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STUDIES OF THE MODE OF ACTION OF ALKYLATING AGENTS—VI
THE METABOLISM OF BIS-2-CHLOROETHYLSULPHIDE (MUSTARD GAS) AND RELATED COMPOUNDS

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Abstract—The metabolism in the rat of ^{35}S-mustard gas (di-2-chloroethylsulphide), the half mustard ^{35}S-2-chloroethyl-2′-hydroxyethylsulphide, and their hydrolysis product ^{35}S-thiodiglycol have been studied and compared.

After intraperitoneal injection of ^{35}S-di-2-chloroethylsulphide, 90 per cent of the injected radioactivity is recovered in the urine during 5 days. At least half of this radioactivity is present as bis-cysteinyl-ethylsulphone (di-β-alanylthioethylsulphone) in a conjugated form. However this is not the sulphone of the cross-linked mustard-glutathione product[1]. Fifteen to twenty per cent of the metabolites present possess the same chromatographic properties as those observed from the hydrolysis product, ^{35}S-thiodiglycol, and a further 10–15 per cent the same as those metabolites formed from the monofunctional ^{35}S-2-chloroethyl-2′-hydroxyethylsulphide, which are not also derived from thiodiglycol.

PART V[2] described evidence for the formation of urinary metabolites of di-2-chloroethylarylamines which are derived by reaction with sulphur-containing compounds. These metabolites were not identified although the accumulated *in vitro* and *in vivo*[2] evidence suggests that derivatives of dicysteinyl compounds rather than thiazan derivatives (analogous to the tetrahydrothiophene derivative formed from Myleran) are the most likely metabolites of difunctional nitrogen mustards. In the hope that alkylating agents of simpler structure and without the complications which result from the presence of an aromatic ring would yield more information on the fate of alkylating agents of the mustard type, the metabolism of bis-2-chloroethylsulphide has been studied. A comparison has been made between the fate of the biologically active mustard gas and that of the less active mono-functional half-mustard 2-chloroethyl-2′-hydroxyethylsulphide.

The distribution and rate of excretion of radioactivity in the rabbit following injection of ^{35}S-mustard gas was studied in detail by Boursnell *et al.*[3] Data for the rate of excretion of radioactivity following administration of the drug to rats was obtained by Smith *et al.*,[4] who showed the appearance of 90 per cent of the radioactivity in the urine and faeces within 24 hr. Later Davison, Rozman and Smith[1] studied the chemical nature of the urinary metabolites and presented evidence that the two major metabolites were identical to the products formed by reacting mustard gas with glutathione, and mustard gas sulphone with glutathione. The results reported here are at some variance with these findings.

MATERIALS AND METHODS

^{35}S-mustard gas, specific activity 290 μc/mg and ^{35}S-2-chloroethyl-2′-hydroxyethyl-sulphide were supplied by the Radiochemical Centre, Amersham. ^{35}S-2-hydroxy-ethylthioethylcysteine was prepared by the method of Kinsey and Grant.[5] The reactions between mustard gas sulphone and cysteine or glutathione were carried out essentially by the methods described by Hartwell.[6].

The reaction between sulphur mustard and cysteine

When the reaction was carried out exactly as described by Hartwell,[6] some difficulty was experienced in isolating a pure sample of the dicysteinyl compound. The product was shown by paper chromatography to be contaminated with a nearly equal amount of the half reaction product, 2-hydroxyethylthioethylcysteine. This was eliminated by using two molecules of cysteine to one of the mustard. A mixture of sulphur mustard (0·243 g), cysteine hydrochloride (0·6 g) and sodium bicarbonate (0·6 g) in water (7 ml) was shaken for 3 hr. The solid was collected, washed with water and ether, reprecipitated from acid solution with ammonia, and shown by paper chromatography and analysis to be the pure dicysteinyl compound.

