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Testicular endocrine effects of alkane methanesulphonates related to the Leydig cell cytotoxic compound, EDS

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Summary. A series of compounds structurally similar to the specific Leydig-cell-cytotoxic substance ethane-1,2-dimethanesulphonate (EDS) were examined for Leydig cell toxicity in the rat. Within 48 h of a single injection of butane-2,3-dimethanesulphonate (BDS), propan-1,3-dimethanesulphonate (P-1,3-DS) or propan-1-chloro-2,3-DS (PCDS) there was a reduction in serum and testicular testosterone levels. The serum luteinizing hormone (LH) concentration was reduced following BDS or P-1,3-DS, and Leydig-cell LH receptors (measured by ^{125}I-labelled hCG binding) were reduced by <15%, from which it is concluded that these compounds are not selectively toxic to Leydig cells. However, PCDS reduced human chorionic gonadotropin (hCG) binding by >70% and could be considered to be a potential toxin. The effects of hydroxyethanemethane-sulphonate (HEMS), 1,5,2,4-dioxadithiepane-2,2,4,4-tetraoxide (cyclic SOSO), PCDS, propan-2,3-DS, α-chlorohydrin and cyclohexane-1,2-dimethanesulphonate were compared with the effects of EDS 7 days after injection. Systemic toxicity, indicated by a loss of body weight, was associated with cyclic SOSO, PCDS and EDS, although only EDS and PCDS reduced both testicular hCG binding and serum and testis testosterone levels consistent with Leydig-cell toxicity. Further studies indicated that the potency of PCDS in reducing testicular hCG binding and serum and intratesticular testosterone levels was similar to that of EDS. However, unlike EDS, PCDS was systemically toxic and also reduced LH, which could at least in part account for changes in testosterone secretion. The experiments confirm the unique cytotoxicity of EDS. Loss of specific Leydig-cell cytotoxicity and an increase in systemic toxicity occurred when the EDS molecule was altered, even if the distance between the alkylating centres was maintained. The mechanism of action of EDS remains elusive.

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Introduction

Alkane dimethanesulphonates [$CH_3SO_2O(CH_2)_nOSO_2CH_3$] were originally investigated because of their potential as antitumour drugs [10, 11]. One of these, busulphan ($n = 4$), is still an established treatment for chronic myeloid leukaemia. Most of these diesters produce infertility in male rats by an inhibitory effect on proliferating germ cells [16]. Ethane-1,2-dimethanesulphonate (EDS; $n = 2$) is, however, unique in that infertility is induced as a result of androgen withdrawal following selective destruction of Leydig cells [12, 21, 23, 24], but no similar action on Leydig cells has been reported for any member of this series. The remarkable specificity of EDS could be a dose-related phenomenon, as the antispermatogenic action of the other alkane sulphonates occurs at doses lower than that at which EDS is cytotoxic to Leydig cells. The object of this study was to examine whether compounds structurally similar to EDS could show specific Leydig-cell toxicity and perhaps provide insight into the mechanism by which the specific cellular response occurs.

The compounds examined are shown in Fig. 1. Propan-2,3-dimethanesulphonate (P-2,3-DS), butan-2,3-dimethanesulphonate (BDS) and propan-1,3-dimethanesulphonate (P-1,3-DS) were used to examine the effects of methyl substitution on the basic EDS structure or increased separation of the alkylating groups.

Propan-1-chloro-2,3-dimethanesulphonate (PCDS) was examined because it has antifertility effects in both mice and rats [13]. It differs from EDS in its additional chloromethyl group. Propan-1-chloro-2,3-diol (α-chlorohydrin) was included because at low doses it causes reversible sterility in a variety of species by its action on spermatozoa [6, 17, 18], and also because PCDS is prepared directly from it. Although differing from PCDS in its absence of mesyl side chains, it still possesses alkylating potential [20].

