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EMBRYOPATHIES IN THE RAT DUE TO ALKANE SULPHONATES

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Summary. Certain alkane sulphonic esters in pregnant rats cause a variety of embryopathies ranging from death *in utero* and congenital malformation to subtle impairment of gonadal form and function. The structure–activity relationship of a homologous series of compounds has been studied.

INTRODUCTION

Gonocytes in the testis of the foetal rat are susceptible to the cytocidal effect of busulphan (Hemsworth & Jackson, 1963). The aim of the present work was as follows:

(a) To determine whether the sensitivity of the rat embryo and selected gonadal tissue to closely related alkane sulphonic esters varies with age. The assessment was based on embryopathies ranging from mortality and teratogenic change to subtle impairment of testicular form and function.

(b) To study the regenerative capacity of the seminiferous epithelium and to investigate the relationship between histological changes and fertility.

(c) To investigate the structure–activity relationship of a series of closely related alkane sulphonates on the above systems.

MATERIALS AND METHODS

Animals

Wistar rats were kept in metal boxes or wire cages and provided with unlimited food and water. Their diet consisted of a commercial preparation, No. 86, supplied by the Scottish North Eastern Agricultural Society. Females were paired with fertile males and the day spermatozoa were found in the vaginal smear was regarded as Day 0 of pregnancy. Males were removed, selected dams allowed to reach term and litters weaned when 4 weeks old.

Treatment

Compounds were administered to dams by the intraperitoneal route (i.p.) on the 13th day of pregnancy. The monoesters of methanesulphonic acid were administered in normal saline. The diesters n = 1 to 3 were dissolved in a

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solution of dimethyl sulphoxide and normal saline. Busulphan (n = 4) was suspended in arachis oil. The animals were killed by ether vapour on the 20th day *post coitum* and foetuses examined for gross anatomical deformities.

Groups, consisting of at least four dams, received a dose of drug (i.p.) between the 5th and 20th days of pregnancy inclusive, and were then allowed to go to term. Selected offspring were killed on Days 1 and 15 *post partum* and their testes fixed for histological investigation. Remaining offspring were kept to maturity when their fertility was assessed; subsequently they were killed when 24 weeks old and their testes fixed for histological study.

Histological techniques

Testes were fixed in formol saline for at least 24 hr and, to aid penetration, gonads from adult rats were halved transversely after 1 hr in fixative. Sections were cut at 7 μ, stained in haematoxylin and mounted in Xam.

Evaluation of changes in the testis

Based on a classification of the tubules. Cross-sections of testis tubule in day-old rats contain supporting cells (i.e. precursors of the Sertoli cells) and gonocytes and these were classified 'normal'; but when gonocytes were absent they were regarded as 'sterile'. Testes from three animals derived from separate litters were used in each estimation and 600 tubules per rat were classified. Tubules in 15-day-old rats normally contain supporting cells, spermatogonia and primary spermatocytes. Testes from three offspring which were not litter mates were again used in each estimation and 600 tubules per rat were classified: 'normal' —containing supporting cells, spermatogonia and primary spermatocytes; 'regenerating'—containing supporting cells and spermatogonia; 'sterile'— only supporting cells present. Three hundred cross-sections of seminiferous tubule from each of six adult rats were classified: 'normal'—containing an entire epithelium; 'sterile'—germ cells absent. No more than two adult rats used in each estimation were litter mates.

Cell counts. One day *post partum*: Supporting cells and gonocytes were counted in 100 cross-sections of tubule sampled, on each occasion, from testes of three rats.

Fifteen days *post partum*: When the characteristic four 'stages' (Clermont & Perey, 1957) were recognizable, supporting cells and germ cells were counted in twenty sections at each 'stage'. Alternatively, supporting cells and Type A spermatogonia were counted from 100 sections of tubule sampled from the testes of three rats.

RESULTS

The monoesters produced a high incidence of limb defects although the pattern after the isopropyl derivative, IMS (50 mg/kg) resembled that of the diesters (n = 2 to 4, Table 1). However, after a higher dose of IMS (75 mg/kg) twenty-one of the thirty-nine embryos had syndactyly. All embryos had limb defects after methyl methanesulphonate (MMS) and twenty-three embryos exposed to ethyl methanesulphonate (EMS) had limb defects and four embryos had cleft

palates. Retardation in the development of the lower jaw was frequent after exposure to EMS; fourteen embryos were affected.

