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The induction of dominant lethal mutations in rats and mice with triethylenemelamine (TEM)

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1. INTRODUCTION

When the work herein described was begun, it was not yet known that TEM (= tretamine = triethylenemelamine = 2 : 4 : 6 tri(ethyleneimino)-1 : 3 : 5 triazine) was a mutagen in mammals: only that it reduced the fertility of injected male rats in the first weeks after injection (Bock & Jackson, 1957). The first task was, therefore, to confirm that this infertility was in fact due to the induction of dominant lethal mutations. This has since been done in the rat by Steinberger *et al.* (1959) and in the mouse by Cattanach & Edwards (1958), who went on to show (Cattanach, 1959) that translocations were also induced. In addition to confirming this published work we have also revealed a pattern of sensitivity in the various germ-cell stages which shows both resemblances to and differences from the sensitivity pattern established for X-rays (Bateman, 1958*a*).

The infertility of the treated male rats could conceivably have been due to numerical or functional oligospermy. By functional oligospermy we mean that most of the sperm inseminated are incapable of fertilization. (Aspermy could be excluded because the criterion of mating was the presence of sperm in vaginal swabs and consequently aspermic matings would not have been recorded as matings.) Severe oligospermy, whether numerical or functional, would reduce the proportion of fertilized eggs. This would reduce the rate of implantation, but all implanted eggs would develop normally.

Dominant lethal mutations in sperm, on the other hand, do not affect their capacity for fertilization, but sooner or later kill the fertilized egg. They are generally assumed to arise from unhealed chromosome breaks, which are freely produced both by chemical mutagens and ionizing rays. As shown by an analysis of X-ray induced mutations in mice (Bateman, 1958*b*), they may express themselves either as unimplanted eggs (which are indistinguishable from unfertilized eggs when dissected after implantation) or as deciduomata. These latter consist of entirely maternal tissue which has been stimulated into growth by an egg which dies shortly afterwards. Though by definition a dominant lethal may kill the embryo carrying it at any time during gestation, in practice those produced by mutagens rarely kill established embryos.

When the mutation rate is high (as following a high dose of mutagen) pre-implantation losses are high, or even complete, simulating failure of fertilization.

For a critical test of dominant lethal induction, therefore, the optimum dose of reagent is the maximum which permits a high proportion of the fertilized eggs to be implanted.

2. ACTION OF TEM ON MALE RATS

A wide range of doses, from 0·4 mg./kg. of body-weight down to 0·025 mg./kg., was administered i.p. to male rats. Single males were placed with single females of proven fertility for weekly periods. In one series an attempt was made to increase the amount of information per male by giving each male two females per week, but it was found that the males generally could not mate effectively with more than one female per week. The doubled consumption of females barely increased the information per male, so we returned to the one female per male per week routine.

As mentioned above, matings were identified by means of daily vaginal swabs, which enabled one to follow oestrus and look for the presence of sperm. The simpler method of inspection for vaginal plugs, so effective for mice, is inapplicable, presumably because the plugs have fallen out before inspection. There is the advantage that aspermic matings, which would result in pseudopregnancy (suspension of oestrus), are excluded from the fertility studies, and the corresponding disadvantage that aspermy induced by the treatment is not distinguished from failure to inseminate. Aspermy is proved only by histological studies of epididymis and testis.

Dissections of uteri were made 14 days from mating. This corresponds to the stage reached at 12 days from mating in the mouse (Bateman, 1958*a*) with one important exception, that in pregnancies without live foetuses the deciduomata of the mouse have already been shed at 12 days though they are still in the uterine lumen, but the deciduomata of the rat at 14 days are still firmly attached to the myometrium.

Slight confusion was encountered at first in counting the rat corpora lutea, as there appeared to be two distinct sizes. It was found to be the large ones that represented the ovulations, so the smaller ones were ignored.

