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THE METABOLISM OF CHLORAMBUCIL

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Abstract—After injection of chlorambucil into rats, metabolites have been isolated from blood and identified by g.l.c.–mass spectrometry as (*E*)-4-[4*N*,*N*-bis (2-chloroethyl)aminophenyl] 3-butenoic acid (3,4-dehydrochlorambucil) and 2-[4-*N*,*N*-bis (2-chloroethyl)aminophenyl] acetic acid (phenyl acetic acid mustard). Analysis of urine 24 hr after administration indicates the absence of chlorambucil, 3,4-dehydrochlorambucil and phenyl acetic acid mustard but the presence of 2-[4-*N*(2-chloroethyl) aminophenyl]acetic acid as the major metabolite. All metabolites isolated have been independently synthesized, allowing confirmation of structures proposed by g.l.c.–mass spectrometry. It has been shown that 3,4-dehydrochlorambucil is an intermediate in the β-oxidation of chlorambucil by injecting an authentic sample into rats and observing the production of phenyl acetic acid mustard in the blood. The contribution that metabolism makes to the anti-tumor activity of chlorambucil is discussed.

Metabolism studies with the alkylating agents cyclophosphamide [1], aniline mustard [2] and the azo mustard, 2′-carboxy-4-bis(2-chloroethyl) amino-2-methyl azobenzene [3] have demonstrated the important contribution that metabolism can make to the activity of the anti-tumour agents. In these studies workers have been successful in isolating metabolites after *in vivo* administration and *in vitro* incubations. At present, chlorambucil is used clinically in the treatment of chronic lymphocytic leukaemia and ovarian carcinoma. Specific interest in the metabolism of chlorambucil was aroused by reports [4, 5] supporting the hypothesis that chlorambucil was transformed metabolically into a more active compound. One group of researchers [4] have experienced an enhancement in the *in vivo* anti-tumour effect of chlorambucil in animals pretreated with phenobarbitone and have postulated that metabolic activation may make a contribution to the anti-tumour activity of chlorambucil. Similarly, a second group of workers [5] have observed an accentuation of chlorambucil cytotoxity *in vitro* by phenobarbitone and postulated a role for metabolic activation. Chlorambucil differs from cyclophosphamide in that it acts directly and does not require metabolic activation to be cytotoxic, but the possibility of metabolism to a more cytotoxic compound must be considered. Studies by Godeneche *et al.* [6] employing radiolabelled chlorambucil have demonstrated that β-oxidation of the butyric acid side chain occurs during the *in vivo* metabolism of chlorambucil; however, no alkylating metabolites were isolated. A preliminary account of certain aspects of work presented in this publication has appeared [7] and describes the isolation of β-oxidation products of chlorambucil from rat blood. Mitoma *et al.* [8] have presented a study of urinary metabolites of chlorambucil and identified ten metabolites by mass spectrometry. Most metabolites had undergone oxidation of the butyric acid side chain. The present report comprises a complete description of the results obtained from a study of the metabolism of chlorambucil in male Sprague–Dawley rats (Fig. 1).

MATERIALS AND METHODS

Radiolabelled chlorambucil

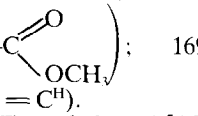
[^{3}H]Chlorambucil (3Ci/mmole) was prepared by tritium exchange of 4[4′-bis(2-chloroethyl)amino-2′-iodophenyl] butyric acid and purified as described previously [9]. [^{3}H]Chlorambucil was stored in benzene (10 ml) as a 2.13 mM solution. Ring ^{14}C-labelled chlorambucil (Radiochemical Centre, Amersham, U.K.: 1.9 mCi/mmole) was stored in benzene (5 ml) as a 9.38 mM solution. The purity of [^{3}H]chlorambucil and [^{14}C]chlorambucil was checked prior to administration by t.l.c. plate-scanning and autoradiography, respectively. Similarly, residual quantities of injection solutions were investigated by this methodology and demonstrated to be free of hydrolysis products. Organic solvents were concentrated by using a Büchi rotary evaporator under reduced pressure.

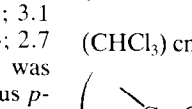
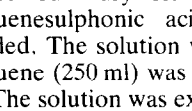
Synthesis of metabolites

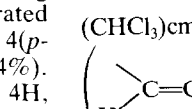
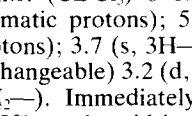
2-[4-N,N-*bis(2-chloroethyl)aminophenyl*] *acetic acid (phenyl acetic acid mustard).* Phenyl acetic acid mustard was prepared by the method of Wall *et al.* [10] and 4-[4′-*N*-(2-chloroethyl)-*N*-(2-hydroxyethyl)]aminophenyl butyric acid was obtained by the method of Jarman *et al.* [11].

