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Methyl methanesulfonate-induced mutations in spermatozoa and spermatids of the mouse

(Mus musculus) *)

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Methylmethansulfonat-induzierte Mutationen in Spermatozoen und Spermatiden der Maus

(Mus musculus)

S y n o p s i s : Dosen von 50 oder 75 mg Methyl-methansulfonat (MMS) pro kg Körpergewicht oder 37 mg NaCl pro kg Körpergewicht (Kontrolle, C) wurden Mäusemännchen des Inzuchtstammes "Tabby" in Form von 1 ml-Volumina intraperitoneal injiziert. MMS-behandelte wie Kontroll-Männchen wurden während der nächsten drei Wochen nacheinander mit unbehandelten C_3H-Inzuchtweibchen gepaart. Die aus dieser Kreuzung hervorgegangene F_1-Nachkommenschaft stammte von MMS-behandelten Spermatozoen (50-I, 75-I), späten (58-II, 75-II) oder frühen (50-III, 75-III) Spermatiden ab. Die F_1-Weibchen wurden mit 101-Inzuchtmännchen ausgekreuzt und mit ihren aus dieser Kreuzung stammenden Söhnen rückgekreuzt. Entsprechend wurden die F_1-Männchen mit 101-Inzuchtweibchen ausgekreuzt und mit ihren aus dieser Kreuzung stammenden Töchtern rückgekreuzt. Die F_1-Männchen der Serien 75-I und 75-III wurden mit C_3H-Inzuchtweibchen anstelle von 101-Weibchen ausgekreuzt. Für die Bestimmung der Häufigkeit toter Embryonen wurde der Uterusinhalt von 101-(75-I, C-I) und C_3H-(75-III, C-III) Weibchen nach deren Paarung mit F_1-Männchen untersucht. Alle Behandlungsgruppen und Generationen wurden mit entsprechenden Kontrollen verglichen.

*) Dedicated to Prof. Dr. H. Janetschek (Innsbruck)

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Ergebnisse: Durch die MMS-Behandlung wurden mehr späte als frühe Spermatiden und Spermatozoen abgetötet. Dominante Letalmutationen traten mit Ausnahme nach Behandlung von frühen Spermatiden mit 50 mg MMS pro kg Körpergewicht in allen Versuchsgruppen auf. Ein Teil der aus MMS-behandelten Spermatozoen (50 und 75 mg/kg) hervorgegangenen F_1-Weibchen war steril oder semisteril. Die Zahl der embryonalen Verluste lag bei den 101- und C_3H-Weibchen, die mit F_1-Männchen gepaart worden waren, die ihrerseits aus mit 75 mg/kg-MMS-behandelten Spermatozoen und Spermatiden abstammten, über der der Kontrollwerte. Hinweise auf die Induktion von autosomalen oder geschlechtschromosomengebundenen rezessiven Letalmutationen durch MMS wurden nicht gefunden.

Introduction:

Methyl methanesulfonate (MMS) belongs to the small number of chemical compounds the metabolism of which is known in the laboratory mouse (*Mus musculus*). This monofunctional alkylating substance reaches the testis in an active form, whereas ethyl methanesulfonate (EMS), a very similar alkylating agent, is rapidly hydrolysed *in vivo*. Thus, MMS is 4 - 6 times more effective in alkylating biological molecules. The amount of genetic damage in the mouse, as expressed by chromosome breakage, was shown to be correlated with *in vivo* alkylation of macromolecules (CUMMING and WALTON, 1970). Furthermore, molecular processes caused by alkylating substances are well documented. Four kind of alterations have been described in DNA treated with monofunctional alkylating agents such as MMS: alkylations of bases or phosphates, depurinations (FREESE, 1961; KRIEG, 1963), single-strand breaks in both bacteriophage and mammalian cells (FOX and FOX, 1969; FOX and FOX, 1972; STRAUSS, 1968) and interstrand crosslinking in methylated DNA after moderate heating at neutral pH (BURNOTTE and VERLY, 1972). The extent of methylation at the N-3 position of guanine was 0.5 m mole/mole DNA-P using MMS with the probable consequence of transition mispairing (LAWLEY, ORR and SHAH, 1972). MMS-induced transition mutations were also claimed in yeast (LOPRIENO et al., 1969) and *Neurospora* (MALLING and DE SERRES, 1969). Single base pair substitutions in yeast were demonstrated by STEWART et al. (1972) to occur through amino-acid replacement in iso-1-cytochrome c after application of MMS as well as after treatment with seven other mutagens. Both MMS and EMS had been shown to initiate repair synthesis in mammalian cells (BRANDT et al., 1972; BUHL and REGAN, 1972; CLARKSON and EVANS, 1971; FOX and AYAD, 1971; ROBERTS et al., 1971). Molecular processes resulting both in breakage of DNA strands and initiating of repair synthesis after MMS treatment are the prerequisite for the appearance of chromosomal rearrangements. Therefore mouse ova fertilized by sperm of MMS treated males were examined at the first cleavage stage for chromosomal aberrations by BREWEN et al. (1975) after intravenous injections with MMS doses of 25, 50, and 100 mg/kg body weight or with isotonic saline. The types of chromosomal aberrations seen were predominantly double fragments (presumably isochromatid deletions), chromatid inter-