The reaction between ^{35}S-sulphur mustard gas and glutathione

A mixture containing glutathione (0·1 g) sodium bicarbonate (0·055 g), sulphur mustard (0·043 g), ^{35}S-sulphur mustard (12·5 mg in ether 0·5 ml—3·6 mc) in water (1·4 ml) and acetone (0·8 ml) was kept at 37° for 2 days. Paper chromatograms were run of the resulting mixture before and after ether extraction and subsequent hydrolysis with 6N HCl. Autoradiographs of these paper chromatograms indicated the presence of many radioactive products in the initial mixture although one of these constituted a major product. In the ether-extracted acid-hydrolysed fraction, there was one major radioactive product which was ninhydrin positive and had the same R_f in three solvent systems as the dicysteinyl product formed from sulphur mustard and cysteine. Chromatographic evidence for the presence of unreacted glutathione was obtained.

All compounds were administered in arachis oil and male Wistar rats (approx. 200 g) were used throughout the experiments. The methods of collection, assay, and chromatography of urine samples have been described.[7, 13]

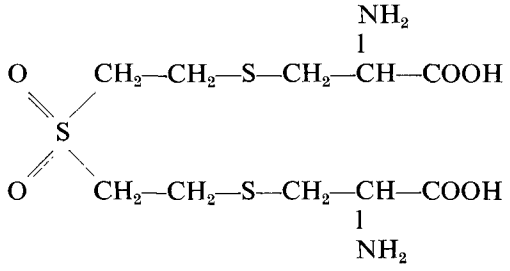
TABLE 1. EXCRETION OF INJECTED RADIOACTIVITY IN THE RAT AFTER I.P. ADMINISTRATION OF ^{35}S-DI-2-CHLOROETHYLSULPHIDE

Days after Injection	% Injected Radioactivity
0–1	52·4
1–2	30
2–3	3·5
3–5	4·7
	90·6

RESULTS

After intraperitoneal injection of ^{35}S-di-2-chloroethylsulphide (1 mg; 290 μc) 50 per cent of the injected radioactivity was excreted in the urine during the next 24 hr and approximately 90 per cent was recovered within 5 days (Table 1).

Figure 1A shows an autoradiograph of a typical paper chromatogram of the urine collected during the first 24 hr after injection. A large number of metabolites were present although one product (Spot a) accounted for at least 50 per cent of the urinary radioactivity. Figure 1B shows a similarly prepared autoradiograph obtained from the urine of a rat which had received the ^{35}S-thiodiglycol (290 μc) obtained by hydrolysing ^{35}S-sulphur mustard (1 mg). The major product in the mustard urine corresponding to spot a in Fig. 1A was thus not derived from the hydrolysis product of the mustard. Heating the urine with an equal volume of concentrated hydrochloric acid for 24 hr converted the major product into one of different R_f (Spot j, Fig. 1D), and this corresponded to approximately 50 per cent of the radioactivity in the hydrolysed urine. This product was compared chromatographically with $\beta\beta'$-dicysteinylethylsulphide, a possible product of metabolism, and found to have a different R_f in several solvents. Furthermore, none of the minor metabolites present in the hydrolysed urine corresponded in R_f to this compound. In view of the tendency for sulphides to be oxidised to sulphones *in vivo*, it was next compared chromatographically with $\beta\beta$-'dicysteinyl-



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ethylsulphone (I), and found to have the same R_f in three solvent systems. Larger amounts of this sulphone were added to the hydrolysed urine and crystallized by addition of sodium carbonate. Repeated crystallisation gave this mustard sulphone-cysteine product of constant specific activity. Paper chromatography of this crystallised sulphone failed to separate the radioactivity from the product. Chromatography of the hydrolysed urine mother liquors after removal of the sulphone showed an absence of the major radioactive product initially present.

