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The induction of dominant lethal mutations in rats by alkane sulphonic esters

By M. PARTINGTON and H. JACKSON
Experimental Chemotherapy, Paterson Laboratories, Christie Hospital, Manchester 20

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1. INTRODUCTION

It has been shown by Cattanach & Edwards (1958) for mice, and by Bateman (1960) for rats and mice, that the sterility resulting from intraperitoneal injection of triethylenemelamine (TEM) is due to the induction of dominant lethal mutations in spermatozoa and spermatids, and in this respect the action of TEM resembles that of X-irradiation (Bateman, 1958*a*).

Extensive data on the fertility patterns of male rats after treatment with alkane sulphonates have been published (Jackson, Fox & Craig, 1961). This information, however, is based on the average size of litters fathered by males at various times after treatment. Under such conditions the causes of sterility are not known, though histological examination of the testis has provided information as to the cell types directly affected.

The alkane sulphonates used here are simple esters of methane and ethane sulphonic acids. In the series methyl ethane sulphonate, ethyl methane sulphonate, isopropyl methane sulphonate and tetramethylene-1,4 dimethanesulphonate (Busulphan), the alkylating portions of the molecules are the monofunctional methyl, ethyl and isopropyl groups and the bifunctional tetramethylene group respectively. Thus they differ from TEM in which the alkylating group is the ethyleneimine part of the molecule. They would therefore be expected to have different biological activity. Of the four compounds chosen, two, methyl ethanesulphonate and ethyl methanesulphonate, have been shown to cause sterility during the first 4 weeks after treatment, whilst isopropyl methanesulphonate and Busulphan have their maximal effect much later (8 weeks onwards; Jackson *et al.*, 1961).

A knowledge of the exact timing of events in spermatogenesis is important in the correlation of the induced periods of sterility with histological data. The duration of the testicular phase in the rat is considered to be 48 days (Clermont, Leblond & Messier, 1959) and investigations of epididymal dynamics (Macmillan & Harrison, 1955) suggest that approximately 15 days are required for transport of spermatozoa through the rete testis, epididymis and vas deferens.

The time of appearance and duration of the sterile periods induced by these drugs were little affected by delay in mating after treatment or changes in its frequency (Jackson *et al.*, 1961). Thus, the present series of experiments was undertaken in an attempt to determine the causes of the sterility observed.

2. METHODS

The technique used for assessing the fertility of male rats after treatment with alkylating agents has been described fully elsewhere (Bock & Jackson, 1957), but more detailed information can be obtained by a modification of this technique. Groups of six proven fertile male rats were given single intraperitoneal injections of various alkylating agents in arachis oil, and control series of six animals were given a single dose of arachis oil alone at 1 ml./kg,. 2 ml./kg. and 4 ml./kg. Each animal was then paired with one female per week and successful matings identified by the presence of sperm in vaginal swabs. The females were killed 14 days post-coitum, their uteri removed and examined for the presence of live offspring and deciduomata (representing embryos which died soon after implantation). Ovaries were examined and the numbers of corpora lutea recorded.

For a critical evaluation of dominant lethal effects the optimum dose of a compound is that which results in subfertility or sterility whilst permitting a high proportion of fertilized eggs to be implanted (Bateman, 1960). A range of dose levels was therefore chosen to include the smallest known to produce definite antifertility effects.

Isopropyl methane sulphonate and Busulphan are thought to act mainly on stages of spermatogonial development, so that treated rats should become aspermic about 60 days after treatment and possibly oligospermic before this time. It is difficult to determine total sperm numbers in the rat, however, since there is no suitable technique for collecting a representative sample of semen. Vaginal smears can provide only a very rough guide, so indirect estimates from spermatid counts were made from histological sections.

