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THE MODE OF ACTION OF ALKYLATING AGENTS—III

THE FORMATION OF 3-HYDROXYTETRAHYDROTHIOPHENE-1:1-DIOXIDE FROM 1:4-DIMETHANESULPHONYLOXYBUTANE (MYLERAN), S-β-L-ALANYLTETRAHYDROTHIOPHENIUM MESYLATE, TETRAHYDROTHIOPHENE AND TETRAHYDROTHIOPHENE-1:1-DIOXIDE IN THE RAT, RABBIT AND MOUSE

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Abstract—The metabolism of Myleran has been investigated in an attempt to explain the mechanism of its biological action. After injection of Myleran into the rat, rabbit and mouse at least 60 per cent of the urinary radioactivity is in the form of 3-hydroxytetrahydrothiophene-1,1-dioxide. S-β-L-alanyltetrahydrothiophenium mesylate, tetrahydrothiophene, and tetrahydrothiophene-1,1-dioxide are also metabolised to this compound. It is concluded that the major reaction which Myleran undergoes *in vivo* is reaction with cysteine or a cysteinyl moiety to form a cyclic sulphonium ion which undergoes decomposition to tetrahydrothiophene. This in turn is converted to tetrahydrothiophene-1,1-dioxide and thence to 3-hydroxytetrahydrothiophene-1,1-dioxide.

INTRODUCTION

EARLIER studies of the metabolism and distribution of Myleran by Peng[1] using the ^{35}S-labelled compound, and by Trams *et al.*[2] using 1:4-^{14}C-Myleran, have revealed the presence of new radioactive compounds in the urine of the human and the rat in addition to a small quantity of unchanged drug. Thus ^{35}S-methanesulphonic acid, the product which would inevitably be formed if Myleran alkylated nucleophilic centres *in vivo*, has been identified, and three ^{14}C-metabolites accounting for 70–80 per cent of the urinary radioactivity have been observed. This paper describes the chemical nature and the mode of formation of the major urinary metabolite.

MATERIALS AND METHODS

2:3-^{14}C-Myleran, specific activity 4 μc/mg was kindly supplied by Dr. P. Brookes of this Institute.

^{35}S-*Tetrahydrothiophene*

^{35}S-sodium sulphide (16 mg, specific activity 26 mc/mM) was dissolved in water and added to an aqueous solution of $Na_2S{\cdot}9H_2O$ (6·6 g) to give a total volume of 50 ml. This was added to a solution of 1:4-dibromobutane (5·4 g) in ethanol (50 ml), and the solution was made up to 180 ml with 50 per cent ethanol. The mixture was heated under reflux for 20 hr, and distilled until the distillate failed to give a precipitate with mercuric chloride. A concentrated solution of mercuric chloride in methanol was then added to the total distillate, and after cooling in ice the precipitated mercurichloride was separated by filtration. A rapid current of steam was

passed through an aqueous suspension of the mercurichloride until all the liberated ^{35}S-tetrahydrothiophene had distilled over. Isolation by ether extraction yielded 1·88 g of product.

Tetrahydrothiophene-1:1-*dioxide*

Redistilled tetrahydrothiophene (8·8 g)was treated with cooling with 30 per cent hydrogen peroxide (30 ml) and after the initial exothermic reaction had subsided the solution was refluxed for 1 hr. The sulphone isolated by continuous extraction with chloroform was distilled under reduced pressure yielding a solid, m.p. $>$ 20 °C (lit. m.p. = 28·86 °C [3]).

^{35}S-*Tetrahydrothiophene*-1:1-*dioxide*

This was synthesized in nearly quantitative yield by the above method using ^{35}S-tetrahydrothiophene (0·5 g) and 30 per cent hydrogen peroxide (1·8 ml).

^{35}S-*Tetrahydrothiophene methiodide*

This was synthesized using ^{35}S-tetrahydrothiophene. Needles from acetone, m.p. 185–190 °C (lit. m.p. = 185–190 °C).[4]

3-*Hydroxytetrahydrothiophene*-1:1-*dioxide dinitrobenzoate*

3-Hydroxytetrahydrothiophene-1:1-dioxide (Sulpholanol) (1 g) kindly obtained by Professor E. Boyland of this Institute, dinitrobenzoyl chloride (3 g) and pyridine (5 ml) were heated together at 100 °C for 0·5 hr. The reaction mixture was treated with 2 N sodium carbonate (100 ml) and the dinitrobenzoate was extracted into chloroform. The chloroform layer was washed successively with 2 N hydrochloric acid, and water, and then dried (sodium sulphate). The residual oil soon solidified and crystallized from methanol in almost colourless prisms, m.p. 195–197 °C. (Found: C, 40·3; H, 3·2; N,8·3; S, 9·5, mol.wt. 340. Calc. for $C_{11}H_{10}O_8N_2S$: C, 40·0; H, 3·0; N, 8·5; S, 9·7%; mol.wt. 330.)