As far as the nature of the derivative of the bis-cysteinylsulphone present in the initial urine is concerned, many possibilities exist. One might anticipate that it was excreted as the mono- or di-acetyl derivative, since alkylated cysteines are invariably excreted as the mercapturic acid. Alternatively the sulphone might have been present initially as a glutathione conjugate[1] or a derivative of this (e.g. mono- or di-acetyl). The product obtained by the reaction between mustard gas sulphone and glutathione was found to differ in R_f value from that of the major urinary metabolites. Consistent with the failure to find any dicysteinylethylsulphide in hydrolysed urine, was the failure of the major product of the glutathione-mustard reaction (which yielded the dicysteinyl compound on hydrolysis) to correspond to this major metabolite in the unhydrolysed urine.

Comparing the autoradiographs of paper chromatograms of urine obtained after administration of ^{35}S-thiodiglycol (obtained from 1 mg of sulphur mustard) and ^{35}S-sulphur mustard, it would appear that only the compounds corresponding to spots

b, c, d, e and f, Fig. 1 which are common to both chromatograms and account for approximately 15 per cent of the urinary radioactivity are derived from the hydrolysis product of the mustard.

In considering *in vivo* reactions of mustard gas it was of interest to determine if possible whether after one arm of the mustard had reacted with a nucleophilic centre such as a thiol group there was a strong tendency for the other arm to hydrolyse. If this were generally the case then one would anticipate similarities in the metabolic products formed from mustard gas and the half hydrolysis product 2-chloroethyl-2′-hydroxyethylsulphide. The radioactive metabolites formed in the urine collected during the first 24 hr after administration of the ^{35}S-labelled half mustard are compared with those of the mustard in Fig. 1C. It was evident that some of the metabolites present in the mustard urine, spots g, h, 1A, and equivalent to approximately 12 per cent of the urinary radioactivity, could correspond to those of the half mustard spots g, h, Fig. 1C, and this view was substantiated by running the urines themselves, and after similar treatments, in a variety of chromatographic solvents. However it was further apparent by treating the urine obtained after ^{35}S-thiodiglycol administration also in a similar way that the majority of the half mustard (80%) is converted *in vivo* into metabolites possessing the same R_f as those formed from its hydrolysis product, ^{35}S-thiodiglycol, (Spots b, c, d, e, f, Fig. 1B and C).

One of the major metabolic products of the mustard, spot i, Fig. 1A ($\equiv$ 10% of the urinary radioactivity) does not correspond to any of the thiodiglycol metabolites. Again since not present in the half mustard urine, it would seem to be a product resulting from a reaction involving both reactive arms. One possibility which suggests itself based on the *in vitro* reactions of the closely related 2-chloroethylamines with cysteine described in the previous paper is that it might possibly be derived by breakdown of an intermediate cyclic sulphonium ion formed in the reaction of the mustards with thiol groups. It is unchanged on hydrolysis and possesses chromatographic properties likely to be associated with a dithian derivative, although further evidence is needed to substantiate such a view.

DISCUSSION

It has been shown that the major product present in the hydrolysed urine of rats which have been administered mustard gas by intraperitoneal injection is bis-cysteinyl-ethylsulphone. The sulphur compound initially present in the urine, while clearly a conjugate of this, is not the mustard gas sulphone–glutathione conjugate *per se*.[1] Although the possibility that such a conjugate had been acetylated cannot be excluded, the alternative and more plausible possibility is that this cysteinyl sulphone was initially present as the mono- or di-acetyl derivative. It was also shown that no mustard gas–glutathione product or conjugate of this was initially present in the urine since no dicysteinylsulphide was produced when the urine was hydrolysed. These results may be contrasted with those obtained by Davison, Rozman and Smith[1] who obtained evidence that the major urinary metabolite following intravenous injection of mustard gas was the glutathione conjugate. The differences found may be accounted for by the different modes of administration, but even so their result is of interest since in general alkylated glutathione derivatives tend to hydrolyse to the cysteinyl derivative or to a conjugate of it[7, 8] before excretion in the urine. Under their conditions there has been

apparently little tendency for the mustard sulphur atom to oxidise or for the glutathione conjugate to be further metabolised. Further comparison of this work with that described here suggests that intraperitoneal injection of the mustard into the rat leads to a much less rapid rate of excretion of radioactivity than does intravenous administration.

