Sensitivity of the *Drosophila* testis to tri-ethylene melamine (TEM)

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When *Drosophila* males are treated mutagenically, different stages of spermatogenesis respond to different degrees. This is true for all mutagens which so far have been tested from this point of view. TEM is one of the most effective mutagens and a potent carcinostatic agent. Its differential action on the male germ-cell stages of *Drosophila* was studied by Fahmy & Fahmy (1955). They came to the conclusion that the highest frequency of recessive lethal and visible mutations is produced in spermatids, probably at the onset of nuclear condensation. Cattanach (1959) scored dominant lethals and translocations in the progeny of male mice that had been injected with TEM. He found that both effects are most frequent in spermatozoa that are utilized from the tenth to the fourteenth day after treatment and presumably had been treated as spermatids. Since X-rays, too, produce their greatest mutagenic effect on spermatids of *Drosophila* (Lüning, 1952; Auerbach, 1954) and mice (Auerbach & Slizynski, 1956), these findings seemed to put TEM into the same class as X-rays as far as differential effects on male germ-cell stages are concerned. Several discrepancies, however, remained to be explained. Firstly, Fahmy & Fahmy (1955) reported that after treatment with TEM Minutes and chromosome fragmentations have their peak sensitivity at a later stage than recessive mutations; but, as shown elsewhere (Auerbach & Sonbati, 1960), this claim is not borne out by the published data. Secondly, also in experiments on *Drosophila* by Fahmy & Fahmy (1954), TEM-induced dominant lethals were found to be most frequent in mature spermatozoa, in contrast to recessive lethals and in contrast to dominant lethals in the mouse. In view of the great difficulties involved in the scoring of dominant lethals in *Drosophila*, it was felt that the mice data are probably more reliable in this regard. Thirdly, Auerbach & Sonbati (1960) found that mustard gas has its strongest mutagenic effect on late spermatogonia of *Drosophila*. This was of interest, since it seemed to point to a difference in action between two closely related chemical mutagens, TEM and mustard gas. The methods of analysis had, however, been different for these two substances. Auerbach & Sonbati analysed sensitivity to mustard gas genetically, using induced crossovers as landmarks for assigning germ-cell stages to successive broods. This method makes use of a dual-purpose stock which allows the scoring of lethals and induced crossovers in the progeny of the same treated males. The latest stage during spermatogenesis when

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crossing-over can be induced is obviously prophase of meiosis. Broods containing induced crossovers must therefore trace back to treated meiotic or premeiotic germ-cell stages, although in the first such brood an admixture of post-meiotically treated germ-cells is possible. In later broods, crossing-over in spermatogonia results in bunches of identical or complementary crossovers. Fahmy & Fahmy (1955) analysed sensitivity to TEM in a different way. They compared the brood pattern of sex-linked lethals with the cytological picture of the testis in males treated with the same dose of TEM; interpretation by this method relies to a considerable extent on assumptions about rate of sperm utilization and amounts of viable spermatozoa formed from different germ-cell stages. It was therefore decided to repeat the brood pattern analysis for TEM by the method used for X-rays and mustard gas.

MATERIAL AND METHODS

One-day-old $\male\male$ of the genotype $y \ sc^{S1} \ In49 \ sc^8$; $\frac{dp \ b \ cn \ bw}{+ \ + \ + \ +}$ were injected with $0.2 \ \mu$l. of TEM at a concentration of 2×10^{-4} M in 0.4% saline. Mortality was between 40 and 50%. Twenty-four hours after injection, the $\male\male$ were mated individually to three virgin $\female\female$ from a strain homozygous for $dp \ b \ cn \ bw$ (dumpy, black, cinnabar, brown; second chromosome). At intervals of two or three days they were remated to three new $\female\female$, and the old $\female\female$ were allowed to produce more progeny in a second vial. Crossovers in F_1 and sex-linked lethals in F_2 were scored separately for each brood and each $\male$. Only $\male\male$ that had given offspring in all broods were used for the test. Care was taken to collect $F_1 \ \female\female$ for lethal tests from among early as well as late hatchers, since Yanders (1958) has shown that late hatchers have a higher proportion of heterozygotes for a lethal than early hatchers.