Ethan-1-hydroxy-2-methanesulphonate (HEMS), the half-hydrolysis product of EDS, was tested for two reasons. Firstly, methane sulphonic acid is a urinary metabolite of EDS [5] and, therefore, HEMS is potentially an

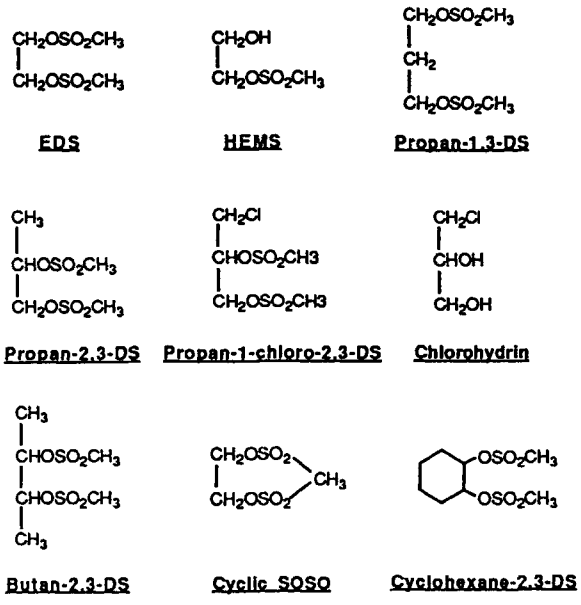

Fig. 1. Structural formulae of compounds investigated for Leydig cell cytotoxicity

active metabolite. Secondly, the importance of two alkylating groups for cytotoxicity would be indicated by the activity of this compound.

The effects of cyclohexyl dimethanesulphonates on male fertility have been described by Jones and Campbell [19], and infertility was produced by some of these compounds. Cyclohexane-*cis*-1,2-DS had a slight antifertility effect in the mouse, but its effect in the rat is unknown. The position of the mesyl groups on this compound are similar to those of EDS, although perhaps more rigidly fixed. The final compound examined was 1,5,2,4-dioxadithiepane-2,2,4,4-tetraoxide (cyclic SOSO). This substance was reported to be an alkylating agent with antitumour properties [8], although its effects on fertility have not been described. It is structurally related to EDS, but the mesyl groupings are held in a rigid cyclic conformation.

Materials and methods

Groups of six adult male Sprague-Dawley rats (300–350 g) were injected intraperitoneally with the various compounds as described in Table 1. The dose of EDS used routinely in this laboratory to destroy Leydig cells is 0.46 mmol/kg (100 mg EDS/kg); thus, equimolar doses of the other alkane sulphonates were given where possible. For some compounds, this exceeded the lethal dose (B. W. Fox, personal communication) and lower amounts were therefore used. At the end of the experimental period, groups of animals were anaesthetised with diethyl ether (BP), blood was collected by cardiac puncture, and the rats were killed by anaesthetic overdose. Serum was prepared and stored at –20°C for testosterone or luteinizing hormone (LH) assays. Testes were dissected and weighed. One testis from each animal was homogenized in 0.1 *M* phosphate-buffered saline (pH 7.4) containing 1% bovine serum albumen. A portion of the homogenate was used immediately for assay of ^{125}I-labelled human chorionic gonadotropin (hCG) binding to LH receptors and the remainder was stored at –20°C for assay of testosterone.

Radioimmunoassays. Serum and testis testosterone concentrations were measured by previously described procedures [7]. The intra- and interassay coefficients of variation were 9.5% and 14.6%, respectively, and the lower limit of detection was 0.11 nmol/l or 1.74 pmol/testis. Serum LH was assayed [1] using reagents provided by the NIADDK (Bethesda, MD) and was expressed in terms of their standard reference preparation rLH-2. The intra- and interassay coefficients of variation were 4.0% and 17.3%, respectively; the limit of detection was 60 pg/ml.

Binding of ^{125}I-labelled hCG in vitro. The binding of ^{125}I-labelled hCG to LH receptors was used as a marker for Leydig cells. For the determination of hCG binding [24], the hormone (hCG, CR 121, 1.345×10^7 IU/g, a gift from NIADDK) was iodinated by a lactoperoxidase enzymatic method [22]; its specific activity [2] was approximately 17 Ci/g. The results are expressed as specific ^{125}I-hCG binding (in cpm/testis), the difference between incubates in the presence or absence of excess cold hCG. Since nonsaturating concentrations of ligand were used, the specific binding was proportional but not identical to the number of Leydig-cell LH (hCG) receptors within the testis.

Unless otherwise indicated, all chemicals were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset). EDS is not commercially available and was prepared by the method of Jackson and Jackson [14]. PCDS was synthesized in this laboratory (H. J.). Cyclic SOSO and *cis-trans* cyclohexane-1,2-DS were gifts from Prof. B. W. Fox, Paterson Institute, (Christie Hospital, Manchester, UK) and propan-2,3-DS was donated by Dr. A. Jones (Department of Biochemistry, University of Sydney, Australia).

