GENETIC EFFECTS OF ETHYL METHANESULFONATE AND GAMMA RAY TREATMENT OF THE PROEMBRYO IN MAIZE

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THE well differentiated meristematic region in the embryo of a mature maize seed limits the classical seed irradiation procedure in the study of induced mutations in this plant. The meristematic region contains up to six embryonic leaves (STEIN and STEFFENSEN 1959). Any mutation induced by irradiating a seed will be produced in only a sector of the plant, and the mutated area is not likely to occur both in the ear and tassel. A mutation thus occurring in either of them would result in a heterozygous plant in the second generation which segregates in the third generation. This difficulty could be overcome by using the one-celled proembryo as the experimental material, which affords an opportunity of obtaining a uniform (nonchimeric) plant.

Moreover, studies on radiosensitivities of developing embryos of plants and animals by different workers (STADLER 1930; BUTLER 1936; RUSSELL and RUSSELL 1954; SARIC 1957; MERICLE and MERICLE 1961; etc.), since the pioneering radiation work of GAGER (1908) with developing embryos of *Oenothera*, have shown that early embryonic stages are more sensitive to radiation than are later stages. The work of STADLER (1930) on the genetic effects of X rays on maize proembryos has indicated the potentialities of the maize proembryo in the study of radiation induced mutations. Work at the Blandy Experimental Farm of the University of Virginia (SINGLETON 1961; VARMA, CASPAR and SINGLETON 1962, 1963) has shown that the 24-48 hour old maize proembryo is a sensitive stage for inducing mutations by gamma radiation.

Since the developing embryos in general, and maize embryos in particular, have proved to be a suitable system in the study of radiation induced mutations, we used these for this comparative analysis of the nature of genetic effects induced by ethyl methanesulfonate (EMS) and gamma rays. It is hoped that this work with linked endosperm and seedling markers in maize may reveal the exact nature of genetic changes induced by EMS in higher plants, and that its action could be compared with that of the gamma rays. Work of a similar nature using mature pollen grains of the plant as the experimental material has been reported (CHATTERJEE, CASPAR and SINGLETON 1965).

MATERIALS AND METHODS

A homozygous inbred line and a hybrid line of field corn were used in all experiments as the male and female parents, respectively. *Sh*, *Bz* and *Wx* at positions 29, 31 and 59, respectively,

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in the short arm of chromosome 9 were used as endosperm markers. V_1 at position 71 in the long arm of chromosome 9 and Lg_1 and Gl_2 at positions 11 and 30 in the short arm of chromosome 2 were used as seedling markers. The male plant was homozygous for lg_1 , gl_2 , V_4 and C , Sh , Bz , Wx , V_1 . The female plant was homozygous for Lg_1 , Gl_2 , V_4 , C , sh , bz , wx and heterozygous for V_1 .

Zygotes or proembryos 24 hours after pollination were treated with either an EMS solution or gamma rays or both. For EMS treatment only, all the husks and silks from an ear were carefully removed. The naked ear was then thoroughly washed in a 2% solution of Tween 20, a wetting agent (source—Amend Drug Co., New York). The ear was wrapped with a thick pad of absorbent cotton which was then completely soaked with the EMS solution and covered with a shoot bag. The cotton pad was replaced after every hour with a fresh one and again soaked with EMS. At the end of treatment, the cotton pad was removed, the ear was washed three or four times with distilled water, wrapped with a fresh pad of dry cotton and covered with a bag. For irradiating proembryos with gamma rays, female plants were grown in pails, which were transported into the Blandy Experimental Farm's gamma field for desired doses. For multiple treatments with gamma rays and EMS, female plants were grown in pails and ears on these plants were first exposed to gamma radiation and then treated with EMS.

The EMS solution was prepared, one hour before each experiment, in deionized distilled water at a pH of 7.2 with phosphate buffer. All treatments were performed in the field and within a temperature range of 27 to 30°C.

From knowledge gained from previous work with proembryos, we selected three doses of gamma rays: 160r, 250r, and 500r. 500r brought about the maximum rate of Lg_1 , Gl_2 losses with little evident injury to the seed. 250r and 160r were intermediate and minimum respectively, in their effect within the present dose range. All these doses were delivered in 20 hours. The different concentrations of EMS were selected after field trials.

