MUTAGENIC ACTION OF ETHYL METHANESULPHONATE IN OOGENESIS OF THE SILKWORM, *BOMBYX MORI* L.¹⁾

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Ethyl methanesulphonate (EMS) is a monofunctional alkylating agent used as a potential mutagen. It brings about intragenic changes by ethylation of guanine or adenine and hydrolysis of these ethylated bases (Fishbein *et al.* 1970; Loveless 1966). Studies on EMS mutagenicity in *Bombyx* have mainly been carried out under conditions of spermatogenesis (Inagaki and Oster 1969; Kondo and Ikenaga 1963; Onimaru and Tazima 1968; Tazima and Onimaru 1968; Tazima *et al.* 1967). The characteristics of EMS mutagenicity in spermatogenic cells of *Bombyx* are that post-meiotic cells were much more sensitive to the chemical than pre-meiotic cells and the majority of mutations recovered was mosaics as observed in *Drosophila* (Jenkins 1967b). Little is known about the mutagenic effects of EMS on the cells in oogenesis of *Bombyx* (Kondo and Ikenaga 1963).

A remarkable biological difference exists in *Bombyx* between female and male in the time required for the post-meiotic stage of gametogenesis. In oogenesis, meiotic divisions in eggs (or oocytes) complete 120 min after oviposition at 25°C. The oocytes enter successively into pronuclear stages by passing through post-meiotic stages (or ootids) for only a few minutes. Syngamy takes place in eggs 150 min after oviposition (Ohtsuki and Murakami 1968). On the other hand, at least two weeks are required for post-meiotic stages in spermatogenesis (e.g., Tazima 1964). Therefore, it is of interest to know whether EMS has similar effects on the spermatogenic and oogenic cells.

Since crossing-over does not normally occur in female *Bombyx* (Tanaka 1913), they can be used to exactly determine the genetical nature of experimentally-induced mutations. The present experiment was carried out with cells in oogenesis in order to elucidate the mechanisms of EMS mutagenicity in *Bombyx*. The results indicate that EMS is a very strong mutagen in pupal oocytes, but not in larval oocytes. A predominance of mosaic mutations occurred in the EMS-treated pupal oocytes, while complete mutations were rare in them. A slight but statistically significant tendency for double and triple mosaics appeared among the recovered mosaic mutants. The present finding and others suggest that EMS is mutagenic in premeiotic oocytes, pronuclei and cleavage nuclei. This paper presents some of the data reported in preliminary communications (Murakami 1971b, 1972a).

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MATERIALS AND METHODS

0.025 ml of 0.1% EMS dissolved in 0.85% NaCl was injected into the dorsal abdomen of C108 wild type female *Bombyx* in the following stages of development: 5th and 7th day larvae of the 5th instar and 6th and 11th day pupae. In addition, 0.05% and 0.5% EMS solutions were administered to 11th day pupae.

According to the cytological observation by Kawaguchi (1928), some germ-cells in *Bombyx* female larvae after the third molting have reached the early growth stages, leptotene, zygotene, pachytene and diplotene. The middle growth stage begins in larvae two or three days before pupation and terminates at the middle pupal stage. The last growth stage beings at the middle pupal stage and extends to the time of the emergence of the adults. Oocytes at this growth stage contain diakinesis and tetrad chromosomes. At the late pupal oocytes enter the first meiotic division.

The egg color specific locus method was used to determine the incidence of mutations. The egg color of the wild type strain is black. The *pe* locus is expressed as yellowish-white eggs (V-0.0) and the *re* locus is expressed as red eggs (V-31.7). The *pe* locus is epistatic to the *re* locus, so that a mutant with a double mutational event at both loci in the same chromosome is expressed as yellowish-white eggs. After the treated larvae and pupae emerged, they were crossed to marked male moths homozygous for *pe* and *re* loci. The number of complete or mosaic mutants in oviposited F₁ eggs colored either yellowish-white or red was counted and used to determine the mutation frequency.

RESULTS AND DISCUSSION

Differential stage sensitivity

The result of experiments is summarized in Table 1. A significant difference was observed in the mutagenic effect of EMS between larval and pupal oocytes as well as between young (6th day) and aged (11th day) pupal oocytes. No remarkable mutagenic effect of EMS was observed in 5th and 7th day larval oocytes in the 5th instar. In contrast, EMS was strongly mutagenic in pupal oocytes. Mutations were observed much more frequently in the 11th day pupae than in the 6th day pupae.

