

Comparative protective actions of gonadotrophins and testosterone against the antispermatogenic action of ethane dimethanesulphonate

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Summary. After a single dose of ethane dimethanesulphonate (EDS) (75 mg/kg) to rats the prolonged antispermatogenic action is due to a temporary elimination of the functional Leydig cell population. Replacement therapy with testosterone propionate (3 mg/day) maintains the spermatogenic epithelium but the EDS effect develops when hormone treatment is discontinued. In contrast, a short treatment with hCG (10–100 i.u./day) or LH (714 µg/day), starting before the EDS dose, permanently protects the spermatogenic epithelium. FSH treatment was completely ineffective. Although histological protection of spermatogenesis appeared complete with testosterone or hCG, effects on fertility remained but over different periods of time. Antispermatogenic and antifertility effects were produced in mice using much higher doses of EDS (5 × 250 mg/kg) but there was no protection from androgen or hCG. It is suggested that EDS binds to Leydig cells irreversibly, interfering with the action of gonadotrophin. At the dose level used the evidence suggests that the degree of reaction renders most of the Leydig cell population non-viable. A direct cytotoxic effect of the compound upon the spermatogenic epithelium might account for the inability of testosterone or hCG alone or in combination to maintain fertility at normal levels.

Introduction

A single intraperitoneal dose (75 mg/kg) of ethane-1,2-dimethanesulphonate (EDS) to male rats induces a period of sterility during Weeks 2–8 after treatment, which is associated with temporary involution of the ventral prostate and seminal vesicles. These changes have been correlated with impaired testicular androgen biosynthetic capacity (Bu'Lock & Jackson, 1975; Morris & McCluckie, 1979) and elevated serum LH levels (Jackson & Morris, 1977). A 9-day course of testosterone, with the EDS given on Day 4, provided temporary protection of the spermatogenic epithelium (histologically assessed) and maintained some degree of fertility (Jackson, Jackson & Jones, 1973); subsequently the characteristic degenerative changes developed followed by a period of infertility. By contrast, 9 daily injections of hCG (100 i.u.) provided complete protection of the seminiferous epithelium against the EDS but infertility again developed although over a time range different from that of the testosterone experiment. These effects prompted further studies of the protective action of hCG and testosterone and a comparison with that of purified FSH and LH.

Materials and Methods

EDS. The compound is not available commercially and may be prepared as follows. To a stirred mixture of dry ethylene glycol (9.4 g) and pyridine (45 ml) cooled in an ice-salt bath, redistilled

methanesulphonyl chloride (34 g) is added drop-wise from a separating funnel, the reaction temperature being maintained below 10°C. The ice bath is next removed and when the reaction mixture reaches room temperature, it is added with stirring to a prepared ice-acid mixture (a 1 litre beaker three quarters filled with crushed ice onto which concentrated sulphuric acid, 45 ml, has been steadily poured). Stirring is continued until the separating oil has solidified, when it is filtered off with suction, washed well with cold water to free from acid and finally with a little chilled methanol. Two crystallizations from chloroform-methanol yields the pure ester (15 g), m.p. 45°C.

Rats. Randomly-bred adult male Wistar rats (300 ± 25 g) were housed under uniform conditions. Groups of 4-6 rats were given 9 consecutive daily subcutaneous injections of testosterone propionate (Sigma Chemical Co., Poole, U.K.), 3 mg/rat in 0.1 ml arachis oil, or hCG (Pregnyl: Organon, Surrey, U.K.) at dose levels of 100, 50, 25, 10 or 5 i.u./rat in 0.2 ml sterile water. On Day 4 of the hormone treatment, each rat also received a single intraperitoneal dose of EDS (75 mg/kg). EDS is insoluble in water and must first be dissolved in dimethylsulphoxide (0.5 ml per 75 mg) and then water (1.5 ml) added drop-wise with agitation to give the required 2 ml dose volume per kg of animal weight. Other rats received a 9-day course of testosterone propionate and hCG (100 i.u./rat) with EDS as above. A further experiment involved the daily injection of testosterone propionate (3 mg/rat) for 27 days, with EDS on Day 4, to determine whether prolonged treatment could lead to complete protection against the EDS dose. Control groups given EDS or hormone only were run in parallel.