Animals

Six-week-old male Wistar rats (approx. 200 g) and 3-month-old doe rabbits were used in the animal experiments.

Collection and assay of radioactive metabolites

The methods used to collect and assay the radioactive urine, faeces and exhaled carbon dioxide have been described.[5] The techniques used to prepare the urinary samples for chromatography were also as described for the study of the metabolism of ethyl methanesulphonate[5] ("half-Myleran").

All fractions were routinely run in the following three solvents: butanol–ethanol–propionic acid–water (20:10:10:4), solvent I; butanol–acetone–di*cyclo*hexylamine–water (20:20:10:4), solvent II; methylethylketone–acetic acid–water (3:1:1), solvent III.

RESULTS

The metabolism of Myleran

1. *Rat.* 2:3-^{14}C-Myleran (20 mg/kg in propylene glycol (2 ml) or arachis oil (2 ml)) was injected intraperitoneally into a male Wistar rat and the urine, faeces and exhaled carbon dioxide were collected and the radioactivity assayed (Table 1).

Autoradiographs of paper chromatograms prepared from urine excreted during the first 24 hr after injection (Fig. 1A) showed that several radioactive metabolites were present, one of these constituting the major fraction (spot *a*, Fig. 1A). It has been shown that at least 60 per cent of the urinary radioactivity was associated with this metabolite by scanning the paper chromatograms with an end window counter. Autoradiographs of paper chromatograms prepared from the second-day's urine again confirmed the presence of the major metabolite in approximately the same relative proportion.

TABLE 1. AVERAGE PERCENTAGE OF INJECTED RADIOACTIVITY EXCRETED FROM THREE MALE WISTAR RATS AFTER INJECTION OF 2:3-^{14}C-MYLERAN

Times after i.p. injection (hr)	Urine (%)	Faeces	CO_2 (%)
6–24	28–35	Negligible	4–8
24–48	12–15	Negligible	Negligible
48–72	Negligible	Negligible	Negligible

The polar nature of the major metabolite was emphasized by the need for prolonged continuous chloroform extraction to isolate it from the aqueous phase: characterization of the major metabolite was precluded at this stage because of the necessarily small quantities of Myleran which could be safely administered.

While the major metabolite has since been identified, it has not been possible to elucidate the nature of the other radioactive compounds present in the urine in much lower concentration.

2. *Rabbit and mouse.* Several rabbits were injected intraperitoneally with 2:3-^{14}C-Myleran (4 mg in arachis oil (2 ml)) and the urines examined separately. Autoradiographs of paper chromatograms again showed that the major proportion of the urinary radioactivity was associated with one metabolite which had chromatographic and chemical properties identical with that excreted by the rat. Subsequent isolation confirmed this identity.

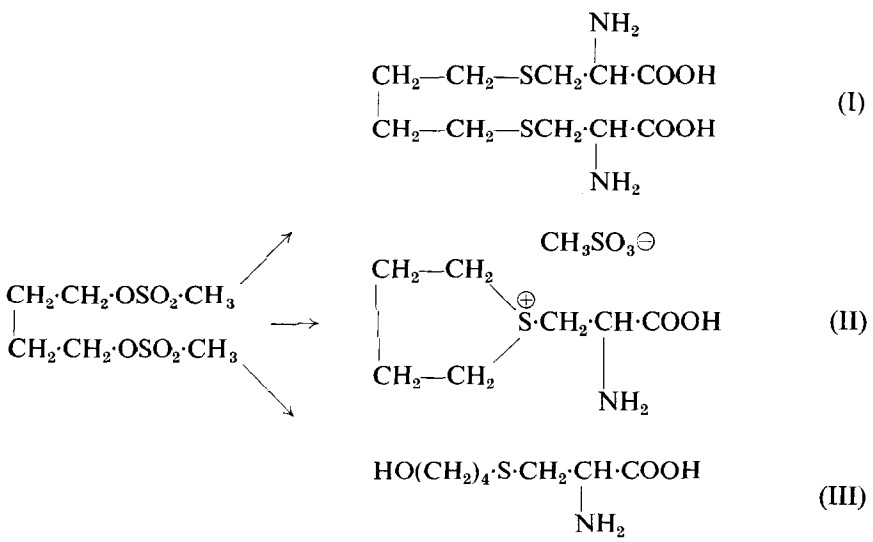
Twenty mice were injected intraperitoneally with 2:3-^{14}C-Myleran (0·6 mg in arachis oil (0·5 ml)) and the bulked urine examined as before. A large proportion of the major metabolite was excreted, but inspection of autoradiographs indicated that the other metabolites differed from those excreted by the rat and the rabbit.