The diester methylene dimethanesulphonate (n = 1) produced a high incidence of limb defects; forty-eight of fifty-one embryos were affected. In contrast, dimethylene dimethanesulphonate (n = 2) and trimethylene dimethanesulphonate (n = 3) did not produce a high incidence of limb defects. Busulphan (n = 4; 15 mg/kg) was not teratogenic by superficial inspection

TABLE 1

TERATOGENIC EFFECT OF ALKYLATING COMPOUNDS

	Dose	*No. dams*	*Viable embryos*	*Non-viable implants*	*Cleft palate*	*Head defects*	*Limb defects*
MONOESTERS	(*mg/kg*)						
$CH_3SO_2OCH(CH_3)_2$ IMS	50	5	40	2	0	1	2
	75	5	39	4	0	1	21
$CH_3SO_2OCH_3$ MMS	100	4	32	10	6	1	32
$CH_3SO_2OCH_2CH_3$ EMS	200	5	37	1	4	14	23
DIESTERS	(*mg/kg*)						
$CH_3SO_2O(CH_2)_nOSO_2CH_3$							
n = 1	15	6	51	3	3	0	48
n = 2	100	4	34	0	0	0	0
n = 3	50	5	52	0	1	0	1
n = 4	15	4	39	1	0	0	0
	25	4	33	1	5	6	16
CONTROLS	(*ml/kg*)						
Untreated	0	4	35	1	0	0	0
Normal saline	1	5	42	0	0	0	0
Dimethyl sulphoxide (0·4 ml DMSO + 0·6 ml saline)	1	4	36	1	0	0	0
Arachis oil	1	8	63	2	0	0	0

Drugs were injected into the peritoneal cavity of pregnant rats on the 13th day of gestation. Dams were killed on the 20th day of pregnancy and the embryos were examined.

but a supra-lethal dose (25 mg/kg) caused gross malformations. Of thirty-three embryos, sixteen had limb deformities, i.e. fusion or absence of digits, six had deformed skulls and five had cleft palates.

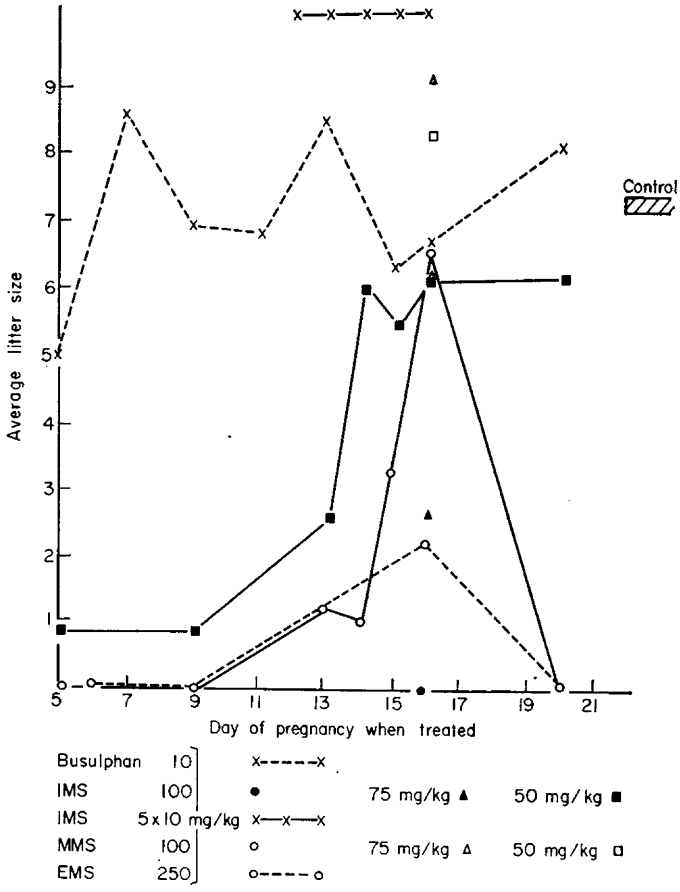
The vehicles used to suspend or dissolve the alkane sulphonate did not produce teratogenic changes.

