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THE METABOLISM OF CHLORAMBUCIL

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Chlorambucil[1], the widely used anti-tumour agent, is under study with a view to investigating the influence of metabolism on the activity of the drug. Recently[2] it has been established that carbon dioxide is a metabolite of chlorambucil and that β-oxidation of the butyric acid is a process which operates at some stage in the metabolism of chlorambucil.

The metabolism of chlorambucil in vivo was investigated by examining the blood of drug-treated animals. Eleven male Sprague-Dawley rats (140-180g) were treated i.p. with ^{3}H chlorambucil (sodium salt[3]) at dose (8mg + 3.17m Ci[4])/kg body weight and after one hour the blood was removed (total = 64.7ml). Scintillation counting gave a total of $7.28 \times 10^{+8}$ dis/min (3.92% injected dose). The blood cells were broken up by a freeze-thawing procedure and extraction of the total blood was carried out by stirring with ethyl acetate (4 vol). After centrifugation the ethyl acetate layer was removed by pipette and found to contain $4.58 \times 10^{+8}$ dis/min (2.45% injected dose) and the aqueous blood layer $2.7 \times 10^{+8}$ dis/min (1.45% injected dose). Extensive analytical examination of the aqueous blood layer indicated that most of the radioactivity was irreversibly bound to biological material and no discrete compound could be isolated from the non-bound material.

The ethyl acetate layer was dried with sodium sulphate, concentrated and analysed by TLC in ethyl acetate-petroleum ether (60-80)-ether, 50 : 46 : 4 (saturated with water). Investigation by radiochromatogram scanning, liquid scintillation counting and UV light showed the presence of two radioactive and UV absorbing areas in the ratio 5 : 1 (Rf 0.3 and 0.53 respectively). The material at Rf 0.53 was chromatographically indistinguishable from chlorambucil in the above mentioned solvent system and in chloroform-methanol, 25 : 3 (Rf 0.50).

Since β-oxidation is a process reported[2] as occurring at some stage in the metabolism of chlorambucil it was considered likely that the metabolite material (Rf 0.3) was 2(4-N, N bis (2-chloroethyl)aminophenyl) acetic acid, (3,R^2=H). This compound was syntnesised by the method of Wall[5] and comparison by TLC revealed it to be indistinguishable from the major component of the ethyl acetate extract both in ethyl acetate-petroleum ether (60-80) ether,

50 : 46 : 4 (saturated with water), (Rf 0.3) and chloroform-methanol, 25 : 3 (Rf 0.48). Preparative TLC was carried out on the ethyl acetate concentrate using the former solvent system and the silica gel from the major radioactive zone centred at Rf 0.3 was segregated, eluted with methanol and treated with excess diazomethane (100 fold) in ether. The solution was concentrated and the product subjected to preparative TLC in ethyl acetate-petroleum ether (20 : 80). The silica gel in the single radioactive area coincident with UV absorbing material (Rf 0.48) was segregated, eluted[6] with methanol and the eluate concentrated. Simultaneously the synthetic 2-(4-N, N bis(2-chloroethyl)aminophenyl)acetic acid was treated with diazomethane allowing the methyl ester to be isolated as above, concentrated and compared with the metabolite material by TLC and mass spectrometry.

Table 1

Thin Layer Chromatographic Investigation of Methylated Metabolite Material Obtained from Blood of Animals at 1hr.

Solvent System	Methylated Metabolite Material	Methyl 2(4 -N, N-bis(2-chloroethyl) aminophenyl)acetate	Methyl Ester Chlorambucil
Ethyl acetate-Petrol (60-80) 20 : 80.	0.48	0.47	0.56
Chloroform-Hexane, 1 : 1	0.37	0.31	0.47
Ether-Benzene, 5 : 95	0.66	0.67	0.77
Acetone-Toluene, 5 : 95	0.78	0.77	0.81
Ethyl acetate-carbon tetrachloride 10 : 90	0.47	0.45	0.57

TLC examination of the methylated material showed it to be one component and indistinguishable from the synthetic methyl ester in five different solvent systems. Mass spectrometry of the methylated metabolite material indicated that methyl 2-(4 -N, N bis(2-chloroethyl)aminophenyl) acetate (3, $R^2 = CH_3$, M^+ 289) was present but it was evident that another compound (M^+ 315) was present. It was thought possible that the peak at 315 was due to the methyl ester of chlorambucil (317) present as an impurity and undergoing dehydrogenation in the mass spectrometer. The methyl ester of chlorambucil was synthesised by reaction of chlorambucil with methanol/HCl and the mass spectrum was recorded. The molecular ion was clearly 317 with characteristic accompanying Cl^{37} and C^{13} peaks and no peak at m/e 315 was present. Therefore the peak at m/e 315 in the spectrum of the methylated metabolite mixture could not be explained as due to the presence of the methyl ester of chlorambucil.

