It was shown by Inchiosa[2] that dopa is not a substrate for the uterine enzyme. Apparently, the presence of the carboxyl group completely precluded substrate activity.

Several adrenergic blocking agents were tested as substrates for the catecholamine oxidase. None of these agents showed substrate activity (Table 3). These same agents have been tested for possible effects on the oxidation of epinephrine by the uterine enzyme. Dichloroisoproterenol and propranolol had no influence on enzyme activity. In preliminary studies, two alpha-adrenergic blocking agents, dibenamine and phentolamine, inhibited the oxidation of epinephrine. Both agents caused a 20 per cent inhibition at 8×10^{-4} M; phentolamine, which is more soluble, was also tested at 3×10^{-3} M where it produced 50 per cent inhibition. However, phenoxybenzamine, another alpha-adrenergic blocking agent, caused no inhibition. It is not possible with the present information to draw conclusions about the pharmacological importance of the findings with the adrenergic blocking agents.

The principal contribution of the substrate specificity studies has been the confirmation of the fact that the initial enzymatic reaction is carried out by a catecholamine oxidase. Thus far, only 3,4-dihydroxy derivatives of phenylethylamine have been found to be substrates for the oxidase reaction.

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Studies with alkylating esters—V. The reactions, metabolism and biological activities of some cyclic dimethanesulphonates; the relevance to the mechanism of action of myleran

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DUE TO its clinical importance in the treatment of certain leukemias,[1] myleran ("Busulphan", 1,4-bis(methanesulphonyloxy)-butane, 1) has been the subject of much investigation related to its mode of action. Timmis and Hudson[2] have reviewed the evidence concerning the myleran and dimethyl-myleran series of dimethanesulphonates and although stating that it "remains open to doubt", concluded that a mechanism of cycloalkylation may explain their biological activities. Confirmation of this hypothesis came from the chemical[3] and metabolic[4] studies of Roberts and Warwick showing[5] that myleran can dethiolate or sulphur-strip a number of sulphur containing compounds through the formation of cyclic sulphonium ions. Recent work, however, indicates that a mechanism involving dethiolation may not be valid since a myleran homologue, 1,3-bis(methanesulphonyloxy)-propane (PDS, 11), producing similar effects as myleran both on haemopoiesis and spermatogenesis,[6] does not cycloalkylate either *in vivo* or *in vitro*.[7] Addison and Berenbaum have also questioned this hypothesis due to the immunosuppressive activity of myleran being potentiated by exogenous cysteine rather than being inhibited.[8]

Further evidence arguing against a cycloalkylation mechanism has emerged from structure–activity

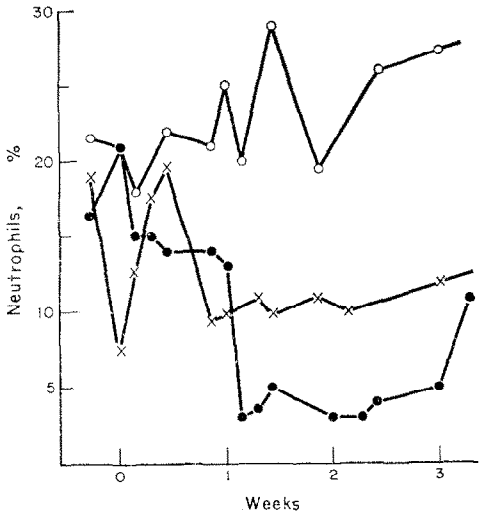
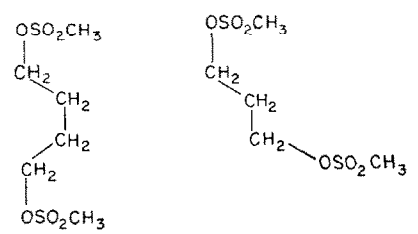


FIG. 1. Neutrophil depressant activity of the isomeric cyclohexane dimethanesulphonates in the rat. The first of five consecutive daily oral doses in arachis oil was given at zero time. ○– – – –○ *cis*-1,2- 5 × 100 mg/kg, ×– – – –× *cis*-1,3- 5 × 100 mg/kg, ●– – – –● *trans*-1,4- 5 × 20 mg/kg.

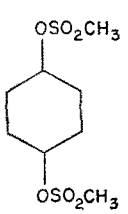
studies with compounds related to myleran and PDS. The alicyclic analogues, cyclohexane-1,4-dimethanesulphonate (III) and cyclohexane-1,3-dimethanesulphonate (IV) possess anti-spermatogenic activity in rats and mice (Table 1) paralleling those of the aliphatic compounds[9,10] and they show similar activity in depressing the circulating neutrophil count in rats (Fig. 1). Unlike myleran and its higher homologues these cyclic dimethanesulphonates do not dethiolate sulphur-containing compounds as they react predominantly by an SN_1 mechanism usually accompanied by an elimination process both *in vivo* and *in vitro*.

