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THE MMS DOSE RESPONSE FOR CHROMOSOMAL ALTERATIONS IN BARLEY

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ABSTRACT

Dose response curves for the chromosomal alterations induced by the monofunctionaly alkylating agent MMS in barley root tip meristem were obtained. The chromosomal alterations scored at anaphase were chromatid and isochromatid fragments, bridges and lagging chromosomes. In addition at the interphase stage,

In most of the cases analyzed, a straight line was found except for the lagging chromosomes and the micronuclei which were fitted the best to an exponential expression. The lagging chromosomes were found not to fit Poisson distribution, implying the non randomnes of the behavior of MMS. The action of MMS on the cellular division of the barley root tip meristems was also investigated finding a direct decrease with the increase of the dose and it was reflected in the number of anaphases per root meristem and in the mitotic index.

RESUMEN

Se obtuvieron curvas de dosis-respuesta para alteraciones cromosómicas inducidas por el agente alkilante MMS en las células de los meristemos de las puntas de la raíz de cebada.

El análisis fue realizado en anafases y las alteraciones observadas fueron: fragmentos cromatídico e isocromatídicos, puentes y cromosomas retardados. Además en la interfase se observaron células con uno o dos núcleos (un micronúcleo). En la mayor parte de las alteraciones se encontró una relación linear excepto para los cromosomas retardados y los micronúcleos, los cuales se ajustaron mejor a una expresión exponencial. Los cromosomas retardados no siguieron la distribución de Poisson, implicando un comportamiento no azarizado del MMS. La acción del MMS sobre la división celular en los meristemos de la raíz de cebada fue también investigada encontrándose una disminución directa con el incremento de la dosis, lo cual se reflejó en la cantidad de anafases por meristemo así como en el índice mitótico.

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Abbreviations: MMS, methyl methanesulfonate; EMS, ethyl methanesulfonate; MNNG N-methyl-N' -nitro-N-nitrosoguanidine; PNNG, N-propyl-N' -nitro-N-nitrosoguanidine; HA, hydroxylamine; MES, methyl ethanesulfonate; EI, ethylen imine; A, adenine; G, guanine; T, thymine; C, cytosine; DNA desoxyribonucleic acid.

INTRODUCTION

Alkylating agents have been widely used as mutagens in several organisms, namely bacterias (Loveless and Howarth, 1959), *Neurospora* (Malling and de Serres, 1973). *Drosophila* (Fahmy and Fahmy, 1961) higher plants (Ehrenberg, 1971) and vertebrates (Ehling and Russel, 1969; Brewen *et al.*, 1970), but their application in mutation breeding programs has been limited because of their toxicity and the genetic damage caused other than mutations. It is known that the toxicity comes from the degradation products of the hydrolysis of the alkylating substances (Whickham *et al.*, 1969) and from the alkylation of macromolecules other than DNA, leading to enzyme inactivation (Osterman-Golkar *et al.*, 1970).

Chromosomal aberrations in the root tip meristems have been used as a test system to evaluate the action of mutagenic agents and they can be a valuable monitor for knowing the moment in which the alkylating agent is active in the cellular cycle (Mikaelsen *et al.*, 1968).

The alkylating agent MMS has not been used very extensively as a mutagen. In fact, Loveless and Howarth (1959) reported it as non-mutagenic. However, it is known that MMS induces mutations efficiently (Drake and Baltz, 1976). At equimolecular basis and at low concentrations, Minocha and Arnason (1962) and Arnason and Minocha (1965) found MMS to be more higly effective in inducing mutations in barley than EMS, which is probably the most frequently used alkylating agent. However, at high concentrations, EMS induces higher mutation frequencies than MMS because the ethylating agents are less toxic than the methylating agents. These findings are also supported by Rao and Natarajan (1965).

Although only few concentrations were used, MMS has been considered as a strong inducer of chromosomal aberrations (Rao and Natarajan, 1965; Brewen *et al.*, 1970; Malling and de Serres, 1973). Malling and de Serres (1973) reported that there is no reason to believe that even very low doses of MMS would not be mutagenic. On this basis it is reasonable that above a certain level, the MMS effectiveness as a mutagen decreases because of the chromosomal aberrations and the toxic effects producer. Therefore it seemed worthwhile to look for the dose-response curves of the aberrations induced by this alkylating agent, in order to determine the overall primary genetic damage.

MATERIAL AND METHODS

Dry resting seeds of *Hordeum vulgare* (var. Común) were used in these experiments. Batches of 50 seeds were washed for two hours in running tap water in order to remove germination inhibitors and buffer the entrance of the mutagen into the seeds. Treatment was carried out by soaking the seeds in fresh solutions of the MMS (Eastman Kodak) concentrations ranging from 0.10 to 0.60% w/vol; for one hour at 20°C in a controlled temperature bath (Grant SS30) equipped with an aeration and bubbling system (Meneses and Rubluo, 1978) to assure a homogeneous treatment. A control without MMS was running in every treatment. Afterwards the seeds were washed for five minutes in running tap water and

immediately sown in plastic boxes provided with a cotton layer and filter paper wet with distilled water (450 ml/30 g of cotton). The treated seeds were allowed to develop in darkness in a germination chamber at 22°C, protected against desiccation with a perforated plastic frame. The control roots reached 1 cm in lenght about 39 hours after the beginning of germination and were ready for the cytological analysis, while at the higher doses, they reached this lenght after 168 hours. The fixations were made when the roots reached 1 cm, cutting 2 mm from the tip and processing it according to a modification of Villalobos-Pietrini method (1965). Permanent slides were made following the Conger and Fairchild technique (1953).

