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METABOLISM OF ANILINE MUSTARD [*N*,*N*-Di-(2-CHLOROETHYL)ANILINE]

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Abstract—The possible metabolic activation of the antineoplastic agent *N*,*N*-di-(2-chloroethyl)aniline (aniline mustard) is discussed. Conversion of aniline mustard into the glucuronide (*p*-di-2-chloroethylaminophenyl-β-D-glucopyranosid)uronic acid was mediated by a rat liver homogenate containing the appropriate cofactors. The glucuronide was a major metabolite in the serum and bile after administration of aniline mustard to rats and after isolation and purification it was identified as its methyl ester by mass spectrometry. The use of Amberlite XAD-2 resin facilitated the isolation from serum of the glucuronide and another metabolite, *N*-(2-chloroethyl)-4-hydroxyaniline. The implication of these findings for the clinical application of aniline mustard is discussed.

IN A recent paper from this laboratory[1] the concept of latent activity, applied specifically to anti-tumour alkylating agents, was illustrated with reference to the activation of an azo-mustard, 2′-carboxy-4-di-(2-chloroethyl)amino-2-methyl-azobenzene by the action of azoreductase in liver. During this study, techniques were developed for the isolation of microgram amounts of drug metabolites from a biological fluid. A basis was thereby provided for the investigation of other problems of this type, and we now report on the metabolism of aniline mustard.

Aniline mustard was one of the earliest of the aromatic nitrogen mustards to be prepared for an experimental evaluation of its anti-tumour activity.[2] It has been used clinically for the treatment of multiple myeloma.[3] Although the metabolism of aniline mustard in the rat has been studied previously,[4,5] no metabolites have hitherto been positively identified. However, later investigations have stimulated a re-examination of the metabolism of the drug, in the hope that this might lead to clinical application on a more rational basis. Connors and Whisson showed aniline mustard to be uniquely effective in curing, with a single dose, the established ADJ/PC5 plasma cell tumour in mice.[6] They compared the uptake of radioactivity into tumour cells *in vivo* following the administration of radio-isotopically labelled aniline mustard with that of a biologically inactive analogue, β-naphthylamine mustard of similar chemical reactivity. No significant difference in uptake was found,[7] but in its ability to bind to cellular DNA, aniline mustard was 50–100 times more effective. Implicit in these findings is the likelihood that the binding was accomplished by a species much more reactive than aniline mustard, and which was metabolically derived therefrom. One possible scheme (Fig. 1) was based on the observation that the tumour had a particularly high level of the enzyme β-glucuronidase, when compared both with other types of tumour and with normal tissues.[8] Conceivably, the drug is first hydroxylated

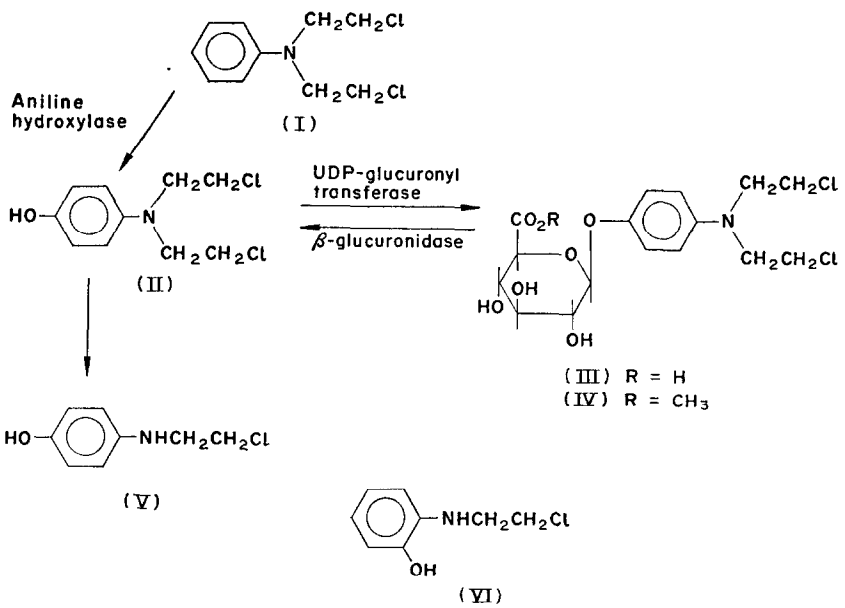

FIG. 1. Scheme for the metabolism of aniline mustard.

