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CHEMICAL TRAPPING OF A REACTIVE METABOLITE
THE METABOLISM OF THE AZO-MUSTARD 2'-CARBOXY-4-DI-(2-CHLOROETHYL)AMINO-2-METHYLAZOBENZENE

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Abstract—A general approach of potential value for the identification of reactive metabolites derived from anti-tumour (or other) agents involves chemical trapping with suitable radioactive reagents. The method, which may preclude the need for radioactively labelled drugs, is exemplified by the identification of the reactive metabolite formed on reduction of the azo-mustard, 2'-carboxy-4-di-(2-chloroethyl)amino-2-methylazobenzene (CB 1414) using a rat liver homogenate. The metabolite was shown to be 4-di-(2-chloroethyl)amino-2-methylaniline since trapping with sodium sulphide-^{35}S gave the corresponding radioactively labelled and chemically stable thiazan, 2-methyl-4-(thiazan-4-yl)aniline which was characterized by mass spectrometry.

The application of radioactive thiazan formation as a general method for the identification of nitrogen mustards in biological systems is discussed.

AS PART of a study of the metabolism of anti-tumour agents, we are investigating the chemical trapping of metabolites as an aid to identification. Such trapping involves for example, modification of reactive functional groups in metabolites in a manner such as to produce relatively unreactive derivatives amenable to structural investigation. This approach has particular value for highly reactive metabolites which, if unmodified, might be decomposed during attempted isolation. Moreover, if a specifically reacting radioactive trapping agent can be used, only the derivatives of the metabolites will be labelled (together with the initial agent if this possesses the appropriate reactive function) so that subsequent isolation and purification procedures may be conveniently monitored. Where this approach can be successfully used, then the need for radioactively labelled drugs for metabolic studies may be eliminated and, when trapping can be effected on samples of tissues or body fluid, a method is provided for the study of the metabolism of drugs in man.

The approach is particularly applicable to certain bifunctional alkylating agents as illustrated by the example which is the subject of this paper.

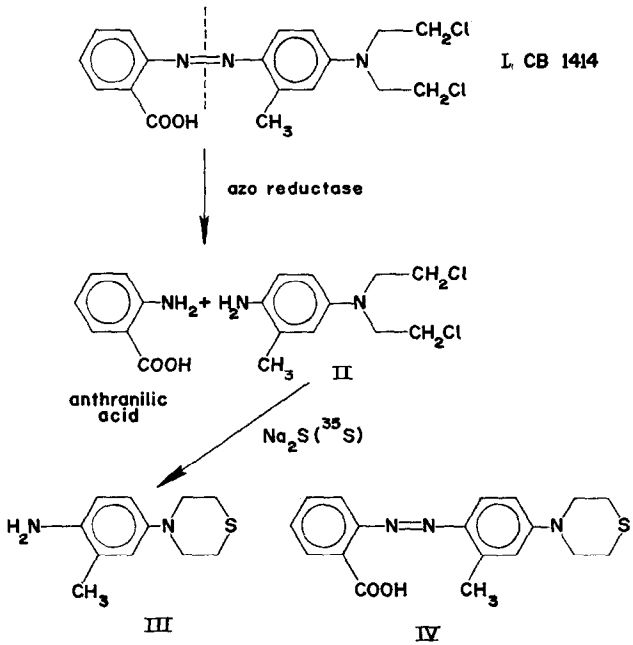
Aromatic nitrogen mustards [*N*,*N*-di-(2-chloroethyl)arylamines] have been widely used clinically in the treatment of cancer, but one disadvantage inherent in their use is cytotoxicity towards all rapidly proliferating tissues. Ross and coworkers[1] have suggested that, in certain cases, this problem might be overcome by the use of "latent" compounds which, although relatively unreactive chemically as alkylating agents (i.e. towards nucleophiles) could be converted by metabolism into more reactive, and

therefore more cytotoxic species. The success of this approach requires that such a species be generated in, or in close proximity to the target tumour, and that it be sufficiently reactive to exert only a localized cytotoxic effect.