It is of interest to note the differential sensitivity of the *Bombyx* oocytes at different stages of oogenesis to the mutagenic effects of EMS. *Bombyx* oocytes approaching meiosis may lack nuclear membranes as revealed in *Drosophila* oocytes (Cummings and King 1969). Such a cytological event may also apply to pronuclei, cleavage nuclei as well as post-meiotic cells in spermatogenesis. Whether the presence or absence of nuclear membranes may account for the differential sensitivity of cells to the mutagenic effects of EMS. In addition, it could not completely rule out the possibility that the differential sensitivity to EMS may be due to a differential repair mechanism with the growth of oocytes (Schewe et al. 1971). In *Drosophila* spermatogenesis, however, the differential sensitivity to the mutagenic effects of EMS was ascribed to a cytoplasmic barrier in the cells (Jenkins 1967a).

Table 1. Frequencies of EMS-induced complete and mosaic mutations in oocytes at different developmental stages of the silkworm, *Bombyx mori*

Stages	Dose in %	Total No. of eggs observed	Number of mutants					
			<i>pe</i> locus		<i>re</i> locus		Complex mosaics	
			Complete	Simple mosaics	Complete	Simple mosaics	Triple (<i>pe:re:+</i>)	Double (<i>pe:re</i>)
5th instar, 5th day larvae (middle growth stage oocytes)	0.1	60,391	7 (11.6)	16 (26.5)	2 (3.3)	0 (0.0)		
5th instar, 7th day larvae (middle growth stage oocytes)	0.1	37,932	6 (15.8)	11 (29.0)	1 (2.6)	5 (13.2)		
6th day pupae (late growth stage oocytes)	0.1	48,403	6 (12.4)	91 (188.0)	3 (6.2)	36 (74.4)		
11th day pupae or the day before emergence (meiotic prophase I oocytes)	0.05	41,850	4 (9.6)	59 (140.8)	2 (4.8)	22 (52.6)	0 (0.0)	0 (0.0)
"	0.1	40,950	6 (14.7)	153 (373.6)	3 (7.3)	57 (139.2)	1 (2.4)	0 (0.0)
"	0.5	24,940	9 (36.1)	603 (2417.8)	2 (8.0)	447 (1792.3)	36 (144.3)	2 (8.0)
Control*	0.0	100,762	11 (10.9)	4 (4.0)	5 (5.0)	0 (0.0)	0 (0.0)	0 (0.0)

The numbers in parentheses indicate mutation frequencies ($\times 10^{-5}$).
* 8th day pupae of the wild type C108 strain silkworm were injected with 0.025 ml of 0.85% NaCl solution (cf. Murakami 1972b).

Another point of interest is the remarkably high incidence of mutations in EMS-treated pre-meiotic pupal oocytes (prophase I). The frequency of EMS-induced mutations in 11th day pupal oocytes was almost equal to that observed in mature sperm which showed the highest frequency of EMS-induced mutations throughout the whole spermatogenesis in *Bombyx* (Tazima et al. 1967; Tazima 1968; Tazima and Onimaru 1968). This may appear to be incompatible with the finding that EMS was less mutagenic in pre-meiotic spermatocytes than in post-meiotic germ-cells. However, a sex difference in EMS mutagenicity may be expected since the time interval required for the post-meiotic stage in *Bombyx* females is much shorter than that in males.

Preponderance of mosaic mutations

Table 2 shows the number of complete, mosaic and complex mosaic mutants found in 11th day pupal oocytes treated with EMS. Mosaic mutants with small (less than 50%) mutant cell areas were four times more frequent than those with large (more than 50%) mutant cell areas. Theoretically, mutations that occur in oocytes would tend to result in either complete mutants or mosaics with the large mutant cell area, while those occur in cleavage nuclei would result in mosaics with the small mutant cell area. Consequently, it can be possible to speculate that either a delayed mutagenic effect or a delayed drug-action effect may have been responsible for the high proportion of small mutant cell area mosaic mutations in the 11th day pupal oocytes treated with EMS.

Table 2. Classification of mutant cell areas in mosaic mutants induced with 0.5% EMS in 11th day pupal oocytes of the silkworm

Locus	Complete	Mutant cell areas in mosaic		Complex mosaics	
		>1/2	<1/2	Triple (<i>pe:re</i> :+)	Double (<i>pe:re</i>)
<i>pe</i>	9	113	490	36	2
<i>re</i>	2	49	398		

The delayed mutagenic effect is a characteristic feature of chemical mutagenesis in various organisms ranging from phage (*E. coli*) (Green and Krieg 1961) to *Drosophila* (Auerbach 1946; Alderson 1965). This effect may be applicable to EMS mutagenicity in *Bombyx* oogenesis as well as spermatogenesis (Kondo and Ikenaga 1963). If so, some of the effect of EMS-induced premutational damage in pupal oocytes would be stabilized as mutations at least after one cycle of DNA replications or cleavage divisions. This would lead to mosaic mutations with small mutant cell areas such as those recovered in the EMS-induced 11th day pupal oocytes. According to Onimaru and Tazima (1968), they indicated that the delayed mutagenic effect can appear in several generations after treatment with EMS or mictomycin C in *Bombyx* males.

