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THE MODE OF ACTION OF ALKYLATING AGENTS—II

STUDIES OF THE METABOLISM OF MYLERAN. THE REACTION OF MYLERAN WITH SOME NATURALLY OCCURRING THIOLS *IN VITRO*

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Abstract—Myleran and 1:4-dibromobutane react with L-cysteine at pH 8 to form the S-β-L-alanyltetrahydrothiophenium ion, and smaller quantities of S-di-L-cysteinylbutane and S-(4-hydroxybutyl)-L-cysteine.

The chemical properties of the sulphonium ion are reported: (a) In the presence of aqueous alkali it rapidly decomposes to tetrahydrothiophene and aminoacrylic acid (in tautomeric equilibrium with iminopyruvic acid); (b) Electrolysis yields tetrahydrothiophene and alanine; (c) Thermal decomposition yields mainly tetrahydrothiophene; (d) Virtually no reaction occurs with cysteine at pH 7 or less; (e) At pH 8–9 it reacts rapidly and almost quantitatively with cysteine yielding S-dicysteinylbutane; (f) At pH > 10 in the presence of cysteine preferential reaction occurs with the hydroxyl ion yielding tetrahydrothiophene and aminoacrylic acid.

Myleran reacts with glutathione in water or in aqueous acetone at pH > 8 yielding 30–35 per cent tetrahydrothiophene and bound lanthionine (but no bound S-dicysteinylbutane or S-hydroxybutylcysteine). Possible mechanisms for these reactions are discussed.

Reduced keratin and egg albumin react with Myleran in the presence of alkali to yield tetrahydrothiophene.

Myleran reacts with β-mercaptoethylamine to form the (S-(β-aminoethyl)) tetrahydrothiophenium ion, and with thioglycolic acid in the presence of alkali to yield tetrahydrothiophene.

The possible relevance of these results to the metabolism of Myleran is discussed.

INTRODUCTION

FROM among the bifunctional alkylating agents which have been synthesized and subjected to laboratory and clinical trial 1:4-dimethanesulphonyloxybutane (Myleran), ((I), $n = 4$) has been selected by the majority of clinicians as the most efficacious compound for the palliative treatment of chronic granulocytic leukaemia.[1]

$$CH_3SO_2O(CH_2)_n{\cdot}OSO_2{\cdot}CH_3$$
(I)

In addition to its effects on the blood elements which have been carefully studied by Elson[2], Myleran is a mutagen[3] and has been shown to be carcinogenic in the rat.[4]

It was hoped that identification of the major urinary constituents formed from the alkylating moiety of Myleran after its administration to animals might contribute to the eventual understanding of the chemical mechanism by which it exerts its pharmacological effects. In this connexion the urinary metabolites excreted after injection of

certain monofunctional methanesulphonyloxy esters have already been examined, and earlier publications[5] described the excretion of derivatives of the corresponding S-alkylcysteine after injection of these compounds into the rat. For example 1-^{14}C-ethyl methanesulphonate (II) was shown to be metabolized in the rat to N-acetyl-S-1-^{14}C-ethylcysteine (III) and other derivatives of S-1-^{14}C-ethylcysteine,

$$CH_3SO_2O\cdot C^{*}H_2\cdot CH_3 \longrightarrow CH_3\cdot C^{*}H_2S\cdot CH_2\cdot CH(NH_2\cdot CO\cdot CH_3)\cdot COOH$$

(II) (III)

compounds which were considered to have been derived by reaction of the drug, not only with endogenous cysteine, but also with glutathione and thiol compounds of higher molecular weight. It was considered that if Myleran underwent similar reactions with thiol groups *in vivo* the identification of its urinary metabolites might be facilitated by an examination of the reaction products formed by reacting it with cysteine and other thiol-containing compounds *in vitro*.

Preliminary reports of this work have already appeared[6, 7] and subsequently some very relevant findings have been published by Parham and Wilbur[8] on the reaction between Myleran and cysteine-ethyl ester.

MATERIALS AND METHODS

Paper chromatography

The solvents used in the paper chromatography of amino acids were butanol–ethanol–propionic acid–water (20:10:10:4) (solvent I), butanol–acetone–di*cyclo*hexylamine–water (20:20:10:4) (solvent II), and methylethylketone–acetic acid–water (3:1:1) (solvent III).

Tanks and spray reagents were as previously described.[5]

2:3-^{14}C-Myleran was kindly supplied by Dr. P. Brookes of this Institute.

