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[STUDIES OF THE MODE OF ACTION OF TUMOUR-GROWTH-INHIBITING ALKYLATING AGENTS—V THE METABOLISM OF 2-CHLOROETHYLARYLAMINES

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Abstract—The metabolism of 2-chloroethylarylamines has been investigated. After intraperitoneal injection of N,N-di-2-chloroethyl-^{14}C-aniline, 60 per cent of the injected radioactivity was excreted in the urine and faeces during the first 24 hr, while almost all the radioactivity was recovered within 10 days.

Little of the excreted radioactivity was associated with metabolites of the hydrolysis product, N,N-di-2-hydroxyethyl-^{14}C-aniline. There was no evidence for the presence of metabolites of 4-^{14}C-phenylthiazan, a possible breakdown product of an intermediate sulphonium ion, and dealkylation of the mustard to ^{14}C-aniline apparently did not occur.

After intraperitoneal injection of N-2-chloro-1,2-^{14}C-ethyl-N-ethylaniline 60 per cent of the radioactivity was excreted in the urine during several days, 70–80 per cent of which was in the form of sulphur-containing derivatives of the mustard, none of which was the N-acetyl-S-alkylated cysteine. A small proportion of the urinary metabolites was derived from the hydrolysis product N-2-hydroxy-1,2-^{14}C-ethyl-N-ethylaniline.

THE metabolism of bis-2-chloroethylarylamines has been investigated in order to find evidence for alkylation *in vivo* with particular reference to the thiol group, and the reactions discussed in the previous paper, which have indicated a possible alternative route of metabolism to that found in the case of 1,4-dimethanesulphonyloxybutane (Myeran).[1]

Earlier studies by Skipper, Bennett and Langham[5] using ^{14}C-HN2 labelled in the methyl group showed that up to 18 per cent of the methyl carbon was exhaled as carbon dioxide after injection into mice. About 30–50 per cent of the agent was attached to tissue after 6 hr and the release from the tissues was slow. Further studies by Trams and Nadkarni[6] indicated that practically the whole of an LD_{50} injected intravenously into a rat was demethylated within 1 hr. Using 4-^{131}iodo-N,N-di-2′-chloroethylaniline Craig and Jackson[7] suggested that it was converted *in vivo* into the hydroxylamine derivative.

MATERIALS AND METHODS

Animals

Six-week old male Wistar rats (approx. 200 g) were used in all the animal experiments.

Collection and assay of radioactive metabolites

Some of the methods used to collect and assay the radioactive urine, faeces, and exhaled carbon dioxide have been described.[2] Later fractions have been assayed using a Tri-Carb liquid scintillation spectrometer (Packard of Illinois). Preparation of urinary samples for chromatography were carried out as before,[2] and the fractions were chromatographed in the following three solvent systems; butanol–ethanol–propionic acid–water (20:10:10:4), Solvent I; butanol–acetone–dicyclohexylamine–water (20:20:10:4), Solvent II; methylethylketone–acetic acid–water (3:1:1), Solvent III. Paper chromatograms were scanned for radioactivity using an Actinograph chromatogram scanner (Nuclear Chicago Co.).