Proven fertile male rats injected with Busulphan (10 mg./kg.), isopropyl methane sulphonate (50 mg./kg.), ethyl methane sulphonate (200 mg./kg.), methyl ethane sulphonate (100 mg./kg.) and arachis oil (2 ml./kg.) were killed in pairs at selected times after treatment. Testes were fixed in formol saline and Helly's fluid and stained in PAS and haematoxylin. Spermatids in stage 8 of spermiogenesis (Clermont, Leblond & Messier, 1959) were counted, ten tubule cross-sections being analysed from each animal. Stage 8, however, is the latest convenient time at which such counts can be made, and consequently a possible loss of spermatids during later maturation stages in the testis and passage of the mature spermatozoa through the rete testis, epididymis and vas deferens will remain undetected.

Failure or delay in fertilization even though normal numbers of spermatozoa are present is a possible result of drug action on spermatogenic cells. This can be studied by examination of ova recovered from the Fallopian tubes of females mated with treated males. Two groups of eight proven fertile males were injected with isopropyl

methane sulphonate (50 mg./kg.) and Busulphan (10 mg./kg.) respectively, a third group of eight males serving as controls. In weeks 5–8 inclusive, the drug-treated males were each paired with one proven fertile female per week. The untreated males were each mated once. Inseminations were recorded by vaginal smears and inseminated females killed 36–40 hr. later. Ova were flushed from the Fallopian tubes under saline and examined in the fresh state by phase contrast microscopy (Austin & Smiles, 1948).

3. RESULTS

(i) *Control series*

In the control series (arachis oil only) the maximum loss of ova recorded was 20% (week 5; Table 1). No histological effects could be detected and spermatid counts were normal during the period studied (Table 5).

Table 1. *'Dominant lethal' mutations after treatment of male rats with arachis oil (control series)*

Weeks after treatment	Treated cell types represented in ejaculate	No. of corpora lutea*	% loss of ova	
			Pre-implant.	Post-implant.
1	Sperm in vas and epididymis	72	3	7
2	Sperm in epididymis	56	0	2
3	Late spermatids	59	11	3
4	Mid spermatids	64	3	6
5	Early spermatids	73	16	4
6	Spermatocytes	49	0	16
7	Spermatocytes	70	7	0
8	Resting spermatocytes and Type B	45	11	4
9	Intermediate and Type A spermatogonia	45	15	0
10	Type A spermatogonia	47	2	0

* Average number of corpora lutea found per mating 11·8.

(ii) *Methyl ethane sulphonate* (MES)

Subfertility after methyl ethane sulphonate (50 mg./kg.) was found to be mainly due to post-implantation deaths with the maximum number occurring from the second to fourth weeks inclusive (Table 2; Fig. 1). Normal numbers of living foetuses were found from 30 days onwards. Spermatid numbers were normal throughout the period after 100 mg./kg. of this compound (Table 5).

(iii) *Ethyl methane sulphonate* (EMS)

Normal numbers of living foetuses were recorded in the first week after treatment (100 mg./kg.) but in week 2 the value was reduced to 61% of the ova shed. A further reduction occurred in the third and fourth weeks when the percentage of live implants fell to 47% and 40% respectively.

Numbers then returned to normal in the fifth week and remained so. The percentage of post-implantation deaths was increased during weeks 3 and 4, corresponding with the fall in numbers of live implants. The number of unimplanted eggs was high in the second week but was not above control levels at other times (Table 2).

At twice the above dose level (200 mg./kg.) there was a high incidence of post-implantation deaths in the first week, whilst in the second and third weeks the number fell and there was an increase in pre-implantation deaths. No living