Since the cracking pattern[7,8] of the compound with M^+ 315 indicated that the bis(2-chloroethyl) amino group was intact the structure of this compound was deduced to be the methyl ester of a dehydro-chlorambucil, either methyl 4-(4-bis(2-chloroethyl)aminophenyl) 2-but

-enoate (2, $R^1 = CH_3$) or the isomer methyl 4-(4-bis(2-chloroethyl)aminophenyl) 3-butenoate (4, $R^1 = CH_3$).

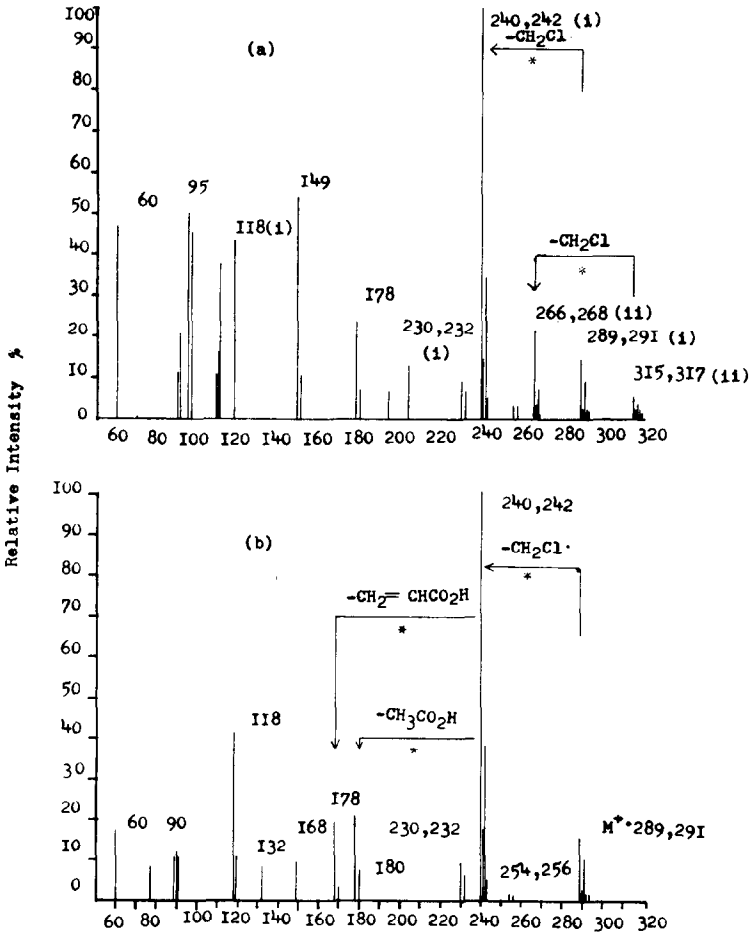

Fig 1: Mass spectra of (a) methylated metabolite material and (b) synthetic methyl 2-(4-N, N bis(2-chloroethyl)aminophenyl)acetate. Major peaks are marked with appropriate m/e values, and with (i) when considered derived from methyl 2-(4- N, N bis(2-chloroethyl)aminophenyl acetate and with (ii) where considered derived from the methyl ester of a dehydro form of chlorambucil. Asterisk denotes a fragmentation accompanied by the appropriate metastable peak.

By analogy with the literature[9] describing the metabolism of fatty acids by β-oxidation chlorambucil is envisaged as being converted to the 2-butenoic acid (2, $R^1 = H$) as an intermediate towards the final product of the B-oxidation cycle 2-(4 bis(2-chloroethyl)amino-phenyl)acetic acid (3, $R^2 = H$). On the basis of literature precedent[10] describing crotonase acting as an isomerase the 3-butenoic acid (4, $R^1 = H$) could be formed from the 2-butenoic acid by an analogous enzyme mediated shift of the double bond. Experimental evidence[11] describing an analogous process is derived from a study of the microbial degradation of 4-phenylbutyric acid to a mixture of 4-phenylbut-3-enoic acid, 4-phenylbut-2-enoic acid and phenylacetic acid.