N-2-chloro-1:2-^{14}C-ethyl-N-ethylaniline

A mixture of ethylenebromohydrin (1·3 g), 1:2-^{14}C-ethylene bromohydrin (50 mg; 2 mc supplied by the New English Nuclear Corporation, Boston 18, Massachusetts), ethylaniline (1·2 g) calcium carbonate (1 g) and water (10 ml) was heated in a sealed tube for 20 hr on a steam bath with occasional shaking. The cooled mixture was extracted with ether (3 × 20 ml) and the combined ether extracts washed with 2N HCl (2 × 30 ml). After neutralisation with cooling of the combined acid extracts with 4N NaOH, the insoluble oil was extracted into ether. The washed and dried (Na_2SO_4) ether extract was evaporated to yield the colourless hydroxy compound (1·41 g). The compound had R_f values comparable to the authentic hydroxy compound in solvents I, II and III. The hydroxy compound was dissolved in dry benzene (15 ml) and after removal of 5 ml benzene from the solution to ensure that the solution was thoroughly dry, it was cooled and treated with freshly distilled phosphorus oxychloride (1 ml) added dropwise during several minutes. The mixture was heated under reflux for 0·5 hr, then evaporated under reduced pressure. Dry benzene (5 ml) was added, the solution evaporated and the residue heated under reflux with concentrated HCl (5 ml) for 0·25 hr. The ice-cold solution was neutralised with 10N NaOH and extracted with benzene (3 × 20 ml). The dried benzene extract was evaporated to dryness under reduced pressure. A solution of the residual oil in petroleum ether 60–80° (20 ml) was shaken vigorously for several minutes with Spence Type H alumina (2 g) filtered, and evaporated to dryness under reduced pressure giving a colourless oil (1 g) which was chromatographically homogeneous when run in the three solvent systems. The oil obtained in an identical manner to that described, but using unlabelled materials, was heated in aqueous acetone until no further hydrolysis occurred when titration of the liberated hydrogen and chloride ions indicated that the mustard was 99 per cent pure. The specific radioactivity of the final product was 0·5 μc/mg.

The reaction of N-2-chloro-1:2^{14}C-ethyl-N-ethylaniline with glutathione

A mixture of glutathione (0·33 g), N-2-chloro-1:2^{14}C-ethyl-N-ethylaniline (0·182 g; 90 μc), N NaOH (25 ml), water (7·5 ml), and acetone (15 ml) was kept at 37° for 24 hr. The neutralised solution was extracted several times with ether. Autoradiographs of paper chromatograms of the aqueous fraction indicated the presence of only one radioactive area which coincided with a UV-absorbing, sulphur-containing, and ninhydrin-positive compound on the paper chromatogram. After hydrolysis with 6N HCl the radioactive compound disappeared giving one new radioactive compound which was again UV-absorbing, sulphur-containing, and ninhydrin-positive, and

which corresponded to the major product of the reaction between cysteine and N-ethyl-N-chloroethylaniline. The hydrolysed mixture also contained glycine, glutamic acid, and a small quantity of cysteine. These findings indicate that the initial aqueous solution while still containing some unreacted glutathione, contained radiochemically pure S-ethylphenylamino-1:2-^{14}C-ethylglutathione. The washed aqueous extract was concentrated to 4 ml. An aliquot (2 ml) of this solution ($\equiv$ 18 μc) adjusted to pH 7·5 was injected intraperitoneally into a male Wistar rat.

To prepare S-ethylphenylamino-1,2-^{14}C-ethylcysteine the remaining solution (2 ml) was heated under reflux for 6 hr with concentrated HCl (2 ml), diluted with water (5 ml), and evaporated to dryness. The final residue was dissolved in water (1 ml), adjusted to pH 7·5, and injected intraperitoneally into a male Wistar rat.

^{35}S-L-cystine was prepared essentially by the method of Williams and Dawson[3] with valuable modifications suggested by Dr. G. A. Maw. To obtain ^{35}S-labelled half mustard metabolites, ^{35}S-L-cystine (100 mg $\equiv$ 100 μc) in water (1 ml), made slightly alkaline with $\bar{N}$ sodium carbonate, was administered by stomach tube into a rat which had been fasted for 24 hr. After 5 min N-2-chloroethyl-N-ethylaniline (50 mg) was given intraperitoneally in arachis oil (2 ml). A second rat received only ^{35}S-L-cystine, and urine was collected from both animals for several days.

N,N-di-2-chloroethyl-^{14}C-aniline. A mixture of aniline sulphate (408 mg.), ^{14}C-aniline sulphate (17·6 mg; 500 μc) [Radiochemical Centre, Amersham], ethylene oxide (4 ml), water (5 ml), and glacial acetic acid (0·5 ml) was shaken intermittently in a stoppered flask during 1 hr, after which time all the aniline sulphate had dissolved. After 16 hr at room temperature excess ethylene oxide was removed under reduced pressure, the aqueous solution was made alkaline with sodium carbonate, and extracted with ether (3 $\times$ 20 ml). The water washed and dried (Na_2SO_4) ether solution when evaporated gave a quantitative yield of the hydroxy compound (m.p. 59°). The hydroxy compound (340 mg) was treated with phosphorous oxychloride (0·5 ml) in dry benzene (5 ml) yielding N,N-di-2-chloroethyl^{14}C-aniline (250 mg), which formed prisms from ethanol m.p. 44°. The mustard had a specific radioactivity of 0·63 μc/mg.