The properties of the major radioactive urinary metabolite excreted after injection of 2:3-^{14}C-Myleran

On the basis of the observed formation of ethyl mercapturic acid from ethyl methanesulphonate (half-Myleran) *in vivo*,[5] it was considered that Myleran might undergo a similar conversion *in vivo* leading to the production and excretion of conjugates of sulphur-containing amino acids.

The formation of S-di-L-cysteinylbutane (I), S-β-L-alanyltetrahydrothiophenium mesylate (II), and S-(4-hydroxybutyl)-L-cysteine (III), by reaction of Myleran with L-cysteine, and the formation of sulphonium compounds from glutathione and proteins *in vitro* has been described earlier.[6]

However, the major metabolite differed in its R_f value from products (I), (II) and (III), and moreover did not contain an amino or carboxyl group as shown by its free passage through ion exchange columns, and its negative response to ninhydrin. Even in high concentration the metabolite did not react with the platinic iodide reagent and was stable to oxidizing agents, indicating the absence of a mercaptan or sulphide grouping, but not ruling out the presence of sulphur in a more oxidized state. The stability of the metabolite to hot concentrated hydrochloric acid showed that it was not a conjugate.



While, therefore, the known products of the reaction between Myleran and L-cysteine were not present as urinary metabolites of Myleran, the possibility of one of these being a precursor was not excluded. Accordingly, the major product of this reaction, S-β-L-alanyltetrahydrothiophenium mesylate (II), was prepared from 2:3-^{14}C-Myleran, and injected into rats to determine whether any of its urinary metabolites were identical to those excreted after Myleran injection. It was considered that if reaction occurred with a thiol-containing peptide or protein *in vivo* to form a sulphonium compound, the final radioactive urinary metabolites might nevertheless be identical to those formed via the cysteine reaction.

The metabolism of S-β-L-*alanyltetrahydrothiophenium mesylate* (II) *in the rat*

The exhaled carbon dioxide and faeces contained negligible quantities of radioactivity while 32 per cent of the injected radioactivity was excreted in the urine during the first 24 hr. Autoradiographs of paper chromatograms of the urine indicated the presence of one major metabolite (spot *a*, Fig. 1B), different from the injected sulphonium compound, but identical to the major Myleran metabolite in both R_f value (solvents I, II, III) and chemical properties. Again continuous extraction of the urine with chloroform separated the major metabolite. It was thus evident that sulphonium ion formation involving either cysteine or a cysteine-containing compound was probably the initial major reaction which Myleran underwent *in vivo*.

