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THE MICROSOMAL METABOLISM OF SOME ANALOGUES OF CYCLOPHOSPHAMIDE: 4-METHYLCYCLOPHOSPHAMIDE AND 6-METHYLCYCLOPHOSPHAMIDE

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Abstract—4-Methylcyclophosphamide and 6-methylcyclophosphamide are, like cyclophosphamide, converted by rat liver microsomes into 4-hydroxy derivatives. 4-Hydroxy-4-methylcyclophosphamide was isolated directly, in admixture with the product [2-(2-chloroethylamino)tetrahydro-4-methyl-2*H*-1,3,2-oxazaphosphorine 2-oxide] of dechloroethylation. P.m.r. data for the hydroxy derivative, which was also formed when 4-methylcyclophosphamide was treated with aqueous $KMnO_4$, indicated that it exists in aqueous solution as the acyclic tautomer, 2-oxopropyl-*N*,*N*-*bis*-(2-chloroethyl)phosphorodiamidate. 4-Hydroxy-6-methylcyclophosphamide was trapped by reaction with ethanol, and afforded two isomeric ethoxy derivatives analogous to those previously reported from cyclophosphamide.

Treatment of the products of metabolism of 4-methyl- and 6-methylcyclophosphamide with 2,4-dinitrophenylhydrazine afforded, respectively, the 2,4-dinitrophenylhydrazones of methyl vinyl ketone and of crotonaldehyde.

4-Methylcyclophosphamide cannot form metabolites analogous to 4-ketocyclophosphamide and carboxyphosphamide, the relatively non-toxic metabolites of cyclophosphamide. The significance of this fact is discussed in relation to a mechanism which could account for the relatively selective cytotoxicity of cyclophosphamide *in vivo* towards neoplastic tissue.

Conventional electron impact mass spectrometry has played an important role in the characterization of the products described in this study. 4-Hydroxy-4-methylcyclophosphamide was additionally characterized by the relatively novel technique of field desorption mass spectrometry.

A detailed study of the metabolism of the antitumour agent cyclophosphamide and some of its analogues is being undertaken in this laboratory. One aim of this programme is to investigate the effect of structural modification of cyclophosphamide on metabolism and antitumour activity. We now report on the metabolism of 4-methylcyclophosphamide {2-[*bis*(2-chloroethyl) amino]-tetrahydro-4-methyl-2H-1,3,2-oxazaphosphorine 2-oxide, I} and of 6-methylcyclophosphamide {2-[*bis*(2-chloroethyl)amino]-tetrahydro-6-methyl-2*H*-1,3,2-oxazaphosphorine 2-oxide, II} by rat liver microsomes.

By analogy with cyclophosphamide [1, 2] the scheme shown in Fig. 1 is a plausible prediction for the metabolic transformations by which 4- or 6-methylcyclophosphamide could yield a common toxic end-product, phosphorodiamidic acid (phosphoramide mustard, V) by way of hydroxylated intermediates (IIIa, IVa) and their acyclic tautomers (IIIb, IVb). Although both 4-hydroxy-4-methylcyclophosphamide and the analogous 6-methyl derivative should be convertible into the cytotoxic end product, phosphoramide mustard [1, 2] the former metabolite (IIIa) and its acyclic tautomer (IIIb) cannot be converted into a 4-keto derivative or a corresponding carboxylic acid whereas the

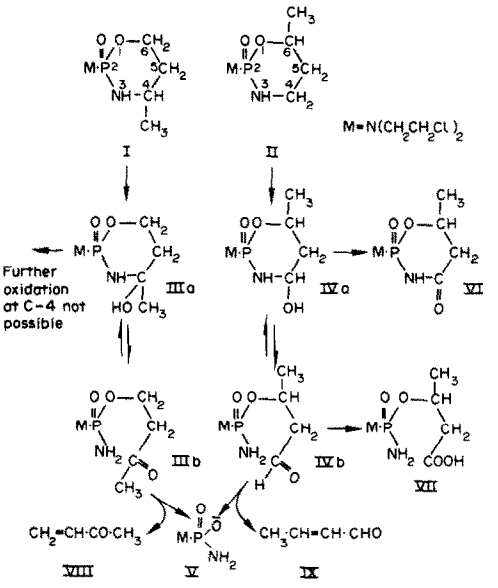

Fig. 1. Scheme for the metabolic activation of 4-methylcyclophosphamide (I) and 6-methylcyclophosphamide (II).

