

INDUCTION OF CHROMOSOME DAMAGE
BY ETHYLENIMINES AND
RELATED COMPOUNDS

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ABSTRACT

INDUCTION OF CHROMOSOME DAMAGE BY ETHYLENIMINES AND RELATED COMPOUNDS

by Thamby Ninan Adangapuram

A number of ethylenimines and related compounds were employed to induce chromosomal aberrations in the primary root meristems of Vicia faba and Pisum sativum. The chromosomal damage was then determined quantitatively by scoring anaphase damage in Pisum, and metaphase damage in Vicia over an extended period of time, consisting of several mitotic cycles. An attempt was made to determine the susceptible stage of the mitotic cycle at which these agents exert their influence in causing chromosome damage and cycle time delay. These studies were aimed towards gaining a better understanding of the type of disruption of the mitotic cycle by this group of drugs, as well as an understanding of the structural organization of the mitotic chromosomes. The comparative mutagenic ability of some of the chemicals were tested on Drosophila melanogaster.

Treatment of the root meristems of Vicia and Pisum was accomplished by dissolving the chemicals in an aqueous nutrient medium used to culture the pea roots under standard conditions. By microscopical examination of the root meristems, analysis were made of the type of damage induced as well as the amount of damage. The relative muta-

genic ability of some of the chemicals were tested by using the Muller 5 system (to detect recessive lethal X chromosome mutants). The two chemicals studied in detail were apholate and metepa. The results obtained are concerned mainly with:

1. delayed effects
2. persistence of effects.

In all of the experiments using metepa and apholate on peas, a maximum effect did not appear until after a period of time equal to one or two complete mitotic cycles. Hence, these are categorized as delayed effects. This delay may be attributed to unusual delay in damaged cells reaching division, or may have to do with the initial unit of breakage. This delay may be explained if we assume that $\frac{1}{2}$ chromatids become chromatids in the next division. However, there are some strong objections to these assumptions. Persistence of effect may have to do with the number of subunits that make up the chromosomes and the type of assortment of the centromeres, or a combination of both. The time of susceptibility was tested using the 5 amino-uracil system. We are forced to the conclusion that no particular stage is especially susceptible to the chemicals, although the possibility exists that they may be retained in sufficient concentration to cause maximum damage at a critical period in the cycle.

In comparing the chemical damage to X-ray damage, it may be seen that these two agents differ in:

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1. the types of damage induced
2. the time of maximum damage
3. the susceptible stage where the damage occurs.

These chemicals thus cannot be called radiomimetic in a strict sense.



INDUCTION OF CHROMOSOME DAMAGE BY
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By

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To my father and mother

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TABLE OF CONTENTS

	Page
DEDICATION.....	ii
ACKNOWLEDGMENTS.....	iii
LIST OF FIGURES, PLATES, AND CHARTS.....	vi
LIST OF APPENDIX TABLES.....	ix
 INTRODUCTION.....	 1
LITERATURE REVIEW.....	4
METHODS.....	27
A. Cytological Experimental System.....	27
B. Genetic Experimental System.....	34
C. Retention of the Chemical.....	35
RESULTS.....	37
A. Azirane.....	37
B. Ethylene Sulfide.....	37
C. Tretamine, or TEM.....	40
D. Aphoxide, or TEPA.....	40
E. Hexamethylphosphoric triamide.....	41
F. Apholate.....	41
G. Metepa.....	47
DISCUSSION.....	67
A. Time of Maximum Damage.....	69

	Page
DISCUSSION, continued	
B. Persistence of effect.....	72
C. Time of Susceptibility.....	75
D. Order of Rearrangements.....	77
E. Comparison of Effect.....	81
1. Chromosomal aberrations.....	81
2. Mitotic inhibition.....	82
3. Cycle time delay.....	82
4. Mutagenic ability.....	83
5. Comparison with X-ray damage.....	83
SUMMARY.....	85
LITERATURE CITED.....	87
APPENDIX.....	99

LIST OF FIGURES, PLATES, AND CHARTS

Figure	Page
1. Azirane damage in which the percentage of anaphase damage is plotted against time in hours.....	38
2. Anaphase damage in <u>Pisum sativum</u> , caused by 3 concentrations of tretamine, plotted against time in hours.....	39
3. Chromosome damage caused by 3.1×10^{-2} M apholate. Percent anaphase damage vs. time shows a 3rd cycle maximum and persistent effect.....	42
4. Dose effect curve using apholate. Percent anaphase damage vs. dose in ppm.....	43
5. Cycle time delay caused by apholate treatment on <u>Pisum sativum</u> . Percent polyploidy is plotted against time.....	45
6. Mitotic index change after 8 hours of 150 ppm of 5 A U on <u>Pisum</u>	46
7. Metepa damage in <u>Pisum sativum</u> . Percent anaphase damage vs. time shows 37 hour (3rd cycle) maximum effect as well as 75 hour (6th cycle) 2nd maximum effect.....	48
8. Metepa damage in <u>Pisum sativum</u> . Percent anaphase damage plotted against time shows 27 hour (2nd cycle) maximum effect which is nonpersistent.....	50
9. Metepa damage in <u>Pisum sativum</u> . Percent anaphase damage vs. time, 27 hour (2nd cycle) maximum effect which is nonpersistent is obtained.....	51
10. Dose effect curve using metepa in <u>Pisum sativum</u> . Percent anaphase damage is plotted against dose. In this graph, the results of two different experiments are plotted.....	52

Figure	Page
11. Cycle time delay caused by metepa treatment on <u>Pisum sativum</u> . Percent polyploidy is plotted against time in hours.....	53
12. X-ray damage (250 r) at anaphase in percent vs. time in hours in <u>Pisum sativum</u>	55
13. Total metaphase damage in <u>Vicia</u> induced by 0.233×10^{-2} M metepa. Percent damage vs. time.....	56
14. Chromatid and chromosome damage in <u>Vicia</u> at metaphase induced by 0.233×10^{-2} M metepa treatment. Percent of damage is plotted against time.....	62
15. Percent polyploidy vs. time in <u>Vicia</u> treated with 0.233×10^{-2} M metepa, showing a delay of 5-6 hours for the 1st cycle and no delay for the second cycle after $\frac{1}{2}$ hour treatment.....	64
16a. Diagram of the mitotic cycle in pea root meristems...	68
16. Model showing half-DNA break in two-stranded chromosome with random distribution of potential chromatids.....	71
17. Dose effect curve, maximum apholate damage in percent vs. dose on log scale.....	78
18. Metepa damage plotted against dose on log scale.....	79
19. Dose effect curve in which the maximum anaphase damage in percent is plotted against the corresponding dose on log scale (metepa treatment).....	80
Plates	
1. Karyotypes of (1) <u>Vicia faba</u> , and (2) <u>Pisum sativum</u>	99
2. Different types of aberrations induced by metepa treatment on <u>Vicia faba</u>	59
3. Aberrations induced by metepa treatment on <u>Vicia faba</u> ..	60
4. Chromosome damage induced by tretamine and apholate on <u>Pisum sativum</u>	61

CHARTS

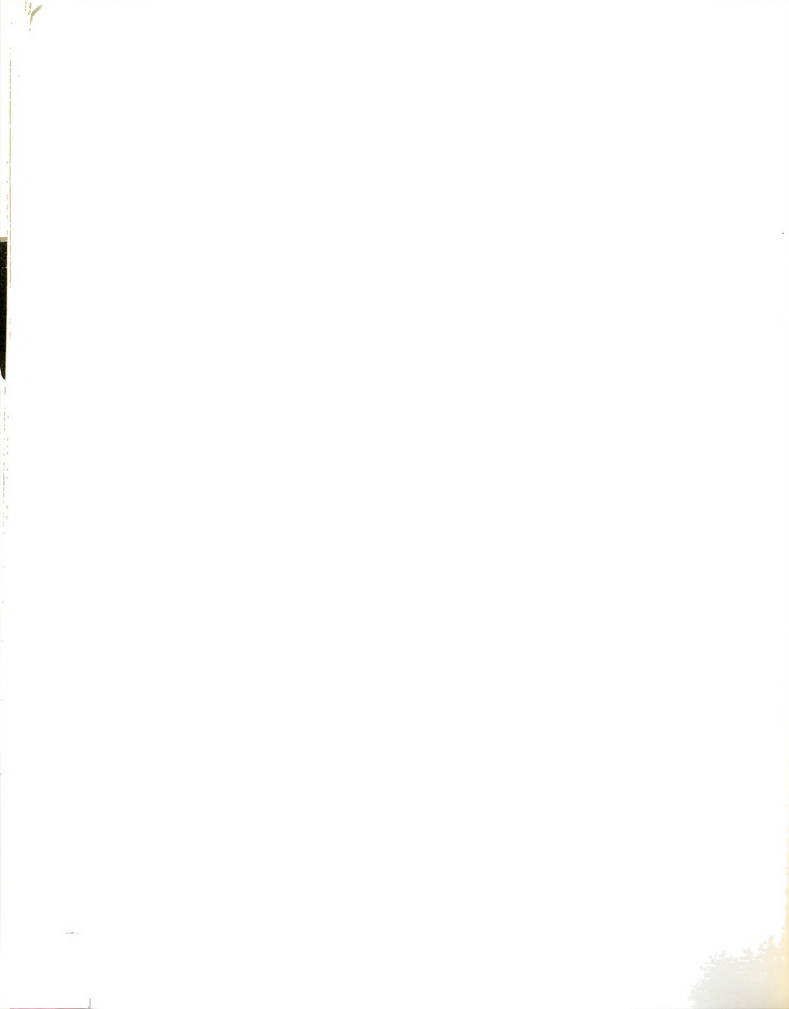
Page

1. Classification of chromosome and chromatid aberrations induced by ethylenimines, and their end results in subsequent cell cycles..... 58

LIST OF APPENDIX TABLES

Table	Page
1. Anaphase damage in percent and mitotic indices at various hours in <u>Pisum</u> induced by azirane.....	100
2. Anaphase damage in percent induced by 3.1×10^{-2} M of apholate in <u>Pisum</u>	101
3. Anaphase damage in percent and mitotic indices at various hours in <u>Pisum</u> , treated with 3.1×10^{-3} M apholate.....	102
4. Maximum anaphase damage in percent at peak hours in <u>Pisum</u> induced by different concentrations of apholate.....	103
5 a. Anaphase damage and M Is at various hours in control <u>Pisum</u>	104
b. Anaphase damage in percent and M Is in <u>Pisum</u> treated with 100 ppm of 5 A U for 8 hours.....	105
c. Anaphase damage in percent and M Is in <u>Pisum</u> , treated first with 5 A U for $7\frac{1}{2}$ hours and immediately transferred to 3.1×10^{-3} M apholate solution.....	106
d. Anaphase damage and M Is in <u>Pisum</u> treated with 5 A U for 8 hours and then transferred to 3.1×10^{-3} M apholate solution at $12\frac{1}{2}$ hours.....	107
e. Anaphase damage in percent and M Is in <u>Pisum</u> , treated with 5 A U for 8 hours and with 3.1×10^{-2} M apholate at 15 hours.....	108
f. Anaphase damage in percent and M Is in <u>Pisum</u> , treated with 5 A U for 8 hours and with 3.1×10^{-3} M apholate at $10\frac{1}{2}$ hours.....	109
6. Anaphase damage in percent and M Is at various hours in <u>Pisum</u> , treated with different concentrations of metepa.....	110

Table	Page
7. Anaphase damage in percent and M Is at various hours in <u>Pisum</u> , treated with 4 different concentrations of metepa.....	112
8. Anaphase damage in percent and M Is at various hours in <u>Pisum</u> after treatment with different concentrations of metepa.....	114
9. Anaphase damage in percent induced by 250 r of X-ray treatment in <u>Pisum</u>	116
10. Percent aberrations at metaphase in <u>Vicia faba</u> , induced by 0.233×10^{-2} M metepa treatment.....	117
11. Comparison of damage.....	118



INTRODUCTION

The study of the effects of drugs on cells and their modes of action assumes considerable importance in this "chemical age" since man uses a larger number of drugs for the treatment of disease as well as for the control of pests than ever before. Plants and animals, including man, are inadvertently exposed to these chemical hazards in their environment. In our search for more effective drugs in combating constantly changing hordes of pathogens and pests, we are unleashing chemicals whose long-term effects are only poorly understood. In selecting drugs for these purposes, it is desirable to gain knowledge concerning the potential metabolic target system, the alterations which the drug is capable of inducing in the system, and the concomitant metabolic lesions that may manifest themselves as gross or submicroscopic abnormalities which may be deleterious to the survival of affected organisms, including man.

The present study is concerned with a group of chemicals known as ethylenimines which are being tested, extensively elsewhere as chemosterilants in controlling insect pests. Ever since the discovery of induced chromosome breakage by nitrogen mustards by Auerbach and Robson (1947), a number of investigators have discovered a wide array of chemicals that could induce chromosome breakage and cause gene mutations. Ethylenimines are one of this group of chemicals which are capable of inducing the above-mentioned alterations. Most of the investigations

with these chemicals, which are also alkylating agents, were performed with a view to simply ascertain their chromosome-breaking ability, and to some extent, their mode of interference with the DNA precursor synthetic system which they are assumed to impair. No quantitative data are available with regard to their dose effect relationships over an extended period of time, involving several cell generations.

Another important aspect of the study of these chemicals is to ascertain the susceptible stage or stages in the mitotic cycle when these chemicals exert their influence. Studies of this sort are generally beset with problems, since our inferences have to be made on extrapolations from data obtained by looking at visible alterations in the mitotic cycle. Knowledge regarding their mode of action and time of action can be gained by using known inhibitors which preferentially hold up stages of the mitotic cycle. Studies of this nature can throw considerable light on not only the effects of these chemicals on specific stages of the mitotic cycle, but also on the structural organization of the chromosome.

To sum up, the investigations described here were carried out in order to:

- A. Ascertain quantitatively how much chromosome damage is produced by different concentrations of several compounds in which the active group is an ethylenimine.
- B. Obtain a clue as to the susceptible stage or stages of the mitotic cycle with regard to the action of the chemicals.

- C. Gain knowledge as to the type of chromosomal aberrations induced by these chemicals.
- D. Gain information on the persistence of the effect of these chemicals over several mitotic cycles, as well as to elicit information regarding the persistence of the chemical as such within the system, which may induce further aberrations in the mitotic cycle at later stages.
- E. Gain information regarding the unit of breakage, and thereby gain an insight into the structural organization of the mitotic chromosome.
- F. Compare the chemical damage by these chemicals to known doses of X-ray damage induced in the same system under controlled conditions.
- G. To determine the mutagenic ability of some of these chemicals.

LITERATURE REVIEW

I. Classification of Chemical Effects

Wilson (1960) classified the effects of chemicals on cells into four types: mutagenic, fragmenting, carcinogenic, and antimitotic activity. Biesle (1958) reviewed the general field of anti-mitosis on the basis of susceptible stages of the mitotic cycle, on observable damage induced, on the physiological changes induced, and on the biochemical reactions affected. According to Levan (1952), lethal and toxic reactions, reversible and physiological reactions, and mutagenic reactions are the three types of chemical effects. Other classifications include: grouping for chromosome breakage, perfect or partial radiomimicry, faulty chromosome separation, and prolongation of metaphase (Biesle, 1958).

II. Types of Chemicals Which Induce Breaks

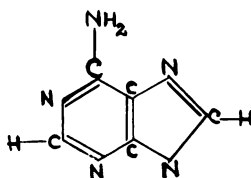
Ever since the studies of Auerbach (1946), and those of Ford (1949) on nitrogen mustards, a large number of chemicals have been reported which produce chromosomal aberrations. These can be classified according to a variety of criteria. These classifications can be based on: the chemical nature of the chromosome-breaking agents; the types of effects produced by the chemicals; the factors, both physical and chemical, that influence the production of aberrations by chemicals; and the chemical, physico-chemical, and

biochemical properties of chromosome-breaking agents likely to be responsible for their cytological effects.

The first type of classification, based on the chemical nature of the chromosome-breaking agents, will be followed here. All the other criteria mentioned above will be discussed as they relate to each of the different types of chemicals.

A. DNA Precursors and Related Compounds

1. Adenine or 6 Aminopurine



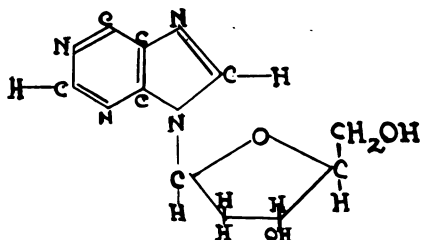
This is one of the bases of DNA, and produces chromosomal aberrations in such plants as Allium, Pisum, Vicia, (Kihlman, 1952), and in mammalian cells in tissue culture (Biesle, et al., 1952 b).

2×10^{-2} M adenine is required to break plant chromosomes. The effect is of the delayed type. The predominant aberrations are chromatid exchanges and isochromatid breaks. Absence of oxygen appears to enhance these aberrations. The following mechanisms of action are suggested: (1) as a chelating agent (Frieden and Allen, 1958); (2) as an inhibitor of P incorporation into DNA and RNA in



Vicia faba, (Odmark and Kihlman, 1965), and (3) as an inhibitor of purine synthesis in ascites tumor cells (Henderson, 1962).

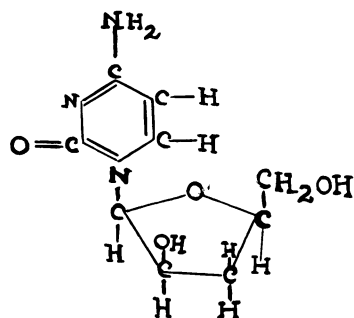
2. 2'Deoxy adenosine (ADR)



2' deoxy adenosine used in late interphase at a concentration above $3 \times 10^{-3} \text{M}$ produced strong fragmentation of chromosomes (Kihlman, et. al., 1963 and Kihlman, 1963 b) which was a nondelayed effect. The types of aberrations were mainly gaps and chromatid breaks. Isochromatid breaks and interchanges were rare, unlike the effects of adenine in which these were present, indicating that normal rejoining is inhibited. Chromatid exchanges localized in the nucleolar constriction were predominant when treatment was at 10^{-3}M concentration for 24 hours, followed by recovery for 24 hours. Chromosome-type aberrations were absent.

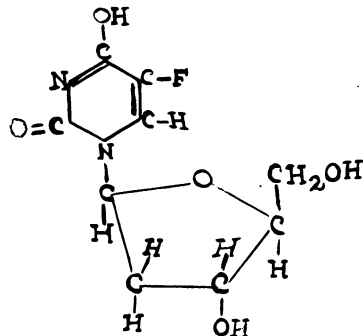
Oxidative phosphorylation inhibitors and anoxia reduced its effects in Vicia. The ADR effect in Vicia is inhibited by adenosine and thymidine. Reichard, et. al., (1961) reported that deoxy adenosine inhibits reduction of ribonucleoside phosphate to deoxy ribose nucleosidediphosphates, and this is assumed to be the cause of its chromosome-breaking ability.

3. Cytosine arabinoside - CA



CA is inactive in Vicia (Kihlman), but nondelayed breaks of the same types as with ADR are produced in human leukocytes with no rejoinings (Kihlman, 1963). Like the previous chemical, CA inhibits the formation of deoxyribonucleotides (Chu and Fischer, 1962).

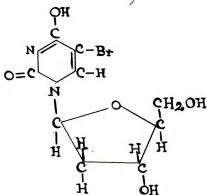
4. 5 Fluorodeoxyuridine - FUDR



During the first 10 hours after a 1 hour treatment with 10^{-6} M FUDR, gaps, chromatid breaks, and nonunion isochromatid breaks are produced in Vicia, and its effects, thus, are nondelayed (Taylor, et. al., 1962). These effects are reduced by anoxia and inhibitors of oxidative phosphorylation (Kihlman, 1962). 10^{-4} M thymidine or thymidine analogue BUDR negates the action of FUDR. FUDR inhibits mitosis to a great extent. This is indicated by very low mitotic in-

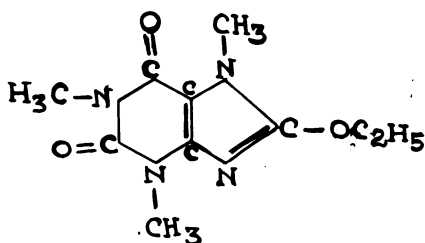
dex after 10 hours following treatment with $10^{-6}M$ in Vicia, which recovers after a day or two. The cells after this period still have a low percentage of damage, composed mainly of chromatid exchanges and sister union isochromatid rejoinings. Hsu et. al. (1964) used FUDR on hamster cells and mouse LM cells in tissue culture, and found that it causes mitotic inhibition and chromosome shattering. They also found that the sensitivity to mitotic inhibition of mice LM cells was higher than that of the hamster cells, and their chromosome shattering susceptibilities to FUDR were just the opposite. It took 100 times as much thymidine as FUDR to reverse the mitotic inhibition and inhibition of DNA synthesis caused by FUDR. Treatment with FUDR during the second half of interphase showed that the chromosomes most seriously fragmented were the late replicating ones, which suggested a correlation between damage and DNA synthetic activity. Cohen (et. al., 1958) showed that the reaction inhibited by FUDR is the methylation of Deoxyuridylic acid to thymidylic acid, which is catalysed by the enzyme thymidylate synthetase. Thus, FUDR resembles the previous two chemicals, not only in its cytological effect, but also in its biochemical action. It is not incorporated into DNA to any appreciable extent. Ahnstrom and Natarajan (1966) hypothesized that the chromosome-breaking effect of ADR and FUDR is due to the reversal of the DNA polymerase reactions caused by the deficiency of deoxyribonucleoside triphosphate.

5. 5 Bromodeoxyuridine - BUDR

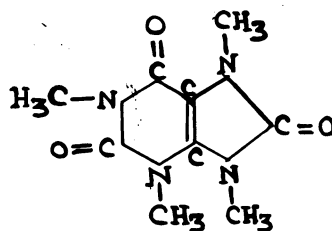


BUDR increases the susceptibility to X-ray damage of Vicia cells, but by itself does not cause any damage in Vicia (Kihlman, 1962, 1963 a). Similar results are obtained with 5 chlorodeoxyuridine and 5 iododeoxyuridine. The effect of BUDR on mammalian cells is delayed, in contrast to that of FUDR (Somers and Hsu, 1962; Hsu, 1963). BUDR has to be incorporated into the DNA to be effective. BUDR preferentially breaks chromosome ends in Chinese hamster cells in tissue culture (Somers and Hsu, 1962), and the rejoining of broken ends is not infrequent. The biochemical effects of BUDR, IUDR, and CUDR are as different from those of FUDR as the cytological effects. FUDR is a deoxyuridine analogue, whereas the other halogenated deoxyuridines are thymidine analogues. Due to the Van der Waals radii of the halogen substitutes, Cl₂, Br₂, and I₂ radii correspond to the methyl group, whereas that of Fluorine has a radius which corresponds more to that of hydrogen (Szybalski, 1962).

6. N-Methylated Oxyapurines



8- Ethoxycaffeine.



1.3.7.9. Tetramethyluric acid.

Kihlman and Levan (1949) described the chromosome-breaking ability of the naturally occurring methylated oxypurines, caffeine, theophylline, and theobromine in Allium cepa. Synthetic oxypurines, like 8 ethoxycaffeine (EOC) and tetramethyluric acid (TMU) used at 2 to 10×10^{-3} M produced nondelayed effects. Exchanges and sister union isochromatid breaks occur with the same relative frequency as produced by X-radiation, indicating noninhibition of rejoining. Woodard et. al. (1961) studied the effect of EOC and compared it with that of X-rays. EOC and X-rays are similar in inducing subchromatid, chromatid, and probably chromosome-type aberrations. On the other hand, alkylating agents produce only chromatid-type aberrations. However, although G_2 (post-DNA synthesis) cells show the highest yield of chromatid-type aberrations with both X-rays and EOC; the cells most sensitive to EOC treatment are cells in late G_2 , whereas those most sensitive to X-ray treatment are mid- G_2 . Besides, a considerable proportion of EOC aberrations are localized in the nucleolar constriction region, and this has led Kihlman (1952 a and 1961 b) to suggest that EOC may be absorbed on the surface of the nucleolus. This suggestion is supported by the high yield of subchromatid changes which could be associated with the release of EOC following the dissolution of the

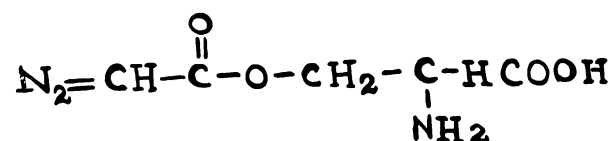
nucleolus at prophase. EOC thus does not interfere with DNA synthesis or chromosome replication, although its radiomimetic effect may in some way be related to other facets of chromosome synthesis.