RESULTS

Two experiments were carried out, and their results are presented in Tables 1 and 2, and in Fig. 1. In both experiments, progeny was sampled over a period of 12 days, that is from the second to the thirteenth day after treatment. In Experiment 1, this period was subdivided into four broods of 3 days each. In Experiment 2, it was subdivided into five broods, lasting successively 2, 2, 3, 3 and 2 days. In both experiments, the peak of mutation frequency was reached in the third brood. Thus in Experiment 2, two broods of 2 days each had exhausted the store of spermatozoa that had been treated at post-sensitive stages, while in Experiment 1 one brood of 3 days had been insufficient for this purpose. The third broods of the two experiments overlap on the eighth day; it is likely that with daily changes of $\female\female$ the highest mutation frequency would have been reached at or around this day. In both experiments, mutation frequency was higher in the second than in the first brood; this may be the expression of a tendency for sensitivity to decrease gradually from the sensitive stage to mature sperm. In both experiments, mutation frequency at the end of the 12-day breeding period was lower than in mature sperm, but this low level was reached abruptly in Experiment 1 and gradually in Experiment 2.

Apparently, spermatozoa treated during the sensitive stage had been used up after 9 days in Experiment 1, but not yet after 7 days in Experiment 2. It may be concluded that under the conditions of these experiments, mutation frequency increased with time available for mating, reached a high level between the sixth and tenth day after treatment, possibly with a peak on the eighth day, and on the eleventh day fell to a level below that in mature sperm.

The crossing-over data can be used for interpreting this temporal pattern in terms of sensitivity differences between stages of spermatogenesis. Only flies in which two marker genes were uncovered can be confidently scored as crossovers. Uncovering of single markers may be caused by mutations or deficiencies; the latter
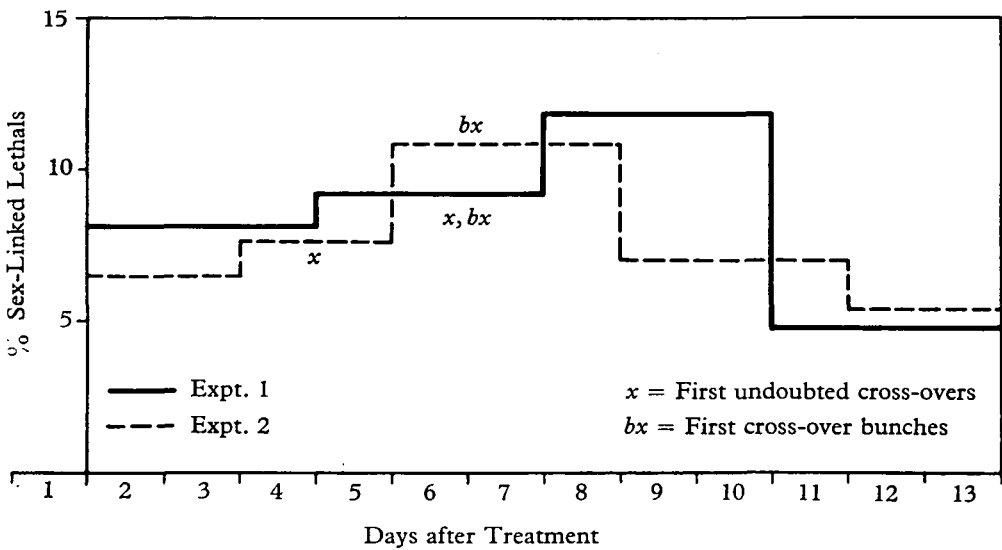

Fig. 1. The brood pattern for sex-linked lethals after treatment of adult males with TEM.

are known to be frequent after treatment with TEM (Fahmy & Fahmy, 1953). Simultaneous uncovering of three genes may happen rarely through reverse mutation or suppressor mutation.