At maturity the ears were harvested and scored for losses of different endosperm and seedling markers. Endosperms and seedlings with recessive phenotypes only were classed as whole losses. Endosperms and seedlings with sectors of recessive and dominant tissues were classed under partial losses. Some partial losses in endosperms and seedlings could be identified as mosaics, which arise as a result of breakage-fusion-bridge cycle typical of endosperms described by McCLINTOCK (1941). In a seedling showing this effect, all the leaves showed many irregular short, narrow, green and white streaks of nonmutant and mutant tissues. Another type of event in scoring a seedling for v_1 losses was classed as streaks, where part or entire length of the leaf blade showed a very thin strip (1 mm or less) of recessive tissue. These streaks were found on the first or second leaf of the seedling.

RESULTS

Table 1 shows the number of ears treated as proembryos, average seed set per ear, and percentage of germination of M_1 seeds for each treatment. The rate of germination of the M_1 seeds decreases with an increase in severity of treatment (concentration and time for EMS; dose for gamma rays).

Tables 2 and 3 show the types of losses of the different endosperm and seedling markers. Table 2 indicates several interesting features: (a) Loss of a single marker (Sh or Bz) is infrequent; (b) whole loss of the multiple markers Sh , Bz and Wx was found only in higher concentrations of EMS; (c) partial losses of the multiple markers Sh and Bz appear to be random with reference to treatment; (d) partial losses of the multiple markers Sh , Bz and Wx show an increase with time and concentration of EMS, and with dose of gamma rays; and (e) the multiple treatments of EMS and gamma rays, in most cases, have decreased the par-

TABLE 1

Types of treatments made on 24 to 48 hour old maize proembryos showing number of ears treated, average seed set per ear and percentage germination of the M_1 seeds

Treatment	Number of ears treated	Average seed set per ear	Percentage germination
Control	19	273	98.8
EMS			
0.0125M for 1.5 hr	10	291	98.7
0.0125M for 3 hr	11	336	97.8
0.0125M for 4.5 hr	9	312	97.8
0.0125M for 6 hr	8	329	97.9
0.025M for 1.5 hr	11	273	96.7
0.025M for 3 hr	10	275	96.3
0.025M for 4.5 hr	10	297	96.6
0.05M for 1.5 hr	9	295	95.6
Gamma rays			
160r	6	250	97.4
250r	5	218	92.4
500r	5	201	86.5
Gamma rays and EMS in combination			
160r + 0.0125M — 1.5 hr	5	237	97.6
160r + 0.0125M — 3 hr	6	229	96.9
250r + 0.0125M — 1.5 hr	7	230	94.6
250r + 0.0125M — 3 hr	6	188	93.1
500r + 0.0125M — 1.5 hr	7	217	93.8
500r + 0.0125M — 3 hr	6	215	91.7

tial losses of the multiple marker (*Sh*, *Bz* and *Wx*) compared with the total losses using EMS and gamma rays alone.

Observed rates for V_1 losses were doubled while scoring the loss of it, since one parent was heterozygous for this marker. It was noted that the phenotypic expression of the different V_1 losses ranged from yellowish green (mostly) to whitish green (in some cases). Table 3 shows that partial V_1 losses, including streaks, exhibited an increase with EMS treatment, but a decrease with gamma rays; the cause of this is not known. Whole losses of V_1 , and Lg_1 and Gl_2 markers also show an increase with gamma-ray dose.

Since the majority of losses of tested markers were partial, we pooled the data for a total rate of loss of these markers for each treatment. It is evident from Figures 2, 3 and 4 that the seedling markers (V_1 , Lg_1 and Gl_2), in general, are affected more than the endosperm markers by EMS. Most observed losses of the seedling markers were, however, at the V_1 locus (Table 3). With increasing gamma doses, the markers V_1 , Lg_1 and Gl_2 showed a linear increase of whole losses (Figure 7).

Figures 5 and 6 show total rates of losses of the endosperm and seedling markers in multiple treatments with gamma rays and EMS. It is evident that the total rates of losses of the different endosperm and seedling markers increased with the

TABLE 2

Different types of losses of the dominant endosperm markers following treatment of 24 to 48 hour old proembryos by EMS and gamma rays