Table 1 summarizes all the rat data except for the controls, which are given in Table 2. The abbreviations used are the same as in a previous paper (Bateman, 1958*a*) but are redefined in the legend to the table. The ratio ED/IMP is only approximately proportional to the incidence of dominant lethal mutations (see Bateman, 1958*b*) but is suitable for our purposes. The main deficiency of ED/IMP is that it cannot exceed unity however high the mutation rate may be. It is most useful therefore at lower mutation rates: up to one mutation per egg. When the mutation rate is high this will affect the implantation rate by increasing (CL—IMP) the eggs which fail to implant. PSP will also reflect mutation rate when this is high enough to cause the death of all the eggs in a pregnancy.

It is clearly established from Table 1 that the reduced fertility of male rats for the first 4 weeks after treatment with TEM is due in part to the induction of dominant lethal mutations represented by ED/IMP, and for the rest to the less specific low IMP and high PSP at higher dose levels. This confirms the conclusions of Steinberger, Nelson, Boccabella & Dixon (1959). Furthermore, the acute sterility

Table 1. *Analysis of matings of treated male rats by dissection at* 14 *days after mating. Week number denotes the week after treatment in which mating occurred*

CL = number of corpora lutea (excluding pseudopregnancy).
IMP = number of implantations alive and dead.
ED = number of deciduomata (i.e. ova dying soon after implantation).
PSP = ratio of pseudopregnancies (no implantations of any kind) to total matings.

Dose (mg./kg.)		Week 1	2	3	4	5	6
0·4	CL	94	72	75	10	73	
	IMP	65	12	21	5	41	
	ED	54	10	21	5	22	
	PSP	1/10	3/9	3/10	5/6	0/6	
	ED/IMP	**0·83**	**0·83**	**1·00**	**1·00**	**0·54**	
0·2	CL	76	75	41	9	54	70
	IMP	64	41	22	1	55	65
	ED	43	38	21	1	15	5
	PSP	0/7	1/9	2/7	2/3	0/5	0/5
	ED/IMP	**0·67**	**0·93**	**0·95**	**1·00**	**0·27**	**0·08**
0·1	CL	21	18	29	43	33	35
	IMP	21	15	17	23	30	31
	ED	4	14	8	20	4	1
	PSP	0/2	0/2	1/4	3/7	2/5	0/3
	ED/IMP	**0·19**	**0·93**	**0·47**	**0·87**	**0·13**	**0·03**
0·05	CL	64	66	66	51	59	53
	IMP	54	64	57	39	55	52
	ED	11	20	16	28	4	4
	PSP	1/7	2/8	0/7	1/5	0/5	0/5
	ED/IMP	**0·20**	**0·31**	**0·28**	**0·72**	**0·07**	**0·08**
0·025	CL	58	52	56	59	39	
	IMP	49	50	55	53	36	
	ED	8	4	2	9	4	
	PSP	0/5	0/5	0/5	0/5	1/4	
	ED/IMP	**0·16**	**0·08**	**0·04**	**0·17**	**0·11**	

experienced in the fourth week is due to a peak sensitivity to induction of mutations in that week. That is, sperm utilized in the fourth week was in a stage hypersensitive to the mutagenic action of TEM at the time of treatment. A break-down of the first week's matings disclosed that mating in the first half of the week had a consistently lower mutation rate than later matings.

A check was made on the possibility that the PSP following high doses was due to a new phenomenon, such as impairment of the fertilizing capacity of the sperm.

Ova from matings in the fourth week after 0·4 mg./kg. (when PSP is very frequent) were examined on the second day after mating. All were fertilized and most had undergone first cleavage. But instead of a single large nucleus in each blastomere there were several nuclei of varying sizes. Evidently the fertilizing capacity of the sperm was unimpaired, the micronuclei being due to multiple breakage of the chromosomes. Hence the failure of the ova to survive to implantation. Thus, in this material, reduced IMP and high PSP were also manifestations of dominant lethal mutations.

There is an abrupt change from the fourth to the fifth week, which except at high doses was similar to the control, with full fertility and almost complete absence of dead embryos. The sixth week was like the controls even at maximum dose levels. These results are in accordance with Bock and Jackson's fertility data (1957).