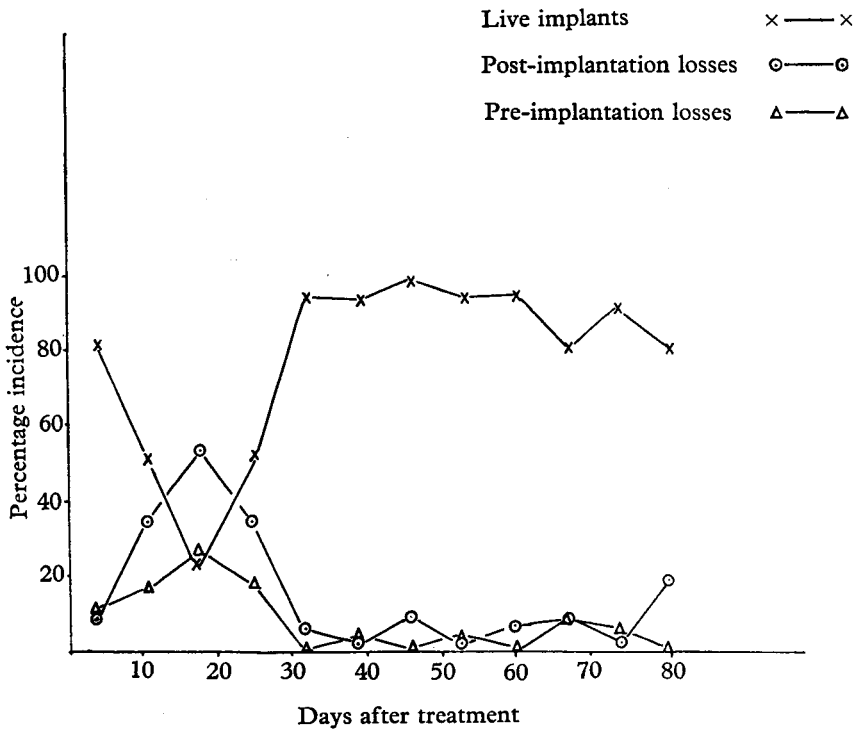

Fig. 1. Fluctuations in percentages of live and dead implantations and pre-implantation losses after methyl ethane sulphonate (50 mg./kg.).

foetuses were seen during the second and 3rd weeks after treatment. By week 4, approximately 60% of the eggs ovulated produced living foetuses and there was then a sharp fall in pre- and post-implantation losses (Table 2). Normal numbers of live offspring were recorded from the fifth week onwards. No changes in spermatid numbers were detected even after the higher dose (200 mg./kg.; Table 5).

(iv) *Isopropyl methane sulphonate* (IMS)

Pre- and post-implantation deaths were at control levels for the first 2 weeks after treatment with this compound (50 mg./kg.) (Table 3, Fig. 2). Subsequently the number of pre-implantation deaths fluctuated considerably, the sixth and eighth

Table 2. '*Dominant lethal*' *mutations after treatment of male rats with methyl ethanesulphonate* (*MES*) *and ethyl methanesulphonate* (*EMS*)

		MES 1 × 50 mg./kg.			EMS 1 × 100 mg./kg.			EMS 1 × 200 mg./kg.		
			% loss of ova			% loss of ova			% loss of ova	
Weeks after treatment	Treated cell types represented in ejaculate	No. of corpora lutea*	Pre-implant.	Post-implant.	No. of corpora lutea	Pre-implant.	Post-implant.	No. of corpora lutea	Pre-implant.	Post-implant.
1	Sperm in vas and epididymis	63	10	9	69	1	6	24	8	71
2	Sperm in epididymis	69	16	33	65	21	17	58	69	31
3	Late spermatids	65	25	52	60	0	53	36	97	3
4	Mid spermatids	57	17	32	47	6	53	44	13	25
5	Early spermatids	65	0	6	68	4	12	57	14	18
6	Spermatocytes	58	5	2	61	4	4	61	5	5
7	Spermatocytes	66	0	8	50	4	10	57	21	0
8	Resting spermatocytes and Type B	50	4	2				40	7	3
9	Intermediate and Type A spermatogonia	53	0	6				33	0	6
10	Type A spermatogonia	55	11	9				39	5	0

* Average number of corpora lutea per mating 10·4.

Table 3. *'Dominant lethal' mutations in male rats after treatment with isopropyl methane sulphonate (IMS)*

Weeks after treatment	Treated cell type in ejaculate	IMS 1 × 50 mg./kg. No. of corpora lutea†	loss of ova % Pre-implant.	Post-implant.
1	Sperm in vas and epididymis	61	5	5
2	Sperm in epididymis	49	0	4
3	Late spermatids	70	24	3
4	Mid spermatids	66	12	0
5	Early spermatids	61	26	5
6	Spermatocytes	53	43	2
7	Spermatocytes	49	16	22
8	Resting spermatocytes and Type B	62	44	3
9	Intermediate and Type A spermatogonia	63	*	0
10	Type A spermatogonia	71	24	6

* Values for pre-implantation losses are not given for week 9, since animals would be aspermic at this time.