Experiment 1 (Table 1)

None of the exceptional flies in the first brood can with certainty be attributed to crossing-over. The seven *dp* flies may have arisen through mutation or deficiency. The single *dp b cn* fly is more difficult to explain. It may have been due to reverse mutation or suppressor mutation; alternatively, it may have arisen through crossing-over in one of the first treated spermatocytes to form mature sperm. The occurrence of crossovers in the second brood is beyond doubt; three of them occurred as a bunch of two identical and one complementary types. These may trace back to a treated spermatogonium, but this is not necessarily true. Apart from the consideration that crossovers within the same chromosome region may

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Table 1. *Sex-linked lethals and apparent crossovers in successive broods from treated males*

Experiment 1

Brood ...	A		B		C			D	
Days ...	3		3		3			3	
	N	X	N	X	N	X		N	X
Crossover data	3541	7 dp	3057	1 dp	2477	2 bw	2 dp	1980	1 bw
		1 dpb cn		1 cn bw		1 cn	{2 cn		2 cn
				1 dp b		{2 dp	{1 cn bw		1 dp
				{2 dp b		{2 cn	{1 bw		1 dpb cn
				{1 cn bw		{1 dp			
						{1 bw			

Sex-linked recessive lethals	n	l	%	n	l	%	n	l	%	n	l	%
	466	38	8·1	348	32	9·2	464	55	11·8	315	15	4·76

N   Non-crossovers.
X   Apparent crossovers (including mutations or deficiencies at the marker loci).
n Number of X chromosomes scored.
l Number of lethals.
{   Exceptional flies bracketed together came from the same male; all other exceptional flies came from different males.

arise by chance coincidence, Parker (1948) has provided evidence for the suggestion that spurious crossover bunches may be formed in synchronously dividing spermatocytes. Independent of whether the crossovers in the second brood came from treated spermatocytes of spermatogonia, it is clear that the following, third, brood was not produced from post-meiotically treated germ-cells. It is in this brood that the peak

Table 2. *Sex-linked lethals and apparent crossovers in successive broods from treated males*

Experiment 2

Brood ...	A		B		C		D		E	
Days ...	2		2		3		3		2	
	N	X	N	X	N	X	N	X	N	X
Crossover data	3222	0	3006	2 cn bw	2907	{2 cn bw	2033	2 dp	2326	1 dp
						1 cn bw		1 cn bw		1 bw
						{2 dp b		3 dpb cn		

Sex-linked recessive lethals	n	l	%	n	l	%	n	l	%	n	l	%	n	l	%
	600	39	6·5	720	55	7·6	760	82	10·8	800	56	7·0	800	42	5·4

N   Non-crossovers.
X   Apparent cross-overs (including mutations or deficiencies at the marker loci).
n Number of X chromosomes scored.
l Number of lethals.
{   Exceptional flies bracketed together came from the same male; all other exceptional flies came from different males.

of mutation frequency was found. Thus, the most sensitive stage to TEM in this experiment occurred in late spermatogonia or early spermatocytes, more probably in the former. Younger spermatogonia, represented in the fourth brood, had already passed the sensitive stage.

Experiment 2 (Table 2)

The results of this experiment confirm the conclusions drawn from the first. Again, the first undoubted crossovers occurred in the brood preceding that with the highest mutation frequency, while relatively few mutations were found in the last brood.

DISCUSSION

The interpretation of brood patterns by the method used here is not free from ambiguities. The first brood in which crossovers occur may still contain progeny from germ-cells treated after meiosis. Bunches may be attributed to treated spermatocytes or spermatogonia. It is, therefore, not possible to decide from the evidence presented above whether the sensitive stage of the *Drosophila* testis to TEM occurs in early spermatocytes or late spermatogonia or both, although it is more likely to occur in the latter. In any case it is certain that in these experiments the sensitive stage was not post-meiotic, for in both of them the first undoubted crossovers were found in the brood *preceding* the peak of mutation frequency.