The aberrations induced by TMU were during prophase, when sub-chromatid exchanges were induced, and during G₂ when chromatid aberrations were induced (Kihlman, 1961 b). The effect of TMU, in contrast to EOC, is dependent on mitotic activity during treatment. Kihlman (1951) suggests that this differential ability of EOC and TMU to affect cells may be due to their differences in ability to penetrate into the nucleus, which, in turn, is dependent on their relative lipid solubility. Only EOC is able to penetrate into the cell nucleus during early and mid-interphase, and only EOC is capable of being absorbed onto G₁ (pre DNA synthesis) and S (DNA synthesis) chromosomes (Kihlman, 1961 b).

Methylated oxypurines are not incorporated into DNA (Koch, 1956; Greer, 1958). They are rather inert biochemically, as well. Lieb (1961) reported a temporal DNA synthesis inhibition with caffeine. Odmark and Kihlman (1965) reported that both DNA and RNA synthesis in Vicia faba is inhibited by EOC.

B. Antibiotics

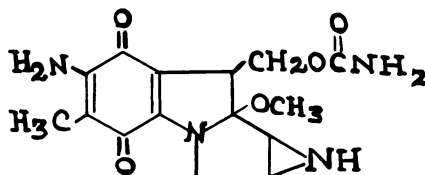
1. Azaserine



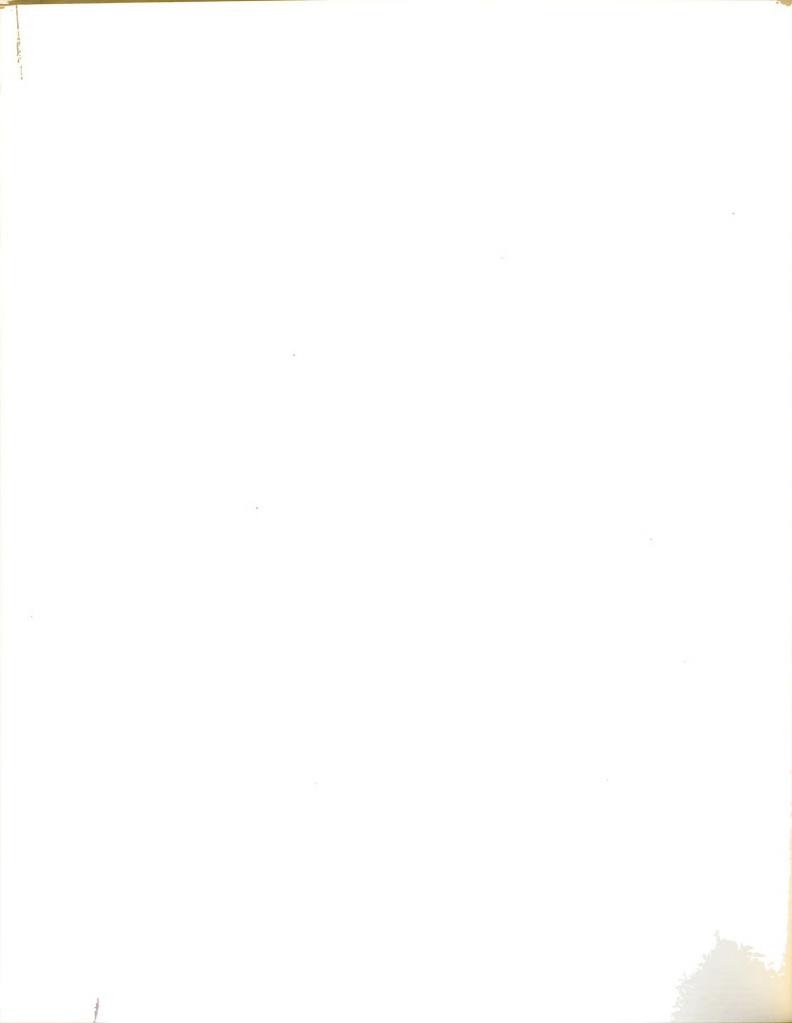


Azaserine is an antibiotic isolated from a strain of *Streptomyces* (Bartz, et. al., 1954). It produces chromosomal aberrations in *Tradescantia paludosa* root tip cells (Tanaka and Sugimura, 1956). It produces delayed chromatid exchanges predominantly in *Vicia* with 10^{-4} M treatment for 1 to 2 hours. This effect is completely suppressed by inhibitors of oxidative phosphorylation and anoxia (Khilman, 1964). Purine biosynthesis, according to Handschumacher and Welch (1960), is inhibited by azaserine, and it also disturbs amino acid metabolism. Freese (1963) suggested that it may also act as an alkylating agent, as well.

2. Mitomycin

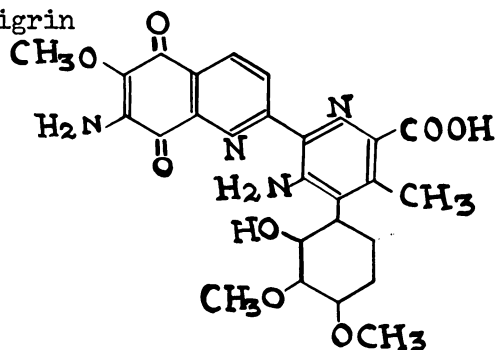


Merz (1961), by treating *Vicia* with .001% solution, produced delayed effects, predominantly chromatid breaks. Anoxia and inhibition of oxidative phosphorylation does not reduce the effect of mitomycin (Merz, 1961), and the effects are independent of pH and temperature. Nowell (1964) found that mitomycin C. causes chromosome breaks in human leukocyte cultures mainly of the chromatid type when treated in the mitotically inactive stage (G_0) of interphase. Treatments of cells in G_2 did not produce any effect, but cells in 'S' showed a low amount of damage. Shiba, et.al. (1959) and Reich, et.al. (1961) showed



mitomycin produces DNA synthesis inhibition and degradation in bacteria. Iyer and Szybalski (1963) showed that mitomycin cross links complimentary DNA strands. Schwartz (1963) suggested that mitomycin may act as an alkylating agent (after activation in Vivo by unmasking the fused aziridine ring).

3. Streptonigrin

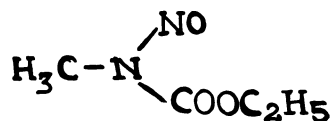


Cohen, et. al. (1963) observed mitotic inhibition and extensive damage to chromosomes of human leukocytes in culture by .0001% to .1 gram/liter of the antibiotic, and these aberrations are produced as early as two hours after treatment, showing that G_2 is the period when the effect is produced. Puck (1964), however, showed that streptonigrin is inactive in mammalian cells in which DNA synthesis is completed. In Vicia faba Kihlman (1964) showed that streptonigrin had a nondelayed effect. It produces chromosome breaks in G_1 , chromatid breaks in "S", G_2 , and subchromatid breaks in prophase. This effect was independent of temperature and pH, and is not reduced by anoxia or by oxidative phosphorylation inhibitors. Rao, et. al.,

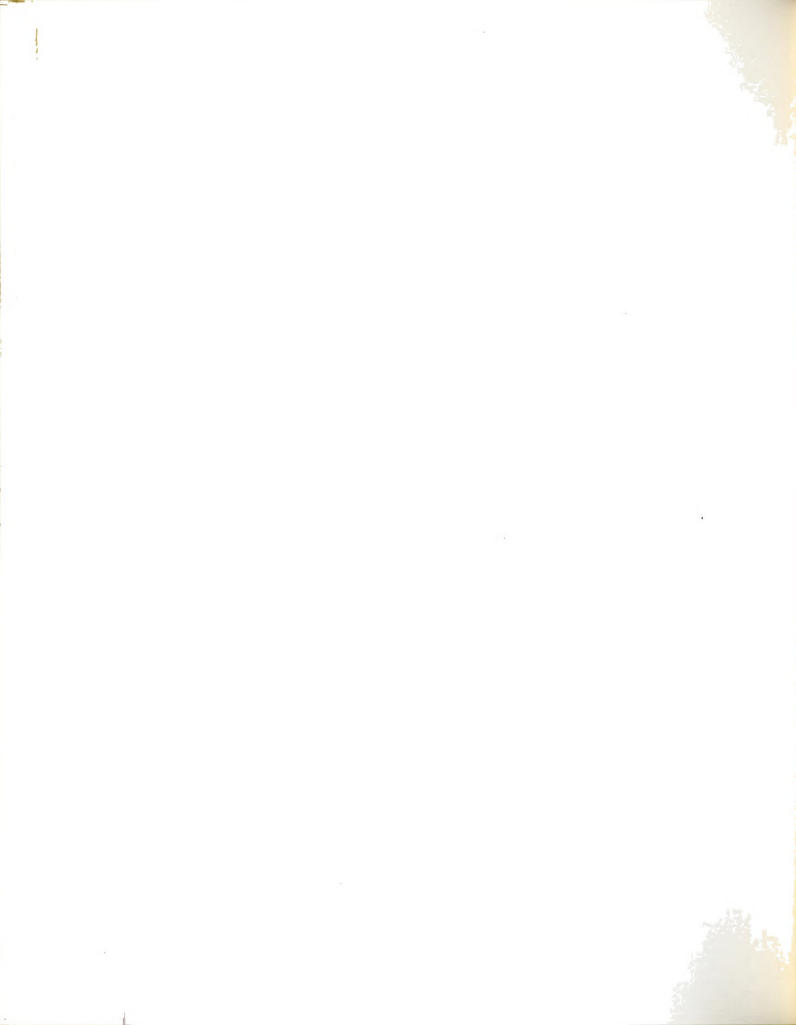
(1963) showed that both streptonigrin and mitomycin contain the same 0-aminoquinone moiety, but the former has a nondelayed effect, and the latter has only a delayed effect, in spite of their chemical similarity. Iyer and Szybalski (1964) pointed out that this similarity is only superficial, since the reactivity of streptonigrin with DNA in Vitro, in contrast to that of mitomycin C, is not influenced by chemical reduction.

C. Nitroso compounds

1. N-Nitroso-N-methylurethan

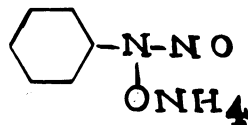


In *Vicia faba*, $0.5 - 1 \times 10^{-3}$ M of this compound produced delayed chromatid-type aberrations with normal rejoining frequency, and these were randomly distributed between S and M chromosomes. This effect is independent of pH and temperature. Respiratory inhibitors and anoxia inhibit its action, but DNP, which uncouples oxidative phosphorylation without affecting respiration, does not influence its effect (Kihlman, 1961 d). The nitrosamines are known as carcinogenic agents (Druckrey, et. al., 1961, a, b, and c). At least one alkyl group is necessary for this carcinogenic effect, which requires the presence of O_2 (Brouwers and Emmelot, 1960). The active carcinogenic groups (alkylating agents) are formed in Vivo by an enzyme-catalyzed oxidative dealkylation. But this mechanism may not explain its chromosome-breaking ability, since cupferron, which is



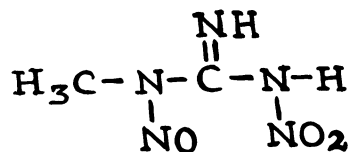
devoid of an alkyl group, is effective in producing chromosome breakage.

2. N-Hydroxylphenylnitrosamine - ammonium or cupferron



Even 10^{-3} M cupferron produced only low effects. Delayed chromatid aberrations (chromatid exchanges and sister union isochromatid breaks) were prevalent, showing normal rejoining. The above effect is enhanced at low pH and high temperature, and so is the toxic effect. It is inactive in the absence of O_2 . The effect of cupferron may well be due to its chelating properties, or to a spontaneous decomposition of this nitrosamine to yield a phenylcarbonium ion (Kihlman, 1959 b and 1961 a).

3. 1-Methyl-3-Nitro-1-Nitrosoguanidine (MNNG)

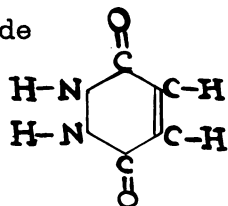


Gichner, et. al. (1963), using Vicia faba, obtained delayed chromatid type aberrations with a peak effect between 24 and 48 hours after 1 hour treatment with 5×10^{-4} M of this chemical. The effect was enhanced by low pH, and temperature from 18 - 24°C. The effect was reduced by anoxia and Sodium azide. DNP uncoupling was ineffectual.



D. Miscellaneous

1. Maleic Hydrazide



Maleic hydrazide produced in Vicia faba a delayed effect, giving chromatid aberrations with normal rejoining upon 2 hour treatment with 10^{-4} M at pH 5.8; the effect being localized in the heterochromatic segment, close to the centromere in the nucleolar arm of the M chromosome (Darlington and McLeish, 1951). The highest frequency was obtained between 24 and 36 hours after treatment. Evans and Scott (1964) found that cells in 'S', exposed to maleic hydrazide, were delayed considerably, and contained chromatid aberrations. The cells in G_1 during treatment had chromatid aberrations, while G_2 cells were not delayed and did not contain any aberrations. Its effect was reduced by anoxia and uncoupling of oxidative phosphorylation, but was increased with temperature increase, and was 4X higher at pH 4.7 than at pH 7.3 (Kihlman, 1956). Its biological activity may be due to its reaction with SH groups in the cell (Muir and Hansch, 1953), inhibiting enzymes requiring free SH groups (Hughes and Spragg, 1958).

2. Potassium Cyanide (KCN)

4×10^{-4} M KCN produces chromatid-type aberrations in Vicia faba of the delayed type with peak frequency between 24 - 36 hours after 1 hour treatment (Lilly and Thoday, 1956). These rejoin



normally. Its effect is independent of temperature, and it is inactive in anoxia. It has only very little effect at O_2 concentration below 10%. Above 10%, the effect increases with increasing O_2 concentrations, and is not influenced by inhibitors of oxidative phosphorylation (Kihlman, 1957). KCN is an effective respiratory inhibitor, since it reacts with iron or copper in cytochromes, and also with catalase and peroxidase. The damaging effect may be due to H_2O_2 accumulation in cells in the presence of KCN.

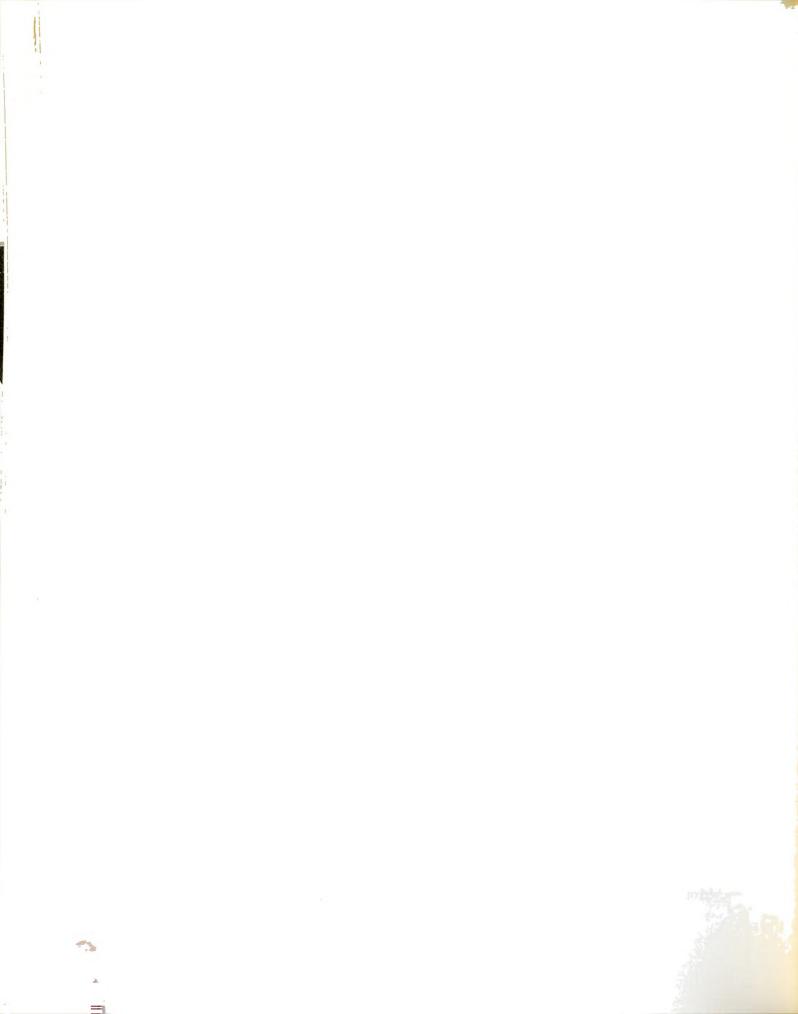
3. Hydroxylamine (NH_2OH)

Hydroxylamine produced nonrandom chromatid and chromosome aberrations localized in the centromere region. Exchanges were frequent (Somers and Hsu, 1962). Borenfreund, et. al. (1964) suggested that chromosomal aberrations induced by this chemical is the result of main chain scission of DNA rather than from a reaction with cytosine in DNA.

Cohn (1964) reported chromosome breakage and shattering 1 - 2 days after treatments of onion and Vicia roots for one-half hour with 10^{-3} M. NH_2OH . Exchanges were rare.

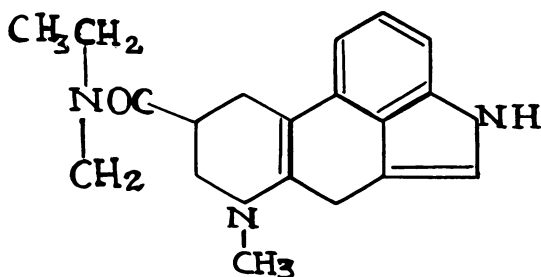
4. Ethyl Alcohol, Coca Cola, Coffee, and Antinauseants

All of the above agents were capable of inducing chromosomal aberrations (Sax and Sax, 1966). A concentration of 0.5% ethyl alcohol was equivalent to about 20 r/day of chronic gamma radiation, or an accumulated dose of 75r. Caffeine has long been known to



be radiomimetic for plant chromosomes (Kihlman, 1949). Strong coffee (2 T. per cup) produced more aberrations than did 50 r per day of chronic gamma radiation. Coca cola had to be diluted 50% to permit growth of onion roots. It is a fourth as potent as weak coffee in producing chromosomal aberrations. Antinauseant drugs like thalidomide produced a small but significant increase in chromosome aberrations. Dramamine produced a slight but consistent increase in chromosome aberrations.

5. Lysergic Acid Diethylamide (LSD)



The effect of LSD 25 was studied in cultures of human leukocytes at concentrations ranging from 10 - 0.001 μ g/ml for 4, 24, and 48 hours. Cytogenetic investigations of a patient extensively treated with this drug over a 4 year period showed similar increase in chromosomal aberrations. It was found to induce different kinds of chromosomal aberrations and showed an affinity for certain regions of chromosomes, like the centromeres and secondary constrictions (Cohen and Marinello, 1967). Chromosomal abnormalities in leukocytes in LSD 25 users was studied by Irwin and Egozcue (1967), who found a significant increase of chromosomal abnormalities as compared to nonuser controls.

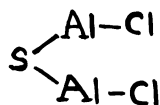


6. Irradiated Carbohydrates

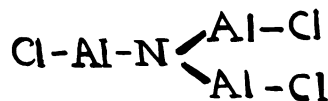
Swaminathan, et. al. (1962) and Holsten, et. al. (1965), found chromosome aberrations such as stickiness, lagging anaphase fragments, nuclear disintegration, etc. induced in plant cells in culture by irradiated carbohydrates in the medium. Shaw and Hayes (1966), studied the effects of irradiated sucrose on the chromosomes of human lymphocytes in Vitro. These showed chromosome breaks of the isochromatid type; gaps, exchanges, stickiness, endoreduplication, etc. Breaks were nonrandom along the chromosomes.

7. Alkylating Agents

Since the ethylenimines belong to the class of compounds known as alkylating agents, these will be discussed in detail. There are many agents that carry one, two, or more alkyl groups in a reactive form. These are called mono, bi, or polyfunctional alkylating agents. The various groups on a polyfunctional agent may either act separately or they may cause the crosslinking of molecules. The chemical structure of the most commonly used classes of alkylating agents are given below:

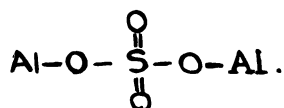


Sulfer mustard

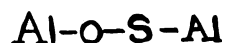


Nitrogen mustard

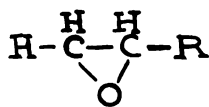




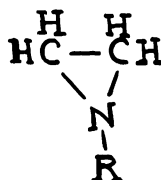
Dialkyl sulfates



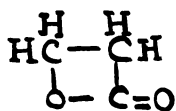
Alkylalkane sulfonates



Epoxides



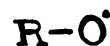
Ethylenimines



-Propiolactone



Diazo compounds

Other
reactive oxygens

Stacey, et. al. (1958) reported that all alkylating agents that prevent cell duplication have in common their reactivity with nucleophylic, i.e., slightly negatively-charged groups. Price (1958) described the various chemical reactions by which alkylation can occur. Ross (1958) described the various chemical groups. Most reactive are sulfhydryl and thioester groups. When isolated DNA is treated in solution, the reactivity of alkylating agents depend on their charge; negatively-charged molecules being least reactive. Alexander and Stacey (1958) proposed that polyfunctional mustards exert their lethal effect by crosslinking DNA molecules, rather than by merely breaking the sugar phosphate backbone of DNA, but the latter mechanism does not seem negligible, since monofunctional alkylating agents also have lethal effects.



Most alkylating agents have a mutagenic effect, whether mono or poly-functional. Whether their effects are due to direct action on DNA or chromosomes, or whether their effects are indirect are not known. In the case of Triethylenemelamine or tretamine, chemical specificity has been correlated with mutagenicity. Tretamine reacts with free pyrimidine bases to form a base analog; the reaction product being mutagenic with thymine and not with cytosine (Lorkiewicz and Szybalski (1961). Fahmy and Fahmy (1958) reported that tretamine induces dominant lethals in Drosophila by causing chromosome aberrations.

DNA can be altered in five different ways by ethylating and methylating agents:

(1) The alkylation of the phosphate groups of nucleic acids has been measured for many alkylating agents (Alexander, 1952; Reiner and Zamenhof, 1951; Stacey, et. al., 1958). The phosphate triester thus formed is unstable, and hydrolyzes mostly to return the free alkyl groups. DNA duplication might be inhibited if enough alkyl groups remain attached up to the time at which DNA attempts to duplicate. The attached alkyl group might interfere with the DNA duplication in such a way that some noncomplimentary base would be incorporated into the new strand.