It is desirable to identify the stage in spermiogenesis which is showing the hypersensitivity; in other words, which stage matures in the fourth week after treatment. There is a superficial resemblance between this hypersensitivity and that found in early spermatids of the mouse following X-rays (Bateman, 1958*a*). A more direct comparison can be made with the data of Craig, Fox & Jackson (1959) on the fertility of male rats following 300 r X-rays. There were two periods of low fertility; the fourth week and a period over weeks 9 and 10. Apart from the extended time-scale, this bears such a close resemblance to the mouse data after 200 r (Bateman, 1958*a*) that the respective sensitive stages may be presumed to be homologous and so also the intervening fertile periods. The timing of spermatogenesis in the mouse has been known for some time from the work of Oakberg (1956) and Sirlin & Edwards (1957). More recently similar studies have been made in the rat (Clermont & Leblond, 1960) and these fully confirm this interpretation. In the terminology of these workers the term 'sperm' is confined to stages outside the testis and 'spermatid' for all post-meiotic stages found in the testis however close their morphology to that of sperm. Since 'spermatid' then covers a wide range of cell types with a wide range of mutagenic sensitivity it is necessary to subdivide them in some way. I shall therefore use the terms 'late', 'mid' and 'early'. Though the classification is based upon the order in which they mature following treatment they will correspond roughly to the morphological types: nuclei resembling mature sperm, elongating nuclei, and spherical nuclei respectively.

From the above considerations it can be concluded that mid and early rat spermatids at treatment will be used for inseminations in the fourth and fifth weeks; spermatocytes in the sixth to eighth week; and spermatogonia type B and intermediate in the ninth and tenth week. It follows from this that in terms of their response to the mutagenic action of TEM, rat sperm and late spermatids are moderately sensitive, mid spermatids are hypersensitive, early spermatids are slightly sensitive and spermatocytes are highly resistant. Using higher doses and mating the rats for longer periods, Jackson (unpublished) found sterility in the ninth and tenth weeks which must have been due to sterilization of spermatogonia type B.

The results presented in Table 2, where data from the first 4 weeks' matings are pooled, suggest an unusual dose-dependence of the mutagenic effects of TEM. The

Table 2. *Analysis of pregnancies initiated by male rats injected with various doses of TEM. Symbols as for Table* 1. *Matings in the first four weeks after treatment have been pooled. The final estimate of mutation rate, —ln (1—ED/IMP), is based, however, on the unweighted mean of the four weeks*

	Dose in mg./kg.					
	0·4	0·2	0·1	0·05	0·025	Control
Number of matings	35	26	15	27	20	10
CL	251	201	111	247	225	93
IMP	103	128	76	214	207	85
ED	90	103	46	75	23	4
PSP	0·34	0·19	0·27	0·15	0·00	0·00
(CL − IMP)/CL	0·59	0·36	0·32	0·13	0·08	0·09
Gross ED/IMP	0·87	0·80	0·61	0·35	0·11	0·05
Mean ED/IMP	0·915	0·888	0·615	0·378	0·113	0·047
− ln (1 − Mean ED/IMP)	**2·465**	**2·185**	**0·954**	**0·474**	**0·119**	**0·048**

effect of 0·025 mg./kg. is disproportionately low, much less than mid-way between the control level and 0·05 mg./kg. As already indicated, the ratio ED/IMP is an imperfect quantitative measure of mutation rate. It has therefore been converted to the parameter advocated by Bateman (1958 *b*): namely −ln[1−(ED/IMP)], and