Chromosomal alterations were scored at anaphase using 30-40 slides per dose (one slide per root tip). They were registered as chromatid breaks, isochromatid breaks, bridges and lagging chrosomomes. The number of micro nuclei per cell were recorded at interphase.

RESULTS AND DISCUSSION

It is known that alkylating agents induce only chromatid aberrations irrespective of the stage of the mitotic cycle at which they are produced, and the cell has to go through and S phase before the aberration are revealed (Evans and Scott, 1964, 1969; Bender *et al.*, 1974; Kihlman *et al.*, 1978; Natarajan and Obe, 1978).

Table I shows the frecuencies and types of chromatid aberrations that were observed, and it can be seen that, in general, as the dose increases the cytological damage also increases. The results are in agrement with those reported by Tomkins and Grant (1972) for EMS. It has been suggested that the production of chromosomal breaks by alkylating agents is due principally to their action on the DNA bases preferentially in the order G, A, C and T (Ramanna and Natarajan, 1966).

Besides, Samanna and Natarajan (1966) and Rao and Natarajan (1967) proposed that methylating agents produce more chromosome breaks than any other ethylating agents or other alkylating agents with more complex molecular structures. This could be due probably because the less complex methylating agents can penetrate more easily into regions rich in G-C. This induces the breaks and as a consequence they cause the formation of fragments.

Moreover, it has been shown that methylating agents, like MMS, react with the phosphate group in the DNA chain (Soyfer *et al.*, 1977) leading ultimately to chromosome breaks (Ramanna and Natarajan, 1966).

When exhibited in a normal axis graphs, chromatid breaks, isochromatid breaks, bridges and also the total number of aberrations are seen to increase as the dose increases (Table I and Figs. 1-4).

These data are in agreement with those obtained with alkylating agents in barley (Swaminathan *et al.*, 1962; Kamra, 1971; Annova, 1973; Nilan, 1973; Veleminsky and Gichner, 1978). Abnormal anaphases (cells in anaphase stage with any kind of cytological alterations) behaved similarly (Fig. 5). It has been pointed out that the damage induced by MMS on denaturated DNA increases with the

TABLE I

FREQUENCIES (MINUS CONTROL) AND TYPES OF CHROMATID ABERRATIONS AND OTHER ALTERATIONS OBSERVED IN ANAPHASE AND MICRONUCLEI IN INTERPHASE CELLS INDUCED BY MMS IN BARLEY ROOT MERISTEMS

*Dose** (% w/y/1 hr)	*Total number of anaphase cells*	*% Abnormal anaphases*	*% Fragments*		*% Bridges*	*% Total of chromatid aberrations*	*% Lagging chomosomes*	*% Micronuclei***
			Chromatid	*Isochromatid*				
0.00	700	0.43	0.43	0.00	0.00	0.43	0.00	0.27
0.10	706	4.10	0.72	0.41	2.27	3.40	0.57	0.47
0.20	681	5.73	1.79	2.49	2.06	6.34	0.73	0.83
0.30	574	12.11	5.19	3.48	5.05	13.72	4.35	1.39
0.40	398	17.15	5.95	6.28	8.04	20.27	5.52	1.67
0.50	369	13.39	4.87	2.70	4.61	12.18	9.48	2.11
0.60	168	32.30	7.75	7.14	10.71	25.60	26.78	3.19

* Control values were substracted in all groups
** From 1000 to 2500 interphase cells were observed for each dose.