latter derivative could be converted into such products (VI and VII). The relatively selective toxicity of cyclophosphamide towards neoplastic tissues could be due [1] to the ability of normal tissue to detoxify the intermediate hydroxy derivative (by further oxidation) coupled with a deficiency of this ability in tumour tissue, which thus allows release of the highly toxic phosphoramide mustard. The theory predicts that 4-methylcyclophosphamide should be less selectively toxic *in vivo* towards tumour tissue, i.e. it should have a lower therapeutic index, than cyclophosphamide and its 6-methyl derivative.

Methyl vinyl ketone (VIII) should also be formed from 4-methylcyclophosphamide and crotonaldehyde (IX) from 6-methylcyclophosphamide analogous to the formation of acrolein from cyclophosphamide [3]. Acrolein was isolated, as its 2,4-dinitrophenylhydrazone, from microsomal incubates of the drug and from the ethoxy derivatives of cyclophosphamide after treatment consecutively with acidic 2,4-dinitrophenylhydrazine and alkali [1].

MATERIALS AND METHODS

4-Methylcyclophosphamide and 6-methylcyclophosphamide were the gifts of Ward Blenkinsop Ltd.

Synthesis of methyl vinyl ketone 2,4-dinitrophenylhydrazone. To a solution of 2,4-dinitrophenylhydrazine (50 mg) in M HCl (25 ml) was added methyl vinyl ketone (VIII) (0·1 ml, 90% solution in water, Koch–Light Laboratories Ltd.). After 15 min at room temperature, the solution was extracted with benzene (20 ml).

The dried (Na_2SO_4) extract was concentrated and the residue crystallized from methanol (3 ml) as orange needles (20 mg), m.p. (corr.) 140–142° (lit. [4] 142°) (Found: C, 47·95; H, 4·35; N, 22·4%. $C_{10}H_{10}N_4O_4$ requires C, 48·0; H, 4·0; N, 22·4%).

The success of the above conventional procedure is contrary to reports [4, 5] that especially mild procedures are required to prepare this compound, owing to the tendency for side reactions to occur.

Reaction of 4-methylcyclophosphamide with aqueous $KMnO_4$: the formation of 4-hydroxy-4-methylcyclophosphamide (IIIa). A solution of 4-methylcyclophosphamide (75 mg) and $KMnO_4$ (150 mg) in water (5 ml) was stirred at room temperature. After 2 hr, the solution was extracted with methylene chloride (20 ml). The dried (Na_2SO_4) organic phase was concentrated to a colourless oil (35 mg) that was almost homogeneous on t.l.c., R_f (chloroform–ethanol, 9:1) 0·54 (cf. 4-methylcyclophosphamide, R_f 0·64) and which was used in the test against the ADJ/PC6 plasma cell tumour (see Table 1). PMR data (Perkin–Elmer R10 60 MHz, 10% solutions in D_2O, using acetonitrile as internal standard. Very similar parameters obtained for solutions in $CDCl_3$): τ 5·88 (quartet, 2H, H-6,6'), 6·45 (AB quartet, 8H, 2 chloroethyls), 6·73 (doublet, 2H, NH_2 protons coupled with P, J = 5 Hz), 7·16 (triplet, 2H, H-5,6, $J_{5,6}$ 5 Hz), 7·83 (singlet, 3H, CH_3CO protons). Infra-red data (Perkin–Elmer 257 grating spectrophotometer, 5% solutions in $CHCl_3$): ν 1715 cm^{-1} (C=O).

Before the determination of electron impact (EI) and field desorption (FD) mass spectra the above product was further purified by t.l.c. in chloroform–ethanol (19:1) on a plate (20 × 20 cm). Material at the appropriate R_f value was eluted with ethanol. Recovery was 18 mg, and the material was stable for at least 1 week when stored at −30°. At room temperature, the odour of methyl vinyl ketone was detected after 1 hr.