The fertility of treated males was assessed over 12 consecutive weeks after the EDS dose by the weekly serial mating procedure. In addition, treated rats (4-6 per group) were killed with ether at various times and their reproductive tracts were examined and processed as described below.

Although predominantly LH-like in its action, hCG contains significant contaminant FSH activity. Rats were therefore also treated with purified ovine preparations of FSH (NIH-FSH-S9) or LH (NIH-LH-S18). Each animal received a daily subcutaneous injection for 9 days of 714 µg FSH or LH in 0.1 ml sterile water. EDS was injected on Day 4 and the animals killed 18 days later.

Mice. Comparable studies were carried out with adult Swiss mice using 5 daily doses of EDS (250 mg/kg), alone or in conjunction with courses of hCG (25 i.u. daily for 11 days) or testosterone propionate (0.5 mg daily for 11 days). The first EDS dose was given on the 4th day of each hormone course and fertility studies covered the ensuing 8 weeks.

Histology. The weights of testes, epididymides, ventral prostate and seminal vesicle glands were recorded. After fixation in Bouin's fluid for 24 h, paraffin wax sections (8 µm) of the gonads and epididymides were prepared, processed and stained with Mayer's haematoxylin.

The significance of differences in organ weights between treated and control groups was determined by Student's *t* test, two-tailed.

Results

Rats

Testosterone propionate and EDS. A single dose of EDS alone induced a period of sterility for 7 weeks in all males (Table 1). In combination with the testosterone treatment for 9 days a low level of average fertility was maintained during Weeks 2-4 but all animals became infertile during Weeks 5-9, after which fertility was gradually restored. Weights of the reproductive and accessory organs also declined progressively after the hormone treatment was discontinued (Text-fig. 1) with a gradual return towards normal by about Week 10 for the testis and prostate. The epididymis and seminal vesicles were still well below normal weight at this time. Histologically, the seminiferous epithelium remained normal for 2 weeks after the EDS-testosterone treatment although the interstitium appeared atrophic (Pl. 1, Fig. 2). Degenerative changes then ensued, resembling those seen

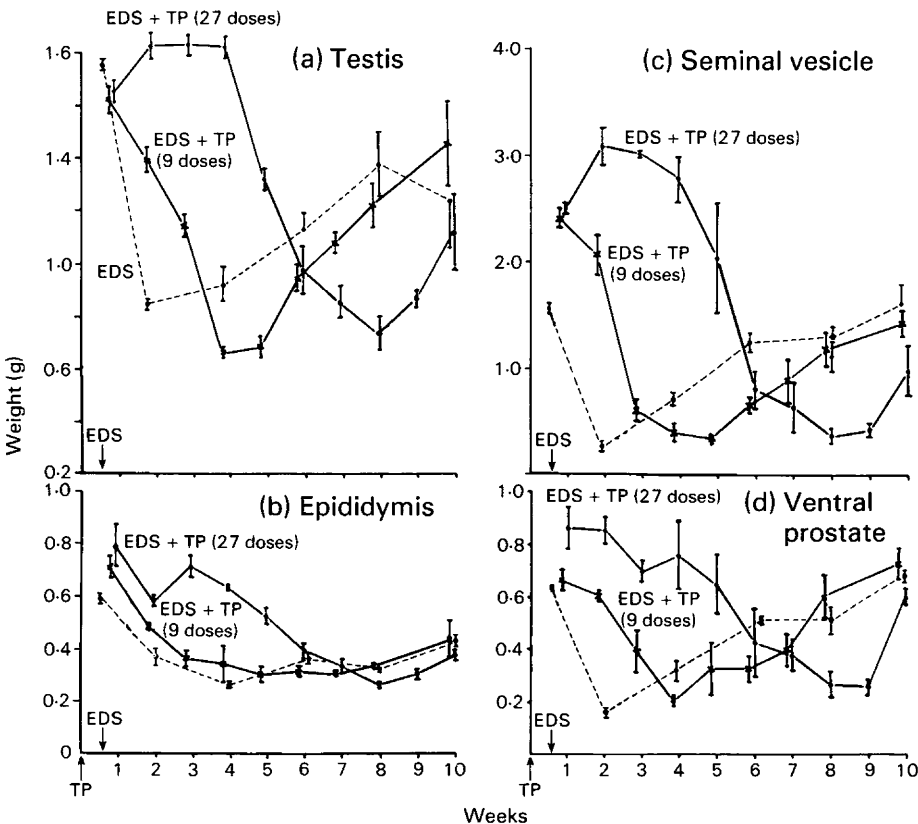
Table 1. Protective effects of testosterone propionate (TP)* and hCG† against the antifertility action of EDS‡ in male rats