in the liver to give *p*-hydroxyaniline mustard [*N*,*N*-di-(2-chloroethyl)-4-hydroxyaniline] (II) and further converted there into the corresponding glucuronide (III). If the conjugate then enters a region of high β-glucuronidase activity, *p*-hydroxyaniline mustard (II) would again be liberated. Since the toxicity in mice, measured as the LD_{50}, is 10-times greater for the latter compound (II) than for aniline mustard,[9] the drug would be potentiated, as a cytotoxic agent, by conversion into its *p*-hydroxy-derivative. A direct comparison between the toxicities of aniline mustard and the synthetic glucuronide (III) has not been made, owing to the disparate solubility properties of the two compounds. However, the LD_{50}, in mice, of the sodium salt of (III), administered in water, was 270 mg/kg, whereas that of aniline mustard, in arachis oil, was 70 mg/kg.[9] Thus the toxicities of the two compounds appear to be favourably related, inasmuch as conversion of aniline mustard into the glucuronide would appear tantamount to a partial detoxification process, in so far as tissues lacking the high β-glucuronidase activity are concerned. If the above explanation for the activity of aniline mustard against the ADJ/PC5 tumour is correct, the routine monitoring of human tumours for high β-glucuronidase activity could indicate where aniline mustard might be therapeutically effective. Many preconditions must be established, however, before this ultimate goal can be attained.

An obvious prerequisite, which is the subject of the present investigation, is a demonstration that aniline mustard can be metabolized in the manner suggested, and that the conjugate is a major metabolite which is circulated in such a way as to permit ready access to the tumour-bearing site. An approach to this problem involved firstly, an investigation of the ability of rat liver enzymes to convert aniline mustard into the glucuronide (III) and, subsequently, an examination of the metabolites in serum and bile in *in vivo* experiments using the rat. In a preliminary investigation of urine of rats treated with aniline mustard no evidence of an alkylating species was found on using

both the Epstein reagent[10] and the previously described chemical trapping procedure with radioactively-labelled sodium sulphide.[11]

MATERIALS AND METHODS

Male Wistar rats of the Chester Beatty strain were used which were 6 weeks old and weighed 200–250 g. Pretreatment with phenobarbitone ensured maximal enzymic activity. Phenobarbitone sodium in water (8 mg/ml) was injected at a dose level of 80 mg/kg into the rats on each of three successive days. The final injection was given *ca.* 18 hr before death. Rat liver microsomes[11] and 9000 g^1 supernatant were prepared as previously described. Aryl hydroxylase activity was determined colorimetrically[12] with aniline as a substrate. In some experiments aniline-^{3}H (94 mCi/mmole, Radiochemical Centre, Amersham) was used in conjunction with thin layer chromatography (TLC) and radiochromatogram scanning to demonstrate the identity of the product in extracts of the incubates. The activity of the microsomes (4 determinations) and 9000 *g* supernatant were respectively 128–216 and 225 nmoles *p*-amino-phenol/g/30 min, without phenobarbitone pretreatment of the rats. With pretreatment the activity of the 9000 *g* supernatant rose to 340 nmoles/g/30 min. UDP glucuronyl transferase activity[13] of the microsomes (4 determinations) using *p*-nitrophenol as substrate was 120–330 nmoles *p*-nitrophenol conjugated/g/30 min.

