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THE ACTION OF TRIETHYLENEMELAMINE ON THE FERTILITY OF MALE RATS

BY

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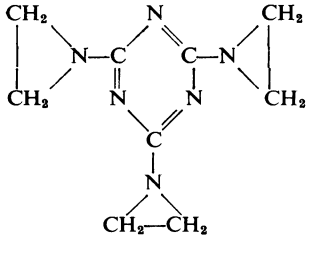
Small doses of triethylenemelamine (TEM) had a selective action on the fertility of male rats. One dose (0.2 mg./kg., i.p.) produced effects ranging from subfertility to sterility during the next 3 weeks. In the fourth week sterility was the rule, but normal fertility was restored in the fifth week. A short course of the drug (5 daily doses, 0.2 mg./kg., i.p.) resulted in sterility lasting about 5 weeks, after which fertility was rapidly regained. Daily doses (0.05 mg./kg., i.p.) caused infertility in about a week which was maintained throughout treatment (7 weeks), and persisted for several weeks after the drug was discontinued. Sexual activity of infertile animals seemed normal and sperm production appeared to continue. Spermatozoa from infertile animals were able to reach and penetrate ova. The results suggest that TEM acts directly on the germinal epithelium. An attempt has been made to provide some explanation for these results and correlate them with the time required for spermatogenesis.

Recent studies of the inhibitory effects of triethylenemelamine (TEM) on the growth of the Walker tumour involved the prolonged administration of this substance to rats (Jackson, 1954). During these experiments tumour-bearing animals of both sexes were kept together, and it was noticed that no litters were produced. It seemed of interest to investigate this effect, with the result that a selective action of triethylenemelamine on the fertility of male rats has been found. In the female rat, the drug prevents the development of the early embryo. A brief account of some earlier results has been published (Jackson and Bock, 1955).



Triethylenemelamine (TEM)

MATERIALS AND METHODS

Triethylenemelamine, a trifunctional ethyleneimine, was prepared according to the method described by Bestian (1950). Both the crystalline compound and its aqueous solution were stored at 5° C. In view of its reputed instability, solutions of the drug were renewed at intervals of about one week as a precautionary measure.

The rats used were an inbred American Wistar strain maintained on a cube diet supplied by the North-Eastern Agricultural Society. In earlier experiments, the fertility of male rats was assessed by group mating—for example, 5 males and 10 females were allowed to remain together in one cage for a period of 14 days, after which the females were removed and housed in separate boxes ; such a technique is only of value when the dose of drug given is sufficient to render all the males infertile. Its limitations led to the use of isolated mating, in which paired animals were housed in separate boxes and the course of events followed by examination of vaginal smears as early as possible each morning. Successful impregnation was almost always associated with the detection of spermatozoa in the vaginal smear ; very occasionally sperm were not seen but litters were produced. Insemination usually occurred within 6 days of pairing, often in the first 4 days. The final routine adopted was to pair off treated males with females and change the latter each week. Insemination by treated, infertile males still producing spermatozoa was followed by pseudopregnancy lasting about 15 days, during which time there was no evidence of further impregnation. As far as possible the female rats used were of established fertility, but on occasions recourse had to be made to virgin animals. For some purposes females in oestrus were selected in order to enhance the likelihood of successful mating on a particular occasion. Control groups of either sex were strictly comparable with those of experimental groups.

RESULTS

Table I shows the combined results of two similar experiments in which persistent treatment of male rats with TEM was carried out (42 doses in 57 days, at the rate of 0.05 mg./kg. each).

TABLE I

EFFECT OF REPEATED ADMINISTRATION OF TRIETHYLENEMELAMINE ON THE FERTILITY OF MALE RATS (42 DOSES, 0·05 MG./KG., I.P., IN 57 DAYS)

Each of 5 treated male rats were paired at various times with females of established fertility. During each period, insemination was detected within the time range indicated in the second column. The table is a composite one derived from two similar experiments.