Treatment (no. of days in parentheses)	No. of males	Average litter size (weeks from EDS dose)											
		1	2	3	4	5	6	7	8	9	10	11	12
None (controls)	15	7.5	9.7	9.2	8.2	7.8	7.0	7.9	10	8.8	7.3	8.8	8.7
EDS	27	6.7	0	0	0	0	0	0	0	3.3	4.7	7.0	5.0
TP(9)	5	8.6	7.8	12	11	8.4	9.4	6.2	9.4	6.2	7.0	12	7.0
TP(9) + EDS	10	8.3	0.6	1.7	1.2	0	0	0	0	0	1.2	3.3	4.4
TP(27) + EDS	5	7.2	0	5.6	3.8	4.2	7.4	1.2	0	0	0	0	2.2
hCG(9)	5	8.4	13	5.8	6.2	9.4	8.0	8.6	5.8	10	4.6	8.0	5.2
hCG(9) + EDS	9	9.2	0	0	0	0.2	4.2	6.9	3.6	5.4	4.9	5.2	2.8
TP(9) + hCG(9) + EDS	5	5.2	0	0	3.6	3.4	9.6	9.8	11	12	10	6.2	8.6

* 3 mg/rat, s.c. daily.

† 100 i.u. s.c./daily for 9 days.

‡ One dose, 75 mg/kg i.p. on Day 4 of the hormone treatment.

**Text-fig. 1.** Changes in the weight of (a) testes, (b) epididymis, (c) seminal vesicle and (d) prostate gland in rats treated daily for 9 or 27 days with testosterone propionate (TP) when EDS (75 mg/kg) was given on Day 4. The broken line indicates weight changes after EDS only. Values are mean \pm s.e.m. for 4 rats/group.

after treatment with EDS alone (Pl. 1, Fig. 1) which correlated with the onset of sterility in Week 5 (Table 1).

Androgen treatment for 27 days extended the period of protection to the seminiferous epithelium and accessory organs (Text-fig. 1), the weights of the prostate and seminal vesicles being normal or greater than in untreated controls for up to 4 weeks after the EDS dose. Apart from sterility in Week 2, fertility was maintained more effectively by prolonged treatment with the testosterone ester (Table 1). Infertility still developed, however, 3–4 weeks after the last dose and persisted for a further 3–4 weeks (i.e. during Weeks 8–11), again correlating with degenerative changes in the seminiferous epithelium and striking weight loss in the testes and accessory organs (Text-fig. 1).

hCG + EDS. When 100 i.u. hCG were given daily for 9 days the seminiferous epithelium was completely protected against EDS (Pl. 1, Fig. 3). Infertility still occurred in Weeks 2–5 (Table 1), but fertility was maintained after Week 5. Treatment with 25 or 10 i.u. hCG daily also afforded complete histological protection of the seminiferous epithelium and the interstitium appeared normal. Unexpectedly, a moderate and dose-related degree of fertility was exhibited during Weeks 2–5 (Table 2). Organ weights in the EDS-treated animals were best maintained by treatment with 25 i.u. hCG (Text-fig. 2). Paradoxically, the 100 i.u. dose regimen afforded least protection to the accessory sexual glands although testicular and epididymal weights were quite well maintained. Dosage below 10 i.u., hCG afforded no protection against the effects of EDS.

Testosterone propionate, hCG and EDS. When rats were treated with testosterone propionate + hCG, sterility occurred only in Weeks 2 and 3, followed by subfertility in Weeks 4 and 5, and normal fertility thereafter (Table 1). Testicular histology and organ weights remained normal.