changes, and some chromatid deletions, as well as shattering effect on the male complement at the highest dose and the time of peak sensitivity to dominant lethal induction. When the frequency of cells containing a cytologically visible aberration was compared to the total dominant lethal data an excellent correlation was obtained. These data strongly suggest that chromosome aberrations seen at the first cleavage stage of dividing zygotes are the basis of MMS-induced dominant lethality. Though many investigations were performed hitherto dealing with the induction of dominant lethal mutations in either male (BATEMAN, 1958; BATEMAN and EPSTEIN, 1971; EHLING, 1971, 1974; EHLING et al., 1968; EPSTEIN et. al., 1972; KRATOCHVILOVA, 1975; MACHEMER, 1975; MACHEMER and HESS, 1971; MOUTSCHEN, 1969), or female (GENEROSO, 1969; STOUT et al., 1972) mice after MMS-treatment, only two experiments were carried out to determine the rate of transmission of MMS-induced mutations to successive generations. These two investigations led to the detection of heritable partial sterility produced by MMS (JACKSON et al., 1964; LANG and ADLER, 1977). Information is also available on the transmissibility of induced mutations for the closely related alkylating compound ethyl methanesulfonate, EMS (CATTANACH et al., 1968; GENEROSO et al., 1974). This is why the present paper mainly concerns the mutagenic response of F_1, outcross and backcross generations in which also MMS-induced gene mutations are expected to become detectable. Because previous studies revealed that alkyl methanesulfonates induced dominant lethal mutations occur only in postmeiotic stages of spermatogenesis (spermatozoa and spermatids) while premeiotic male germ cells were not affected (EHLING, 1973), the present investigations are dealing with the mutagenic effects in offspring derived from the first three mating weeks after the treatment of male mice with MMS. According to the duration and timing of the mouse spermatogenesis (OAKBERG, 1956), these mating weeks correspond to the treatment of spermatozoa, late and early spermatids, respectively (Table 1). Spermatozoa and spermatids are known to be the most sensitive stages to the induction of mutations by various kinds of mutagens.

Table 1: Stages of spermiogenesis either treated with two dosages of MMS or NaCl

Dose of MMS or NaCl (1 ml)	Successive mating weeks after treatment of tabby males	Stage of male germ-cells treated	Symbols for the treatment groups
50 mg/kg of MMS, or 75 mg/kg of MMS, or 37 mg/kg of NaCl, respectively	1st week (1–7 days)	spermatozoa	50-I, 75-I, C-I
	2nd week (8–14 days)	mature spermatids	50-II, 75-II, C-II
	3rd week (15–21 days)	young spermatids	50-III, 75-III, C-III

Material and Methods:

I. Maintenance and Origin of the Mice:

All the mice were bred and kept under conventional conditions in Macrolon boxes of type no. 2. They were 10 - 12 weeks old when mated for the first time. The Macrolon boxes contained the animals which were collected for further breeding tests as well as the couples or dams with their young. The stable rooms were completely (Neuherberg) or partly (Innsbruck) air-conditioned, at a temperature of 24 ± 2 °C and an atmospheric humidity of 55 - 60%. The artificial illumination was set automatically to a 12-hour rhythm. By this means, the seasonally-caused variation in average litter-size did not reach a significant level. Inbred tabby males originally derived from the Edinburgh tabby stock and C_3H or 101 inbred mice and their offspring were used for the present experiments. Investigations carried out at the Zoological Institute of the University of Innsbruck were designated with the abbreviation IBK in brackets (IBK). The breeding conditions at both locations (Neuherberg and Innsbruck) were almost identical.

II. Mating Scheme:

Because of the semi-dominant marker gene «tabby» (Ta) located on the X-chromosome of the mice of the tabby strain, all female descendants of the cross C_3H-♀ (+/+) x tabby-♂ (Ta/Y) were expected to be heterozygous (Ta/+) while the male offspring were wild-type (+/Y) animals (Fig. 1). Thus, the F_1 females (Ta/+) contained a tabby-X chromosome symbolized by Ta which was treated in the germ cells of the father (Ta/Y). Among the progeny of F_1 females outcrossed with wild-type 101-males (+/Y) four phenotypes were expected to appear: wild-type (+/+) and heterozygous (Ta/+) daughters and tabby (Ta/Y) and wild-type (+/Y) sons. In the case of the presence of a recessive sex-linked lethal gene located on the replicas of treated tabby-X's, no tabby male (Ta/Y) would occur in this generation as described elsewhere (SCHRÖDER, 1971).

For the detection of recessive autosomal lethal mutations, however, F_1 females were backcrossed with their own sons and F_1 males with their own daughters, produced both through outcrossing of F_1 mice with non-related 101 mates (Fig. 1). This is because induced recessive mutations which are heterozygous and therefore undetectable in F_1 animals are expected to become homozygous in the backcross generations (LÜNING, 1971) causing a reduction of litter-size and/or an increase in preweaning mortality between birth and weaning at an age of about 4 weeks.

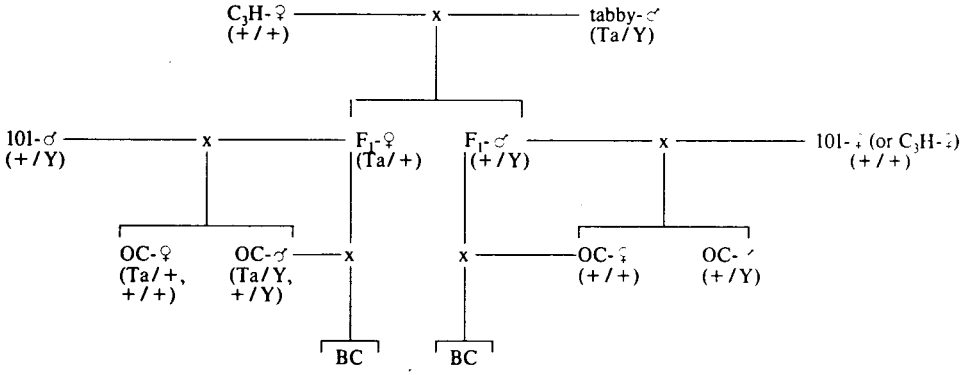

Fig. 1: Scheme of the experimental crosses performed in this study. OC, autcross generation; BC, backcross generation