† Average number of corpora lutea per mating 10·8.

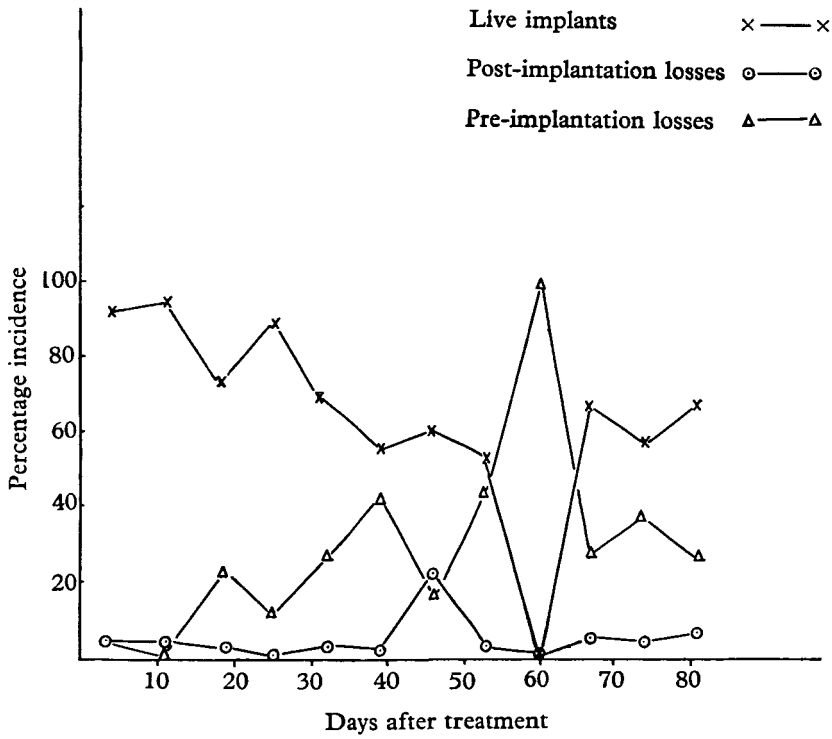

Fig. 2. Fluctuations in percentages of live and dead implantations and pre-implantation losses after isopropyl methane sulphonate (50 mg./kg.).

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weeks showing the highest values (Table 3). Throughout the experimental period, post-implantation deaths remained at control levels (Table 3 and Fig. 2). Normal numbers of stage 8 spermatids were recorded until 20 days after treatment. At 26 days the count was approximately 50% of normal and by 30 days had fallen to zero (Table 5).

The results obtained from the study of eggs recovered 36–40 hours after matings in the fifth to the eighth weeks after treatment are summarized in Table 6. The percentage of penetrated eggs remained high throughout the period studied and the majority of these were two-celled and of normal appearance (Plate 1 *a* and *b*). Of the remaining eggs some had blastomeres of uneven shape and size and small cytoplasmic extrusions in addition to the second polar body, whilst others showed multiple fragmentation of blastomeres (Plate 1 *c* and *d*). A number of ova were found in which the second maturation division and syngamy had occurred but no further development (Plate 1 *e*). These were particularly frequent in the fifth and eighth weeks. In the eighth week, 3 eggs were seen in which sperm penetration had occurred, but the second polar body had not been extruded. The two pronuclei could be seen in the cytoplasm, one considerably larger than the other.