(E)-4-[4N,N-*bis(2-chloroethyl)aminophenyl*] 3-*butenoic acid (3,4-dehydrochlorambucil) (3).* (E)-Methyl 4-(paracetylaminophenyl)-3-butenoate.β-(4-acetylaminobenzoyl) propionic acid (53.5 g, 0.23 mole), prepared as previously described [12], was stirred with water (250 ml) containing sodium hydroxide (9.0 g, 0.25 mole) and the pH of the solution adjusted to 13 with 2 M sodium hydroxide. To

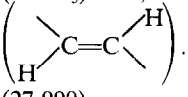
this solution sodium borohydride (5.35 g, 0.14 mole) was added and stirred for 2 hr. The solution was chilled to 0° and cold concentrated hydrochloric acid was added to bring the pH to 4.0, causing precipitation of a white solid which was dried over P_2O_5 to yield the hydroxy acid (7, 43.1 g). To a solution of the hydroxy acid (43.1 g) in methanol (650 ml) was added *p*-toluene-sulphonic acid (3.8 g, 0.02 mole) followed by refluxing for 2 hr. Evaporation of the solvent gave a gum which was dissolved in ethyl acetate (600 ml). This solution was shaken with saturated sodium bicarbonate solution (300 ml) and then dried (Na_2SO_4) before evaporation to give a gum (40.0 g). T.l.c. (ethyl acetate) showed a principal product (R_f 0.25) plus an impurity (R_f 0.5) adjudged to be a deacetylated product. This impurity was removed by dissolving the gum in methanol (50 ml) and reacting with acetic anhydride (50 ml). After 1 hr at room temperature the solution was evaporated to yield a brown solid (40.0 g) adjudged, on the basis of p.m.r., to be the methoxyester (8). P.m.r. ($CDCl_3$): δ 8.1 (bs, 1H, NH exchangeable); 7–7.5 (AB q, 4H, aromatic protons); 4.1 (5, J = 6 Hz, 1H, —CH $(OCH_3)CH_2$—); 3.6 (s, 3H, $—CO_2CH_3$); 3.1 (s, 3H, OCH_3), 1.8–2.6 (m, 4H, $—CH_2CH_2—$); 2.7 (s, 3H, $—NHCOCH_3$). The methoxyester, 8, was dissolved in dry toluene (500 ml) and anhydrous *p*-toluenesulphonic acid (1.0 g, 0.0058 mole) was added. The solution was raised to the boil and the toluene (250 ml) was distilled off over 40 min.

The solution was extracted with saturated sodium bicarbonate (200 ml) and the toluene layer dried (Na_2SO_4) and concentrated to give a brown solid (38.0 g). Recrystallization from ethyl acetate produced methyl 4-(*p*-acetylaminophenyl)-3-butenoate (9), (28.0 g, 53 per cent overall yield from 6) m.p. 105–106°. Anal. calcd. for $C_{13}H_{15}NO_3$: C, 67.0; H, 6.43; N, 6.0. Found: C, 66.99; H, 6.49; N, 5.99. P.m.r. ($CHCl_3$): δ 8.3 (s, 1H exchangeable NH); 7–7.5 (ABq, J_{A-B} = 8.5 Hz, 4H, aromatic protons); 6–6.6 (m, ABX, J_{A-B} = 8.5 Hz, 4H, aromatic protons); 6–6.6 (m, ABX, J_{A-B} = 15 Hz, J_{B-X} = 6.1 Hz, $J_{A-X} < 1$, 2H, $—CH_A=CH_B—CH_{2X}$); 3.7 (s, 3H, $—CO_2CH_3$), 3.24 (d, J_{B-X} = 6.1 Hz, 2H, $—CH_A=CH_B—CH_{2X}$); 2.1 (s, 3H, $—NCOCH_3$). I.r. ν max ($CH\ Cl_3$)cm^{-1}: 3450 (N–H); 1740  ; 1695 (—$NHCOCH_3$); 970 ($_HC=C^H$).