Treatment	Number of seeds	Single and whole <i>sh</i> or <i>bz</i>	Partial <i>sh bz</i>	Whole <i>sh bz wx</i>	Partial* <i>sh bz wx</i>	Total rate of loss of different markers†
Control	5179	0	0	0	6(0.10)	0.10 ± 0.02
EMS						
0.0125M — 1.5 hr	2915	0	0	0	18(0.60)	0.60 ± 0.26
0.0125M — 3 hr	3696	0	1(0.02)	0	66(1.76)	1.78 ± 0.28
0.0125M — 4.5 hr	2804	0	0	0	52(1.85)	1.84 ± 0.34
0.0125M — 6 hr	2638	0	2(0.07)	0	56(2.11)	2.18 ± 0.40
0.025M — 1.5 hr	3012	0	1(0.03)	1(0.03)	45(1.47)	1.53 ± 0.23
0.025M — 3 hr	2747	0	0	1(0.03)	60(2.16)	2.19 ± 0.28
0.025M — 4.5 hr	2975	1(0.03) <i>bz</i>	1(0.03)	3(0.10)	72(2.41)	2.57 ± 0.35
0.05M — 1.5 hr	2654	1(0.03) <i>bz</i>	0	1(0.03)	59(2.21)	2.27 ± 0.39
Gamma rays						
160r	890	0	4(0.44)	0	8(0.89)	1.33 ± 0.14
250r	1039	0	1(0.09)	0	54(5.17)	5.26 ± 0.60
500r	1002	0	3(0.29)	0	67(6.67)	6.96 ± 0.77
Gamma and EMS in combination						
160r and						
0.0125M — 1.5 hr	400	0	1(0.25)	0	9(2.25)	2.50 ± 0.16
160r and						
0.0125M — 3 hr	836	0	0	0	20(2.37)	2.37 ± 0.29
250r and						
0.0125M — 1.5 hr	1660	0	2(0.12)	0	54(2.88)	3.00 ± 0.27
250r and						
0.0125M — 3 hr	1128	0	0	0	35(3.09)	3.09 ± 0.29
500r and						
0.0125M — 1.5 hr	999	0	4(0.40)	0	46(4.60)	5.00 ± 0.30
500r and						
0.0125M — 3 hr	1807	0	8(0.44)	0	101(5.57)	6.01 ± 0.56

* (a) Includes mosaics also. (b) In case of some minute losses of the multiple markers, *Sh*, *Bz* and *Wx*, scoring of the *Sh* losses was doubtful.

† In percent. The loss per hundred seeds is given in parentheses.

dose. The curve representing the loss of seedling markers (Figure 6), however, shows a saturation effect at higher doses, the cause of which is not known.

DISCUSSION

RANDOLPH (1936) observed that in maize it takes approximately 15 to 24 hours for the pollen tube to grow down the silk and fertilize the egg, and the first division of the proembryo occurs during the next 10 to 12 hours. Thirty-six hours after pollination, proembryos consist of 3 or 4 cells, and by 42 hours a 7 or 8 cell structure is formed. Therefore, in the present study, mutagens were applied 24 hours after pollination to treat one-celled proembryos. All exposures to gamma rays were of 20-hour duration. For multiple treatments with gamma rays and EMS, the treatment time did not exceed 24 hours. EMS treatments were continued for a

FIGURE 1.—Total rates of loss (by whole and partial events) of the dominant endosperm (solid line) and seedling (broken line) markers following different doses of gamma rays on 24 to 48 hour old maize proembryos.

FIGURE 2.—Total rates of loss (by whole and partial events) of the dominant endosperm (solid line) and seedling (broken line) markers following treatments of the 24 to 48 hour old maize proembryos by different concentrations of EMS for 1.5 hours.

maximum period of 6 hours. Thus all the treatments were completed within a maximum period of 24 hours after fertilization. Or in other words, the proembryos developed from the one- to the eight-cell stage during the time through which the treatments were made.

Loss of a dominant marker was identified from the phenotypic expression of its corresponding recessive allele after it had been lost following the mutagen treatment.

These data show that EMS, like gamma rays, is very effective in inducing losses of dominant markers; and the rates of losses of different endosperm and seedling markers, in most cases, increase with an increase in the treatment (Figures 1 to 7, Tables 2 and 3). A direct comparison of the gene losses induced by the two mutagens could not be made, because comparable dose levels have not been established. However, it is evident in Figures 1 to 4 and Tables 2 and 3 that seedling markers are lost much more frequently than the endosperm markers after EMS treatment but not after gamma ray treatment. It is not known if this apparently differential action is due to different modes of action of the mutagens on the genetic material.