Purified gonadotrophins. With the quantities of hormones available, it was not possible to carry out fertility studies of rats treated with LH and FSH. However, testicular weight was well maintained in the EDS-treated rats given purified ovine LH, whereas FSH afforded no protection (Text-fig. 3). Similarly, although LH treatment did not fully maintain the accessory glands, their weights were substantially higher than those of rats given EDS alone or in combination with FSH.

Table 2. Dose–response–protective effect of hCG daily for 9 days against the antifertility action of EDS* in male rats

Treatment	No. of rats	Average litter size (weeks from EDS dose)											
		1	2	3	4	5	6	7	8	9	10	11	12
100 i.u. hCG	5	5	8.4	13	5.8	6.2	9.4	8.0	8.6	5.8	10	4.6	8.0
100 i.u. hCG + EDS	9	9.2	0	0	0.2	4.2	6.9	3.6	5.4	4.9	5.2	2.8	
25 i.u. hCG + EDS	5	3.4	0.4	1.8	3.6	2.2	7.0	4.2	7.6	9.2	8.6	5.0	7.4
10 i.u. hCG + EDS	5	9.4	0.8	2.6	4.0	6.2	3.6	6.0	9.6	12	6.4	13	11

* 75 mg/kg i.p. injected on Day 4 of the hormone treatment.

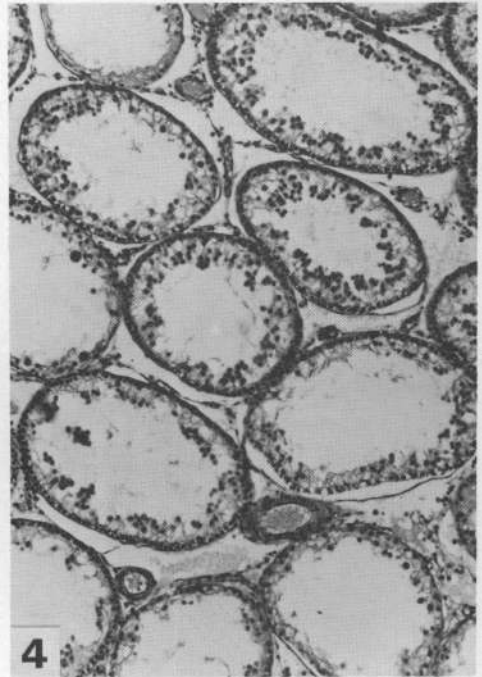
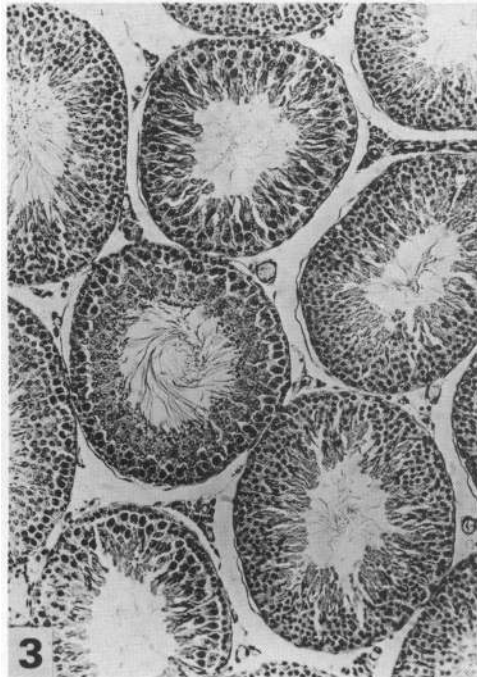
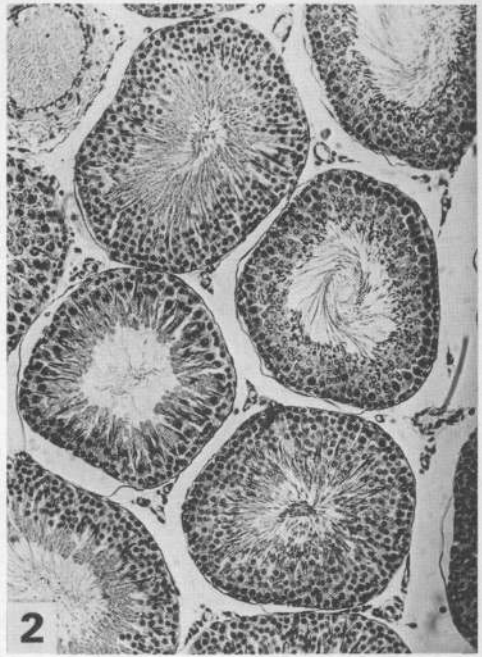
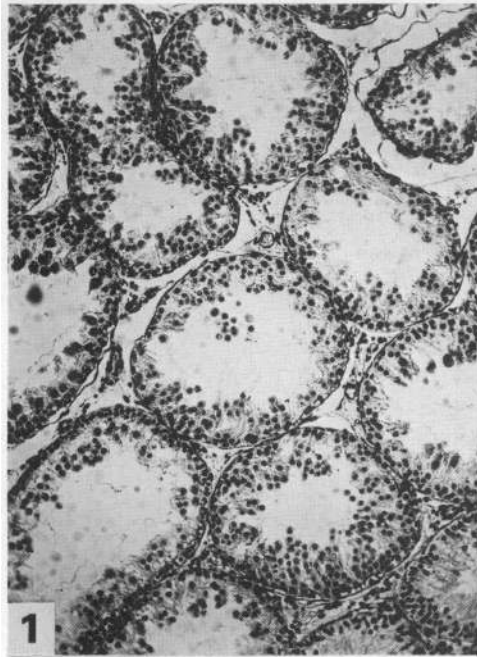
PLATE 1

