginning of HAS treatment	Total No. of anaphases scored	% anaphase aberrations	No. of anaphase nuclei		% labelled anaphases	No. of fragments per 100 cells	No. of bridges or pseudochiasmata per 100 cells
			L*	U*			
4	112	8.0 ± 2.64	2	110	1.8 ± 1.00	11.61	8.0
8	34	17.7 ± 7.14	2	32	5.9 ± 4.00	26.47	8.8
12	26	30.8 ± 10.86	5	21	19.2 ± 7.68	34.62	7.7
16	176	17.1 ± 3.00	126	50	71.6 ± 3.31	22.16	3.4
20	148	12.8 ± 2.62	103	45	69.6 ± 3.44	23.65	0.7
24	173	8.7 ± 2.23	49	124	28.3 ± 3.46	9.24	1.4
28	191	8.9 ± 2.00	87	104	45.6 ± 3.46	13.09	2.6
32	187	8.0 ± 2.00	93	94	49.7 ± 3.60	12.83	1.1

* L = Labelled; U = Unlabelled.