Rotary evaporations were at 30° and 10 mm. Assays for radioactivity were carried out with a Packard Tricarb 3375 scintillation counter, with naphthalene, POP and POPOP in ethanol, dioxan and toluene as scintillation fluid.

Aniline mustard-^3H

This was prepared either by the method of Ball and Wade[14] or by a shorter procedure[15] involving the direct iodination of aniline mustard with iodine in fuming sulphuric acid (20% SO_3),[16] followed by reductive tritium-halogen exchange on the resulting *N,N*-di-(2-chloroethyl)-3-iodoaniline. The material used for the present experiments had a specific activity of 20 Ci/mmole, and a radiochemical purity of 93 per cent. It was stored as a 3 mM solution in benzene (100 ml). Aliquots were purified before use, where necessary, by passage through a column of silicic acid (Merck, Kieselgel G) with benzene as eluant. For metabolic studies, 1 ml of the stock benzene solution was diluted with ethanol (usually 100 ml) and the required volume then concentrated to dryness before dissolution in the injection or incubation medium. The quantities of radioactively-labelled materials used in the following experiments are given, for simplicity, as equivalent volumes of the original 3 mM benzene solution.

Isolation and chromatography of metabolites

The precautions taken to ensure minimal contamination of samples eluted from thin layer chromatograms were those previously described.[1] The preparation of extracts is described individually. They were subjected to thin layer chromatography on silicic acid (Merck, Kieselgel GF_{254}) using plates of 20 × 20 cm, or 20 × 5 cm. The previously described use of grooved plates[1] was here unnecessary, owing to the preliminary purification of metabolites achieved by extraction processes or by chromatography on Amberlite XAD-2 resin (BDH). The developed plates were scanned for radioactive bands using either a Panax or a Berthold radiochromatogram scanner.

Mass spectrometry

The mass spectrum of the synthetic (*p*-di-2-chloroethylaminophenyl-β-D-glucopyranosid)uronic acid methyl ester (IV) was determined with an AEI MS-12 spectrometer using the direct insertion technique, an ionizing voltage of 70 eV and a source temperature of *ca.* 150°. Slightly higher temperatures (160–170°) were used to obtain the spectra of the corresponding metabolite derivatives, in order to maximize the ion-currents obtainable from the smaller amounts of sample available. A lower source temperature (105°) was used to obtain the mass spectrum of *N*-(2-chloroethyl)-4-hydroxyaniline (V), *N*-(2-chloroethyl)-2-hydroxyaniline (VI) and the corresponding metabolite.

In vitro *metabolism of aniline mustard*

The incubation mixture contained 9000 *g* supernatant (for preparation, see Ref. 1) from 20 g of liver, 7 mg of aniline mustard together with the ^{3}H-derivative (equivalent to 20 μl of the benzene solution) in ethanol (2 ml) and cofactors [600 μmoles of glucose-6-phosphate, 600 μmoles of $MgCl_2$, 30 μmoles of NADP and 32 μmoles of UDPGA (Boehringer)] in a final volume of 100 ml of Tris-HCl buffer at pH 7·5. Incubation was for 45 min at 37°.