Fig. 1. Degenerating rat spermatogenic epithelium 14 days after EDS (75 mg/kg).

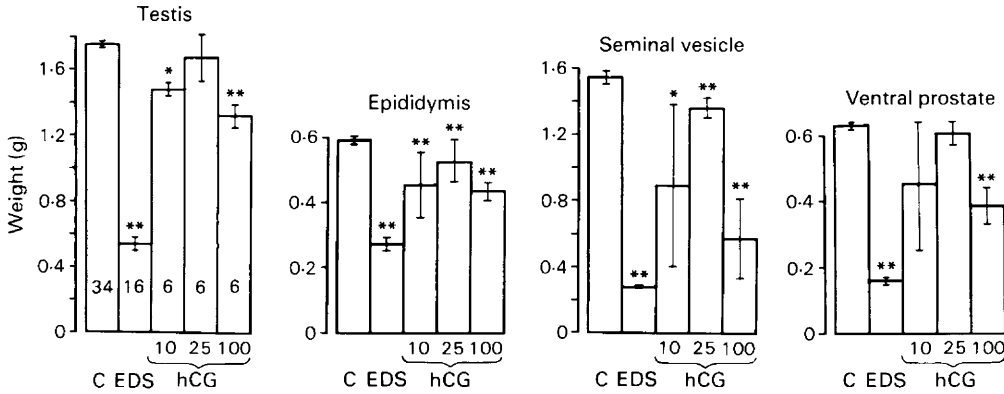
Fig. 2. Normal appearance of the spermatogenic epithelium 14 days after EDS (75 mg/kg), protected (temporarily) by 9 daily doses of testosterone propionate (3 mg/rat).

Fig. 3. Permanent protection provided by hCG injections, 9 daily doses, shown 14 days after EDS (75 mg/kg). See text for details.