Table 1. Dose of each compound administered intraperitoneally to adult male rats

Experimental design: Drug	Vehicle	Dose (mmol/kg)
Dimethyl sulphoxide (DMSO)	25% DMSO	1.60
Ethane-1,2-dimethanesulphonate (EDS)	25% DMSO	0.46
Butane-2,3-dimethanesulphonate (BDS)	25% DMSO	0.46[a]
Propan-1,3-dimethanesulphonate (P-1,3-DS)	60% DMSO	0.46
Propan-2,3-dimethanesulphonate (P-2,3-DS)	25% DMSO	0.46
1,5,2,4-dioxadithiepane-2,2,4,4-tetraoxide (SOSO)	50% DMSO	0.23[b]
Propan-1-chloro-2,3-dimethanesulphonate (PCDS)	60% DMSO	0.46
Cis-trans 1,2-Cyclohexane dimethanesulphonate (C/T-CDS)	100% DMSO	0.34[b]
Propan-1-chloro-2,3-diol (α-chl)	0.9% NaCl	0.46
Ethan-1-hydroxy-2-methanesulphonate (HEMS)	25% DMSO	0.34[b]

Most of the compounds are insoluble in water and were dissolved in DMSO before addition of water to the appropriate concentration. Wherever possible the final concentration of DMSO was 25% (v/v), although higher concentrations (up to 100%) were required for some of the drugs. Abbreviations used in the text are shown in parentheses after the name of each compound

[a] $n = 3$, dose = LD_{50}

[b] Higher doses lethal

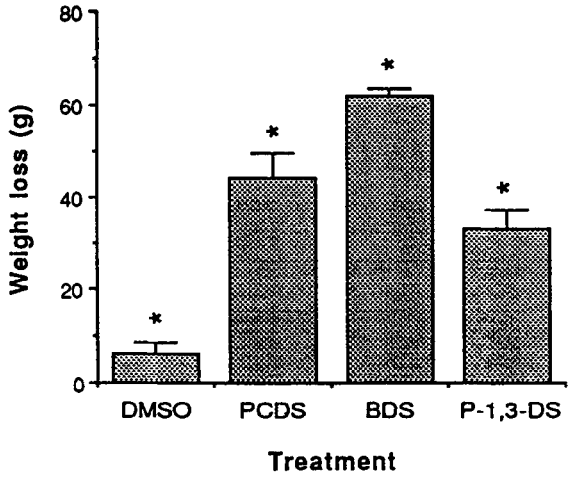

Fig. 2. Loss of body weight over 48 h following an intraperitoneal injection of injection vehicle (*DMSO*) or 0.46 mmol/kg propan-1-chloro-2,3-DS (*PCDS*), butan-2,3-DS (*BDS*) or propan-1,3-DS (*P-1,3-DS*). * $P < 0.05$ in comparison to weight at injection (Student's *t*-test for paired samples)

Statistical analysis. Results are expressed as the means ± SEM. Data were analyzed by the Kruskall-Wallace non-parametric analysis of variance followed by multiple comparisons using the two-tailed Mann-Whitney *U*-test. Differences in mean values were considered significant if $P < 0.05$.

Results

In the first study, the effects of P-1,3-DS, BDS and PCDS were examined. The doses were toxic to the rats, causing considerable weight loss (Fig. 2). Three of the rats given BDS ($n = 6$) died or were killed within 24 h of injection, and the experiment was terminated after 48 h. Testes appearance and weights were normal, but the serum and testis testosterone levels were markedly reduced by all three treatments (Fig. 3a, b). The serum LH concentration was reduced after P-1,3-DS and BDS (Fig. 3c) but was unaffected by PCDS. Although P-1,3-DS and BDS caused only slight reductions (<15%) in the number of LH receptors, with PCDS the *in vitro* binding was reduced by >70% (Fig. 3d).