Effect of alkane sulphonates during pregnancy on the number of offspring produced

IMS (50 mg/kg) was administered to dams on the 5th or 9th day of gestation and the average litter size was less than one (Text-fig. 1). When dams were treated on Day 14 *post coitum* or later, near normal numbers of offspring were born. Higher dosage with IMS, 75 mg/kg or 100 mg/kg on the 16th day of pregnancy reduced the average litter size to 2·7 and to nil respectively. Five consecutive daily doses of IMS (10 mg/kg/day) were administered to dams from the 12th day *post coitum* and a normal number of offspring was born. MMS (100 mg/kg) on Day 5 or 9 of gestation produced effects on litter size

similar to those caused by the isopropyl derivative, although later in pregnancy (Day 16) MMS was less destructive. Near to term (Day 20) MMS was lethal to the embryo. Ethyl methanesulphonate (250 mg/kg) produced effects on litter size resembling those of the methyl monoester.

Methylene dimethanesulphonate (n = 1; 15 mg/kg) on either Day 5, 9, 13 or 14 of pregnancy reduced the average litter size to one, but when the compound was administered on Day 15 or 20 normal numbers of offspring were



TEXT-FIG. 1. Drugs were injected into the peritoneal cavity of dams on a selected day during gestation and at least four rats received similar treatment. The monoesters of methanesulphonic acid were administered in normal saline whilst the diesters n = 1 to 3 were dissolved in a solution of dimethyl sulphoxide and normal saline. Busulphan (n = 4) was suspended in arachis oil.

produced (average litter size eight). Dimethylene dimethanesulphonate (n = 2; 100 mg/kg) on Day 5 or 9 reduced the average litter size to two, but after treatment on Day 13, 14 or 15 a normal number of offspring was produced. Treatment on Day 20 reduced the average litter size to 4·8. Trimethylene dimethanesulphonate (n = 3; 15 mg/kg) on Day 5 or 9, reduced the average litter size to one and 2·3 respectively. The compound was less destructive when

administered on Day 13 or 14, average litter size 3·3 and 5·3 respectively. A normal number of offspring was produced when the drug was administered on Day 15 or 20 *post coitum*. Busulphan (n = 4; 10 mg/kg) exerted its maximal effect early in pregnancy (Text-fig. 1).

Effect of alkane sulphonates on spermatogenesis

Based on a classification of testis tubules. MMS (100 mg/kg) or EMS (200 mg/kg) in near-lethal dose, administered to dams on the 16th day of pregnancy, had no apparent permanent effect on the development of the seminiferous epithelium in offspring (Table 2). IMS (50 mg/kg) administered on the same day had a

TABLE 2

PERCENTAGE OF TUBULES LACKING GERM CELLS

	Dose (mg/kg, i.p.)	*Percentage of tubules lacking germ cells*		
		Day-old	15 *days*	6 *months*
MONOESTERS				
Isopropyl methanesulphonate IMS	50	24	86	17
Methyl methanesulphonate MMS	100	1	0	0
Ethyl methanesulphonate EMS	200	1	0	0
DIESTERS				
n = 1	15	1	87	7
n = 2	100	1	0	0
n = 3	50	2	81	98
n = 4	10	28	100	100