Falling into this category of latent compounds is a series of di-(2-chloroethyl)amino derivatives of azobenzene[2] (azo-mustards), of which 2′-carboxy-4-di(2-chloroethyl)-amino-2-methylazobenzene (CB 1414, I) was selected as a model compound for the present studies. CB 1414 was found[2] to be one of the most effective members of the azo-mustard series against the Walker 256 carcinosarcoma in rats. The chloroethyl groups of CB 1414 are relatively unreactive, but reductive cleavage of the azo linkage would yield the reactive and more cytotoxic nitrogen mustard 4-di-(2-chloroethyl)-amino-2-methylaniline (II).[3] Since liver possesses high azo reductase activity, azo-mustards are being investigated for selective activity against hepatomas.

The high chemical reactivity of the 2-chloroethylamino functions in metabolites such as (II) prevents their isolation by conventional procedures since not only will their lifetime under the metabolising conditions be transient but they may be destroyed during attempted isolation. Ross[4] has shown that a wide range of *N,N*-di-(2-chloro-ethyl)arylamines react with sodium sulphide to give the corresponding thiazans which are stable towards hydrolysis. Hence, in the azo-mustard series it seemed feasible to trap the metabolite (II) of CB 1414 as the thiazan (III). The use of sodium sulphide-^{35}S would then yield a radioactively labelled thiazan thereby allowing the isolation and purification procedures to be monitored.

Conditions for the trapping of azo-mustard metabolites generated by a liver homo-genate have been evaluated using CB 1414 and, because of the small amounts of material isolated, mass spectrometry has been used to identify the derived thiazan (III).



MATERIALS AND METHODS

Preparation of "9000 g supernatant"

All tissues were homogenized at 0° in 4 vol. of 0·1 M tris(hydroxymethyl)methylamine–HCl buffer, (pH 7·4) with a Teflon-glass homogenizer and centrifuged at 9000 *g* for 20 min at 0°; 4 ml of the supernatant were equivalent to 1 g wet wt. of the original tissue.

CB 1414*-reductase assay of liver*

A solution of CB 1414 in dimethyl sulphoxide at a concentration of 5 mg/ml was used.

The incubation mixtures contained 9000 *g* supernatant from 250 mg of liver, 100 μg/ml of CB 1414, cofactors [15 μmoles of glucose-6-phosphate and 0·7 μmole of NADP (Boehringer), 17·5 μmoles of $MgCl_2$, and 50 μmoles of nicotinamide] and when required, 0·1 mg/ml of sodium sulphide-^{35}S (2·3 mc/mM, from the Radiochemical Centre, Amersham) in a final volume of 3 ml of 0·1 M tris–HCl buffer at pH 7·4. Incubation was for 15 min at 37° under nitrogen.

The incubates were then shaken with 10 ml of chloroform to extract the excess of substrate and centrifuged briefly. To 2 ml of the aqueous layer 3 ml of aqueous 20% TCA were added and the precipitated protein was removed by centrifugation. The supernatant was assayed for anthranilic acid by a modification of the method of Bratton and Marshall.[5] To 3 ml of supernatant was added 1 ml of aqueous 0·05% sodium nitrite. After 3 min, 1 ml of aqueous 0·5 per cent ammonium sulphamate was added and, after a further 3 min, 1 ml of aqueous 0·1 per cent *N*-naphthylethylenediamine dihydrochloride. The optical density at 545 nm was determined after 30 min.

CB 1414*-reductase assay of other tissues*

Rat kidney, spleen and gut. The 9000 *g* supernatant from 1 g of tissue was used in each incubation and twice the amount of cofactors noted above was added. When gut metabolism of CB 1414 was examined, it was found that centrifugation at 9000 *g* resulted in a loss of metabolic activity. This was probably due to the removal of the intestinal flora which have high azo-reductase activity.

Tumour. Ascites cells of the Walker 256 carcinosarcoma were harvested in 0·3 per cent saline to haemolyse red cells. The tumour cells were centrifuged gently. They were not homogenized before incubation with the drug.

Human liver. Human liver was obtained at autopsy 4 hr after death and was kept at the temperature of solid carbon dioxide until use. The procedure was then the same as that for rat kidney.

Isolation of metabolites

In order to minimize the contamination of metabolites intended for study by mass spectrometry, it was essential to observe the following precautions throughout the incubation and isolation procedures. All glassware was cleaned with chromic acid before use. The use of silicone and other greases was carefully avoided and all solvents were of Analar grade. Except for the preparation of the 9000 *g* supernatant, glass centrifuge tubes were used.