(*E*)-*methyl* 4-[4-N,N-*bis* (2-*hydroxyethyl*)-*aminophenyl*] 3-*butenoate* (10). Methyl 4-(*p*-acetylaminophenyl)-3-butenoate (4.5 g, 0.19 mole) was refluxed under nitrogen for 3 hr with 0.75 M sulphuric acid in methanol (63 ml). The solution was concentrated to 20 ml before equilibrating between dichloromethane (50 ml) and saturated sodium sulphate and evaporated to give methyl 4(*p*-aminophenyl)3-butenoate as a red oil (3.5 g, 94%). P.m.r. ($CDCl_3$) δ 6.5–7.3 (ABq,J_{A-B} = 9 Hz, 4H, aromatic protons); 5.7–6.3 (m,ABX, 2H, olefinic protons); 3.7 (s, 3H—CO_2CH_3); 3.8 (s, 2H —NH_2, exchangeable) 3.2 (d, J = 6 Hz, 2H, —CH = CH—CH_2—). Immediately the amine was mixed with 12.5% acetic acid in water (3.5 ml) under nitrogen and the mixture was frozen by immersing in dry ice–acetone at −20°, ethylene oxide (5.5 ml, 0.11 mole) was added and the reaction vessel sealed under nitrogen. After 15 hr at room temperature the reaction mixture had become one phase and the product was extracted into dichloromethane (100 ml). The organic layer was washed with sodium bicarbonate (100 ml), separated, dried (Na_2SO_4), and evaporated to yield a brown oil (5.0 g). Purification on silica gel 60 (250 g), eluting with 1 litre ethyl acetate–petroleum ether (b.p. 60–80) (4:1), then ethyl acetate allowed isolation of methyl 4-[4-*N*,*N*-bis (2-hydroxyethyl)aminophenyl]-3-butenoate, 10, as a gum (3.5 g, 65%) which solidified on standing. Recrystallization from petroleum ether (b.p. 60–80) and ethyl acetate (1 : 1) gave pure material, m.p. 58–60°. Anal. calcd. for $C_{15}H_{21}NO_4$: C, 64.5; H, 7.5; N, 5.0. Found: C, 64.29; H, 7.68; N, 4.97. P.m.r. ($CDCl_3$): δ 6.5–7.3 (ABq, J_{A-B} = 9 Hz, 4H, aromatic protons); 5.8–6.5 (m, 2H, olefinic protons); 4 (s, 2H, exchangeable $—CH_2CH_2OH$); 3.7 (s, 3H, $—CO_2CH_3$); 3.7–3.8 (m, 4H, —N—CH_2—); 3.5–3.6 (m, 4H —O—CH_2—CH_2—OH); 3.15 (d, J = 6.3, 2H, —CH=CH—CH_2—). Ir ν max ($CHCl_3$) cm^{-1}: 3400 (OH); 1740  ; 970  .

(*E*)-*methyl* 4-[4-N,N-*bis* (2-*chlorethyl*)-*aminophenyl*] 3-*butenoate*. Methyl 4-[4-*N*,*N*-bis(2-hydroxyethyl)aminophenyl]3-butenoate (2.5 g, 0.00896 mole) was dissolved in dry pyridine (25 ml) and cooled to −20° before slowly adding methane sulphonyl chloride (1.625 ml, 0.02 mole). The reaction solution was allowed to come to room temperature and stirred for 3 hr before adding lithium chloride (5.0 g, 0.12 mole) and heating the solution at 75° for 35 min with stirring. The product was isolated by pouring into ethyl acetate (500 ml) and water (250 ml). HCl (6N) was added whilst stirring the mixture until the pH was 4.0 and the organic layer separated. After washing with sodium bicarbonate the ethyl acetate layer was dried (Na_2SO_4) and evaporated to give a brown oil (1.9 g), and this was purified by prep. h.p.l.c. (Waters Prep. 500) using hexane, ethyl acetate (20 : 4) to yield methyl 4-[4-*N*,*N*-bis(2-chloroethyl)aminophenyl] 3-butenoate (11) as an oil (1.3 g, 46%). Anal. calcd. for $C_{15}H_{19}Cl_2NO_2$: C, 56.9; H, 6.0; N, 4.5. Found: C, 56.5; H, 6.10; N, 4.3. P.m.r. ($CDCl_3$): δ 6.5–7 (ABq J_{A-B} = 9Hz, 4H, aromatic protons); 5.9–6.4 (m, ABX, 2H, $CH_A=CH_B—CH_{2X}$—); 3.7 (s, 3H, CO_2CH_3); 3.5–3.7 (m, 8H, —N—CH_2CH_2Cl): 3.2 d, J = 6.3Hz, 2H, —CH=CH—CH_2—). I.r. ν max ($CHCl_3$)cm^{-1}: 1740  ; 965  .

(*E*)-4-[4-N,N-*bis*(2-*chloroethyl*)*aminophenyl*] 3-*butenoic acid*. Methyl 4- [4-*N*,*N*-bis(2 chloroethyl)aminophenyl] 3-butenoate (2.0 g, 0.0063 mole) was reacted with potassium hydroxide (1.62 g, 0.289 mole) in analar acetone–water medium