The conditions for obtaining washed rat liver microsomes, and for incubating these with the methylcyclophosphamides were those previously described for cyclophosphamide [1].

Isolation and identification of metabolites. Ethanol was used to precipitate protein, thus terminating the incubations, and subsequent extraction of the products into chloroform and thin-layer chromatography (t.l.c.) of the extracts was also conducted as previously described for the microsomal metabolites of cyclophosphamide. Since non-radioactive substrates were used in the present experiments, the metabolites were detected on the developed chromatograms either by exposure to iodine vapour or by the 2,4-dinitrophenylhydrazine spray reagent (see below). Thus, an unmasked portion of the developed plate was sprayed with the reagent. Silicic acid in the masked area was removed from regions corresponding in R_f value to those of the detected substances, eluted with ethanol and the mass spectra of eluates were determined [1].

Mass spectrometry. Electron impact (EI) mass spectra were obtained with an AEI-MS12 spectrometer operating at an ionizing voltage of 70 eV, a source temperature of 100° and a trap current of 100 μA.

Table 1. Screening against the ADJ/PC6 mouse plasma cell tumour

Compound	LD_{50} (mg/kg)	ED_{90} (mg/kg)	T.I. (LD_{50}/ED_{90})
Cyclophosphamide	335	3·6	93
4-Methylcyclophosphamide (I)	430	8	54
6-Methylcyclophosphamide (II)	340	3·7	92
4-Hydroxy-4-methylcyclophosphamide (IIIa)	225	10	22·5

Table 2. Concentration of compounds required to kill 75% Walker tumour cells *in vitro*

Compound	Concn (μg/ml)
Cyclophosphamide	400
4-Methylcyclophosphamide (I)	200
4-Hydroxy-4-methylcyclophosphamide (IIIa)	0·75

Field desorption (FD) mass spectra were obtained on a Varian CH5-D spectrometer at the University of Cardiff, using a source temperature of 75° and a heating current of 0–15 mA.

Measurement of anti-tumour activities in vivo *and* in vitro. The effectiveness of cyclophosphamide, 4- and 6-methyl derivatives and synthetic 4-hydroxy-4-methylcyclophosphamide against the advanced ADJ/PC6 tumour in female BALB/C-mice (Table 1) was tested by previously described methods [6, 7].

4-Methylcyclophosphamide and 4-hydroxy-4-methylcyclophosphamide were also tested against Walker ascites tumour cells *in vitro* (Table 2), the cell kill being measured by a bioassay technique [1].

RESULTS

(a) *Metabolism of 4-methylcyclophosphamide*

The appropriate chloroform extract was subjected to t.l.c. in chloroform–ethanol (9:1). Only one product (R_f 0·54) was reactive to the 2,4-dinitrophenylhydrazine spray reagent (saturated solution in 2M HCl; aldehydes and ketones give orange spots). 4-Methylcyclophosphamide (R_f 0·64) was detected on chromatograms by the Epstein reagent [8] (alkylating compounds give blue spots) and by exposure to iodine vapour. The product of R_f 0·54 gave a mass spectrum (Fig. 2a) containing signals appropriate to a mixture of 4-hydroxy-4-methylcyclophosphamide (IIIa) (or its acyclic tautomer) and an *N*-monodechloroethyl derivative of 4-methylcyclophosphamide (X).

(b) *Metabolism of 6-methylcyclophosphamide*

Thin-layer chromatography of the chloroform extract containing the metabolites was conducted in chloroform–ethanol (9:1). Three products were reactive to the 2,4-dinitrophenylhydrazine reagent. The least mobile component (R_f 0·35) gave only a weak mass spectrum (see Discussion). The component of R_f 0·55 was incompletely resolved from residual 6-methylcyclophosphamide, but was separated therefrom by two developments in diethyl ether–ethanol (9:1). Both this component and the component of R_f 0·63 gave