S-(4-*hydroxybutyl*)-L-*cysteine*

L-Cysteine hydrochloride (3·14 g) in 4 N sodium hydroxide (15 ml) was added rapidly to a hot solution of tetramethylenechlorohydrin (2·13 g) in 50 per cent ethanol (30 ml), and the solution heated under reflux for 0·5 hr. After allowing the mixture to stand overnight at room temperature the precipitated S-(4-hydroxybutyl)-L-cysteine was collected by filtration.

The product crystallized from aqueous acetone as microprisms, m.p. 203° (d.). (Found: C, 44·0; H, 8·0; N, 7·2. Calc. for $C_9H_{15}O_3NS$: C, 43·5; H, 8·0; N, 7·4%.)

S-*β*-L-*alanyltetrahydrothiophenium chloride*

S-(4-hydroxybutyl)-L-cysteine was heated under reflux with an excess of concentrated hydrochloric acid for 0·5 hr. The sulphonium compound formed a picrate which separated from water as small prisms, m.p. 135 °C (d.). (Found: C, 38·3; H, 3·9; N, 13·8; S, 7·5. Calc. for $C_{13}H_{16}O_9N_4S$: C, 38·6; H, 4·0; N, 13·8; S, 7·9%)

The reaction of Myleran with L-*cysteine*

Myleran (2·46 g) was dissolved in boiling water (20 ml) and treated with a solution of L-cysteine hydrochloride (3·14 g) in 4 N sodium hydroxide (8·4 ml), added dropwise

during 2 min. After heating for a further minute the mixture was quickly chilled in ice and the pH adjusted to 6–7. The solid (0·2 g) which separated was a sulphur-containing amino acid. It was characterized as S-di-L-cysteinylbutane by preparation of its bis-uramino derivative by the general method of Loring and du Vigneaud,[9] plates from a mixture of ethanol, chloroform and petroleum (b.p. 60–80 °C), m.p. 158 °C. (Found: C, 54·1; H, 5·8; N, 9·8; S, 11·7. Calc. for $C_{24}H_{30}O_6N_4S_2$: C, 53·9; H, 5·7; N, 10·5; S, 12·0%.)

The filtrate was treated with a large excess of acetone and the precipitated heavy oil containing salt, cysteine and the sulphonium compound, solidified on standing at 0 °C. Treatment of an aqueous solution with sodium picrate yielded S-β-L-alanyltetrahydrothiophenium picrate, m.p. 135 °C, undepressed on admixture with an authentic sample prepared from S-(4-hydroxybutyl)-L-cysteine as described previously.

Paper chromatography of the mother liquors indicated a variety of ninhydrin positive compounds, one present in relatively high concentration having the same R_f value as S-(4-hydroxybutyl)-L-cysteine in three solvent systems although no further proof of its constitution has been obtained.

The reaction of dibromobutane with cysteine

Dibromobutane (4·32 g) in ethanol (65 ml) and water (30 ml) was treated with a solution of L-cysteine hydrochloride (3·14 g) in 4 N sodium hydroxide (10 ml). The clear solution was allowed to stand at room temperature. Next day a small quantity of S-di-L-cysteinylbutane, characterized by chromatography and as its bisuramino derivative, which had separated out, was removed by filtration. Careful addition of acetone (1 l.) to the liquors then gave the sulphonium compound which formed large colourless plates from aqueous acetone, m.p. 134 °C (d.). (Found: C, 32·7; H, 5·6; N, 5·7; S, 12·0; Br, 30·5. Calc. for $C_7H_{14}O_2NS$ Br: C, 32·8; H, 5·5; N, 5·5; S, 12·5; Br, 31·2%.)

Reactions of the S-β-L-*alanyltetrahydrothiophenium ion*

(a) *With alkali.* The ion was very unstable above about pH 9 rapidly decomposing at room temperature to tetrahydrothiophene (80–90 per cent), characterized as its mercurichloride,[10] needles from methanol, m.p. 129–130 °C (lit. m.p. 129–130 °C), and its methiodide, m.p. 185–188 °C (lit. m.p. 185–190 °C[11]), not depressed on admixture with authentic samples.

Paper chromatography of the aqueous solution indicated the presence of a mixture of unidentified amino acids. However, when the solution was neutralized and electrolysed, L-alanine was detected on paper chromatograms. This was characterized by preparing its phenyluramino derivative by the general method of Loring and du Vigneaud.[9] It formed small plates from ethanol, chloroform and petroleum (b.p. 60–80 °C), m.p. 160 °C (d.), undepressed on admixture with an authentic sample. (Found: C, 59·2; H, 5·4; N, 13·3. Calc. for $C_{10}H_{12}O_3N_2$: C, 57·7; H, 5·8; N, 13·4%.)

(b) *Stability in water and acid.* The compound appeared to be relatively stable in hot water and at lower pH values.

