Biochemical Pharmacology, 1963, Vol. 12, pp. 1315–1319. Pergamon Press Ltd., Printed in Great Britain.

STUDIES OF THE MODE OF ACTION OF TUMOUR-GROWTH-INHIBITING ALKYLATING AGENTS—IV *IN-VITRO* REACTIONS OF 2-CHLOROETHYLARYLAMINES

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(*Received* 12 *June* 1963; *accepted* 28 *August* 1963)

Abstract—The reactions of mono- or di-2-chloroethylarylamines with cysteine or glutathione have been studied. The major products of reaction of the bifunctional mustards were bis-cysteinyl or bis-glutathione derivatives, although in the reaction between NN-di-2-chloroethylaniline and cysteine there was evidence that a cyclic sulphonium ion was formed initially.

THE bifunctional cytotoxic alkylating agent 1,4-dimethanesulphonyloxybutane (Myleran) reacted *in vitro* with the thiol group of cysteine or glutathione to form cyclic sulphonium ions which decomposed to tetrahydrothiophene under a variety of conditions.[1] Since the major urinary metabolite formed from Myleran was a derivative of tetrahydrothiophene (3-hydroxytetrahydrothiophene-1,1-dioxide)[2] it was evident that analogous reactions occurred *in vivo*. In the following papers the possibility that the bifunctional chloroethylarylamines might undergo similar reactions with the thiol group has been investigated *in vitro* and *in vivo*. As a guide to subsequent *in vivo* studies it was of interest to determine whether chloroethylarylamines react with the thiol group of cysteine to form sulphonium ions, and if so to determine their subsequent stability when treated with water, alkali, excess cysteine, and electrolysed. In view of the differences in the end products observed in the reaction of Myleran with cysteine and with glutathione[1] it was important also to study the reaction of the aromatic nitrogen mustards with this compound.

That chloroethylarylamines are capable of forming sulphonium ions with thiols was demonstrated by Ross[3] using thiophenol and mercaptoethanol. Burnop *et al.*[4] showed that nitrogen mustard (HN2) reacted rapidly with cysteine at neutrality, causing an abolition of the SH titre and to some extent the amino group titre, although the product was an unidentified oil. These workers in an extensive study, also showed that the —SH and other groups of certain proteins are alkylated by HN2 which confirmed and extended earlier studies by Barron, Bartlett and Miller[5] using denatured egg albumin.

Past studies on the reaction between mustard gas and cysteine by Hartwell[6] have showed that the S-dicysteinyl compound was the major product formed, while reaction with glutathione was shown to lead to the S-bis-glutathione derivative. Boursnell, Francis and Wormall[7] showed that the —SH groups of human serum albumin were capable of reaction with mustard gas but that other groups also

effectively competed. Peters and Wakelin[8] in studying the reaction of mustard gas with the thiol groups of reduced wool obtained evidence for the formation of a sulphide in which one arm of the mustard had reacted while the other remained free,

$$\left.\begin{matrix}\\\\\end{matrix}\right\} -S\ CH_2CH_2SCH_2CH_2Cl$$

somewhat surprisingly not showing any tendency to ring close into a sulphonium compound. In fact there appears to be no evidence available to implicate the formation of sulphonium ions in the reaction of sulphur mustard with cysteine-containing compounds.

In this paper the reaction of bifunctional aromatic nitrogen mustards with cysteine and glutathione has been studied, and evidence has been sought to implicate the intermediary participation of sulphonium ions.

EXPERIMENTAL

Paper chromatography

This was carried out as described before[9], solvents 1, 11 and 111 being butanol–ethanol–propionic acid–water (20:10:10:4), butanol–acetone–water–dicyclohexylamine (20:20:10:4), and methylethylketone–acetic acid–water (3:1:1).

The reaction of NN-di-2-chloroethylaniline with cysteine

A mixture of NN-di-2-chloroethylaniline (6·54 g), cysteine (3·63 g), ethanol (520 ml), water (180 ml), and 4N NaOH, (7·5 ml) was kept at 37° for 3 days. The precipitated solid was collected by filtration, the mother liquors extracted with ether and then concentrated to about 15 ml under reduced pressure. The precipitated solid was collected and combined with the first crop. (Total wt 4·1 g). Paper chromatography of this solid showed it mainly to consist of one ninhydrin-positive, sulphur-containing, UV-absorbing compound, contaminated with smaller amounts of cystine and another UV-absorbing amino acid. The major product was isolated by chromatography on 3MM filter paper (Solvent 1) followed by elution from the paper with 1N HCL. The amino acid which precipitated with ammonia was NN-di-2-cysteinylethylaniline.