III. Application of Methyl Methanesulfonate:

Methyl methanesulfonate (MMS), kindly provided by Dr. Ehling's laboratory, was intraperitoneally injected into each tabby male at doses of either 50 or 75 mg of MMS per kg body weight. The amounts were adjusted according to the weights of the animals. Injections were made with 1-ml volume of a 0.01225-molar MMS solution corresponding to 50 mg/kg of MMS or with 1-ml volume of 0.0186-molar solution corresponding to 75 mg/kg of MMS. For the controls, 1 ml of a 0.0186-molar saline solution corresponding to about 37 mg/kg of NaCl was intraperitoneally injected into each male mouse. Immediately after the injection, the treated and control tabby males were mated successively for three weeks to untreated C_3H inbred females. Each male was mated to two virgin females per week. The females were replaced by new ones for each mating week. Thus, three different stages of treated and control male germ cells fertilized the untreated ova of the C_3H females, i.e., spermatozoa, mature (late) and young (early) spermatids, corresponding to the first, second and third mating week, respectively (OAKBERG, 1956). Symbols for the three mating weeks and the different treatment groups are given in Table 1, breeding parameters scored for the present experiments are summarized by Table 2.

IV. Dissection of Pregnant Females:

To detect embryonic mortality in pregnant 101 and C_3H females which were mated to F_1 males (Fig. 1), the uterine contents of the females of the different treatment groups were compared to each other (cf. Table 1 and 2). 13.5 - 17.5 days after the appearance of a vaginal plug, the females were dissected. The numbers of corpora lutea (CL), implants (IMP), resorbed embryos (RES), early deaths (ED), late deaths (LD), and living embryos (LE) were determined as described elsewhere (SCHRÖDER, 1969; SCHRÖDER and HUG, 1971).

Table 2: Breeding parameters examined in the different treatment groups and generations.

Parameters examined	Treatment groups	Generations	
		Origin	Designation
Percentage fertile matings, mean litter-size, sex-ratio, preweaning mortality	50-I, 75-I, C-I; 50-II, 75-II, C-II; 50-III, 75-III, C-III	C_3H-♀ x tabby-♂	P-offspring (F_1)
	50-I, C-I	F_1-♀ x 101-♂	F_1-♀ offspring (outcross, OC)
Mean litter-size, sex-ratio, preweaning mortality	75-I, C-I; 50-II, 75-II, C-II; 75-III, C-III		
Percentage fertile matings, mean litter-size, sex-ratio	50-I, C-I	F_1-♂ x 101-♀	F_1-♂offspring (outcross, OC)
Percentage fertile matings, uterine content	75-I, C-I		
	75-III, C-III	F_1-♂ x C_3H-♀	
Percentage fertile matings, mean litter-size, sex-ratio	50-I, C-I	F_1-♀ x son (OC)	(F_1 x OC) - offspring (backcross, BC)
		F_1-♂ x daughter (OC)	

V. Statistical Calculations:

Analyses of variance were performed to compare the distribution frequency of females according to the number of living young at birth, corpora lutea (CL), implants (IMP), resorbed embryos (RES), early deaths (ED), late deaths (LD), and living embryos (LE) at dissection as well as with respect to the segregation ratio of ♀♀ ♂♂, ♀♀ tabby-♂♂, ♀♀ wild-type ♂♂, and tabby-♂♂ wild-type ♂♂ (SCHRÖDER, 1971) among the progeny of F_1 females. Mean values were compared by the use of 95%-confidence limits according to the t-test (WEBER, 1967). The statistical significance of differences concerning mean litter-size, sex-ratio, percentages of different phenotypes and preweaning mortality was also calculated by the standard chi square method or by chi square in a 2 x 2 table. Yates' correction was applied for small sample sizes (SACHS, 1973).

Results:

I. P-offspring (F_1 generation from C_3H-♀ x tabby-♂):

The percentage of *infertile matings* indicating the sterility of tabby males increased significantly only in 75-II as compared to C-II (Table 5). This effect may be due to cell killing of late (mature) spermatids which seem to be more sensitive to MMS-induced cell killing than early (young) spermatids and spermatozoa. With the only exception of 50-II (young spermatids), a significant *reduction of litter-size* was found in all treatment groups when compared with the corresponding controls (Table 3 - 6). However, a significant litter-size reduction of 25.6% in 50-I was detected only in the IBK experimental series (Table 3 and 4) but not in 50-I of the Neuherberg experiment (Table 5 and 6). Comparing the percentages of litter-size reduction relative to the control values for the different treatment groups, the following order of decreasing effectiveness was obtained: 75-II (70.98%) > 75-I (34.74%) > 75-III (28.92%) > 50-I (25.61%) > 50-II (24.55%). There were also significant differences of litter-sizes between the treatment groups 50-II *vs.* 75-II and 50-III *vs.* 75-III (Table 3 - 6).

No change of sex-ratio which would indicate dominant sex-linked lethal and detrimental mutations was found in either treatment group. Statistically significant *decreases of preweaning mortality* in 50-II, 50-III, and 75-III (Table 5) were probably due to the drastically reduced litter-size in these treatment groups which led to a better nutrition of the young by their dams than it was possible in the larger control litters. Only insignificant decreases of preweaning mortality were found in 50-I and 75-I (spermatozoal treatment) though there was also a significant reduction of litter-size in 75-I as compared to C-I.

Table 3: P offspring (F_1 from C_3H-♀x tabby-♂). Litters scored only at birth (IBK).

