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SOME STUDIES OF THE ACTIVE INTERMEDIATES FORMED IN THE MICROSOMAL METABOLISM OF CYCLOPHOSPHAMIDE AND ISOPHOSPHAMIDE

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Abstract—Evidence is presented in support of the following metabolic pathways, in the liver, of the antitumour agent cyclophosphamide 2-[bis(2-chloroethyl)amino]-tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide. The drug is first converted, presumably by the mixed-function oxidases, into 4-hydroxycyclophosphamide which may then break down by elimination of acrolein from its tautomeric form, aldophosphamide, to yield phosphoramide mustard [*N*,*N*-bis(2-chloroethyl)phosphorodiamidic acid], a known cytotoxic agent. In competition with this process is the enzymic conversion of 4-hydroxycyclophosphamide (by dehydrogenation) and aldophosphamide (by oxidation) into the known *in vivo* metabolites of cyclophosphamide, 4-ketocyclophosphamide and carboxyphosphamide, respectively, each of which has low cytotoxicity.

4-Hydroxycyclophosphamide, which was too unstable to allow identification directly by conventional procedures, was trapped by reaction with ethanol. The resulting two, apparently isomeric, ethyl derivatives, (1) were amenable to mass spectrometry, (2) yielded acrolein 2,4-dinitrophenylhydrazone on treatment with acidic 2,4-dinitrophenylhydrazine, (3) were hydrolysed in water (pH 4·3), each isomer apparently regenerating 4-hydroxycyclophosphamide, (4) were highly toxic to Walker tumour cells in culture.

Phosphoramide mustard was also isolated after *in vitro* metabolism of cyclophosphamide. On the basis of a bioassay involving Walker tumour cells in whole animals it appeared that, of the known metabolites of cyclophosphamide, only phosphoramide mustard possesses the cytoxicity and biological half-life appropriate to the active antitumour metabolite.

Four other metabolites of low cytotoxicity were isolated and identified, namely, 4-ketocyclophosphamide, carboxyphosphamide, 2-(2-chloroethylamino)tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide, and 3-hydroxypropyl-*N*,*N*-bis(2-chloroethyl)phosphorodiamidate.

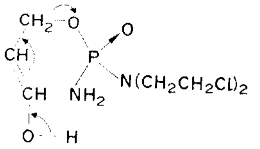
The significance of metabolic detoxification processes in relation to the selective cytotoxicity of cyclophosphamide towards tumour cells *in vivo* is discussed.

The metabolic activation of isophosphamide appears to follow a pathway similar to that of cyclophosphamide.

AS PART of a study in this laboratory of the metabolism of antitumour alkylating agents[1,2] we have investigated the metabolism of cyclophosphamide {2-[bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide, 1}. This compound is a widely used antitumour agent,[3] which is non-toxic to cancer cells *in vitro*. The nature of the activation process whereby it exerts its cytotoxic action *in vivo* has been extensively investigated, and is the subject of the present report.

The original basis for the design of cyclophosphamide[4] was the hope that certain tumour cells, known to be rich in the enzyme phosphoramidase, might mediate the hydrolysis of the *P–N* linkage, either exocyclically or endocyclically, with the liberation of a reactive alkylating species. Activation is now known to occur primarily in the liver[5] and not in cancer cells and is thought to involve the mixed-function oxidases of the liver to yield 4-hydroxycyclophosphamide (IIa) which then equilibrates with its acyclic tautomer, aldophosphamide (IIb). Neither IIa nor IIb, nor derivatives in the same oxidation state have yet been conclusively identified as metabolites of cyclophosphamide. However, evidence for the formation of the metabolites IIa $\rightleftharpoons$ IIb was the observation,[6] that incubation of cyclophosphamide with rat liver microsomes gave two cytotoxic intermediates which were transformed into the known *in vivo* metabolite carboxyphosphamide [2-carboxyethyl-*N*,*N*-bis(2-chloroethyl)phosphorodiamidate, III] either by the soluble fraction of liver cells, or by purified aldehyde oxidase. It seems likely that aldophosphamide (IIb) is the precursor of III. Moreover, another known *in vivo* metabolite of cyclophosphamide, 4-ketocyclophosphamide {2-[bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine-4-one oxide, IV} could be derived by dehydrogenation of 4-hydroxycyclophosphamide.[7]

Apart from the phosphorus-containing metabolites, acrolein was formed when cyclophosphamide was incubated with liver microsomes.[8] Although the relevance of this finding to the activation process was discussed, one unmentioned possibility was the formation from aldophosphamide (IIb) of acrolein and phosphoramide mustard [*N*,*N*-bis(2-chloroethyl)phosphorodiamidic acid, V] by a β-elimination reaction from the enol form (IIc). Compound V (as its cyclohexylammonium salt) is a potent antitumour agent[9] and is thus a candidate for consideration as the cytotoxic product ultimately responsible for the antitumour properties of cyclophosphamide.



(IIc)

Thus, a scheme for the metabolic processes leading to the activation of cyclophosphamide (Fig. 1) would encompass three experimental findings not previously integrated, namely, the implication of aldophosphamide as a key intermediate,[6] the production of acrolein from cyclophosphamide under activating conditions,[8] and the known[9] cytotoxicity of phosphoramide mustard (V).

In seeking to validate this unifying scheme, an *in vitro* system was used in which cyclophosphamide was shown[10] to be activated by rat liver microsomes to the same extent as was observed *in vivo* with experimental animals. The metabolites were isolated and identified by mass spectrometry, using procedures described in relation to previous metabolic studies.[1,2] In addition to the conventional bioassay technique,[11] used to estimate the toxicity of synthetic metabolites on a milligram scale, a novel cell culture bioassay procedure (described in the following paper by B. J. Phillips) was used which allowed a quantitative measure of the cytotoxicity of the microgram amounts of metabolites isolated after thin-layer chromatography.

