Biochemical Pharmacology, Vol. 19, pp. 1783–1789. Pergamon Press. 1970. Printed in Great Britain

# STUDIES WITH ALKYLATING ESTERS—II

## A CHEMICAL INTERPRETATION THROUGH METABOLIC STUDIES OF THE ANTIFERTILITY EFFECTS OF ETHYLENE DIMETHANESULPHONATE AND ETHYLENE DIBROMIDE

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(*Received* 4 *September* 1969; *accepted* 2 *December* 1969)

**Abstract**—The metabolism of 1,2-$^{14}$C-ethylene dimethanesulphonate (EDS) has been followed in the rat and mouse and compared with that of 1,2-$^{14}$C-ethylene dibromide (EDB). EDS is excreted unchanged in urine together with S-(2-hydroxyethyl)-cysteine-*N*-acetate and S-(2-hydroxyethyl)-cysteine-*N*-acetate-S-oxide. EDB is not excreted unchanged but is metabolised mainly to S-(2-hydroxyethyl)-cysteine and its *N*-acetate.

The distribution of radioactive label in mouse tissues is different for both compounds which may indicate a difference in the origin of cysteinal units for alkylation. Both ethylene dimethanesulphonate and ethylene dibromide react efficiently *in vitro* with SH containing compounds by a reaction analogous to the "sulphur stripping" action of Myleran, although the metabolism of EDS and EDB would not indicate such a reaction takes place *in vivo*. The antispermatogenic actions of EDS differ from those of its homologues, such as Myleran, but show some resemblance to those of EDB. These effects of EDS and EDB may indicate that both compounds have latent "mustard-like" activity, and this possibility is discussed in terms of the pharmacological effects produced by EDS and its chemical reactions with nucleophiles.

STUDIES with $^{35}$S-ethylene dimethanesulphonate (EDS) in rodents demonstrated differences between its metabolic fate and that of Myleran.[1] Besides methanesulphonic acid (MSA) two other metabolites were found from 1,2-$^{14}$C-EDS accounting for 10% of the urinary radioactivity. The mode of formation of these metabolites is discussed and compared to the metabolism and fate of 1,2-$^{14}$C-ethylene dibromide (EDB). From chemical and metabolic studies a possible interpretation of the differing antifertility effects of the diester homologues Myleran and EDS is suggested. Some preliminary metabolic results have been published elsewhere.[2]

## MATERIALS AND METHODS

### A. *Preparative methods*

1,2-$^{14}$*C-ethylene dimethanesulphonate* was prepared as previously described[1] with a specific activity of 71 $\mu$c/mM.

1,2-$^{14}$*C-ethylene dibromide* (Radiochemical Centre, Amersham) had a specific activity of 1·04 mc/mM.

$^{35}$*S-(2-hydroxyethyl)-cysteine* was prepared from $^{35}$S-cysteine (Radiochemical

Centre, Amersham) by the method of Nachtomi *et al.*[3] Acetylation gave $^{35}S$-(2-*hydroxyethyl*)-*cysteine*-N-*acetate*.

*S,S'-ethylene-bis-cysteine* and its *bis*-N-*acetate* were also prepared as previously described.[3]

$^{35}S$-(2-*hydroxyethyl*)-*cysteine-S-oxide*

Hydrogen peroxide (30%, 1 ml) was added to a solution of $^{35}$S-(2-hydroxyethyl)-cysteine (500 mg) in water (5 ml) and the mixture kept at room temperature for 24 hr. A further quantity of peroxide (1 ml) was added and after another 24 hr the solution evaporated to dryness *in vacuo*. The residue was dissolved in water (2 ml), filtered, and acetone (5 ml) added to the filtrate to precipitate the S-oxide. The precipitation procedure was repeated several times and the product dried. M.p. 136–138°, specific activity 200 $\mu$c/mM.

| | | | |
|---|---|---|---|
| Found: | C 29·90 | H 6·81 | S 15·86%. |
| $C_5H_{11}NSO_4$ requires | C 30·14 | H 6·55 | S 16·05%. |

$^{35}S$-(2-*hydroxyethyl*)-*cysteine*-N-*acetate-S-oxide*

After acetylation of $^{35}$S-(2-hydroxyethyl)-cysteine-S-oxide, the oil was dissolved in hot acetone and treated with an excess of petroleum ether (40–60°). The precipitated oil was removed by centrifugation and shown to be radiochemically homogeneous by paper chromatography. At room temperature it decomposed to $^{35}$S-(2-hydroxyethyl)-cysteine-S-oxide within a few days.