^{35}S-4-phenylthiazan, specific activity 6·32 μc/mg, was prepared according to the method of Ross[4] by the reaction between ^{35}S-sodium sulphide (Radiochemical Centre, Amersham) and N,N-di-2-chloroethylaniline.

The metabolism of N,N-di-2-chloroethyl-^{14}C-aniline

The amounts of radioactivity excreted in the urine, faeces and as exhaled carbon dioxide after administration of N,N-di-2-chloroethyl-^{14}C-aniline (20 mg; [12·6 μc] in arachis oil [2 ml]) are recorded in Table 1. Figure 1A shows an autoradiograph of a paper chromatogram of the urine collected during the 24 hr after administration of the mustard which indicates the presence of a major product and a number of other products in lesser amount. That the major area represents a reaction product and not a product derived by hydrolysis of the mustard was shown by comparing its R_f value with that of N,N-di-2-hydroxyethyl-^{14}C-aniline and with radioactive metabolites obtained from it during the first day after its administration, (Fig. 1B). Furthermore there were differences in the routes of excretion of the corresponding metabolites of the mustard and its hydrolysis product, the radioactive products from the hydroxy

compound being excreted mainly in the urine (55 per cent of the injected radioactivity was found in the urine collected during the first 24 hr after injection and only a total of 5 per cent was found in the faeces after 4 days) in contrast to those from the mustard which was excreted in the urine and the faeces in approximately equal amounts, (Table 1). No evidence for the presence of sulphur-containing metabolites was found

TABLE 1. PERCENTAGE OF INJECTED RADIOACTIVITY EXCRETED FROM A MALE WISTAR RAT AFTER INJECTION OF N,N-DI-2-CHLOROETHYL^{14}C-ANILINE

Times after i.p. injection (days)	Urine	Faeces	Exhaled carbon dioxide	
0–1	39	22	0	
1–2	10·2			
2–4	2·4	22·6		
4–6	0·7			
7–11	0·4			
	52·7%	44·6%	0%	Total: 97·3%

on paper chromatograms of the urine using either the platinic iodide reagent or the potassium dichromate-silver nitrate reagent. Hydrolysis of the urine led to a variety of new products none of which could be characterised as a sulphur-containing amino acid, using the usual tests. It seemed possible from the *in vitro* studies previously described that the difunctional mustard N,N-di-2-chloroethylaniline could react with a thiol group to form a cyclic sulphonium ion, capable under some conditions, notably an absence of another thiol group, of decomposing to 4-phenylthiazan which would probably be metabolised *in vivo* with oxidation of the sulphur atom to a sulphone. The R_f values of the radioactive metabolites formed from the mustard were therefore compared with those of 4-phenylthiazan and with the radioactive metabolite formed from ^{35}S-4-phenylthiazan after injection of an amount equivalent to that which could be formed from the injected mustard. A large number of radioactive products were excreted in the urine and these had different R_f values from the mustard metabolites.

Another possible metabolic pathway for the mustards which was considered was dealkylation analogous to the conversion of dibenamine to dibenzylamine. None of the radioactive metabolites formed from ^{14}C-aniline sulphate (2 mg in water [1 ml] 57 μc) however corresponded to those formed from the mustard when compared on identically prepared chromatograms.

As further examination of the properties of the mustard metabolites failed to indicate their identity or the nature of any functional group with which the mustard had reacted, it was felt that examination of a monofunctional compound might eliminate complicating secondary reactions due to the presence of a second reactive chloroethyl group, although any initial reaction with a particular group would be the same as with the difunctional compound.

Furthermore, the decreased toxicity of monofunctional compounds would enable larger quantities of the metabolites to be obtained, facilitating their detection on paper chromatograms.