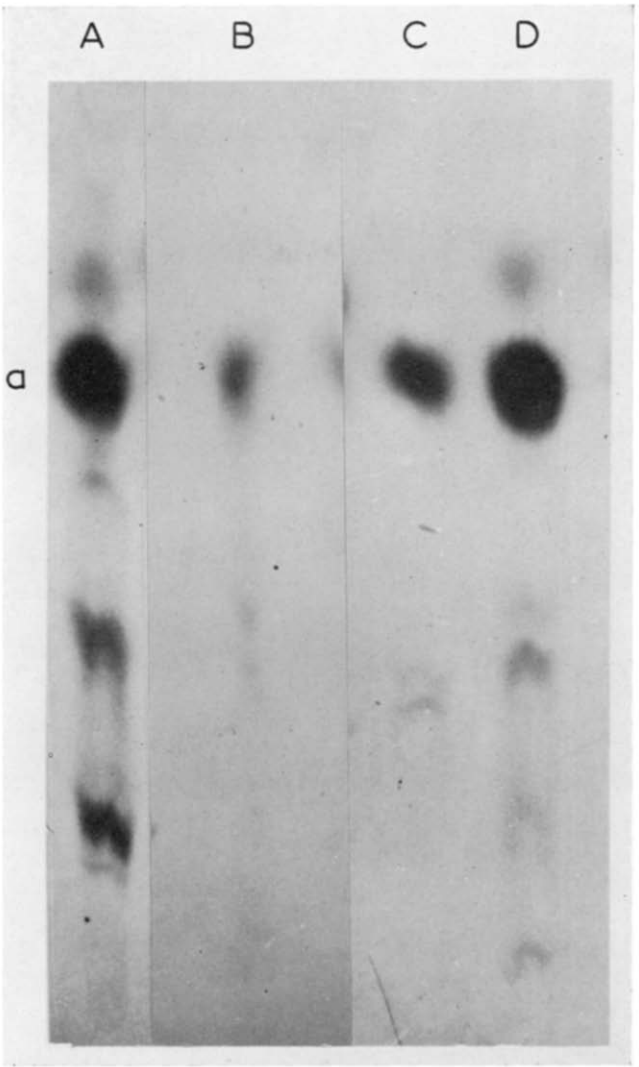


FIG. 1. Autoradiograph of a unidimensional paper chromatogram (solvent I) of urine excreted from the rat after injection of 2:3-^{14}C-Myleran (A), 2:3-^{14}C-S-β-alanyltetrahydrothiophenium mesylate (B), ^{35}S-tetrahydrothiophene (C), and ^{35}S-tetrahydrothiophene-1:1-dioxide (D). Spot *a* is radioactive 3-hydroxytetrahydrothiophene-1:1-dioxide.

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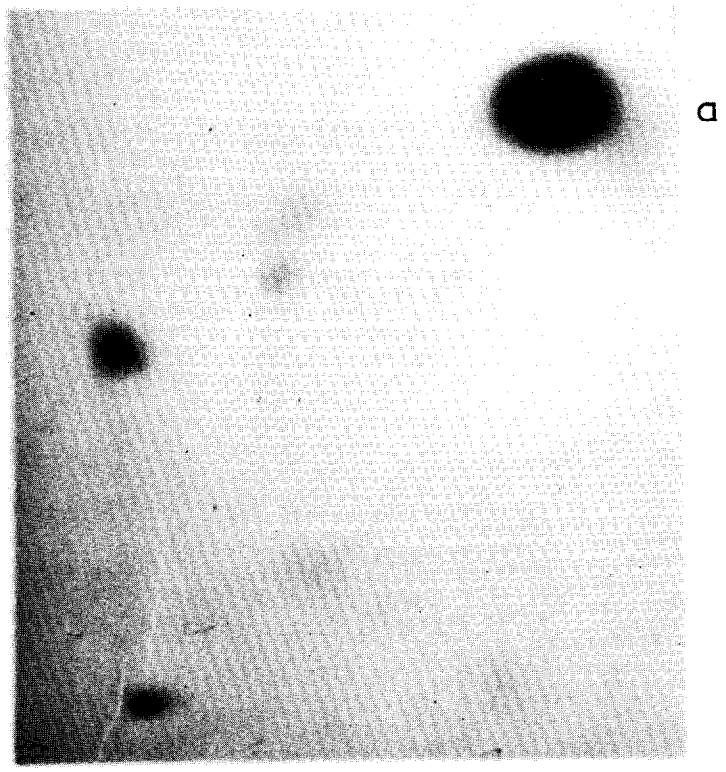
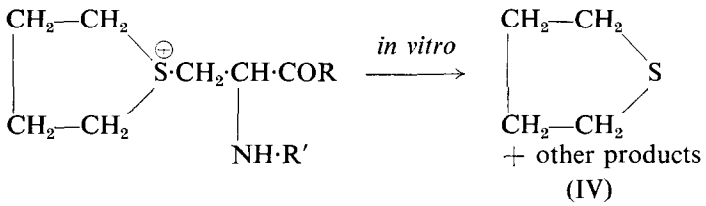


FIG. 2. Autoradiograph of a two-dimensional paper chromatogram (solvents I and II) showing the position and relative concentration of radioactive 3-hydroxytetrahydrothiophene-1:1-dioxide. (Spot *a*) excreted after injection of 2:3-^{14}C-Myleran. Solvent I →. Solvent II ↑.

The metabolism of ^{35}S-*tetrahydrothiophene in the rat*

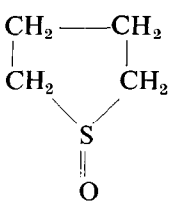
The ease of formation of tetrahydrothiophene (IV) under mild conditions from the S-β-L-alanyltetrahydrothiophenium cation *in vitro* and from the sulphonium compounds derived from the reaction of Myleran with glutathione and proteins *in vitro* led to a consideration of the possibility of its being formed also *in vivo*.