*S*-(2-*hydroxy*-1,2-$^{14}$*C-ethyl*)-*cysteine*

Urine from rats treated with 1,2-$^{14}$C-ethylene dibromide was chromatographed in solvent 1 on Whatman No 17 paper and the areas corresponding to S-(2-hydroxy-1,2-$^{14}$C-ethyl)-cysteine and its *N*-acetate separately eluted with water. The latter was treated with acylase (Sigma Chemicals) for 3 hr at 37°, rechromatographed and the de-acetylated metabolite eluted. Reduction of the combined extracts to low volume *in vacuo* and treatment with excess acetone gave the required compound, recrystallised from aqueous acetone, m.p. 189°, (specific activity 38 $\mu$c/mM). Peroxide oxidation gave S-(2-hydroxy-1,2-$^{14}$C-ethyl)-cysteine-S-oxide.

*Ethylene sulphide*

Ethylene sulphide (thiiran) was prepared by the method of Furukawa *et al.*[4]

B. In vitro *chemical reactivity of EDS*

L-Cysteine ethyl ester hydrochloride (4·3 g) in absolute ethanol (50 ml) was added to a solution of sodium ethoxide (2·7 g). This suspension was rapidly mixed with a hot solution of EDS (2·3 g) in ethanol (170 ml), heated to 60° and *n*-hexane (200 ml) added dropwise over 0·5 hr, during which time it was distilled through a fractionating column into a receiver cooled with carbon dioxide. The residue was vigorously refluxed for 2 hr, cooled, filtered and the filtrate reduced *in vacuo* to a colourless gum. This was dissolved in ether (300 ml) and dry HCl passed through the solution. The white precipitate formed on cooling was recrystallised from ethanol: ether (m.p. 139°) and characterised as 2-methyl-2,4-carbethoxythiazolidine HCl, identical with the product from interaction of Myleran with L-cysteine ethyl ester hydrochloride.[5]

The *n*-hexane solution of thiiran was dried over sodium sulphate and analysed quantitatively by GLC.

Yield of thiiran based on EDS 7%

Yield of 2-methyl-2,4-carbethoxythiazolidine HCl based on EDS 5%.

*Reaction with egg albumin*

Egg albumin (3 g) in water (100 ml) was mixed with a suspension of EDS (1·5 g) and 1,2-$^{14}$C-EDS (19·6 mg) in water (30 ml). After addition of sodium hydroxide (2N, 20 ml) the mixture was boiled and then steam passed to collect about 50 ml of distillate. Acetone (50 ml) was added to the latter, the thiiran (6·5% of the initial radioactivity) collected by co-distillation and isolated as the mercuric chloride complex, recrystallised from acetone.

Found: C 20·99 H 3·52 S 28·02%.

Thiiran: mercuric chloride, (5:1 complex) requires:—

C 20·29 H 3·75 S 26·69%.

C. *Analytical methods*

*Ascending chromatography* was carried out on Whatman paper (No. 1 for qualitative, No. 2 for quantitative). Solvent systems and $R_f$ values are indicated in Table 1. Radiotracer detection and assay techniques have been previously described.[1]

TABLE 1. CHROMATOGRAPHY OF EDS, EDB AND THEIR METABOLITES

| Compound | $R_f$ in solvent* 1 | 2 |
|---|---|---|
| EDB | 0·74 | 0·73 |
| EDS | 0·85 | 0·87 |
| S-(2-hydroxyethyl)-cysteine | 0·34 | 0·30 |
| S-(2-hydroxyethyl)-cysteine-*N*-acetate | 0·72 | 0·41 |
| S-(2-hydroxyethyl)-cysteine-*N*-acetate-S-oxide | 0·46 | 0·21 |
| S-(2-hydroxyethyl)-cysteine-S-oxide | 0·17 | 0·28 |
| S,S′-ethylene-bis-cysteine | 0·08 | 0·07 |
| S,S′-ethylene-bis-(cysteine-*N*-acetate) | 0·78 | 0·16 |

Whatman No. 1 paper.

* Solvent 1: butanol–acetic acid–water, 4–2–1; solvent 2: butanol–pyridine–3N ammonia, 4–3–3.