(1 : 1) containing lithium chloride (2.12 g, 0.05 mole) under nitrogen for 1 hr at room temperature. The acetone was evaporated from the solution under reduced pressure and the aqueous residue was adjusted to pH 4 with 1N HCl before extracting the product into dichloromethane (200 ml). The organic layer was dried (Na_2SO_4) and evaporated to give a red oil (1.8 g) which was chromatographed on silica gel H (25.0 g) eluting with 10% ethyl acetate in petroleum ether (b.p. 60–80) under a nitrogen pressure of 5 lb/in^2. The eluates were evaporated to yield 3,4 dehydrochlorambucil as an oil (1.5 g, 79%), which solidified on standing. Recrystallization was carried out from ethyl acetate–petroleum ether 60–80 (1 : 1) and white crystals were obtained, m.p. 77–78°. Anal. calcd. for $C_{14}H_{17}Cl_2NO_2$: C, 55.6; H, 5.6; N, 4.6, Cl, 23.5. Found: C, 55.7; H, 5.7; N, 4.5; Cl, 23. P.m.r. ($CDCl_3$): δ10.5 (s, 1H, exchangeable $CO_2\underline{H}$); 6.6–7.3 (AB_q, J_{A-B} = 9HZ, 4H, aromatic protons); 5.9–6.6 (m, ABX, J_{A-B} = 15.8Hz, J_{B-X} = 6.4HZ, J_{A-X} = 0, 2H, $—\underline{CH}_A=\underline{CH}_B—CH_{2X}—$); 3.5–3.8 (m, 8H, $—N—\underline{CH_2}\,\underline{CH_2}—Cl$); 3.25 (d, J_{B-X} = 6.4Hz, 2H, $—CH_A=CH_B—\underline{CH_2}_X$). I.r. ν max ($CHCl_3$)cm^{-1}; 3500 (OH); 1720 (C=O); 960  . U.v. λ max. (CH_3OH)nm: 290 (27,990).

2[4-N(2-*chloroethyl*)*aminophenyl*] *acetic acid*. Ethyl *p*-aminophenylacetate (5.0 g, 0.028 mole) was dissolved in dry toluene (25 ml) and anhydrous potassium carbonate (7.8 g, 0.057 mole) was added. Bromochloroethane (4.0 g; 0.028 mole) was added and the mixture was refluxed for 3 hr, whereupon another quantity of bromochloroethane (4.0 g, 0.028 mole) was added and refluxing under nitrogen was continued for 12 hr. The carbonate was filtered off and evaporation of solvent gave a brown oil (4.45 g), which was purified by chromatography (150 g, Kieselgel 60) eluting with petroleum ether (b.p. 60–80)–ethyl acetate (80–20) to yield ethyl 2-[4-*N*(2-chloroethyl)aminophenyl] acetate (13) as an oil (1.9 g, 32%). This was dissolved in concentrated hydrochloric acid (18 ml) in a pressure bottle and heated for 5 hr at 110° before pouring into salt water solution. (400 ml, sat.) at pH 8.5. The solution was extracted with ethyl acetate (500 ml) and the aqueous layer was adjusted to pH 4 using cold concentrated hydrochloric acid to give a white precipitate which was filtered and dried over P_2O_5 (1.2 g, 71%). Recrystallization from acetone gave the acid (5), m.p. 160–161°. Anal. calcd. for $C_{10}H_{12}ClNO_2$; C, 56.2; H, 5.6; N, 6.55. Found; C, 56.3; H, 5.6; N, 6.45. P.m.r. ((CD_3COCD_3): δ 6.5–7.1 (m, 4H, aromatic protons); 3.4–3.7 (m, 8H, $\underline{NH}—\underline{CH_2}—\underline{CH_2}—Cl$, $—\underline{CH_2}CO_2\underline{H}$). I.r. ν max (nujol)cm^{-1} 3200 (N—H); 1695 (—C=O).

OH

Incubation of chlorambucil with rat liver microsomes

Male Sprague–Dawley rats (150–200 g) were treated i.p. with 100 mg/kg sodium phenobarbitone on three consecutive days and killed by cervical dislocation 24 hr after the final injection. The microsomal fraction of the liver was then prepared by homogenization and differential centrifugation by the method of Shenkman [13] and the cytochrome P-450 content assayed by the procedure of Sato [14]. Chlorambucil was used as the sodium salt and the complete system contained, in a 3 ml volume, 0.1M Tris–HCl buffer (pH 7.4), NADP 1 μmole, 3 μmoles $MgCl_2$, 25 μmoles glucose 6-phosphate, 1.2 units glucose-6-phosphate dehydrogenase, 7.8 μmoles chlorambucil + tritiated chlorambucil (25 μCi) and 2 mg microsomal protein from phenobarbital induced animals. Samples were incubated for 20 min at 37° and the reaction terminated by freezing. Drug derived materials were extracted with ethyl acetate (8 vol.). The dried extracts (Na_2SO_4) were concentrated under reduced pressure and analysed by t.l.c. using chloroform–methanol (25 : 3).