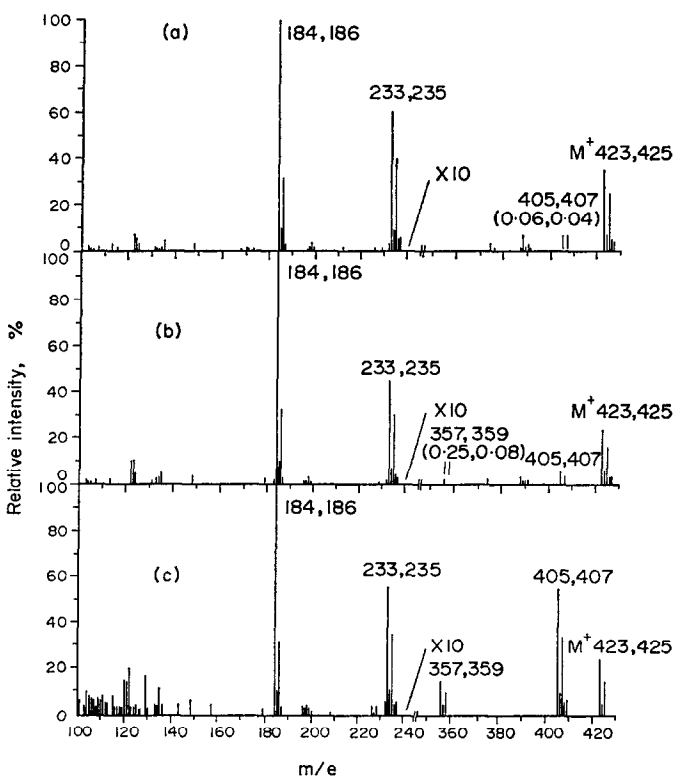

FIG. 2. Mass spectra: (a) synthetic methyl ester of (*p*-di-chloroethylaminophenyl-β-D-glucopyranosid), uronic acid (IV); (b) methylated *in vitro* metabolite of aniline mustard; (c) methylated *in vivo* metabolite of aniline mustard (from serum).

After incubation, protein was precipitated with redistilled ethanol (30 ml) and collected by centrifugation at 2000 *g* for 10 min. The supernatant was concentrated to 15 ml. After adjustment to pH 2 (HCl) the solution was added slowly to a rapidly stirred suspension of $MgSO_4$ (30 g) in ethyl acetate (150 ml). After filtration, the solution was concentrated, then subjected to TLC in chloroform–methanol (3 : 2). The developed plate was scanned and the silicic acid in the single radioactive area (R_f 0·45) which corresponded in R_f value with the synthetic glucuronide (III)[9] was removed and eluted with methanol (*ca.* 1 ml). The eluate was treated with a solution of diazomethane in ether[17] (5 ml) at 4°. After standing overnight, the solution was concentrated and the product was subjected to TLC (ethyl acetate). The silicic acid in the single radioactive area was segregated, eluted[18] with methanol, and the eluate subjected to mass spectrometry (Fig. 2b). The mass spectrum of a synthetic sample of the methyl ester (IV) was obtained for comparison purposes (Fig. 2a). The methylated metabolite was identical on TLC with the synthetic ester (IV) both in the above-mentioned solvent and in chloroform–methanol, 9 : 1 (R_f 0·48).

In vivo *metabolism of aniline mustard:* (a) *serum*

The solution for injection contained aniline mustard (125 mg) together with the ^{3}H-derivative (equivalent to 0·4 ml of the benzene solution) in dimethyl sulphoxide (2·25 ml). Aliquots (0·25 ml) were injected intraperitoneally (i.p.) into 8 rats. After 1 hr the blood was removed and centrifuged at 2000 *g* for 10 min to give 30 ml of serum. Protein was precipitated with acetone (4 vol.) and collected by centrifugation at 2000 *g* for 7 min. The supernatant was concentrated to 2·8 ml. Removal of 10 μl

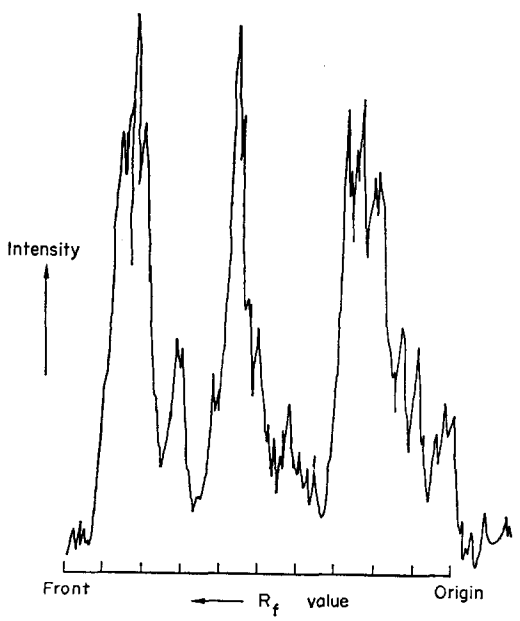

FIG. 3. Radiochromatogram scan of thin layer chromatogram (chloroform–methanol, 1 : 1) of aniline mustard metabolites from serum: ethyl acetate extract from a methanol eluate off an Amberlite XAD-2 column.