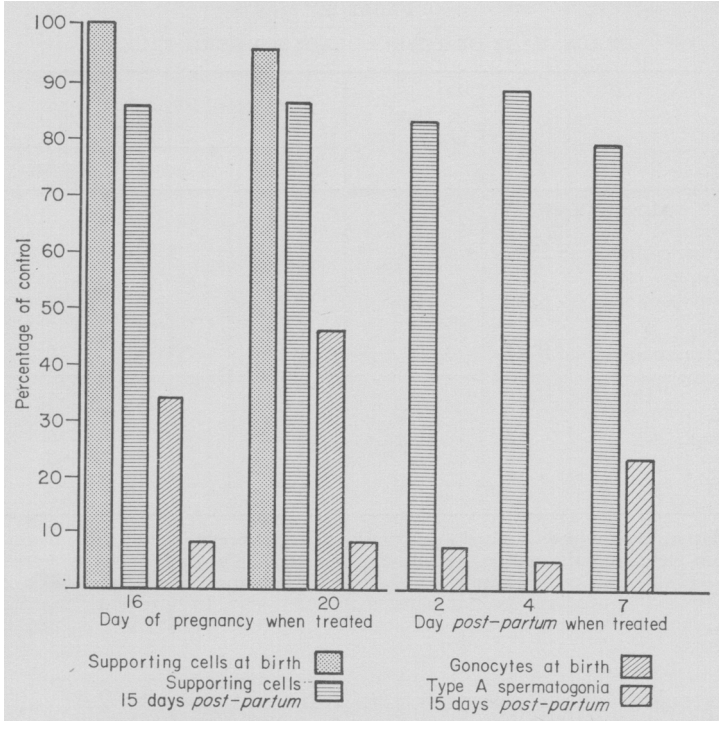
Drugs were injected into the peritoneal cavity of pregnant rats. The monoesters were administered on the 16th day *post coitum*. The diesters were injected on Day 15 *post coitum*. Dams were allowed to go to term and the effect of treatment on offspring assessed when they were 1 day, 15 days and 6 months old. Each value is a mean derived from 1800 transverse sections of testis tubule.

powerful cytocidal effect on gonocytes and consequently 24% of the testis tubules in day-old offspring were 'sterile'. When offspring were 15 days old, 86% of the sections lacked germ cells, but by the time related rats were 6 months old only 17% of the sections were 'sterile'.

Methylene dimethanesulphonate (n = 1; 15 mg/kg) was administered to dams on the 15th day of pregnancy. Although only 1 % of the tubules in day-old offspring were 'sterile', when rats were 15 days old 87% lacked germ cells. By the time the offspring were 6 months old only 7% of the tubules were 'sterile'. A near-lethal dose of the diester n = 2 (100 mg/kg) had virtually no effect on the testes although the related compounds, trimethylene dimethanesulphonate (n = 3; 50 mg/kg) and busulphan (n = 4; 10 mg/kg) caused extensive damage. Neither dimethyl sulphoxide, which was used to dissolve the diesters (n = 1 to 3) nor arachis oil in which busulphan was suspended, had any effect on the histology of the testis.

Based on cell counts. IMS (50 mg/kg) was administered to dams on Day 16 or 20 of gestation. Supporting cells were present in normal numbers in day-old offspring, but when rats were 15 days old they were below normal values (Text-fig. 2). Gonocytes and Type A spermatogonia were greatly reduced in number, the latter to less than 10% of normal. The isopropyl derivative was administered to young rats (i.p.) and its effect on supporting cells and Type A spermatogonia assessed when rats were 15 days old (Text-fig. 2).

A near-lethal dose of MMS (100 mg/kg) or EMS (200 mg/kg) administered to dams on Day 16 *post coitum* had no effect on the number of supporting cells in offspring, although a lower dose of the methyl monoester (50 mg/kg)



TEXT-FIG. 2. Effect of isopropyl methanesulphonate (50 mg/kg, i.p.) administered to pregnant or neonatal rats on the number of supporting cells and gonocytes in day-old offspring and supporting cells and type A spermatogonia in 15-day-old animals. Each value is a mean based on cell counts from 100 cross-sections of testis tubule sampled from three rats.

reduced the number of gonocytes to 32% of normal. The subsequent effect of MMS (100 mg/kg) was studied in 15-day-old offspring. The first generation of spermatocytes was reduced to 33% of control values, whilst the remaining germ cells provided evidence of re-population. Type A spermatogonia were present in normal numbers. The second generation of intermediate type spermatogonia, Type B spermatogonia and resting primary spermatocytes were present in 84%, 65% and 61% of control values respectively. Treating

dams with EMS (200 mg/kg) reduced the first generation of spermatocytes in 15-day-old offspring to 70% of control values, the remaining germ cells were present in normal numbers.

Effect of alkane sulphonates on the fertility of male offspring

The monoesters and diesters n = 1 and 2 had no effect on the fertility of male offspring, a result which contrasts with the sterility caused by the related compounds n = 3 and 4 (Table 3). Neither dimethyl sulphoxide nor arachis oil had any effect on the fertility of offspring.