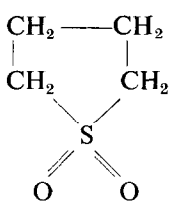
(IV)

(R = OH, peptide or protein residue)
(R′ = H, peptide or protein residue)

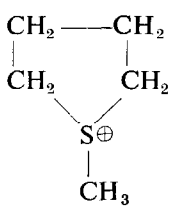
The chemical properties of the major metabolite excreted after injection of Myleran or S-β-L-alanyltetrahydrothiophenium mesylate differed from those of tetrahydrothiophene. It was possible, however, that tetrahydrothiophene was formed initially by the decomposition of the sulphonium compound, and then further metabolized. When ^{35}S-tetrahydrothiophene was injected intraperitoneally into the rat (10 mg in arachis oil (2 ml)) one major radioactive metabolite was present in the first-day's urine, (spot *a*, Fig. 1C) accounting for at least 85 per cent of the urinary radioactivity. This was shown to possess the same chromatographic and chemical properties as the major metabolite formed from either Myleran or S-β-L-alanyltetrahydrothiophenium mesylate (spot *a*, Fig. 1A and 1B). No unchanged tetrahydrothiophene was detected either in the urine or in the exhaled air. Since the major metabolite from ^{35}S-tetrahydrothiophene failed to give a positive test for a thioether, thiol or disulphide (platinic iodide reagent), but clearly contained sulphur, it was evident that the sulphur atom was in some way modified. Possible mechanisms included oxidation to form tetrahydrothiophene sulphoxide (V), tetrahydrothiophene-1:1-dioxide (VI), or *in vivo* methylation to form the S-methyltetrahydrothiophenium cation (VII) (by analogy with the *in vivo* methylation of pyridine).



(V)



(VI)



(VII)

However, ^{35}S-tetrahydrothiophenium methiodide had a different R_f value from the major metabolite, and moreover when injected into rats (5 mg in water (2 ml)) it was

apparently excreted unchanged, a finding consistent with the stability of the methiodide when heated with caustic soda solution (cf. S-β-L-alanyltetrahydrothiophenium mesylate and bromide).

The stability of the major metabolite to hot concentrated hydrchloric acid and to powerful oxidizing agents proved that the compound was not a sulphoxide, and strongly favoured its being a sulphone; although it was not tetrahydrothiophene-1:1-dioxide the possibility of its being a derivative of this was next investigated.

The metabolism of ^{35}S-*tetrahydrothiophene*-1:1-*dioxide* (VI)

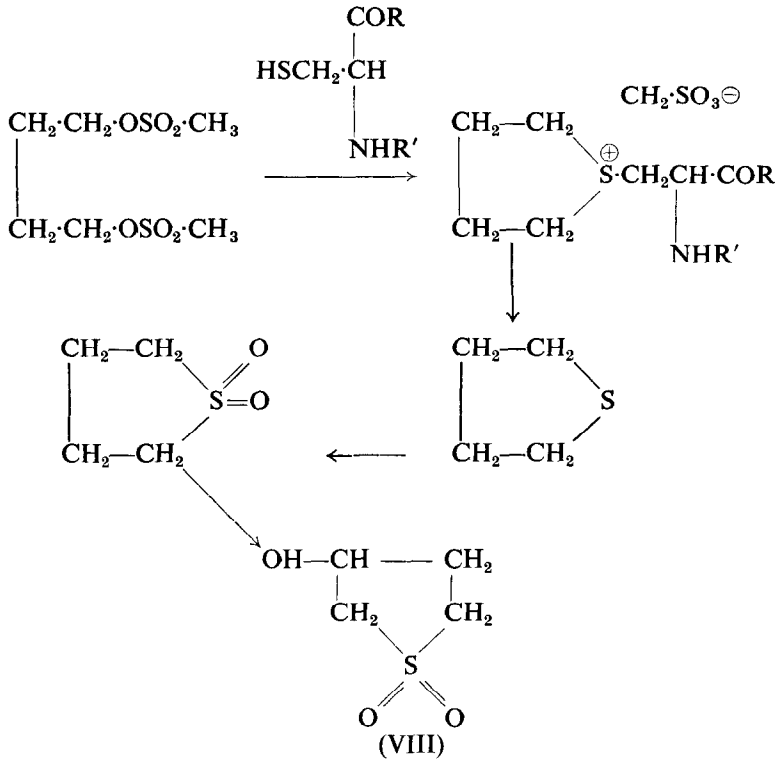
^{35}S-Tetrahydrothiophene-1:1-dioxide was injected intraperitoneally into three male Wistar rats (100 mg/rat in water (2 ml)) and the 24-hr urinary samples were examined separately. Again one major metabolite was excreted, constituting at least 85 per cent of the urinary radioactivity (spot *a*, Fig. 1D). It was shown to be identical in its chromatographic and chemical properties to the major metabolite excreted after injection of 2:3-^{14}C-Myleran, 2:3-^{14}C-S-β-L-alanyltetrahydrothiophenium mesylate, and ^{35}S-tetrahydrothiophene. The chemical properties of the major metabolite were not consistent with its being a further oxidation product such as a ketone, a diacid or a dehydro compound, and hence a remaining likely possibility was that ring hydroxylation had occurred, particularly in view of the enhancement of the reactivity of the ring carbon atoms towards nucleophilic reagents by the powerfully electron-attracting sulphone group. The presence of one or more hydroxyl groups in the metabolite was demonstrated by its reactivity towards acetyl chloride or benzoyl chloride in pyridine, leading to the formation of new products from which the metabolite could be regenerated by hydrolysis with mineral acid.