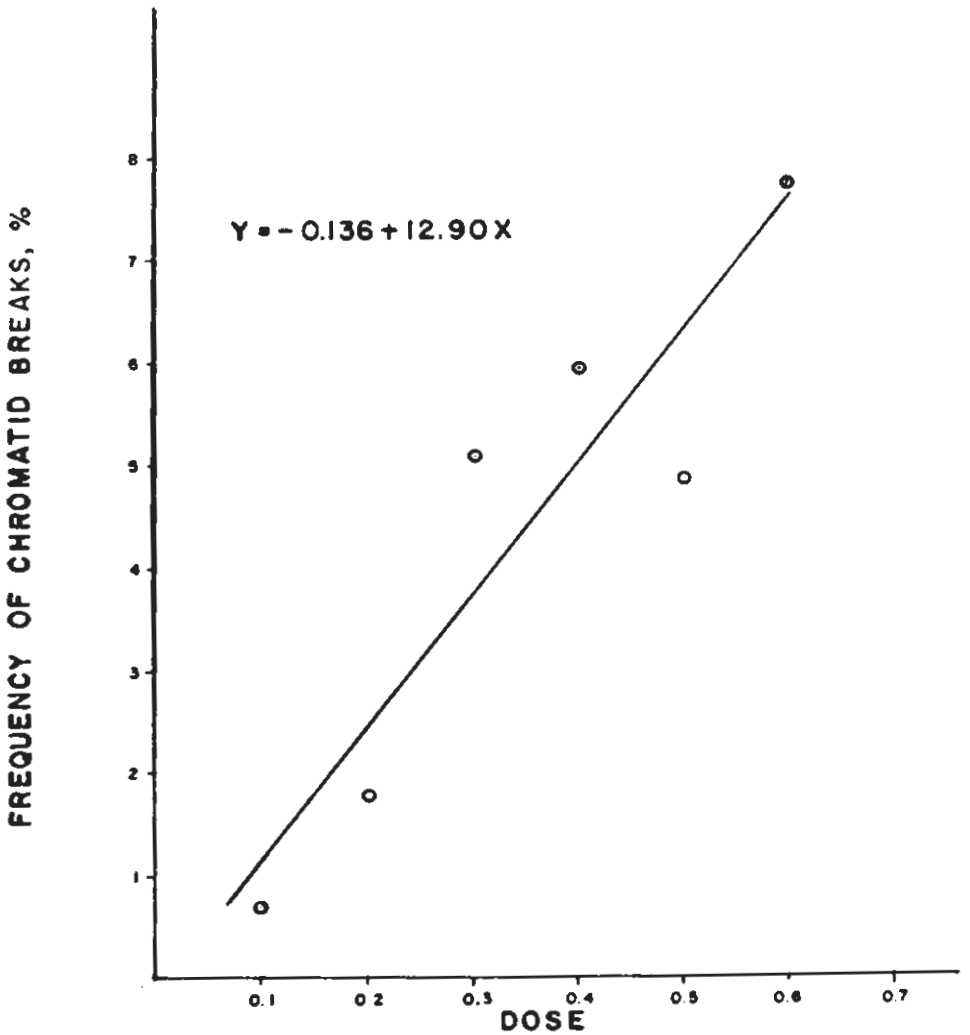

Fig. 1. Chromatid breaks induced by MMS in barley.

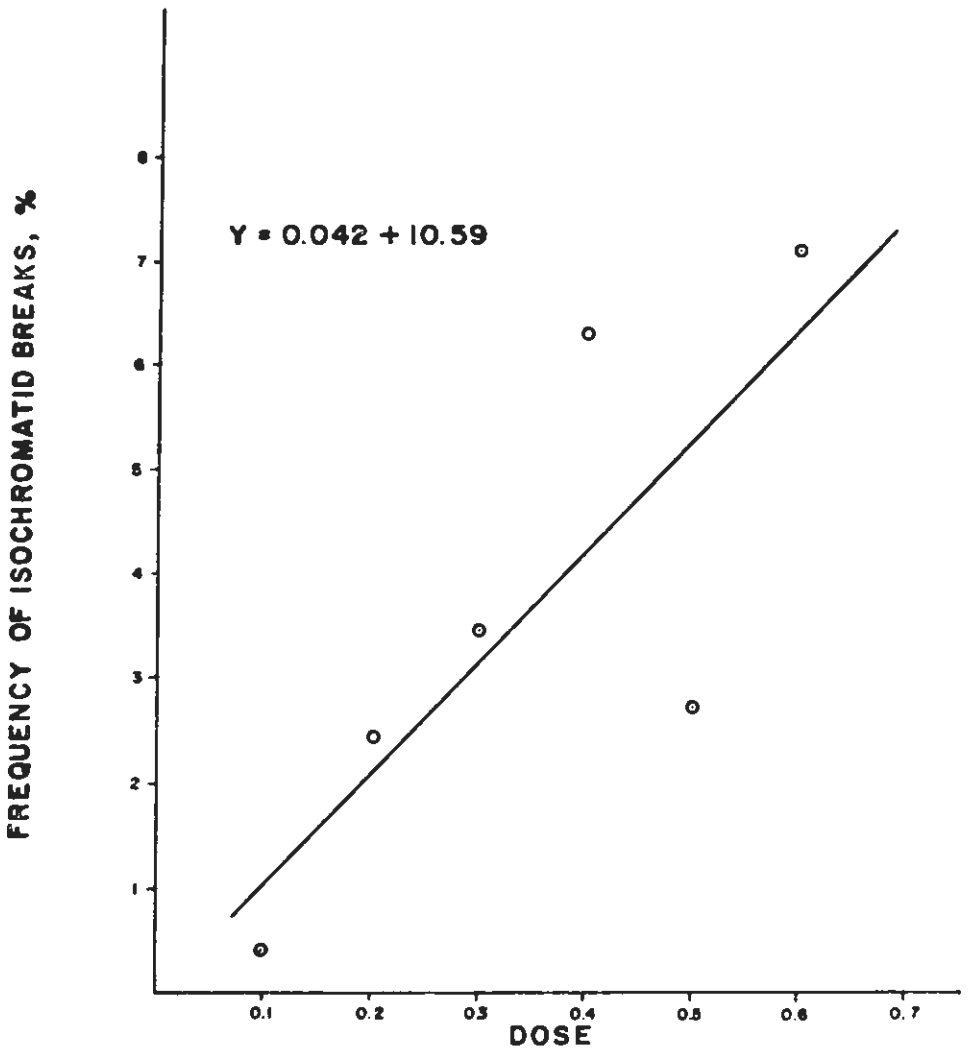

Fig. 2. Isochromatid breaks induced by MMS in barley.