Treatment	No. of mated females	No. of pregnant females	% pregnant females	No. of litters	No. of offspring at birth			Mean litter size ± S.E.	♂♂ / (♂♂ + ♀♀) (%)
					total	♂♂	♀♀		
50-I	9	7	77.8	7	48	24	24	6.86 ± 0.83[a)]	50.00
C-I	9	9	100.0	9	83	40	43	9.22 ± 0.49[a)]	48.19

a) Significant difference (cf. Table 4).

Table 4: Distribution of P females according to the number of their offspring (IBK).

Treatment	Newborn mice per litter									
	3	4	5	6	7	8	9	10	11	12
50-I [a)]	1	0	1	0	2	1	2	0	0	0
C-I [a)]	0	0	0	0	0	4	2	1	1	1

[a)] Significant litter size reduction of **25.61%** in 50-I: $F = 6.6$ with $0.05 > p > 0.01$

Table 5: P offspring (F_1 from C_3H-♀ x tabby-♂). Litters scored at birth and weaning.

Treatment group	No. of mated males	No. of mated females	No. of pregnant females	% pregnant females	Living offspring at birth					Preweaning mortality			
					total	♂♂	♀♀	Mean litter-size + S.E.	♂♂ / (♂♂ + ♀♀) (%)	♂♂		♀♀	
										No.	%	No.	%
50-I	10	20	16	80.00	123	59	64	7.69 ± 0.66	47.97	3	5.09	7	10.94
75-I	10	20	12	60.00	71	42	29	5.92 + 0.78 [a)]	59.16	2	4.76	2	4.76
C-I	10	20	15	75.00	136	75	61	9.06 ± 0.55 [a)]	55.15	15	20.00	12	19.67
50-II	10	19	10	52.63	65	37	28	6.50 ± 0.70 [b)d)]	56.92	0 [h)]	0.00	0 [i)]	0.00
75-II	10	21	4 [g)]	19.05	10	5	5	2.50 ± 1.50 [c)d)]	50.00	2	40.00	1	20.00
C-II	10	20	13 [g)]	65.00	112	63	49	8.62 ± 0.38 [b)c)]	56.25	24 [h)]	38.10	12 [i)]	24.49
50-III	10	19	13	68.42	113	48	65	8.69 ± 0.56 [f)]	42.48	2 [j)]	4.17	3 [k)]	4.62
75-III	10	19	12	63.16	76	35	41	6.33 ± 0.68 [e)f)]	46.05	[l)] 1	2.86	[m)] 0	0.00
C-III	10	20	11	55.00	98	51	47	8.91 ± 0.87 [e)]	52.04	[l)] 19 [j)]	37.26	[m)] 15 [k)]	31.92

[a) - m)]: Significant differences; [a) - f)]: for p values cf. Table 6; [g)]: $\chi^2 = 5.22$ with $p = 0.02$; [h)]: $\chi^2 = 16.52$ with $p = 6.10^{-5}$; [i)]: $\chi^2 = 6.37$ with $p = 0.01$; [j)]: $\chi^2 = 14.28$ with $p = 0.0001$; [k)]: $\chi^2 = 11.29$ with $p = 0.0009$; [l)]: $\chi^2 = 11.90$ with $p = 0.0006$; [m)]: $\chi^2 = 13.59$ with $p = 0.00025$.

Table 6: Distribution of P females according to the number of their neonatal offspring in lst litters (cf. Table 5).

Treatment group	Newborn mice per litter												
	1	2	3	4	5	6	7	8	9	10	11	12	13
50-I	0	1	0	1	1	0	4	4	2	1	1	0	1
75-I [a)]	1	0	2	1	1	1	2	1	3	0	0	0	0
C-I [a)]	0	0	1	0	0	0	2	0	5	3	4	0	0
50-II [b) d)]	0	0	1	2	0	1	2	3	0	1	0	0	0
75-II [c) d)]	3	0	0	0	0	0	1	0	0	0	0	0	0
C-II [b) c)]	0	0	0	0	0	1	3	0	5	4	0	0	0
50-III [f)]	0	0	0	1	0	0	2	3	2	2	3	0	0
75-III [e) f)]	0	1	1	1	0	2	4	1	1	1	0	0	0
C-III [e)]	0	1	0	0	0	0	1	3	1	1	2	2	0

a) - f): Significant differences, i.e.
a): litter-size reduction of 34.74% in 75-I ($F = 11.50$; $p < 0.005$);
b): litter-size reduction of 24.55% in 50-II ($F = 7.86$; $0.01 < p < 0.05$);
c): litter-size reduction of 70.98% in 75-II ($F = 34.27$; $p < 0.005$);
d): litter-size reduction of 61.54% in 75-II ($F = 7.86$; $0.01 < p < 0.05$);
e): litter-size reduction of 28.92% in 75-III ($F = 5.57$; $0.01 < p < 0.05$);
f): litter-size reduction of 27.14% in 75-III ($F = 7.30$; $0.01 < p < 0.05$);

Table 7: F_1-♀offspring (OC from F_1-♀ x 101-♂). Litters scored only at birth (IBK).

Treatment	No. of mated females	No. of pregnant females	% pregnant females	No. of litters	No. of offspring at birth			Mean litter size ± S.E.	$\frac{♂♂}{♂♂+♀♀}$ (%)
					total	♂♂	♀♀		
50-I	11	6	54.5[a]	6	42	17	25	7.00 ± 1.53[b]	40.48[c]
C-I	12	12	100.0[a]	12	126	35	91	10.50 ± 0.31[b]	27.78[d]

a) Significant reduction (45.5%) of fertility in 50-I: $\chi^2 = 5.5$ with $p = 0.01$
b) Significant reduction (33.33%) of litter-size in 50-I: $\chi^2 = 5.3$ with $p = 0.02$
c) 1 F_1 dam with a sex-ratio amongst their offspring significantly different from the 1:1-ratio (10♀♀/2♂♂)
d) 2 F_1 dams with a sex-ratio amongst their offspring significantly different from the 1:1-ratio (11♀♀/0♂ and 9♀♀/1♂)

Table 8: Distribution of F_1 females according to the number of their offspring (IBK).