*Gas–liquid chromatography* was performed on a Varian Aerograph Autoprep 705 equipped with a flame-ionisation detector. Analysis for ethylene sulphide was carried out at ambient temperatures using a stainless steel column (1/8 in. o.d. × 10 ft) packed with XF-1150 cyanosilicone on a stationary phase of 85–100 mesh SCB 104 (Phase Separations Ltd., Rock Ferry, Cheshire).

D. *Animals and administration of compounds*

Male Wistar rats (ca. 250 g) and male RF/Hiraki mice (ca. 25 g), maintained on standard pellet diets, were kept in metabolic cages permitting separation of urine and faeces and, when required, collection of expired gases. Tissue distribution of EDB was performed on mice dosed with 1,2-$^{14}$C-ethylene dibromide intraperitoneally

(40 mg/kg) in arachis oil. Animals were killed at 1, 2 and 3 hr after injection and tissue samples digested in perchloric acid and hydrogen peroxide. Aliquots were counted in an IDL Tritomat liquid scintillation counter with phosphor solutions as previously described.[1] The results, given as per cent administered dose per g wet weight of tissue (Table 2), are expressed as total radioactivity, both bound and unbound. Ethylene sulphide was administered intraperitoneally in olive oil (5×20 mg/kg) and aqueous solutions of S-(2-hydroxyethyl)-cysteine by gavage to male mice (5×1 g/kg). Ethylene dibromide was given in arachis oil to male rats (5×10 mg/kg). Bile was collected by cannulation and labelled EDB or EDS (100 mg/kg) administered by deep subcutaneous injection. Animals were maintained for a period of 2·5 hr after surgery, the bile being collected in an ice-cooled ampoule.

*Fertility studies* were performed by a serial mating technique.[6]

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN MOUSE TISSUES AFTER INJECTION OF $^{14}$C-EDB

| Tissue | Hours after administration 1 | 3 | 24 |
|---|---|---|---|
| Bone* | 1·4 | 2·1 | 0·47 |
| Brain | 0·25 | 0·57 | 0·14 |
| Cauda epididymis | 3·1 | 4·4 | 0·66 |
| Fat | 4·9 | 2·4 | 0·30 |
| Heart | 1·1 | 1·1 | 0·21 |
| Large intestine† | 5·3 | 15 | 0·33 |
| Small intestine† | 34 | 5·8 | 0·39 |
| Kidney | 13 | 12 | 1·0 |
| Liver | 12 | 6·6 | 0·42 |
| Lung | 2·5 | 3·3 | 0·42 |
| Muscle‡ | 0·90 | 1·0 | 0·14 |
| Skin | 1·2 | 2·1 | 0·31 |
| Spleen | 4·1 | 4·7 | 0·61 |
| Stomach† | 1·7 | 4·6 | 1·0 |
| Testis | 1·1 | 1·5 | 0·23 |
| Whole blood | 7·2 | 7·4 | 6·2 |
| Plasma | 12 | 12 | 2·6 |
| Residue of animal | 1·3 | 2·4 | 0·24 |

Results expressed as % administered dose/g wet tissue after an intraperitoneal dose of 40 mg/kg in arachis oil.
* Whole femur.
† Including contents.
‡ Gastrocnemius.

## RESULTS

Oral administration of 1,2-$^{14}$C-ethylene dibromide to rats and mice (40 mg/kg) gave two labelled urinary metabolites which, in agreement with previous studies,[3] correspond to S-(2-hydroxyethyl)-cysteine (I, Fig. 1) and S-(2-hydroxyethyl)-cysteine-*N*-acetate (II). Intraperitoneal administration also gave S-(2-hydroxyethyl)-cysteine-*N*-acetate-S-oxide (III). From the absence of volatile radioactive material it was inferred that no unchanged ethylene dibromide was present in the urine. Confirmatory evidence for the nature of the metabolites was obtained by treatment of their eluates from preparative chromatograms with acetic anhydride, thioglycollic acid or by acid hydrolysis. The products gave $R_f$ values in agreement with those of authentic samples under identical conditions. Acetates were further confirmed by treatment with acylase.