Mating Period	Time Range of Inseminations (Days from 1st Dose Incl.)	No. of Females Found Inseminated	No. Producing Litters	Individual Litter Size
1	9–11	5/5	1/5	0, 0, 0, 3, 0
2	15–17	4/5	0/5	0
3	35–37	4/5	0/5	0
	TREATMENT DISCONTINUED ON DAY 57			
4	62–63	3/5	0/5	0
5	74–75	3/5	0/5	0
6	87–90	4/5	0/5	0
7	92–94	5/5	3/5	0, 5, 3, 0*,
8	107–108	5/5	4/5	10, 5, 11, 0, 8
9	109	5/5	4/5	0, 12, 7, 6, 12

* Unknown number of young eaten.

Sterility was soon induced and was maintained not only during treatment but persisted for about 30 days after the last dose. Thus inseminations occurring between 87 and 90 days from the commencement of treatment were unproductive, whilst those taking place between 92 and 94 days indicated that fertility was returning. By the 109th day 8/10 (8 of 10) animals showed normal fertility. Throughout the period of sterility, both during and after treatment, sperm production apparently continued as indicated by their presence in vaginal smears. The sperm content of vaginal smears was similar in females mated with either treated or untreated males. No change in the sexual behaviour of the treated animals was observed.

The administration of one dose of TEM (0.2 mg./kg., i.p.) produced complex effects (Table II). Treated males were mated after a lapse of 24 hr. to allow clearance of the drug, when apparently normal insemination occurred in 9/10 pairs between the third and sixth day according to the oestrous cycles of the females concerned. Only 5/10 produced litters compared with 5/5 in the corresponding control series, although the actual litter sizes were comparable in each group (Table II, week 1). In a subsequent experiment, again using 10 treated males, insemination occurred in 9/10 females between the second and fourth day, but only three litters were produced. In neither of these experiments was there any relation between time after the dose when insemination occurred and the successful production of litters. Referring again to the experiment shown in Table II, 8/10 females were impregnated between day 8 and 10 after the dose of TEM (week 2). Of these, 7 produced litters, but the number of offspring in each was much reduced compared with the controls; this subfertile state also extended into the third week of the experiment. It was noteworthy that during these three weeks of mating sterility was not a constant feature in any one male. A striking change occurred in the fourth week of the experi-

TABLE II

EFFECT OF ONE DOSE OF TRIETHYLENEMELAMINE (0·2 MG./KG., I.P.) ON THE FERTILITY OF MALE RATS

Each vertical column shows the results of pairing 10 treated male rats for six consecutive weeks with females of established fertility. They are to be compared with those from 5 untreated males in a concurrent experiment. Each rat was numbered and the litter data are to be read horizontally at the appropriate level. Weekly fluctuations in fertility are pronounced in each treated male during the first 3 weeks, with uniform sterility in the 4th and restoration of normal fertility in the matings of the 5th and 6th weeks.

	TREATED						CONTROLS					
Week	1	2	3	4	5	6	1	2	3	4	5	6
Time range of inseminations (days after dose)	3–6	8–10	Not examined	23–26	30–33	36–39	3–6	8–11	Not examined	23–25	30–33	36–38
No. of females found inseminated	9/10	8/10	—	9/10	9/10	9/10	4/5	4/5	—	5/5	5/5	5/5
No. of litters produced	6/10	7/10	8/10	1/10	9/10	8/10	5/5	4/5	4/5	5/5	5/5	5/5
Individual litter size:												
Rat No. 1	0	4	1	0	8	9	9	4	11	3	9	9
2	0	0	2	0	0	0	9	0	12	12	6	9
3	5	0	5	0	6	11	8	11	13	11	12	12
4	8	3	3	0	7	0	5	13	0	11	9	9
5	0	0	5	0	7	4	8	10	13	4	9	9
6	8	0	3	0	10	8						
7	†	3	6	0	10	3						
8	9	5	5	1 (d)	5	9						
9	0	3	0	0	6	7						
10	5	2	0	0	5	12						

†=Unknown number of young eaten. (d)=Dead foetus.

TABLE III

FREQUENCY OF MATING OF MALE RATS IN RELATION TO THE ACTION OF TEM (ONE DOSE, 0·2 MG./KG., I.P.)