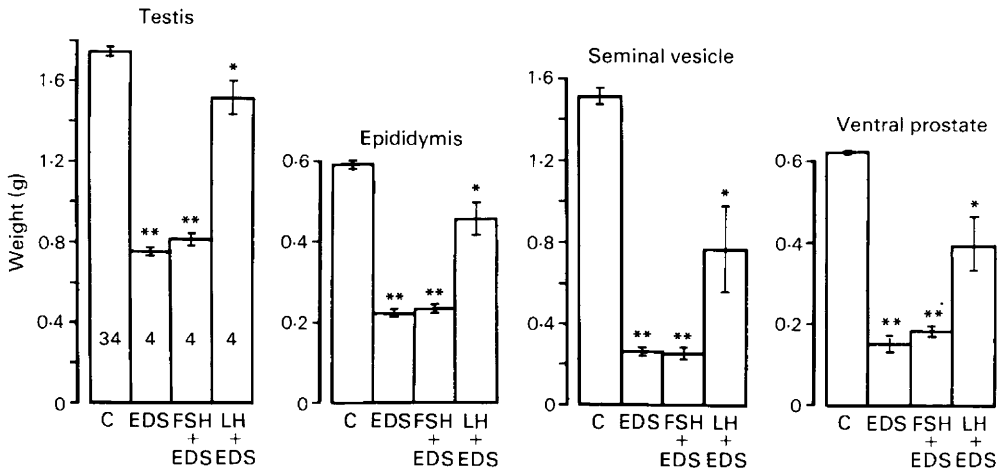
Fig. 4. No protection to spermatogenesis afforded by purified FSH as seen 18 days after the EDS dose. The appearance is identical to that produced by the latter alone at this time.



(Facing p. 396)



Text-fig. 2. Relative protective action of various dose levels of hCG (9 daily doses) against EDS (75 mg/kg) on the rat testis and accessory organs. The assessment was made on Day 18 after the first gonadotrophin dose, with EDS given on Day 4. Values are mean \pm s.e.m. for the no. of rats indicated in the testis panel. * $P < 0.05$, ** $P < 0.001$ compared to control value.



Text-fig. 3. Comparative effects of purified (ovine) FSH and LH (714 μ g daily for 9 days) and the action of EDS (75 mg/kg) on rat testis and accessory structures. Values are mean \pm s.e.m. for the no. of rats indicated in the testis panel. * $P < 0.05$, ** $P < 0.001$ compared with control value.

In rats treated with LH the spermatogenic epithelium appeared normal whereas in those given FSH degenerative changes were seen typical of EDS treatment alone (Pl. 1, Fig. 4).

Mice

Infertility after EDS alone occurred in Weeks 2, 3 and 4; fertility was then restored but remained below control levels (Table 3). Mating was not affected, vaginal plugs being consistently seen even during the sterile weeks. Although testis and epididymal weights were reduced due to the antispermatogenic action, there was no notable change in seminal vesicle and prostate weights. Treatment with testosterone propionate or hCG did not provide any protective action (Table 4).

Table 3. Antifertility effects of EDS (5 daily doses of 250 mg/kg) in adult male Swiss mice

	Weeks from first dose															
	Controls (n = 16)								Treated (n = 12)							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
Av. litter size	6	9	8	9	8	7	7	8	4	0	0	0	4	5	4	6
No. of vaginal plugs	7	10	11	8	11	9	10	11	6	10	11	11	12	9	10	9
No. of litters	8	10	9	9	10	10	8	10	10	0	0	0	7	9	7	8

Table 4. Antifertility effect of EDS in Swiss mice and concomitant hormonal therapy

	No. of males	Average litter size (weeks from first dose of EDS)							
		1	2	3	4	5	6	7	8
EDS*	12	4	0	0	0	4	5	4	6
hCG†	6	9	8	8	8	7	10	9	11
hCG† + EDS*	6	5	0	0	0	3	7	6	6
TP‡	6	5	10	6	9	8	11	10	6
TP‡ + EDS	6	2	0	0	0	3	9	8	4

* 250 mg/kg i.p. for 5 days or on Days 4-8 of hormone regimen.

† 25 i.u. daily for 11 days.

‡ 0.5 mg/mouse s.c. daily for 11 days.

Discussion

The methanesulphonic di-ester of ethylene glycol (EDS) has remarkable pharmacological properties. Unlike the other members of the homologous series of glycol di-esters (general formula $\text{CH}_3 \cdot \text{SO}_2 \cdot \text{O}(\text{CH}_2)_n \cdot \text{O} \cdot \text{SO}_2 \text{CH}_3$), EDS ($n = 2$) does not produce their typical toxicological action in rats on primary cells of the bone marrow and testis. The changes induced in the latter organ are essentially of an endocrine nature. The present work was directed towards elucidating the mode of action and explaining the protection afforded by hCG or androgen (Jackson *et al.*, 1973).