TABLE 3
*Different types of losses of the dominant seedling markers following treatment of
 24 to 48 hour old proembryos by EMS and gamma rays*

Treatment	Number of seedlings	Whole V_1	Partial* V_1	Streaks V_1	Whole $Lg_1 G l_2$	Partial $Lg_1 G l_2$	Total rate of loss of different markers†
Control	5106	6(0.11)	0	10(0.18)	0	0	0.29 ± 0.03
EMS							
0.0125M — 1.5 hr	2859	6(0.20)	24(0.82)	54(1.87)	0	1(0.03)	2.92 ± 0.19
0.0125M — 3 hr	3551	20(0.56)	54(1.51)	106(2.98)	0	0	5.06 ± 0.31
0.0125M — 4.5 hr	2709	6(0.22)	62(2.27)	170(6.26)	0	0	8.75 ± 0.82
0.0125M — 6 hr	2533	4(0.15)	64(2.52)	194(7.65)	0	3(0.11)	10.43 ± 0.88
0.025M — 1.5 hr	2868	18(0.62)	94(3.27)	160(5.57)	0	0	9.46 ± 0.61
0.025M — 3 hr	2588	34(1.31)	104(4.00)	184(7.10)	0	0	12.41 ± 0.66
0.025M — 4.5 hr	2798	16(0.57)	100(3.57)	230(8.21)	1(0.03)	6(0.21)	12.59 ± 0.83
0.05M — 1.5 hr	2475	10(0.40)	110(4.44)	228(9.21)	0	10(0.40)	14.45 ± 0.95
Gamma rays							
160 r	852	2(0.23)	4(0.46)	22(2.57)	2(0.23)	0	3.49 ± 0.28
250r	910	6(0.65)	6(0.65)	16(1.73)	4(0.43)	0	3.46 ± 0.15
500r	902	10(1.10)	4(0.44)	6(0.66)	11(1.21)	0	3.41 ± 0.20
Gamma rays and EMS in combination							
160r and 0.0125M — 1.5 hr	324	0	0	6(1.80)	0	1(0.30)	2.10 ± 0.29
160r and 0.0125M — 3 hr	651	0	6(0.91)	20(3.06)	2(0.30)	0	4.27 ± 0.31
250r and 0.0125M — 1.5 hr	1600	2(0.12)	6(0.36)	34(2.10)	1(0.06)	2(0.12)	2.76 ± 0.29
250r and 0.0125M — 3 hr	1056	0	8(0.75)	24(2.26)	4(0.37)	1(0.09)	3.47 ± 0.25
500r and 0.0125M — 1.5 hr	890	4(0.44)	12(1.34)	40(4.48)	3(0.33)	0	6.59 ± 0.46
500r and 0.0125M — 3 hr	1572	8(0.50)	10(0.62)	24(1.52)	9(0.57)	0	3.21 ± 0.10

* This column includes mosaics also. The loss per hundred seedlings is given in parentheses.

† In percent.

FIGURES 3 and 4.—Sensitivity of the 24 to 48 hour old maize proembryos to EMS as shown by total rates of loss (by whole and partial events) of the dominant endosperm (solid line) and seedling (broken line) markers.

FIGURES 5 and 6.—Total rates of loss (by whole and partial events) of the dominant endosperm (solid line) and seedling (broken line) markers following combination treatments of gamma rays and EMS of the 24 to 48 hour old maize proembryos.

FIGURE 7.—Rates of whole loss of the dominant markers V_1 (solid line) and $Lg_1 Gl_2$ (broken line) by different doses of gamma rays on 24 to 48 hour old maize proembryos.

Loss of a single marker such as *Sh* and *Bz* is infrequent following both EMS and gamma ray treatments (Table 2). (This column does not include the loss of the marker *Wx* since its loss in every seed was difficult to score.) Most observed losses of the endosperm markers after EMS and gamma ray treatments included the region from *Sh* to *Wx*. These losses were mostly fractional (in many cases occupying a very small portion of the endosperm) and of nonmosaic type. There were, however, a few partial mosaics also. This type of loss of multiple endosperm markers of the *Sh* to *Wx* region of chromosome 9 of maize, due to deficiencies, has been reported by GIBSON *et al.* (1950) using mustard gas on maize pollen. Among the seedling markers (V_1 , Lg_1 and Gl_2), V_1 also showed losses mostly in the form of partial types (which include many nonmosaic and few mosaics) and streak types in all the treatments of both the mutagens. Lg_1 and Gl_2 , on the other hand, showed few partial losses after EMS treatment (possibly because they were difficult to detect), and mostly whole losses after gamma ray or gamma ray and EMS multiple treatments. Of the different types of losses, an increase in the rate of loss with an increase in dose within a group of treatments could be noted in case of partial losses of *Sh*, *Bz* and *Wx*, and V_1 , and whole losses of V_1 , and Lg_1 and Gl_2 (Tables 2 and 3).