for scintillation counting gave a total of $1{\cdot}45 \times 10^8$ dis/min for the entire supernatant, whereas the total dose administered was equivalent to $1{\cdot}57 \times 10^{10}$ dis/min.

The concentrate was applied to a column ($16 \times 1{\cdot}5$ cm) of Amberlite XAD-2 resin which was eluted with water (40 ml) followed by methanol (56 ml).

The aqueous eluate contained $4{\cdot}93 \times 10^7$ (30 per cent) and the methanol eluate $1{\cdot}13 \times 10^8$ dis/min (70 per cent). A concentrate of the methanol eluate was dissolved in water (1·8 ml), and the solution was adjusted to pH 2 with 0·1 N HCl (0·3 ml) then extracted with ethyl acetate (2×2 ml). The aqueous phase contained $3{\cdot}72 \times 10^7$ (35 per cent) and the organic phase $6{\cdot}85 \times 10^7$ dis/min (65 per cent).

(i) *Composition of the organic phase.* The dried ($MgSO_4$) organic phase was concentrated and subjected to TLC (chloroform–methanol, 1 : 1) as developing solvent. A radiochromatogram scan (Fig. 3) revealed that one of the three radioactive components which was coincident with u.v.-absorbing material, and which accounted for *ca.* 46 per cent of the total radioactivity had an R_f value (0·24) corresponding to that of the synthetic glucuronide (III). The silicic acid in this area was segregated and eluted with methanol (*ca.* 0·25 ml) and the eluate was allowed to react overnight at 4°

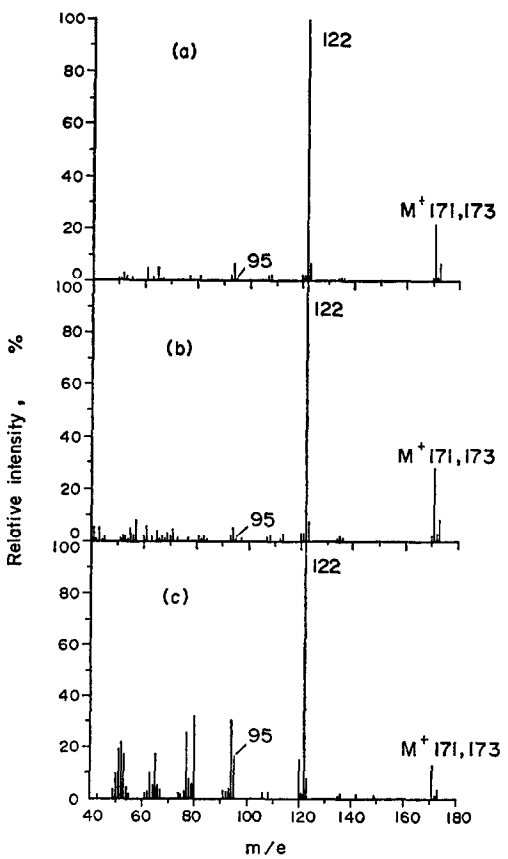

FIG. 4. Mass spectra: (a) synthetic *N*-(2-chloroethyl)-4-hydroxyaniline; (b) metabolite from serum, of same M.W.; (c) synthetic *N*-(2-chloroethyl)-2-hydroxyaniline.

with a solution of diazomethane in ether (0·5 ml). TLC (chloroform–methanol, 19 : 1) revealed a single radioactive component (R_f 0·14) which, after elution, gave a mass spectrum (Fig. 2c) similar to that of a synthetic sample of the methyl ester (IV) and had a mobility in TLC identical to that of the latter compound.