Tetrahydrothiophene-1:1-dioxide was found to be relatively non-toxic to the rat and the rabbit, and could thus be injected in sufficient quantity to enable isolation and characterization of the major metabolite. Three rabbits were each injected intraperitoneally with unlabelled tetrahydrothiophene-1:1-dioxide (1 g) and ^{35}S-tetrahydrothiophene-1:1-dioxide (100 mg), and after continuous extraction of the bulked urines with chloroform, the residual extract was treated with 3:5-dinitrobenzoyl chloride, yielding the dinitrobenzoate of 3-hydroxytetrahydrothiophene-1:1-dioxide (VIII). The ester formed pale yellow plates from methanol, m.p. 195–197 °C, undepressed on admixture with an authentic sample.

The same radioactive ester was prepared from the combined chloroform extracts of the urine obtained from two rabbits, one of which had received a small dose of 2:3-^{14}C-Myleran to act as carrier, and the other a relatively high dose of unlabelled tetrahydrothiophene-1:1-dioxide to enable isolation and characterization of the derivative. It was also obtained from the chloroform extract of urine from rats injected with high doses of S-β-L-alanyltetrahydrothiophenium mesylate (II) containing a radioactive tracer. Each derivative had m.p. 195–197 °C alone, and on admixture with an authentic sample. These latter experiments constituted proof that 3-hydroxytetrahydrothiophene-1:1-dioxide was produced *in vivo* from both Myleran and S-β-L-tetrahydrothiophenium mesylate.

It seems likely from the foregoing experiments that the major route of metabolism of Myleran *in vivo* is as follows:

(R = OH, amino acid, peptide or protein residue.)
(R′ = H, amino acid, peptide or protein residue.)



(VIII)

DISCUSSION

Urinary radioactivity

Since the urinary radioactivity was estimated by evaporating samples on plastic planchettes with an infra-red lamp, and in view of the steam volatility of both tetrahydrothiophene-1:1-dioxide and its 3-hydroxy derivative, the values recorded must represent a minimum value for the total radioactivity present.

In comparing the metabolic fate of ethyl methanesulphonate and Myleran it is of interest that while 30 per cent of the injected ^{14}C-ethylmethanesulphonate was converted to ^{14}C-carbon dioxide, only 4–7 per cent of the 2:3-^{14}C-Myleran was metabolized in this way. This result indicates that *in vivo* hydrolysis of Myleran was not a major metabolic pathway, since its hydrolysis product 1:4-(2:3-^{14}C)-butanediol was converted almost quantitatively to exhaled ^{14}C-carbon dioxide *in vivo*.

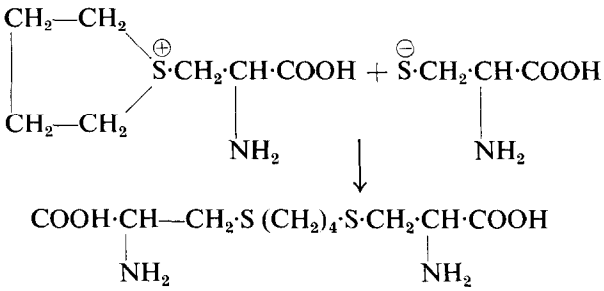
The metabolism of S-β-L-*alanyltetrahydrothiophenium mesylate*, (II), *tetrahydrothiophene*, (IV), *and tetrahydrothiophene*-1:1-*dioxide*, (VI)

Comparatively little is known of the nature of the intermediate products formed during the metabolism of sulphonium compounds in the mammal, since sulphate is the only urinary metabolite which has been reported following administration of certain sulphonium compounds to animals.[7] However, some sulphonium compounds are capable of survival since Challenger has reported the presence of sulphonium compounds in dog urine,[8] and S-methyltetrahydrothiophenium iodide, (VII), appears to be excreted unchanged after administration to the rat.