(a) *Metabolism.* The incubation mixture to be used for the isolation of metabolites consisted of 9000 *g* supernatant from 15 *g* of liver, 100 μg/ml CB 1414, 1 mg/ml radioactive Na_2S, and cofactors (450 μmoles glucose-6-phosphate, 42 μmoles NADP, and 525 μmoles $MgCl_2$) in a total volume of 75 ml of 0·1 M tris–HCl buffer (pH 7·4). Nicotinamide was omitted as it had the same R_f value as the thiazan in the solvent systems used. Incubation was for 45 min at 37° under nitrogen.

(b) *Extraction and chromatography.* After incubation, protein was precipitated with 200 ml of redistilled ethanol and collected by centrifugation at 2000 *g* for 10 min. The supernatant was concentrated to 25 ml at 30°/10 mm. After storage for 20 hr at room temperature the concentrate was extracted with chloroform and the dried ($MgSO_4$) extract was concentrated to 3 ml.

The concentrated extract was subjected to thin-layer chromatography (TLC) on silicic acid (Merck, Kieselgel GF_{254}) using a grooved plate [May & Baker (Dagenham) Ltd.] and development with butan-1-ol–acetic acid–water (5 : 2 : 3). The chromatogram was scanned for radioactive bands using a Panax radiochromatogram scanner. The silicic acid in the area of radioactivity corresponding in R_f value to that of the synthetic thiazan (III) was eluted with methanol and the product contained therein was subjected to further TLC using ethyl acetate as the developing solvent.

(c) *Mass spectrometry.* The silicic acid in the appropriate radioactive area of the final chromatogram in (b) was extracted with methanol and the extract applied to the probe of an AEI MS-12 spectrometer following the procedure described by Rix, Webster and Wright.[6] The mass spectrum was obtained by the direct insertion technique at a source temperature of 100° and an ionizing potential of 70 eV. The mass spectrum of the synthetic thiazan (III) was obtained for comparison purposes.

Synthesis of thiazans

The progress of reactions and the homogeneity of products was routinely monitored by TLC on silicic acid and detection with ultraviolet radiation using a Hanovia Chromatolite portable u.v. lamp. Melting points were determined on a Kofler block and are corrected.

2′-*Carboxy*-2-*methyl*-4-(*thiazan*-4-*yl*)*azobenzene* (*IV*). A stirred suspension of 2′-carboxy-4-di-(2-chloroethyl)amino-2-methylazobenzene[2] (CB 1414, I, 0·1 g) in water (25 ml), containing sodium sulphide (0·5 g), was treated with sodium hydroxide until dissolution occurred. After 9 days, little starting material remained (TLC, benzene–chloroform, 1 : 1). The coloured components were extracted with chloroform, and the extract was dried ($MgSO_4$) and concentrated. A solution of the residue in benzene–chloroform (1 : 1) was added to a column of silicic acid (30 g) and eluted with the same solvent mixture. Combination and concentration of the fractions which contained a component of R_f 0·2 (TLC, cf. starting material, R_f 0·3) yielded dark red needles (51 mg, 57%), m.p. 174·5–175·5° (from acetone). (Found: C, 62·9; H, 5·7; N, 12·0; S, 9·7%. $C_{18}H_{19}N_3O_2S$ requires C, 63·35; H, 5·55; N, 12·3; S, 9·4%.)

4-(4-*Amino*-3-*methylphenyl*)*thiazan* (*III*). A solution of 4-di-(2-chloroethyl)amino-3-methylaniline hydrochloride[3] (II, 2 g) in acetone (25 ml) was added to a vigorously stirred solution of sodium sulphide (5 g) in water (100 ml) at room temperature. After 24 hr, the solution was extracted with chloroform (300 ml) and the dried ($MgSO_4$) extract was concentrated. The oily residue crystallized from petroleum ether (b.p.

40–60°) as colourless rods (1·43 g, ~100%), m.p. 77–78°. (Found: C, 63·2; H, 7·65; N, 13·6; S, 15·6%. $C_{11}H_{16}N_2S$ requires C, 63·45; H, 7·7; N, 13·45; S, 15·4%.)