TABLE 3

STERILE MALE OFFSPRING

	Dose (mg/kg, i.p.)	*Day of pregnancy when dams were treated*				
		13	14	15	16	20
MONOESTERS						
Isopropyl methanesulphonate	50	0/6	0/5	0/6	0/6	0/6
Methyl methanesulphonate	100	—	—	0/5	0/6	—
Ethyl methanesulphonate	200	—	0/6	0/6	0/6	—
DIESTERS						
n = 1	15	—	—	0/5	—	0/6
n = 2	100	0/5	0/5	0/5	—	0/5
n = 3	50	3/3	6/6	6/6	—	6/6
n = 4	10	6/6	6/6	6/6	6/6	6/6

Dams were allowed to go to term and the fertility of offspring was assessed when they were at least 4 months old. The numerator in each fraction shows the number of animals which were sterile and the denominator indicates the number of rats which were tested.

Effect of alkane sulphonates on body and selected organ weights

Although the monoesters had no effect on body weight of offspring, they caused a reduction in the weight of the testis (Table 4). In contrast, the diesters (n = 1 to 4) caused an appreciable reduction in body weight although attention must be drawn to the finding that so did dimethyl sulphoxide and arachis oil. The decrease in testicular weight due to the diesters n = 3 and 4 was accompanied by a substantial decrease in the weight of the seminal vesicles and coagulating glands.

DISCUSSION

Teratogenicity

The diester busulphan (n = 4; 15 mg/kg) was not teratogenic by superficial inspection; however, a supra-lethal dose (20 mg/kg) produced teratogenic changes in the rat (Murphy, Del Moro & Lacon, 1958) and similarly in the present experiments (Table 1). Although methylene dimethanesulphonate (n = 1) produced a high incidence of limb defects, the related compounds n = 2 and 3 did not.

The effect of the branched chain monoester IMS (50 mg/kg) resembled

that of the diesters n = 2 to 4. Methyl methanesulphonate and EMS produced a high incidence of limb defects although neither caused sterility when administered to adult rats due to interference with proliferating germ cells (Jackson, Fox & Craig, 1961). Neither dimethyl sulphoxide (DMSO) nor arachis oil (used to dissolve or suspend the diesters n = 1 to 3 and n = 4 respectively) was teratogenic. In high dosage (2500 mg/kg) DMSO induced congenital malformations in the hamster (Ferm, 1966) but recent studies have shown that, in the Sprague-Dawley strain of rat, 10·25 g DMSO/kg/body weight/day for 3 consecutive days from Day 8 of pregnancy did not cause skeletal malformation among live foetuses (Juma & Staples, 1967). The data obtained in the present experiments suggest a possibility of finding structure–activity relationships in homologous series of compounds.

TABLE 4

PERCENTAGE REDUCTION IN WEIGHT OF BODY, GONADS AND ACCESSORY SEX ORGANS OF 6-MONTH-OLD MALE OFFSPRING

	Dose	*Percentage reduction in weight*		
		Body	*Testes*	*Seminal vesicles + coagulating gland*
MONOESTERS	(*mg/kg, i.p.*)			
Isopropyl methanesulphonate	50	0	21	0
Methyl methanesulphonate	100	0	8	8
Ethyl methanesulphonate	200	0	15	0
DIESTERS	(*mg/kg, i.p.*)			
n = 1	15	14	36	0
n = 2	100	11	6	0
n = 3	50	31	87	43
n = 4	10	19	86	29
CONTROLS	(*ml/kg, i.p.*)			
Dimethyl sulphoxide (0·4 ml DMSO + 0·6 ml saline)	1	9	3	0
Arachis oil	1	9	0	0

Drugs were injected into the peritoneal cavity of pregnant rats on the 15th day of gestation and then dams were allowed to go to term. The values recorded are means derived from groups of rats, each group contained at least five animals.

Embryotoxicity

Busulphan (10 mg/kg) administered to dams of an inbred strain of rat on Day 8 of gestation caused death of embryos due to interference with the development of the allantois and subsequent failure in placentation (Alexandrov, 1966). The embryotoxicity of the monoesters and diesters n = 1 to 3 shortly after implantation may be associated with similar changes. After mid-term, alkane sulphonates generally were less destructive, although susceptibility to methyl and ethyl monoesters and the diester n = 2 increased sharply during the ante-natal period, which may be due to interference with the control of parturition.