The radioactive urinary metabolites of ^{35}S-cyclohexane-1,4-dimethanesulphonate (III) in the mouse are methanesulphonic acid (V), the methanesulphonate of cyclohexene-4-ol (VI) and cyclohexane-1,4-diol monomethanesulphonate (VII). Both *cis*- and *trans*-cyclohexane-1,3-dimethanesulphonate (IV) give analogous metabolites indicating that this SN_1 mechanism is operating *in vivo*. No cysteine conjugates could be detected and the recent work of James *et al.*[11,12] suggests that this is not a major pathway in the detoxification of cyclohexyl compounds. We find that there is no reaction of III or

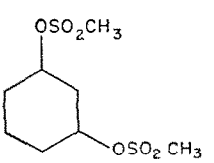


I

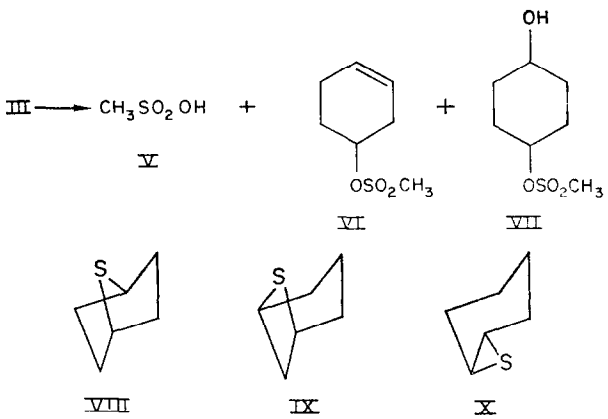
II



III



IV



IV with either ^{35}S-cysteine or ^{35}S-cysteine-*N*-acetate under conditions in which myleran[3] and PDS[7] both react in high yield. The chemical reactivity of the cyclic analogues confirms their SN_1-elimination reactivity[13,14] and our attempts to alkylate either cysteine ethyl ester[15] or sodium sulphide,[16] reactions in which myleran and its homologues undergo cycloalkylation, gave unsaturated compounds and not the cyclic excission products. Indeed the cyclic thiols derived from III and IV, 7-thiabicyclo(2.2.1)heptane (VIII) and 6-thiabicyclo(3.1.1) heptane (IX) are known not to be formed by such an alkylation reaction.[17,18] Furthermore cyclohexane-1,2-dimethanesulphonate, possessing no activity on haemopoiesis or spermatogenesis, readily dethilolates cysteine ethyl ester[15] and sodium sulphide to give cyclohexene sulphide (7-thiabicyclo(4.1.0)heptane, X).

Considered with the reactivity of PDS,[7] these results suggest that the cycloalkylation of thiol groups by myleran represents a normal detoxification route[19] rather than a mechanism of action. Preliminary studies[20] on the rate of entry of dimethanesulphonates into rat rete testis fluid show that accessibility depends to some extent on their oil–water solubility ratio (a criterion mentioned by Timmis and Hudson[2]) and that the alkyl moiety influences transport to the site of action (i.e. the germinal epithelium). The action of methanesulphonates in general could be explained by the mechanism of cleavage of the methanesulphonyloxy group. This theory, at present under investigation, involves the formation of sulphene ($CH_2{=}SO_2$), the highly reactive intermediate produced by the reaction of methanesulphonyl chloride with bases.[21] If this species is liberated *in vivo* by a base-activated reaction, then the similar actions of these esters could result from the extreme reactivity of sulphene with specific cellular nucleophiles,[22] a possibility inferred by Addison and Berenbaum.[8] The relative biological activities of methanesulphonates, therefore, should parallel their rates of reactivity; a comparison of hydrolysis rates confirms this for the cyclohexyl, myleran and dimethylmyleran[2] series as antispermatogenic agents.