In matings before the drug was administered the male remained each night with 5 females in oestrus, and in the post-treatment matings two females in oestrus/male were used on each occasion. The females used were of established fertility, and all the positive vaginal smears showed masses of spermatozoa. In spite of repeated matings the changes in fertility followed a similar sequence as when single weekly matings were used (Table II).

					Drug Given ↓		POST-TREATMENT										
	Days	4	3	2	1	0	2	4	5	7	8	11	15	20	22	24	28	34	42
MALE A	No. of females inseminated	1	2	0	1	0	1	1	0	2	1	1	1		0	1	1	1
	No. producing litters	1	2	0	1	0	1	0	0	0	1	1	0		0	1	1	1
	Litter size	6	4, 5	0	11	0	6	0	0	0	4	1	0		0	6	9	13
MALE B	No. of females inseminated	1	0	2	2	1	1	0	0	2	1	1	0	1	1	1	1	1
	No. producing litters	1	0	1	1	1	1	0	0	0	0	0	0	0	0	1	1	1
	Litter size	4	0	10	6	4	2	0	0	0	0	0	0	0	0	8	8	12

ment, for, in spite of "normal" insemination of 9/10 females, between the 23rd and 26th day one animal only gave birth to a dead foetus. None of the others produced litters or, indeed, showed any signs of pregnancy. However, impregnation of 9/10 females during the 5th week (between days 30 and 33) resulted in normal-size litters from each animal, and a similar result was obtained from the final period of mating (week 6). Comparable results have emerged from other experiments using the same dose of the drug. In these experiments, each female remained with the male for a week, insemination occurring at oestrus when ovulation takes place spontaneously. The manifest effects of TEM on fertility might depend on the frequency of mating with the removal of available mature spermatozoa, as well as on the rate of replenishment. Table III records the changes in fertility of two male rats paired frequently with females in oestrus after the injection of one dose of the drug (0.2 mg./kg.). For several nights before the drug was given, each male was mated with groups of five females in oestrus in an attempt to deplete any sperm reservoir, but surprisingly few were inseminated. After the drug, matings were continued, using 2 new females per male at the times shown. Inseminations continued at a similar rate as in the pretreatment period, and the changes in fertility were comparable to those observed when one mating each week was the routine (Table II). The sperm content of vaginal smears was similar in pretreatment and post-treatment matings.

The effect on fertility of five daily doses of TEM (0.2 mg./kg., i.p.) has been examined on a number of occasions. The combined results of two experiments are shown in Table IV. Such a course of treatment effectively inhibits the growth of certain rat tumours (Walker carcinosarcoma, Jensen sarcoma) which accounts for its application in the present work. Before commencing the series of matings, it was thought prudent to allow at least 48 hr. to elapse after the last dose of TEM to ensure adequate clearance of the drug. Sterility of the males was the rule from the eighth day after the first dose, and this condition persisted for 25 to 29 days after the final dose. Fertility had returned in 2/5 animals 33 days from the last dose and recovery was complete 38 to 40 days from the end of treatment. These figures are based on the known dates of insemination by individual males. Throughout these experiments the sperm content of vaginal smears was similar in both control and treated groups of animals. Repeated injections of TEM (0.2 mg./kg.) distributed over a prolonged period of time (21 doses in 57 days) produced infertility in a group of rats which was maintained long after the drug had been discontinued (Jackson and Bock, 1955). Even 75 days after the last dose, fertility had returned in only one animal. Spermatozoa were found in

TABLE IV

EFFECT OF TRIETHYLENEMELAMINE (5 DAILY DOSES, 0·2 MG./KG., I.P.) ON THE FERTILITY OF MALE RATS

Results of post-treatment pairings of 5 treated males with females of established fertility. Note the prolonged infertility associated with seemingly normal inseminations. The restoration of normal fertility ultimately occurs quite rapidly.