Treatment	Newborn mice per litter							
	3	4	5	7	9	10	11	12
50-I [a]	1	1	1	1	0	0	1	1
C-I [a]	0	0	0	0	2	5	2	3

a) Significant difference: $F = 9.45$ with $0.005 < p < 0.01$

Table 9: F_1-♀offspring (OC from F_1-♀ x 101-♂). Litters scored at birth and weaning (cf. Table 10).

Treatment group	No. of litters	Mean litter size ± S.E.	F_1-♀ offspring at birth							Preweaning mortality					
			Total	Ta/Y		+/Y		Ta/+; +/+		Ta/Y		+/Y		Ta/+; +/+	
			No.	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
75-I	36	7.53 ± 0.64[a)]	271	77	28.41	60	22.14	134	49.45	0	0.00	0	0.00	0	0.00
C-I	59	9.53 ± 0.40[a)]	562	162	28.83	138	24.56	262	46.62	1	0.62	0	0.00	1	0.38
50-II	36	9.28 ± 0.53	334	70	20.96	95	28.44	169	50.60	11[b)]	15.71	16[c)]	16.84	11[d)]	6.51
75-II	4	8.00 ± 1.35	32	5	15.63	9	28.13	18	56.25	0	0.00	0	0.00	0	0.00
C-II	46	8.85 ± 0.49	407	95	23.34	114	28.01	198	48.65	0[b)]	0.00	0[c)]	0.00	1[d)]	0.51
75-III	53	8.23 ± 0.39	436	114	26.15	110	25.23	212	48.62	4	3.51	1	0.91	0	0.00
C-III	34	9.29 ± 0.41	316	79	25.00	87	27.53	150	47.47	0	0.00	1	1.15	1	0.67

a) - d): Significant differences; [a)] $F = 7.76$ with $0.05 > p > 0.01$; [b)] $\chi^2 = 13.6$ with $p = 0.0002$;
[c)] $\chi^2 = 48.2$ with $p < 10^{-10}$; [d)] $\chi^2 = 8.6$ with $p = 0.0035$

Table 10: Distribution of F_1 females according to the number of their offspring at birth (cf. Table 9).

Treatment group	Newborn mice per litter															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
75-I [a)]	3	4	1	2	1	3	0	3	5	4	5	3	2	0	0	0
C-I [a)]	1	1	1	0	3	2	5	8	5	9	8	10	1	2	2	1
50-II	0	2	1	0	2	2	2	2	6	6	2	5	5	1	0	0
75-II	0	0	0	1	0	0	0	0	2	1	0	0	0	0	0	0
C-II	3	0	0	3	2	2	3	3	7	6	8	5	2	2	0	0
75-III	1	2	2	1	2	5	4	5	14	7	7	1	0	2	0	0
C-III	0	1	1	0	0	0	4	4	5	9	6	3	0	1	0	0

[a)] Significant litter size reduction of 20.97% in 75-I: $F = 7.76$ with $0.05 > p > 0.01$

Table 11: F_1-♂offspring (OC from F_1-♂x 101-♀). Litters scored only at birth (IBK).

Treatment	No. of mated females	No. of pregnant females	% pregnant females	No. of litters	No. of offspring at birth			Mean litter size ± S.E.	$\frac{♂♂}{♂♂ + ♀♀}$ (%)
					total	♂♂	♀♀		
50-I	19	19	100.0	19	129	75	54	6.79 ± 0.54	58.14
C-I	6	6	100.0	6	37	18	19	6.17 ± 0.65	48.65

Table 12: Distribution of 101 females according to the number of their offspring at birth (IBK).

Treatment	Newborn mice per litter								
	1	2	4	5	6	7	8	9	10
50-I	1	1	1	0	3	5	5	1	2
C-I	0	0	0	3	1	1	0	1	0

Table 13: F_1-♂-offspring (OC). Numerical values of the uterine contents of 101- or C_3H-females after mating them to F_1-males (cf. Table 14).

Treatment group	CL		IMP		RES		ED		LD		LE		POSTIMPLANTATIONAL LOSSES		PREIMPLANTATIONAL LOSSES	
	No.	per ♀ ± S.E.	No.	per ♀ ± S.E.	No.	per ♀ ± S.E.	No.	per ♀ ± S.E.	No.	per ♀ ± S.E.	No.	per ♀ ± S.E.	No.	per ♀ ± S.E.	No.	per ♀ ± S.E.
75-I [a]	383	9.82 ± 0.25	230	5.90 ± 0.38	19	0.49 ± 0.14	6	0.15 ± 0.07	0	0.00	205	5.26 ± 0.40	25	0.64 ± 0.16	153	3.92 ± 0.45
C-I [b]	592	10.76 ± 0.26	420	7.64 ± 0.24	23	0.42 ± 0.10	13	0.24 ± 0.06	1	0.02 ± 0.02	383	6.96 ± 0.27	37	0.67 ± 0.12	172	3.13 ± 0.35
75-III [c]	242	10.52 ± 0.26	215	9.35 ± 0.32	17	0.74 ± 0.20	6	0.26 ± 0.13	2	0.09 ± 0.06	190	8.26 ± 0.37	25	1.09 ± 0.25	27	1.17 ± 0.41
C-III [d]	280	9.03 ± 0.34	229	7.39 ± 0.33	2	0.07 ± 0.05	6	0.19 ± 0.07	0	0.00	221	7.13 ± 0.32	8	0-26 ± 0.08	51	1.65 ± 0.48

[a] 40 mated females, 39 pregnant females
[b] 57 mated females, 55 pregnant females
[c] 23 mated females, 23 pregnant females
[d] 31 mated females, 31 pregnant females

Table 14: Distribution for the dissection data of 101 (75-I and C-I) and C_3H (75-III and C-III) females after mating them to F_1 males (cf. Table 13).