In the case of the S-β-L-alanyltetrahydrothiophenium ion, (II), it is certain that cleavage to tetrahydrothiophene, (IV), is the major, if not the exclusive, primary route of metabolism in the rat, rabbit and the mouse. An analogy can thus be drawn between the metabolism of this sulphonium compound and its hydrolytic decomposition with alkali (pH > 10). It will be recalled, however, that the sulphonium compound (II) was relatively stable in aqueous solution at physiological pH even at 100 °C, and this would strongly suggest that decomposition *in vivo* is controlled enzymatically. In this connexion the work of Cantoni and Anderson[9] is very relevant since they have isolated an enzyme from a marine alga which rapidly decomposes dimethylpropiothetin to dimethyl sulphide and acrylic acid, the same products as are formed by treatment with hot sodium hydroxide.

$$(CH_3)_2\overset{\oplus}{S}\cdot CH_2\cdot CH_2\cdot COOH \xrightarrow[\text{or hot NaOH}]{\text{enzyme, pH 5}} (CH_3)_2\cdot S + CH_2{=}CH\cdot COOH$$

It was shown in Part II[6] that the S-β-L-alanyltetrahydrothiophenium ion reacted rapidly and almost quantitatively with cysteine at about pH 8, yielding S-di-L-cysteinylbutane, and this reaction might be expected to occur *in vivo*.



However, metabolites of S-di-L-cysteinylbutane were not detectable in the urine from rats which had been injected with large doses of (II).

In general organic sulphides appear to be excreted with the sulphur atom intact, or else completely oxidized to sulphate.[10] The excretion of small quantities of a conjugate of S-ethyl-L-cysteine-1:1-dioxide after injection of ethyl methanesulphonate or S-ethylcysteine,[5] and the conversion of tetrahydrothiophene to 3-hydroxytetrahydrothiophene-1:1-dioxide, appear to be the first demonstrations of the conversion of sulphides to sulphones *in vivo*.[10]

Studies of the reactivity of tetrahydrothiophene-1:1-dioxide towards nucleophilic reagents have revealed that substitution normally occurs in the 2-position.[11] While steric effects are of secondary importance in these reactions, they will exert greater influence as the size of the attacking reagent increases, so that in the case of an enzyme-mediated reaction the less reactive, but less sterically-hindered 3-position obviously becomes the more vulnerable to attack.

In general phenols and alcohols are excreted as glucuronides and sulphates,[12] but 3-hydroxytetrahydrothiophene-1:1-dioxide was either not esterified *in vivo*, or formed an extremely labile ester which was not detectable by the methods employed.

The origin of the sulphur in 3-hydroxytetrahydrothiophene-1:1-dioxide

Since the reaction between Myleran and an ionized sulphydryl group to form a cyclic sulphonium compound appears to be general,[6] the sulphur atom in the Myleran

metabolite (VIII) could conceivably be derived from any naturally occurring sulphydryl compound such as coenzyme-A or homocysteine as well as from cysteine and cysteine-containing compounds. However, ethylmethanesulphonate (half-Myleran) reacted almost exclusively with the sulphydryl group of a cysteinyl moiety[5] and it would seem, therefore, by analogy that this is most probably the major source of the sulphur in the Myleran metabolite.

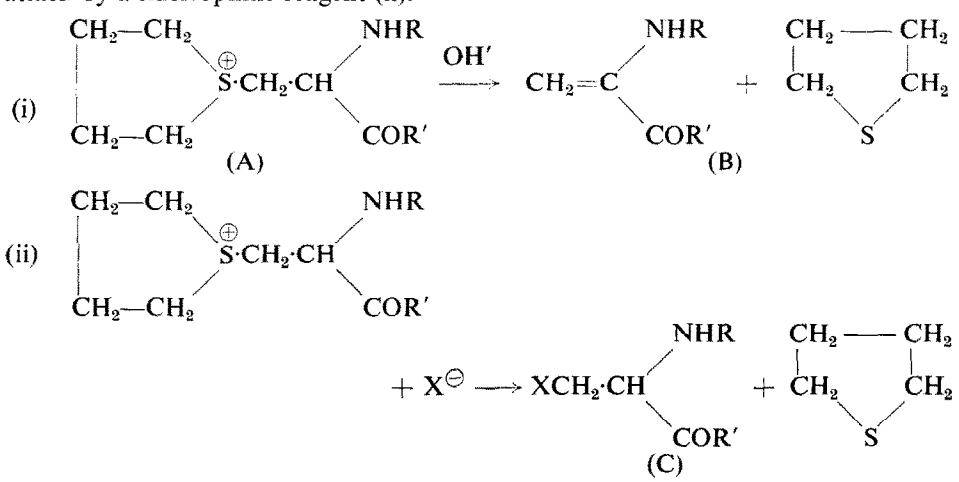
Possible relevance of these findings to the mode of action of Myleran

As stated earlier[5] the objective throughout the work described here and that described earlier on the metabolism of ethyl methanesulphonate[5] was to provide a comparison between the *in vivo* and *in vitro* reactions of two compounds, one possessing one alkylating centre and the other two, since compounds having two reactive centres separated by an optimum distance often manifest enhanced biological effects compared with their monofunctional analogues. For example Myleran, unlike ethylmethanesulphonate, exerts a profound effect on the bone marrow,[13] and has been shown to be a carcinogen in the rat.[14]