(c) *Thermal decomposition.*[12] S-β-L-Alanyltetrahydrothiophenium bromide (3 g) was heated in an oil bath at (140–160 °C) when immediate decomposition occurred yielding an oil (0·9 g) which was largely tetrahydrothiophene as shown by preparation of its mercurichloride.

(d) *Electrolysis.* S-β-L-Alanyltetrahydrothiophenium bromide (1 g) in water (10 ml) was electrolysed using a current of 0·5–0·8 A in a Shandon Desalter. The tetrahydrothiophene which was liberated was extracted into ether and estimated as its mercurichloride (yield = 80–90 per cent). The aqueous phase was found to contain only L-alanine (0·45 g) which was characterized as its phenyluramino derivative as previously described. (Found: C, 57·2; H, 5·4. Calc. for $C_{10}H_{12}O_3N_2$: C, 57·7; H, 5·8%.)

(e) *Reaction with L-cysteine at pH 7.* S-β-L-Alanyltetrahydrothiophenium bromide (0·51 g) was dissolved in water (2 ml) and treated with a solution of L-cysteine (0·24 g) in 4 N sodium hydroxide (0·5 ml). The pH of the solution was adjusted to 7 if necessary with dilute acid, and from then on the pH of the mixture was frequently tested and never allowed to exceed 7. After heating on the steam bath for 3 hr the mixture, which contained a small quantity of solid, was extracted with ether. After drying and evaporating the ether the residue was treated with a concentrated solution of mercuric chloride in methanol, and the resulting solid (20 mg) was found to be tetrahydrothiophene mercurichloride. The solid (0·09 g) which separated from the original reaction mixture was shown by paper chromatography to consist of cysteine and S-dicysteinylbutane.

A similar result was obtained when the reaction was carried out in 50 per cent acetone.

(f) *Reaction with cysteine at pH 8–9.* S-β-L-Alanyltetrahydrothiophenium bromide (2·56 g) was dissolved in water (5 ml) and treated with a solution of L-cysteine (1·21 g) in 4 N NaOH (2·5 ml). After heating at 100 °C for a few minutes a copious solid separated. After 0·5 hr the mixture was cooled, treated with methanol (5 ml), and the solid was separated by filtration and washed with methanol. The solid (1·8 g) formed a phenyluramino derivative, plates from a mixture of chloroform, ethanol, and petrol (b.p. 60–80 °C), m.p. 158 °C, undepressed on admixture with a sample of bis-phenyluraminodi-L-cysteinylbutane prepared from S-di-L-cysteinylbutane obtained by reacting Myleran directly with L-cysteine. The filtrate was extracted with ether, and after washing with water and drying (Na_2SO_4) the ether was evaporated. The residue yielded tetrahydrothiophene mercurichloride (0·1 g) on treatment with mercuric chloride in methanol.

The filtrate from the original reaction mixture was found to contain mainly cystine, while no lanthionine could be detected on paper chromatograms.

(g) *Reaction with cysteine in the presence of aqueous sodium hydroxide.* S-β-L-Alanyltetrahydrothiophenium bromide (0·75 g) was dissolved in water (3 ml) and treated with a solution of L-cysteine (0·12 g) in 4 N NaOH (0·75 ml), giving a solution of pH > 10. After heating on the steam bath for 0·5 hr, the oil which had separated was extracted with ether. After drying (Na_2SO_4) and evaporating the ether, the residue was treated with a concentrated solution of mercuric chloride in methanol. The tetrahydrothiophene mercurichloride (0·84 g) corresponding to 0·21 g tetrahydrothiophene (approx. 80 per cent based on sulphonium compound), had m.p. 128 °C, undepressed on admixture with an authentic sample. The aqueous phase was neutralized with dilute hydrochloric acid and a solid (0·13 g) separated. Chromatography and co-chromatography in three solvent systems showed the mixture to consist of S-dicysteinylbutane and cystine, while no lanthionine was detected.

Paper chromatograms of the remaining aqueous solution indicated that no sulphonium compound remained unchanged, while no other identifiable ninhydrin-positive

compounds were present. When the solution was electrolysed, and chromatographed, alanine was detected, and characterized as its phenyluramino derivative as described earlier.

The run proceeded similarly in 50 per cent acetone, and at room temperature if the mixture was allowed to stand for a longer period.

Electrolysis of a mixture of pyruvic acid and ammonia in water

Pyruvic acid (0·5 g) was treated with an excess of 4 N ammonia and the mixture electrolysed for 0·5 hr. Alanine was the only ninhydrin-positive compound which was produced. Evaporation yielded alanine (0·48 g) characterized as its phenyluramino derivative.