The metabolism of N-2-chloro-1:2-^{14}C-ethyl-N-ethylaniline

Table 2 shows the rate of excretion of radioactivity in the urine and faeces after administration of N-2-chloro-1:2-^{14}C-ethyl-N-ethylaniline (60 mg [30 μc] in arachis oil [2 ml]). Autoradiographs of paper chromatograms of the urine collected during the first 24 hr after injection of the mustard indicated the presence of one major metabolite, which accounted for 50 per cent of the urinary-radioactivity, and a number of

TABLE 2. PERCENTAGE OF INJECTED RADIOACTIVITY EXCRETED AFTER INJECTION OF N-2-CHLORO-1:2-^{14}C-ETHYL-N-ETHYLANILINE

Times after i.p. injection (days)	Urine	Faeces	
0–1	42·5		
1–2	10		
2–3	2·6	21	
3–4	1·3		
4–5	1·3		
	57·7%	21%	Total: 78·7%

TABLE 3. PERCENTAGE OF INJECTED RADIOACTIVITY EXCRETED AFTER INJECTION OF N-1:2-^{14}C-2-HYDROXYETHYL-N-ETHYLANILINE

Times after i.p. injection (days)	Urine	Faeces	
0–1	60		
1–2	5	13	
2–3	1		
3–4	1		
	67%	13%	Total: 80%

minor metabolites (see for example Figs. 2A, 3C and 4C). The major metabolite was not derived by hydrolysis as shown by comparing, on identically prepared chromatograms, the metabolites of N-2-hydroxy-1:2-^{14}C-ethyl-N-ethylaniline (55 mg [28 μc] in arachis oil [2 ml]) (Fig. 2B) with those of the mustard. It was apparent that only a small proportion of the mustard was converted into metabolites of the corresponding hydroxy compound.

As in the case of the difunctional mustard more radioactivity was excreted in the faeces than with the corresponding hydroxy compound.

By analogy with earlier work with the monofunctional alkylating agent, ethyl methanesulphonate[2] which was excreted as N-acetyl-S-ethylcysteine, it would seem that reaction of the mustard with thiol groups and subsequent excretion of a mercapturic acid was the most plausible metabolic pathway for the 2-chloroethylarylamines. However no evidence for sulphur reaction was found by examining paper chromatograms prepared from the urine before or after acid hydrolysis with sulphur detecting reagents.

It was possible however that secondary changes led to products other than the mercapturic acid, and this was investigated by administering the product formed from the radioactive half-mustard and glutathione, S-ethylphenylamino-1:2-^{14}C-ethylglutathione (17·3 μc) and comparing the radioactive metabolites so formed with those of the half mustard by means of an autoradiograph of similarly prepared chromatograms of the urines obtained during the first 24 hr after injection of the compounds.

TABLE 4. PERCENTAGE OF INJECTED RADIOACTIVITY EXCRETED AFTER INJECTION OF S-ETHYLPHENYLAMINO-1:2-^{14}C-ETHYLGLUTATHIONE

Time after injection (days)	Urine	Faeces	
0–1	41·7		
1–2	10·6		
2–3	4·4	35	
3–4	1·5		
4–5	—		
	58·2%	35%	Total: 93·2%

It was evident that an entirely different metabolic picture was obtained, and this was confirmed by subjecting both urines to a variety of similar treatments. There was almost complete recovery of the half-mustard-glutathione radioactivity in the metabolites in the urine and faeces during the course of several days (Table 4). S-Ethylphenylamino-1:2-^{14}C-ethylcysteine also failed to give the same radioactive metabolites as the half-mustard or somewhat surprisingly as the half-mustard-glutathione product. That considerable modification of the alkylated glutathione or cysteine products had occurred was apparent by the failure to find a new amino acid with a sulphide linkage on chromatograms of the hydrolysed urines. These findings were in contrast to earlier experiments with other monofunctional alkylating agents when it was found that ethyl methanesulphonate, S-ethylcysteine and S-ethylglutathione all gave ethyl mercapturic acid and autoradiographs of paper chromatograms of urine obtained after administration of S-1-^{14}C-ethyl methanesulphate and S-1-^{14}C-ethylcysteine were identical.[2]