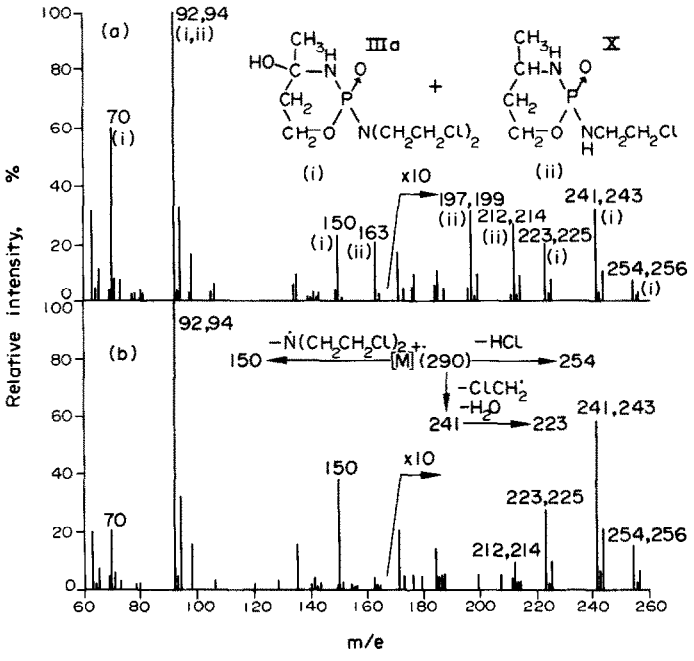

Fig. 2. Mass spectra: (a) Metabolites of 4-methylcyclophosphamide produced with washed microsomes [signals attributable to 4-hydroxy-4-methylcyclophosphamide (IIIa) are marked (i), those attributable to the N-monochloroethyl derivative (X) are marked (ii)]. (b) Product from the oxidation of 4-methylcyclophosphamide with $KMnO_4$.

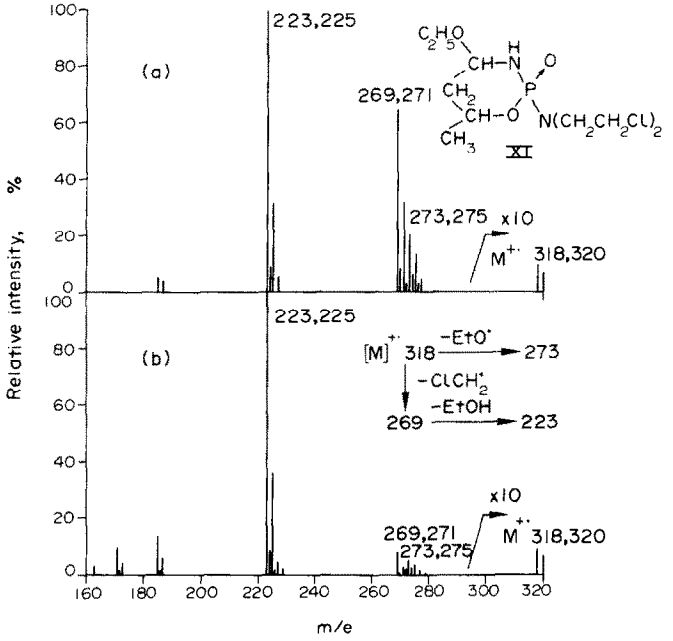

Fig. 3. Mass spectra of ethoxy derivatives isolated after the metabolism of 6-methylcyclophosphamide with washed microsomes: (a) fast ethoxy derivative, (b) slow ethoxy derivative.

mass spectra (Fig. 3) appropriate for 4-ethoxy derivatives of 6-methylcyclophosphamide (see Discussion). They are hereinafter referred to as the "slow" and the "fast" products from 6-methylcyclophosphamide, according to their relative mobilities in chloroform–ethanol (9:1).

(c) *Reaction with 2,4-dinitrophenylhydrazine of the products from 4- and 6-methylcyclophosphamide*

(i) *Extract from 4-methylcyclophosphamide.* A concentrate of the chloroform extract containing the metabolites [see (a) above] was treated with a saturated solution of 2,4-dinitrophenylhydrazine in 2M HCl (0·4 ml). After 10 min, the solution was adjusted to pH 9–10 (M NaOH) and extracted with chloroform. Thin-layer chromatography of the concentrated extract in benzene afforded an orange component of R_f value identical with synthetic methyl vinyl ketone 2,4-dinitrophenylhydrazone, and which gave, after elution, a mass spectrum identical with that of the authentic sample (Fig. 4: for preparation, see Materials).