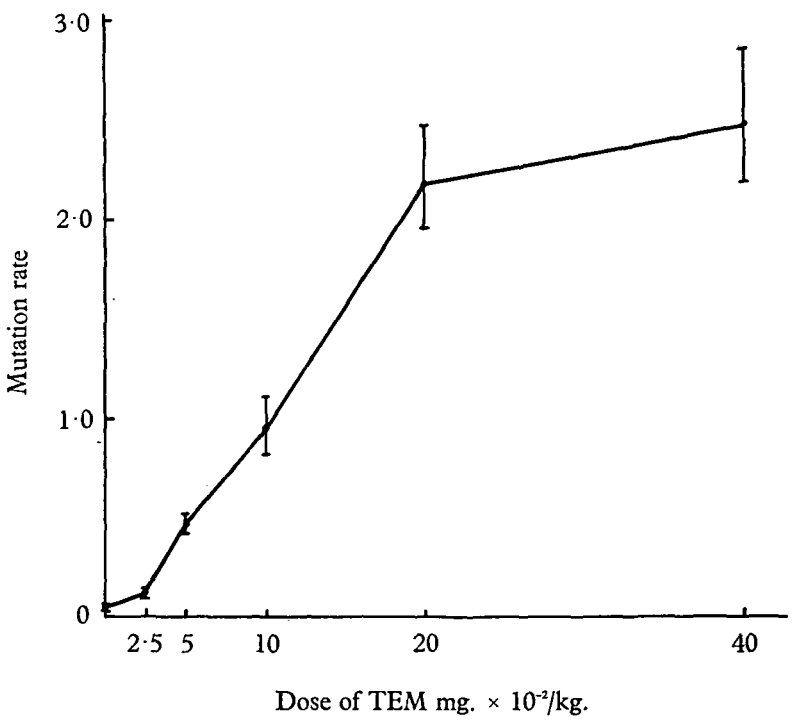

Fig. 1. Dependence of mutation rate [−ln(1−ED/IMP)] in the rat on dose of TEM. The depression at the lowest dose (0·025 mg./kg.) might indicate a threshold effect. The lines representing the standard errors are derived from those calculated for ED/IMP using the binomial [$\sqrt{(pq/k)}$].

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this has been plotted against dose in Fig. 1. It would be premature to discuss whether this signifies a threshold effect for chemical mutagens.

3. ACTION OF TEM ON MALE MICE

The pattern of sensitivity to TEM with successive sperm samples, as published by Cattanach & Edwards (1958) and Cattanach (1959), appeared to contrast strongly with that for TEM in rats as presented above, and also for X-rays in mice (Bateman, 1958*a*). It was therefore felt necessary to repeat the work in our own laboratory, with the same TEM as had been used on the rats and the same strain of mice as had been irradiated.

Owing to the freer mating of mice it was possible to obtain a day-by-day analysis of sperm instead of the week-by-week analysis obtainable with rats. Two dose levels were administered: single injections i.p. of 0·2 mg./kg. to eight mice and 0·8 mg./kg. to four mice. Each male at the high dose was given one female per week starting with the second week. The purpose of this latter was merely to time the return of fertility at a high dose level. Matings were timed by the appearance of vaginal plugs.

Table 3. *Dominant lethal mutations induced in male mice by a single injection of 0·2 mg./kg. of TEM on day* 0. *Symbols as in Table* 1. *Though data were obtained for each day separately, for the purposes of the table adjacent days have been combined so that each estimate of ED/IMP is based on a minimum of thirty IMP.*

	Week 1			Week 2				Week 3				Week 4		
Day	0–2	3	5–6	7–8	9–10	11–12	13–14	15–17	18	19	20–21	23	24–28	Control
CL	47	58	55	67	84	71	115	59	67	67	85	29	65	187
IMP	48	40	45	37	75	55	68	51	58	60	71	29	61	179
ED	8	18	18	17	35	30	36	11	7	4	5	1	8	8
ED/IMP	0·17	0·44	0·40	0·46	0·44	0·55	0·53	0·22	0·12	0·07	0·07	0·03	0·13	0·05
Week's ED/IMP		0·331			0·505				0·195			0·092		

The data presented in Table 3 confirm the results of Cattanach & Edwards. The features of the sensitivity pattern are as follows. The mutation rate rises rapidly to a steady level from the third day after treatment to the tenth. Days 11–14 show a slight further rise which is followed by a sudden drop to the fifteenth day which develops into a steady decline, reaching the control level by the twenty-second day. The contrast with the sensitivity pattern following X-rays is clearly seen in Fig. 2, where 500 r is compared with 0·2 mg./kg. of TEM. The two mutagens give the *same* mutation rate from days 3–12, but very different results before and after. The absence of values beyond the nineteenth day for X-rays is due to the onset of complete sterility in contrast to the full fertility of TEM. The high value for day 3 with X-rays, though not significant in itself, is confirmed by more extensive data at other doses (Bateman, 1958*a*).