	Treatment groups	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
CL	75-I	–	–	–	–	–	0	0	4	3	10	7	9	6	0	0	0
	C-I	–	–	–	–	–	0	0	0	6	10	12	8	8	6	3	2
	75-III	–	–	–	–	–	0	0	0	0	4	9	7	1	1	1	0
	C-III	–	–	–	–	–	2	0	2	9	8	3	3	3	1	0	0
IMP	75-I	–	2	0	3	6	6	5	11	2	1	1	1	1	–	–	–
	C-I	–	0	1	0	1	3	11	8	12	10	8	1	0	–	–	–
	75-III	–	0	0	0	0	1	1	0	1	9	7	3	1	–	–	–
	C-III	–	0	0	0	2	4	4	4	8	7	1	0	1	–	–	–
RES	75-I	26	10	1	1	1	–	–	–	–	–	–	–	–	–	–	–
	C-I	38	13	2	2	0	–	–	–	–	–	–	–	–	–	–	–
	75-III	12	7	2	2	0	–	–	–	–	–	–	–	–	–	–	–
	C-III	29	2	0	0	0	–	–	–	–	–	–	–	–	–	–	–
ED	75-I	34	4	1	–	–	–	–	–	–	–	–	–	–	–	–	–
	C-I	43	11	1	–	–	–	–	–	–	–	–	–	–	–	–	–
	75-III	19	2	2	–	–	–	–	–	–	–	–	–	–	–	–	–
	C-III	25	6	0	–	–	–	–	–	–	–	–	–	–	–	–	–
LD	75-I	39	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	C-I	54	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	75-III	21	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	C-III	31	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–
LE	75-I	–	3	1	7	5	4	6	9	1	1	0	1	1	–	–	–
	C-I	–	0	1	2	0	10	11	10	7	8	5	1	0	–	–	–
	75-III	–	0	0	0	1	0	2	5	5	4	3	3	0	–	–	–
	C-III	–	0	0	0	4	2	4	6	9	4	1	1	0	–	–	–

Table 15: Analysis of variance made for the dissection data of 101 (75-I and C-I) and C_3H (75-III and C-III) females after mating them to F_1 males (cf. Tables 13 and 14).

Comparison between treatment groups	CL		IMP		RES		ED		LD		LE	
	F	p	F	p	F	p	F	p	F	p	F	p
75-I *vs.* C-I	6.48	> 0.01 < 0.05	73.20	< 0.005	0.17	> 0.05	0.75	> 0.05	0.64	> 0.05	13.68	< 0.005
75-III *vs.* C-III	3.71	> 0.05	17.07	< 0.005	14.01	< 0.005	0.23	> 0.05	2.86	> 0.05	5.24	> 0.01 < 0.05
75-I *vs.* 75-III	3.46	> 0.05	39.32	< 0.005	1.10	> 0.05	0.64	> 0.05	3.63	> 0.05	25.29	< 0.005
C-I *vs.* C-III	16.59	< 0.005	0.37	> 0.05	6.67	> 0.01 < 0.05	0.18	> 0.05	0.50	> 0.05	0.15	> 0.05

II. F_1-♀ offspring (outcross generation from F_1♀ x 101-♂):

Unsuccessful matings caused by the sterility of F_1 females increased significantly only in 50-I (Table 7). No change of the percentage of fertile matings was found in all the other treatment groups. Significant *reductions of litter-sizes* were observed both in 50-I and 75-I relative to C-I. No statistically relevant difference for the *segregation ratio* of the three phenotypes distinguishable at birth among the offspring of F_1 females was detected between the treatment groups (Table 9). An increase of *preweaning mortality* for both female and male offspring was observed in 50-II. No change of *sex-ratio* in either direction could be obtained between the different groups. The significant difference in sex-ratio found between 50-I and C-I was caused by one F_1 female in 50-I with 10 daughters and only 2 sons, and two F_1 females in C-I with a progeny of 9 daughters and 1 son in one case and with only female offspring in the other case (Table 7). No significant effect for any breeding character was found in 75-II and 75-III.

III. F_1-♂ offspring (outcross generation from F_1 - ♂ x 101 - or C_3H - ♀)

No effect on *fertility of F_1 males* was found in all treatment groups tested (50-I, 75-I, and 75-III). *Litter-size* and *sex-ratio* were scored only in 50-I, where no difference relative to C-I could be detected (Table 11 and 12). The determination of the *uterine content* was performed in 75-I, 75-III and the corresponding controls (C-I and C-III). Significant differences between the different treatment groups were found as follows: *Corpora lutea (CL):* reduction (8.74%) in 75-I *vs.* C-I, increase (19.16%) in C-I *vs.* C-III; *implants* (IMP): reduction (22.78%) in 75-I *vs.* C-I, increase (26.52%) in 75-III *vs.* C-III, decrease (36.90%) in 75-I *vs.* 75-III; *resorbed embryos* (RES): increase (957.14%) in 75-III *vs.* C-III; increase (500%) in C-I *vs.* C-III; *living embryos (LE):* reduction (24.43%) in 75-I *vs.* C-I, increase (15.85%) in 75-III *vs.* C-III, increase (57.03%) in 75-I *vs.* 75-III; *postimplantational losses* (RES + ED + LD): increase (70.31%) in 75-III *vs.* 75-I; *preimplantational losses* (CL-IMP): increase (235.04%) in 75-I *vs.* 75-III, increase (137.58%) in 75-I *vs.* C-III (Table 13 - 15). KRATOCHVILOVA (1975) has recently shown that preimplantational losses of mouse ova fertilized by MMS - treated sperm were really caused by dominant lethal mutations. Two formulae were used to calculate the all-over dominant lethal rate in mice, i.e.,