(ii) *Isolated product from 4-methylcyclophosphamide.* The product of R_f 0·54 isolated after t.l.c. of the chloroform extract containing the metabolites [see (a) above] was eluted with ethanol and a concentrate was allowed to react with 2,4-dinitrophenylhydrazine as in (c)(i) above. Methyl vinyl ketone 2,4-dinitrophenylhydrazine was similarly isolated and characterized.

(iii) *Extract from 6-methylcyclophosphamide.* A concentrate of the chloroform extract was treated as in (c)(i) above. An orange component was isolated with the R_f value and mass spectrum [9] of crotonaldehyde 2,4-dinitrophenylhydrazone.

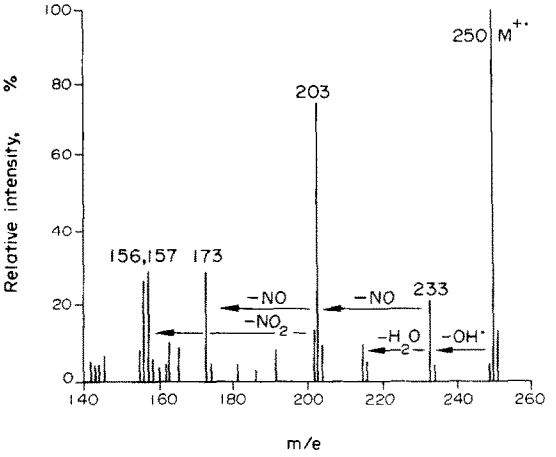

Fig. 4. Mass spectrum of methyl vinyl ketone 2,4-dinitrophenylhydrazone.

(iv) *Isolated products from 6-methylcyclophosphamide.* Each of the three components isolated by t.l.c. from the chloroform extract as described in (b) above afforded crotonaldehyde 2,4-dinitrophenylhydrazone when treated as in (c)(i).

(d) *Effect of water on the fast and slow products of 6-methylcyclophosphamide*

Ethanolic solutions of the fast and slow products prepared as in (c) above, were each concentrated to dryness and solutions of the residues in water (pH 4·5) were monitored by t.l.c. in either chloroform–ethanol (9:1) or diethyl ether–ethanol (9:1), followed by the use of the 2,4-dinitrophenylhydrazine spray reagent [see (a)].

The results are shown diagrammatically in Fig. 5.

DISCUSSION

When 4-methylcyclophosphamide (I) and the isomeric 6-methyl compound (II) were incubated with washed rat liver microsomes and the appropriate cofactors each afforded products (for isolation, see Methods) which were reactive towards a 2,4-dinitrophenylhydrazine reagent. 4-Methylcyclophosphamide apparently afforded only one such product, which was less mobile on t.l.c. than the parent compound. However, the mass spectrum (Fig. 2a) was consistent with a mixture of 4-hydroxy-4-methylcyclophosphamide (IIIa) and an *N*-monochloroethyl analogue (X) of 4-methylcyclophosphamide.

Thus, although no molecular ion for the 4-hydroxy derivative (IIIa, MW 290) was observed, signals attributable to loss of CH_2Cl (*m/e* 241) or HCl (*m/e* 254) were present. Other prominent signals were *m/e* 150 $(M-N(CH_2CH_2Cl)_2]^+$, *m/e* 92 $[ClCH_2CH_2NH{=}CH_2]^+$ and *m/e* 70, probably attributable to methyl vinyl ketone, MW 70, which could be formed thermally by β-elimination. The intensities of the signals at *m/e* 92 and 141 $[NH(CH_2CH_2Cl)_2]^+$, relative to those of higher *m/e* values noted above increased as the temperature was raised, indicating thermal decomposition of (IIIa) or its acyclic tautomer (IIIb), with *N,N-bis*(2-chloroethyl)amine as a major product. Signals attributable to the *N*-monochloroethyl derivative (X) included the molecular ion at *m/e* 212 and the ion (*m/e* 163) formed from it by loss of CH_2Cl. Thin-layer chromatography of the mixture in a variety of solvents failed to resolve the two components.