Reaction of dimethanesulphonyloxypropane with L-*cysteine*

1:3-Dimethanesulphonyloxybutane (4·64 g) was dissolved in 50 per cent aqueous ethanol (150 ml) and treated with a mixture of L-cysteine hydrochloride (6·28 g, 2 Eq.) and 4 N caustic soda (20 ml, 4 Eq.) in ethanol (20 ml). After standing for 24 hr at room temperature, the precipitated solid was filtered off. Paper chromatography indicated the presence of a new ninhydrin-positive compound which was characterized as S-di-L-cysteinylpropane by preparation of its bisphenyluramino derivative. This formed small prisms from ethanol. m.p. = 176 °C. (Found: C, 53·1; H, 5·4; N, 10·8. Calc. for $C_{23}H_{28}O_6N_4S_2$: C, 53·6; H, 5·4; N, 11·1%.)

Reaction between Myleran and mercaptoethylamine

Preparation of aminoethyltetrahydrothiophenium mesylate. A solution of mercaptoethylamine hydrochloride (0·46 g) in water (4 ml) and 2 N NaOH (4 ml) was added to a hot solution of Myleran (0·96 g) in acetone (20 ml). The clear solution was warmed for 2 hr on a steam bath and then evaporated to dryness under reduced pressure. Dilution of an ethanolic extract of the residue with dry ether gave an oil, which formed aminoethyltetrahydrothiophenium dipicrate, m.p. 203 °C, with sodium picrate. (Found: C, 36·7; H, 3·2; N, 17·0; S, 5·4. Calc. for $C_{18}H_{19}O_{14}N_7S$: C, 37·0; H, 3·5; N, 17·5; S, 5·1%.)

Paper chromatography of a solution of the original oil showed the presence of a ninhydrin-positive compound which disappeared, giving rise to a new spot when the solution was electrolysed. When the sulphonium picrate was converted to the chloride and run on a similar two-way chromatogram only one compound was present which corresponded in R_f value to the original compound.

The reaction of Myleran with glutathione

(a) *In water.* Myleran (0·62 g) was dissolved in hot water (10 ml) and treated with a solution of glutathione (0·76 g) in 4 N sodium hydroxide (2·5 ml). The mixture was heated on a steam bath for 1·75 hr. The cooled solution was extracted with ether and the tetrahydrothiophene estimated as its mercurichloride. The yield was usually about 35 per cent. Paper chromatography of the aqueous layer indicated that some breakdown of the peptide had occurred. Hydrolysis with an equal volume of concentrated hydrochloric acid yielded glycine, glutamic acid, lanthionine, and a small quantity of cystine.

Lanthione was also produced at shorter reaction times, but if a greater excess of alkali was used the yield of lanthionine was greatly reduced.

Lanthionine was not isolated from the reaction mixture and so its concentration could not be estimated. Traces of lanthionine were sometimes produced when glutathione (0·76 g) was heated with 4 N NaOH (2·5 ml) in the absence of Myleran and then hydrolysed.

(b) *In aqueous acetone.* Myleran (62 mg) was dissolved in acetone (3 ml) and treated with a solution of glutathione (153 mg) in a mixture of 1 N NaOH (1·5 ml) and water (2 ml). After standing overnight the tetrahydrothiophene which had separated was extracted with ether and estimated as its mercurichloride (Yield $< 50\%$).

Paper chromatography of the aqueous layer indicated that some breakdown of the peptide had occurred. The aqueous layer was hydrolysed by refluxing with an equal volume of concentrated hydrochloric acid for 6 hr. Chromatography and co-chromatography indicated that glutamic acid, glycine, and lanthionine were virtually the only ninhydrin-positive products present.

Identical runs carried out in the absence of Myleran indicated that lanthionine was not produced under these conditions.

The effect of alkali on cystine

A solution of cystine (240 mg) in 4 N NaOH (0·5 ml) was heated at 100 °C for 2 hr. Aliquots taken at intervals were neutralized and chromatographed. No lanthionine was formed, the cystine remaining apparently unchanged.

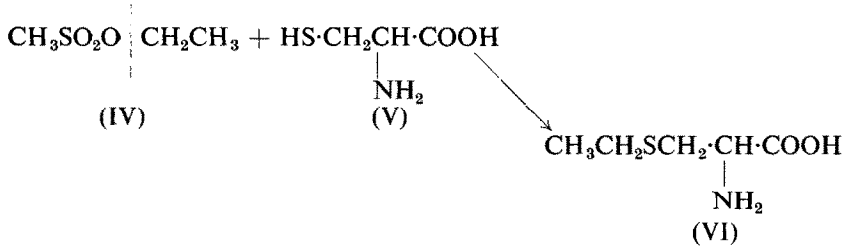
Reaction of Myleran with proteins

(a) *Wool.* Washed wool (25 g) reduced with calcium thioglycollate was suspended in water (200 ml) and 2 N NaOH (20 ml) and treated with a hot solution of Myleran (5 g) in water (300 ml). Steam distillation of the mixture yielded tetrahydrothiophene (in the initial runnings) which was characterized as its mercurichloride, m.p. 128–129 °C. Yield = 2·8 g (40 per cent of theoretical assuming that approximately 10 per cent of the reduced wool is cysteine).