$$100 - \left(\frac{\text{L. E. per pregnant female in the experimental group}}{\text{L. E. per pregnant female in the control group}} \times 100 \right)$$

according to EHLING et al. (1968), and

$$\frac{\text{L.E. per C.L. for experimental females}}{\text{L. E. per C. L. for control females}} \times 100$$

according to GENEROSO (1969).

Taking these two formulae to determine the *frequency of embryonic losses* in 101 or C_3H females mated to F_1 males from the dissection data (Table 13), different percentages were obtained: 24.4% for 75-I and -15.8% for 75-III using the first formula, and 82.69% for 75-I and 99.49% for 75-III according to the second formula. The differences between 75-I and 75-III as well as between C-I and C-III are likely due to the different females used in these treatment groups (101 in 75-I and C-I, and C_3H in 75-III and C-III, respectively).

IV. Recessive sex-linked lethal and detrimental mutations:

As described previously (SCHRÖDER, 1971), calculations concerning the ratio of ♀♀/♂♂, ♀♀/tabby-♂♂, ♀♀/wild - type ♂♂, and tabby-♂♂ wild-type ♂♂ may be useful to search for recessive sex-linked lethal and detrimental mutations amongst the offspring of F_1 dams. Altogether 135 X-chromosomes of the MMS-groups (6 in 50-I, 36 in 75-I, 36 in 50-II, 4 in 75-II, and 53 in 75-III) and 151 X's of the controls (71 in C-I, 46 in C-II and 34 in C-III) were screened by this method for significant deviations between the treatment groups. Because no significant change could be detected between any of the different groups, these data were not included in Table 7 - 12. Accordingly, the search for recessive X-linked lethal and detrimental mutations after the treatment of spermatozoa and late and early spermatids with 50 and 75 mg/kg of MMS was not successful.

V. Backcross generations (F_1 x OC-offspring):

F_1 - ♀ x son (Table 16)

F_1 females of the 50-I and C-I series (IBK) were backcrossed with their own sons and were allowed to produce at least 20 offspring. The corresponding sons were obtained from the preceding progeny of the same F_1 females after outcrossing them with 101 males. Sterility, litter-size and sex-ratio were determined in the backcross generation but no significant difference between 50-I and C-I was observed.

F_1 - ♂ x daughter (Table 17)

F_1 males of the 50-I and C-I series (IBK) were backcrossed with their own daughters and were allowed to produce at least 20 offspring. The daughters were derived from the preceding crosses of the same F_1 males with 101 females. No changes in fertility, litter-size and sex-ratio between the 50-I and C-I backcross generations were found.

Table 16: Backcross-generation derived from the mating of the F_1-♀ to her OC-son. Litters scored only at birth (IBK).

Treatment	No. of mated F_1-females	No. of pregnant F_1-females	No. of mated OC-males	No. of fertile OC-males	No. of litters	No. of offspring at birth			Mean litter size ± S.E.	$\frac{♂♂}{♂♂+♀♀}$ (%)
						total	♂♂	♀♀		
50-I	7	6	8	8	18	167	97	70	9.28 ± 0.82	58.08
C-I	5	5	5	5	11	109	68	41	9.91 ± 0.97	62.39

Table 17: Backcross-generation derived from the mating of the F_1-♂ to his OC-daughter. Litters scored only at birth (IBK).

Treatment	No. of mated OC-females	No. of pregnant OC-females	No. of mated F_1-males	No. of fertile F_1-males	No. of litters	No. of offspring at birth			Mean litter size ± S.E.	$\frac{♂♂}{♂♂+♀♀}$ (%)
						total	♂♂	♀♀		
50-I	24	24	11	11	63	589	310	279	9.35 ± 0.30	52.63
C-I	6	6	4	4	18	158	82	76	8.78 ± 0.69	51.90

Discussion:

I. Killing of male germ cells:

A reduction of the proportion of pregnant females after mating them to MMS-treated males indicated cell killing of male germ cells. Such an effect was obtained at a significant level only after treatment of mature spermatids with 75 mg/kg of MMS. No cell killing effect of MMS on spermatozoa and young spermatids was found.

II. Induction of dominant lethal mutations:

Since in irradiation experiments the reduction of the mean litter-size completely corresponds to the frequency of dominant lethal mutations as determined by the examination of the uterus contents (SCHRÖDER, 1969, 1971), the litter-size reductions found in the present investigation may also reflect the rate of dominant lethals induced by the treatment of spermatozoa and spermatids with MMS. Significant reductions of the mean litter-size relative to the corresponding controls were obtained in all treatment groups (50-I, 75-I, 50-II, 75-II, 75-III) except after 50 mg/kg of MMS to early spermatids (50-III). These results agree sufficiently with previous data found in a collaborative study done in six different European laboratories (MACHEMER, 1975; LANG and ADLER, 1977).