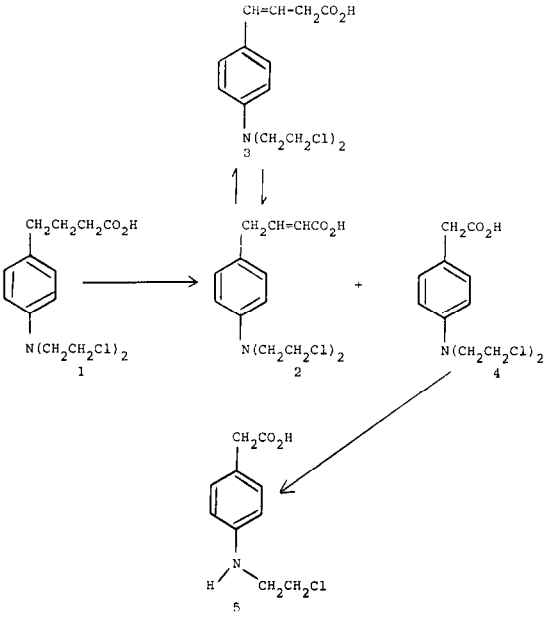


Fig. 1. Scheme for metabolism of chlorambucil.

In vivo *Metabolism of chlorambucil*

Blood 1 hr. The sodium salt of chlorambucil (16 mg) prepared as described by Ross [15] was dissolved in phosphate buffered saline (10 ml, pH 8). An aliquot of the [^{3}H]chlorambucil benzene stock solution (1 ml ≡ 6.33 mCi) was concentrated and the residue redissolved in the non-radioactive chlorambucil solution producing an injection solution of tritiated chlorambucil (1.6 mg/ml, 0.4 mCi/mg). Eleven male Sprague–Dawley rats (140–180 g) were treated i.p. with the chlorambucil injection solution (8 mg/kg). After 1 hr blood (total 65 ml 7.3×10^8 d.p.m.) was obtained from the animals as previously described [7].

(a) Composition of organic phase. The ethyl acetate extract contained 4.58×10^8 d.p.m. (63 per cent of total blood radioactivity) and prep. t.l.c. allowed purification and isolation of metabolites (R_f 0.3; 3.77×10^8 d.p.m.), which were methylated with diazomethane [7]. Unmetabolized chlorambucil (R_f, 0.53; 6.13×10^7 d.p.m.) was also recovered from the plate. The methylated metabolites were analysed by g.l.c.–mass spectrometry (Figs. 2 and 3).

(b) Composition of aqueous phase. The aqueous

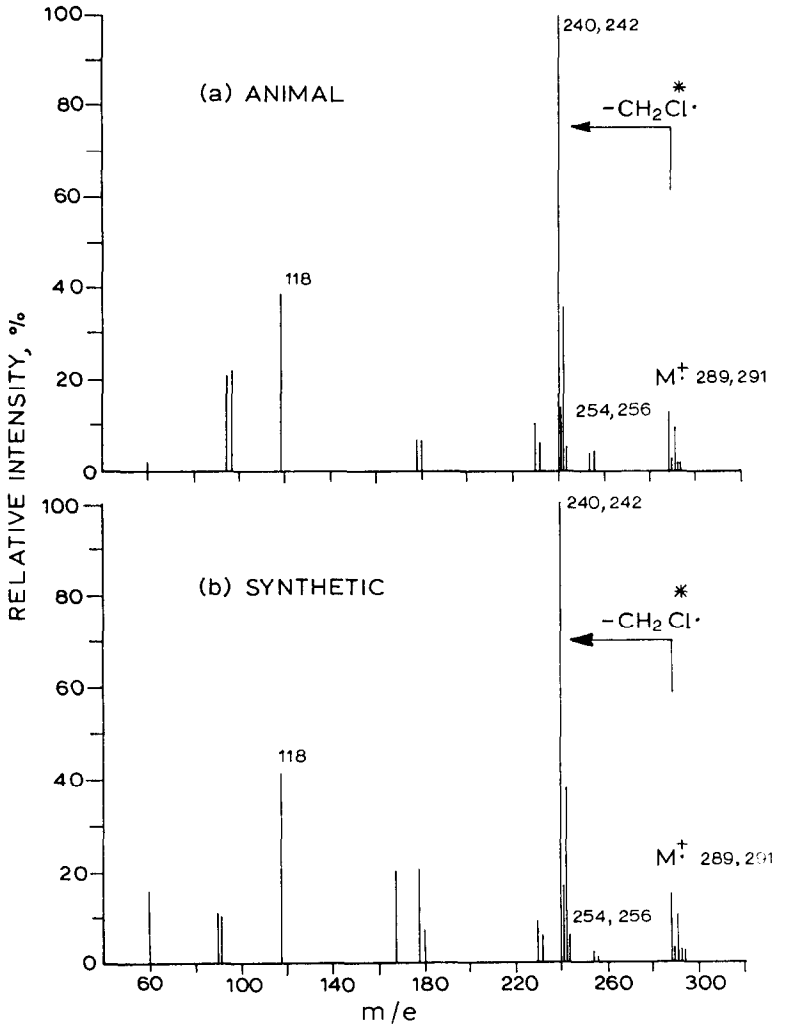


Fig. 2. Mass spectra of: methylated metabolite with g.l.c. retention time 7 min from methylated metabolite mixture 1 hr after administration (panel a); and synthetic methyl 2-[4-*N*,*N* bis(2-chloroethyl)aminophenyl] acetate (panel b).