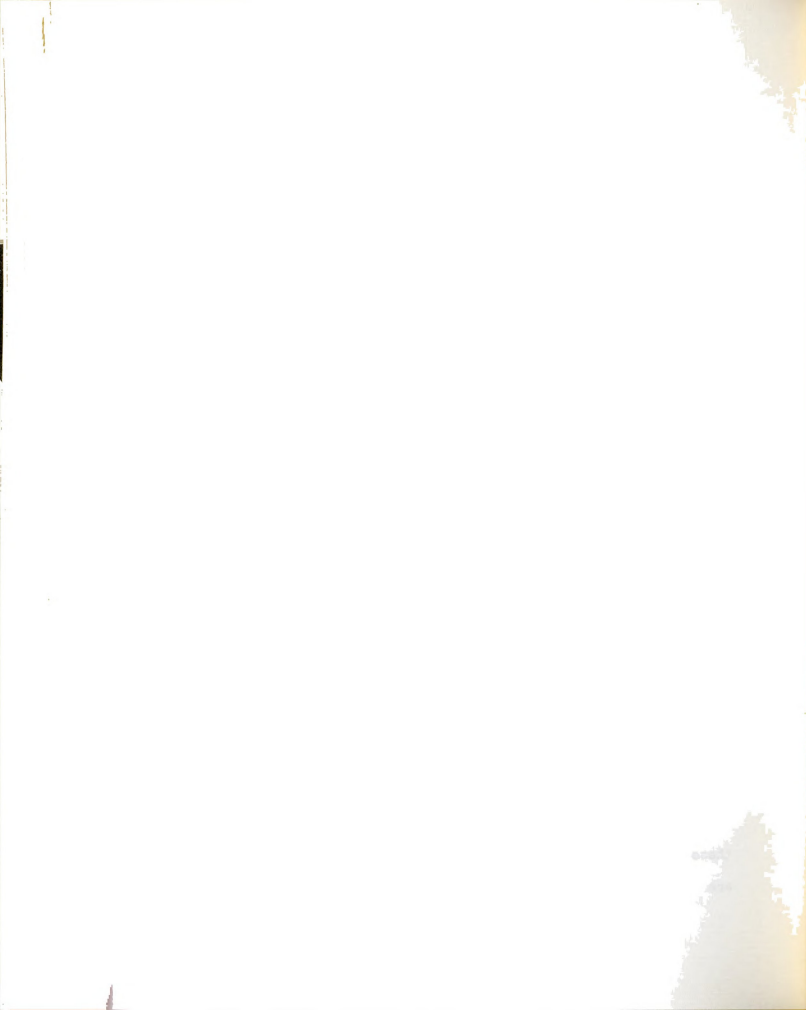
(2) The DNA "backbone" is broken if the phosphate triester hydrolyzes between the sugar and the phosphate. The relative frequencies with which the alkyl group becomes removed, or if the chain is broken, are not known. This kind of chain breakage might induce larger

alterations to be lethal.

(3) Some of the DNA bases are alkylated. The preferential formation of 7-alkylguanine has been found for nitrogen mustard (Brookes and Lawley, 1960 a). Dimethyl sulfate produced mainly seven methyl guanine (Reiner and Zamenhof, 1957). Lett, et. al. (1962) agreed that alkylation of the base does not occur directly, but transalkylation, the alkyl group changing from the phosphate to the base, takes place. Thus, the alkylated base might inhibit DNA duplication or cause base pairing mistakes during DNA duplication.

(4) Depurination: Unstable quaternary nitrogen are produced by the alkylation of the purines in the 7-position, in which case the alkyl group itself hydrolyzes away from the purines, or else the alkylated purine separates from the deoxyribose, leaving it depurinated (Freese, 1963). Bautz and Freese (1960) observed the liberation of ethylated and methylated purines from DNA. The gap might interfere with DNA duplication, or cause the incorporation of a wrong base.

(5) At high pH, the depurinated DNA is labile, and may occasionally break even at neutral pH, thus inducing larger alterations or be lethal (Freese, 1963). The majority of the known chromosome-breaking agents are believed to act by one or the other of these mechanisms. The cytological effects produced by these agents are mainly exchanges of the chromatid type, and isolocus breaks of



the delayed type. Base analog and alkylating agents may, according to Lorkiewicz and Szybalski (1961) produce aberrations in chromosomes by rather similar mechanisms, namely, incorporation into DNA during "S" period. It was pointed out earlier that tretamine causes chromosome aberrations by this mechanism. Thus, breakage by alkylating agents occur during "S" period (Scott and Evans, 1964). But this mechanism of aberrations production by alkylation of DNA precursors rather than of DNA is not widely accepted. It is generally agreed that DNA is the most sensitive material to alkylation within the cell, and is probably the primary site of alkylation (Wheeler, 1962). It is then difficult to understand why alkylating agents are effective only during "S", if it is true that DNA rather than DNA precursors is the primary site of alkylation.

Kihlman (1966) suggests several reasons why this could be so:

(1) It may be that alkylation of DNA is possible only during "S" because the chromosomes are protected at other stages by other substances

(2) It is also possible that although DNA may be alkylated at any stage, the alkylation results in chromosome aberrations only during "S"

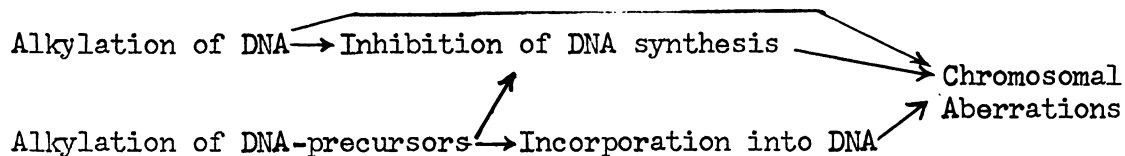
(3) It is possible that the direct cause of aberrations is not the alkylation of DNA, but the inhibition of DNA synthesis, which the alkylation has produced. This inhibition could be due to the reduced primer activity of alkylated DNA, or due to competition for sites in the DNA polymerase enzyme between normal and alkylated

100

100

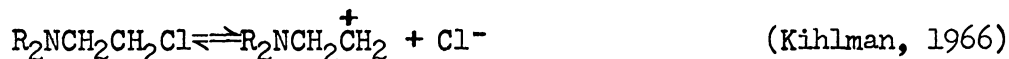
deoxyribonucleotide triphosphates.

The above possibilities are shown schematically in the following (Kihlman, 1966):



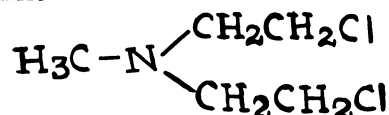
However, for alkylating agents, there appears to be no correlation between the ability to inhibit DNA synthesis and production of chromosomal aberrations (Wheeler, 1962).

The alkylating agents are very reactive, and combine readily with nucleophilic centers in other molecules, such as SH groups, ionized acid groups, and nonionized amino groups, and their affinity towards nucleophilic groups is a result of their ability to form positive carbonium ions in polar solvents; e.g.,



Some of the alkylating agents are discussed below with regard to their chromosome-breaking ability.

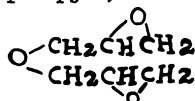
- a. Di (2-chlorethyl) methylamine
or Nitrogen mustard



Darlington and Koller (1947) studied the chromosome-breaking effects of nitrogen mustards on mitotic cells in wheat, and Ford (1940) studied their effects on roots of Vicia faba. With 10^{-5} M HN_2 produced delayed nonrandom chromatid-type aberrations that first appeared between 8 - 10 hours, and these rejoined normally. The

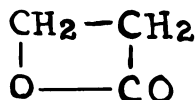
effect was independent of O_2 tension during treatment (Kihlman, 1955).

b. Di (-2-3-Epoxypropyl) ether (DEPE)



Loveless and Revell (1949), and Revell (1953), studied the chromosome-breaking effect of epoxides. Revell (1953), and Kihlman (1956) treated Vicia root tips with 2×10^{-4} M DEPE for one hour and obtained chromatid aberrations ten hours after treatment with a maximum effect between 24 and 36 hours. Rejoining frequency was normal. The heterchromatic segment in the middle of the long arm of "S" chromosomes appeared to be more often affected (Revell, 1953). This effect is temperature dependent and is independent of O_2 tension (Kihlman, 1956).

c. β -Propiolactone



Smith and Srb (1951), and Swanson and Merz (1959) studied its effect. Delayed chromatid-type aberrations with high rejoining frequency with a peak effect at 48 hours after treatment, were obtained. The breakage was nonrandom within the chromosomes. Higher temperature increases the effect, but it is independent of pH and O_2 tension.

d. Ethylenimines and other
Pesticides

The effect of apholate on Aedes aegypti was studied by Rai (1964). It was found to induce aberrations such as stickiness,



deletions, ring chromosomes, dicentrics, and anaphase bridges in somatic cells.

Wu and Grant (1966), carried out a study on the root tips of C_1 and C_2 generations of Hordeum vulgare, after treating seeds (C_0 generation) with one of 15 pesticides, including metepa, sevin, and cytol. In the C_1 generation, the percentage of affected cells ranged from 2.9 to 17.7%. Treatments with metepa induced 17.7% aberrations, which exceeded the frequency of chromosome aberrations (9.1%) found in root tip cells from X-rayed seeds (5500 R). In the C_2 generation, chromosome aberrations were found in over half of the different treatments. A number of 'mutant' C_2 seedlings were found, which included albino and yellow seedlings, dwarfs, and striped and narrow thick-leaved seedlings.

Comparative mutagenic ability of TEPA and HEMPA was tested in the sperm of Bracon hebetor by Palmquist and LaChance (1966). They found that TEPA is 100 times more effective than its nonalkylating analog, HEMPA, in inducing recessive lethal mutations.



METHODS

I. Cytological Experimental System

A standardized experimental system was employed to determine the cytological effects of ethylenimines. The root apical meristem of the pea, Pisum sativum Variety Alaska, (obtained from the Farm Bureau, Lansing, Michigan), and the broad bean Vicia faba, (obtained from Sutton and Company of England) were used in the study.

The dried peas were rinsed (three times) and soaked in deionized double distilled water for five hours at 22.5° C. The soaked peas were then spread evenly on absorbant paper toweling, moistened with deionized double distilled water (DDW), rolled, and then wrapped with wax paper. The rolls were placed in an upright position in 600 ml beakers containing about one inch of DDW. The beakers were then placed in an incubator at 22.5°C., and the peas allowed to germinate for 37 hours. At the end of this period, the paper toweling was unrolled and the seedlings were selected for the modal length of the population. This length was usually between 2 to 2.5 cms. These seedlings were suspended by paraffin-coated grids and the roots immersed in nutrient solutions contained in 350 ml crystallizing dishes, 100 mm X 50mm. The nutrient solution was modified Hoaglands, made according to the following schedule. Each stock solution was made in 500 ml of DDW.

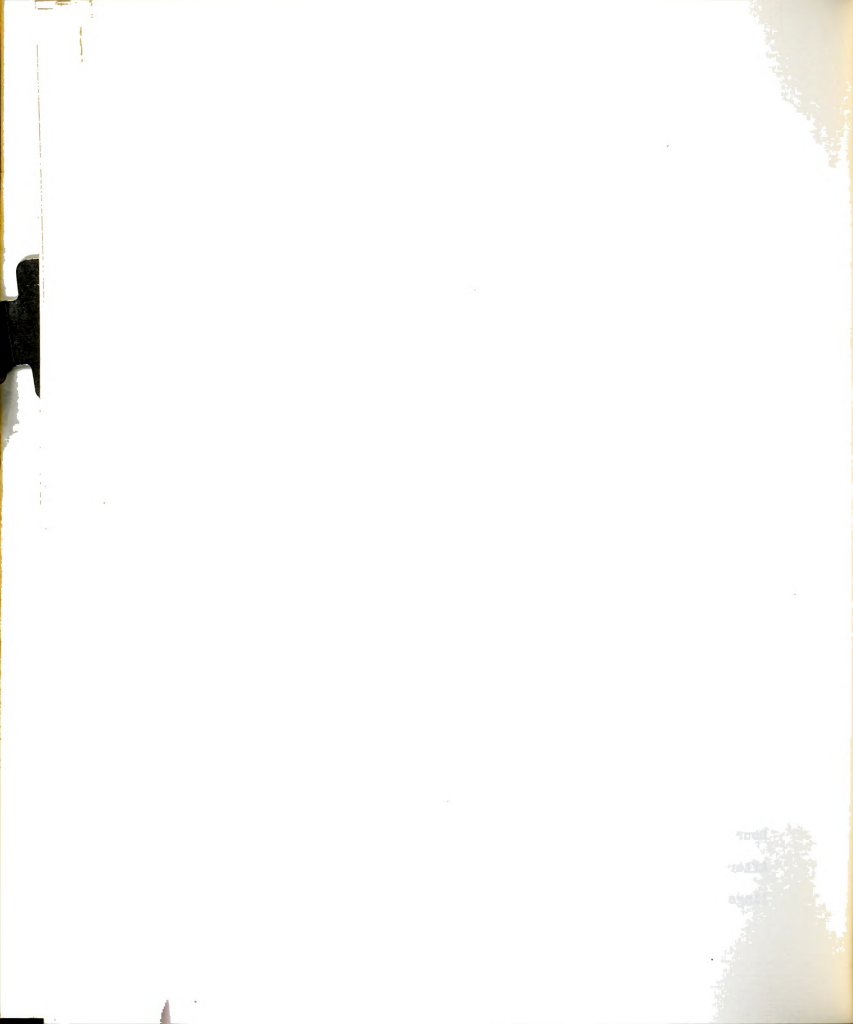


- A. 3.80 grams of Calcium Nitrate $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$
- B. 5.16 gms. of Ammonium Nitrate NH_4NO_3
- C. 7.20 gms. of Magnesium Sulfate $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
- D. 5. gms. of Potassium monobasic Phosphate KH_2PO_4
- E. 0.28 gms. of Potassium dibasic Phosphate K_2HPO_4

12.5 ml of each of the above stock solutions were used, and 970 ml of DDW was added to make 1,000 ml of nutrient. The pH of this solution was found to be between 5.6 and 5.7. In order to keep the nutrient solution well-oxygenated and stirred, filtered air was constantly bubbled through the solution. The dishes containing the peas were kept in a constant temperature water bath at 22.5°C ., located in an air-conditioned room kept at 22.5°C . The temperature of the water bath was monitored by means of a recording thermograph.

The peas were allowed to equilibrate in the culture conditions for at least 4 hours. After this period, the wire grids carrying the peas were transferred to the treatment solutions contained in similar dishes. The chemical under investigation was dissolved in the above nutrient solution according to the desired concentrations and placed in the treatment dishes. All of the ethylenimines were readily soluble in water to the extent required.

The time that the treatments were begun was considered to be the zero hour. The chemical treatments were given for only one half hour, since prolonged treatment was likely to produce complex results. After the one half hour period, the wire grids containing the pea seedlings were thoroughly rinsed in DDW and returned to the nutrient solu-



tions. Samples were taken at predetermined hours that coincided with the mitotic cycle times, determined by experimentation; the cycle time delay being known (from experiments described on page 32) for each chemical studied in detail. Samples taken at critical hours in peas were scattered. Scattering was accomplished by transferring the seedlings to 120 ppm of rulene solution mixed in water, using a wetting agent. They were allowed to stay in that solution for 45 minutes before they were fixed and squash preparations made as described below. The scattered metaphases and anaphases were studied for chromatid and chromosome damage, and were photographed (Plates I and IV). The first three cm. of the pea root was cut off by means of tweezers, placed in a small vial containing 5 ml of Pienaar's fixative (Pienaar, 1955) (6 parts of methanol, 3 parts chloroform, and 2 parts of propionic acid). Five pea root tips were placed in vacuum for ten minutes, after which time they were refrigerated for 12 hours before slides were made.

The Feulgen technique (Lillie, 1951) was employed to stain the fixed material. This technique consisted of pouring off the fixative and refilling the vials with one normal HCL, kept in an oven at 60° C., and hydrolyzing the tips for 16 to 17 minutes. After this time, the acid was poured off, and leucobasic fuchsin was added. The meristematic region developed a deep purplish color in 15 to 20 minutes. Squash preparations were made from the darkly-stained region. The slides were then transferred to 90 parts tertiary butyl alcohol and 10 parts 100% ethyl alcohol solution for dehydration. After 10 to 12 hours in the TBA solution, the slides were made permanent, using

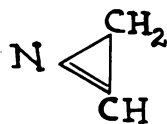


diaphane as the mounting medium. These slides were then scored for the type of data desired.

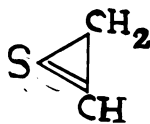
The dried Vicia faba seeds were soaked 15 to 20 hrs. in DDW. The soaked seeds were peeled in order to facilitate germination. These were then placed in large dishes containing moistened absorbant paper toweling, and stacked one over the other to prevent drying. Occasionally, water was added to the dishes. After 48 hrs., the primary roots acquired a length of 1 to 2". During this period, the seeds were thoroughly washed several times and returned to fresh absorbant paper in clean dishes. These washings helped to remove secretions from cotyledons that inhibited growth of the roots. They were then transferred to wire grids, placed over 600 ml beakers containing modified Hoagland solution, and equilibrated for at least 4 hrs. After equilibration, the seedlings were transferred to other 600 ml beakers containing the chemicals dissolved in the nutrient solution. The treatments were given for $\frac{1}{2}$ hr., using the desired concentrations of chemicals. After this time, the seedlings were transferred to the nutrient solution. Samples were taken at predetermined hours from these dishes. Only 2 root tips were taken from each group and fixed in Pienaar's fixative. 1 hr. prior to the predetermined sampling times, the seedlings were transferred to a saturated paradichloro benzene solution (PDB), and allowed to remain there for 1 hr. before the tips were excised and fixed.

The Feulgen technique was employed to stain the slides. Each root apex was split into 3 equal parts longitudinally, and transferred to 3 slides, macerated, and squash preparations were made from them. These were made permanent according to the procedure used for Pisum.

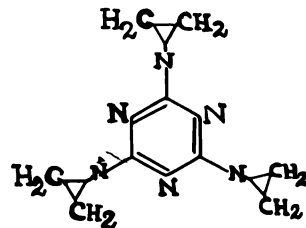
The chemicals under investigation belong to the general group viz, ethylenimines. The active group in these chemicals is the aziridinyl ring. They are:



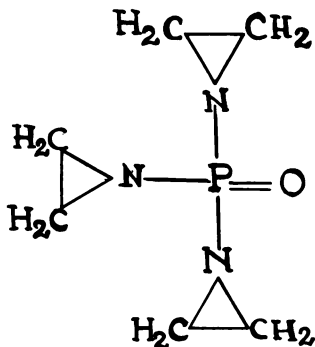
1. Azirane



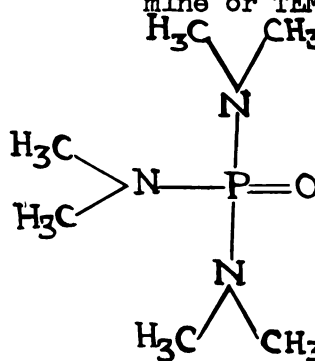
2. Ethylene sulfide



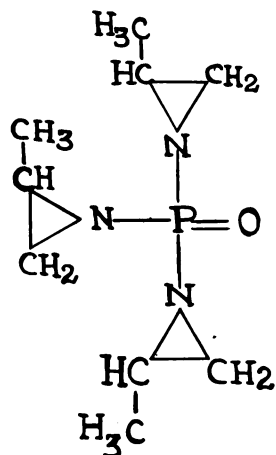
3. Triethylenemelamine or Tretamine or TEM.



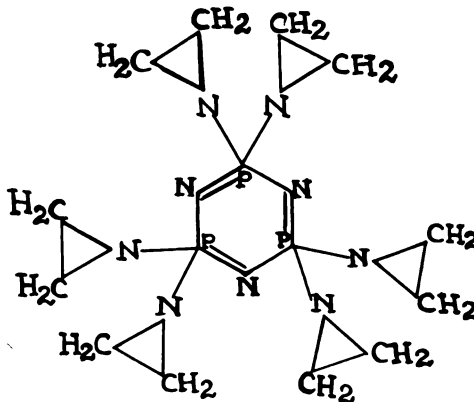
4. Tris (1-aziridinyl) phosphine oxide or TEPA



5. Hexamethyl phosphoric triamide or HEMPA



6. Metepa



7. Apholate



Detailed studies were made using apholate and metepa. Most of the quantitative data using peas as test organisms were obtained by scoring anaphase bridges and fragments. A total of 200 anaphases were scored whenever possible from each of the five slides, and these were then averaged and converted to percent damage. In beans, the quantitative data were obtained by analyzing metaphases that were scattered, using PDB treatment for one hour prior to the predetermined sampling times. Chromosome and chromatid fragments and exchanges were scored, and good preparations were photographed. Mitotic index, which is the total number of dividing cells for a total of 1,000 cells, was also determined for each slide whenever those data were deemed necessary. Random fields were chosen, and in each field the total number of dividing and interphase cells were counted. These counts were continued until 1,000 cells were obtained. Mitotic indices were usually obtained from at least four slides, and the average computed.

For determining the cycle time delay caused by treatment with the chemicals under investigation, a continuous colchicine method was employed. The peas were first treated with the chemical with the desired concentration for one half hour, and immediately rinsed and transferred to a colchicine solution (75 ppm), and allowed to remain there for the rest of the experiment. Controls with and without colchicine treatments were also run simultaneously. Samples were taken at one, two, and four hours to check the colchicine effect. These root tips were fixed, and squash preparations were made and scored for clumps, scatters, and normal post-prophase stages. A colchicine



index (Greenberg, 1966) was then calculated, using the formula

$$CI = \frac{2X \# \text{ of clumps} + 1 X \text{ the } \# \text{ of scatters} + 0 X \text{ normal postprophase cells}}{\text{postprophase cells}}$$

The index value has a peak at about two hours. At 1.8 or above the colchicine is assumed to be good. If the effect was low, a new batch of colchicine was obtained for experimentation. Samples were taken at predetermined hours. Slides were made from these, and scored for percent polyploidy. As time increases, the percent polyploidy also increases.

On a plot of time versus ploidy, at least four points were obtained, and these were then joined to meet the X-axis. Where they meet is assumed to be the minimum cycle time (Bekken, 1966).

For determining the duration of the second cycle after treatment, the roots were treated with the chemical for one half hour, rinsed, and transferred to the nutrient solution until the end of the first cycle, already determined by experimentation. These were then transferred to a continuous colchicine solution (75 ppm), and were allowed to stay there for the rest of the experiment. Samples were taken from these dishes at predetermined time intervals. The results were plotted as before, and cycle time delay was thus determined.

The 5 amino-uracil (5 AU) system was employed in order to ascertain the susceptible stage of the mitotic cycle at which the ethylenimines exert their influence in causing chromosome breakage. The pea system was exclusively employed in these experiments. After

four hours of equilibration, the peas were transferred to dishes containing 100 - 150 ppm of 5 AU suspended in the nutrient solution. Since 5 AU was not readily soluble in the nutrient solution, the temperature was raised to about 40°C with constant stirring. This improved solubility. The peas were left in 5 amino-uracil for 8 hours, at which time they were taken out and thoroughly rinsed in DDW, and returned to the nutrient solution. Treatment with apholate 3.1×10^{-3} M was made at various times after the 5 AU treatment. These treatments were made at $7\frac{1}{2}$, $10\frac{1}{2}$, 12, 15, 16, and 17 hours after treatment with 5 AU. Samples were taken up to 63 hours after treatment, and slides were scored for anaphase damages as well as for mitotic indices.

X-ray standards, using peas, were also run. This was done at Brookhaven National Laboratories by Dr. Van't Hoff. A dose (250 r) was given, and samples were taken at 3, 6, 9, 12, 15, and 27 hours. Slides were made and scored for damage.

2. Genetic Experimental System

The Muller 5 system was used to study the mutagenic ability of certain of the chemicals. Cultures of Oregon R Drosophila melanogaster were started in fresh culture media. A cylindrical piece of white bond paper, 3"X 4", was placed in contact with the medium in the bottles. The larvae climbed this paper before pupation. When the larvae reached the third instar stage, the paper with the larvae attached to it was lifted from the medium and dipped into a 1%

1001
1002
1003
1004

1005
1006
1007
1008
1009
1010

solution of the chemical for one minute. It was then dried with absorbant paper toweling and transferred to fresh media. When the flies emerged, they were etherized, and the males were mated to virgin Muller 5 females. The F_1 progeny were then pair-mated in smaller vials to get the F_2 . These were screened for eye color. If in any F_2 progeny the full red-eyed males were missing, this was considered to be induced by a lethal mutation. Such cultures were retested by mating the red-eyed females, which are the carriers of the lethal, to wild type red-eyed males. The progeny of these retested pair matings were then screened for eye color. If no full red-eyed males occurred in this progeny, then the existence of a lethal mutation was considered confirmed.