Alkane sulphonates during pregnancy and their effect on testes from offspring

Extra-gonadal gonocytes in the rat embryo are susceptible to the destructive action of busulphan (n = 4; 10 mg/kg, Hemsworth & Jackson, 1962; Forsberg & Olivecrona, 1966). Due to the embryotoxicity of the monoesters and diesters (n = 1 to 3) it was not feasible to assess whether they would be destroyed by these compounds.

Methyl and ethyl monoesters, like busulphan (Hemsworth & Jackson, 1963), did not affect the number of supporting cells, i.e. precursors of the Sertoli cells, which emphasizes the selective nature of their effect on germ cells. Although MMS and EMS depleted germ cells in offspring, by the time they were 15 days old re-population was apparent in the testis and, when offspring were adult, neither spermatogenesis nor development of the seminiferous epithelium had been impaired (Table 2). The same applies to the diesters n = 1 and 2, although the former impaired the development of the seminiferous epithelium so that 7% of the tubules in adult offspring were 'sterile' (Table 2).

In contrast, the diesters n = 3 and 4 had a powerful destructive action on gonocytes in the foetal testis, resulting in virtually complete elimination of germ cells in offspring. Furthermore, they presumably impaired the development of the interstitial tissue for accessory sex organs were greatly reduced in weight (Table 4). The branched chain monoester IMS (50 mg/kg) like the diesters n = 3 and 4, destroyed gonocytes although some survived and produced Type A spermatogonia. Although these were reduced to less than 10% of normal number (Text-fig. 2) in young offspring they presumably proliferated, for when offspring were 6 months old the extent of damage to the seminiferous epithelium had been greatly reduced (Table 2).

It is not known why gonocytes are susceptible to the cytocidal effect of certain alkane sulphonates; use of suitably labelled compounds may enable one to determine whether certain destructive compounds are selectively incorporated in specific locations.

Alkane sulphonates during pregnancy and their effect on fertility and libido of male offspring

Monoesters and diesters n = 1 and 2 had no effect on the fertility of male offspring, which contrasts with the sterility caused by the diesters n = 3 and 4 (Table 3). Presumably sterile males did mate, judged by the formation of corpora lutea of pseudopregnancy and the corresponding vaginal smears in inseminated females. Methyl and ethyl monoesters (Jackson, Fox & Craig, 1961) and diesters n = 1 (Fox & Jackson, 1965) and n = 2 (Jackson, 1965) in adult male rats cause sterility due to impairment of spermatids and spermatozoa. Their failure to cause sterility referable to interference with gonocytes emphasizes the selective nature of their action on post-meiotic germ cells. Busulphan (n = 4) in adult male rats causes sterility due to suppression of spermatogonial mitoses (Jackson, Fox & Craig, 1959) and clearly has a range of action extending to gonocytes. Due to the cytocidal effect of the related compound (n = 3) on gonocytes there is little doubt that its administration to adult rats would cause sterility referable to spermatogonial damage.

It is possible that the alkane sulphonates used in the present study or related compounds may have an antifertility action which is not due to the destruction

of foetal germ cells and subsequent oligo- or aspermia. Cattanach & Edwards (1958) have shown for mice and Bateman (1960) for rats and mice that sterility, due to triethylenemelamine (TEM), is due to the induction of 'dominant lethal mutations' in spermatids and spermatozoa. Methyl methanesulphonate and EMS can also induce similar changes in the rat (Partington & Jackson, 1963). Furthermore, these authors have shown that, although IMS and the diester n = 4 exert their maximal effect on spermatogonia and spermatocytes, susceptibility to the induction of 'lethal mutations' by IMS seems to extend to spermatids. It is possible that interference with gonocytes may cause changes compatible with their ability to produce spermatozoa yet rendering the latter impaired. Methyl methanesulphonate in adult male rats causes a small degree of transmissible damage resulting in the partial sterility of offspring (Jackson, Partington & Walpole, 1964), which draws attention to further possible hazards associated with the use of related compounds during pregnancy.

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