In further studies, the endocrine effects of α-chlorohydrin, MEDS, PCDS, and lower doses of *cis-trans* 1,2-cyclohexane-DS (C/TCDS), HEMS and cyclic SOSO were compared at 7 days after injection with those produced by EDS. This time was chosen in an effort to avoid the acute toxic phase and to enhance possible changes in Leydig-cell activity. By 48 h, the testicular ^{125}I-labelled hCG binding in EDS-treated rats does not reach its nadir [24]. With cyclic SOSO, the mean weight of the group was significantly lower than that of the vehicle-treated group 7 days after injection (Table 2). Relative to that in vehicle-treated

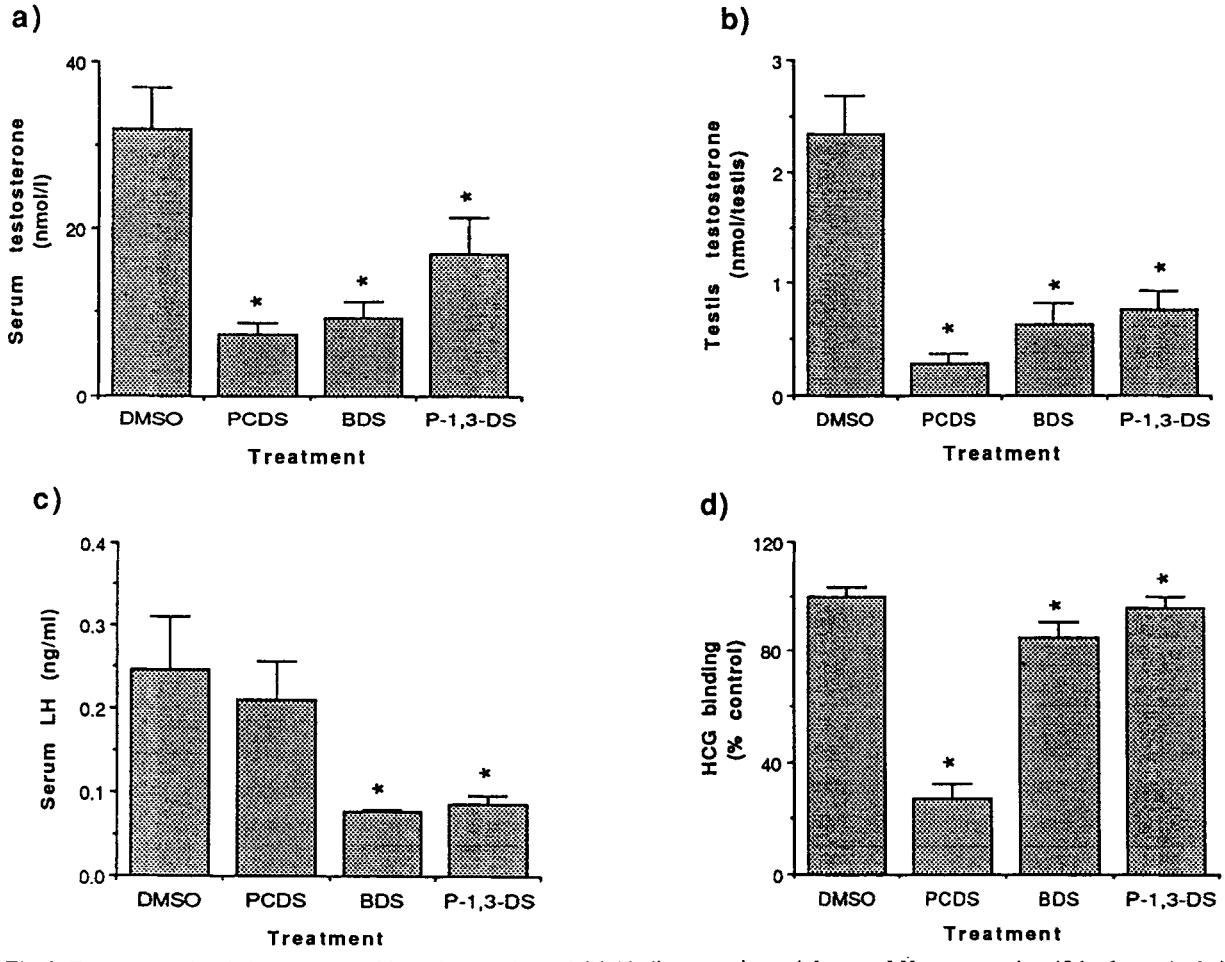

Fig. 3. Testosterone levels in **a** serum and **b** testis, **c** testicular hCG binding capacity and **d** serum LH concentration 48 h after a single intraperitoneal injection of vehicle (**DMSO**) or 0.46 mmol/kg propan-1-chloro-2,3-DS (*PCDS*), butan 2,3-DS (*BDS*) or propan-1,3-DS (*P-1,3-DS*). Groups were compared with controls using the Mann-Whitney U-test (two-tailed); * $P < 0.05$