Our earlier studies indicated that EDS essentially affects rat Leydig cells, as shown by a profound inhibition of in-vitro androgen biosynthesis by testicular tissue from rats given a single dose of the compound (Bu'lock & Jackson, 1975). Serum androgen levels were likewise temporarily reduced over a period of 7 weeks, with maximum suppression comparable to levels in castrated animals after 24 h (Morris & McCluckie, 1979). Histological protection of the seminiferous epithelium against the EDS action by daily treatment with testosterone propionate is a temporary phenomenon; the same dose rate will maintain spermatogenesis after hypophysectomy (Boccabella, 1963). In spite of the normal appearance of the spermatogenic epithelium during Weeks 2-4 in rats treated with EDS + androgen for 9 days, the functional ability of spermatozoa available at this time was severely impaired (Table 1). This may reflect the need for direct gonadotrophin support during spermatid metamorphosis and sperm maturation in the epididymis, or an antifertility action of EDS on post-meiotic cells. The latter seems more likely since infertility remains when the testosterone and hCG are given for protection (Table 1).

Leydig cell damage by EDS still occurred in testosterone-treated rats and there was no recovery even during 1 month of continued daily administration of the androgen. Evidently the latter continues to suppress the secretion of pituitary gonadotrophin necessary for the regeneration of Leydig cells. This, and the consequent lack of endogenous androgen, explains the regression of the

spermatogenic epithelium, changes in organ weights (Text-fig. 1) and appropriately delayed onset of infertility (Table 1) when exogenous testosterone support was withdrawn. The delayed regression is thus a consequence of failure of recovery of the Leydig cell population whilst androgen therapy continues.

By contrast, hCG or LH treatment for 9 days covering a single EDS dose provides permanent histological protection to the spermatogenic epithelium, maintenance of testicular weight and substantial protection to the accessory organs (Text-figs 2 & 3). The present dose-response experiments indicate that this can be achieved with one tenth of the amount of hCG formerly used (Jackson *et al.*, 1973). In fact, according to the fertility data (Table 2) and organ weights (Text-fig. 2), a high daily dose of hCG in conjunction with EDS had some detrimental effects on the accessory organs and functional activity of spermatozoa, although hCG alone at the same dose rate had no notable adverse effect (Table 1). Pharmacological doses of hCG or LH can cause a rapid and profound reduction ('down regulation') in the number of binding sites for gonadotrophin on the Leydig cells of the rat. After single s.c. doses of 100–500 i.u., for example, all detectable LH/hCG receptors were reported lost and there was no testosterone secretion in response to a subsequent hCG challenge (Hsueh, Dufau & Catt, 1976; Hasuer & Saez, 1977; Sharpe, 1980). Two consecutive doses of human LH (32 i.u., i.p) to adult rats also resulted in negligible specific binding of ^{125}I -labelled LH in testicular homogenates by 48 h, and 10–12 days were required for restoration to normal levels (Gnanaprakasam, Chen, Sutherland & Bhalla, 1979). On a longer time scale, daily administration of 100 i.u. hCG caused a striking decrease in receptors by 1 week but values returned to normal by 3 weeks in spite of continued treatment (Risbridger & de Kretser, 1979). Plasma testosterone levels had risen markedly over controls by 1 week and were in the normal range by 3 weeks. The fact that 9 daily doses of hCG alone failed to influence male rat fertility (Table 2) indicates that any down-regulatory effect, with resultant reduction in testosterone production, was not detrimental to the spermatogenic process. The effects of one or two doses of gonadotrophin and rapid fall in biosynthetic capacity for testosterone production do not facilitate interpretation of the protective action of hCG against EDS Leydig cell damage. In fact, the presumed fall in testosterone production when hCG is given 3–4 days before EDS, as in the present study, might augment potential damage by the sulphonic ester. The normal testis histology in hCG-protected rats, according to current views, implies maintenance of sufficient testosterone output to the seminiferous epithelium, although testicular tissue from rats treated with 100 i.u. hCG + EDS was reported to be unable to synthesize testosterone *in vitro* from ^{14}C -labelled progesterone (Bu'Lock & Jackson, 1975). This suggests a direct trophic action of hCG on the seminiferous epithelium. Permanent histological protection of the seminiferous epithelium by hCG in EDS-treated rats implies that destruction of the interstitial cells does not occur. Therefore, a temporary hCG dose-dependent impairment of testosterone secretion may occur in the hCG/EDS-treated rats, of insufficient duration to damage spermatogenesis histologically. Since spermatozoa were seen in vaginal smears throughout fertility studies with these rats, the infertility and subfertility during Weeks 2–5 suggest that the post-meiotic maturation of cells to fully mature functional spermatozoa must still be impaired. Additional treatment with testosterone (Table 1) failed to overcome this deficiency. Two aspects merit further study: (1) the possibility that hCG or a metabolite exerts a trophic action on post-meiotic stages and (2) whether EDS produces a functional antifertility effect on these cells ordinarily masked by the Leydig cell damage.