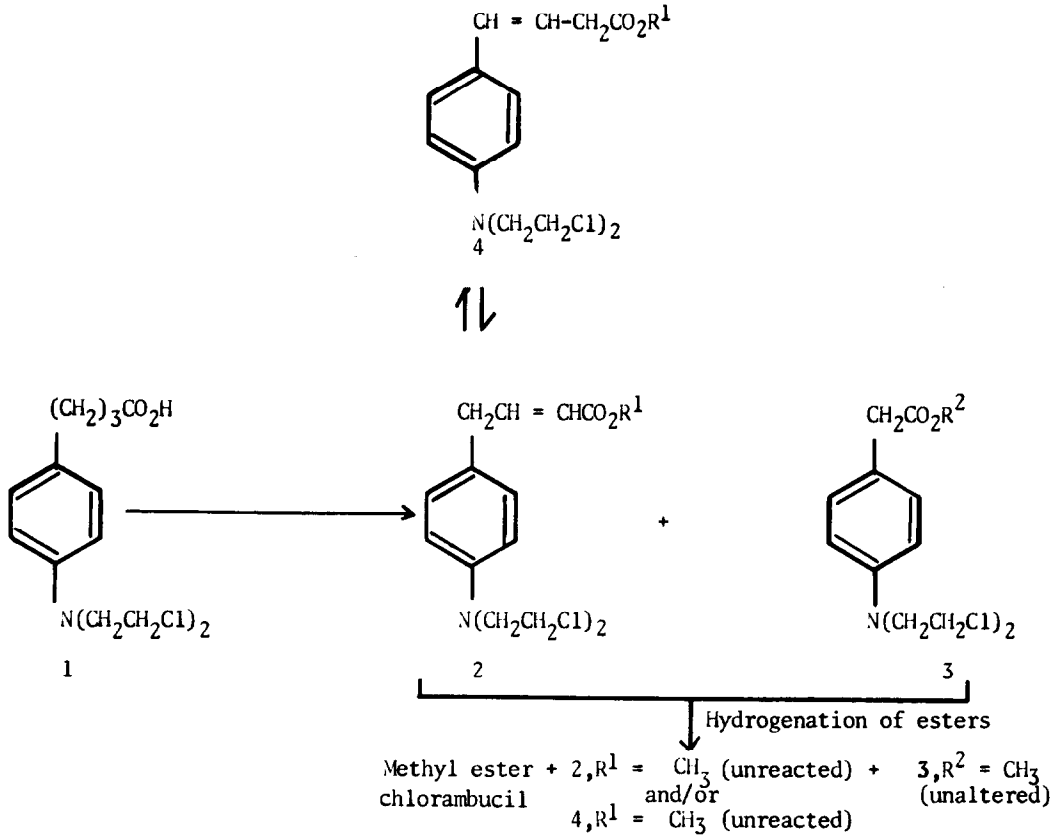

Fig 2: The β-oxidation of chlorambucil and catalytic hydrogenation of methylated metabolite mixture.

The presence of a methyl ester of a dehydro-chlorambucil was demonstrated by hydrogenating the methylated metabolite mixture (approx 50µg) using 5% palladium on carbon causing the production of a compound indistinguishable on TLC from the methyl ester of chlorambucil (Rf 0.56) using petroleum ether - ethyl acetate, 80 : 20. This hydrogenated product was separated from the metabolite mixture (Rf 0.48) by preparative TLC and isolated as above. Since the mass spectrum of this product and the mass spectrum of the synthetic methyl ester of chlorambucil were identical the presence of a methyl ester of a dehydro-chlorambucil as a component of the methylated metabolite mixture was established. Mass-spectral investigation of the material (Rf 0.48) obtained from silica gel indicated that there was some unreacted dehydro methyl ester ($M^{\ddagger}$ 315) in addition to the unchanged methyl 2-(4 bis(2-chloroethyl) amino-phenyl)acetate (3, $R^2 = CH_3$). This material (Rf 0.48) was hydrogenated again to produce more methyl ester chlorambucil which was again separated by preparative TLC. The material (Rf 0.48) was again recovered from the gel and examined by mass spectroscopy but again unreacted dehydro methyl ester ($M^{\ddagger}$ 315) was evident in addition to the unchanged methyl 2-(4-bis(2-chloroethyl)aminophenyl)acetate (3, $R^2 = CH_3$). This hydrogenation procedure was

repeated another two times in an effort to cause complete conversion of the methyl ester dehydrochlorambucil to methyl ester chlorambucil but mass spectral analysis of the material (Rf 0.48) finally isolated by preparative TLC still showed a trace of dehydro methyl ester ($M^{\ddagger}$ 315) in addition to unchanged methyl 2-(4-bis(2-chloroethyl)amino-phenyl)acetate. The failure to obtain a quantitative hydrogenation was attributed to technical difficulties associated with the small scale of the reaction.

Previously[12] chlorambucil has been tested against the Walker 256 transplanted rat tumour employing an i.p. single dose and found to have an LD_{50} of 17.8mg/kg with ID_{90} of 1.5mg/kg. The chemotherapeutic index is thus 11.9. The final product of the β-oxidation of chlorambucil, 2-(4- N, N bis(2-chloroethyl)aminophenyl) acetic acid (3, R^2 = H), is a compound first isolated by Ross[1], and when examined according to the protocol used for the above chlorambucil test was found to have LD_{50} 11.5mg/kg and ID_{90} 2.0mg/kg (C.I. 5.8).

Present research is orientated towards isolating sufficient quantity of the methylated metabolite material to allow an examination of the mixture by NMR. The synthesis of both methyl 4-(4-bis(2-chloroethyl)aminophenyl)2-butenoate (2, $R^1 = CH_3$) and methyl 4-(4bis(2-chloroethyl)aminophenyl)3-butenoate(4, $R^1 = CH_3$) is in progress and it is anticipated that NMR studies will allow a structure to be assigned for each component of the methylated metabolite mixture.

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