In contrast to 4-methylcyclophosphamide, 6-methylcyclophosphamide yielded three products which reacted with 2,4-dinitrophenylhydrazine. One of these was less mobile on t.l.c. than the parent drug and gave a very weak mass spectrum in which *m/e* 92/94 were the only signals corresponding to chlorine-containing fragments. The remaining two products were more mobile on t.l.c. than 6-methylcyclophosphamide, and each gave a strong mass spectrum (Fig. 3) appropriate to an ethoxy derivative (XI) of the parent compound. These spectra could be interpreted by analogy with those of the ethoxy derivatives obtained from cyclophosphamide [1]. Thus, a molecular ion, *m/e* 318, gave fragments at *m/e* 273 (loss of C_2H_5O) and 269 (loss of CH_2Cl) and the last-mentioned ion afforded *m/e* 223 by loss of C_2H_5OH. The explanation already presented [1] for the existence of two such ethoxy derivatives of cyclophosphamide could apply to the present case.

The contents of the chloroform extracts obtained after the metabolism of 4-methylcyclophosphamide (I) and 6-methylcyclophosphamide (II) were allowed to react with acidic 2,4-dinitrophenylhydrazine [see Results, (c)] affording, respectively, the 2,4-dinitrophenylhydrazones of methyl vinyl ketone and cro-

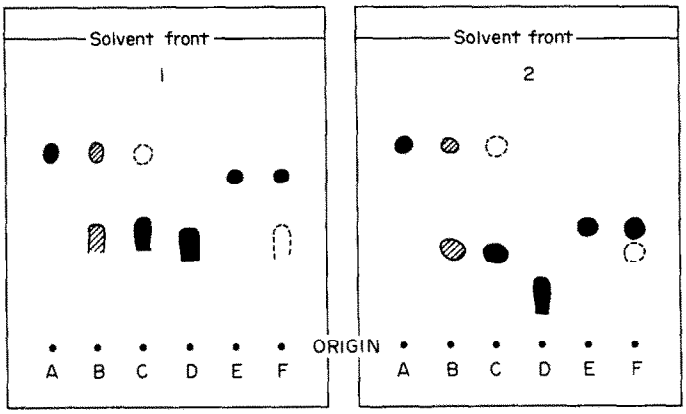

Fig. 5. Diagrammatic representation of thin-layer chromatograms in (1) chloroform–ethanol (9:1) and (2) diethyl ether–ethanol (9:1) showing the hydrolytic behaviour of the ethoxy derivatives of 6-methylcyclophosphamide: A—fast product, B—fast product in water (1·5 hr), C—fast product in water (4·5 hr), D—least mobile component isolated from metabolism extract, E—slow product, F—slow product in water (2·5 hr). Intensity of the reaction with acidic 2,4-dinitrophenylhydrazine: solid areas—strong, hatched areas—medium, dotted areas—weak.

tonaldehyde. The present results are thus consistent (see Fig. 1) with metabolic 4-hydroxylation of (I) and (II) by liver microsomes. The ethoxy derivatives (XI) could yield 4-hydroxy-6-methylcyclophosphamide (IVa) on acidic hydrolysis as postulated for the corresponding cyclophosphamide derivatives [1].

The hydrolytic behaviour of the ethoxy derivatives of 6-methylcyclophosphamide (Fig. 5) differed from that previously reported for the analogous cyclophosphamide derivatives. Formation of the products (Fig. 5, B, C, F), of mobilities on t.l.c. lower than those of the parent compounds, apparently occurred more rapidly than was the case for the product similarly formed from the ethoxy derivatives from cyclophosphamide [1]. The product of hydrolysis of ethoxycyclophosphamide was chromatographically identical with a metabolite of cyclophosphamide which was isolated under ethanol-free conditions, and which, therefore, was probably 4-hydroxycyclophosphamide or its acyclic tautomer. In contrast, the product (Fig. 5, B, C, F) formed by treatment of each of the 4-ethoxy-6-methylcyclophosphamides (Fig. 5, A, E) with water was not chromatographically identical with the least mobile component isolated from metabolism extracts (Fig. 5, D). These two substances were, however, closely related, since all attempts to isolate the products of hydrolysis of the ethoxy derivatives (e.g. freezing and thawing of the aqueous solutions, lyophilization, and extraction into methylene chloride) caused conversion into the unethylated metabolic product (D). They may therefore be the 4-hydroxy derivative (IVa) and its acyclic tautomer (IVb).