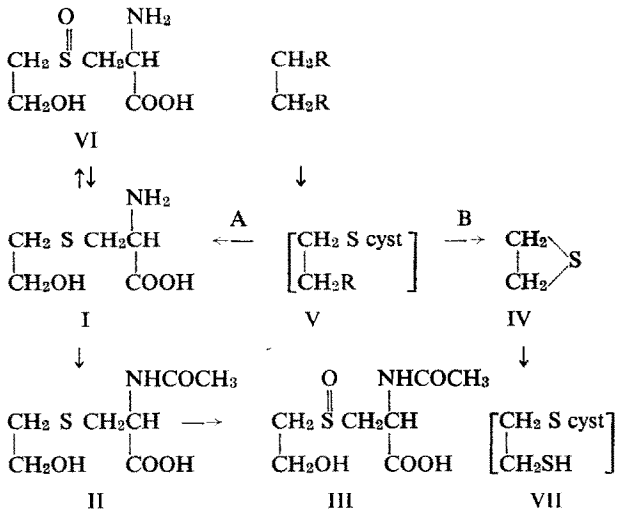


FIG. 1. Metabolic pathway (A) of ethylene dimethanesulphonate (R = $OSO_2CH_3$) and ethylene dibromide (R = Br) in the rat. Though chemically feasible, there was no indication of detoxification by route (B). Intermediates from alkylation of cysteine-derived thiol moieties are shown in brackets.

Administration of either $^{14}C$- or $^{35}S$-(2-hydroxyethyl)-cysteine to rats produced metabolites identical to those obtained from either EDS or EDB. $^{35}S$-(2-Hydroxyethyl)-cysteine produced inorganic sulphate (2% of the dose) though $^{35}S$-(2-hydroxyethyl)-cysteine-S-oxide (VI) was degraded more extensively to sulphate (15%). Both of these metabolites are interconvertible *in vivo* and their presence in urine shows that enzymic acetylation, thought to occur in the liver,[7] is rate limiting. The isolation of S-(2-hydroxyethyl)-cysteine as a metabolite of EDB probably indicates that EDB conjugates with cysteine too rapidly for the acetylation stage to be completed. From an oral dose of $^{14}C$-EDS (40 mg/kg) 5–8 per cent of the radioactive dose was expired by the rat in 24 hr. Both rat and mouse urine contained S-(2-hydroxyethyl)-cysteine-*N*-acetate (II) and its S-oxide (III), although the majority of radioactive material was EDS (confirmed by isotope dilution). Intraperitoneal administration, besides producing these two metabolites, gave a minor amount of S-(2-hydroxyethyl)-cysteine (I).

Tissue distribution of 1,2-$^{14}C$-EDB (40 mg/kg i.p.) in mouse tissues after 1 hr showed that the small intestine, liver, kidney and fat contained an appreciable proportion of the radioactive dose (Table 2). The circulating label was mainly accounted for as S-(2-hydroxyethyl)-cysteine-*N*-acetate with less than 1% of the dose being present in the blood as a volatile component (by inference EDB). Tissue levels at 3 and 24 hr remained appreciable and reflected the low recovery of radioactivity in the urine (40% in 24 hr). In the mouse $^{14}C$-EDS showed a uniform distribution of radioactive material with a relatively slow rate of metabolism[1] compared to EDB. The major difference is that EDB was rapidly removed from the circulation in the mouse *via* the liver and concentrated in the small intestine. This raised the possibility that EDS and EDB were being excreted in the bile, although in the rat by 2·5 hr after administration, only 5 per cent of a dose of $^{14}C$-EDB and 1 per cent of a dose of $^{14}C$-EDS were excreted by this route.

*In vitro* reactivity of EDS and EDB with cysteine was demonstrated by the production of S,S′-ethylene-bis-cysteine and S-(2-hydroxyethyl)-cysteine. These reactions were accompanied by the production of ethylene sulphide (thiiran, IV) indicating that both compounds are capable of performing 'sulphur excision' reactions similar to those observed for Myleran.[8] Further indication of the sulphur stripping ability of EDS was obtained by procedures analogous to those adopted for Myleran,[5] the yield of thiiran being 7 per cent. However, ethylene dibromide also produced a similar amount of the episulphide. No evidence was obtained of thiiran formation following EDS administration to rats or mice since no volatile metabolites appeared in either