3. Retention of the Chemical

Pea seedlings were solely employed in this study. A large quantity of peas were equilibrated for four hours in modified Hoaglands nutrient solution, and treated with 1.4×10^{-2} M of the chemical (metepa) for a half hour. These were rinsed thoroughly in DDW. Samples of 75 roots were taken at $\frac{1}{2}$, 1, 2, and 4, 8, 12 and 24 hours after treatment and ground in a homogenizer, using 1.5 ml of acetone as extracting medium. This extract was then centrifuged at 8,000 rpm. The supernatant was then carefully poured into a small test tube and injected into a gas chromatographic column for detecting the presence of the phosphate group, using a hydrogen flame ionization detector with a sodium thermionic shield (Giuffrida, 1964) in a Packard 800

gas chromatograph. Extracts from control pea roots were also run simultaneously. Metepa standards of known concentrations were also run as comparisons, and quantitative estimations could be made by comparing the peaks made by the recorder on the graph paper.

RESULTS

Azirane

The damage caused by different concentrations of azirane treatment on the pea root meristem was scored at anaphase. It was found that azirane damage, as indicated by the presence of bridges and fragments, was highest at 1.83×10^{-2} M. The maximum effect was found at 39 hours. Low doses up to 0.73×10^{-2} M failed to show appreciable damage above control level. Using 1.22×10^{-2} M, the damage reached a maximum at 27 hours, and a high level was maintained at 39 hours. Doses above 1.83×10^{-2} M were lethal, and mitotic inhibition as shown by mitotic indices at different sample times was very great. Even 1.22×10^{-2} and 1.83×10^{-2} M treatments showed mitotic inhibition. The M Is (mitotic indices) at 15 hours were low (14). At 27 and 39 hours the M Is were at control level. At later hours (51 and 63) it was of the order of 20 (see Figure 1). The effects of azirane with respect to M I and damage are summarized in Table 1 in the appendix.

Ethylene Sulphide

This was tested on the pea system at concentrations ranging from 0.42×10^{-3} M to 8.32×10^{-2} M. There was no significant damage in any of the concentrations employed. The solutions of this chemical turned cloudy. It seems that this compound is very unstable and disso-



38.

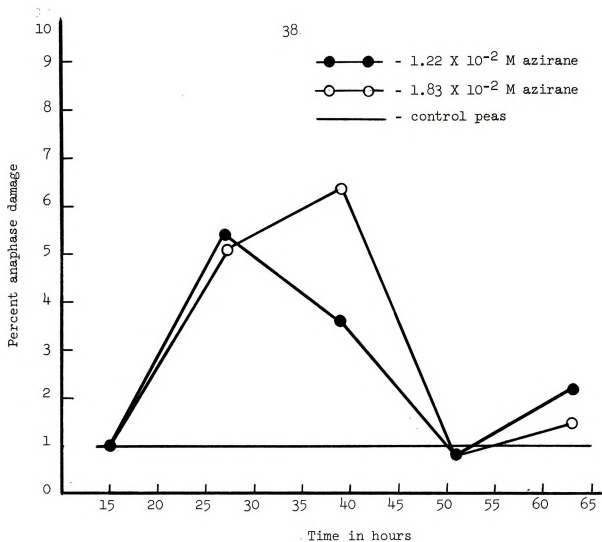


Figure 1. Azirane damage in which the percentage of anaphase damage is plotted against time in hours.

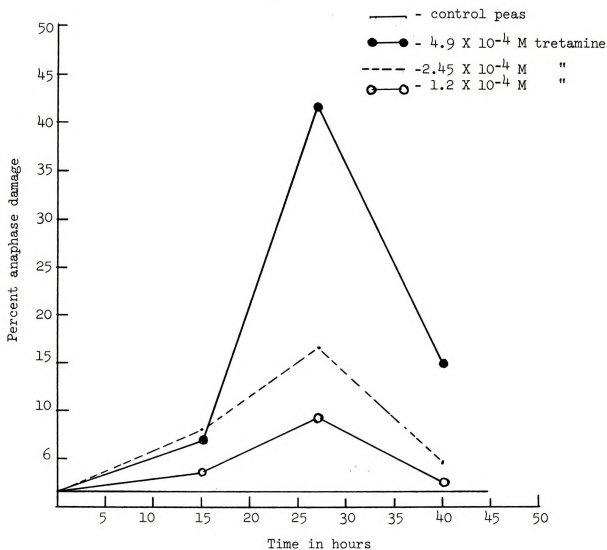


Figure 2. Anaphase damage in Pisum sativum, caused by 3 concentrations of tretamine, plotted against time in hours.



ciates easily at the pH employed, which might account for its insignificant damaging ability.

Tretamine or TEM

The chromosomal damage induced by tretamine treatment in peas was measured by scoring anaphase damage. Of the concentrations used, the range between $1.23 \times 10^{-4} \text{ M}$ - $4.9 \times 10^{-4} \text{ M}$ was found to induce the maximum damage without causing lethality. The maximum effect was at 27 hours, and dropped off at later hours, as shown in Figure 2.

The maximum effect (60% damage) was obtained with $3.68 \times 10^{-4} \text{ M}$ at 27 hours. With $4.9 \times 10^{-4} \text{ M}$, the maximum effect obtained at 27 hours was 40%, and at 39 hours, the damage was only 15%. The maximum damage using $2.45 \times 10^{-4} \text{ M}$ was 16.5%, and at 40 hours the damage dropped to 4%. Exposure to $1.23 \times 10^{-4} \text{ M}$ produced the lowest percentage of damage; 15, 27, and 40 hours had respectively; 3.6%, 9.6%, and 2.4% damage (Fig. 2)

M Is were also determined at various hours at different treatment levels. The M Is were considerably lower at later hours. The M I showed a decline at 39 hours, and at 51 and 63 hours, M Is were of the order of 15 - 20.

Aphoxide or TEPA

The effect of TEPA was studied using the pea system. The maximum damage was found at 27 hours, and showed lower values at 39 and subsequent hours.



Hexamethyl phosphoric triamide or HEMPA

Various concentrations of HEMPA, ranging from 5.58×10^{-3} M - 1.675×10^{-2} M were tested on the pea root meristem. None of the concentrations tested had any significant chromosome-breaking effect. Inhibition of mitosis as shown by M I was present.

HEMPA was tested on the Muller 5 system by treating Drosophila larvae. It was found to induce lethal mutations.

<u># of pair matings</u>	<u># of lethal mutations</u>	<u>$\frac{\%}{1.95}$</u>
153	3	

The percentage of lethal mutations was considerably higher than can be accounted for by spontaneous mutations, since our particular stock of Oregon R flies have a rate of less than .01% X chromosome recessive lethals.)

Apholate

The chromosome-damaging effect of apholate was studied more extensively on the pea root system. The apholate used had only 40% active ingredient, and doses were based on this. The damaging effect of apholate was independent of pH between the range of 5 - 7. Two types of results were obtained by using apholate:

1. two experiments showed maximum damage at 39 hours, and the damage persisted for a considerable period of time, viz up to 95 hours, and
2. in other experiments the maximum damage was at 27 hours, and did not persist at such high levels as before, although a level of damage above control was still present up to 95 hours.



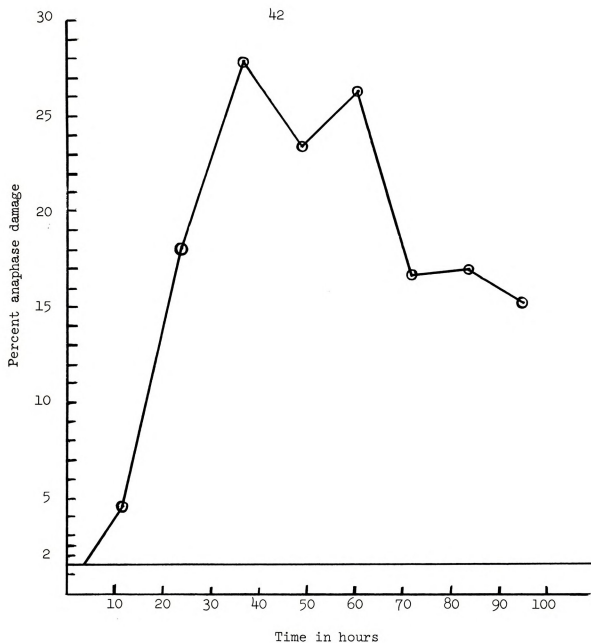


Figure 3. Chromosome damage caused by 3.1×10^{-2} M apholate. Percent anaphase damage vs. time, shows the 3rd cycle maximum and persistent effect.

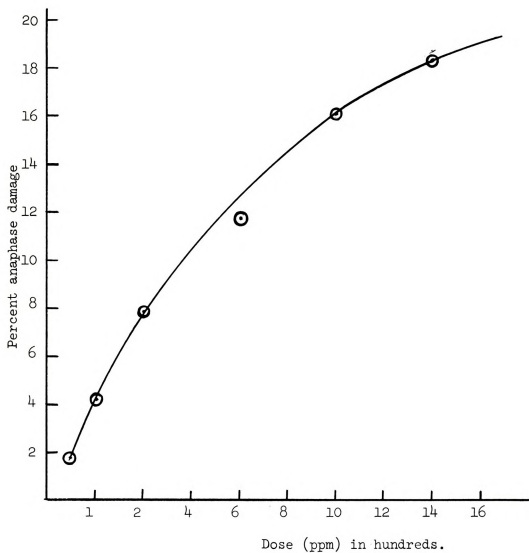


Figure 4. Dose effect curve using apholate. Percent anaphase damage vs. dose in parts per million.



The concentrations of apholate employed and their corresponding maximum damages were: 0.26×10^{-3} M, 2.5%; 0.52×10^{-3} M, 4.5%; 1.03×10^{-3} M, 8.6%; 2.1×10^{-3} M, 11.6%; 3.1×10^{-3} M, 16.4%; and 4.1×10^{-3} M, 18.4%.

Another experiment in which 3.1×10^{-3} M concentration was employed, the damage at 12 hours was 4.5%. At 24 hours, the damage rose to 18%, and at 36 hours reached a peak at 27.6%. This level of damage then persisted up to 61 hours, after which it dropped to 16% and 16.9% at 72 and 84 hours, respectively. A high percent of damage (15.3%) was still present at 95 hours. The average control damage was of the order of 2% or less (See Figure 3 and Table 2).

In a different experiment using 3.11×10^{-3} M of apholate, it was found that the amount of damage at 15 hours was still at control level, i.e., below 2%. However, at 24 hours the maximum damage of 15% was obtained. At 36 hours the amount of damage was lower (7.5%). At 48 and even 61 hours, the damage was persistent at the level of 5% (Table 3).

Mitotic indices were determined in both control and treated pea roots. It was found that the M I remained rather steady at control level (M I 60) for up to 30 hours. It then showed a rise, and again fluctuated around the mean 60. The dose effect relationship for the peak hours are shown in Figure 4 and Table 4.

Mitotic cycle time delay caused by treatment with apholate (3.1×10^{-3} M) was also determined (see Figure 5). Continuous treatment with colchicine was employed in this experiment, and percent polyploidy was plotted against time. In control peas the polyploids

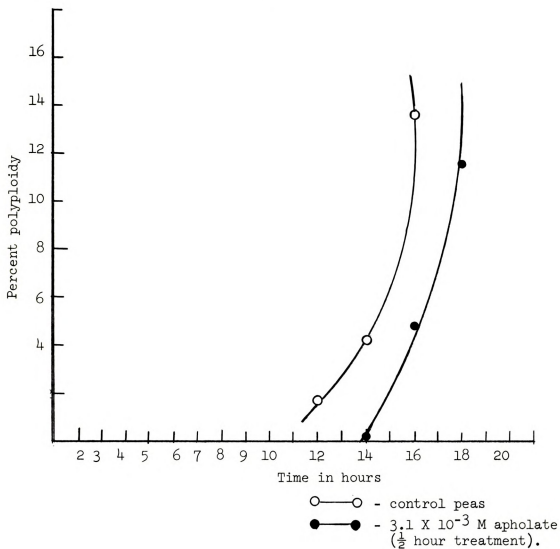


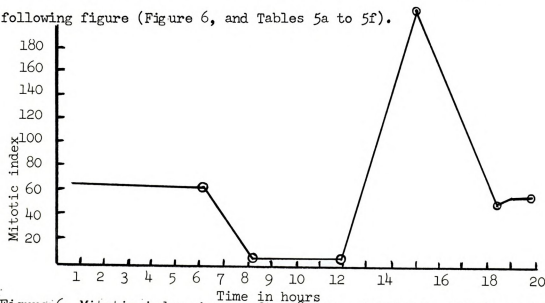
Figure 5. Cycle time delay caused by apholate treatment on *Pisum sativum*. Percent polyploidy is plotted against time. Polyploids did not appear until 15 hours in treated peas.



appeared earlier. Significant amounts of polyploids appeared in the apholate-treated peas only at 15 hours, and the delay was estimated to be about 2.5 to 3 hours.

5 Amino-Uracil System

The 5 amino-uracil system was utilized in order to determine the particular stage or stages or interphase or active mitosis where the damage is taking place. 5 A U is known to inhibit DNA synthesis (Perensky and Smith, 1965). It is also known that it stops cells from passing through the mitotic cycle, at the end of G_1 , and also in S (Van't Hoff, 1966). The roots were treated with 5 A U continuously for 8 hours, after which the roots were removed from the 5 A U solution, rinsed, and returned to the nutrient solution. The pattern of M I change after 8 hours of 100 ppm of 5 A U is shown in the following figure (Figure 6, and Tables 5a to 5f).



[Figure 6. Mitotic index change after 8 hrs. of 150 ppm of 5 AU on *Pisum*. The roots were treated with apholate for $\frac{1}{2}$ hour at various times with

8 hours in 5 A U. The different times were chosen so that the cell



population undergoing treatment was in several different stages of the mitotic cycle. These treatment times and the corresponding stages that the cell population was assumed to be in were: $7\frac{1}{2}$ hours, G_1S (actually the end of G_1); $10\frac{1}{2}$ hours, midsynthesis, 'S' stage; $12\frac{1}{2}$ hours, early G_2 ; and at 15 hours, active mitosis. The peak M I (200) at 15 hours (as shown in Figure 6) was obtained because of the synchrony induced by the 5 A U treatment. This synchrony, however, does not persist even for the next cycle. The damages induced at these treatment times were then scored, to determine whether there was any significant difference between them. The data for each time of treatment, showing both M I and the percentage of damage, are given in the tables 5a through 5f.

Drosophila larvae were treated with apholate, and were tested against the Muller 5 system to detect any lethal mutations induced by treatment. These data are as follows:

LATE LARVAE TREATED BY DIPPING IN 1% APHOLATE FOR 30-60 SECONDS

Negative:	104 + 132 + 49 + 103 + 97	=	485	
Positive:	8 + 2 + 1 + 9 + 0	=	20	3.9% mutations

Metepa

Extensive studies were made using Metepa. These studies were conducted on both Pisum sativum and Vicia faba.

A. Peas

The anaphase damage using metepa at different concentrations was determined for the system. In this case, two types of results were obtained, as well:



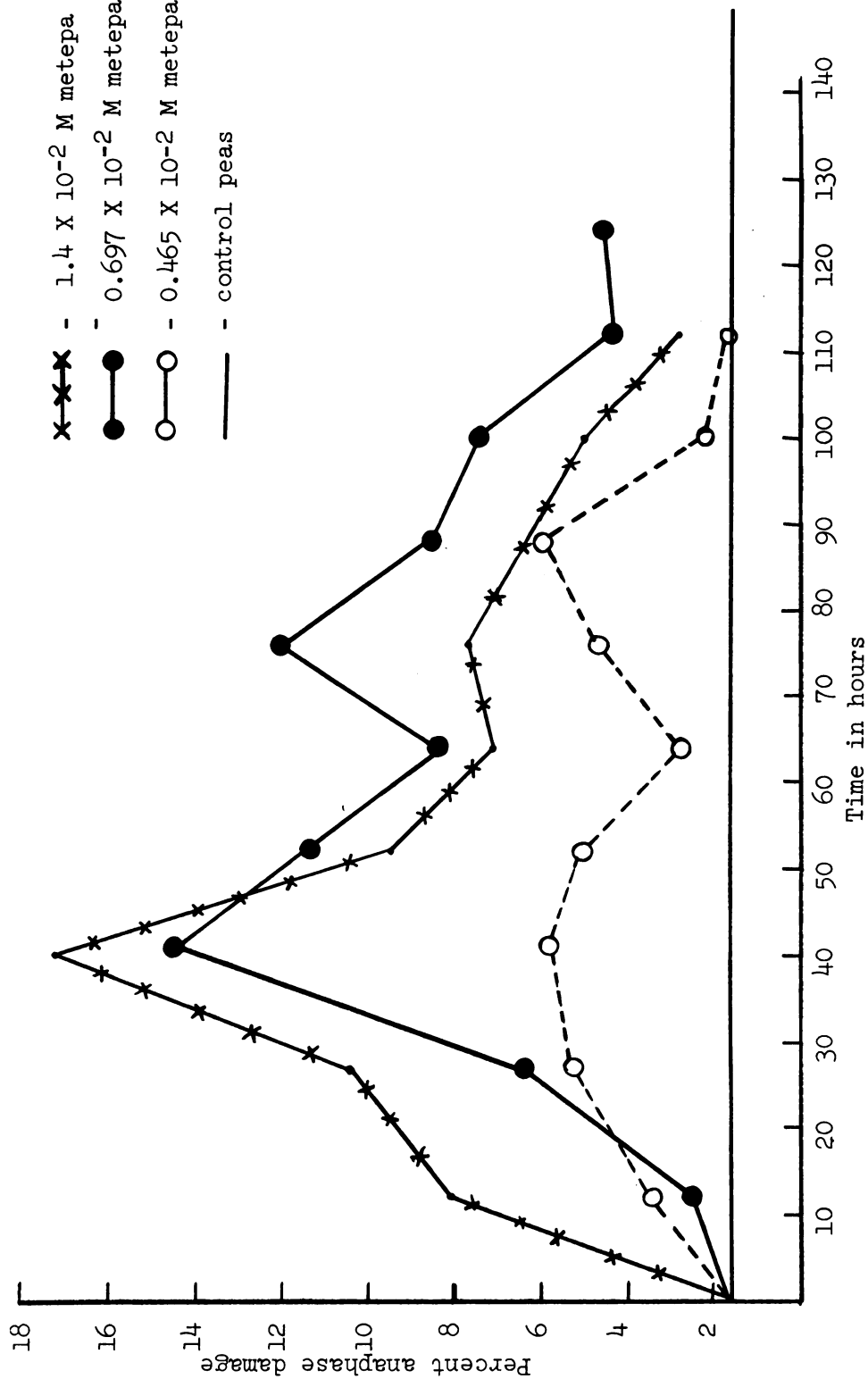
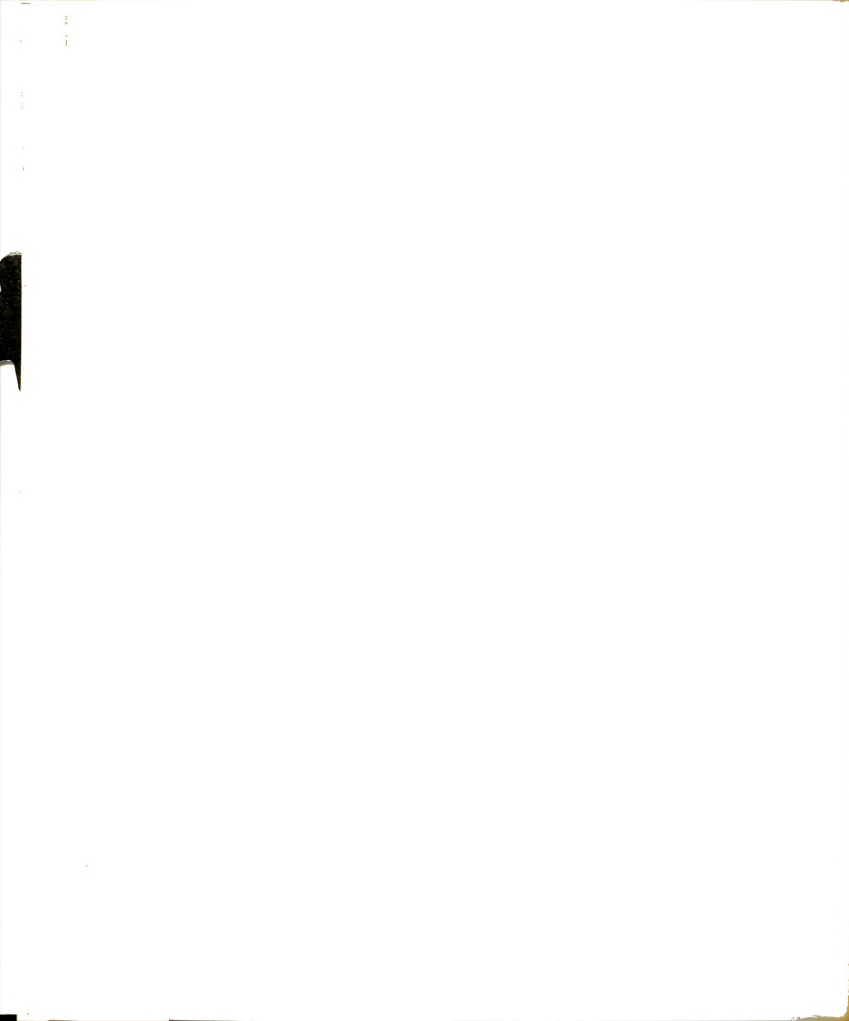


Figure 7. Metepa damage in Pisum sativum. Percent of anaphase damage versus time shows 37 hour (3rd cycle) maximum effect, as well as 75 hour (6th cycle) 2nd maximum effect. The effect is persistent.

Squares under the curve, 0.465×10^{-2} M (thousand ppm)=23; 0.697×10^{-2} M (1500 ppm)=68; 1.4×10^{-2} M (3000 ppm)=64. Fragments vs. bridges at concentrations of 1500 ppm and 3000 ppm were the same.



1. in which the maximum damage was at 27 hours, corresponding to the second cycle after treatment, and

2. in which the maximum damage was at 39 hours, corresponding to the 3rd cycle after treatment. The concentration of metepa used in these experiments ranged from $.465 \times 10^{-2}$ M to 1.4×10^{-2} M.

In one experiment where three different concentrations were employed, the maximum damage in all three concentrations appeared at 39 hours (see Figure 7). These concentrations and their corresponding peak damages were: 0.465×10^{-2} M, 5.9%; 0.697×10^{-2} M, 14.4%; 1.4×10^{-2} M, 17.2%. In this experiment a second maximum was obtained at the 6th cycle, although with the highest concentration it was not so pronounced. With the lower concentrations this second maximum was very well pronounced, however, and nearly equaled the first (see Table 6).

The number of squares under the curves were also determined. Concentrations of $.465 \times 10^{-2}$ M, $.697 \times 10^{-2}$ M, and 1.4×10^{-2} M had values of 23, 68, and 64, respectively. These last two values are not significantly different from each other, which indicates that these two concentrations induced nearly equal amounts of damage (see Figure 7 and Table 6).

The ratios of fragments to bridges were determined at $.465 \times 10^{-2}$ M and 1.4×10^{-2} M, and these, also, were found to be the same.