Table 2. Body weight at injection and at death and the weight of testes and seminal vesicles

	DMSO	EDS	P-2,3DS	SOSO	PCDS	C/TCDS	HEMS	αChlor
Body weight at injection (g)	324.4 ± 8.9	323.8 ±8.2	325.7 ±7.0	326.8 ±6.2	321.7 ±8.4	363.3 ±6.4	345.5 ±7.1	307.9 ±6.7
Body weight at death (g)	364.3 ±11.9	329.3 *±8.2	360.7 ±6.8	325.0* ±5.0	224.2* ±7.1	377.2 ±8.9	374.8 ±7.1	327.9 ±9.7
Wt, seminal vesicles (g)	1.16± 0.06	0.28*±0.05	1.18±0.08	0.84*±0.06	0.32*±0.05	1.2 ±0.04	1.2 ±0.06	0.96 ±0.07
Wt. paired testes (g)	2.91± 0.10	2.43*±0.03	2.85±0.18	2.89 ±0.07	1.26*±0.04	3.35±0.10	3.26±0.05	5.53*±0.20

Rats were killed 7 days after a single intraperitoneal injection of 0.46 mmol/kg EDS, propan-2,3-DS (P-2,3-DS), propan-1-chloro-2,3-DS (PCDS) or α-chlorohydrin (αChlor), 0.34 mmol/kg ethan-1-hydroxy-2-methanesulphonate (HEMS) or *cis/trans*- cyclohexane-1,2-DS (C/TCDS), 0.23 mmol/kg cyclic SOSO (SOSO), or the injection vehicle, DMSO. Data represent the means ± SEM; $n = 6$. Groups were compared with controls using the two-tailed Mann-Whitney U-test; * $P < 0.05$