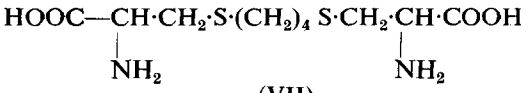
(b) *Egg albumen.* Myleran (5 g) in water (300 ml) was added to a solution of egg albumen (100 g) in water (200 ml) and 2 N NaOH (50 ml). The resulting mixture was steam distilled and tetrahydrothiophene appeared in the first runnings. Yield 120 mg, m.p. of mercurichloride 128–129 °C.

RESULTS AND DISCUSSION

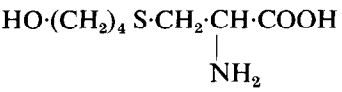
Ethyl methanesulphonate (IV) reacted readily with L-cysteine (V) in a slightly alkaline medium forming S-ethyl-L-cysteine (VI).[5]



It seemed likely therefore that Myleran (I) would react with L-cysteine in an analogous manner yielding S-di-L-cysteinylbutane (VII), if both alkylating centres reacted with cysteine, or S-(4-hydroxybutyl)-L-cysteine (VIII), if one alkylating centre reacted with cysteine while the other was hydrolysed.

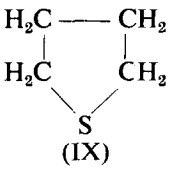


(VII)



(VIII)

However, when the reaction was carried out in hot aqueous solution at pH 8 using either 1 or 2 moles of cysteine, only relatively small quantities of these products were formed, the major product being a water-soluble amino acid which was isolated by the addition of a large quantity of acetone to the reaction mixture. While the product isolated by this method was contaminated with considerable quantities of salt, examination of its chemical properties was not precluded. In attempting to desalt the amino acid by electrolysing its aqueous solution, decomposition occurred giving rise to a strong-smelling water-insoluble oil identified as tetrahydrothiophene (IX):

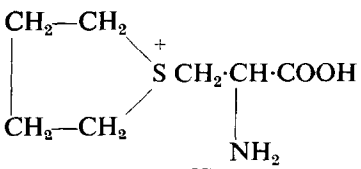


(IX)

Paper chromatograms of the aqueous phase showed alanine to be the only ninhydrin-positive product present, and this was later isolated and characterized as its phenyluramino derivative.

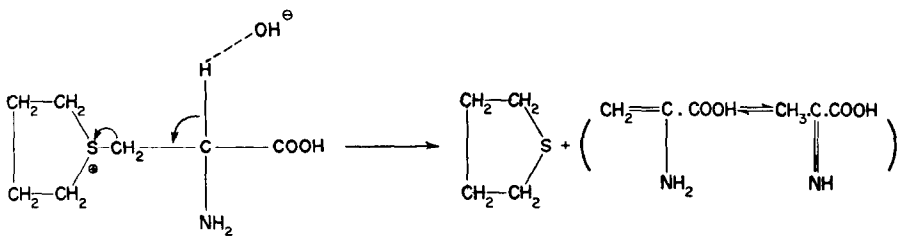
While the compound appeared to be relatively stable in aqueous solution, treatment with aqueous alkali again led to rapid decomposition to tetrahydrothiophene and some ninhydrin-positive material which on electrolysis in water was converted to alanine. Pyrolysis at 140 °C again yielded tetrahydrothiophene. The extreme water solubility of the product and the fact that it formed an immediate precipitate with sodium picrate (unlike alkylated cysteine) suggested the presence of a positively charged ion.