In another experiment in which a series of concentrations were used, ranging from 0.58×10^{-2} M to 1.16×10^{-2} M, the maximum effects were obtained at 27 hours, which corresponds to the second cycle after treat-

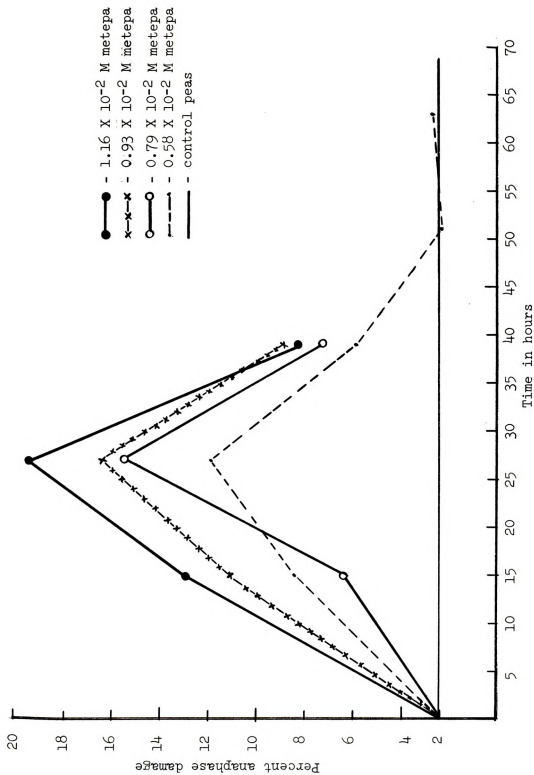


Figure 8. Metepa damage in *Pisum sativum*. Percent anaphase damage plotted against time shows 27 hour (2nd cycle) maximum effect, which is nonpersistent.

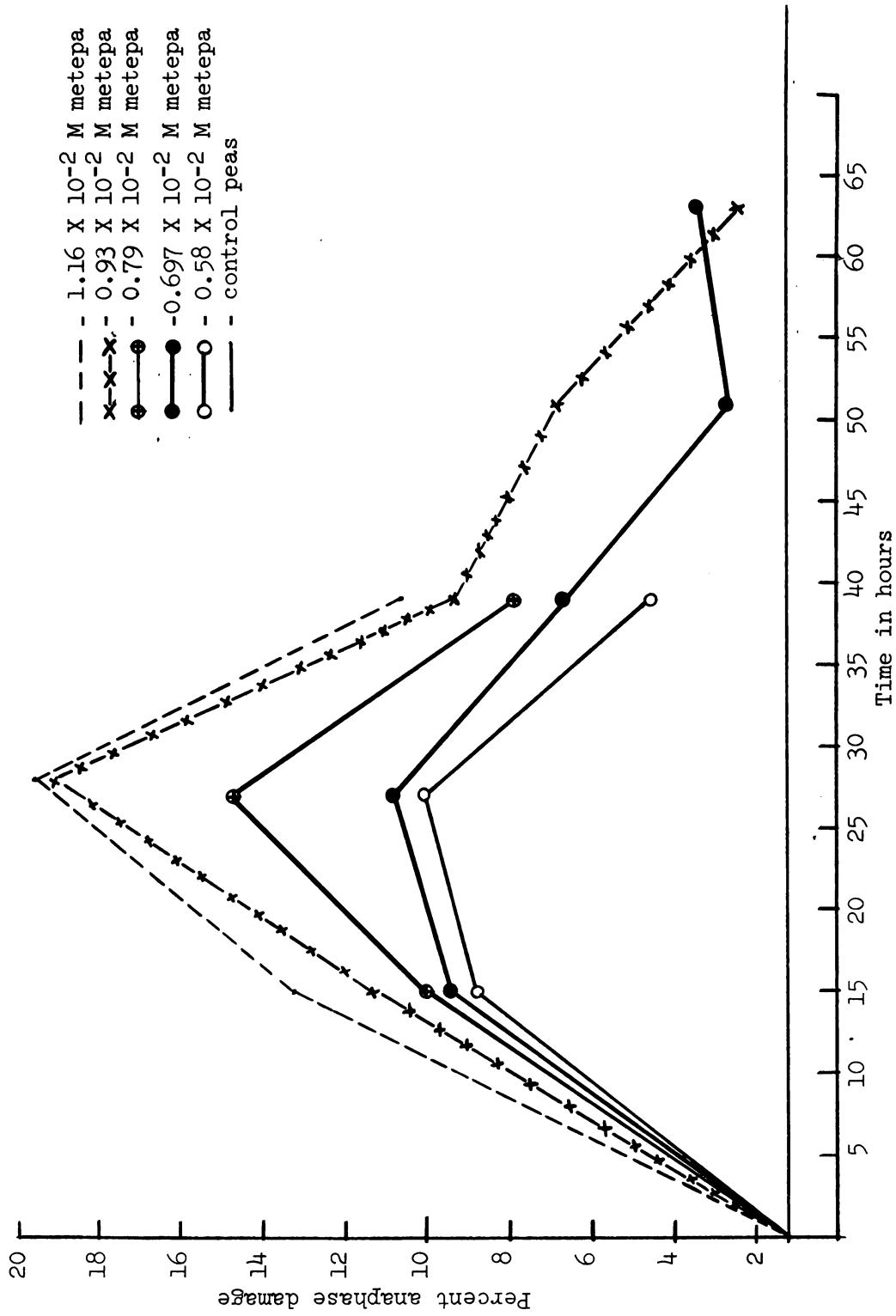


Figure 9. Metepa damage in Pisum sativum. Percent anaphase damage vs. time. 27 hour (2nd cycle) maximum effect which is nonpersistent is obtained.



- - peak anaphase damage
from Figure 8.
● - peak anaphase damage
from Figure 9.
Both of the above had a
peak effect at 27 hrs.

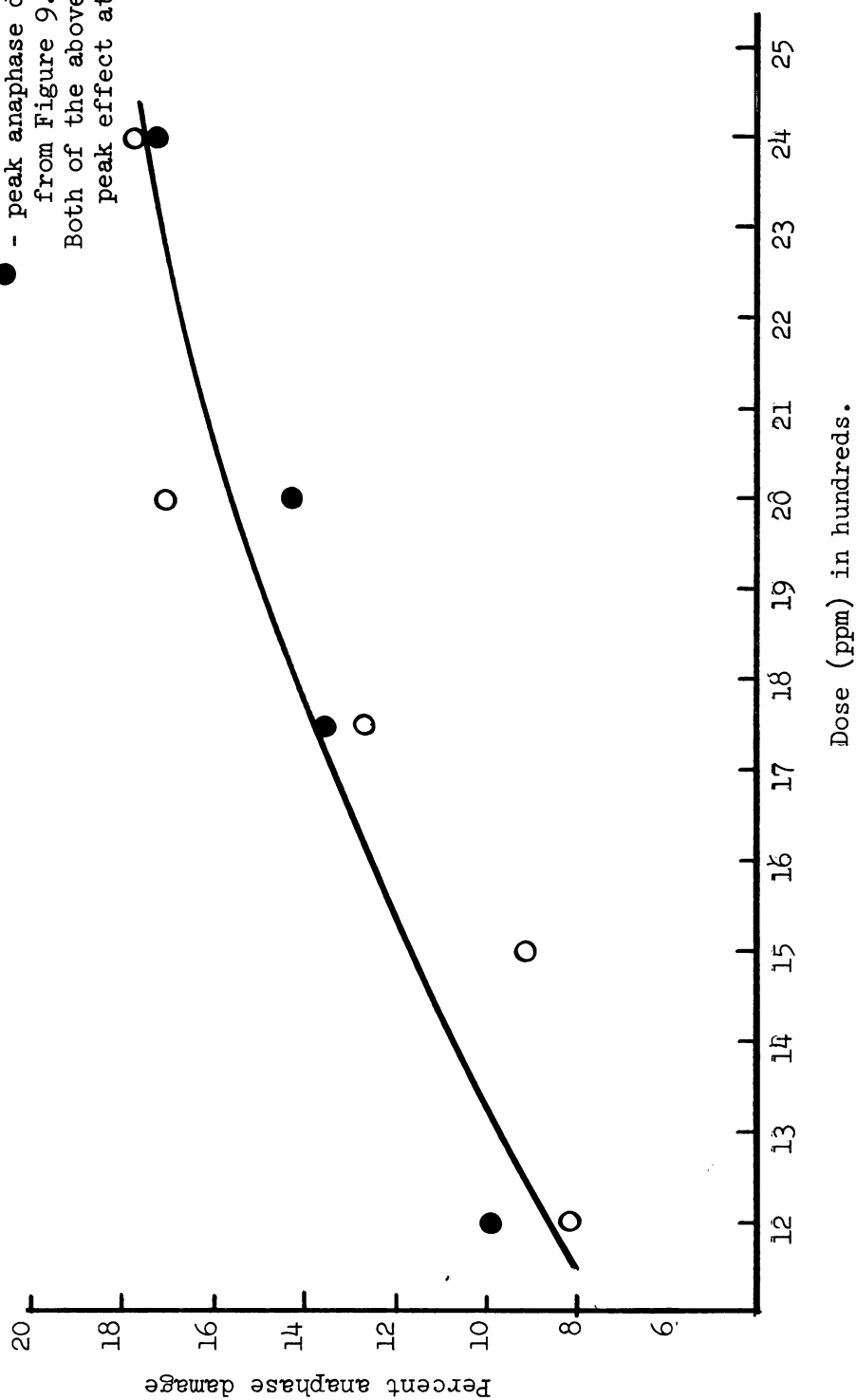


Figure 10. Dose effect curve using metepa in Pisum sativum. Percent anaphase damage is plotted against dose. In this graph the results of two different experiments are plotted.

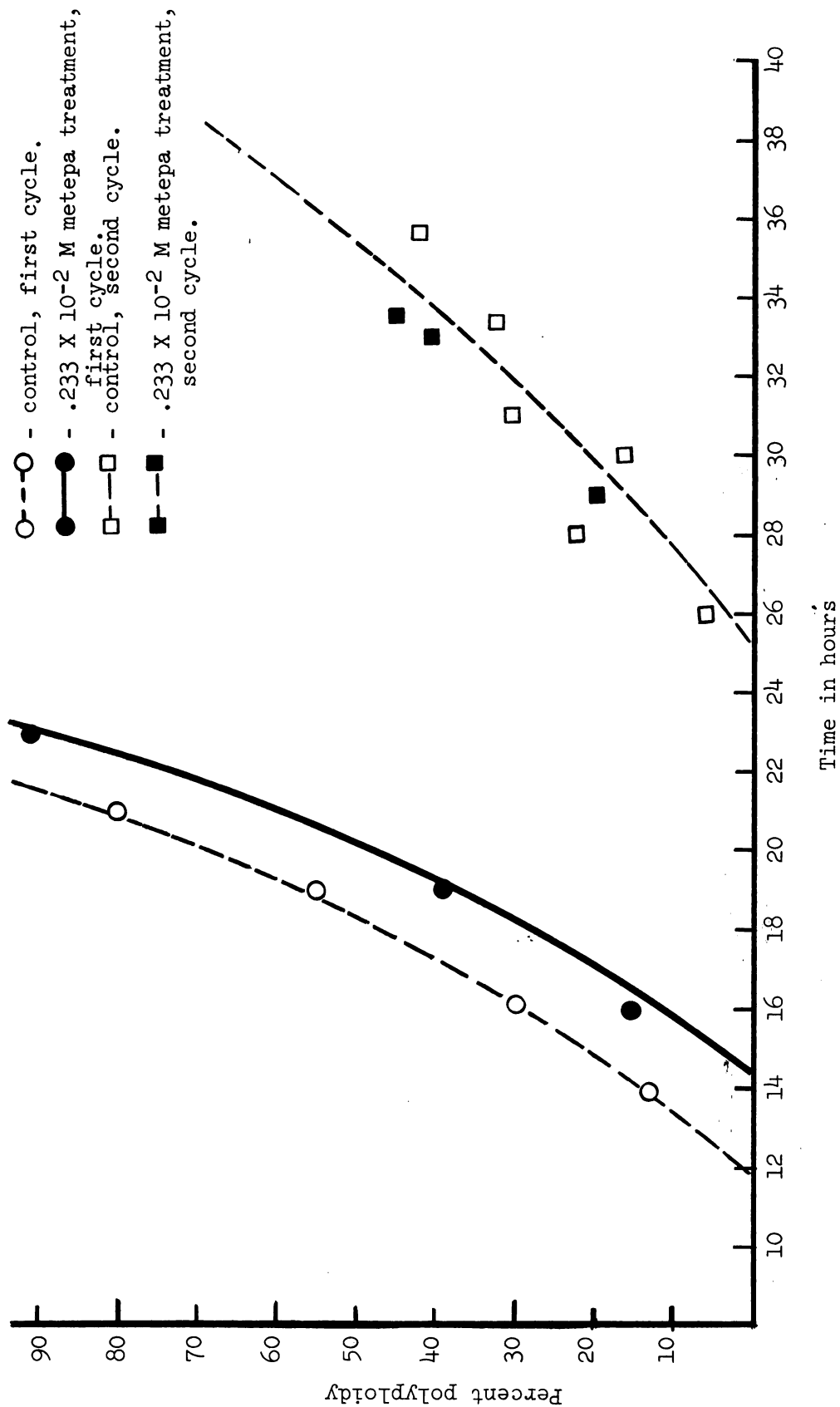


Figure 11. Cycle time delay caused by metepa treatment on Pisum sativum. Percent polyploidy is plotted against time in hours. In treated peas, polyploids did not appear until 16 hours. The delay is about 2 hours in the first cycle. The polyploids appear at about the same time in the 2nd cycle in both control and treated peas, indicating no significant delay in this cycle.



ment. These peaks were 11.6%, 15.6%, 16.4%, and 19.5% for the respective concentrations of 0.58×10^{-2} M, 0.79×10^{-2} M, 0.93×10^{-2} M, and 1.16×10^{-3} M (see Figure 8 and Table 7).

In another experiment which duplicated the previous one (except that one more concentration of metepa [0.697×10^{-2} M] was used), the results obtained were comparable. Second cycle nonpersistent effect was obtained in this case, also (see Figure 9 and Table 8).

The peak effects were plotted against the respective doses, as shown in the dose effect curve in Figure 10.

Mitotic indices were determined in all of the above experiments (see Tables 6, 7, and 8 [M Is]). It was observed that the M Is remained rather steady (at about control level) in the experiment in which a third cycle peak was obtained. However, in two experiments which showed a second cycle peak damage, the M Is dropped considerably below control level after 39 hours.

Cycle time delay for both the first and second cycle after treatment with $.692 \times 10^{-2}$ M of metepa were determined (see Figure 11). In the control peas, polyploids appeared earlier than in the treated ones. In both treated and control peas, the percentage of polyploidy was determined for at least three different hours. The delay in the first cycle, due to 0.692×10^{-2} M treatment, was between 2.5 and 3.5 hours. When percent polyploidy was determined for the second cycle, it was observed that in both control and treated peas the polyploids appeared at about the same time. Thus, there was no delay during the second cycle after metepa treatment.

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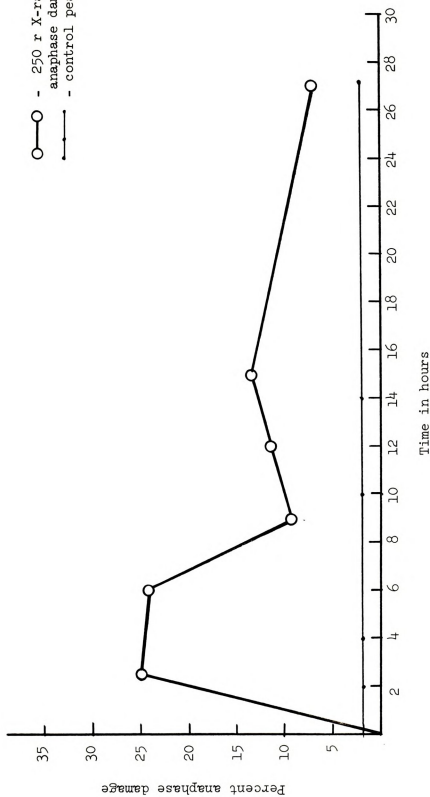


Figure 12. X-ray damage (250 r) at anaphase in percent versus time in hours in peas.

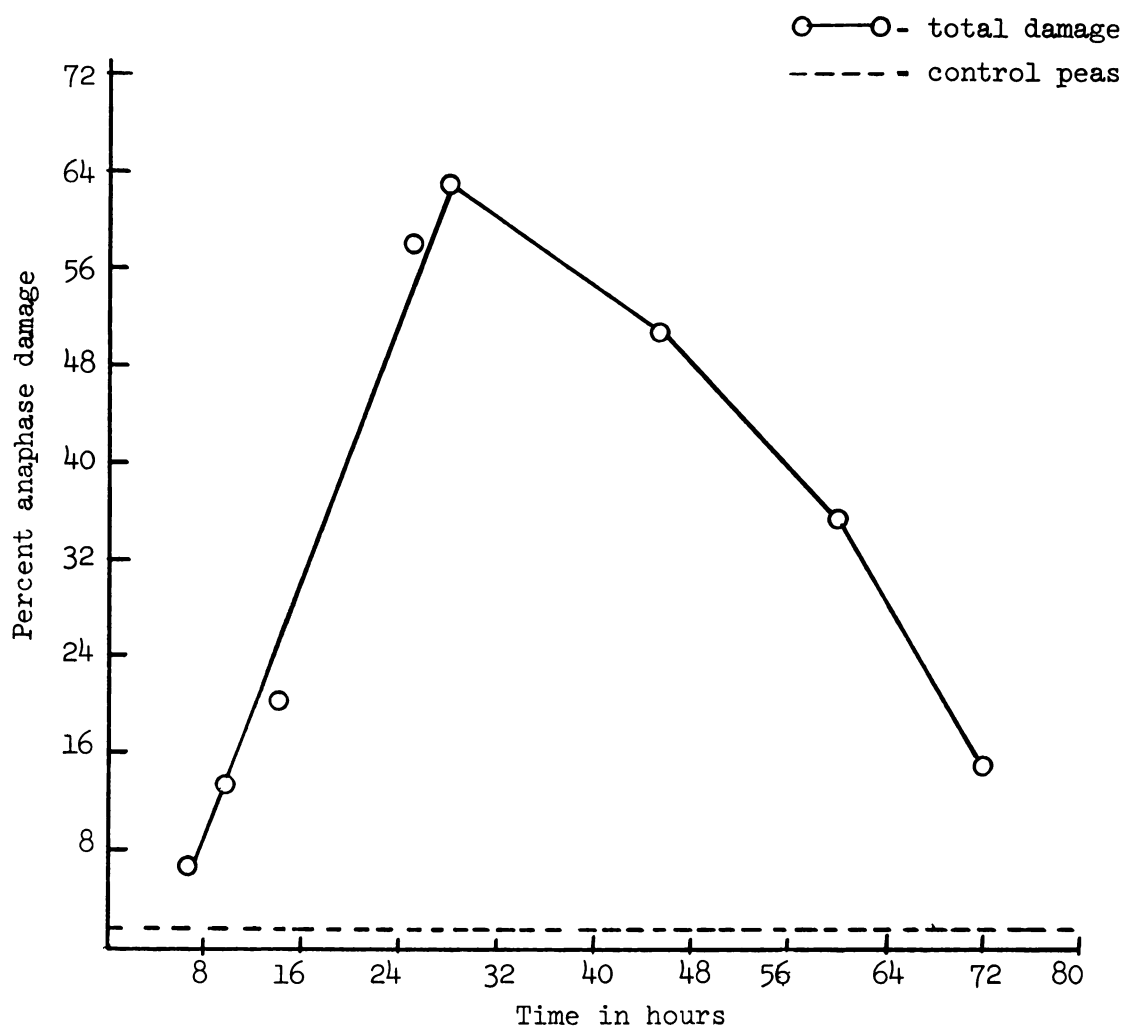


Figure 13. Total metaphase damage in Vicia, induced by 0.233×10^{-2} M metepa. % damage vs. time.

Retention of Metepa

Acetone extracts were made of the treated roots at $\frac{1}{2}$, 1, 2, 4, 8, 12, and 24 hours. These were then injected into a column in a gas chromatograph to detect the presence of metepa. It was found that within the limits of the detecting mechanism, .004 micrograms of metepa was present per root tip at 8 hours. No metepa could be detected beyond 8 hours after treatment.

X-Ray Data

250 r of X-ray was used and sampled at 3, 6, 9, 12, 15, and 27 hours. The percent corresponding damages for the above hours were respectively; 25%, 24.2%, 9.9%, 11.3%, 13.3%, and 7% (see Figure 12 and Table 9).

Vicia

Vicia primary roots were used mainly to determine the type of damage, time of first appearance, and cycle time delay. The concentration used in these studies were $.233 \times 10^{-2}$ M. The damage was scored at metaphase. The total damage at 10, 25, 28, 45, 61, and 72 hours were respectively; 13, 57, 63, 57, 33, and 15% (see Figure 13). The total damage was classified into:

1. chromatid exchanges
2. chromatid breaks
3. chromosome exchanges, and
4. chromosome breaks (see chart on page 58).

10/10/10

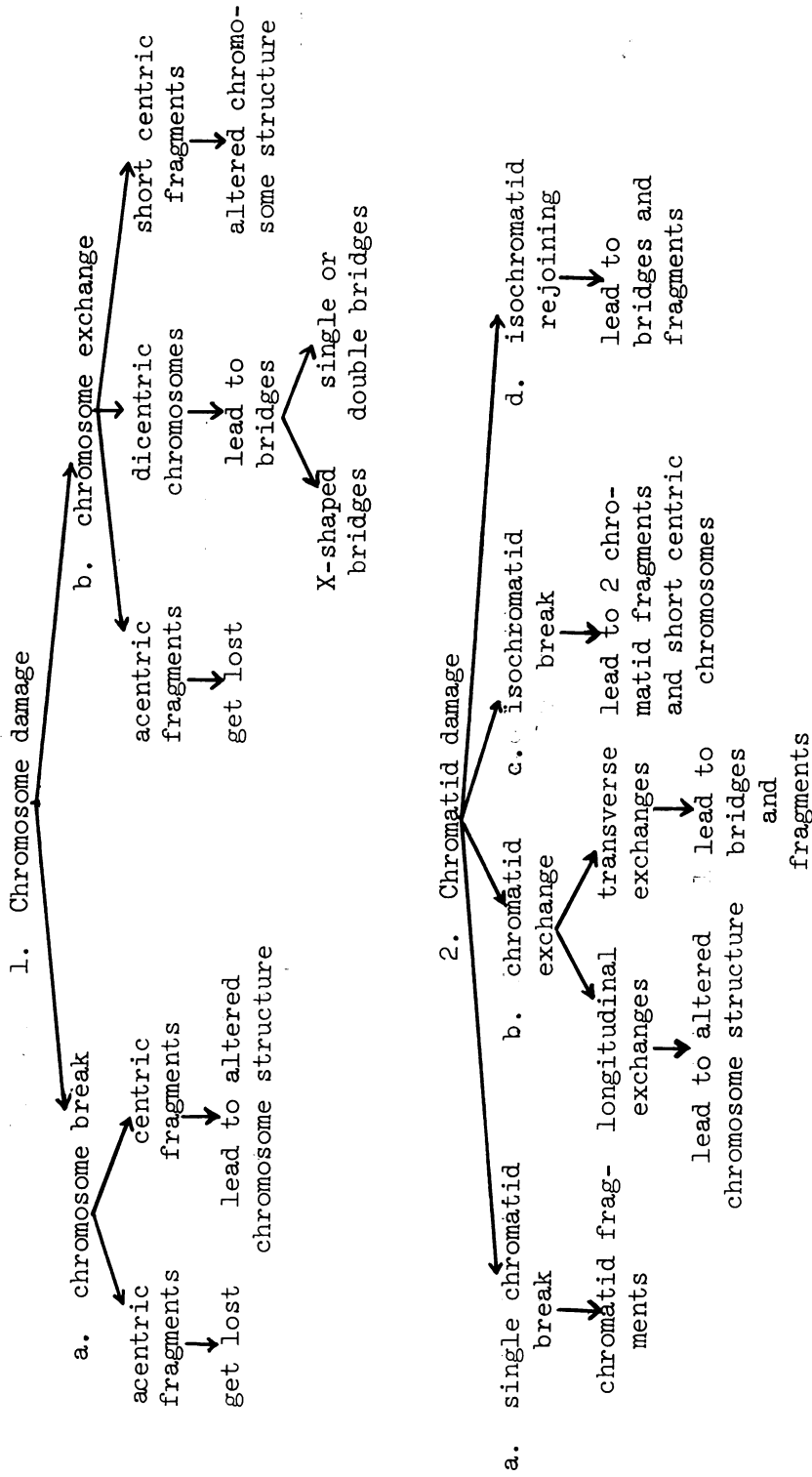
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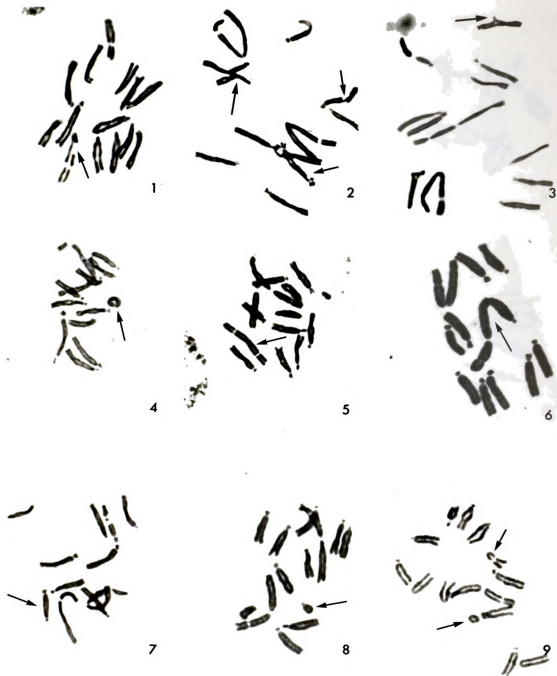
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CHART 1



Classification of chromosome and chromatid aberrations induced by ethylenimines, and their end results in subsequent cell cycles.