Conventional acetylation of (III) using acetic anhydride–pyridine gave 4-(4-acetylamino-3-methylphenyl)thiazan as white needles, m.p. 164·5–165·5° (from ethanol). (Found: C, 62·2; H, 7·25; N, 10·95; S, 12·5%. $C_{13}H_{18}N_2O_2S$ requires C, 62·4; H, 7·2; N, 11·2; S, 12·8%.)

Hydrolysis of 2'-carboxy-4-di-(2-chloroethyl)amino-2-methylazobenzene (I) and 4-di-(2-chloroethyl)amino-2-methylaniline (II)

Hydrolysis rates were determined with a radiometer automatic titrator. The compound (II-hydrochloride or I, 0·02 m-mole) was added to 0·1 M potassium perchlorate (30 ml) in a thermostatically controlled vessel (V-512) at 37° and pH 7. The acid liberated was titrated continuously with 0·1 N sodium hydroxide added automatically from a micrometer burette. The rate of addition was recorded on a chart using a radiometer titrigraph (SBR-2).

The velocity constants were determined by the usual first-order plot.

RESULTS AND DISCUSSION

When the azo-mustard CB 1414 (I) was incubated with 9000 *g* supernatant from rat liver, a metabolite was produced which gave a positive reaction for primary aromatic amines in the Bratton–Marshall test. This suggests that on metabolism, the azo linkage of CB 1414 was reductively cleaved and the metabolites expected were therefore anthranilic acid and (II). The coloured product formed when the diazonium coupling reaction of the above test was applied to the metabolite mixture had the same spectrum (200–700 nm) as that derived from anthranilic acid. Moreover, when the second expected metabolite (II) was synthesized it failed to diazotise under the conditions of the Bratton–Marshall test. Thus, the amount of anthranilic acid produced by metabolism was directly equivalent to the amount of CB 1414 reduced and Table 1 shows the rate of metabolism of CB 1414 in different tissues.

TABLE 1. RATE OF METABOLISM OF CB 1414 IN DIFFERENT TISSUES OF THE RAT

Organ	Anthranilic acid (nmoles/g/15 min)
Liver	2955 ± 313
Liver + Na_2S	3013
Kidney	879 ± 112
Spleen	848 ± 44
Gut	108
Gut without centrifugation	472
Walker 256 tumour cells	573
Human liver	497

As expected, the liver had the highest level of CB 1414-reductase activity and it was found that most of the activity was in the soluble fraction of the cell. The activity of human liver was low in comparison with that of rat liver. However, the human liver

was obtained 4 hr after death during which time the enzyme activities would have decreased.

In view of the liability to hydrolysis of the metabolite (II) it was necessary to have the trapping agent, sodium sulphide, present in the incubate. The presence of sodium sulphide at a concentration of 1 mg/ml in the incubate had no significant effect on metabolism. Moreover, it was found in separate experiments that sodium sulphide at this concentration quantitatively converted synthetic (II) into the thiazan (III). Hydrolysis products of (II) were not detected in this reaction reflecting the high competition factor of the sulphur nucleophile (hydrosulphide ion at this pH value[7]) in the reaction with the alkylating functions of (II). It was also shown that sodium sulphide alone did not convert CB 1414 into (II): it has been reported that sodium sulphide may reduce certain azo-mustards[8] but the necessary conditions were relatively severe and, moreover, the N–N linkage was not cleaved.

When sodium sulphide-^{35}S was present in the incubate, both unchanged CB 1414 and the metabolite (II) reacted to form thiazans. Since at pH 7 and 37°, the half-life for the hydrolysis of CB 1414 is greater than 12 hr whereas that for (II) is 8·5 min, the more rapid reaction of the latter compound with sodium sulphide was as expected. Unmetabolized CB 1414 reacted during the interval of 20 hr between incubation and extraction and the only radioactive products detected by thin-layer chromatography