Mating Period	Time Range of Inseminations (Days from 1st Dose Incl.)	No. of Rats Inseminated	No. Producing Litters	Individual Litter Size
1	8–12	5/5	0/5	0
2	12–17	5/5	0/5	0
3	20–24	5/5	0/5	0
4	30–34	5/5	0/5	0
5	37–42	5/5	2/5	0, 0, 5, 3, 0
6	43–45	5/5	5/5	4, 6, 10, 4, 7
7	52–53	5/5	4/5	9, 0, 6, 10, 8

vaginal smears from 4/5 females 15 to 20 days after the last dose, but their numbers were obviously reduced compared with the corresponding controls. In subsequent matings sperm were encountered only sporadically. This treatment produced obvious toxic effects such as failure to gain weight and general lack of well-being.

It has been shown that spermatozoa produced by rats rendered infertile with TEM can reach and penetrate ova, although no evidence of embryonic development was seen in the uterine horns of females examined at intervals up to 14 days after insemination. For example, five treated male rats (after 25 doses of TEM, 0.05 mg./kg. in 30 days) were mated separately with females in oestrus. The next morning (12 hr. later) vaginal smears indicated that insemination of four had occurred, so these animals were killed and shed ova removed from the Fallopian tubes. From each animal ova were recovered into which sperm had penetrated. In all 11/25 ova contained spermatozoa. Since the male rats used had previously been shown to be infertile, they were re-mated 24 hr. after the above experiment. In spite of apparently normal inseminations during the next few days no litters were produced (Table I, period 3). The initial fertility of these males is established by the recovery of fertility some weeks after treatment was discontinued. In a comparable experiment with untreated males, 38 ova were recovered, 33 of which contained spermatozoa. Similar results were obtained during the infertile period after five daily doses of TEM (0.2 mg./kg., i.p.). Five treated males were paired with females in oestrus on the 28th night after the first dose. By the next morning two animals had been inseminated and were killed about 12 hr. after mating. Shed ova were recovered with the following results:

Female 1. R tube: Four ova found, all containing sperm. In two ova the nuclei had fused; in the others the sperm were lying free in the cytoplasm. L. tube: Two ova found, both containing sperm.

Female 2. R. tube: Five ova seen, four containing sperm. L. tube: Four ova found, one containing a spermatozoon.

The two females killed were replaced and the five paired animals then constituted the group referred to in Table IV (mating period 4), from which no litters resulted. All the males of the group later recovered their fertility.

When litters were born after mating with treated males, the period of gestation was within normal limits and no superficial abnormalities were seen in the offspring, which were usually kept for three weeks.

Discussion

Triethylenemelamine is a highly toxic substance, its pharmacological effects being generally ascribed to its ability to react chemically with undetermined but vital cell components and thus interfere with the process of cell division. The results may be manifest, for example, as toxic effects on the susceptible bone marrow and alimentary tract or as inhibition of the growth of some varieties of experimental tumour. On the other hand, mutagenic changes may be induced or neoplastic transformations brought about (Hendry, Homer, Rose, and Walpole, 1951; Walpole, Roberts, Rose, Hendry, and Homer, 1954). The great susceptibility of the human subject to the action of TEM is well recognized (Haddow, 1953; Nabarro, 1953) although rodents are more tolerant, the mouse more so than the rat. It may be that these differences are related to the ability of the recipient species to metabolize the drug, and that too much emphasis has been laid previously on its chemical reactivity (Craig and Jackson, 1955).

In view of the rapid rate of cell division in the testis, the susceptibility of this organ to damage by TEM is not unexpected and is, at first sight, in keeping with the well-known radiomimetic properties of the drug. The histological picture of destruction in the testis of the dog and the rat after TEM was noted by Hendry *et al.* (1951) during an extensive survey of the tumour-inhibiting properties of ethyleneimines. They observed that a lethal dose of TEM to rats produced effects on the germinal epithelium ranging from inhibition of spermatogenesis to complete atrophy. The present work indicates that TEM can produce more subtle effects on spermatogenesis than is suggested by the picture of inhibition and destruction referred to above. Evidence has been presented which shows that the entire sequence of sperm production may be affected, possibly by interference at certain key positions. Spermatogenesis is a process of great complexity (Fig. 1) and little is known about the factors which initiate and regulate its many stages. However, a working hypothesis has been developed which provides some explanation for the effects produced by TEM. It has been necessary to assume that spermatogenesis goes on continually and that, over the testis as a whole at any given time, constant proportions of the germinal epithelium are occupied in particular phases of the process. In this connexion, it has been reported that mitotic activity in the testis of the mouse and rat is constant throughout the day (Bullough, 1948; Clermont and Leblond, 1953), whilst close agree-