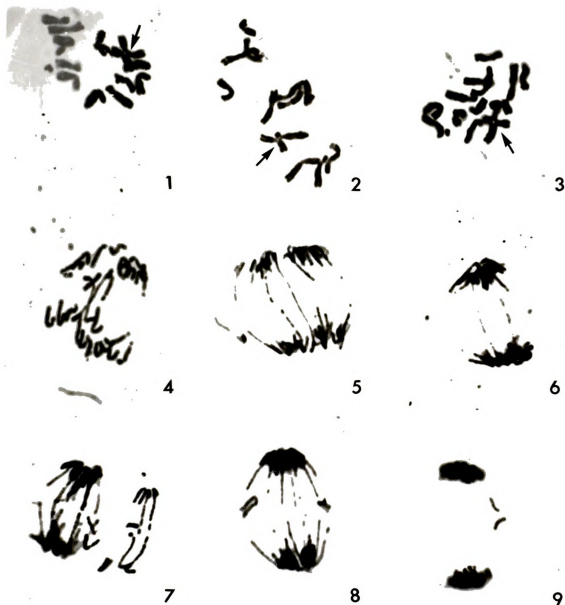
Different types of aberrations induced by metepa treatment on Vicia faba. 1, 2, and 3, chromatid breaks; 4, ring chromosome; 5, 6, and 7, chromosome exchanges and dicentrics; and 8 and 9, isochromatid rejoinings.





Aberrations induced by metepa treatment on *Vicia faba*. 1, longitudinal exchanges, involving 2 'S' chromosomes, and 8, involving the 2 'M' chromosomes, which do not lead to bridges and fragments. 2, 4, and 5, transverse exchanges, involving 2 'S' chromosomes, and 3, 6, and 7, involving one 'M' and one 'S' chromosome, which lead to bridges and fragments. 9, complex chromatid exchange, in which 3 'S' chromosomes take part.





Chromosome damage induced by tretamine and apholate on Pisum sativum. 1, 2, and 3 chromatid exchanges at metaphase, involving 2 chromosomes; 4, X-shaped bridge; 5, single bridge; 6, double bridge; 7, bridge and fragments; 8, U-shaped fragment; and 9, two fragments.



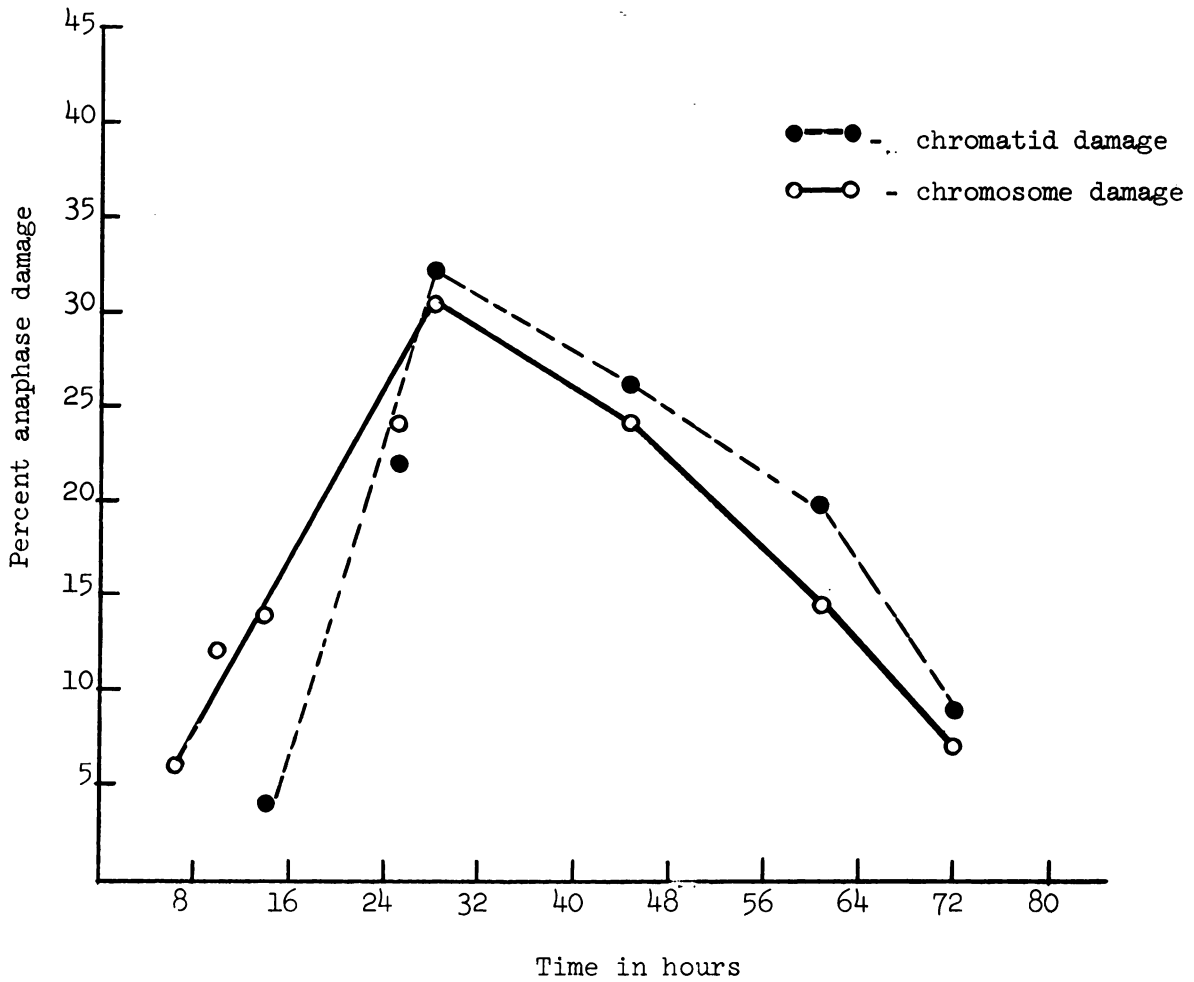


Figure 14. Chromatid and chromosome damage in Vicia at meta-phase, induced by 0.233×10^{-2} M metepa treatment. Percent damage is plotted against time. Chromosome damage appeared first, followed by chromatid damage. Both reached a peak at 28 hours, and then dropped off.

The types of damages are shown in Plates 2 to 4.

Time of Appearance

Samples were taken at 1 and 2 hour intervals after treatment, and scored for damage. The first significant damage appeared at 6 hours, (6%), and these were 100% chromosome fragments. Chromatid damage did not appear until 10 hours, and chromatid exchanges started appearing only at about 12 hours. Total chromatid breaks as well as chromosome breaks increased rapidly, attained a maximum at 28 hours, and then declined. A low level of chromatid and chromosome damage was maintained at later hours (Figure 14 and Table 10). Chromosome exchanges were not observed before 25 hours, and these showed a gradual rise, and maintained high values as late as 60 hours (19%). The total damage also showed a rapid rise, reached a maximum at 28 hours (63%). It then declined to 57% at 54 hours, and dropped off considerably at later hours (see Figure 13, and Table 10).

Cycle Time Effect

The delay in cycle time due to metepa treatment was studied using Vicia. Continuous colchicine treatment was employed, and percent ploidy was plotted against time. Only concentrations of 0.233×10^{-2} M or lower could be employed in this study. When higher concentrations were used, polyploid cells could not be obtained, indicating noncycling of the treated population of cells. When 0.233×10^{-2} M was used, polyploids did not appear in any significant number until 23 hours after treatment. In control roots, a significant number



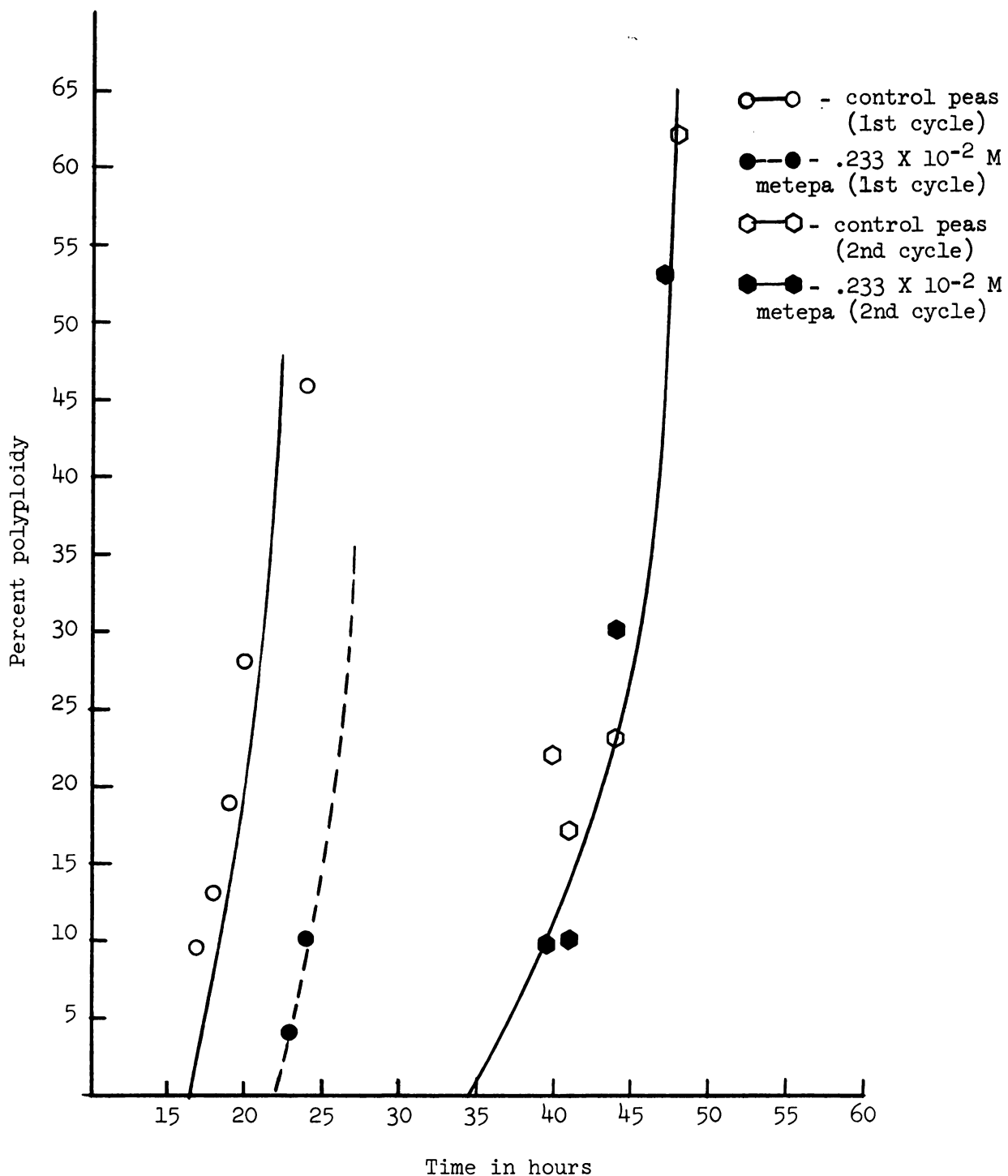


Figure 15. Percent polyploidy vs. time in *Vicia*, treated with 0.233 X 10⁻² M metepa, showing a delay of 5 to 6 hrs. for the 1st cycle, and no delay in the 2nd cycle after $\frac{1}{2}$ hr. treatment.

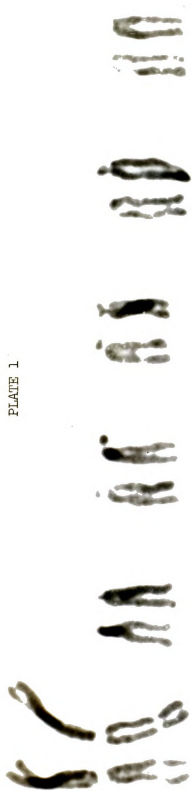


of polyploids appeared as early as 17 hours, or even earlier (see Figure 15). Thus, the cycle time delay due to metepa treatment was between 5.5 and 6.5 hours.

Cycle time delay was determined for the second cycle, as well (see Figure 15). In this case, polyploids appeared in both treated and control roots at about the same time, indicating little or no delay. The comparison of damage caused by different concentrations of the various chemicals at peak hours are shown in Table 11.



PLATE 1



1



2

This plate shows the karyotypes of:- (1) Vicia faba, and (2) Pisum sativum.



DISCUSSION

Before any fruitful discussion of the questions presented in the introduction can be attempted in the light of the results obtained, it is pertinent to present certain findings regarding the experimental system of both Vicia faba and Pisum sativum that are relevant to the present discussions.

The mitotic cycle in Vicia faba and Pisum sativum have been studied extensively both in this laboratory and at Brookhaven National Laboratory by Dr. Van't Hoff (1963 and 1966 b). The karyotypes of both these plants have been worked out in this laboratory (see Plate 1). Pisum sativum has 7 pairs of chromosomes, consisting of 2 pairs of satellited chromosomes, of which one pair has larger satellites than the other. There are three pairs with median centromeres, one pair with subterminal centromeres, and one pair with submedian centromeres.

In Vicia faba there are 6 pairs of chromosomes. One pair, the M chromosome, has a satellite and submedian centromere with a short and a long arm. The other five pairs have subterminal centromeres, and can with difficulty be distinguished from each other by the relative lengths of the shorter arms.

The following is a schematic drawing of the mitotic cycle in the pea root meristem (Van't Hoff, 1963).

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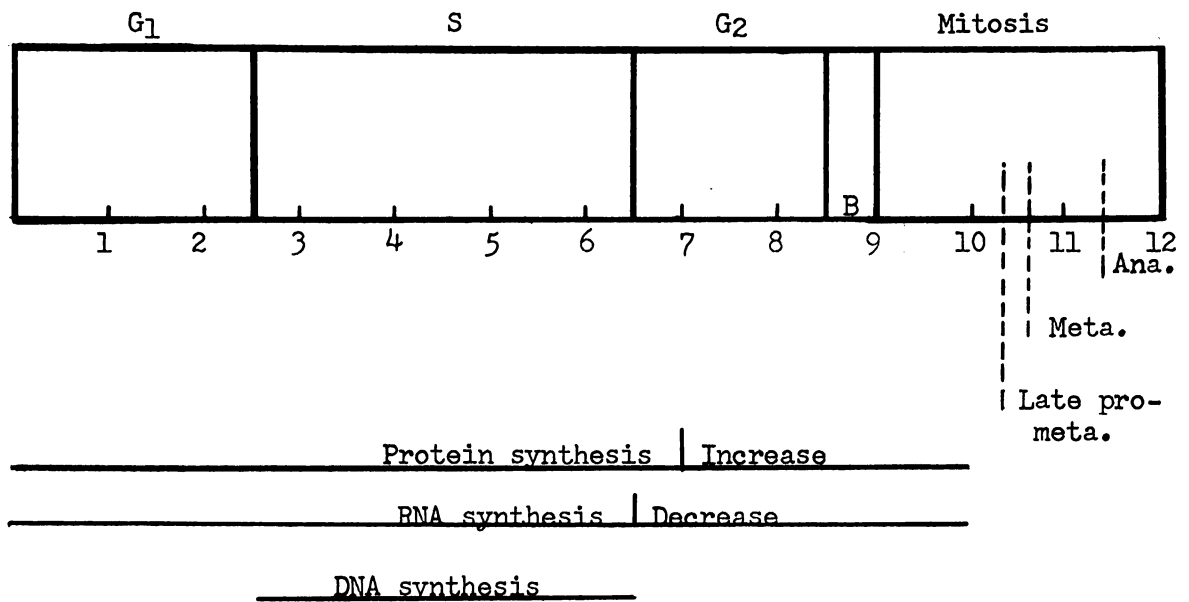


Figure 16 a. Diagram of the mitotic cycle in pea root meristems.



The cycle time durations were determined both by the continuous colchicine method (Bekken, 1966), as well as by the tag method, and also by the tritiated thymidine labelling technique. The average duration of the complete cycle is 12 hours under our conditions. Indirect studies in this laboratory, together with direct ones by Van't Hof, indicate that the bulk of DNA synthesis occurs during the middle 4 hours of interphase, and that both RNA and protein synthesis are continuous into early prophase. Active mitosis itself lasts for between 2 hours, 45 minutes to 3 hours according to a variety of estimates made in this laboratory (Wilson, 1965), of which a little more than half of this time is spent in prophase. G_1 and G_2 last for between $2\frac{1}{2}$ and 2 hours, respectively. In Vicia faba the mitotic cycle time is estimated by Van't Hof (1966) to be between 17 and 18 hours (which agrees with our findings), consisting of 9 hours for the 'S' period, 4 hours for G_1 , 3 to 5 hours for G_2 , and 1.9 hours for M.

With respect to the experiments described here, we are primarily concerned with two questions; namely, (1) time of maximum damage, and (2) persistence of effect.

Time of Maximum Damage

From the results presented earlier, it is obvious that the ethylenimines and related compounds employed in the present study show their maximum effect after a certain period of delay; i.e., only after 20 hours after treatment. If the maximum effect does not appear within the duration of time equivalent to one mitotic cycle after treat-

ment, taking into consideration also the delay caused by treatment, then it may be considered a delayed effect. In Pisum, the first mitotic cycle after treatment with apholate or metepa is delayed for about 3 hours. However, the second and subsequent cycles are not delayed to any extent. The maximum effect in Pisum appears at about 27 hours, which corresponds to two complete mitotic cycles, and hence should be considered delayed. In other experiments, however, the maximum effect appeared at 39 hours, which corresponds to three complete cycles. This delay in appearance of the maximum effect may be attributed to several different causes. Among these are the following:

1. the damaged cells may be unusually delayed in reaching division. This, however, seems unlikely, since we found a significant number of polyploid cells 23 hours after treatment in Vicia, in which chromatid exchanges were especially present. These polyploid cells were induced by colchicine after a $\frac{1}{2}$ hour treatment with metepa. Thus, the damaged cells are entering division with nondamaged cells, indicating that both kinds of cells are cycling normally, although delayed to a certain extent.

2. Delay may also have to do with the initial unit of chromosome breakage. It is generally assumed that the half-chromatid of one division is the chromatid of the next division. The one-fourth chromatid of one division is assumed to be the half-chromatid of the next, and the chromatid of two divisions hence. However, the question

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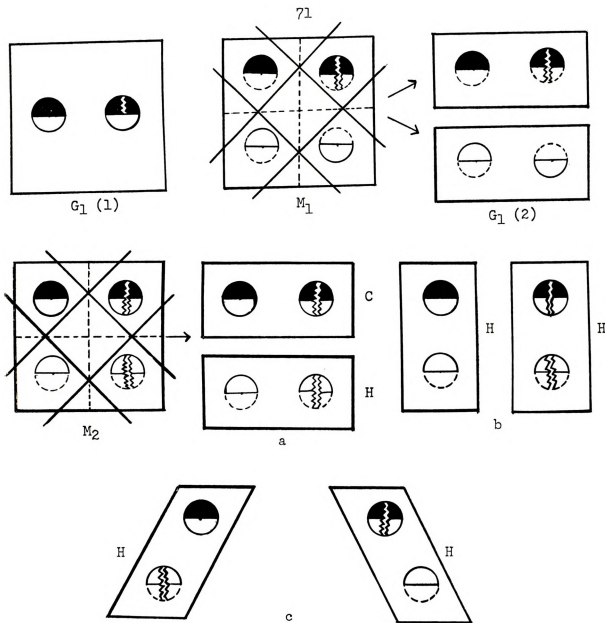


Figure 16. Half DNA break in two stranded chromosome with random distribution of potential chromatids. a = two half chromatid breaks; b = chromatid break; and c = 2 half chromatid breaks. Labelling of chromosomes is done during the 1st G_1 stage, when the breaks occur. C = cold, (nonlabelled); H = hot, (labelled) chromatids.

The assumptions made are the following: (1) the chromosome at G_1 is bipartite; (2) initial damage is to $\frac{1}{2}$ the DNA molecule, i.e., one component strand; (3) semiconservative replication of chromosomes (as shown by Taylor); and (4) half chromatids assort at random into chromatids.



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of whether or not the association of different elements in a chromatid is entirely nonrandom, has not been resolved. Marimuthu and Threlkeld (1966) presented evidence for a nonrandom assortment of chromatids in Haplopappus. Thus, if the major part of the damage is done at the subchromatid or lower level, there might be a delay before the original damage becomes a chromatid effect. This is illustrated in Figure 16. The main objections to this are:

1. The unlikelihood of breaks remaining open in order to form chromatid exchanges, and
2. the delay in appearance of some chromosome breaks which should have been deleted earlier as chromatid events. However, the possibility exists that the initial damage is not an actual break, but a point of weakness which may later become a break. Thus, this variability in appearance of the damage is rather difficult to explain.

Persistence of Effect

From the experiments described, it is obvious that the effect may be persistent for a duration of time equivalent to 8 cycles or more (95 hours), or may be nonpersistent, i.e., lasting for only 3 cycles (36 hours), and then drops to control level. It was observed in several experiments that the persistence of the effect was usually associated with the third cycle maximum effect. Mitotic index counts indicated that in the experiments showing persistent effect, the M Is

remained rather steady, or fluctuated around the mean of 60 for the duration of the experiment. In these experiments, we also obtained a second peak effect. This would indicate that the damaged cells are cyclically appearing in division. If the percent of damage is too great, due to a high concentration, then this second peak effect is not so pronounced. This strengthens the suggestion that at high doses a significantly high percent of damaged cells do not return to the cycle, whereas at optimum doses the affected cells could go on for several cycles.

In the experiments showing nonpersistent effect, which was associated with a second cycle peak, the mitotic indices were invariably low in cycles subsequent to the one that showed peak damage, suggesting a lack of dividing cells going through the cycle.