Cyclophosphamide is converted by aqueous $KMnO_4$ into 4-ketocyclophosphamide [10] and carboxyphosphamide [11], presumably by way of 4-hydroxycyclophosphamide. The oxidation of 4-methylcyclophosphamide by $KMnO_4$ was therefore investigated as an approach to the synthesis of 4-hydroxy-4-methylcyclophosphamide, since further oxidation should be resisted. 4-Methylcyclophosphamide, when so treated, afforded only one significant product (see Materials), with chromatographic and mass spectral characteristics [Fig. 2(b)] virtually identical with those found for the single component isolated following the microsomal metabolism of 4-methylcyclophosphamide, with the exception that the mass spectrum of the chemical oxidation product contained only weak signals attributable to the *N*-monochloroethyl derivative (X). Thus it would appear that the signals at *m/e* 212 and *m/e* 163 present in the mass spectrum of the metabolite were correctly ascribed to this second component, since the metabolic and chemically derived products had otherwise identical properties. The latter product likewise afforded methyl vinyl ketone 2,4-dinitrophenylhydrazone when appropriately treated with acidic 2,4-dinitrophenylhydrazine.

The chemical oxidation product, being unstable at room temperature, was not amenable to elemental analysis. Therefore, although the conventional EI mass spectrum (Fig. 2b) was consistent with the proposed structure (the corresponding spectrum for the metabolite has been discussed earlier) additional evidence was sought using FD mass spectrometry [12]. A limitation of the EI technique is that the sample must be volatilized without extensive decomposition, since ionization and fragmentation of the molecule occur in the vapour phase. Moreover, because of the high energy of the impacting electrons, some molecules do not afford signals for the molecular ion, so that the molecular weight, and the structure of the molecule, must be deduced from the nature of the ionized fragments. Both these potential disadvantages can often be circumvented by the FD technique since vaporization of the sample is avoided. Thus, the sample is adsorbed from solution on to a specially prepared tungsten wire. When a high positive potential (10 kV) is applied to the wire, electrons are lost by tunnelling and the resulting positive ions are emitted. The principles of the method have been discussed in detail elsewhere [12, 13], but the important point is that for ionization to occur, the sample temperature, which can be raised above ambient by passage of a current (between 0 and 50 mA) through the wire, need not attain the value required to produce vaporization of the sample. The technique is therefore particularly suited to the analysis of thermally unstable compounds. Also, since the molecular ion carries little excess energy, fragmentation is often limited or absent. This factor proved particularly important in the application of the FD technique described here.

The FD mass spectrum (Fig. 6) of the product of chemical oxidation of 4-methylcyclophosphamide contained the molecular ion appropriate to (IIIa) at *m/e* 290, accompanied by a stronger signal at *m/e* 291 due to an ion $[M + H]^+$ formed by surface reactions between the adsorbed molecules, which often occur with the FD mode (see, e.g. Ref. 13). There were no other signals in this spectrum definitely attributable to 4-hydroxy-4-methylcyclophosphamide. Extraneous signals due to volatile impurities have often been seen in the EI mass spectra of samples isolated from t.l.c. [14] but corresponding signals which might appear in FD mass spectra have not yet been documented. These may include signals from involatile impurities, which would not afford signals in the electron impact mode.