TABLE 3. ANTIFERTILITY EFFECTS OF EDS, EDB AND THIIRAN IN THE MALE RAT

| Compound | Dose (mg/kg, i.p.) | Average live litter size (from six rats) in week | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| EDS | 100 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| EDS | 50 | 8 | 0 | 0 | 0 | 6 | 3 | 4 | 5 | 5 | 7 |
| EDB | 50* | 5 | 7 | 4 | 0 | 7 | 5 | 6 | 6 | 5 | 8 |
| Thiiran | 100* | 8 | 7 | 0 | 6 | 7 | 7 | 6 | 6 | 5 | 0 |
| Treated cell type sampled at mating | | Epididymal spermatozoa | | | Spermatids | | | Spermatocytes | | Spermatogonia | |

* Divided into five doses on successive days.

urine or expired gases. Ethylene dibromide, unlike EDS, showed a selective predisposition to damage spermatogenic cells (Table 3) since a short course of treatment only produced transient sterility in rats resulting from spermatid damage. The primary metabolite of both EDS and EDB, S-(2-hydroxyethyl)-cysteine (1 g/kg p.o. daily for 5 days), had no effect on male mouse fertility, so that none of the isolated metabolites appeared to be responsible for the antifertility activity of EDS. Thiiran produced a transient phase of infertility corresponding to spermatid damage (Table 3).

## DISCUSSION

From the structural resemblance of the two alkylating compounds EDS and EDB, some similarity in their antifertility effects might be expected. EDB has an effect directed at spermatids (third week subfertility and fourth week infertility), whereas for EDS (Table 3) the action readily extends to spermatocytes.[9] The differences between testicular effects of EDS and EDB may be due to distribution differences *in vivo* if interference with spermatogenesis follows chemical reaction of the compounds within the testis. An earlier study[1] of the distribution of $^{14}$C-EDS showed generalised distribution in mouse tissues with a long *in vivo* life and relatively little metabolism. EDB is rapidly removed from the circulation with little generalised distribution (Table 2) and rapid metabolism, facts which may account for its weaker antifertility effects relative to EDS.

Myleran owes its antifertility effects in the male rat mainly to a selective destructive action on spermatogonia whereas EDS exerts maximal damage on spermatids and spermatocytes according to histological and fertility studies.[9] The relative haematological and tumour growth inhibitory effects of the Myleran homologues have been

explained in terms of solubility and chemical reactivity.[10] Clearly, the biological effects of EDS and Myleran are exerted on cells in the same environment but at different stages of development and differentiation, with which it is difficult to correlate solubility influences. The ability of Myleran to remove thiol groups from cysteinal units has been demonstrated *in vitro*[5, 8] and *in vivo*[11] whilst its effectiveness as an anti-tumour agent has been attributed to this action. EDS and EDB readily remove sulphur from both cysteine ethyl ester and egg albumin leaving a reactive carbonium ion at the site of excision. The efficiency with which this occurred was unexpected, particularly as the excision product, thiiran, is itself a reactive compound towards nucleophiles. This observation showed that EDB and EDS are capable of reacting chemically in a bifunctional manner analogous to Myleran. The formation of thiiran *in vivo* might be a significant process in the biological activity of EDS, since besides carbonium ion formation, this episulphide could be capable of reactions with biological nucleophiles (see Fig. 1). An alkylation reaction between thiiran and cysteine produces S-(2-thiolethyl)-cysteine (VII) analogous to the formation of S-(2-hydroxyethyl)-cysteine from ethylene oxide.[12] Only weak antifertility activity was shown by large doses of thiiran which argues against its biological role in the effects of EDS or EDB although it might be effective if produced in intracellular sites.

The variation in biological effects seen by closely related alkylating compounds, such as EDS and Myleran, suggests that different chemical modes of action are involved. Reaction of EDS or EDB with cysteinal units or the amino-nitrogens of nucleic acids would greatly enhance the reactivity of the remaining alkylating group by producing a monofunctional mustard (V). Evidence for mustard-type activity with EDS is seen in its antifertility effects, which resemble those of the aziridines and nitrogen mustards, spermatids being the most sensitive spermatogenic cells.[9, 13] Interference with earlier cell types, spermatocytes and even spermatogonia, may result from mechanisms other than direct alkylation of components within seminiferous cells.[14]

*Acknowledgements*—This work was supported by grants to the Christie Hospital by the British Empire Cancer Campaign and to the Unit of Reproductive Pharmacology by the Ford Foundation and the Wellcome Trust.

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