The translation of days from treatment on which mating occurred into stage at which the germ-cells were treated is shown in Table 4. Taking the sensitivity of the

period 3 12 days as standard (late spermatids and epididymal sperm) ripe sperm and early spermatids have a high sensitivity to the induction of mutation by X-rays, and a low sensitivity to TEM. The sensitivity of mid and early spermatids to TEM is not uniform but steadily decreases with immaturity. Finally spermatocytes, completely sterilized (i.e. incapable of producing viable sperm) by the X-rays, are resistant both in terms of sterilisation and of mutation to TEM.

The resistance of spermatocytes to TEM was further tested at the 0·8 mg./kg. dose level. Though matings occurred regularly each week, no litters were obtained from second-week matings, nor from the third-week, though blood in the vagina of one

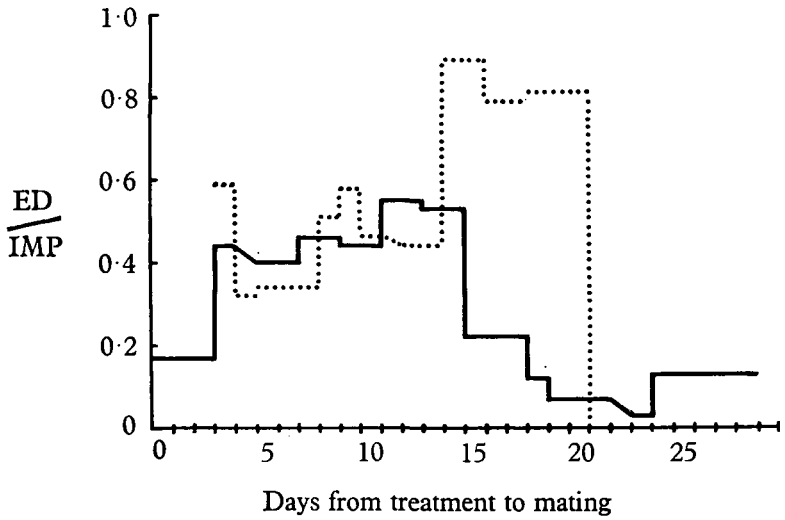

Fig. 2. Histogram showing daily variation in the ratio ED/IMP in matings of treated male mice. Entire line: 0·2 mg. TEM/kg. (present data): Dotted line: 500 r X-rays (from Bateman, 1958*a*). Where there are less than thirty implants per day, neighbouring days have been pooled to make up a total of at least thirty.

female 12 days after mating indicated pregnancy with abortion of the entire litter. Matings in the fourth week produced litter sizes of 3, 10, 13 and 11. In the fifth week the corresponding litters were 13, 17, 16 and 13. The litters conceived in the fourth week appear to be slightly subnormal in size. It can be concluded that spermatocytes are much more resistant than spermatids to TEM and early spermatocytes more resistant than late ones.

4. DISCUSSION

There are three possible comparisons to be made: between X-rays and TEM, between rats and mice, and interactions between species and mutagen. One could make this on a purely temporal basis, according to the time from treatment to mating. But a much more meaningful comparison, because of the different timing of spermatogenesis in rats and mice, would be according to the germ-cell treated. The tran-

slation of period from treatment to mating into stage treated is shown in Table 4, together with, in semi-quantitative terms, the sensitivity of each stage to mutagen in respect of the induction of dominant lethals on the one hand and in respect of the sterilization of the cell on the other. The table combines the author's data with those from several sources previously cited in the text. By 'sterilization' is meant here, the inability of the cell to form viable division products, whether of type A spermatogonia to form type B, type B to form spermatocytes, or of spermatocytes to form viable sperm. The term 'sterilization' is used in preference to 'cytotoxic' or 'cell lethal' because it covers not only death of the treated cell but also the inability of that cell to produce functional sperm. The sterilization of sperm would mean the destruction of its fertilizing ability, something which no mutagen has been found to do.