Complete histological protection of the spermatogenic epithelium was also achieved by purified LH but not by FSH. After EDS alone, the blood LH rises to levels similar to those of castrate males within a few days (Jackson & Morris, 1977), FSH being only slightly elevated. The LH increase presumably develops too late to prevent the EDS-induced regression of the Leydig cells. Exogenous gonadotrophin treatment has to precede and extend for several days after the EDS dose to ensure protection. The action of EDS might involve combination (by alkylation) with membrane receptors for LH, so that prior saturation of these with exogenous gonadotrophin could deny access to EDS molecules. Our current studies indicate, however, that specific receptor binding of ^{125}I -labelled

gonadotrophin is unimpaired for at least 24 h after administration of EDS. It has also been reported that EDS *in vivo* prevents the subsequent release of cyclic AMP by testicular tissue *in vitro* in response to hCG stimulation (Bu'Lock & Jones, 1976). Deprivation of LH support to Leydig cells by specific LH antiserum did not seem to cause cell death but a reduction in cell volume (Dym & Madhwa Raj, 1977). A precipitous fall in serum testosterone occurred within 24 h to 17% of controls, and after 5 daily doses of antiserum, the level was 10% of normal. These results correlate with suppression of in-vitro testosterone production after EDS (Bu'Lock & Jackson, 1975).

The proposed interaction of EDS with Leydig cells has no apparent counterpart in the female rat, in which neither the oestrous cycle nor fertility is impaired by comparable treatment with EDS (Jackson, 1975). The reason for the selective action of this particular compound on the Leydig cell of the rat and Japanese quail (Jones, Kominkova & Jackson, 1972) remains obscure. However, neither testosterone nor hCG were protective in the quail. In the mouse, high doses of EDS were antispermatogenic (Table 3) with sterility in Weeks 2–4. Testicular and epididymal weights were reduced but there was no notable effect on those of the accessory organs, whilst no histological change was seen in Leydig cells and mating was unimpaired; an endocrine action therefore seems unlikely. This conclusion was further substantiated by the absence of a protective action with hCG or testosterone (Table 4), favouring a direct EDS action on the spermatogenic epithelium.

The evidence presented in this paper favours direct damage to the rat Leydig cell as the specific major pharmacological action of EDS. The protective role of hCG or LH appears to be associated with Leydig cell membrane receptor occupancy preventing an interaction with EDS rather than by a cytotoxic action of the compound involving internal cellular mechanisms. The metabolic pathway of EDS is similar in rats and mice (Edwards, Jackson & Jones, 1970), yet the Leydig cells of mice are resistant to the high dosage. Possibly some structural membrane receptor component of the rat Leydig cells, not present in the mouse, is vulnerable to EDS. Since restoration of the Leydig cell population does occur, either some precursor cells are responsible or a sufficient number of functional cells have their receptors occupied during the EDS exposure, and are thus physiologically protected and able to repopulate the interstitium. An inferred direct action of EDS on the rat seminiferous epithelium, which is not counteracted by hormonal treatment, is supported by the mouse data. EDS is clearly of special interest in relation to its novel and selective action on Leydig cell function as shown in the rat and Japanese quail. If primate species were adequately susceptible to the action of this compound there might be a potential use in the treatment of hormone-dependent prostatic carcinoma.

This research was supported by the Medical Research Council and the Ford Foundation. We thank Dr A. F. Parlow, NIAMDD, U.S.A., for the purified gonadotrophins.

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Received 1 September 1983