(v) *Busulphan* (Myleran)

After a single dose of Busulphan (4 mg./kg.) normal numbers of living embryos were recorded in the earlier weeks (compare 6 mg./kg. also). A decrease in the seventh week was followed by a normal value in week 8 but this was followed by a further fall in weeks 9 and 10. Pre-implantation losses increased correspondingly in weeks 7 and 9–10, but post-implantation deaths remained at control levels throughout the period (Table 4). Normal numbers of viable implantations were recorded during the first 6 weeks after treatment with 6 mg./kg. of this drug and pre- and post-implantation losses were around control levels (Table 4). A progressive rise in pre-implantation deaths occurred in the seventh to tenth weeks inclusive, with a corresponding fall in live implantations. Post-implantation deaths were at all times below 10%.

After the higher dose (10 mg./kg.) a slight reduction in numbers of viable embryos was evident in weeks 5 and 6 followed by a sharp fall to 13% of normal in week 7. No implantations were recorded from week 8 onwards. The spermatid counts (Table 5) fell between 34 and 39 days, thus aspermia would occur at approximately 62 days after treatment (28 days are required for stage 8 spermatids to reach the ejaculate). Loss of embryos in the ninth and tenth weeks at this dose must therefore be due to aspermia.

During the fifth and sixth weeks after treatment with Busulphan (10 mg./kg.) the majority of eggs recovered were fertilized and had completed first cleavage (Table 6). In the seventh and eighth weeks, however, the number of fertilized eggs decreased to 70%, and of these, only 4/27 and 4/30 respectively had undergone first cleavage. In many of the eggs in these weeks (7 and 8) a sperm tail could be seen in the egg cytoplasm, but the second maturation division and syngamy had apparently not

Table 4. *'Dominant lethal' mutations after treatment of male rats with Busulphan*

Weeks after treatment	Treated cell types represented in ejaculate	4 mg./kg.			6 mg./kg.			10 mg./kg.		
		No. of corpora lutea†	% loss of ova		No. of corpora lutea	% loss of ova		No. of corpora lutea	% loss of ova	
			Pre-implant.	Post-implant.		Pre-implant.	Post-implant.		Pre-implant.	Post-implant.
1	Sperm in vas and epididymis				50	0	6			
2	Sperm in epididymis				50	4	4			
3	Late spermatids				50	4	6			
4	Mid spermatids				53	2	0			
5	Early spermatids	50	8	10	47	6	2	55	11	9
6	Spermatocytes	51	8	6	52	4	8	58	17	9
7	Spermatocytes	69	29	19	46	20	9	54	83	4
8	Resting spermatocytes and Type B	69	11	3	41	22	7	39	100	0
9	Intermediate and Type A spermatogonia	59	22	10	43	32	2	46	*	0
10	Type A spermatogonia	56	43	4	36	66	6	41	*	0

* Values for pre-implantation losses are not quoted for weeks 9 and 10 after 10 mg/kg., since animals would be oligospermic at this time.

† Average number of corpora lutea per mating 9·7.

occurred (Plate 1*f*). No ova showing multiple fragmentation were seen. The proportion of eggs showing a normal second polar body but no further development was similar to that in the isopropyl methane sulphonate series.

Table 5. *Spermatid numbers (stage 8 of spermiogenesis) after various treatments of male rats, expressed as percentage of numbers in untreated animals*

Days after treatment	Arachis oil, 2 ml./kg.	Methyl ethane sulphonate, 100 mg./kg.	Ethyl methane sulphonate, 200 mg./kg.	Isopropyl methane sulphonate, 50 mg./kg.	Busulphan, 10 mg./kg.
1	—	96	94	—	—
5	—	89	90	—	—
10	—	—	—	99	—
12	—	—	—	94	88
14	93	92	97	96	—
18	—	95	—	88	—
19	—	—	—	—	84
20	—	96	80	88	—
24	—	—	—	—	93
26	96	94	100	57	—
30	83	98	95	0	72
34	—	—	—	—	52
39	—	—	—	—	19
42	89	96	92	—	0

Normal average number of spermatids per tubule 197 ± 5·7.