phase (63 ml, 2.7×10^8 d.p.m.) was diluted with an equal volume of phosphate buffered saline (63 ml). Four volumes ethanol (500 ml) were added and the mixture was homogenized with a Silverson high speed mixer at 4° for 70 min. The precipitated protein was removed by centrifugation and the ethanol : water supernatant (555 ml, 5×10^7 d.p.m.) decanted, prior to concentration by rotary evaporation at 0–10° and freeze drying. The lyophilysate was redissolved in water (5 ml) and applied to an Amberlite XAD-II column (19 × 1.5 cm). Elution was carried out with water (100 ml) and then methanol (90 ml). The water fraction contained 2.1×10^6 d.p.m. and the methanol fraction contained 2.8×10^7 d.p.m. The methanol fraction was investigated by t.l.c. (solvent system A); however, the radioactive material was immobile in the system. A second aliquot of the methanol eluate was treated with diazomethane and the product examined by t.l.c. (solvent system B), but again only immobile material was observed.

Blood 4 hr. Eight male Sprague–Dawley rats (240–265 g) were injected i.p. with [^{3}H]chlorambucil sodium salt at 8 mg/kg body wt (injection solution = 1.6 mg/ml, 1.01 mCi/mg). The total quantity injected was 16.2 mg (1.43×10^{10} d.p.m.). Four hours later blood was removed and combined to give a total of 60 ml (1.47×10^8 d.p.m., 1.02 per cent injected material). As described previously [7], an ethyl acetate extract was prepared and contained 7.48×10^7 d.p.m.

Treatment of the organic phase. The ethyl acetate extract was purified as described for 1 hr blood extract. T.l.c. indicated one radioactive zone (R_f 0.42) and the metabolite material (2.68×10^7 d.p.m.) was isolated, methylated and purified by t.l.c. as previously described [7]. The methylated metabolite material (7.2×10^6 d.p.m.) was analysed by g.l.c.–mass spectrometry.

Treatment of aqueous phase. The aqueous phase (60 ml) was processed as described for the corresponding 1 hr aqueous phase. The aqueous eluate from the XAD-II column comprised 125 ml, 2.29×10^6 d.p.m. and the methanol eluate 100 ml, 9.97×10^6 d.p.m. Extensive t.l.c. analysis of the methanol eluate gave results analagous to those obtained with 1 hr material.