Synthesis. *Cis-* and *trans*-cyclohexane-1,3-dimethanesulphonates were prepared from the corresponding isomeric diols; *cis*-m.p. 126° (reported[13] m.p. 125°) *trans*-m.p. 96–7° (reported[13] m.p. 97·5°). Cyclohexane-1,4-dimethanesulphonate has been prepared previously though the products are isomeric mixtures. Levshina *et al.*[23] give *cis*-m.p. 117–8° and *trans*-m.p. 119–20° but comment that treatment of the isomeric diols in triethylamine with methanesulphonyl chloride gave only one compound which we have confirmed as a mixture of *cis-* and *trans*-isomers. Wiley and Kraus[24] give m.p. 148° though they do not mention which isomer has been prepared. To the *cis*-1,4-diol[14] (5·75 g) in dry pyridine (30 ml) at −20° was added methanesulphonyl chloride (11 g) dropwise with stirring over 2 hr. The mixture was acidified with ice-cold 20% sulphuric acid, the product filtered from the mother liquors A and recrystallized from excess acetone. The first crop of crystals was recrystallized from aqueous acetone to give *cis*-cyclohexane-1,4-dimethanesulphonate (1·7 g), m.p. 138–40° decomp. Found, C 35·54, H 5·80, S 22·53 per cent. $C_8H_{16}S_2O_6$ requires C 35·30, H 5·92, S 23·60 per cent. The product isolated from the mother liquors A was recrystallized three times from aqueous acetone to give the *trans*-isomer (8·5 g), identical with that produced by mesylation of pure *trans*-1,4-diol[14] m.p. 160–62° decomp. Found, C 35·48, H 6·07, S 23·35 per cent. $C_8H_{16}S_2O_6$ requires C 35·30, H 5·92, S 23·60 per cent. Comparative infrared fingerprint analysis of the two isomers with the respective diols agreed with the assigned configurations. As a consequence of the *cis*-isomer possibly being converted *in vivo* to the *trans*-isomer and the fact that both isomers produce identical biological effects at equivalent doses, it is not certain whether the *cis*-possesses is own integral activity.

Treatment of the *trans*-1,4- and the isomeric 1,3-diols with ^{35}S-methanesulphonyl chloride in pyridine gave the respective ^{35}S-cyclohexane dimethanesulphonates of s.a. 350 μc/mM.

Metabolism. ^{35}S-cyclohexane-*trans*-1,4-dimethanesulphonate (III) was administered orally to male

TABLE 1. ANTIFERTILITY ACTIVITY OF THE ISOMERIC CYCLOHEXANE-DIMETHANESULPHONATES

Compound	Species	Dose (mg/kg) 7	Average weekly litter size 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Myleran	Mouse	7 × 5			8	10	3	0	0	0	3	2						
	Rat	5 × 2					7	6	7	5	0	0	0	0	0	0	4	5
Cyclohexane-*trans*-1,4-dimethanesulphonate	Mouse	5 × 10	7	4	3	4	0	0	0	0	0	5						
	Rat	5 × 20	3	0	2	1	4	7	2	0	0	0	0	0	0	0	0	1
Cyclohexane-*cis*-1,3-dimethanesulphonate	Mouse	5 × 200	1	3	1	3	2	0	0	0	0	3						
	Rat	5 × 100	5	1	2	6	7	10	5	0	2	0	0	0	0	1	7	6
Cyclohexane-*trans*-1,3-dimethanesulphonate	Mouse	5 × 200	3	2	3	2	2	0	0	0	0	0	0	0	0	0	0	0
	Rat	5 × 100	5	2	2	6	5	3	2	0	0	0	0	0	0	0	3	3
Cyclohexane-*cis*-1,2-dimethanesulphonate	Mouse	5 × 200	7	4	3	5	10	12	11	10								

Apart from Myleran, which was given intraperitoneally, all compounds were administered orally as suspensions in arachis oil. Fertility was assessed weekly in groups of six mice and five rats by the serial mating technique (Ref. 31).

I.C.I. mice (about 50 g) as a solution in 25% dimethyl sulphoxide in arachis oil (50 mg/kg). Total urinary radioactivity (50 per cent after 16 hr and 91 per cent after 100 hr) was assayed as previously described.[25] Chromatograms of 0–16 hr urine were developed in *n*-butanol; glacial acetic acid; water 4;2;1 on Whatmans No. 17 papers and scanned on a Packard 7201 Radiochromatogram Scanner. Four radioactive areas were detected and identified as follows. The compound at R_f 0·28–0·31 was confirmed as methanesulphonic acid (V) by gas-chromatography of its methyl ester.[26] The metabolite at R_f 0·45 possessed the same R_f as ^{35}S-cyclohexane-1,4-diol monomethanesulphonate (VII), prepared in 40 per cent yield by attempted half-mesylation of the *trans*-1,4-diol which gave a mixture of the mono- and di-methanesulphonates. Elution of the metabolite with water and steam-distillation of the concentrated eluate from 5 N sulphuric acid produced ^{35}S-methanesulphonic acid and, by continuous ether extraction of the steam distillate, cyclohexene-4-ol, identified by gas-chromatography (Table 2). This indicates VII to be the metabolite as the sequence of reactions is

TABLE 2. G. L. C. RETENTION TIMES OF METABOLIC DERIVATIVES AND REACTION PRODUCTS

	Retention time (mins)*
Cyclohexane-*cis*-1,4-diol	6·92
Cyclohexane-*trans*-1,4-diol	6·92
Cyclohexane-*cis*-1,3-diol	6·33
Cyclohexane-*trans*-1,3-diol	6·00
Cyclohexene-3-ol	1·50†
Cyclohexene-4-ol	1·50†

* Determined on a Varian Aerograph Autoprep 705 with flame ionization detector. The column was $\frac{1}{8}$″ × 6′ of 10% silicone XL 60 on acid-washed DMCS Chromosorb W (80–100 mesh) at 155° with nitrogen carrier gas flow rate of 18 ml min^{-1}.