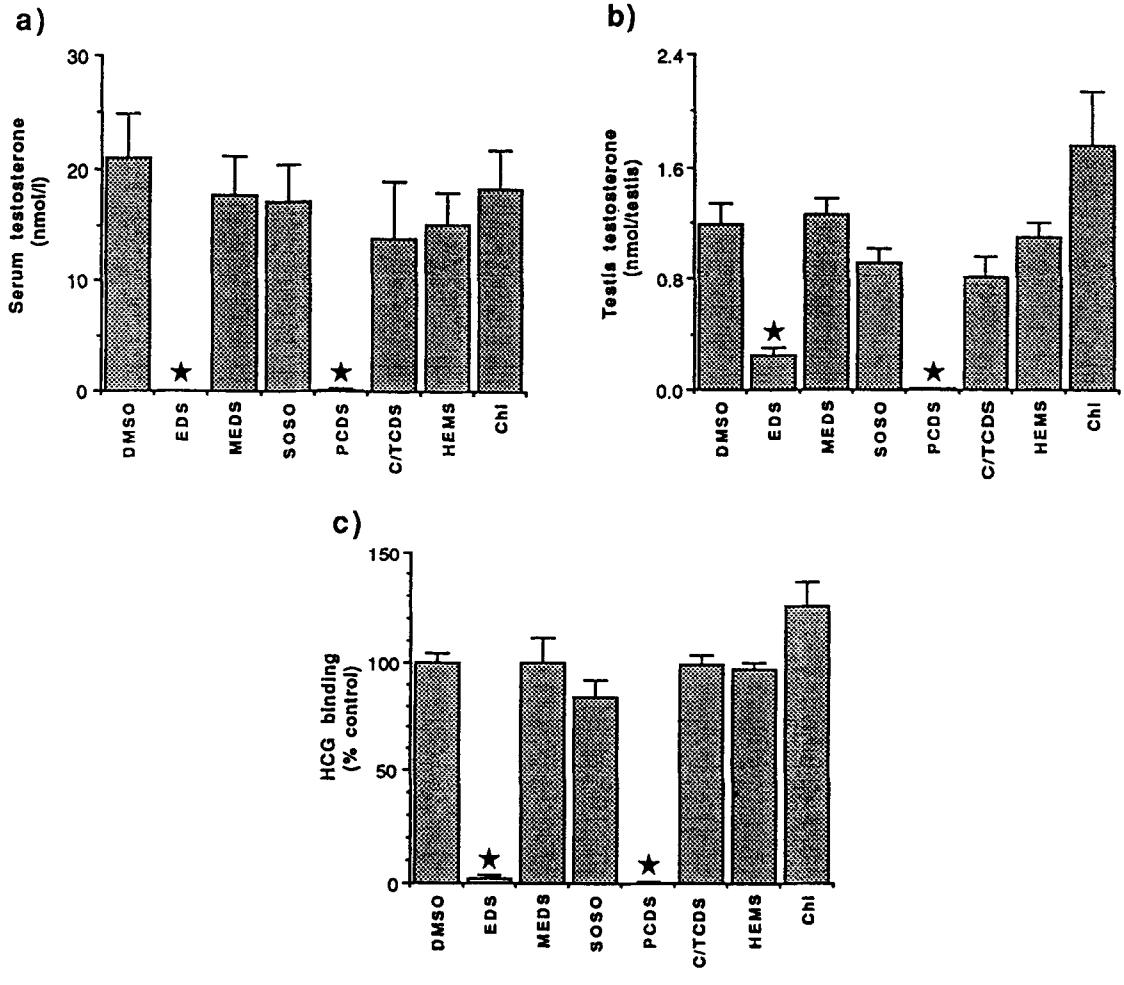

Fig. 4. Testosterone levels in **a** serum or **b** testis and **c** hCG binding capacity of the testis 7 days after a single intraperitoneal injection of 0.46 mmol/kg EDS, propan-2,3-DS (*MEDS*), propan-1-chloro-2,3-DS (*PCDS*) or chlorohydrin (*Chl*), 0.34 mmol/kg ethan-1-hydroxy-2-methanesulphonate (*HEMS*) or *cis/trans*-cyclohexane-1,2-DS (*C/TCDS*) or 0.23 mmol/kg cyclic SOSO (*SOSO*). Control groups received a single injection of vehicle, DMSO. Groups were compared with controls using the Mann-Whitney U-test (two-tailed); * $P < 0.05$

Table 3. Body weight at the time of injection and at death and testes weights

	PEG	EDS	Propan-1-chloro-2,3-DS		
Dose (mmol/kg)		0.17	0.11	0.17	0.23
Body weight at injection (g)	383.7 ±6.1	374.0 ±7.1	375.3 ±10.8	370.3 ±5.7	381.8 ±5.4
Body weight at death (g)	387.2 ±6.7	377.7 ±7.5	359.7* ±10.5	343.5* ±7.3	352.7* ±6.2
Wt. paired testes (g)	3.08 ±0.04	0.2 ±0.07	2.95 ± 0.04	2.84* ±0.08	2.73* ±0.08

Rats were killed 48 h after a single intravenous injection of EDS, propan-1-chloro-2,3-DS or the injection vehicle, 50% PEG. Data represent the means ± SEM; $n = 6$.
Groups were compared with controls using the Mann-Whitney U-test; * $P < 0.05$