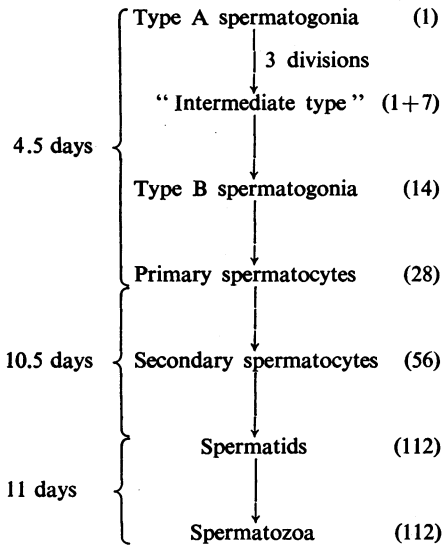


FIG. 1.—Outline of the development of spermatozoa. (Adapted from work by Leblond and Clermont (1952) and Oakberg (1955).) The estimated number of cells originating from one spermatogonium is shown in brackets for each stage. The population of spermatogonia is said to be maintained by one out of the 8 cells arising from three initial divisions of a type A spermatogonium. The duration of the stages (Leblond and Clermont, 1952) is based on an estimate of 26 days as the time required for spermatogenesis.

ment in % of tubules present in any given stage of the seminiferous cycle has been observed in different rats (Leblond and Clermont, 1952).

The most notable feature which follows a single injection of TEM is a period of sterility associated with inseminations occurring between 23 and 26 days after the dose (Table II). This suggests a well-defined action of the drug on an early stage of the spermatogenic process, its specific nature being emphasized by the production of normal-size litters after the next inseminations between 30 and 33 days. It follows that generations of cells succeeding those affected by the drug are unharmed and implies that waves of spermatogenic activity passing along the many seminiferous tubules are co-ordinated and in phase. The rapid restoration of normal fertility also indicates that the available mature sperm was used at each impregnation. Other changes in fertility after one dose of TEM show that later stages in spermatogenesis are affected. The marked reduction in litter size following matings of the second and third weeks (Table II) appears to be best explained by inseminations with mixtures of normal and infertile spermatozoa. Since the latter have been shown to be able to reach and penetrate ova, the subfertility could be due to competition for available ova by the two kinds of sperm. The normal sperm may originate from a closely associated phase of spermatogenesis which is less susceptible to the drug. This is not unreasonable, since the development of spermatozoa involves many stages and morphological changes. In fact, the results of a short exposure to the compound may well correlate with the time required to complete various stages of spermatogenesis. It is difficult to understand why so many inseminations occurring during the first six days after the drug were non-productive, whilst others resulted in litters of normal size. This action of the drug is presumably associated with late stages of spermatogenesis or even with mature sperm, but there is no apparent relation between the sterility of the mating and the time after the dose when impregnation occurred. An antimitotic effect is excluded since the formation of mature sperm from spermatids requires 11 days and does not involve cell division. Another unexplained result is the significant but irregular variation from fertility to sterility occurring in individual males from week to week (Table II).

The question of the influence of frequency of effective mating in experiments of this nature is an important one. How far any existing store of mature sperm in the rat is utilized during insemination is, so far as we are aware, unknown. From experiments carried out during the present investigations, it appears that the rat rarely inseminates more than one or two females in oestrus per night ; also that periods of one or two nights recur during which there is no evidence of insemination. It is interesting that the overall effects on fertility of one dose of TEM seem to be independent of previous copulations or the frequency of mating after the treatment. Physiological considerations appear to govern the results, so that insemination of one female each week (Table II) reflects the changes induced in the spermatogenic process as effectively as more frequent matings (Table III). This matter is being examined in more detail.