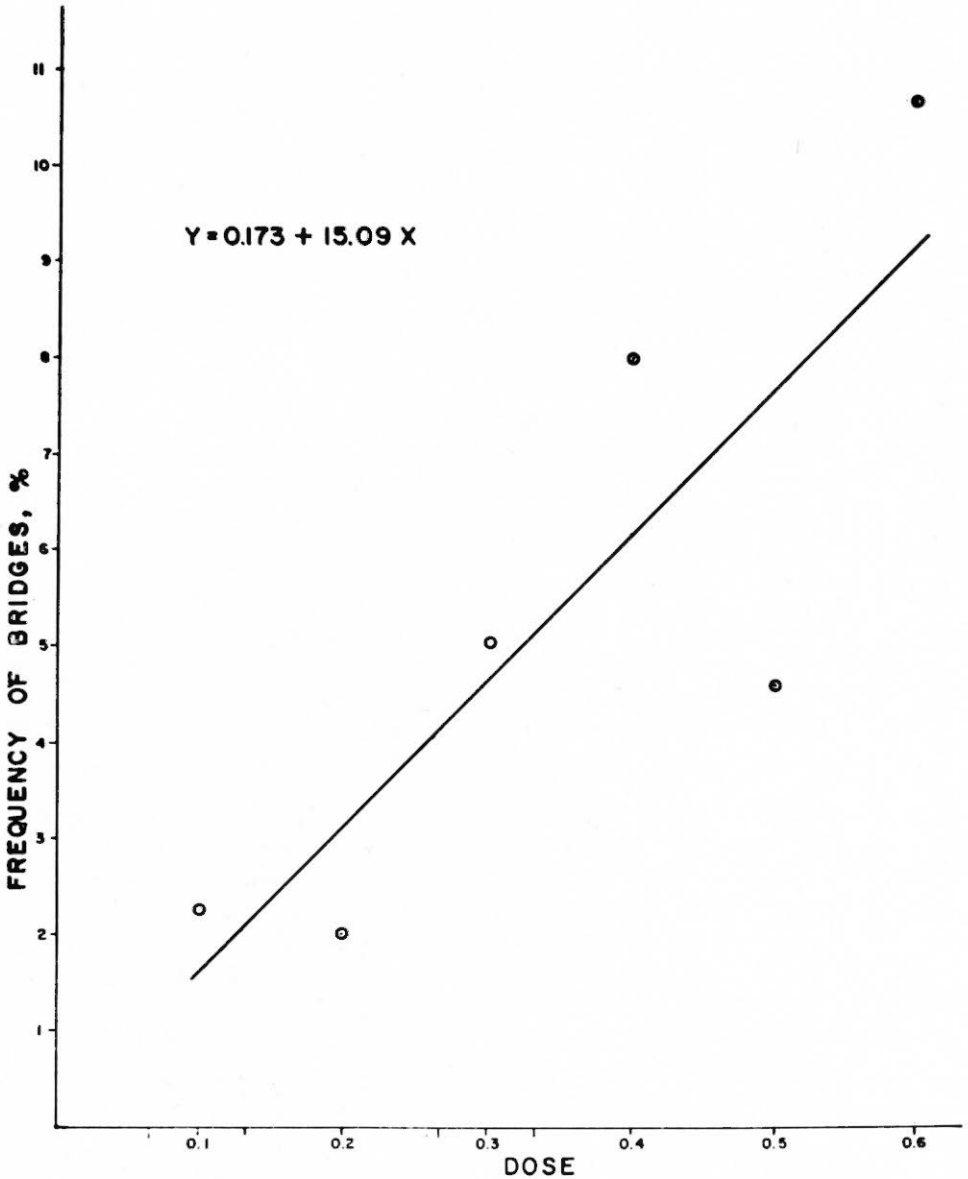

Fig. 3. Bridges induced by MMS in barley.

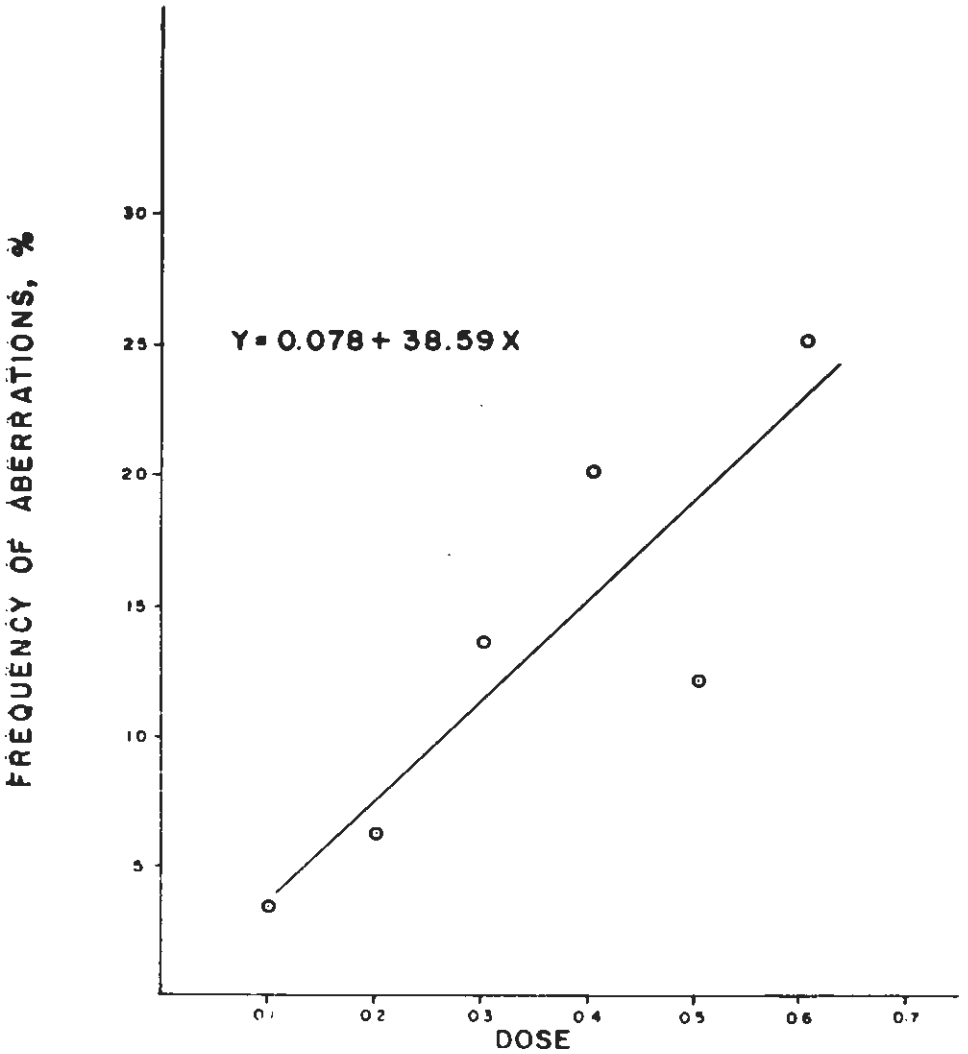

Fig. 4. Chromatid aberrations induced by MMS in barley.