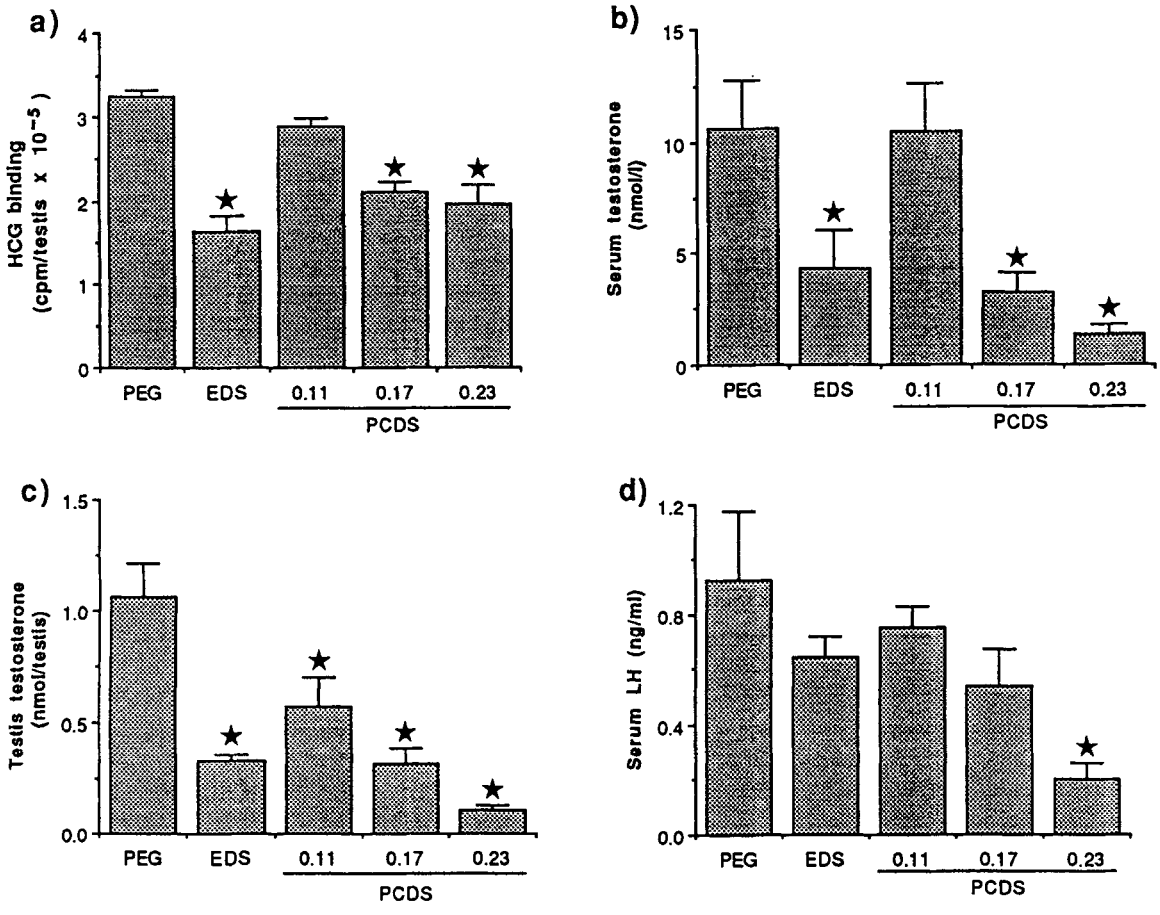

Fig. 5. Effects on **a** hCG binding capacity, testosterone levels in the **b** serum and **c** testis and **d** serum LH concentration of intravenous injections of propan-1-chloro-2,3-DS (mmol/kg, as indicated) with those of 0.17 mmol EDS/kg and the injection vehicle (*PEG*). Animals were killed 48 h after injection. Groups were compared with controls using the Mann-Whitney U-test (two-tailed); * $P < 0.05$

animals, EDS also reduced the amount of weight gained in the 7 days post-treatment. Substantial loss of body weight occurred with PCDS, but weights increased after treatment with all other compounds.

Reduction in the weights of the seminal vesicles and testes occurred with EDS and PCDS (Table 2). Both compounds also reduced the serum concentration and testicular content of testosterone (Fig. 4a, b), whereas the *in vitro* binding capacity of the testis for ^{125}I-labelled hCG was almost totally depleted (Fig. 4c). Cyclic SOSO had no effect on testis weight (Table 2), although the weight of the seminal vesicles was reduced. Serum and testis testosterone levels were not significantly different from those of controls (Fig. 4a, b), and the ^{125}I-labelled hCG binding capacity of the testis was also unaffected (Fig. 4c).

α-Chlorohydrin did not affect the weight of the seminal vesicles but produced a significant increase in the weight of the testes (Table 2) due to spermatocoele induction in the caput epididymis, which blocks the outflow of testicular fluid [18]. Both the testicular ^{125}I-labelled hCG binding capacity (Fig. 4c) and the serum testosterone concentration (Fig. 4a) remained within normal range, although the

testicular testosterone content was elevated (Fig. 4b). HEMS, BDS or C/TCDS did not cause Leydig-cell depletion, as judged by normal testosterone levels and testicular hCG binding (Fig. 4).

Comparison of the effects of EDS and PCDS

Of the compounds tested, only PCDS produced effects resembling those of EDS. At the high dose levels used in the initial study, this compound was extremely toxic. However, since maximal effects were produced, it was not possible to determine whether PCDS was as potent as EDS. Further experiments were carried out using sub-maximal doses to determine whether the effects of the chloro-compound were dose-dependent and could be equated with those of EDS. Animals were killed 48 h after a single intravenous dose of either compound. During this period, the body weight of animals treated with either EDS or vehicle (50% PEG) was unchanged. However, at each dose level PCDS caused a significant loss of body weight (Table 3). In comparison with those of controls, testes weights were unaffected by EDS but were significantly reduced by the two high doses of PCDS (Table 3).