III. Sterility of F_1 mice:

Sterility of F_1 mice leads to an increase of infertile matings in those groups the parents of which were treated with effective mutagens. Only after 50 mg/kg of MMS to spermatozoa the incidence of sterile matings of F_1 females relative to the control - F_1 was significantly enhanced. No change was found for F_1 males of the same group as well as for F_1 mice derived from MMS - treated early and late spermatids.

IV. Semisterility of F_1 mice:

Like sterility also semisterility of F_1 mice originated from MMS - treated male germ cells is due to reciprocal translocations (EPSTEIN et al., 1972; LANG and ADLER, 1977; LEONARD, 1973; GENEROSO et al., 1974) and results in less young per litter than produced by the corresponding controls. Thus, F_1 females derived from MMS - treated spermatozoa (50-I and 75-I) produced significantly smaller litters than the corresponding F_1 - control females. However, no reduction of the litter-sizes was found when F_1 males were used as mates though the incidence of chromosomal rearrangements of F_1 males in 75-II exceeded that of the controls (LEONARD, 1973). On the other hand, the examination of the uterine contents of untrea-

ted 101 (75-I) and C_3H (75-III) females crossed with F_1 males revealed an increase of embryonic losses.

V. Preweaning mortality of the outcross generations:

Heterozygosity for chromosome mutations could also cause a reduction of the viability of the animals in question which can ben expressed as an increase of preweaning mortality during the first four weeks since birth. This was found to be true only in the outcross generation from the cross of F_1 females with 101 males of 50-II, i.e., after 50 mg/kg of MMS to late spermatids.

VI. Differential reproductive capacity of female mice:

As shown by the comparison of the numbers of CL and RES between the control series, i.e., C-I *vs.* C-III (cf. Table 15), the different results of the examination of the uterus contents between C_3H and 101 females both mated to F_1 males (cf. Table 2 and 13-15) perhaps reflect not only different mutagenic response of spermatozoa and early spermatids to the induction of heritable translocations but also differences in the reproductive capacity of the females of the two inbred strains used.

VII. Recessive mutations:

According to LÜNING's scheme, F_1 mice were backcrossed with their own children in order to detect possibly induced recessive autosomal mutations which were expected to become homozygous in the backcross generations. However, this procedure was carried out only for 50-I and the sample size was too small to allow general conclusions. No changes in fertility, litter-sizes and sex-ratio among the 50-I and C-I backcrosses could be observed indicating the lack of induced recessive autosomal lethals. Of course, not only recessive lethals but also recessive visibles should become homozygous and therefore detectable in this generation. As it was also true for the induction of recessive sex-linked lethal and detrimental mutations, no recessive visible mutation was found.

Thus, the only type of mutation which occurred after MMS - treatment of spermatozoa, late and early spermatids in this study was highly probable due to chromosomal rearrangements. BREWEN et. al. (1975) demonstrated that double fragments, chromatid interchanges, and some chromatid deletions as well as shattering effects on the male complement were induced in ova at the first cleavage stage fertilized by MMS - treated mouse spermatozoa and spermatids. Because the amount of genetic damage expressed as chromosome breakage in the mouse is correlated with *in vivo* alkylation of macromolecules (CUMMING and WALTON, 1970), the molecular mechanisms of DNA changes by MMS (cf. Introduction) may also be responsible for the mutagenic effects reported in the present study. Moreover, the extensive

experiments carried out by EHLING et al. (EHLING, 1973, 1974; EHLING and NEUHÄUSER, 1972; EHLING and RUSSELL, 1969; EHLING et al., 1968) with alkyl methanesulfonates and by CATTANACH et al. (1958, 1966, 1967, 1971) with TEM (2,3, 6-tris(1-aziridinyl)- s-triazine) support the assumption that both mutations at seven specific loci from wild-type alleles to the corresponding recessive alleles, or, more probably, to the null alleles (i.e., to small deletions) and chromosome aberrations (LANG and ADLER, 1977; LEONARD and LINDEN, 1972) were induced in the same germ-cell stages of the mouse. Accordingly, all mutational changes so far induced by MMS (such as specific locus mutations, dominant lethals, sex-chromosome losses, reciprocal translocations, sterility and semisterility) seem to have the same cytological basis, i.e., they are due to chromosome breaks.

Summary:

Inbred male tabby mice were intraperitoneally injected with 50 or 75 mg of methyl methanesulfonate (MMS) per kg body weight or with 37 mg/kg of NaCl (controls, C). Treated and control males were mated to untreated C_3H females for three successive mating weeks corresponding to the treatment of spermatozoa (50-I, 75-I), late (50-II, 75-II) and early (50-III, 75-III) spermatids with MMS. The resulting F_1 females were outcrossed with 101 inbred males producing an outcross generation (OC), and then backcrossed with their own OC sons. Correspondingly, the F_1 males were outcrossed to either 101 inbred females, also producing an outcross generation, and then backcrossed with their own OC daughters, or outcrossed with C_3H inbred females instead of 101 females. For the determination of the frequency of dead embryos, the uterus contents of 101 (75-I, C-I) and C_3H (75-III, C-III) females were examined after mating them to F_1 males. All treatment groups and generations were compared with the corresponding controls.

Results: Late spermatids were more sensitive to cell killing by MMS than early spermatids and spermatozoa. Dominant lethal mutations expressed by the reduction of mean litter-size were obtained in all treatment groups except after 50 mg/kg of MMS to early spermatids. Sterility and semisterility was found in F_1 females conceived after 50 and 75 mg/kg of MMS to spermatozoa. The amount of embryonic losses exceeded that of the controls in 101 and C_3H females after mating them to F_1 males derived from spermatozoa and young spermatids treated with 75 mg/kg of MMS. No indication for the induction of autosomal or sex-linked recessive lethal mutations was found.

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