dose, and also that there is a mutagen-dose dependence of the frequency of chromosomal aberrations induced by this agent (Veleminsky and Gichner, 1978). Our results are in good agreement with those reported by this authors (Table I and Fig. 4).

Furthermore, if the lagging chromosome frequency is taken into account, a rise in the frequency of the chromosomal alterations per cell is noted (Table I). Other have reported similar results with alkylating agents like EMS (Tomkins and Grant, 1972), MNNG and PNNG (Kamra, 1971).

The high frequency of lagging chromosomes obtained (Table I and Fig. 6) was probably due to the action of the MMS on the centromeric region which apparently became inactive. This is in agreement with those results obtained with EMS, EI, HA and MES (Natarajan and Upadhya, 1964; Ramanna and Natarajan, 1966; Tomkins and Grant, 1972; Nicoloff and Gecheff, 1976).

In interphase stage only cells with one and two nuclei (one micronuclei) were observed. Micronuclei were the result of acentric fragments and lagging chromosomes (Ramanna and Natarajan ,1966; Schmid, 1973). The dose responses of lagging chromosomes and micronuclei were fitted to an exponential expression (Table I and Figs. 6 and 7). This similar behaviour suggest that the former could be due the main constituent of the latter.

The distribution of lagging chromosomes among anaphase cells induced by MMS, did not adjust to the Poisson formula (Table II). This nonrandom distribution could be due to an unequal penetration of the chemical within the root (Kihlman, 1966). There has also been found a nonrandom distribution of chromatid aberrations induced by alkylating agents in barley (Nicoloff *et al.*, 1975).

A dormant seed is in a G_0 stage (Fousová *et al.*, 1974) and some authors have determined the DNA synthesis stage in the barley embryo, but their results are in disagreement: Arnason *et al.* (1966), established that it occurs 6 hours after the beginning of germination; Savin *et al.* (1968) after 17 hours; and Fousová *et al.* (1974) after 22 hours. In any case, as the mutagen was applied in a one hour pulse to G_1 cells, and only chromatid aberrations were observed a delayed action of this alkylating agent has been shown. This is in agreement with some other workers (Kihlman, 1966; Loveless, 1966; Rieger and Michaelis, 1967; Kunzel, 1971; Bender *et al.*, 1974).

Evans and Scott (1969) considered that the lesions induced by alkylating agents were chromatid aberrations as the consequence of errors in chromosome replications (mis-replications). It is known that the main DNA alteration site by MMS is the N-7 atom of guanine (Lawley and Shah, 1972) and Prakash and Strauss (1978) found a degradation of the methylated DNA after treatment at high MMS doses consequently producing breaks. The action of MMS on the cell division process is showed in our results by the analysis of the number of anaphases per root meristem (Fig. 8) and the mitotic index (Fig. 9).