On such a definition the sensitivities of rat and mouse spermatogonia to the sterilizing effects of X-rays are similar, even though the histological results (cf. Mandl, 1959, and Oakberg, 1955) are so contrasting. Apparently, whilst rat and mouse spermatogonia are equally damaged by, say, 500 rad, the death of the cell is within a few hours in the mouse but is delayed for several days in the rat. This much is certain, that after 500 rad to the male rat (Craig *et al.*, 1959) as with the male mouse, there is complete sterility during the interval corresponding to irradiation of spermatogonia and spermatocytes.

The most imperfect of the four sets of data on which the comparisons are based is the effect of X-rays on the rat (Craig *et al.*, 1959), since it is concerned only with mean litter sizes, and cannot therefore discriminate between dominant lethals and cell sterilization. It can safely be assumed, however, that reductions in litter size for matings in the first five weeks are due to dominant lethals. After allowing for the difference in timing, the parallelism with the mouse data is so close that it is justifiable to conclude that mouse and rat germ-cells show the same sensitivity pattern to X-rays.

In making comparisons of sensitivity it is necessary to adopt a standard as the basis of comparison. I have taken as this standard the production of an ED/IMP of 0·5 in epididymal sperm. This is produced by 500-rad X-rays in rats and mice, by 0·2 mg./kg. of TEM in the mouse and 0·1 mg./kg. of TEM in the rat. These doses are only approximate, but this is good enough for our purposes as we are only concerned with major fluctuations in sensitivity from the standard. The standard dose for X-rays in the rat is based on a small-scale unpublished experiment in this laboratory, which is consistent with the fertility changes reported by Craig *et al.* (1959).

Let us first consider the comparison between X-rays and TEM, confining ourselves to features common to both species. Similarities are the immunity of post-meiotic stages to any sterilizing effect, and the absence of dominant lethals from treated spermatogonia. This latter is due to a failure to recover them in the sperm, rather than failure to induce them. That is, it lies in the nature of the mutations rather than of the mutagen. That TEM can induce chromosome breaks in type A spermatogonia has been shown by Ockey (1959), who observed aberrations in these

Table 4. *A comparison of the relative sensitivities of the various stages of spermatogenesis to the mutagenic and cell-sterilizing actions of X-rays and TEM in the mouse and rat. The dominant lethal sensitivity of spermatogonia is entered as nil, not because the mutations cannot be induced, but because they are not recoverable in the sperm*

		Stage of germ-cell treated						
		Sperm		Spermatid			Spermatogonium	
		in vas deferens	in epididymis	late	mid : early	Spermatocyte	Type B	Type A
Week after treatment in which it is used as sperm	Mouse	½	½–1	2	3	4–5	6–7	8 onwards
	Rat	½	½–2	3	4–5	6–8	9–10	11 onwards
Dominant lethals								
X-rays	Mouse and Rat	High	STANDARD	Standard	High	Standard	NIL	NIL
TEM	Mouse	Low	STANDARD	Standard	Low	Almost immune	NIL	NIL
	Rat	Low	STANDARD	Standard	High : Low			
Cell sterilization								
X-rays	Mouse and Rat	Immune	Immune	Immune	Immune	Moderate	Very high	Moderate
TEM	Mouse and Rat	Immune	Immune	Immune	Immune	Immune	Almost immune	Immune

spermatogonia in the field vole (*Microtus agrestis*) after TEM, though the maximum rate observed was low (1·8 per 100 cells).

One difference between mutagens is that mature sperm is more sensitive than the standard, immature sperm, to X-rays but less sensitive to TEM. A second difference is that pre-meiotic stages are sterilized by the standard X-ray dose, but there is no detectable effect of the standard TEM dose. Of the three pre-meiotic cell types listed in Table 4, type B spermatogonia are the most sensitive to both reagents. At lower doses of X-rays (e.g. 100 rad to mouse; Oakberg, 1955) and at higher doses of TEM (e.g. 1 mg./kg. to rat; Jackson, unpublished), they are the only stage to be sterilized.