Table 6. *Details of eggs recovered from normal females* 36–40 *hr. after mating with treated males*

		Isopropyl methane sulphonate (50 mg./kg.)				Busulphan (10 mg./kg.)			
		Weeks after treatment:				Weeks after treatment:			
	Control (untreated)	5	6	7	8	5	6	7	8
Number of ♀♀ from which eggs were recovered	7	5	5	6	7	8	4	5	4
Total number of eggs examined	50	33	32	42	47	53	29	27	30
Total number fertilized	44	30	26	38	45	48	29	18	21
Normal, two-celled	38	18	22	28	33	37	24	4	4
Two-celled, unequal blastomeres and/or fragments	2	2	3	5	2	4	4	2	2
Second polar body present, not cleaved	3	7	1	3	7	7	0	5	4
Pronucleate, no polar body	0	0	0	0	3	0	0	7	11
Multicellular	1	3	0	2	0	0	1	0	0

4. DISCUSSION

Cattanach & Edwards (1958) have shown that the sterile period following treatment of male mice with TEM is due to induction of dominant lethal mutations, more of which are produced in developing spermatids than in mature spermatozoa. More recently, Cattanach (1959) has shown that the decrease in fertility of TEM treated males is associated with an increase in the frequency of translocations among their F_1 offspring. The frequency of translocations among these F_1 individuals is thought to represent the proportion of viable eucentric rearrangements between chromosomes, so that a similar proportion of non-viable aneucentric rearrangements might be expected to occur. These, together with single breaks and deficiencies, will probably cause death of the F_1 embryos and may be considered to be dominant lethal mutations.

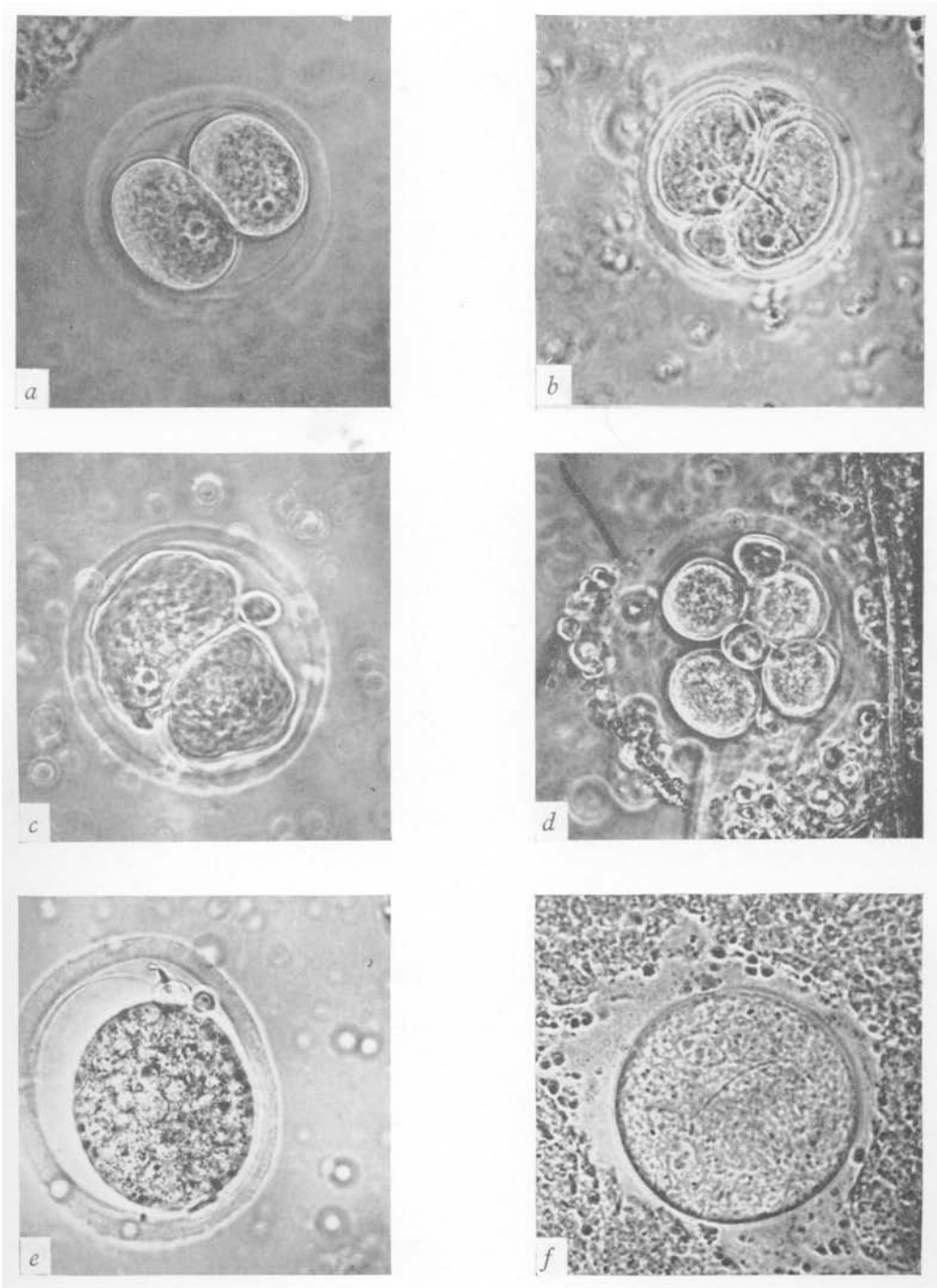
Analysis of X-ray induced dominant lethals in mice (Bateman, 1958*b*) and TEM induced lethals in rats and mice (Bateman, 1960) has shown that they are expressed either as unimplanted eggs or deciduomata. Thus, in the rat and mouse, deficiencies resulting from the loss of small acentric fragments will cause breakdown of the embryos after implantation; larger deficiencies will account for embryonic death in the early cleavage stages.