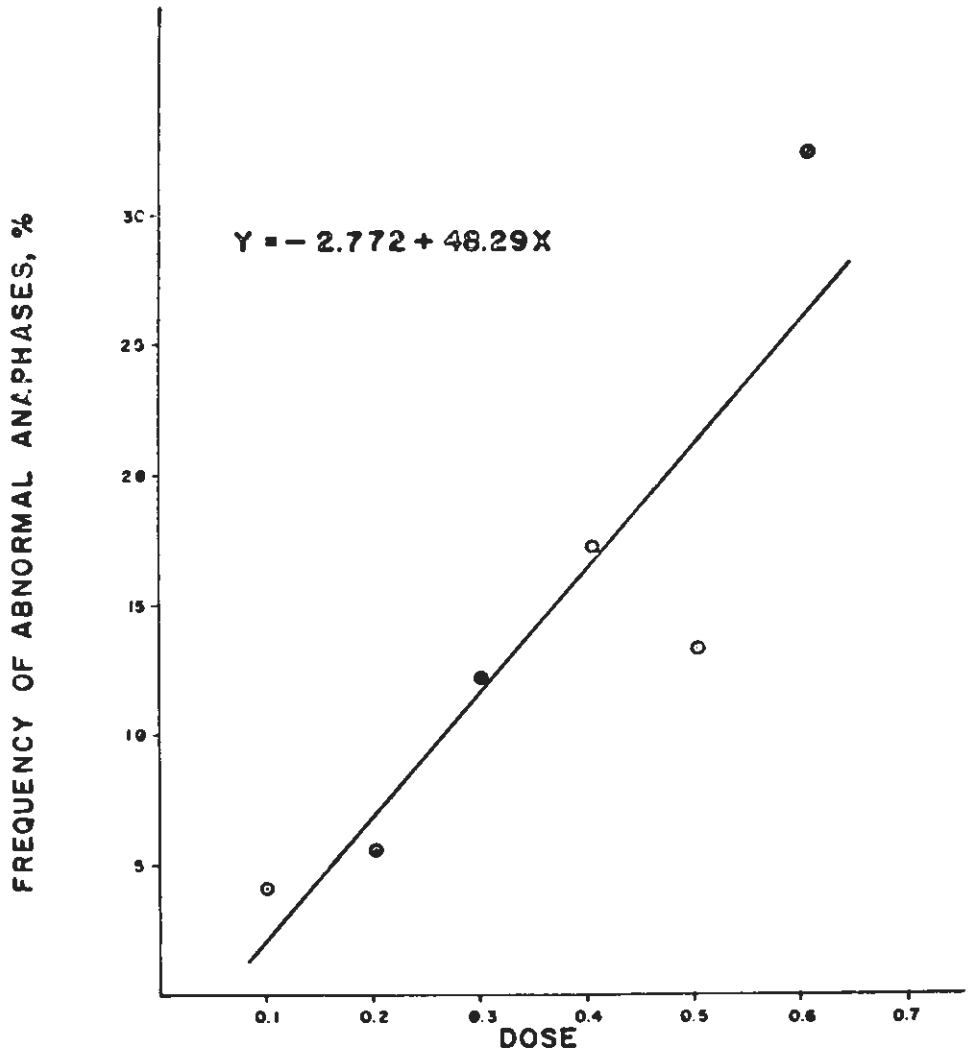

Fig. 5. Abnormal anaphases induced by MMS in barley.

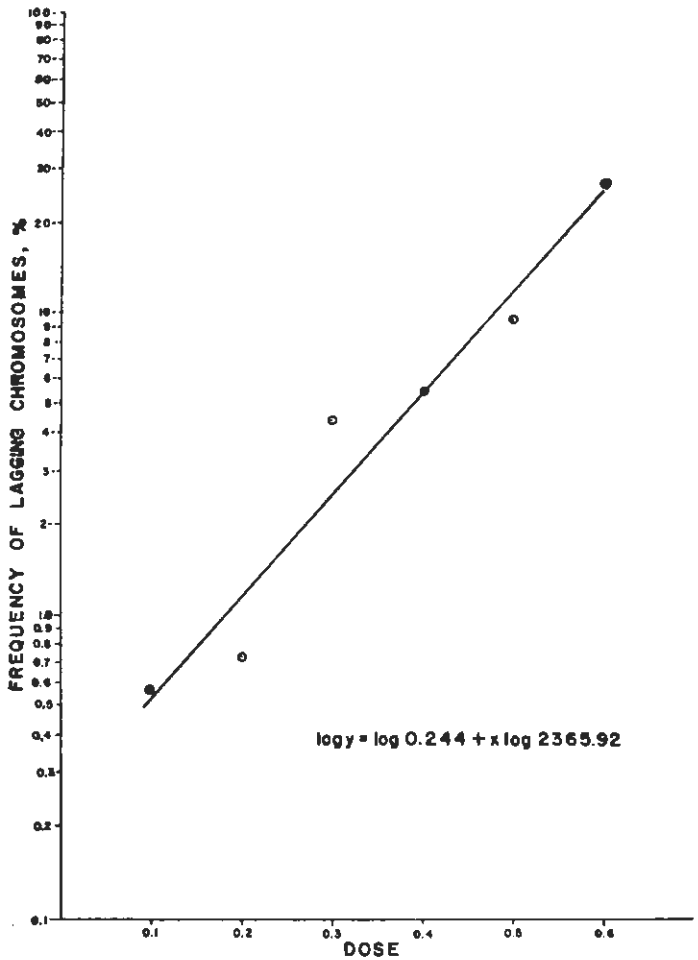

Fig. 6. Lagging chromosomes induced by MMS in barley.