It would appear that only a relatively small proportion of the mustard (15%) is hydrolysed *in vivo* to thiodiglycol and excreted as metabolites of such, a somewhat surprising result in view of the rapid hydrolysis of mustard gas in water. Further comparisons between the metabolism of mustard gas and its half hydrolysis product which revealed certain similarities showed that there is, after one arm of the mustard has reacted with a thiol group, a greater tendency for the second arm to react with another thiol group than for it to hydrolyse. This was also apparent in the *in vitro* reactions of mustard gas with thiols.

The results reported in part V and this paper show that aromatic nitrogen mustards and sulphur mustard combine predominantly with the thiol group *in vivo* and are excreted as cysteinyl derivatives which have been further conjugated or further degraded. However it is not yet known whether in addition to reaction with glutathione (and possibly cysteine) alkylation of protein thiol groups has also occurred. It is known from extensive *in vitro* studies of the reaction between mustards and proteins[9, 10] that many functional groups other than the thiol group also react. Furthermore the administration of radioactive mustards, radioactivity has been found to be bound to isolated protein fractions.[11] It can be inferred that the breakdown products of such reactions do not contribute grossly the urinary radioactivity, and so it seems unlikely that much of the urinary sulphur metabolites have been derived by reaction with protein thiol groups. The most likely candidate for reaction is therefore glutathione in view of the comparatively low levels of free cysteine in the animal body. In this connection it was deduced from the studies of Myleran[12, 13] that the reaction leading to the formation of 3-hydroxytetrahydrothiophene sulphone most likely involved an initial reaction with the thiol group of bound, rather than free, cysteine since the sulphonium ion formed by reaction between Myleran and cysteine reacted readily with a further molecule of cysteine yielding dicysteinylbutane which could not be detected as a urinary metabolite.

The reaction with glutathione is unlikely to be implicated in the cytotoxic or other pharmacological properties of these alkylating agents, and therefore judgment must be reserved when considering the role of thiol alkylation in determining pharmacological properties until an examination has been made of isolated alkylated proteins from *in vivo* sources. These studies will be facilitated by the knowledge of the manner in which the various alkylating agents interact chemically with thiol groups. When protein reaction is being considered other functional groups must be considered also as candidates for reaction in view of the earlier *in vitro* studies already mentioned.

In attempting to assess the relative importance of drug interaction with various nucleophiles *in vivo* useful clues might be obtained by studying the relative extents of drug binding in both sensitive and resistant cell- or tumour-lines. At the same time such an approach would be of potential importance clinically.

The interaction of alkylating agents with nucleic acids is of undoubted importance in determing some of the pharmacological effects of the alkylating agents, but very little is known about the importance of the simultaneous protein reactions occurring

either in the cytoplasm or the nucleus. Alteration of key proteins by direct interaction may yet prove to be a vital link in the carcinogenic process, for instance. There is probably no carcinogen which does not interfere with both nucleic acid and protein integrity simultaneously, and hence there is no evidence to suggest that both reactions are not essential.

SUMMARY

1. The major product of metabolism of mustard gas corresponding to 50 per cent of the urinary radioactivity is a conjugate of $\beta\beta'$-dicysteinylethylsulphone, which is liberated on acid hydrolysis of urine.
2. Probably only 15 per cent of the urinary radioactivity corresponds to metabolites derived from the hydrolysis product thiodiglycol.
3. Evidence was obtained that some urinary metabolites, corresponding to 12 per cent of the urinary radioactivity, were the products of the metabolism of the half mustard 2-chloroethyl-2′-hydroxyethylsulphide.
4. The majority (80%) of the metabolites of the half mustard ^{35}S-2-chloroethyl-2′-hydroxyethylsulphide possess the same R_f as those derived from ^{35}S-thiodiglycol.
5. Another metabolite present in the mustard urine accounting for 10 per cent of the urinary radioactivity would appear to be the result of a two-arm reaction.

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