In this study most of the endosperm losses could be noted as partials. Such an excess of partial endosperm mutants over whole ones has been reported earlier with ultraviolet radiation (KONZAK and SINGLETON 1956 and others), mustard gas (GIBSON *et al.* 1950), diepoxybutane (KREIZINGER 1960), and EMS and ultraviolet radiation (NEUFFER and FISCOR 1963). KONZAK and SINGLETON, working with gamma rays on maize pollen, noted a low frequency of partial endosperm mutations compared to whole ones, whereas we observed exclusively par-

tial endosperm losses with gamma rays on maize proembryos. The cause of the absence of whole endosperm losses in the present study is not known.

Various hypotheses for explaining the origin of sectoring in maize and *Drosophila* following ultraviolet or chemical treatment have been summarized by KREIZINGER (1960) in the study of diepoxybutane as a chemical mutagen in maize as follows. A mutagen may (1) affect a portion of the subunits of a multi-stranded chromosome; (2) interfere with chromosome duplication resulting in subsequent chromosome or chromatid breakage; (3) induce an unstable genic state; or (4) produce some effect on the chromosome matrix. She has suggested that diepoxybutane may exert an effect on the chromosome matrix which consequently renders it more difficult for chromatids or acentric fragments to separate. This effect would increase the frequency of restitution and sectoring when the entire chromosome is broken by the treatment. In this study, most of the EMS-induced endosperm losses were minute, occupying less than one sixth of the entire seed, whereas all the gamma ray-induced endosperm losses were larger, occupying more than one third of the entire seed. Moreover, there was a high proportion of minute V_1 losses of the streak type following EMS but not gamma-ray treatment (see Table 3). This type of minute loss of the dominant markers suggests that EMS operates at a finer level of chromosome organization or produces delayed effects in the plant. In this connection, it is noteworthy to mention that our cytological observations (unpublished) from mitotic divisions following aceto-carmine squash of stem tips of the EMS-treated seeds did not show any significant increase in chromosome aberrations over the control. This also could indicate that the chemical, having operated at a finer level of chromosome organization, does not produce sufficient large changes in chromosome structure to be detected microscopically.

The effect of EMS in producing mutations in crop plants such as barley and wheat has been studied (HESLOT *et al.* 1959; EHRENBERG *et al.* 1961; KONZAK *et al.* 1961; D'AMATO *et al.* 1962; and others). These authors have found it to be an efficient mutagen, producing many mutations and few chromosome aberrations. Present data show two important features of the EMS-induced losses of genetic markers: most endosperm losses included the multiple markers *Sh*, *Bz* and *Wx*, and most losses of the endosperm and seedling markers were very small. This type of EMS-induced multiple loss of endosperm markers, in higher proportion than single endosperm-marker losses, has also been noted in maize by NEUFFER and FISCOR (1963).

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SUMMARY

Maize proembryos 24 to 48 hours old were treated with ethyl methanesulfonate (EMS) and gamma rays to study genetic losses of several endosperm (*Sh*, *Bz*, *Wx*) and seedling (V_1 , Lg_1 , Gl_2) markers. EMS and gamma rays were applied

alone and in combination. Losses of the dominant marker genes were used to evaluate the genetic losses induced by these mutagens. Of the endosperm markers, single losses of either *Sh* or *Bz* were infrequent, and most of the losses included all the three markers. These multiple endosperm losses were partial rather than whole losses of the entire endosperm. Of the seedling markers, *V*₁ showed whole losses as well as partial and streak type losses in all the treatments; *Lg*₁ and *Gl*₂ showed mostly whole losses increasing with an increase in gamma dose. Multiple treatments with EMS and gamma rays showed partial losses of multiple endosperm markers, and whole and partial losses of seedling markers. The total rates of loss of the endosperm and seedling markers increased with EMS concentration and time of treatment. With gamma rays, however, only the loss of endosperm markers showed an increase with dose. With multiple treatments, losses of the endosperm and seedling markers, in most cases, increased with the treatment. Seedling markers are lost much more frequently than endosperm markers after EMS treatment but not after gamma ray treatment.

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