These data were consistent with the major product from the reaction of Myleran with L-cysteine being a sulphonium compound derived from the S-β-alanyltetrahydrothiophenium ion (X):



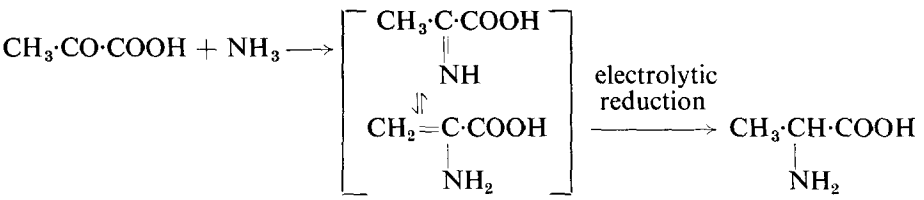
(X)

The formation of tetrahydrothiophene by the action of alkali probably occurred by the normal β-elimination of a proton



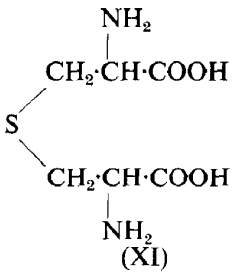
(XI)

The other product formed in this reaction was almost certainly aminoacrylic acid (in tautomeric equilibrium with iminopyruvic acid). This would account for the formation of alanine on electrolysis of the aqueous phase of the reaction mixture, since a mixture of pyruvic acid and ammonia was also converted to alanine on electrolysis.



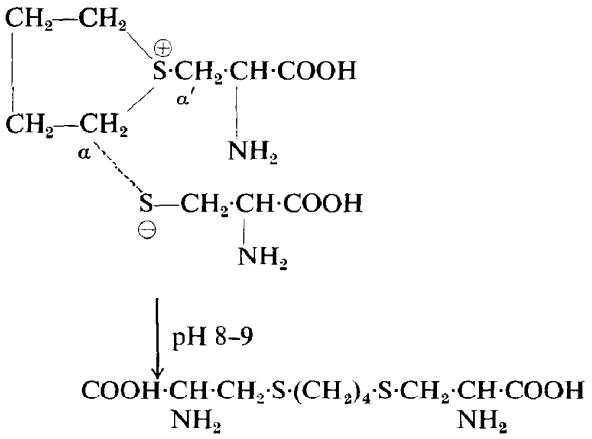
The sulphonium compound could not be freed from salt, but treatment of its aqueous solution with sodium picrate yielded a picrate, m.p. 135 °C, which was not depressed on admixture with the picrate prepared from S-β-alanyltetrahydrothiophenium chloride, made unambiguously by heating S-(4-hydroxybutyl)-L-cysteine (VIII) with hydrochloric acid. When 1:4-dibromobutane was reacted with L-cysteine in aqueous acetone a similar reaction occurred to that with Myleran, yielding S-β-alanyltetrahydrothiophenium bromide m.p. 134 °C (d.), and since it was easily isolated in a pure state and underwent similar chemical reactions to the Myleran product, it was used in many of the *in vitro* studies.

Since it was considered that this or a related sulphonium ion might be formed *in vivo* during the metabolism of Myleran, it was relevant to determine whether the sulphonium compound was itself an alkylating agent. As previously reported[7] this sulphonium compound does not react with water or cysteine at pH 7 or produce S-(4-hydroxybutyl)-cysteine or S-dicysteinylbutane *in vivo*. It has since been established that it does react with nucleophilic reagents under appropriate experimental conditions, the pH of the medium being the major controlling factor. S-β-L-alanyltetrahydrothiophenium bromide was relatively stable at pH 7 and when treated with cysteine under these conditions, either in water or in aqueous acetone, almost no reaction occurred, probably due to there being virtually no ionized cysteine at this pH. However, when the pH of the medium was raised to 8–9 a rapid reaction occurred even at room temperature, and S-dicysteinylbutane separated in high yield. An examination of the reaction mixture indicated the absence of lanthionine (XI) which would have



(XI)

been produced had the ionized cysteine attacked the α'-carbon atom instead of one of the α-carbon atoms.



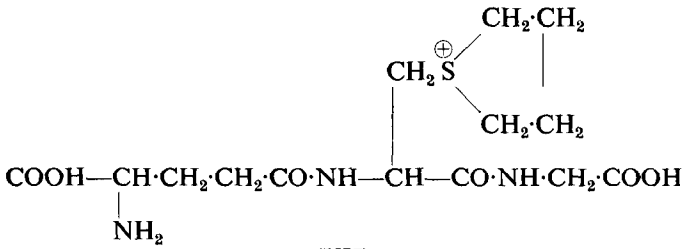
There was virtually no tetrahydrothiophene liberated under these conditions, while formation of lanthionine would have involved its simultaneous liberation in equivalent amount. The method is suggested as very satisfactory for making S-dicysteinylbutane.

In the presence of an excess of strong alkali very little reaction occurred between L-cysteine and the sulphonium compound, as judged by the liberation of an 80 per cent yield of tetrahydrothiophene together with only a small quantity of S-di-L-cysteinylbutane. The tetrahydrothiophene was produced by hydroxyl ion attack on the β-hydrogen atom as discussed earlier, rather than by attack of the ionized cysteine on the α'-carbon atom (see above scheme) since no lanthionine was formed. Paper chromatography of the remaining aqueous phase showed that no naturally occurring amino acids were present. However, electrolysis produced L-alanine indicating the presence of the pyruvic acid–ammonia tautomeric mixture, and supporting the postulated mechanism for the formation of tetrahydrothiophene.