A delayed drug-action effect may have contributed to the induction of predominant mosaic mutations in the pupal oocytes. EMS which incorporated into oocytes during vitellogenesis and/or before completion of egg-shell formation may be an effective

mutagen in both egg pronuclei and cleavage nuclei. However, the half-life of EMS in aqueous media is 48.5 hr at 25°C (Froese-Gertzen et al. 1963). A much long half-life is required for EMS in the cytoplasm of oocytes in order to induce mutations by a delayed drug-action effect.

Other explanations to account for the predominance of mosaic mutations in *Bombyx* females are that EMS acts on dominant genes only during the developmental stages after fertilization (Kondo and Ikenaga 1963) or that the EMS-induced mutation is due to a change in one of the paired complementary polynucleotide chains (Kondo and Ikenaga 1963). On the other hand, the minority of recovered mosaic mutations having large mutant cell areas may have arisen from mutational events in either meiotic stage oocytes or very early cleavage cells. Since germ-cell mutations that occur at the stage of the oocytes would be generally expressed as large mutant cell areas.

Transmissibility of induced mutants

The recovered mosaic mutants were checked for transmission and gonadal mosaicism by remating to marked strain moths homozygous for the *pe* or *re* gene locus. According to this test, the gonadal mosaic moths would produce more yellowish-white or red eggs than non-mutant black eggs, while non-gonadal mosaic moths would produce both yellowish-white or red eggs and non-mutant black eggs with a segregation ratio of 1 to 1.

Table 3 shows the results of the transmission and gonadal mosaicism test for mosaic mutants derived from the EMS-treated 11th day pupae. Approximately one-third (11/31) of the tested mosaic mutants produced significantly more yellowish-white or red eggs than non-mutant black eggs. This class of gonadal mosaic mutants was almost of the large mutant cell area type as expressed as the mutational incidence depending on either the stage of oocytes or the earlier cleavage stage. While non-transmitted mosaic mutants were of the small mutant cell area type as expressed as the mutational incidence depending on the later cleavage stage. The mutant character of the complete mutations was transmitted, without exception, into the next generation. The results of the transmission test suggest that a number of EMS-induced mosaic mutants in pupal oocytes would have been resulted from mutational events (either induction of mutations or fixation of premutational damages) at the cleavage stage.

Origin of multiple mosaic mutants

This section will discuss the origin of complex mosaic mutants and the time of occurrence of the final mutational event induced by EMS in the late pupal oocytes. Two different types of mosaics were observed. One type was a double mosaic mutant composed of both yellowish-white (*pe*) and red (*re*) cell-lines simultaneously. The other was a triple mosaic mutant made up of all three phenotypes simultaneously: i.e., yellowish-white (*pe*), red (*re*) and black (+) cell-lines.

It is now well accepted that a gene consists of one paired complementary polynucleotide chain on one chromatid in any type of higher organisms. Ito and his co-workers proposed that changes in both of the paired complementary chains of a gene lead to whole-body (complete) mutations, while changes in one of the paired complementary polynucleotide chains lead to fractional-body (mosaic) mutations (Ito 1967). If

Table 3. Transmission and gonadal mosaicism tests for EMS-induced mosaic mutants

Expt.	Locus	Sex	Mutant cell area	Number of eggs observed				χ^2
				+	<i>pe</i>	<i>re</i>	Total	
1	<i>pe</i>	male	L	72	71		143	0.007
2	"	"	"	190	212		402	1.204
3	"	"	"		260		260	260.000*
4	"	"	S	94	90		184	0.087
5	"	"	"	136	160		296	1.946
6	"	"	"	101	218		319	142.912*
7	"	"	"	11	13		24	0.167
8	"	female	L	132	345		477	95.113*
9	"	"	"	314	343		657	1.280
10	"	"	"	195	349		544	43.595*
11	"	"	"	171	314		485	42.163*
12	"	"	S	274	345		619	8.144*
13	<i>re</i>	male	L	15		60	75	27.000*
14	"	"	S	103		97	200	0.180
15	"	"	"	41		37	78	0.205
16	"	"	"	183		196	379	0.446
17	"	"	"	52		46	98	0.367
18	"	"	"	192		221	413	2.036
19	"	"	"	104		264	368	69.565*
20	"	"	"	114		158	272	7.118*
21	"	"	"	144		125	269	1.342
22	"	"	"	179		175	354	0.045
23	"	female	L	113		369	482	135.967*
24	"	"	"	65		311	376	160.947*
25	"	"	S	264		240	504	1.143
26	"	"	"	260		246	506	0.387
27	"	"	"	204		214	418	0.239
28	"	"	"	274		260	534	0.367
29	"	"	"	180		211	391	2.458
30	"	"	"	311		301	612	0.163
31	"	"	"	292		286	578	0.062

L: Large (more than 1/2) mutant cell area. S: Small (less than 1/2) mutant cell area.