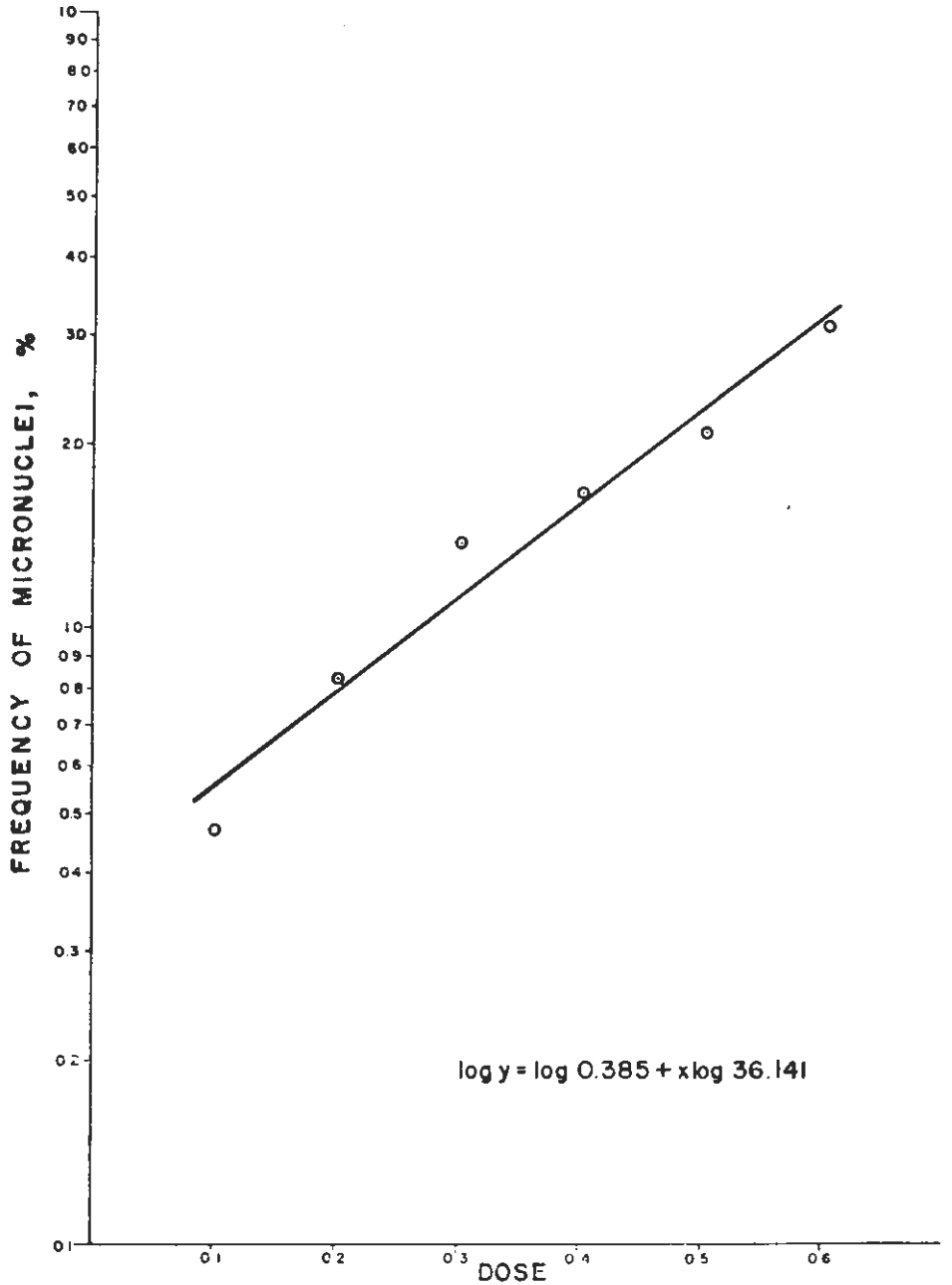

Fig. 7. Micronuclei induced by MMS in barley.

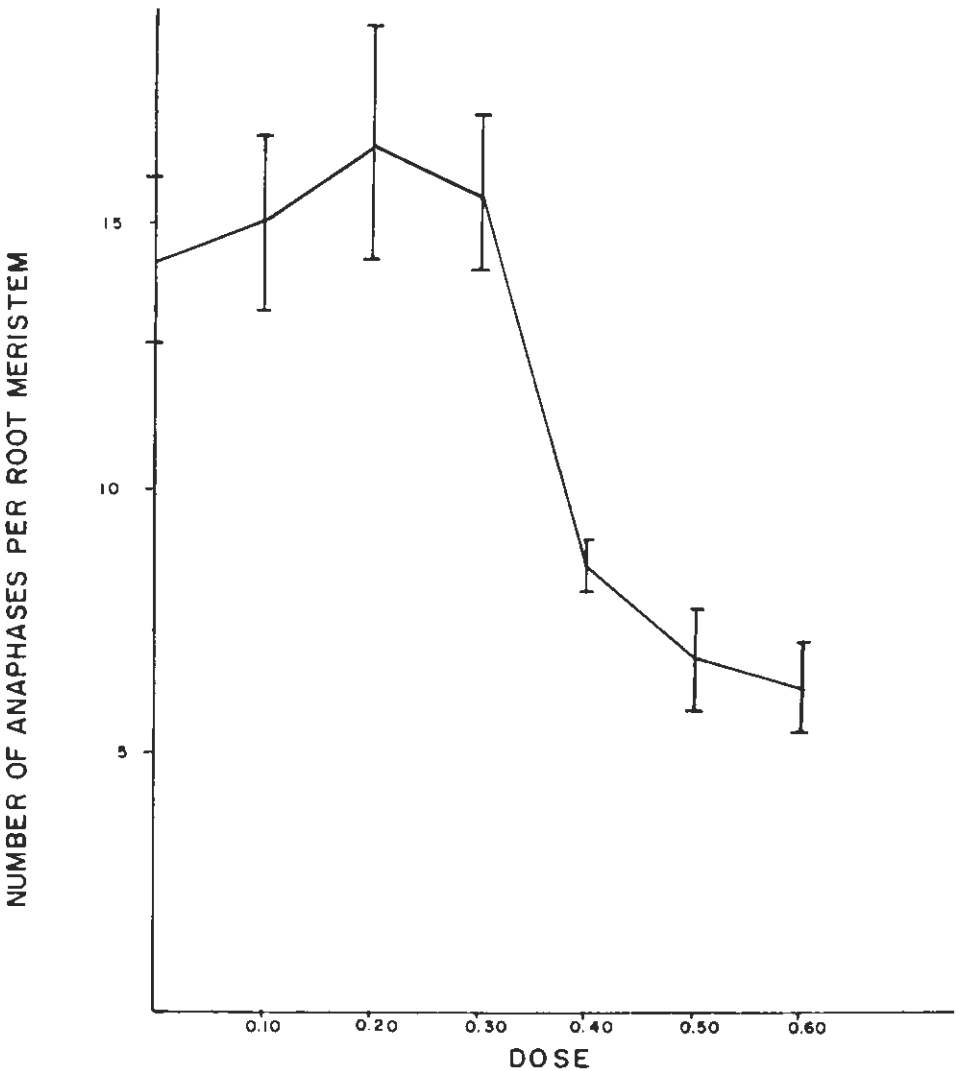

Fig. 8. Number of anaphases per root meristem ± S.E. treated with MMS.