The study of the *in vivo* and *in vitro* reactions of Myleran with the thiol group has indicated three mechanisms by which the drug might modify the function of thiol-containing compounds in the cell. These include the initial sulphonium ion formation (A) which occurs *in vivo*, and which is obviously dependent on the bifunctionality of the drug, and the number of atoms in the chain separating the alkylating centres. Rings of greatest stability will be formed from diesters with a chain length of four or five carbon atoms. In this connexion it has been observed that maximum biological activity in the series of bismethanesulphonyloxy esters is associated with these particular chain lengths. Sulphonium ion formation of this type involves not only alkylation of the thiol group, which can also be accomplished by the monofunctional alkylating agents *in vivo*,[5] but also an alteration of the electrical properties of the molecule.

Further modification of the original amino acid, peptide, or protein would result from the subsequent formation of tetrahydrothiophene, a reaction occurring *in vivo*, and involving removal of the sulphur atom from the amino acid moiety.

In vitro studies have indicated that the tetrahydrothiophene formed from sulphonium compounds can be produced either by hydrolytic cleavage (i), or by direct attack by a nucleophilic reagent (ii).

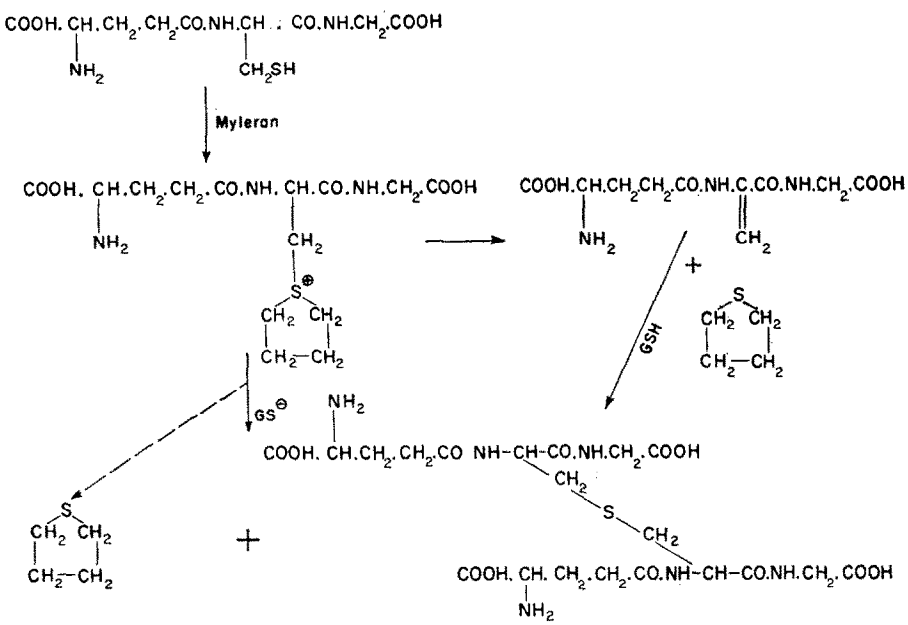


(where R and R′ represent H and OH, respectively, or amino acid, peptide, or protein residues, and X⊖ represents a nucleophilic reagent such as an ionized thiol).

In the first case the remaining residue would be a dehydropeptide (or dehydroprotein) (B), and in the latter a new saturated amino acid or amino acid chain would appear in place of the original cysteinyl moiety (C).

The chemical reactivity and stability of model dehydropeptides has been studied by Greenstein[15] and Nicolet[16] and it seems likely that in the body they may undergo either destruction by dehydropeptidases[17] or addition reactions with compounds such as thiols and amines. In each case new amino acids would appear in the molecule in place of the original cysteinyl moiety.

One such change which has been shown to occur very readily *in vitro* is the replacement of the cysteinyl moiety of glutathione by bound lanthionine (IX) after Myleran treatment (cf. Part II [6]). This transformation could have resulted by either of the two mechanisms outlined ((i) and (ii)), and indicated in the following scheme.



The reaction which might be general for peptides and proteins may be regarded as either the incorporation of a potential antimetabolite or as the cross-linking of two peptide chains.

Changes such as those indicated could be regarded as a type of chemical mutation, and attempts are now in progress to determine the nature of the modifications which occur *in vivo* following Myleran administration.

Other bis-aklylating agents such as the sulphur and nitrogen mustards or epoxides could conceivably undergo *in vivo* sulphonium ion formation with cysteine moieties in an analogous manner, and in this connexion studies with dichloroethylaniline have shown that a reaction of this type does occur *in vitro*.

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