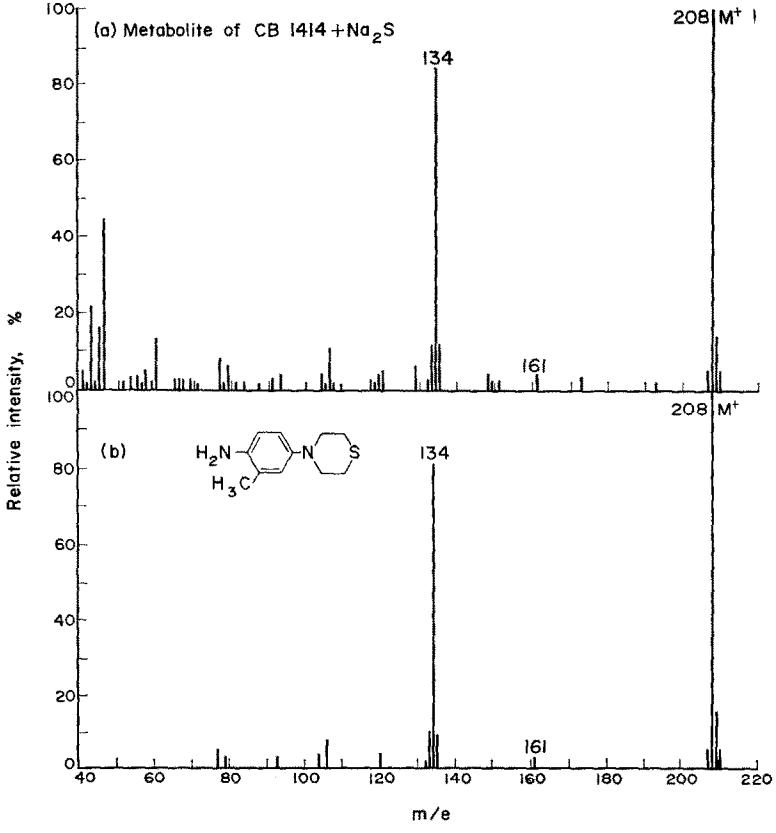
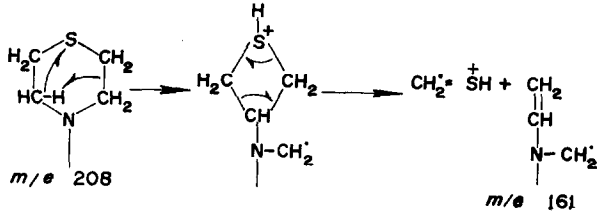


FIG. 1.

were the thiazans (III and IV, respectively) of CB 1414 and the metabolite (II). However, depending on the extent of metabolism, the relative radioactive intensities associated with (III) and (IV) altered and, occasionally, owing to complete metabolism, (IV) was absent.

Since the amount of the thiazan (III) conveniently isolable from the incubate was too small for identification by conventional procedures, mass spectrometry was used to effect characterization.[9] Figure 1 includes the line diagrams for the authentic thiazan (III) and the thiazan derived from the metabolite (II) of CB 1414. All the peaks present in the spectrum of the synthetic compound, especially those of the molecular ion (M^+, *m/e* 208) and the fragment ions *m/e* 161 and 134, were present in the same relative intensities as in that of the metabolite thiazan derivative. The additional small peaks in the latter spectrum are due to unidentified contaminants which are difficult to eliminate completely in spite of the special precautions taken (see Materials and Methods). Mass spectra of the quality shown in Fig. 1 could be obtained from 20 μg of the material.

The sole major fragment (*m/e* 134) in the mass spectrum of (III) was probably formed in two stages from the molecular ion (*m/e* 208) since a metastable peak at *m/e* 124·6 confirms the loss of 47 mass units to give the minor fragment *m/e* 161. Such a loss (elimination of CH_3S) has been observed[10] for the related compound 1,4-dithiane and the mechanism suggested[11] for the fragmentation of 3-thiapentane could apply to (III) as shown below. However, the origin of the fragment of mass 27, which must



cleave from the intermediate fragment *m/e* 161 to give the fragment ion *m/e* 134 is unclear since it could come either from cleavage of the vinyl side chain or by the elimination of HCN from the aromatic moiety (cf. aniline[12]).

The method developed for the identification of the reactive metabolite of CB 1414 should apply to other investigations into the metabolism of compounds containing a reactive, or potentially reactive, di-(2-chloroethyl)amino function. The application of the thiazan reaction to metabolites in the serum, the urine, or in tissue homogenates should extend the scope of the method to the investigation of metabolism in the whole animal and in man.

Where the thiazan method yields products, the homogeneity and identity of which can be established, a more reliable analytical procedure is provided than by the use of the Epstein colorimetric reaction[13] for the determination of alkylating compounds.

The effectiveness of CB 1414 and related compounds in the treatment of hepatomas will be reported elsewhere.

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