The reaction of Myleran with glutathione

The course of the reaction between Myleran and glutathione was studied since the latter is present in high concentration in the mammal, and moreover it provides an example of a compound containing "bound" cysteine. Since the pK of the thiol group of glutathione is higher than that of cysteine, it was not surprising that the rate of reaction was relatively slow until the pH of the solution was made greater than 8. A sulphonium compound of structure (XII) was almost certainly formed, but its

isolation was precluded since subsequent rapid decomposition to tetrahydrothiophene also occurred at the pH of the reaction mixture.

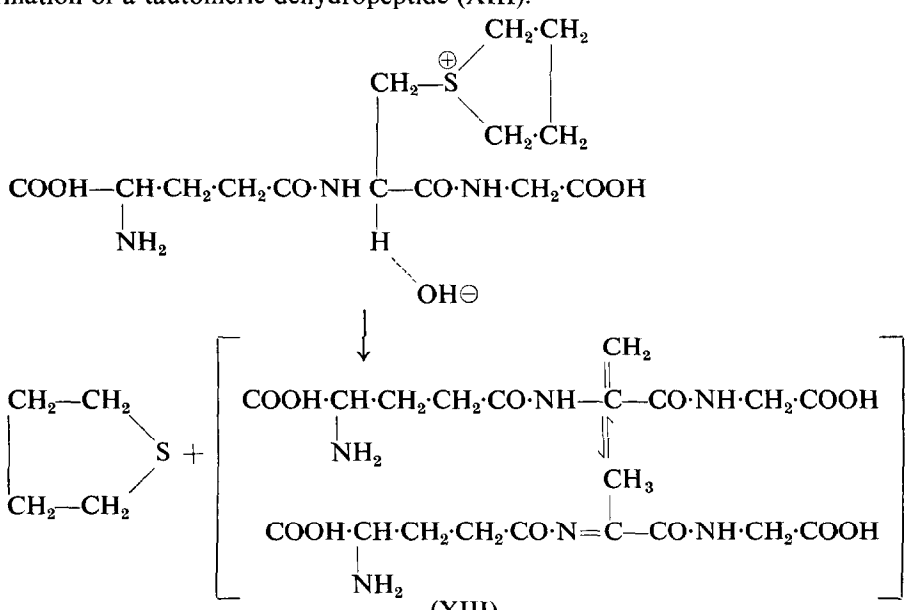


(XII)

However, the yield of tetrahydrothiophene was less than 50 per cent, probably partly due to alkaline hydrolysis of some of the Myleran.

Paper chromatography of the aqueous solution revealed that some decomposition of the peptide had occurred during the Myleran treatment. After hydrolysis with hydrochloric acid paper chromatography revealed that glycine, glutamic acid and lanthionine were the only major ninhydrin-positive compounds present. There was a striking absence of S-(4-hydroxybutyl)-L-cysteine and S-di-L-cysteinylbutane in the hydrolysed reaction mixture.

Two possible mechanisms are suggested for the formation of lanthionine, although it has not been possible to determine which was operative. If the sulphonium compound was attacked by alkali in a manner analagous to the S-β-L-alanyltetrahydrothiophenium ion then tetrahydrothiophene would be liberated with the simultaneous formation of a tautomeric dehydropeptide (XIII).

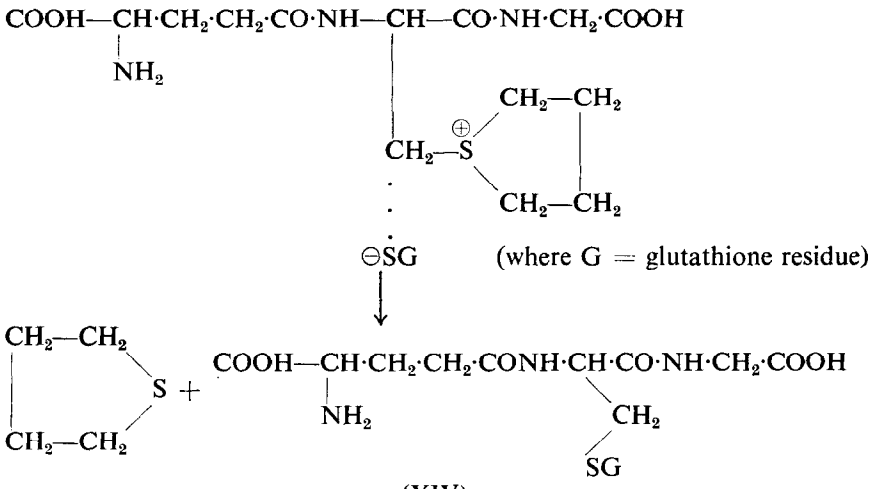


(XIII)