(ii) *Composition of the aqueous phase.* The aqueous phase was lyophilized. A solution of the residue in methanol was subjected to TLC (chloroform–methanol, 88 : 12). The sole significant radioactive component which was mobile in this solvent (R_f 0·60), accounted for 50 per cent of the total activity of the aqueous layer. The TLC mobility of this component and a mass spectrum (Fig. 4) of a total lyophilizate obtained similarly in a subsequent experiment were identical with those of an authentic sample of *N*-(2-chloroethyl)-4-hydroxyaniline (V).

In vivo *metabolism of aniline mustard:* (b) *bile*

The solution for injection contained aniline mustard (125 mg) together with the ^{3}H-derivative (equivalent to 0·015 ml of the benzene solution) in dimethyl sulphoxide (1·2 ml). Aliquots (0·45 ml) were injected i.p. into 2 rats. The bile ducts were cannulated and a total volume of 4·1 ml of bile was collected during 2·3 hr. Scintillation counting [cf. (a) above] gave $3{\cdot}07 \times 10^8$ dis/min for the recovered bile, compared with $7{\cdot}72 \times 10^8$ dis/min for the administered drug. A part (2 ml) of the bile was adjusted to *ca.* pH 2 with 0·1 N HCl (0·1 ml), and the solution was extracted with ethyl acetate (3 × 2 ml).

The aqueous layer contained 60 per cent and the organic phase 40 per cent of the total radioactivity in the bile. The dried ($MgSO_4$) organic phase was subjected to TLC as in (a) above. A single radioactive area coincident with a u.v.-absorbing band was present with an R_f value appropriate for the glucuronide (III). Methylation of this bile metabolite using the procedure described above gave a product with the same chromatographic properties and mass spectrum as the corresponding derivative from the serum.

(i) *Synthesis of* N-(2-*chloroethyl*)-4-*hydroxyaniline* (*V*). A solution of 4-aminophenol (0·25 g) in dimethyl sulphoxide (0·25 ml) and 1,2-dichloroethane (0·5 ml) was heated on a steam bath for 20 min. Two principal products resulted, of which the more mobile (TLC, ethyl acetate) gave a positive reaction with the Epstein reagent. After addition of water (25 ml) the reaction mixture was extracted with chloroform (25 ml). The dried ($MgSO_4$) extract was concentrated, and the concentrate was applied to a column (20 × 1·5 cm) of silicic acid (10 g) which was eluted with chloroform. The required product (30 mg R_f 0·60, chloroform–methanol, 88 : 12) appeared in fractions 8–12 (10 ml fractions). Crystallization from benzene gave V as colourless feathery needles (10 mg), m.p. (corr.) 103–104°. (Found: C, 55·5; H, 5·85; Cl, 21·05; N, 7·95%. $C_{18}H_{10}ClNO$ requires C, 55·95; H, 5·85; Cl, 20·65; N, 8·15%.)

(ii) *Synthesis of* N-(2-*chloroethyl*)-2-*hydroxyaniline* (*VI*). The above preparative conditions were applied to 2-aminophenol (1 g). The product (125 mg R_f 0·68 in chloroform–methanol, 88 : 12) was eluted from a column (23 × 2 cm) of silicic acid (35 g) with benzene (800 ml) after a forerun of 800 ml of this solvent. Crystallization of the product from benzene–petroleum ether (b.p. 30–40°, 1 : 4) gave VI as colourless plates (30 mg), m.p. (corr.) 82–84°. (Found: C, 55·75; H, 5·9; Cl, 20·5; N, 8·0.)