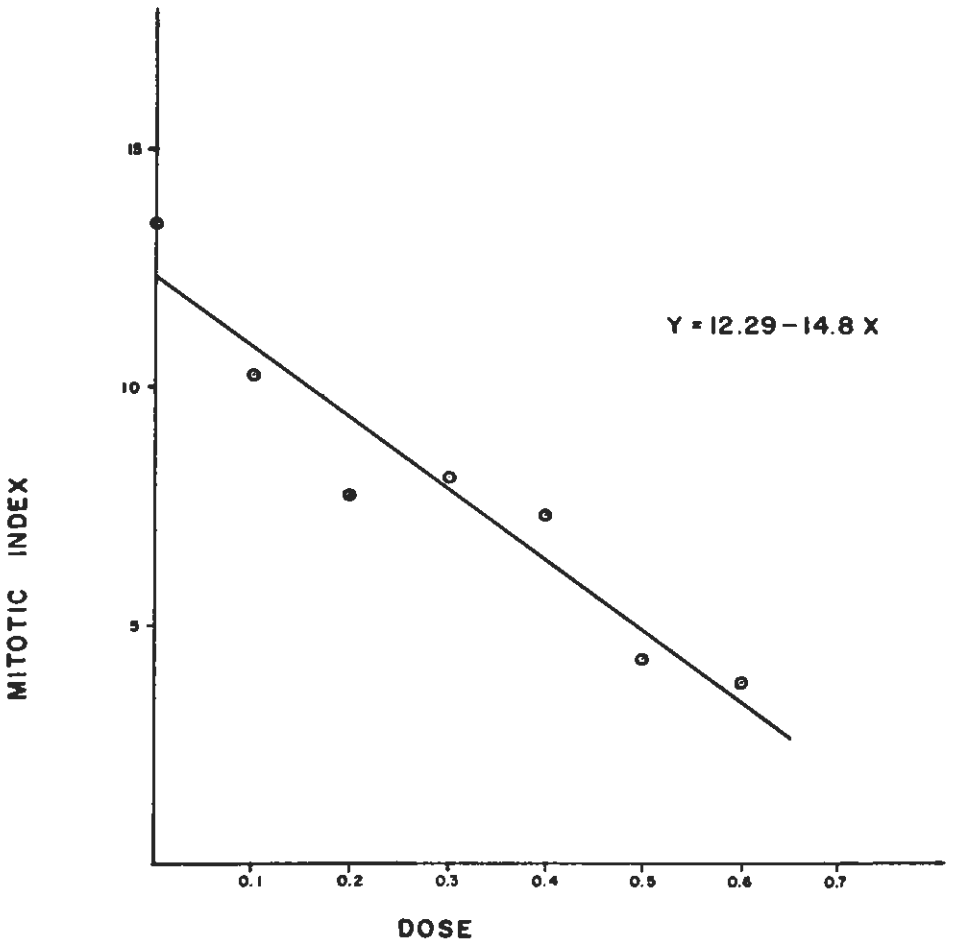

Fig. 9. Mitotic index in cells of barley root tip meristems treated with MMS.

TABLE II

NUMBER OF CELLS IN ANAPHASE CONTAINING 0, 1, 2 or 3 LAGGING CHROMOSOMES INDUCED BY MMS BARLEY ROOT MERISTEMS. EXPECTED VALUES CALCULATED ASSUMING A POISSON DISTRIBUTION.

Dose (% w/v/1 hr)	*Total number of anaphase cells*	*Distribution*	*Frequencies of anaphases with indicated No. of lagging chromosomes. 0*	*1*	*2*	*3*	*X^2 Test*
0.10	706	obs	702.00	4.00			0.010
		exp	702.10	4.21			
0.20	681	obs	677.00	3.00	1.000		
		exp	676.02	4.96	0.017		57.618*
0.30	574	obs	552.00	18.00	4.00		
		exp	548.58	24.85	0.56		23.041*
0.40	398	obs	380.00	15.00	2.00	1.00	
		exp	376.59	20.81	0.58	0.11	12.330*
0.50	369	obs	345.00	14.00	9.00	1.00	
		exp	335.60	31.81	1.51	0.05	65.437*
0.60	168	obs	136.00	21.00	11.00		
		exp	130.06	33.28	4.26		15.466*

* $P < 0.01$

The data in both cases exhibited a direct decrease with increasing dose, however when the results were analyzed by way of the Student Newman-Keuls test looking for the multiple comparison among means of the number of anaphases per root meristem it showed up the formation by way of two groups one high doses and one low doses group (Table III).

TABLE III

MULTIPLE COMPARISON AMONG MEANS OF THE NUMBER OF ANAPHASES PER ROOT MERISTEM ACCORDING TO THE STUDENT-NEWMAN-KEULS TOST.

RANK	1	2	. 3	4	5	6	7
DOSE	0.6	0.5	0.4	0.0	0.1	0.3	0.2
$\bar{X}$	6.27	6.87	8.50	14.26	15.07	15.51	16.50
RANK	DOSE	$\bar{X}$	Ni	26	52	46 50	46 37 40
1	0.6	6.27	26	——			
2	0.5	6.87	52	0.60	——		
3	0.4	8.50	46	2.23	1.63	——	
4	0.0	14.26	50	7.99*	7.39*	5.76*	——
5	0.1	15.07	46	8.80*	8.20*	6.57*	0.81 ——
6	0.3	15.51	37	9.24*	8.64*	7.01*	1.25 0.44 ——
7	0.2	16.50	40	10.23*	9.63*	8.00*	2.24 1.34 0.99 —

* Significant at < 0.05.

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