A third difference only becomes apparent at lower X-ray doses than the standard. It is only at doses below the sterilizing dose for spermatocytes (e.g. 200 rad mouse) that one can expect to detect any mutagenic effect of X-rays. It is then found that spermatocytes have a sensitivity to X-rays equal to immature sperm (Bateman, 1958*a*), contrasting with their high resistance to TEM.

The next comparison is between rats and mice. Both species react similarly to X-rays but differently to TEM. There is thus no consistent difference between the species.

We come therefore to the third comparison: the interaction between species and mutagen. Both species show the same pattern of resistance to the sterilizing effect of TEM, which is almost confined to type B spermatogonia at the doses used in this work, though really high doses (five consecutive daily doses of 0·4 mg./kg. rats; Jackson, personal communication) can produce permanent sterilization. The species-mutagen interaction is confined to the yield of dominant lethals from spermatids treated with TEM. Mid-stage rat spermatids are hypersensitive whilst the earliest stages are resistant. Both mid and early mouse spermatids, on the other hand, are highly resistant.

The main consistent differences between X-rays and TEM concern the pre-meiotic stages. The simplest explanation would be one of penetrance. The penetrance of X-rays is of course uniform throughout testis and epididymis, but it might be that TEM has much readier access to post-meiotic stages in testis and epididymis than elsewhere. This would explain the parallel immunity of all pre-meiotic stages to sterilization, and of spermatocytes to mutation. The only further assumption that has to be made is that mid-stage mouse spermatids, unlike mid-stage rat spermatids, are largely sheltered from TEM. Only experiments with labelled TEM could test this hypothesis.

It is not easy to compare the sensitivity pattern in mouse and rat with that obtained in *Drosophila* by Fahmy & Fahmy (1954). This is because, in *Drosophila*, unlike rats and mice, mature sperm which is not used in matings is accumulated in the seminal vesicles. A fast mating rate is therefore necessary before one can attempt to equate interval from treatment to mating with stage of treated germ-cell being sampled. The Fahmys provided only one female per male every 3 days, and this is certain to produce accumulation of earlier maturing sperm which would be used with later maturing sperm. It is not possible, therefore, to equate successive 'broods' (3-day mating periods) with particular germ-cell stages except that the

first brood would consist of sperm which was mature at treatment, whereas the sperm in later broods would be, on average, less mature at treatment. In that case, the fall in mutation rate in *Drosophila* from the first to the second brood must mean that mature sperm is more sensitive than immature sperm, which is contrary to our mammals. No further comparison is justified.

SUMMARY

1. TEM resembles X-rays in inducing dominant lethal mutations in the sperm of rats and mice and sterilizing type B spermatogonia. Beyond this, however, there are several important differences.

2. The relative ease with which TEM and X-rays affect sperm and spermatogonia varies greatly. The X-ray dose which produces 50% dominant lethals in sperm (500 rad) sterilizes spermatocytes and type B spermatogonia, and has such a drastic effect on type A spermatogonia that recovery of fertility is delayed for $2\frac{1}{2}$ months. The TEM dose which produces the same mutation rate in sperm (0·1 mg./kg. rat) has no detectable effect on pre-meiotic stages. Even 1 mg./kg. only sterilizes the most sensitive stage, type B spermatogonia.

3. Taking immature sperm as the standard, mature sperm are more sensitive to X-rays, but less sensitive to TEM, and early spermatids, the most sensitive stage to X-ray-induced dominant lethals, are highly resistant to TEM.

4. Spermatocytes, in which X-rays yield a mutation rate equal to immature sperm, are highly resistant to TEM.

5. To produce the same mutation rate in immature sperm, mice require twice as much TEM as the rat in mg./kg., though approximately the same X-ray dose.

6. In contrast to mid-stage rat spermatids, which are the most sensitive stage to TEM, mouse mid-stage spermatids are resistant.

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