RESULTS AND DISCUSSION

When aniline mustard was incubated with the 9000 *g* supernatant in the presence of UDPGA and NADPH a product was obtained which appeared to be identical with the synthetic glucuronide (III) inasmuch as methylation of the metabolite gave a derivative with chromatographic properties and mass spectrum (Fig. 2) appropriate for the synthetic methyl ester (IV). In the mass spectra, ions containing chlorine each give rise to two peaks due to the presence of the pair of isotopes ^{35}Cl and ^{37}Cl. Differences between the relative intensities of ions in the high mass region, specifically *m/e* 423 (M^+) and 405 $(M\text{-}H_2O)^+$ and the accompanying changes in the relative intensities of *m/e* 374/376 $(M\text{-}CH_2Cl)^+$ and 356/358 $(405\text{-}CH_2Cl)^+$ are attributable to variations in the extent of thermal elimination of water. In these mass spectra the most abundant fragments namely *m/e* 233/235 and 184/186 were also the principal components in the mass spectrum of *p*-hydroxyaniline mustard (II). These fragments arise by cleavage of the acetal linkage to the aglycone, accompanied by capture by the latter of a proton from the glucuronate moiety. Despite the evidence for minor thermal decomposition processes, the very simple structural modification needed to convert the glucuronide (III) into a derivative (IV) amenable to structural determination by mass spectrometry is a vindication of the usefulness of the direct insertion technique, in conjunction with thin layer chromatography, for the identification of metabolites. The application of combined gas chromatography–mass spectrometry (GC–MS) to the identification of drug glucuronides has hitherto involved the protection, additionally, of the hydroxyl functions on the glucuronide moiety with the trimethylsilyl (TMS) group.[19] Even where direct insertion has been previously employed in mass spectral studies of glucuronic acid conjugates, these hydroxyl functions were first protected by methylation.[20]

Since the objective of the *in vitro* experiment was simply to demonstrate the feasibility of the conversion of aniline mustard (I) into the glucuronide (III) a very simple isolation procedure[21] was adopted which did not, however, lend itself to accurate quantitation. In the subsequent *in vivo* studies it was necessary to determine the extent of formation of the glucuronide, and the isolation procedures were appropriately modified. Since a tumour would receive the drug and its metabolites via the blood supply, the additional quantitation and identification of other potentially alkylating, and therefore cytotoxic species in the serum is important. An additional and important step, utilizing a procedure previously applied to the urinary metabolites of certain alkaloids[22] was the separation of the serum metabolites into two fractions by passage of the serum through a column of Amberlite XAD-2 resin. An alkylating metabolite, additional to the glucuronide (III) was identified in the methanol eluate. Background contamination, probably biologically-derived, prevented a structural evaluation by mass spectrometry, of other metabolites of aniline mustard present in the serum.

Of the total radioactivity present in the serum, which represented only *ca.* 1 per cent of the total dose administered, *ca.* 21 per cent was present as the postulated glucuronide (III). The other identified metabolite, *N*-(2-chloroethyl)-4-hydroxyaniline (V) represented *ca.* 12 per cent of the serum metabolites. Metabolic elimination of a 2-chloroethyl group from an *N,N*-di-(2-chloroethyl)amine has also been reported for cyclophosphamide.[23]

In contrast to the low percentage of radioactivity recovered from the serum, the bile contained *ca.* 40 per cent of the administered radioactivity. Of this, 40 per cent

was extractable at pH 2 by ethyl acetate, and the sole significant component in the extract was the glucuronide (III). Thus the glucuronide contained in the bile represented *ca.* 16 per cent of the administered dose.

The postulate that the glucuronide (III) would be a major metabolite of aniline mustard was therefore validated. Although the proportion of administered drug (measured as drug together with metabolites) which is present in the blood supply was small, a substantial proportion is present as the glucuronide. The larger amount excreted in the bile represented a reservoir whereby a continuous supply of the glucuronide could be made available via enterohepatic circulation.