Both EDS and the high doses of PCDS reduced ^{125}I-labelled binding to testis homogenate (Fig. 5a) and the serum testosterone concentration (Fig. 5b). The testosterone content of the gonad was reduced by EDS and by all doses of PCDS (Fig. 5c). Equimolar concentrations of EDS and PCDS produced similar effects on serum and testis testosterone and on *in vitro* testicular hCG binding. Dose-related reductions in serum LH were apparently produced by PCDS, although this effect was only significant after a dose of 0.23 mmol/kg (Fig. 5d). EDS did not significantly affect the serum LH concentration. However, in a further experiment a supramaximal dose of EDS, which unequivocally destroys all Leydig cells (0.46 mmol/kg), produced a 4-fold rise in serum LH concentration within 48 h (vehicle, 0.5 ± 0.1 ng/ml; EDS, 3.5 ± 0.9 ng/ml; $n = 5-6$, $P < 0.01$).

Discussion

The structural specificity of EDS in destroying Leydig cells in the absence of general systemic toxicity was confirmed. To date, no other compound investigated has been found to be selectively toxic to the rat Leydig-cell population. However, PCDS produced changes in the biochemical indices of Leydig-cell function consistent with Leydig-cell death, although this compound also appears to produce direct cytotoxic effects on the spermatogenic epithelium, as judged by fertility studies (unpublished observations).

Increasing the length of the carbon chain to 3 or 4 with or without separation of the mesyl groups, as in P-1,3-DS and BDS, respectively, resulted in some reduction of testis and serum testosterone levels within 48 h; however, the hCG binding capacity of the testis was hardly affected. After EDS these parameters are substantially decreased [24]. Perhaps the systemic toxicity of MEDS and, particularly, BDS, reflected by a large fall in body weight, was responsible for these effects, rather than a direct action on the gonad. Busulphan, the 1,4-dimesyl compound, was reported not to affect the weights of androgen-dependent organs or the serum testosterone concentration when given at near-lethal doses to rats [4, 9, 25], implying a lack of Leydig-cell cytotoxicity. Busulphan had no effect on testosterone production by isolated Leydig cells at a concentration at which EDS would cause inhibition [26]. Both PDS and busulphan show antifertility and antitumour effects related to their alkylating DNA [3, 10, 11, 15]. There is therefore no simple link between potency as an alkylating agent and the ability to deplete Leydig cells of the testis. Substitutions on (or increasing length of) the carbon backbone appeared to reduce toxicity towards Leydig cells, although the general systemic toxicity was increased.

The major metabolic product formed after EDS administration is methane sulphonic acid [5]. HEMS, a potential half-hydrolysis product of EDS, was ineffective in depleting Leydig cells and caused no change in either serum or testis testosterone levels. Since it is a monofunctional alkylating agent, the conclusion is that both adjacent alkylating groups are involved in the action of EDS.

Two other compounds with structural similarity to EDS are cyclic SOSO and C/TCDS. Cyclic SOSO produces DNA-protein cross-links and DNA strand breaks but not DNA-DNA interstrand cross-links [8]. Metabolic hydrolysis might cause the ring structure to open, enabling alkylation by one side chain. The second alkylating group could then produce cross-links as does EDS. However, its failure to simulate the effects of EDS negates this idea as the basis of the Leydig-cell destruction.

The potency of PCDS was similar to that of EDS, both in reducing ^{125}I-labelled hCG binding to testis homogenate and in the reduction in serum and testis testosterone levels. However, unlike EDS, PCDS reduced the serum concentration of LH. Indeed, the fall in the serum and testis testosterone levels seemed to parallel that of serum LH. It therefore seems possible that PCDS could reduce testosterone secretion by an effect on both the Leydig cell and the anterior pituitary gland. Thus, it does not appear to show entirely the specific Leydig-cell cytotoxicity of EDS.

The appearance of one other compound producing Leydig-cell toxicity adds to the mystery surrounding the mode of action of EDS. The chloromethyl EDS molecule now possesses a third group with alkylating potential, but it seems unlikely that this group would be eliminated completely, with the production of EDS to explain its mode of action. The additional antispermatogenic action of the PCDS must be related to the third alkylating group or to its influence on the reactive alkylating capacity of the molecule as a whole. Metabolic studies could throw some light on these speculations. Otherwise, EDS still retains the unique capacity for the destruction of Leydig cells in certain species, concerning which a considerable volume of biochemical studies have yet failed to identify a mechanism.

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