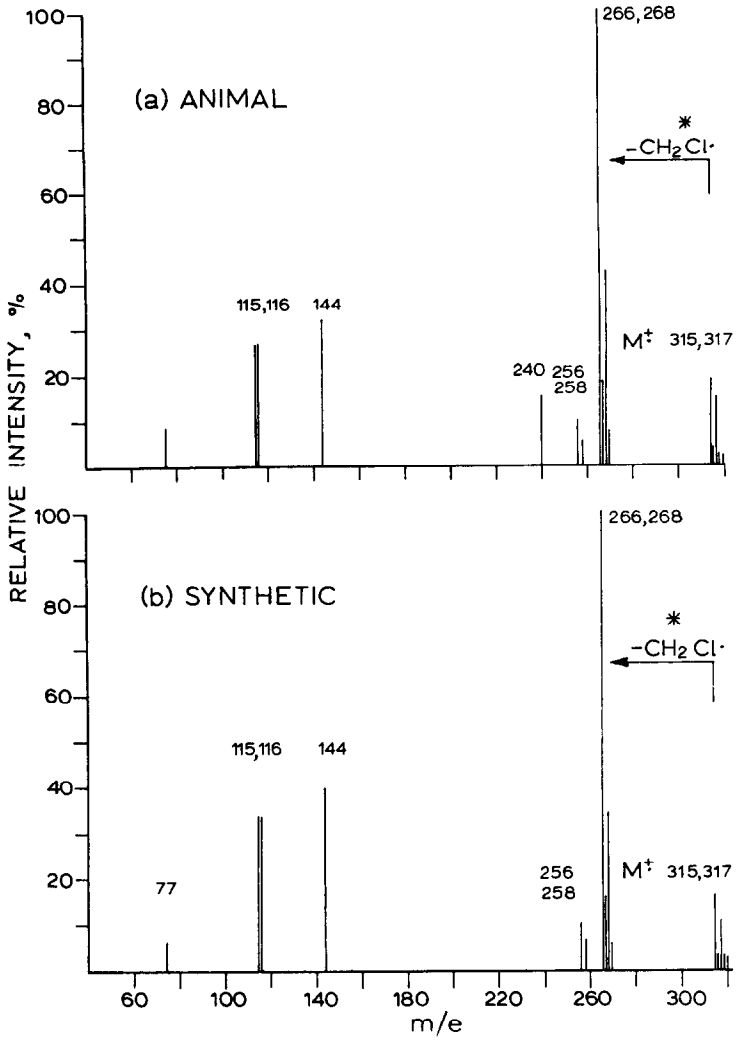


Fig. 3. Mass spectra of: methylated metabolite with g.l.c. retention time 19 min from methylated metabolite mixture 1 hr after administration (panel a); and synthetic methyl ester of 3,4-dehydrochlorambucil (panel b).

Urine 24 hr. Three male Sprague–Dawley rats (200 g) in a suitable cage were injected i.p. with [^{14}C]chlorambucil sodium salt at 10 mg/kg body wt (injection solution = 2 mg/ml, 2.72×10^6 d.p.m./mg). Animals were given access to food and water and 24 hr after injection the total urine (15 ml, 4.93×10^6 d.p.m. = 30.2 per cent injected dose) was removed from the cooled flask (0–10°). After saturation of the urine with sodium chloride extraction was carried out with two portions of ethyl acetate (4 vol.). The combined extracts (120 ml, 3.65×10^6 d.p.m.) were dried over sodium sulphate, concentrated and analysed by t.l.c. (solvent system A). Radiochromatogram scanning and overnight autoradiography exposure of the plate indicated one principal u.v. active metabolite of coincidental R_f (0.5) with the synthetic 2-[4-*N*(2-chloroethyl)-aminophenyl] acetic acid. The radioactive metabolite zone was segregated and the methanol eluate of the silica gel contained 1.11×10^6 d.p.m. (30.5 per cent total urine extract). The metabolite was converted to the methyl ester and purified by t.l.c. (solvent system B) prior to examination by mass spectrometry (Fig. 4).

In vivo *metabolism of* 3,4 *dehydrochlorambucil*

The male Sprague–Dawley rats (200–250 g) were injected i.p. with 8 mg/kg of 3,4-dehydrochlorambucil dissolved in ethanol (1 vol.) and diluted with phosphate-buffered propylene glycol (9 vol.; 0.12 M K_2HPO_4 in 45% v/v propylene–glycol). After 1 hr the blood was removed as described previously and plasma (27 ml) obtained by centrifugation at 500 *g* for 10 min. The separated blood cells and plasma were stored at −20° prior to analytical investigation. The plasma was adjusted to pH 4 and extracted with ethyl acetate (4 vol.). The extract was concentrated and the volume adjusted to 5 ml. The extract was investigated qualitatively for the presence of chlorambucil, dehydrochlorambucil and phenyl acetic mustard at 20, 40 and 60 min after injection by a recently developed h.p.l.c. analysis [16].

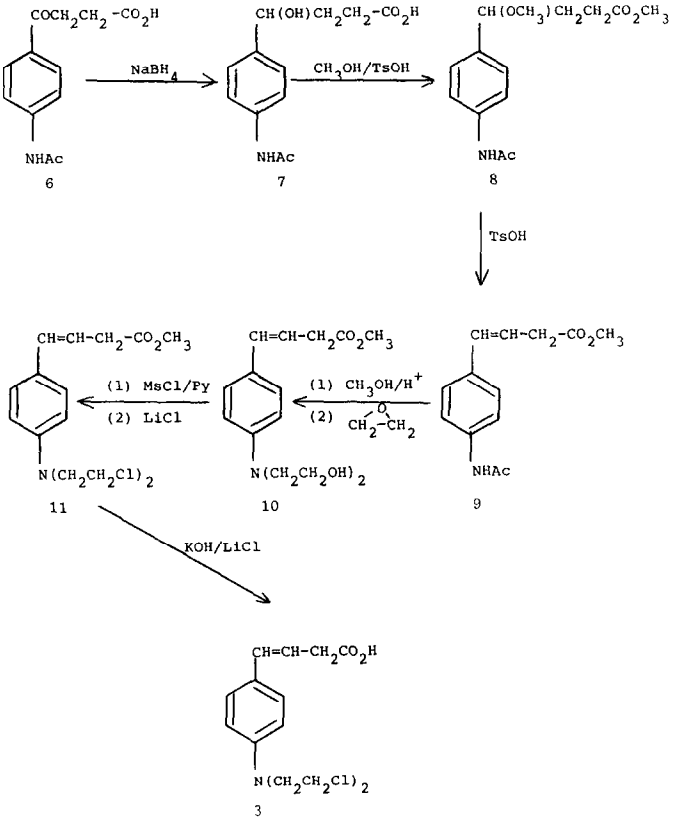


Fig. 5. Synthesis of (E)-4-[4-*N*,*N*-bis(2-chloroethyl)aminophenyl]3-butenoic acid.