This is in contrast to the claim by Fahmy & Fahmy (1955) that peak sensitivity to TEM occurs in late spermatids. Actually, the observational data are the same in both cases; for in the experiments by the Fahmys, as well as in those reported here, the highest mutation frequency occurred in the third brood, which the Fahmys assume to have been drawn from treated post-meiotic germ-cells. Although it is not impossible that differences between strains (Strømnaes, 1959) or experimental conditions may affect the sensitivity pattern, the discrepancy is more easily explained by the difference between the genetical method of 'landmarks' used in the present experiments and the much more inferential and speculative method of correlating genetical and cytological evidence, used by the Fahmys. In fact, where Fahmy & Fahmy mention genetical 'landmarks' in support of their own interpretation (1960), these agree much better with the one presented here. In support of their claim that the first four broods trace back to treated post-meiotic stages, they adduce the observation that the first clusters of visible mutations were found in the fifth brood. Since only large clusters are likely to be detected in Muller-5 tests, in which the tested progeny per male per brood is small, this finding indicates that at least some of the spermatozoa utilized for the fifth brood were treated in early spermatogonial stages. This is confirmed by the further statement that germinal selection became first apparent in the fifth brood; for there are good reasons for believing that germinal selection acts mainly or only on young spermatogonia which have not yet reached the stage of synchronous division within cysts (Pontecorvo, 1944; Auerbach & Moser, 1953; Auerbach, 1954; Auerbach, unpublished). If, then, the fifth brood already contained spermatozoa that had been treated as early spermatogonia, the third brood may well have been derived from treated

spermatocytes or late spermatogonia. It is therefore probable that also in the experiments by Fahmy & Fahmy, TEM (1955) and other mutagens (1960) that produced peaks of mutation frequency in the third brood did do by virtue of a preferential effect on meiotic or late pre-meiotic stages.

While the present results remove a suspected difference between TEM and mustard gas, they reveal an unsuspected one between the testes of *Drosophila* and the mouse. Although the exact location of the most sensitive stage to TEM in mouse spermatogenesis is still under debate (Cattanach, 1959; Bateman, 1960), there can be little doubt that it occurs after meiosis and before the formation of fully mature sperm. We are still completely ignorant of the causes that determine the differential response of germ-cells to mutagens. Almost certainly many factors are involved, including penetration, general toxicity, oxygenation and other changes in the biochemical pattern of the affected cells, and these may act at various levels of the process of mutagenesis. It is therefore hardly surprising that the sensitivity pattern can differ between species. That it can also be affected by slight differences in mutagenic treatment has recently been shown by Ives (1960), who found that in *Drosophila* males peak sensitivity to gamma-radiation from a cobalt source occurs at a somewhat earlier stage than peak sensitivity to X-rays.

In spite of these minor variations, X-rays, gamma-rays, TEM, mustard gas, and several other alkylating agents studied by Fahmy & Fahmy (1958, 1960) resemble each other in the broad outlines of their differential effects on male germ-cells. All these mutagens are effective throughout spermatogenesis; early spermatogonia are least affected; mature spermatozoa are more sensitive than early spermatogonia; and peak sensitivity occurs at some intermediate stage: in spermatids, spermatocytes, or late spermatogonia. Certain other mutagens, including urethane (Vogt, 1950), formaldehyde used as injection (Sobels, 1956) and ethyl methane sulphonate (Fahmy & Fahmy, 1957) act most strongly on mature spermatozoa. Whether the difference between the two groups of mutagens is due to some essential difference of action is doubtful. There appears to be more justification for suspecting that mutagens that, like formaldehyde food (Auerbach & Moser, 1953) and chloroethyl methane sulphonate (Fahmy & Fahmy, 1957), act exclusively or almost exclusively on pre-meiotic stages may do so by virtue of some special interaction with the synthetic processes going on during these stages. For formaldehyde food, this assumption finds support in Alderson's observation (1960) that the presence of ribonucleic acid is a necessary condition for the production of mutations.

SUMMARY

The sensitivity pattern of the *Drosophila* testis to TEM was analysed by means of a dual-purpose strain that allows the scoring of induced crossovers and sex-linked lethals in the progeny of the same flies. It was found that TEM produces the highest frequency of mutations in spermatocytes or late spermatogonia, while early spermatogonia are even less sensitive than mature spermatozoa. The discrepancy between this conclusion and that obtained by Fahmy & Fahmy (1955) is attributed to a difference in the method of analysis. The sensitivity pattern of the *Drosophila*

testis to TEM resembles that to mustard gas and differs from that to X-rays. The sensitivity pattern of the mouse testis to TEM differs from that of the *Drosophila* testis.

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