Shaver (1953) has estimated that, in the rat, about 27 days are required for mature sperm to be formed from spermatogonia. This figure was derived from a histological study of the effect of whole-body radiation on the testis, which caused complete inhibition of mitosis in spermatogonia (and possibly in more primitive cells) lasting for about four weeks. Later stages were apparently unaffected by the treatment, so that a maturation depletion of the germinal epithelium occurred, which was complete in 27 days. Thereafter a re-

population of the epithelium occurred, requiring 4 to 5 months for completion. The parallel between Shaver's estimate of 27 days and the occurrence of an infertile period 23 to 26 days after one dose of TEM is very striking. In the drug experiment, however, it is evident that only sterile sperm were available for copulations between 23 and 26 days after the dose and that these were rapidly replaced by fertile ones; thus, without an intermediate mating, full fertility was present at the latest 30 to 33 days after the injection. By analogy with Shaver's work, it appears that a comparable early stage in spermatogenesis is most susceptible to TEM. The available evidence suggests that small doses of TEM impose modifications on the sperm-producing mechanism so that normal-looking but infertile sperm are produced. There is no indication of a maturation depletion such as Shaver describes after irradiation, for the speedy restoration of normal fertility could not then occur.

Short courses of TEM (Table IV) produce more drastic effects on fertility. All animals were sterilized for 3 to 4 weeks after the last dose, but during this period inseminations continued at seemingly normal levels. Ova can be reached and penetrated by these sterile sperm, but, owing to some change due to the drug, embryonic development does not proceed. In this connexion it is known that spermatozoa of *x*-irradiated rats can retain motility and fertilizing power, but are unable to initiate normal development of the ovum (Brennecke, 1937). The return of fertility after several doses of TEM is also quite rapid; from the dates of insemination of individual animals fertility had returned 35 to 42 days after the last dose, no remarkable prolongation over the re-establishment of normal fertility after one dose of TEM. The development of sterility in about a week with small daily doses of TEM (Table I) emphasizes the overall sensitivity of spermatogenesis to the drug and shows that the effect is cumulative. In these prolonged experiments, sterility is associated with continued sperm production as evidenced by insemination findings and the sperm complement of the cauda epididymis from animals killed during and after the course of treatment. Frequent administration of the drug should ensure that successive generations of cells passing through susceptible phases of spermatogenesis become modified. Once the entire sequence has been affected discontinuation of treatment must be followed by persistence of sterility. The time required for fertility to return should be a measure of that needed for the generation of spermatozoa from the earliest site of action of the drug. After two months of infertility during treatment (Table I), fertility had returned in 2/5 animals 35 days after the last dose. Unfortunately more exact data are not available owing to the timing of the mating periods. It seems clear, however, that more intense or prolonged treatment in these various experiments does not unduly retard the recovery process and points to a common focus of action on an early stage of spermatogenesis. It is hoped that further experiments now in progress combined with histological studies will give more precise information and help to locate the sensitive phases. The possible rôle of oligospermia caused by the drug has also to be considered, but an accurate evaluation of this can only be made by sperm counts—a difficult problem in these small animals. However, the normal sperm content of the vagina after insemination by treated sterile animals and the ability of these sperm to reach and penetrate ova suggest that a reduced sperm output is not a major factor.

Although TEM is a highly toxic substance, the lower doses used produced little evidence of untoward effects apart from some interference with gain of weight. Blood examinations were not carried out, but Crossley, Allison, Wainio, and Muenzen (1951) found a similar dose rate (0.05 mg./kg. daily) caused a mild leucopenia in their rats; half this dose produced neither leucopenia nor other demonstrable toxic effect. Walpole *et al.* (1954) have shown that injections of TEM in arachis oil are actively carcinogenic, but no tumours were obtained after the administration of aqueous solutions. In the present work only aqueous solutions have been used and treated rats have not shown any noticeable tendency to develop tumours.

The relation between chemical structure and antifertility action is being investigated. So far, some other ethyleneimines have been shown to produce similar effects to triethylenemelamine, but comparable activity has not been observed among a wider range of antimitotics examined, including other radiomimetic compounds of the alkylating type.

The authors are much indebted to S. Muldal, Cand. real., for demonstrating that sterile sperm from TEM-treated male rats were able to reach and penetrate ova.

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