Found: C, 49·1., H, 6·3., N, 10·5., S, 16·2%. $C_{16}H_{25}N_3S_2O_4$ requires:
C, 49·6., H, 6·5., N, 10·8., S, 16·5%.

The mother liquors were evaporated to dryness and the residue suspended in water (3 ml) and filtered (solution [a]). Paper chromatography showed the presence of another UV-absorbing ninhydrin-positive compound. Addition of sodium picrate to an aliquot of [a] gave an insoluble red oil which failed to solidify. When a further aliquot was heated under reflux for 4 hr, a solid M.P. 32° collected in the condenser. This was 4-phenylthiazan (mixed melting point with an authentic sample). Paper chromatography of the remaining aqueous solution showed that the original ninhydrin-positive compound had disappeared giving rise to a new compound with Rf value identical to 4-phenylthiazan in three solvent systems. Treatment of [a] with hot 2N NaOH gave 4-phenylthiazan, while electrolysis led to the formation of alanine (same Rf value in 3 solvent systems) and 4-phenylthiazan. Hence, the amino acid present in [a] was almost certainly S-β-alanyl-4-phenylthiazanium chloride (II). Attempts to prepare this sulphonium compound from β-chloroalanine with 4-phenylthiazan were unsuccessful.

4-Phenylthiazan could not be detected in the initial reaction mixture even when the reaction was carried out under a variety of conditions. Paper chromatography of the initial reaction mixture indicated that the presumed sulphonium salt was present in considerable quantity, although only a minute amount of 4-phenylthiazan was isolated by the method described, indicating that under these conditions the sulphonium salt decomposed to products other than the thiazan. Attack by cysteine to form the dicysteinyl compound seems likely by analogy with the formation of dicysteinylbutane from S-β-alanyl-tetrohydrothiophenium bromide and cysteine at pH 8.[1]

The large number of products present in the initial reaction mixture together with the failure to isolate 4-phenylthiazan except by the method described suggests that reactions also occurred between the thiazan and the mustard, and evidence for the presence of new products was obtained when equimolecular amounts of 4-phenylthiazan and dichloroethylaniline were mixed in 60% ethanol at room temperature.

Reaction of p-*methoxy-N,N-dichloroethylaniline with cysteine*

N,N-Dichloroethyl-*p*-anisidine (4·96 g) in ethanol (150 ml) was mixed with cysteine hydrochloride (6·24 g) in water (75 ml) and 4N NaOH (20 ml), and the mixture kept overnight at 37°. The precipitated solid (5·25 g) was recrystallised from hot water, m.p. 246–250° and was shown by paper chromatography to be homogeneous.

The hydrochloride, prepared by treating the amino acid (0·8 g) with N HCl (4 ml), and evaporating to dryness, was recrystallised from ethanol–ether m.p. 218–220° (d)

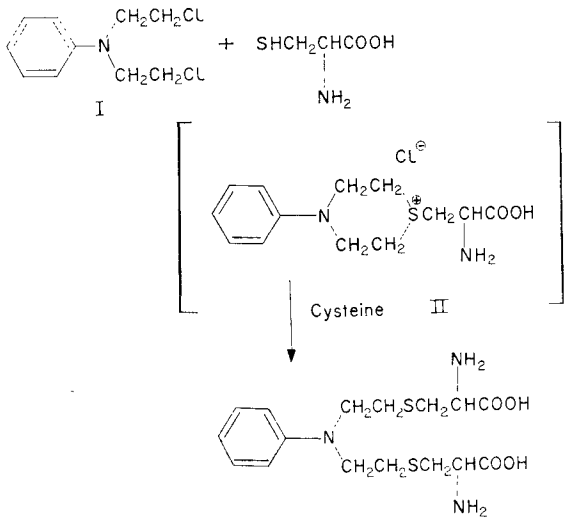
Found: C,41·6; H,5·7; N,9·0%.
Required for $C_{17}H_{29}O_5N_3S_2Cl_2$: C, 41·6; H, 5·9; N, 8·6%.