Considerable variation in sensitivity of spermatogenic cells to mutagenic agents has been reported. Bateman (1960) considered that spermatids, the cells most sensitive to X-rays, were 'highly resistant' to TEM, whilst Cattanach (1959) reported a similar pattern of sensitivity in mice to both TEM and X-rays.

The reduced fertility of male rats after treatment with methyl ethanesulphonate and ethyl methanesulphonate is presumably due to the induction of 'dominant lethal' mutations in spermatozoa and spermatids which are expressed mainly as post-implantation deaths (Table 2). The lowest litter sizes and hence the highest mutation rates occurred about the third week after treatment with both compounds (Table 2). Thus, spermatids in the acrosome phase (stages 8–14 in the classification of Clermont, Leblond & Messier, 1959) are the most susceptible cells.

On the present evidence, the partition of 'dominant lethals' between pre- and post-implantation deaths is dose-dependent. After ethyl methanesulphonate (100 mg./kg.) the majority of losses occurred post-implantation. A higher dose (200 mg./kg.) reduced the implantation rate considerably (weeks 2–4) and extended the effects to mature spermatozoa (week 1). At this dose, sterility was due almost entirely to pre-implantation deaths (Table 2). A similar dose-response relationship has been described by Bateman (1960) for TEM.

Isopropyl methanesulphonate and Busulphan exert their maximal effect on earlier stages of spermatogenesis, i.e. spermatogonia and spermatocytes (Tables 3 and 4), but sensitivity to the former compound also appears to extend to spermatids (Table 3). Both compounds caused a high proportion of pre-implantation losses from 30 and 42 days onwards, respectively, after a single dose. Recently, quantitative cell population studies in the rat testis after Busulphan indicated that this drug imposed a specific block to the earliest Type A spermatogonial divisions (Partington,



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Fox & Jackson, 1963). Isopropyl methanesulphonate was apparently less specific, its action also extending to subsequent Type A divisions. Neither compound directly affected the numbers of Type B spermatogonia or spermatocytes, and the time of fall in spermatid counts observed in the present study (Table 5) confirms this finding. There was no histological evidence of interference with the dynamics of spermatogenesis, i.e. no disturbance could be detected in the relative frequency of the fourteen stages of spermatogenesis.