290 nm were due to phenyl acetic acid mustard (λ max 264) and dehydrochlorambucil, respectively. On the basis of molecular structure, 2,3 dehydrochlorambucil (2) would be expected to have a similar λ max in the u.v. to phenyl acetic acid mustard and chlorambucil, but 3,4 dehydrochlorambucil (3) has a double bond conjugated with the *N*-bis 2-(chloroethyl)amino group through the benzene ring and would be expected to have a u.v. absorbance at longer wavelength. Independent synthesis of 3,4-dehydrochlorambucil (Fig. 5) demonstrated that the chromophore of the synthetic compound (290 nm) was coincidental with the longer wavelength absorbance of the metabolite mixture. G.l.c.–mass spectrometry confirmed that the metabolite was indistinguishable from the synthetic compound, whilst additional evidence was provided by cochromatography on h.p.l.c.

Comparison of blood metabolites at 1 and 4 hr is of interest since it allows proposals to be made regarding the mechanism of formation of 3,4-dehydro isomer. At 4 hr the 3,4-dehydro isomer was absent, but a minute quantity of another dehydro isomer (also present at 1 hr) persisted. It is postulated that this metabolite is the 2,3 isomer which is formed directly by dehydrogenation. The 2,3 isomer is processed further by the β-oxidation cycle and results in the formation of the phenyl acetic mustard but it is envisaged that a competing isomerization of the double bond occurs to form the 3,4-dehydro isomer. On the basis of the recorded chemical stability of 4-phenyl but −2,3-eneoic acid under neutral conditions [18] and the known action of the isomerase, crotonase [19], it is envisaged that this isomerization is enzyme-catalysed. The metabolic generation of the phenyl acetic acid mustard from 3,4-dehydro chlorambucil indicates in an unambiguous fashion that 3,4-dehydrochlorambucil is an intermediate towards the production of phenyl acetic acid mustard (Fig. 1).

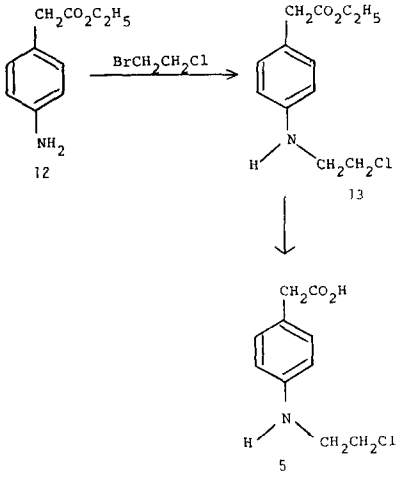


Fig. 6. Synthesis of 2-[4-*N*(2-chloroethyl)aminophenyl] acetic acid.

Although the dechlorethylation of chlorambucil was not observed *in vitro*, the isolation of 2[4-*N*-(2-chloroethyl)aminophenyl]acetic acid mustard as the major urinary metabolite (confirmed by independent synthesis; Fig. 6) demonstrates that *in vivo* the phenyl acetic acid mustard is subjected to biological hydroxylation. It is postulated that in an analogous fashion to 4-methyl-cyclophosphamide [20], loss of chloroacetaldehyde follows hydroxylation and the dechlorethylated metabolite results. No attempt has been made to identify the large number of minor metabolites. The observation of 2-[4-*N*(2-chloroethyl)aminophenyl]acetic acid mustard as a major metabolite was consistent with the work of Mitoma [8] who has identified this compound in rat urine by g.l.c.–mass spec.

The conversion of chlorambucil to phenyl acetic acid mustard is regarded as production of a compound which is more polar and water soluble but of similar *in vivo* activity to chlorambucil (Table 1), when tested against the Walker tumour [21]. Not surprisingly, the metabolic intermediate 3,4 dehydrochlorambucil, when tested *in vivo*, had similar activity to chlorambucil. The dechlorethylation of phenyl acetic acid mustard is a process producing a mono 2-chloroethylamine derivative which is inactive as an antitumour agent and considerably reduced in toxicity. Previously [22], monofunctional alkylating agents were often found to be as toxic as the corresponding difunctional analogue without possessing their antitumor action. Therefore, comparison of the biological activity of 2-[4-*N*(2-chloroethyl)aminophenyl] acetic acid and 2-[4-*N*,*N*-bis (2-chloroethyl) aminophenyl] acetic acid provides an example where the reduction in antitumour activity is accompanied by a corresponding reduction in toxicity. The *in vitro* testing of the synthesized metabolites is relevant since it will provide information about the intrinsic cytotoxicity of each compound and presently the activity of these metabolites is under investigation in a human stem cell assay. (D. S. Alberts and A. McLean, unpublished results).

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