The persistence of the effect may depend on any of several of the following:

1. The persistence of the chemical within the system
2. The kinetics of the cell population in the pea root meristem
3. The number of subunits that make up the chromosome
4. The assortment of the kinetochores
5. The cycling of the cells in the system

The last point was discussed earlier. The persistence of the chemical was tested on a gas chromatograph, but there was no evidence of persistence of the chemical within the system beyond 8 hours. Gel-fant's work (1963) on mouse epidermis and Van't Hof's work (1966) on

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on the excised pea root system, suggested that any proliferative system may consist of two subpopulations. If this is so, then we could conceive of one affected population, and one nonaffected one, both cycling at different cycle times. At certain points in the cycle these cell populations would coincide, and at other times we would see either population separately. This would possibly account for a fluctuating persistent effect. Our data, however, do not support this assumption.

Persistence of effect can also be obtained by assuming a large number of strands for a chromosome. As many as 128 strands have been proposed by Ris (1961). If this is the case, then chromatid fragments will be given off for several cycles, depending on the number of strands involved in the initial breakage.

On the basis of our assumption (see Figure 16) that two strands are present in G_1 , and that random assortment of centromeres occurs, fragments would be given off indefinitely, thus giving a persistent effect. This would find support with Wu and Grants' finding in barley; that the damage is carried over into the next generation. On the other hand, if nonrandom assortment is present, fragments will be produced almost exclusively at the second cycle, and no persistent effect would be observed. Thus, the persistence of effect may be due to either the number of subunits that make up the chromosome, or the type of assortment of the centromeres, or a combination of both.

Time of Susceptibility

To find the susceptible stage of the mitotic cycle during which the aberrations are produced, the 5 amino-uracil system was employed. This enabled us to treat the cells in G_1 - S, S, G_2 , and M. The damage induced by these different treatments were compared to see whether there was any significant difference between them. Our results show that there was no significant difference between the percent of damage induced at these various treatment times. It might, however, be that there was enough of the chemical still present at the critical stage when the damage is induced to produce the maximum effect.

The results of the experiments are summarized in the tables. It was found that a significantly high amount of damage is obtained at the second cycle in all of the treatments. It is noteworthy that this high level of damage was persistent for a number of hours. This is due to the fact that in the 5 A U treatment, a second peak of M I is not obtained, but cells return over a spread of several hours, producing a somewhat smeared effect.

The following sequences of the mitotic cycle gives the different stages the cells have passed through after treatment, and before the highest damage was obtained.

Treatment Times of Apholate after the Beginning of 5 AU Treatment

7½
10
12½
15

Stages of the Cycle Cells Passed Through Before Peak Effect was Obtained

S-G₂-M-G₁-S-G₂-M
G₂-M-G₁-S-G₂-M
G₂-M-G₁-S-G₂-M
M-G₁-S-G₂-M

Table of Contents

Page

Introduction

Chapter I

Chapter II

Chapter III

Chapter IV

Chapter V

Chapter VI

Chapter VII

Chapter VIII

Chapter IX

Chapter X

Chapter XI

Chapter XII

Chapter XIII

Chapter XIV

Chapter XV

Chapter XVI

Chapter XVII

Chapter XVIII

Chapter XIX

Chapter XX

It can be seen from p. 75 and Table 5 that the significantly high effects were obtained only after the treated population passed through one complete cycle, in addition to the one during which treatment was given. This is the common factor in all of the treatments. The time of delay before this high effect is obtained also corresponds with this time period, i.e., 27-30 hours. However, a certain amount of damage was present prior to this maximum effect. This could have possibly come from cells that are cycling at a much faster rate than the normal cycle time of 12 hours. It can also be concluded that there is no significant difference in the amount of damage induced, no matter what stage of the mitotic cycle the cell population undergoing treatment is in. This observation is at variance with findings reported in the Literature Review by other workers; i.e., that the G_1 S stage is the susceptible stage. Therefore, it seems likely that there is no specific stage at which the cells are particularly susceptible to chromosome breakage.

The only stage at which we could not treat the cells with apholate with certainty was G_1 . It is extremely difficult to obtain cells precisely in G_1 using this system, so this was not attempted. Thus, G_1 may be the stage when the effect is first induced. This is suggested in the diagram showing the initial breakage to $\frac{1}{2}$ the DNA strand (see Figure 16) in G_1 . Thus, the delay in cycle time might also be caused in this segment of the mitotic cycle. The absence of a significantly higher effect with any of the treatment times may not, however, warrant this conclusion. The ethylenimines and related com-

pounds could lock onto chromosomes and cause potential breaks to be realized at a future, but variable time.

Order of Rearrangements

In Vicia where the damage was quantitatively studied at metaphase, the types of damage were classified as shown on page 58 (see Plates 2 and 3).

The data on Vicia shows that chromosome damage appeared first, followed by chromatid aberrations of various kinds (see Table 10). Both chromosome and chromatid damage increased very rapidly. The appearance of chromosome damage ahead of chromatid damage suggests that the chromosomes at the susceptible stage act as a single unit to the action of the chemical. It is interesting to note that, unlike X-rays which produce chromatid damages in G_2 , ethylenimines produce chromosome damages in G_2 . It may be that at this specific stage when ethylenimines exert their influence, the presence of a chromosome coat is responsible for the fact that they act as a single unit to chemical breakage. The breaks produced prior to these are chromatid breaks, when this chromosomal coating is not synthesized. Then they act as separate units. The data in Table 10 are compatible with this interpretation. Thus, the chromosome breaks that are first observed were produced by the above mechanism, although this does not explain the occurrence of apparent chromosome breaks at later cycles. These are probably chromatid fragments that are held over from an earlier cycle which duplicated, and gave the appearance of chromo-

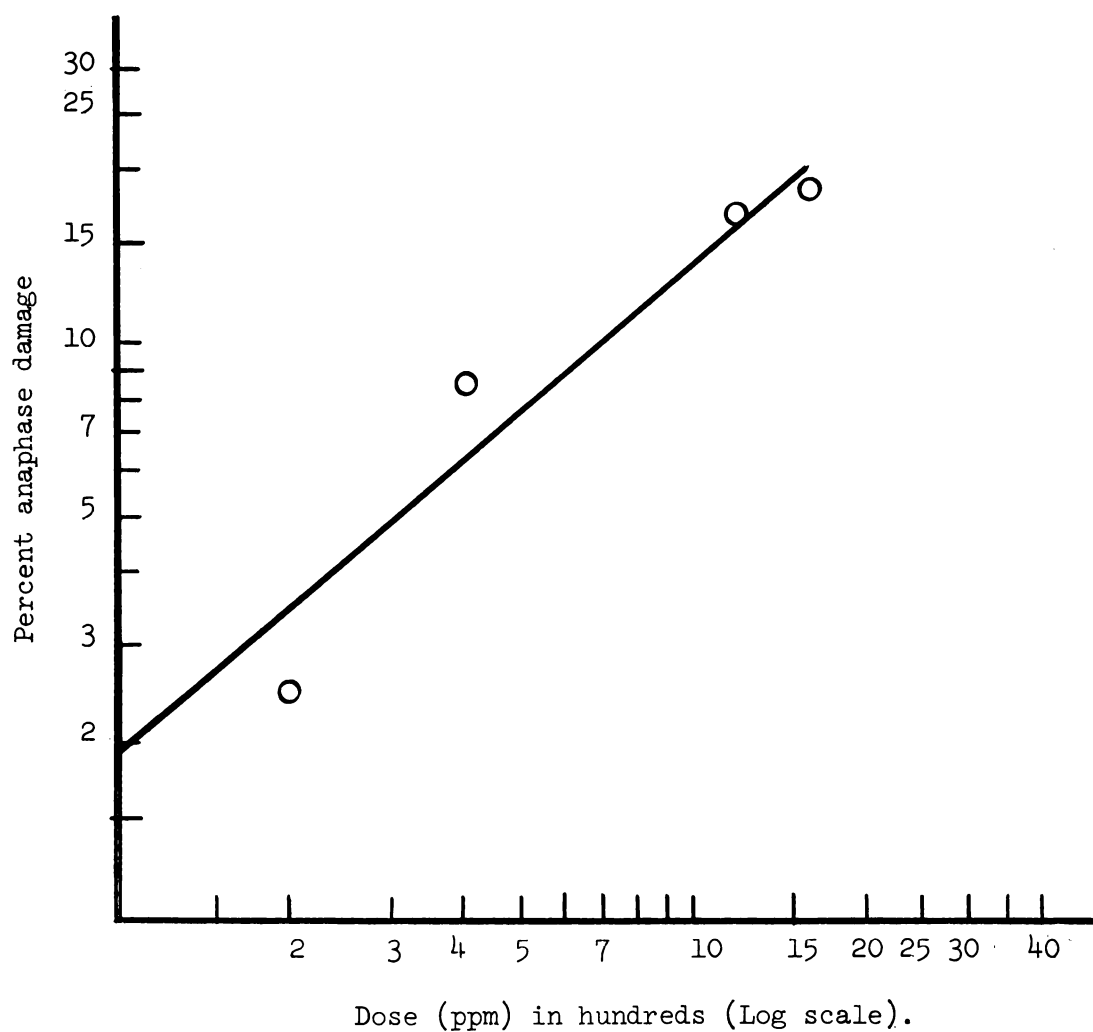


Figure 17. Dose effect curve, maximum apholate damage in percent versus dose on log scale.

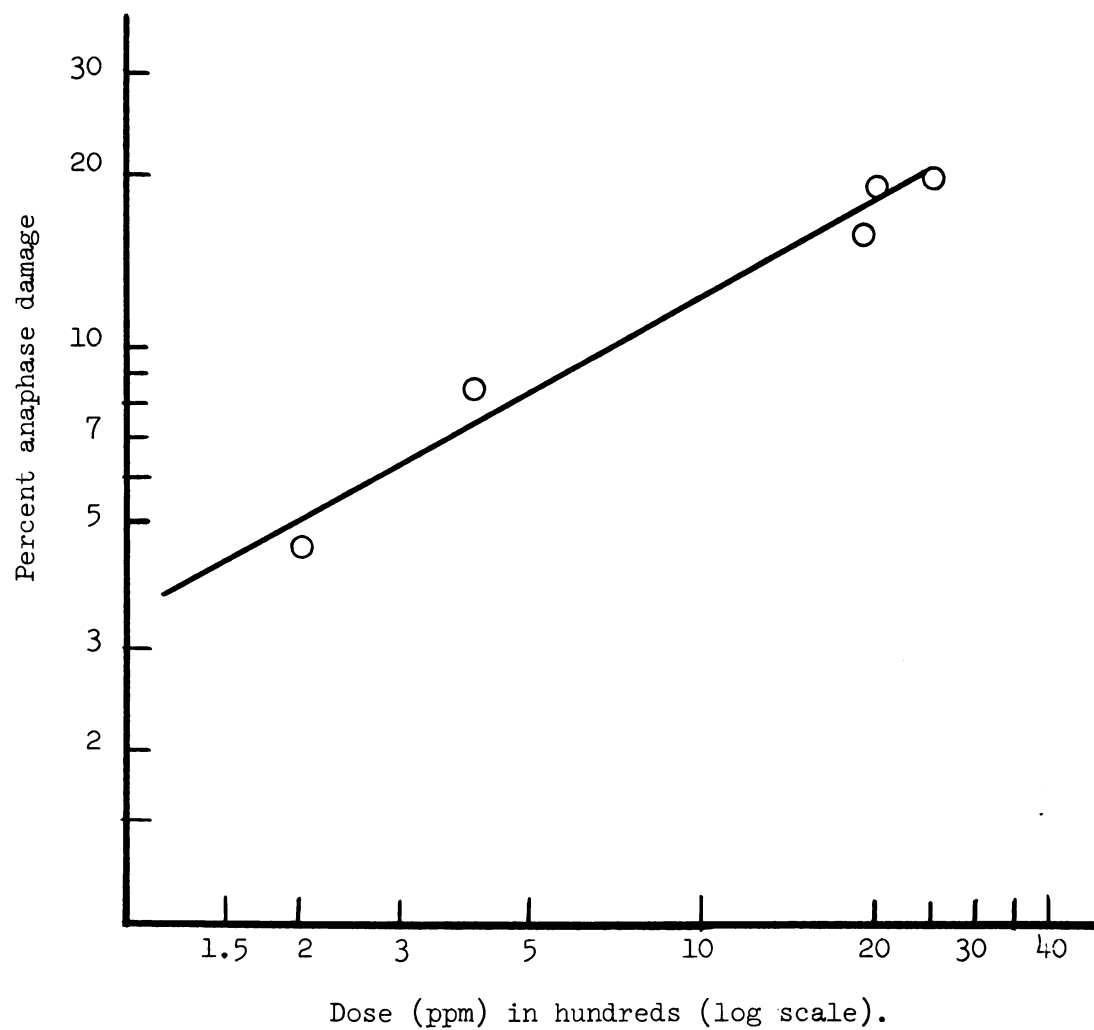


Figure 18. Metepa damage plotted against dose on log scale.

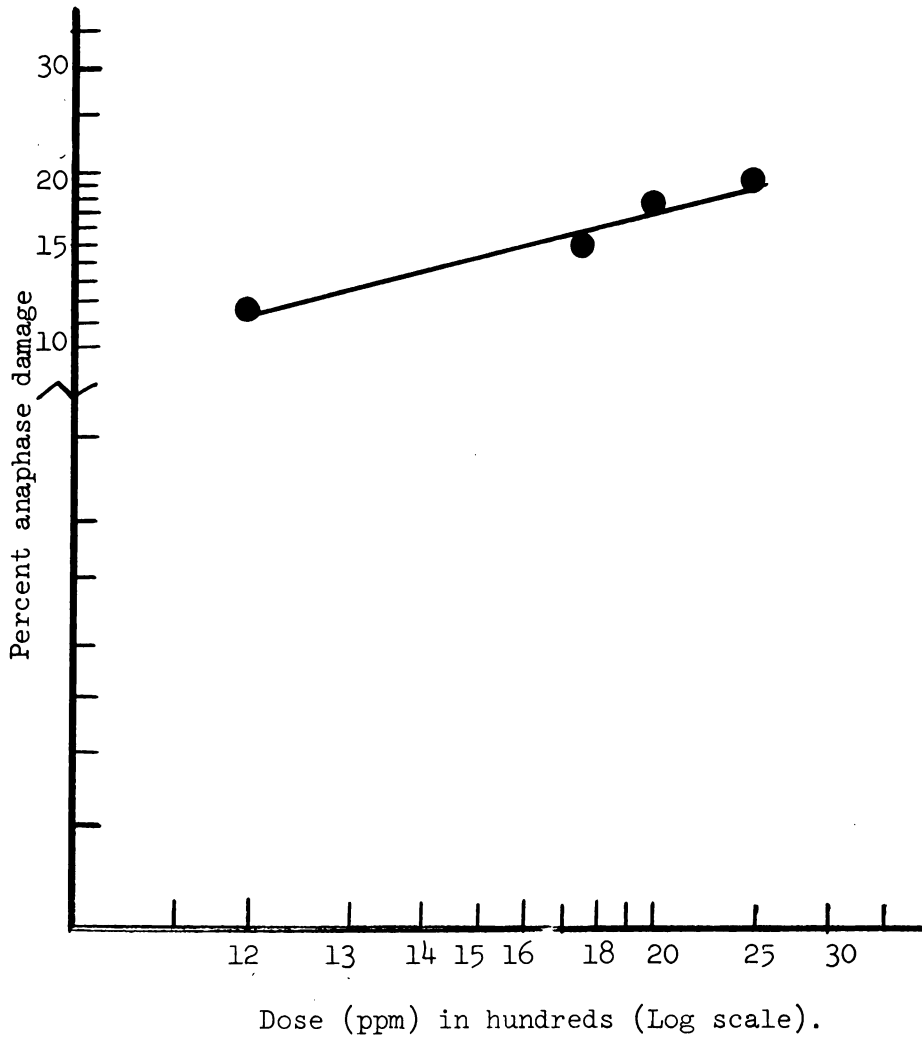


Figure 19. Dose effect curve, in which the maximum anaphase damage in percent is plotted against the corresponding dose on log scale (metepa treatments).



some breaks. Similarly, the appearance of a large number of centric fragments at later cycles may also be explained, by the same mechanism.

Comparison of Effects

A. Chromosomal aberrations

Table 11 summarizes the comparative anaphase damage induced by all of the chemicals employed. Vicia faba is far more sensitive to damage than Pisum sativum. Tretamine is the most potent in causing chromosome damage, and the next in order of potency are apholate, metepa, TEPA, azirane, ethylenesulfide, and HEMPA. Ethylenesulfide easily dissociates at the pH employed, and is noneffective. On the basis of comparisons made in terms of molar concentrations related to damage, we found that tretamine is about 6 times as effective as apholate. Apholate is about 2 to 2.5 times as effective as metepa. Azirane is only 1/17 times as effective as apholate in causing chromosomal damage. The poor chromosome-breaking ability of azirane is perhaps, due to its inability to cause cross linking of DNA, in addition to alkylation.

When the dose versus effect was plotted on Log Log paper (see Figures 17, 18, and 19) it is observed that in the case of both metepa and apholate we get a straight line, indicating the exponential nature of the curve, which is the type of effect we would expect from a chemical reaction of this nature involving a biological system. The straight line aspect of the Log Log curve in both of



these cases may also justify the assumption that both apholate and metepa have a similar mechanism of action. This is not surprising, since both are polyfunctional alkylating agents, and their reactive groups consist of the aziridinyll ring structures.

B. Mitotic Inhibition

Mitotic inhibition caused by the different chemicals was measured by means of mitotic index change after treatment. It was found that mitotic inhibition produced by tretamine and azirane is quite comparable, although the anaphase damage caused by the two were quite different. Apholate and metepa are next in order of their ability to induce mitotic inhibition. The high potency of azirane and tretamine in causing mitotic inhibition is probably due to their low molecular weight and consequent ease with which they can enter the treated cells. It is probably due to the extreme toxicity of azirane, (which is a monofunctional alkylating agent,) that its chromosome-breaking ability is not so pronounced as that of the polyfunctional alkylating agents.

C. Cycle Time Delay

In peas, the first cycle was delayed for $2\frac{1}{2}$ to 3 hours, with $\frac{1}{2}$ hour apholate treatment. In Vicia, metepa caused a delay of 6 to 7 hours in the first cycle. The second cycle was not delayed in either Vicia or Pisum to any appreciable extent. It is suggested that this delay in the first cycle is caused in the G_1 stage of interphase owing to the actual presence of the chemical within the system.

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D. Mutagenic Ability

In comparing the mutagenic ability of HEMPA and apholate, it is observed that 1% solution of both chemicals used for 1 minute on Drosophila larvae showed 3.9% lethal mutations in the X chromosome with apholate and only 1.9% in the case of HEMPA. This is not surprising when we consider the molecular structure of both the chemicals. Apholate is a polyfunctional alkylating agent with a very reactive aziridynl ring structure. In HEMPA this ring structure does not exist, since the CH₂ group in the structure is replaced by CH₃ groups which are far less reactive. This would also account for the difference in chromosome-breaking ability of these two chemicals.

E. Comparison with X-ray Damage

When we compare the damage caused by ethylenimines to X-ray induced damage, it soon becomes apparent that the term "radiomimetic" as applied to this group of chemicals loses its significance. The only similarity between the two types of damage is that both cause chromosomal aberrations, but the time of peak effects, the time of first appearance and the kinds and persistence of damage induced, are very different in the two cases. Probably the conditions affecting the production of damage and the susceptible stages of damage are also quite different. Wilson and Sparrow (1960) showed that irradiation of early interphase nuclei produces chromosomal aberrations, late interphase produces chromatid aberrations, and late prophase induces subchromatid aberrations, indicating the four-stranded condition of

the chromosome. It has already been pointed out that this type of sequence is not found when these chemicals were used. So the peak effects due to X-ray treatment is obtained in the immediate mitosis following treatment. In our comparison to X-ray damage, about 250 r of X-ray treatment is comparable to 2500 ppm of apholate, and 3,000 ppm of metepa in causing chromosome damage (see Figure 12).

SUMMARY

Ethylenimines and related compounds were employed in the present study to determine the amount and type of damage induced on Pisum sativum and Vicia faba chromosomes.

It was observed that these chemicals produce two main types of effects. These are primarily concerned with time of maximum damage and persistence of damage. Using apholate and metepa on Pisum, a maximum damage did not appear until a delay equal to at least one or two complete mitotic cycles; the average mitotic cycle time being 12 hours. The delay could be caused by unusual delay in treated cells entering division, or due to the initial unit of breakage. There are, however, some objections to these explanations. The time of susceptibility was tested using the 5 A U system. We are forced to conclude from the results that there is no particular stage which is especially susceptible to these chemicals.

Cycle time delay caused by treatment was determined for the first and second cycle after treatment in both Pisum and Vicia. It was found that the first cycle in Vicia was delayed approximately 6 to 7 hours, and in Pisum this delay was approximately 3 hours. In both Vicia and Pisum the second and subsequent cycles after treatment were not delayed to any extent.

Mutagenic ability of both apholate and HEMPA was tested on the Muller 5 system. Both were mutagenic, although HEMPA does not



contain the aziridinyl ring structure, and did not cause any observable aberrations in Pisum.

The effect of these chemicals are different from X-ray damage in the type of aberrations produced, the susceptible stage of damage, and the time of peak damage.

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APPENDIX



Table 1. Anaphase damage in percent and mitotic indices at various hours in Pisum, induced by azirane: Ex. 6 - control (no treatment); Ex. 7 - 1.22×10^{-2} M azirane; Ex. 8 - 1.83×10^{-2} M azirane.

M I T O T I C I N D E X									
F R A G M E N T S									
Slide #									
Hrs.	1	2	3	4	5	Frag. %	1	2	Av. M. I.
Ex. 6	15	-	-	-	-	-	58	24	59.5
	27	1.5	1	-	-	1.3%	79	67	72
	39	-	-	-	-	-	-	-	-
	51	-	-	-	-	-	-	-	-
	63	-	-	-	-	-	-	-	-
Ex. 7	15	-	-	-	1.1	1.1%	3	8	14
	27	2.2	8.2	5.5	-	5.3%	74	94	84
	39	3.6	0.5	7.3	0.8	3.6%	72	73	71
	51	2.2	-	-	-	2.2%	29	5	18
	63	2.3	-	-	-	2.3%	18	38	28
Ex. 8	15	1	-	-	1	1.0%	25	1	13
	27	4.7	4.8	5.8	-	5.1%	60	82	62
	39	7.8	6.2	5.1	-	6.4%	30	10	20
	51	1.1	-	-	-	1.1%	23	36	20
	63	0.5	1	-	-	0.8%	3	36	19

Table 2. Anaphase damage in percent, induced by 3.1×10^{-2} M of apholate in Pisum.

FD 19						
FRAGMENTS						
Hrs.	Slide #					Frag. %
	1	2	3	4	5	
11	3.7	1.1	-	2.1	-	4.5%
12	-	-	-	-	-	
13	4.2	-	11.6	-	-	
23	-	23.1	-	-	18.3	18.0%
24	-	-	-	-	15.3	
25	26.1	-	7.5	-	-	
35	23.8	31.4	17.3	-	-	27.6%
36	22	34.5	-	-	-	
37	25	39.7	-	-	-	
47	31.5	22.4	-	-	-	23.4%
48	34.6	-	-	-	-	
49	14.8	13.8	-	-	-	
59	31.3	-	-	34	-	26.2%
60	14.5	-	-	-	-	
61	22.6	28.4	-	-	-	
71	14.5	14	-	-	-	16.6%
72	22.2	15.1	-	-	-	
73	17.2	-	-	-	-	
83	12.8	11	-	-	-	16.9%
84	10.2	-	-	-	-	
85	32.3	14.7	9.5	27.8	-	
95	8.2	37.8	11.1	7.2	12.2	15.3%



Table 3. Anaphase damage in percent and mitotic indices at various hours in Pisum,
treated with 3.1×10^{-3} M apholate.