In a further attempt to determine whether the 2-chloroethylarylamines reacted with sulphydryl groups, the body sulphur pool was labelled by oral administration of ^{35}S-L-cystine prior to the intraperitoneal injection of the unlabelled N-2-chloroethyl-N-ethylaniline. Figures 3A and 4A show the radioactive metabolites formed from ^{35}S-cystine alone while Figs. 3B and 4B show the additional radioactive compounds (spots a, b) formed when the mustard is subsequently injected. These compounds clearly correspond with the carbon-labelled metabolites formed from the carbon-labelled half mustard Figs. 3C and 4C. This was confirmed by separating the sulphur-labelled aromatic metabolites derived from the mustard from those derived from the radioactive cystine, by absorbing them onto charcoal. Subsequent elution of the washed charcoal with phenol and acetic acid gave a mixture of metabolites which corresponded to those obtained by treating the half mustard urine in a similar manner. It was concluded that 70–80 per cent of the half mustard metabolites were derived by reaction with sulphur compounds.

Little can be said with regard to the precise chemical nature of the urinary metabolites of the half mustard. The compounds corresponding to areas a, b Fig. 2A. which account for 50 per cent of the urinary radioactivity, are clearly closely related since the proportion of one to the other was found to vary in different urinary samples, In solvent III both compounds have the same R_f value (Spot a Fig. 3B and C) and it is possible that they represent the same compound running in different ionic forms in Solvent I.

When the urine was heated under reflux for 0·5 hr with 10% HCl the major urinary metabolite underwent decomposition and gave rise to a number of new compounds, although no further decomposition occurred on heating for 12 hr with 6N HCl, conditions which would be expected to hydrolyse N-acetyl groups. This lability with acid and the fact that the sulphur atoms present were in an oxidised state might suggest the presence of a sulphoxide grouping.

DISCUSSION

The accumulated evidence indicates that the major reaction which the 2-chloroethylamines undergo *in vivo* is one involving reaction with sulphydryl groups. While no precise evidence of the nature of the urinary metabolites has been obtained it may be presumed that alkylation of the thiol groups of glutathione or other cysteine-containing compounds are the initial major reactions which occur. It might have been anticipated that such reactions would eventually result in the excretion of mercapturic acids in the case of the monofunctional 2-chloroethylamines, although it would appear that secondary metabolic changes occurred involving oxidation of the sulphur atom and possible loss of the amino acid moiety.

It is likely that esters formed by reaction of the mustards with ionised acid groups *in vivo* would hydrolyse to the corresponding hydroxy compounds, N-2-hydroxyethyl-N-ethylaniline in the case of N-2-chloroethyl-N-ethylaniline, and result in the same urinary metabolites as formed from the hydrolysis products. The failure to obtain an appreciable quantity of metabolites in the urine corresponding to those formed from the hydroxy compounds obtained after injecting either the mono- or di-functional 2-chloroethylarylamines, suggest that these undergo little hydrolysis or reaction with ionised acids *in vivo*. This presupposes that transalkylation from an ester to a thiol group does not occur.

In the case of the difunctional aromatic nitrogen mustards the *in vitro* reactions with cysteine and glutathione combined with the *in vivo* experiments, (comparing the metabolism of the mustard with that of the corresponding thiazan which could be formed from a cyclic sulphonium salt) would indicate that a dethiolation reaction comparable to that found in the case of Myleran metabolism is only a minor or non-existant metabolic pathway. The intermediate formation of a cyclic sulphonium ion which might cross-link with another thiol group to give dicysteinyl derivatives seems likely. A reaction of this type involving nucleophilic centres in proteins and nucleic acids provides a possible mechanism for the cross-linking of proteins to nucleic acids.

A comparison of the *in vitro* and *in vivo* reactions of the difunctional 2-chloroethylamines with those of Myleran indicate basic differences in the type of reaction occurring with thiol groups, and might be to some extent responsible for the differences in the pharmacological properties of these two classes of bifunctional alkylating agents.

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