On this basis, aspermia should occur about 55 days after isopropyl methanesulphonate (50 mg./kg.) and 60 days after Busulphan (10 mg./kg.). Thus, the high pre-implantation losses during weeks 8 and 9, and week 9 onwards respectively, could be the result of oligo- or aspermia. However, eggs recovered from eighth week matings of rats treated with isopropyl methanesulphonate, were fertilized in normal numbers and developmentally similar to those recovered in earlier weeks (Table 6). In the Busulphan series, fewer ova were fertilized and in some there appeared to be interference with syngamy, since development did not proceed beyond the early pronucleate stage (Plate 1*f*). A similar abnormality was observed by Edwards (1957*a* and *b*) in ova (4 of 46) fertilized by spermatozoa exposed *in vitro* to high doses of X-rays. The suggestion, that this effect was the result of damage to a non-chromosomal factor, seems unlikely to apply in the present experiments where effects on early spermatogenic cells are involved.

In the earlier weeks (5–7) after both compounds, eggs were fertilized in normal numbers and the percentage completing first cleavage (Plate 1*a* and *b*) was generally greater than the percentage of viable implantations (cf. Tables 3 and 4 with 6). Thus, fertilized ova which had not undergone first cleavage at the time of sampling, and embryos which had cleaved abnormally, must contribute to the pre-implantation losses during this period. Abnormalities, similar to those observed here, have been reported after radiation (Brenneke, 1937) and TEM (Cattanach & Edwards, 1958; Bateman, 1960). They are thought to be the result of chromosome breakage in treated spermatozoa and spermatids.

The reduction in implantation rate after isopropyl methanesulphonate and

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EXPLANATION OF PLATE

(*a*) Apparently normal two-celled egg recovered from a mating in the sixth week after treatment of the male with isopropyl methane sulphonate (50 mg./kg.).

(*b*) Apparently normal two-celled egg with persistent polar bodies recovered from a mating in the sixth week after treatment of the male with Bulsulphan (10 mg./kg.).

(*c*) Degenerating two-celled rat egg recovered from mating in the seventh week after treatment of male with isopropyl methane sulphonate (50 mg./kg.). Note the unequal size of the two blastomeres.

(*d*) Fragmenting egg recovered from a mating in the sixth week after isopropyl methane sulphonate.

(*e*) Uncleaved egg recovered from a mating in the fifth week after isopropyl methane sulphonate. Two polar bodies can be seen but there is no development beyond syngamy.

(*f*) Egg recovered from a mating during the seventh week after Busulphan. The sperm tail and two pronuclei are visible.

Busulphan prior to the onset of aspermia could therefore be due to the induction of dominant lethal mutations in spermatids and spermatocytes. A similar low yield of 'dominant lethals' from irradiated spermatocytes was described by Bateman (1958*a*). In both cases this may be due to the elimination of damaged cells by selection rather than to a lack of sensitivity.

The effects of these alkylating agents on the spermatogenic epithelium may have a common explanation in terms of chromosome damage and in some cases cell death. The outstanding difficulty remains, however, to explain the high degree of selective action of Bulsuphan on Type A spermatogenia, and the fact that such closely related compounds (ethyl methanesulphonate and isopropyl methanesulphonate) exert their maximal effects on pre- and post-meiotic stages respectively.

5. SUMMARY

Sterility resulting from treatment of male rats with methyl ethane sulphonate and ethyl methane sulphonate was found to be due to the induction of dominant lethal mutations in spermatozoa and spermatids. Spermatids in the acrosome phase of development were found to be the most sensitive cells and at low doses of both compounds the majority of deaths occurred after implantation. The earlier cell stages, i.e. spermatogenia and spermatocytes, were the most sensitive to isopropyl methane sulphonate and Busulphan (Myleran). The action on spermatogonia was confirmed by spermatid counts and the time of occurrence of oligospermia or aspermia was calculated. Pre-implantation deaths occurred mainly in the early cleavage stages after both isopropyl methane sulphonate and Busulphan; at least part of these losses can be attributed to the induction of dominant lethal mutations in spermatocytes and early spermatids. Isopropyl methane sulphonate appears to be the more effective of the two drugs in this respect.

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