Addition of an unreacted molecule of glutathione across the ethylenic double bond would yield bound lanthionine (XIV) from which lanthionine would be liberated on hydrolysis. Greenstein[13] has demonstrated that in general dehydropeptides readily undergo addition reactions of this type. Birch[14] considers that dehydropeptides are

relatively unstable and this might account for the presence of decomposition products in the Myleran–glutathione reaction mixture prior to acid hydrolysis.

The other possible mechanism for the production of lanthionine would involve attack of the sulphonium compound by ionized glutathione as indicated below.

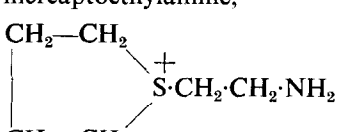


(XIV)

In this case attack of the carbon atom indicated rather than the ring carbon atom such as was observed when L-cysteine reacted with the S-β-L-alanyltetrahydrothiophenium ion, would probably be favoured because of the electron-attracting substituents attached to the neighbouring carboxyl and amino groups. Either mechanism would account for the low yield of tetrahydrothiophene formed.

These studies provide an interesting comparison between the reactivities of two sulphydryl compounds, cysteine and glutathione, towards Myleran. Sulphonium compounds were readily formed, and both yielded tetrahydrothiophene under appropriate conditions. The ease of formation of S-di-L-cysteinylbutane by reaction of the S-β-alanyltetrahydrothiophenium ion with L-cysteine would suggest that if 2:3-^{14}C-Myleran reacted with free L-cysteine *in vivo* then this compound might be formed as a radioactive metabolite. However, the dicysteinyl compound would probably not be formed if reaction occurred *in vivo* with glutathione, or presumably with higher peptides or proteins containing fully bound cysteine.

The thiol proteins kerateine and egg albumen both yielded tetrahydrothiophene on treatment with Myleran in the presence of alkali, indicating the general nature of this reaction with cysteine-containing compounds. The more general nature of the reaction was established by showing that a sulphonium compound (XV) was produced when Myleran was reacted with β-mercaptoethylamine,



(XV)

and that tetrahydrothiophene was liberated when thioglycolic acid was treated with Myleran in the presence of alkali.

On the basis of these results it seemed likely that if 2:3-^{14}C-Myleran reacted with the thiol group of cysteine or cysteine-containing compounds *in vivo* then the radioactive urinary metabolites would probably be derived from S-dicysteinylbutane (VII), S-(4-hydroxybutyl)-cysteine (VIII) or the major product of reaction, the S-β-alanyltetrahydrothiophenium ion (X). Secondary changes could also result in the replacement of cysteinyl residues by lanthionine or other amino acids.

SUMMARY

(1) The reaction between Myleran and L-cysteine yielded mainly S-β-L-alanyltetrahydrothiophenium mesylate. S-di-L-cysteinylbutane was also produced in low yield.

(2) Dibromobutane reacted in an analagous manner with L-cysteine yielding S-β-L-alanyltetrahydrothiophenium bromide and S-di-L-cysteinylbutane.

(3) Myleran reacted with glutathione at pH 8–9 to form tetrahydrothiophene and bound lanthionine.

(4) Myleran reacted with both kerateine and egg albumin at pH $>$ 10, liberating tetrahydrothiophene.

(5) Myleran reacted with β-mercaptoethylamine to form β-aminoethyltetrahydrothiophenium mesylate, and with thioglycolic acid in alkali to form tetrahydrothiophene.

(6) Treatment of the S-β-L-alanyltetrahydrothiophenium ion with aqueous alkali caused decomposition to tetrahydrothiophene and aminoacrylic acid.

(7) Thermal decomposition of S-β-L-alanyltetrahydrothiophenium bromide yielded mainly tetrahydrothiophene.

(8) Electrolysis of the S-β-L-alanyltetrahydrothiophenium ion yielded tetrahydrothiophene and L-alanine.

(9) No reaction occurred between the S-β-L-alanyltetrahydrothiophenium ion and L-cysteine at pH 7. At pH 8 a high yield of S-di-L-cysteinylbutane was produced, while at pH $>$ 10 a high yield of tetrahydrothiophene was formed together with some S-di-L-cysteinylbutane.

(10) Aminoacrylic acid was converted quantitatively to alanine on electrolysis.

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