* Indicates significance at the 1 per cent level.

this is the case in *Bombyx*, the progeny of crosses between the mosaic type mutant and the marker strain would be expected to produce both mutant (*pe* or *re*) and non-mutant (+) cell-lines, whereas complete mutants would produce only one mutant cell-line. According to Ito's model, double mosaic mutants having both yellowish-white and red cell-lines would result from two independent mutational events that occur at the *pe* locus in one of the paired complementary polynucleotide chains and at the *re* locus in the other chain in *Bombyx* oocytes. This explanation is analogous to that for double mosaics in lethal mutations in *Drosophila* (Epler 1966). The egg-color com-

plete mutations may result from one of the double mosaic mutations occurring within a given locus. This may agree in Jenkins' model for double mosaic mutations (Jenkins 1969) and apparent whole-body mutations in the *dumpy* locus of *Drosophila* (Carlson and Oster 1962).

If this model is applied to the egg-color specific locus mutations induced by EMS in *Bombyx* oocytes, the frequency of the double mosaic mutations should be equal to that of the complete mutations. However, the double mosaic mutations were observed less frequently than the complete mutations. This departure from the expected ratio may have been due to the fact that double mosaic mutations were more difficult to detect than complete mutations. It is also possible that changes in a single strand of the paired DNA molecules in the oocytes are converted into changes involving both strands by passing through the pronuclear stage, since this stage cell may replicate DNA (Murakami 1971a). The mechanism responsible for the greater frequency of complete than double mosaic mutants remains to be elucidated. Nevertheless, the significant increase in the occurrence of both kinds of mutations indicates that EMS was mutagenic in meiotic oocytes.

Triple mosaic mutants may arise from oocytes which incorporates EMS into their cytoplasm and the EMS remains as an effective mutagen for DNA molecules in cleavage nuclei: mutational events that occur at the *pe* locus of one polynucleotide chain in one nucleus and at the *re* locus of one polynucleotide chain of the other nucleus would be expected to produce mosaic mutants with black (+), yellowish-white (*pe*) and red (*re*) cell-lines. If this is the case, the existence of the triple mosaic mutant in late pupal oocytes treated with EMS provides a suggestive evidence that the chemical is mutagenic in cleavage nuclei. A similar explanation was given for the triple transmission pattern of mosaic mutations induced by nitrosodimethylamine in *Drosophila* spermatozoa (Corwin et al. 1971).

Another possible explanation for the occurrence of triple mosaic mutations in EMS treated *Bombyx* oocytes is that the gamete in this organism is a multistranded DNA structure (Tazima and Onimaru 1969). But it is difficult to account for the occurrence of complete mutations by this multistranded DNA model (e.g., Auerbach and Kilbey 1971). Therefore, the definite mechanism responsible for the occurrence of triple mutations in *Bombyx* oocytes treated with EMS at the late pupal stage also remains to be elucidated.

In conclusion, the results of experiments show that in *Bombyx* EMS is strongly mutagenic when administered to pre-miotic oocytes (prophase I) at the late pupal stage. A predominance of mosaic mutations occurred in the EMS-treated pupal oocytes indicates that the chemical appeared to have either the delayed mutagenic effect or the delayed drug-action effect.

SUMMARY

Mutagenic effects of ethyl methanesulphonate (EMS) on the cells in oogenesis of the silkworm (*Bombyx mori* L.) have been studied by a larval and pupal injection method. Germ-cells at the time of injection were from middle growth stage oocytes

to pre-meiotic prophase I oocytes. Frequencies of mutations were estimated by the egg-color specific locus method using *pe* and *re* gene loci. A remarkable high mutagenicity was observed after EMS treatment of the late pupal prophase I oocytes. No marked mutagenicity of EMS was observed in larval growth stage oocytes. Simple mosaic mutants having mutant and non-mutant cells predominated in the progeny derived from EMS-treated late pupal prophase I oocytes. In this stage oocytes other types of mutations such as double mosaics having two mutant phenotypes, yellowish-white (*pe*) and red (*re*) cells, triple mosaics having three possible phenotypes, *pe*, *re* and +, and complete mutants were also observed. The results indicate that EMS might be mutagenic for pre-meiotic oocytes, egg pronuclei and cleavage nuclei.

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