In the present and previous studies in this laboratory on the metabolism of cyclophosphamide and its analogues, the position of the tautomeric equilibrium of the hydroxy derivatives [e.g. III (a or b), IV (a or b)] has not been specified. The i.r. spectrum of the synthetic 4-hydroxy-4-methylcyclophosphamide in chloroform solution contained the C=O stretching absorption appropriate for the acyclic tautomer, 2-oxopropyl *N,N-bis*(2-chloroethyl)phosphorodiamidate (IIIb). Moreover, the PMR spectrum both in D_2O and in $CDCl_3$ also indicated almost total preponderance of the acyclic tautomer. Thus the signal for N—H (doublet, τ 6·73) integrated for 2 protons, indicating an NH_2 group. Also the signal for the methyl protons was a singlet at τ 7·83, whereas two singlets would be expected for the cyclic structure (IIIa) where the

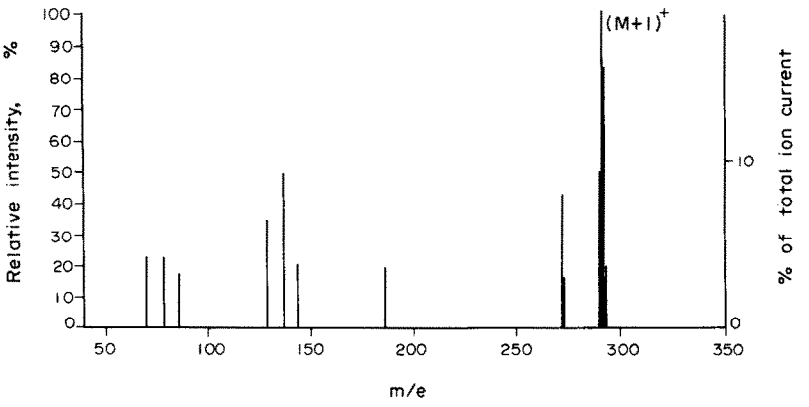

Fig. 6. Field desorption mass spectrum of 4-hydroxy-4-methylcyclophosphamide.

methyl group can take up both axial and equatorial orientations (see Ref. 1).

The existence of the 4-hydroxylation product of 4-methylcyclophosphamide in aqueous solution as its acyclic tautomer lends further credence to the activation scheme (Fig. 1) proceeding via the acyclic tautomer (IIIb) of the 4-hydroxy derivative (IIIa) to phosphoramide mustard (V), since the acyclic tautomer must be present before β-elimination [1] of methyl vinyl ketone can occur. Since the preferred tautomeric form of metabolically derived 4-hydroxycyclophosphamide and its 4- and 6-methylated analogues is not known, the designation of these compounds as 4-hydroxy derivatives as opposed to aldehydo-derivatives is purely arbitrary.

The results of tests against the ADJ/PC6 plasma cell tumour in mice (Table 1) accord with the prediction (see Introduction) that 4-methylcyclophosphamide should be less selective than the 6-methyl derivative (II) and cyclophosphamide. These compounds had been previously tested against the Yoshida ascites sarcoma in rats [15, 16], against which both the 4- and 6-methyl derivatives were inferior (T.I. 12·5 and 12·0, respectively) to cyclophosphamide (T.I. 20·0). Whereas the inferior selectivity of 4-methylcyclophosphamide again accorded with prediction, 6-methylcyclophosphamide and cyclophosphamide should have similar selectivities, as was the case in the ADJ/PC6 test. Clearly, these compounds must be evaluated on other experimental tumours before firm conclusions about the effect of the methyl substituents on biological activity can be made.

The inferior therapeutic index against the ADJ/PC6 tumour of the 4-hydroxy-4-methyl derivative was mainly due to its greater toxicity, and this finding lends support to the earlier stated view that this compound cannot be detoxified by metabolism. The cytotoxicity towards Walker tumour cells in culture (Table 2) was consistent with the presumed role of the 4-hydroxy derivative as an activated form of 4-methylcyclophosphamide, since synthetic 4-hydroxycyclophosphamide [17] and aldophosphamide [18] were also highly toxic towards neoplastic cells in culture. Since completion of this work, Thomson and Colvin [19] have reported that Fenton oxidation of 4-methylcyclophosphamide generated methyl vinyl ketone and that the involatile material remaining after removal of this compound was toxic to the L1210 mouse leukaemia. These workers also characterized methyl vinyl ketone as its dinitrophenylhydrazone by mass spectrometry, but did not report on the characterization of the initial oxidation product from which methyl vinyl ketone was presumably derived.

The effect on biological activity of structural alterations designed to influence the β-elimination reaction, which appears to be the terminal step in the activation pathway for cyclophosphamide and its analogues, is under investigation.

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