The previously described chemical trapping technique[1] in which a compound containing a reactive *N,N*-di-(2-chloroethyl)amino function was converted into the corresponding thiazan by reaction with radioactively-labelled sodium sulphide has not so far been applied to the appropriate metabolites of aniline mustard. The ready availability of highly radioactive aniline mustard-^{3}H, coupled with the relative stability to hydrolysis of the alkylating functions in the glucuronide (III) obviated the need for this technique in the studies with experimental animals. However, the logical extension of this work, namely the investigation of the metabolism of aniline mustard in man, will involve longer time scales in the isolation procedures, as well as an ethical problem associated with the use of a radioisotopically-labelled drug. Hence, the chemical trapping technique, in which serum containing the non-radioactive drug and its metabolites would be treated with sodium sulphide-^{35}S, may well be important in any clinical extension of the present studies. Thiazans derived from *p*-hydroxyaniline mustard and its glucuronide are readily obtainable.[24]

It has been tacitly assumed so far that in the metabolism of aniline mustard the principal hydroxylation and conjugation processes have involved the position *para* to the alkylating substituent. This assumption is in part justified by the relative unimportance of *ortho*-hydroxylation in the metabolism of aniline by the male rat, the ratio of *o*- to *p*-hydroxyaniline formed being 1 : 6.[25] The *ortho*-isomer corresponding to the glucuronide (III) is not known, and is unlikely to prove readily accessible by chemical synthesis. Hence, any attempt to determine the presence and assess the percentage of the *ortho* glucuronide is prevented by the lack of information concerning its chromatographic properties and the chromatographic and mass spectral parameters of its methyl ester. In contrast, both *N*(2-chloroethyl)-4-hydroxyaniline (V) and its 2-hydroxy-isomer (VI) proved readily accessible, albeit in poor yield, from a single stage reaction of the appropriate aminophenol with 1,2-dichloroethane. The R_f-values (see Materials and Methods) of the *ortho*- and the *para*-isomers were sufficiently different to allow ready detection of the *o*-isomer as a significant product of metabolism; only the *p*-isomer was detected as a metabolite. A mass spectrum of a lyophilizate of the solution containing the appropriate serum metabolite afforded conclusive evidence for the absence of the *o*-isomer. Thus a comparison (Fig. 4) of the mass spectrum with those of the synthetic isomer V and VI clearly shows that the spectrum of the metabolite resembles in detail that of the *p*-isomer (V) and differs from that of the *o*-isomer (VI). More specifically, the intensity of the fragment of *m/e* 95 was much greater (10 per cent) in the case of the *o*-isomer than it was for the *p*-isomer and the metabolite (both 1 per cent). This ion was selected for this comparison because its formation from the primary fragment *m/e* 122 $(M\text{-}CH_2Cl)^+$ was supported by the presence of the appropriate metastable peak at *m/e* 74·0, and because the contrast

between its intensity in the spectra of the two isomers was maximal. The failure to detect the *ortho* derivative (VI) among the metabolites of aniline mustard does not exclude *ortho* hydroxylation of aniline mustard as a metabolic process. Thus, assuming that the metabolic route to the *p*-isomer (V) is via the initial formation of *p*-hydroxyaniline mustard, and not of *N*-(2-chloroethyl)aniline, it may be that any similarly-formed *o*-hydroxyaniline mustard will not undergo subsequent elimination of a 2-chloroethyl substituent. Conversely, there may be further metabolic transformations of the *ortho* derivative (VI) which do not occur for the *p*-isomer (V).

Although the absence of significant *ortho*-hydroxylation was indicated, although not conclusively established, the occurrence of such processes would not invalidate the basic hypothesis that activation of aniline mustard could be mediated by hydroxylation followed by a potentially reversible formation of a glucuronide since *o*-hydroxyaniline mustard is itself a potent cytotoxic agent. Its LD_{50} in Wistar rats is some 6-fold less than that of aniline mustard, although some 4-fold greater than that of *p*-hydroxyaniline mustard.[26] Thus a small extent of *ortho*-hydroxylation and conjugation should not detract significantly from the extent of the *in vivo* activation of aniline mustard by the mechanisms outlined herein.

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