FD 42

<u>F R A G M E N T S</u>						<u>M I T O T I C I N D E X</u>						
Hrs.	<u>Slide #</u>					Frag. %	<u>Slide #</u>					Av. M. I.
	1	2	3	4	5		1	2	3	4	5	
14	1	1	-	-	-	1%	50	60	40	-	-	50
24	14	16	12	18	-	15%	60	45	55	-	-	52
38	7	6	9.5	-	-	7.5%	35	28	-	-	33	32
48	4	-	-	8	12	6%	-	-	20	22	40	26
61	5	-	-	4	6	5%	25	-	-	30	23	21



Table 4. Maximum anaphase damage in percent, at peak hours in Pisum induced by different concentrations of apholate: FD 24 - control (no treatment); FD 25 - 4.1×10^{-5} M apholate treatment; FD 26 - 2.1×10^{-3} M apholate treatment; FD 27 - 1.03×10^{-3} M apholate treatment; FD 28 - 0.52×10^{-3} M apholate treatment; FD 29 - 0.26×10^{-3} M apholate treatment; and FD 30 - 0.13×10^{-3} M apholate treatment.

<u>FRAGMENTS</u>						
		<u>Slide #</u>				
	<u>Hrs.</u>	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>Frag. %</u>
FD 24	35	6.0	2	3.6	-	2.1%
	36	0.5	-	-	-	
	37	0.5	-	-	-	
FD 25	35	18.5	32	-	-	18.4%
	36	29.5	18	12.6	15.4	
	37	20.3	27.6	15.6	6.1	
	38	17	19.6	9.8	14.2	
FD 26	35	8.5	-	-	-	11.6%
	36	10.5	14.8	10	13.9	
	37	8.3	18.8	15.3	3.4	
FD 27	35	11	13.2	10.8	-	8.6%
	36	5.8	-	6.8	-	
	37	6.6	6	9.4	-	
FD 28	35	6	-	-	-	7.9%
	36	5.4	14.3	4.0	-	
	37	8.7	-	-	-	
	38	9.2	-	-	-	
FD 29	35	5.1	-	3	-	4.5%
	36	5.3	-	4.3	3.3	
	37	-	4.5	-	-	
	38	-	4	-	6.5	
FD 30	35	-	2	1	-	1.9%
	36	-	1.5	-	-	
	37	-	2	-	-	



Table 5 a. Anaphase damage and mitotic indices at various hours in Pisum. FD 47-control.

FD 47

F R A G M E N T S										M I T O T I C I N D E X				
		<u>Slide #</u>					<u>Slide #</u>							
Hrs.	1	2	3	4	5	Frag. %	1	2	3	4	5	Av. M. I.		
7 $\frac{1}{2}$	2	1	-	-	-	1.5%	70	70	50	-	-	63		
12 $\frac{1}{2}$	1	1	-	-	-	1%	56	68	77	78	76	71		
15	-	-	-	-	-	-	-	-	-	-	-	-		
16	-	-	-	-	-	-	-	-	-	-	-	-		
17	-	-	-	-	-	-	-	-	-	-	-	-		
18	-	-	-	-	-	-	-	-	-	-	-	-		
19	2	1	1	-	-	1%	65	72	-	-	-	68		
20	-	-	-	-	-	-	-	-	-	-	-	-		
21	-	-	-	-	-	-	-	-	-	-	-	-		
22	-	-	-	-	-	-	-	-	-	-	-	-		
23	-	-	-	-	-	-	-	-	-	-	-	-		
24	2	2	-	1	-	1.6%	50	70	60	-	-	60		
25	-	-	-	-	-	-	-	-	-	-	-	-		
26	-	-	-	-	-	-	-	-	-	-	-	-		
27	-	-	-	-	-	-	-	-	-	-	-	-		
32	2	2	1	-	-	1.6%	65	70	86	-	-	73.7		
34	-	-	-	-	-	-	-	-	-	-	-	-		
36	-	-	-	-	-	-	-	-	-	-	-	-		
39	-	-	-	-	-	-	-	-	-	-	-	-		
44	-	-	-	-	-	-	-	-	-	-	-	-		
51	-	2	2	2	-	2%	62	53	55	-	-	50		

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Figure 5 b. Anaphase damage in percent and mitotic indices in *Pisum* treated with 100 ppm. of 5 A U for 8 hours. FD 48 - 5 A U control

FD 48

F R A G M E N T S

M I T O T I C I N D E X

Hrs.	<u>Slide #</u>					Frag. %	<u>Slide #</u>					Av. M. I.
	1	2	3	4	5		1	2	3	4	5	
7 $\frac{1}{2}$	-	-	-	-	-	-	6	7	11	20	27	14.2
12 $\frac{1}{2}$	-	-	-	-	-	-	32	37	54	38	41	40.1
15	-	-	-	-	-	-	131	93	74	99	214	122
16	0.5	1	2.5	-	-	1.3%	215	230	180	140	130	179
17	-	-	-	-	-	-	130	136	173	84	97	123
18	-	-	-	-	-	-	84	75	-	-	-	79
19	-	-	-	-	-	-	25	63	69	64	-	55
20	-	-	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	99	60	-	-	-	80
23	-	-	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	42	80	-	-	-	61
26	-	-	-	-	-	-	60	90	94	-	-	81.3
27	-	-	-	-	-	-	72	72	96	123	129	98
32	-	-	-	-	-	-	81	80	39	-	-	67
34	-	-	-	-	-	-	22	23	-	-	-	23
36	-	-	-	-	-	-	-	-	-	-	-	-
39	-	-	-	-	-	-	-	-	-	-	-	-
44	-	-	-	-	-	-	55	78	-	-	-	67
51	-	-	-	-	-	-	40	54	75	60	-	57

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Figure 5 c. Anaphase damage in percent and mitotic indices in *Pisum*, treated first with 5 A U for 7½ hours and immediately transferred to 3.1 X 10⁻³ M apholate solution. FD 49 - treated with apholate at end of G₁, and in S.

FD 49	F R A G M E N T S					M I T O T I C I N D E X					Av. M. I.	
	Slide #					Slide #						
Hrs.	1	2	3	4	5	Frag. %	1	2	3	4	5	
7½	-	-	-	-	-	-	11	13	21	15	11	14
12½	-	-	-	-	-	-	26	43	11	73	-	38
15	3	-	6	5.5	-	5%	90	61	97	71	-	80
16	3.5	5	7.5	7	-	6%	91	104	125	170	-	123
17	5	2	3	-	-	3%	101	105	105	-	-	104
18	6.5	3.6	7	7.5	-	6%	113	82	91	108	-	99
19	-	1.1	1	3	-	2%	51	77	109	66	-	76
20	2.5	1.5	1.3	1.5	-	2%	72	74	71	63	-	70
21	2.7	2.5	3.3	5	-	3%	72	75	44	29	-	55
22	3.9	3	-	-	-	3%	-	58	41	-	-	50
23	-	-	-	-	-	-	28	62	17	1	1	22
24	-	-	4	2	4.5	4%	45	24	46	77	58	50
26	-	-	-	-	-	-	40	44	44	71	-	39
27	13.5	4.5	6.8	16	-	10%	-	66	82	40	63	57
32	-	-	-	9	-	9%	32	66	83	-	-	60
34	13	16.6	8	-	-	12.5%	59	78	83	-	-	73
36	13	-	-	-	-	13%	56	30	-	-	-	43
39	15	-	-	-	-	15%	32	-	32	-	-	32
51	11	-	-	-	-	11%	40	72	45	-	70	57



Table 5 d. Anaphase damage and mitotic indices in Pisum treated with 5 A U for 8 hours and then transferred to 3.1×10^{-3} M apholate solution at $12\frac{1}{2}$ hours. FD 50
- treated with apholate at S and G_2 .

FD 50

F R A G M E N T S						M I T O T I C I N D E X						
Slide #						Slide #						
Hrs.	1	2	3	4	5	Frag. %	1	2	3	4	5	Av. M. I.
7½	-	-	-	-	-	-	21	14	13	24	17	18
12½	-	-	-	-	-	-	28	26	26	30	-	28
15	1	1.5	1	-	-	1.1%	201	61	205	202	84	110
16	4.5	1	-	-	-	2.7%	174	89	182	168	-	153
17	0.7	2	-	-	-	1.3%	172	136	132	122	-	141
18	2	2	-	-	-	2%	113	107	59	111	-	98
19	-	-	-	-	-	-	46	27	37	93	44	49
20	0.5	0.5	1	1	-	0.75%	60	50	91	97	79	75
21	0.9	1	0.7	-	-	0.9%	44	56	53	-	51	.51
22	1.2	0.37	-	-	-	0.78%	-	22	45	26	-	31
24	4.6	4	5.5	-	-	5%	73	30	43	57	64	53
26	-	-	-	-	-	-	-	44	55	-	-	50
27	12.5	2	14.5	-	-	10%	36	44	24	39	-	36
32	14.5	12	4	-	-	10%	29	36	58	58	45	45
34	12	-	10.5	-	-	11%	125	-	75	-	-	100
36	11.5	-	11.5	-	-	11.5%	67	26	-	-	-	47%
39	14	12	-	-	-	13%	64	55	91	69	-	70
51	11	11.5	11	9	8	10%	88	48	65	46	54	60

Table 5 e. Anaphase damage in percent and mitotic indices in *Pisum* treated with 5 A U for 8 hours and with 3.1×10^{-2} M apholate at 15 hours. FD 53 - treated with apholate during active mitosis.

MITOTIC INDEX												
FRAGMENTS						Slide #						
Hrs.	1	2	3	4	5	Frag. %	1	2	3	4	5	Av. M. I.
8	-	-	-	-	-	-	1	1	3	1	-	1.5
12	-	-	-	-	-	-	2	1	1	2	-	1
15	-	-	-	1	2	1.5%	260	275	285	298	211	265.8
16	2	1	1	-	-	1.0%	232	260	194	131	125	188.4
17	-	-	-	-	0.3	0.3%	122	122	128	180	375	185.4
30	3.8	15.3	8.5	7.1	2.8	5.8%	73	55	67	53	68	63.2
31	10	5.5	14	8.3	18	11.7%	43	47	39	60	55	48.8
32	10.8	10.5	10.2	9	7.5	9.8%	59	29	34	72	45	48
42	11.6	9.3	-	13.2	5.2	10.2%	44	44	44	46	45	44
44	-	-	-	-	-	-	31	21	-	-	-	23
54	16.7	-	-	-	-	16.7%	16	8	7	5	1	9
55	10.4	-	-	-	-	10.4%	21	24	5	5	-	13.8
56	-	-	-	-	-	-	20	5	-	-	-	12.5
66	12.5	-	-	-	-	12.5%	10	3	8	9	11	5



Table 5 f. Anaphase damage in percent and mitotic indices in Pisum treated with 5 A U for 8 hours and with 3.1×10^{-2} M apholate at $10\frac{1}{2}$ hours. FD 56-apholate treatment during 'S' stage.

FD 56

FRAGMENTS

M I T O T I C I N D E X

Hrs.	1	2	3	4	5	Frag. %	1	2	3	4	5	Av. M. I.
8	-	-	-	-	-	-	3	5	5	9	6	5.6
10	-	-	-	-	-	-	4	4	-	-	-	4
12	-	-	-	-	-	-	9	2	-	-	-	5.5
15	2.4	0.5	1.7	1.6	3	1.8%	267	213	192	179	184	207
16	-	-	-	-	-	-	160	174	-	-	-	167
17	3.5	1.8	3	-	-	2.8%	118	170	130	-	-	139.3
19	6.3	2.8	1.8	1	-	2.9%	27	56	31	58	-	43
21	5	5.3	-	-	-	5.2%	51	66	-	-	-	58.5
24	-	-	6.2	5	-	5.7%	-	12	51	22	-	18.3
26	8.9	-	-	-	-	8.9%	35	46	41	38	-	40
27	-	12.1	7.1	8.7	-	9.7%	28	78	38	70	-	53.5
30	-	-	-	14.1	13.2	13.6%	57	46	39	46	30	43.6
32	-	15.3	13.1	10.4	-	12.9%	13	67	91	-	-	57
39	15.8	12.1	9.5	-	-	12.8%	53	78	20	-	-	50.3
42	12.5	13	-	-	-	12.8%	56	59	20	31	44	42
51	10.3	7.5	-	-	-	8.9%	25	27	23	-	18	23



Table 6. Anaphase damage in percent and mitotic indices at various hours in Pisum, treated with different concentrations of metepa: EU 73 - control (no treatment); EU 74 - 0.465×10^{-2} M metepa treatment; EU 75 - 0.697×10^{-2} M metepa treatment; EU 76 - 1.4×10^{-2} M metepa treatment.

F R A G M E N T S													M I T O T I C I N D E X												
Slide #													Slide #												
Hrs.		1	2	3	4	5	Frag. %		1	2	3	4	5	Av. M. I.											
EU 73	0	1.5	1	-	-	-	1.3%		53.5	-	-	-	-	-	53.5										
	12	-	-	-	-	-	2.0%		77	47	96	-	-	-	76										
	27	2.0	1.5	3	1.5	-			-	-	-	-	-	-	-										
	41	-	-	-	-	-			-	-	-	-	-	-	-										
	52	-	-	-	-	-			-	-	-	-	-	-	-										
	64	-	-	-	-	-			-	-	-	-	-	-	-										
	76	2	1	-	-	-	1.5%		57	48	-	-	-	-	53										
	88	-	-	-	-	-			-	-	-	-	-	-	-										
100	-	-	-	-	-			-	-	-	-	-	-	-											
112	-	-	-	-	-			-	-	-	-	-	-	-											
EU 74	0	-	-	-	-	-			44	54	-	-	-	-	46										
	12	2	3	-	-	-	2.5%		39	68	-	-	-	-	53.5										
	27	4.5	6	-	-	-	5.3%		56	-	-	-	-	-	56										
	41	7.2	6	4	5.3	-	5.7%		30	47	-	-	-	-	38.5										
	52	5	7.5	3.5	-	-	5.0%		55	52	-	-	-	-	53.2										
	64	3	3	2	-	-	2.7%		40	45	-	-	-	-	42.5										
	76	5	5.5	3.5	-	-	4.7%		85	87	-	-	-	-	86										
	88	2.8	12	2	8	-	6.0%		58	63	-	-	-	-	60.5										
	100	3	2.5	1	-	-	2.2%		53	70	-	-	-	-	61										
	112	2.5	1.5	1	-	-	1.7%		37	45	-	-	-	-	41										

Table 7. Anaphase damage in percent and mitotic indices at various hours in Pisum, treated with 4 different concentrations of metepa; FG 37 - control (no treatment); FG 38 - 0.58×10^{-2} M metepa treatment; FG 39 - 0.79×10^{-2} M metepa treatment; FG 40 - 0.93×10^{-2} M metepa treatment; and FG 41 - 1.16×10^{-2} M metepa treatment

F R A G M E N T S						M I T O T I C I N D E X						
						Slide #						
Hrs.	1	2	3	4	5	Frag. %	1	2	3	4	5	Av. M. I.
FG 37	15	2	1.4	3.5	-	-	78	70	-	-	-	74
	27	2	0	6	-	2.3%	72	64	-	-	-	69
	39	-	-	-	-	2.7%	48	82	-	-	-	65
	51	-	-	-	-	-	41	59	40	37	-	44.1
	75	-	-	-	-	-	13	20	-	-	-	16.5
FG 38	15	8	8.5	8.4	-	8.3%	48	46	-	-	-	47
	27	12.5	14	11.5	9	11.8%	69	61	-	-	-	65
	39	4.5	8	3.5	5.5	5.8%	32	30	-	-	-	31
	51	1.3	2.5	3	-	2.3%	27	18	-	-	-	22.5
	63	.6	3.8	1.3	-	2.7%	31	34	16	-	-	27
	75	-	-	-	-	-	18	18	-	-	-	18

Table 7. Continued

FG 39	15	6.5	7	8.6	3	-	6.3%	33	59	47	-	46
	27	13.5	17.5	16.1	-	-	15.6%	77	-	-	77	77
	39	8.5	6	7	-	-	7.2%	29	-	54	-	31
	51	-	-	-	-	-	-	7	8	15	-	11.5
	63	-	-	-	-	-	-	21	9	-	-	8
	75	-	-	-	-	-	-	6	1	-	-	14
FG 40	15	13.5	11	9	-	-	11.2%	57	48	-	-	52.5
	27	20	16	12	17.5	-	16.4%	66	54	-	-	60
	39	6	5.5	11.5	12.4	-	8.9%	-	-	63	74	68.5
	51	-	-	-	-	-	-	-	7	27	10	22
	63	-	-	-	-	-	-	-	17	19	-	18
	75	-	-	-	-	-	-	-	8	11	-	9.5
FG 41	15	17	12.4	13.4	9.4	-	13.1%	27	-	35	-	31
	27	12	32.5	16.5	17	-	19.5%	15	46	54	-	57.5
	39	8.6	7.6	8.6	-	-	8.2%	-	59	49	-	54
	51	-	-	-	-	-	-	9	21	4	-	17
	63	-	-	-	-	-	-	8	15	-	-	11.5
	75	-	-	-	-	-	-	62	66	-	-	64





Table 8. Anaphase damage in percent and mitotic indices at various hours after treatment with different concentrations of metepa in Pisum: FG 50 - control (no treatment); FG 51 - 0.58×10^{-2} M metepa treatment; FG 52 - $.697 \times 10^{-2}$ M metepa treatment; FG 53 - 0.79×10^{-2} M metepa treatment; and FG 54 - 0.93×10^{-2} M metepa treatment; and FG 55 - 1.16×10^{-2} M metepa treatment.

F R A G M E N T S										M I T O T I C I N D E X									
Hrs.	1	2	Slide #		3	4	5	Frag. %	1	2	Slide #		3	4	5	Av. M. I.			
			1	2							1	2							
FG 50	15	1	2	-	-	-	-	1.0%	44	-	-	-	-	-	-	44			
	27	1	2	-	-	-	-	1.0%	50	60	-	-	-	-	-	55			
	39	-	-	-	-	-	-	-	9	17	-	-	-	-	-	13			
	51	-	-	-	-	-	-	-	27	19	-	-	-	-	-	23			
	63	-	-	-	-	-	-	-	10	15	-	-	-	-	-	12.5			
FG 51	15	8.5	11.9	7	-	-	-	8.9%	49	78	69	-	-	-	-	65.1			
	27	11	9.1	10	-	-	-	10.0%	58	73	-	-	-	-	-	65.5			
	39	3.5	6	4	-	-	-	4.5%	57	37	-	-	-	-	-	47			
FG 52	15	10.5	7	-	-	-	-	9.5%	64	24	27	-	-	-	-	39			
	27	12	9.5	11.2	10.5	-	-	10.8%	40	-	-	-	48	-	-	44			
	39	2.7	13.7	5.5	-	-	-	6.8%	29	-	38	-	-	-	-	32.5			
	51	0.1	2.9	4.4	-	-	-	2.5%	15	8	-	-	-	-	-	11.5			
	63	0.9	3.5	1.9	-	-	-	3.6%	17	23	-	-	-	-	-	20			

Table 8. Continued

FG 53	15	8	11.1	12.2	-	-	10.0%	40	19	-	-	-	29.5
	27	15	13	16	-	-	14.7%	46	38	68	-	-	50.6
	39	8	5	9.3	-	-	7.4%	83	75	-	-	-	79
FG 54	15	8.2	14.5	-	-	-	11.5%	22	50	-	-	-	36
	27	22.4	18.5	17	-	-	19.3%	59	46	-	-	-	52.5
	39	9.4	13.5	5.5	-	-	9.5%	60	47	-	-	-	53.5
	51	8	4.5	8	-	-	6.8%	61	-	49	-	-	55
	63	2	2.9	-	-	-	2.4%	43	37	-	-	-	40
FG 55	15	15.4	9.7	-	-	-	13.3%	29	28	-	-	-	28.5
	27	21	15.5	21.5	19.5	-	19.4%	30	-	-	32	-	31
	39	12	6.9	-	-	-	10.5%	82	61	46	-	-	63



Table 9. Anaphase damage in percent, induced by 250 r of X-ray treatment in Pisum.

<u>FRAGMENTS</u>						
	<u>Slide #</u>					
<u>Hrs.</u>	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>Frag. %</u>
3	24	29.7	22.9	-	-	25.0%
6	28	17.2	22.5	19.1	-	24.2%
9	12	-	12.6	1	14	9.9%
12	12	12	13	8	-	11.3%
15	-	11	15	14	-	13.3%
27	-	7	10	6	5	7.0%



Table 10. Percent aberrations in Vicia faba, induced by 0.233×10^{-2} M metepa treatment.

EU 100									
Hrs.	Chroma- tid Break	Chromatid Exchange	Total Chroma- tid Damage	Chroma- Break	Chromosome Exchange	Centric Fragments	Total Chromo- some Damage %	Total Cells Counted	Total Damage
$2\frac{1}{2}$	1	-	1%	-	-	-	-	100	1%
6	-	-	-	3	-	-	6%	50	6%
10	-	2	2%	11	-	-	11%	100	13%
12	-	-	-	8	-	-	8%	100	8%
14	3	1	4%	14	1	-	15%	100	19%
25	14	8	22%	22	2	-	24%	80	57.5%
28	20	12	32%	25	6	-	31%	100	63%
$45\frac{1}{2}$	14	13	27%	14	10	-	24%	100	51%
61	2	1	20%	8	6	17	14%	100	34%
72	2	-	9%	6	-	7	6%	100	15%





Table 11. Comparison of damage.

Name of chemical used	Concentration	Test organism	Time of maximum damage	% of maximum anaphase damage	% of maximum metaphase damage	Persistence
<u>Tretamine</u>						
	1.23 X 10 ⁻⁴	Pisum	27 hours	10.0%	-	no
	2.45 X 10 ⁻⁴	Pisum	27 hours	20.0%	-	no
	3.68 X 10 ⁻⁴	Pisum	27 hours	60.8%	-	no
	4.9 X 10 ⁻⁴	Pisum	27 hours	40.0%	-	no
<u>Apholate</u>						
Exp. #1	3.1 X 10 ⁻³	Pisum	39 hours	25.0%	-	yes
Exp. #2	.26X 10 ⁻³	Pisum	36 hours	2.5%	-	yes
	.52X 10 ⁻³	Pisum	36 hours	4.5%	-	yes
	1.03X 10 ⁻³	Pisum	36 hours	8.6%	-	yes
	2.1 X 10 ⁻³	Pisum	36 hours	11.6%	-	yes
	3.1 X 10 ⁻³	Pisum	36 hours	16.4%	-	yes
	4.1 X 10 ⁻³	Pisum	36 hours	18.4%	-	yes
Exp. #3	3.1 X 10 ⁻³	Pisum	27 hours	15.0%	-	no
<u>Metepa</u>						
Exp. #1	.465 X 10 ⁻²	Pisum	39 hours	5.9%	-	yes
	.697 X 10 ⁻²	Pisum	39 hours	14.4%	-	yes
	1.4 X 10 ⁻²	Pisum	39 hours	17.2%	-	yes
Exp. #2	.58 X 10 ⁻²	Pisum	27 hours	11.6%	-	no
	.79 X 10 ⁻²	Pisum	27 hours	15.6%	-	no
	.93 X 10 ⁻²	Pisum	27 hours	16.4%	-	no
	1.16 X 10 ⁻²	Pisum	27 hours	19.5%	-	no

Table 11. Continued

Exp. #3	.58 X 10 ⁻²	Pisum	27 hours	10.0%	-	no
	.697 X 10 ⁻²	Pisum	27 hours	10.8%	-	no
	.79 X 10 ⁻²	Pisum	27 hours	14.7%	-	no
	.93 X 10 ⁻²	Pisum	27 hours	19.0%	-	no
	1.16 X 10 ⁻²	Pisum	27 hours	19.5%	-	no
Exp. #4	2.33 X 10 ⁻²	Vicia	28 hours	-	63%	yes
<u>Azirane</u>	1.22 X 10 ⁻²	Pisum	27 hours	5.3%	-	yes
	1.83 X 10 ⁻²	Pisum	39 hours	6.4%	-	no
<u>X-ray damage</u>	250 r	Pisum	3 - 6 hours	25.0%	-	-











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