† Flow rate 10 ml min^{-1}.

typical of this class of compound.[14] At R_f 0·89–91 were trace amounts of unchanged cyclohexane-*trans*-1,4-dimethanesulphonate, confirmed by methanol extraction, reverse isotope dilution with inactive material, recrystallization to constant specific activity and chromatographing to give an identical R_f value. At the solvent front, R_f 0·98–1·00, was the methanesulphonate of cyclohexene-4-ol (VI). Synthesis of this compound by meslyation of cyclohexene-4-ol[14] gave a viscous water soluble oil decomposing on attempted distillation though when prepared from ^{35}S-methanesulphonyl chloride, the crude product had an R_f of 0·98–1·00. When given intraperitoneally in 30% dimethyl sulphoxide in water to mice at 750 mg/kg, the synthetic ^{35}S-compound (VI) was absent from 0 to 2 hr urine though both methanesulphonic acid and cyclohexane-1,4-diol monomethanesulphonate (VII) were present in nearly equal amounts.

Analogous results were obtained after intraperitoneal administration to mice of; (a) ^{35}S-cyclohexane-*trans*-1,3-dimethanesulphonate [at 75 mg/kg urinary excretion of label (50 per cent in 29 hr, 64 per cent in 100 hr) was accounted for by four radioactive areas; methanesulphonic acid, cyclohexane-1,3-diol monomethanesulphonate (R_f 0·45), unchanged compound (R_f 0·85) and the methanesulphonate of cyclohexene-3-ol (R_f 0·93–0·95) and (b) ^{35}S-cyclohexane-*cis*-1,3-dimethanesulphonate (at 50 mg/kg urinary excretion of label (50 per cent in 24 hr, 70 per cent in 100 hr) was accounted for by the same three metabolites and unchanged compound at R_f 0·86)]. The configurations of the cyclohexane diol monomethanesulphonates could not be ascertained.

The mercapturic acid conjugate of cyclohexane-1,4-diol could not be detected as a metabolite of III. Urine from treated mice was extracted by the method of Bray *et al.*[27] and after diazomethylation examined by gas-chromatography for *N*-acetyl-*S*-(4-hydroxycyclohexyl)-L-cysteine methyl ester. Authentic material was prepared from 4-chlorocyclohexanol[14] and *N*-acetyl cysteine and isolated according to James *et al.*[11]

Chemical reactivity. Cyclohexane-*cis*- and *trans*-1,3- and *trans*-1,4-dimethanesulphonates were separately reacted with L-cysteine ethyl ester HCl[15] and ethanolic sodium sulphide.[16] In neither case did the ethanolic distillate contain the expected cyclic sulphur compound (negative mercuric chloride test), though when the reactions were carried out under nitrogen and the effluent gas passed through a solution of bromine in carbon tetrachloride, each compound gave reasonable yields (35–45 per cent) of the tetrabromide of cyclohexa-1,4-diene, white prisms from aqueous acetone, m.p. 188° (reported[13] m.p. 188–190°). When cyclohexane-*cis*-1,2-dimethanesulphonate[28] was reacted under identical

conditions, cyclohexene sulphide (7-thiabicyclo(4.1.0)heptane, X) was isolated from the ethanolic distillate as the mercuric chloride complex, white needles from ethanol, m.p. and mixed m.p. with the mercuric chloride complex of authentic material[29] 108–110° (decomp).

Hydrolysis of cyclohexane-*cis*- or *trans*-1,3-dimethanesulphonates in refluxing 50% aqueous acetone[2] gave a mixture of isomeric diols determined by g.l.c. (Table 2). The 1,4-analogues may give an isomeric mixture on hydrolysis or equilibrate to the more stable *trans*-1,4-diol; however g.l.c. characterization of cyclohexane-*cis*- and *trans*-1,4-diols could not be achieved.[11]

Hydrolysis of the ^{35}S-compounds in water gave the ^{35}S-monomethanesulphonates of the diols and methanesulphonic acid, the half-lives at 38° being 2 hr for the *trans*-1,4-, 11 h for the *trans*-1,3- and 7·5 hr for the *cis*-1,3-. Cyclohexane-*cis*-1,2-dimethanesulphonate is stable in water, no hydrolysis occurring at 38° over 10 days.

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