*Reaction of N,N-dichloroethyl-*p*-anisidine with glutathione*

p-Methoxy-N,N-dichloroethyl-aniline (0·83 g) in acetone (100 ml) was mixed with glutathione (1·08 g) in 4N NaOH (2·5 ml) and water (66 ml). The mixture, pH 8, was kept at 37° for 24 hr, treated with 10N HCl (66 ml), evaporated almost to dryness, made alkaline, and extracted several times with ether. The alkaline solution was made neutral, treated with an equal volume of 10N HCl, and refluxed for 6 hr. The mixture was evaporated to dryness, and the excess hydrogen chloride removed by evaporating to dryness several more times after adding water. The amino acid was absorbed into charcoal using the method of Partridge,[4], and the phenol eluate (350 ml) after extraction several times with ether was evaporated to 10 ml, and adjusted to pH 5 with dilute NaOH. The solid (0·22 g) had m.p. 246–250° (d) not depressed on admixture with the authentic sample of N,N-di-2-cysteinylethyl-*p*-anisidine prepared in the previous experiment.

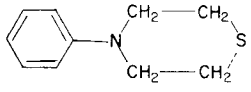
RESULTS AND DISCUSSION

The major products formed by reacting N,N-dichloroethylaniline (I) and N,N-dichloroethyl-*p*-anisidine with cysteine at pH 7–8 were the corresponding biscysteinyl derivatives, and evidence has been obtained which indicates that the formation of an intermediate sulphonium ion (II) might be an essential step in the formation of these cross-linked products, e.g. (III).



I II III

When Myleran or dibromobutane were treated with cysteine at pH 8 it was possible to isolate the sulphonium compounds and to study their properties.[1] However, it has not been found possible to isolate sulphonium compounds under similar conditions using the above mustards, but convincing circumstantial evidence implicates their intermediate formation. Thus, after removing the precipitated dicysteinyl compound from a reaction mixture containing dichloroethylaniline and cysteine at pH 8, paper chromatography revealed the presence of another ninhydrin-positive compound in the filtrate, which was present in quite high concentration. The behaviour of this compound when its solution was treated in various ways was consistent with its being a sulphonium compound. Thus, an oily picrate was formed on treatment with sodium picrate, while 4-phenylthiazan (IV) was detected when the solution was refluxed



IV

(obtained as a condensate in the condenser), when it was treated with alkali, and when it was electrolysed. The simultaneous formation of alanine in the latter case is also consistent with this conclusion.[1] All attempts to isolate 4-phenylthiazan by extraction of the original reaction mixture at pH 8 failed, probably because of preferential decomposition of the sulphonium compound in different ways, such as attack by an ionized cysteine molecule to form the biscysteinyl derivative, or because the thiazan itself was attacked by an unreacted mustard molecule. In this connection it was shown that new derivatives of the thiazan were formed when 4-phenylthiazan was treated with N,N-dichloroethylaniline in aqueous ethanol. The possiblity cannot be precluded however that some of the biscysteinyl derivatives

were formed by independent attack by cysteine molecules on each of the chloroethyl arms without the participation of an intermediate sulphonium ion.

The reaction of glutathione with Myleran is in direct contrast to its reaction with N,N-dichloroethylamino-*p*-anisidine. In the former case the peptide was converted into bound lanthionine with the simultaneous liberation of tetrahydrothiophene,[1] while in the latter no bound lanthionine was detectable, the major product being the bound biscysteinyl derivative. The nature of the products formed from the Myleran reaction indicated the initial formation of a sulphonium ion. The absence of bound lanthionine and 4-phenylthiazan in the mixture obtained by reacting bis-chloroethylamines with glutathione does not preclude sulphonium ion formation as the initial step, because evidence has been previously obtained that under appropriate conditions cyclic sulphonium ions can undergo ring fission when attacked by nucleophilic reagents.[1] In the case of the Myleran reactions, however, ring fission was observed in the reaction with cysteine but not with glutathione.

These results suggest that under *in vivo* conditions when reaction with glutathione and cysteine-containing compounds of higher molecular weight can be anticipated, the major urinary products formed from Myleran on the one hand and derivatives of bis-chloroethylaniline on the other, should be different, derived from tetrahydrothiophene in the former case, and from the bis-cysteinyl compounds in the latter.

In the following papers evidence has been obtained that the aromatic nitrogen mustards react extensively with the thiol group *in vivo*, and it has been shown that bis-chloroethyl sulphide (mustard gas) in fact forms a derivative of the bis-cysteinyl compound.[6]

In the studies with ethyl methanesulphonate,[9] Myleran[1, 2] and the mustards, a striking analogy has been found between the *in vitro* reactions of these compounds with cysteine or cysteine-containing compounds, and the structure of the new sulphur-containing urinary metabolites obtained after their injection into animals.

Acknowledgements—We wish to thank Professor A. Haddow, F.R.S. and Dr. L. A. Elson for their interest in the work. We are grateful to Miss R. Cotter for technical assistance.

This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council, the British Empire Cancer Campaign, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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