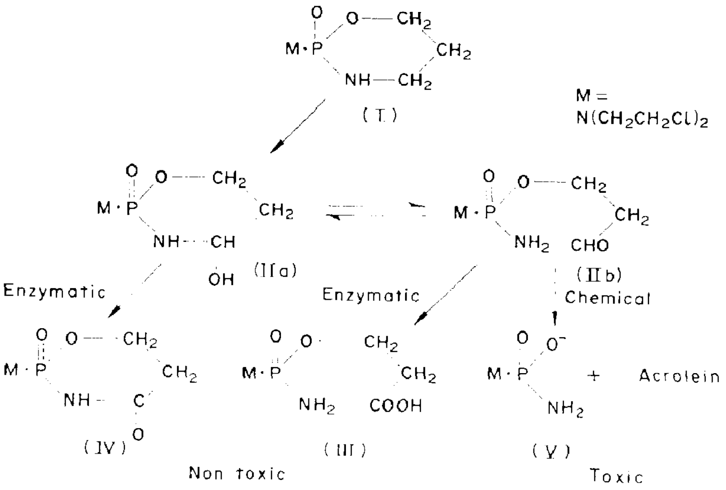
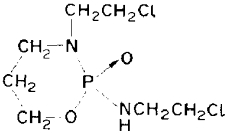


FIG. 1. Scheme for the metabolic activation of cyclophosphamide.

The metabolism of isophosphamide [3-(2-chloroethyl)-2-(2-chloroethylamino)tetrahydro-1,3,2-oxazaphosphorine 2-oxide, VI][12] by rat liver microsomes was also investigated. The metabolic conversion of VI into a cytotoxic metabolite and the accompanying detoxification mechanisms were expected to resemble those for cyclophosphamide, since similar kinetics were obtained for the microsomal activation of the two compounds,[13] and acrolein is also produced during the metabolism of isophosphamide.[14]



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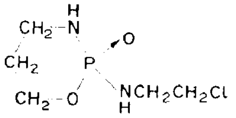
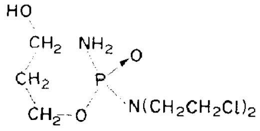


(VII)



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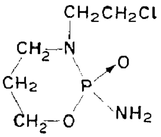
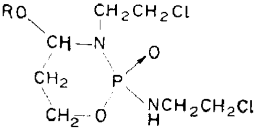
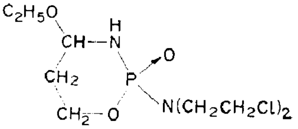


(IX)



(X) R = H
(XII) R = C_2H_5



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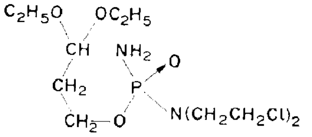


(XIII)

METHODS AND RESULTS

Metabolism. Male Wistar albino rats (inbred colony) weighing 200–250 *g* were used for metabolism studies. Sodium phenobarbital was introduced into their drinking water at a level of 500 mg/l for at least 10 days prior to each experiment to enhance mixed-function oxidase activity in the liver.

9000 g_{av} supernatant was prepared by centrifugation of 25% liver homogenates prepared in 0·1 M Tris–HCl buffer, pH 7·4. Liver microsomes were obtained by centrifugation of the 12,000 g_{av} supernatant, prepared in 0·15 M KCl, for 35 min at 190,000 g_{av}. The microsomal pellet was washed by suspension in 0·15 M KCl and recentrifugation at 190,000 g_{av} and was then suspended in 0·2 M Tris–HCl buffer, pH 7·4, such that 1 ml of suspension was equivalent to 1 g of original liver. The microsomes were thoroughly washed since it has been suggested[6] (see Introduction and Fig. 1) that aldophosphamide (IIb) is converted into carboxyphosphamide (III) by an enzyme present in the soluble fraction of the liver cell.

Incubations were carried out at 37° for 45 min in 25 ml conical flasks, which were shaken gently. Each flask contained 8 ml of 9000 g_{av} supernatant or 3 ml of microsomal suspension, 2·75 μmole of NADP$^+$, 55 μmole of glucose 6-phosphate, 49·5 μmole of $MgCl_2.6H_2O$ and 3·5 units of glucose 6-phosphate dehydrogenase, in a total volume of 10 ml, buffered at pH 7·4 with 0·1 M Tris–HCl. Substrate was added in 0·2 ml of aqueous solution. The microsomal metabolism proceeded to a greater extent if cofactors and microsomes were each added in three portions, at 0, 15 and 30 min.

Bioassay. The bioassay technique was essentially that previously described,[11] the principal difference in this work being that 10^6 Walker ascites cells/ml were used. Cyclophosphamide and various synthetic metabolites at a range of concentrations, dissolved in either water or dimethylsulphoxide (0·1 ml) were added to the cell suspension (10 ml). Solvent only was used in the control bioassays. After incubation at 37° for 2 hr, 10^6 cells were injected into rats, using groups of five animals for each dose or treatment. Survival times were recorded and hence percentage cell kill could be calculated, taking the mean generation time for Walker cells to be 18 hr.

To measure the stability of phosphoramide mustard (V, cyclohexylammonium salt) in the presence of microsomes and an NADPH-generating system, a method similar to that used to demonstrate the activation of cyclophosphamide[10] was utilized. Phosphoramide mustard (V) (26·4 μg/ml) was incubated with microsomes and cofactors, and aliquots (1 ml) were bioassayed at various time intervals. Microsomes and cofactors incubated in the absence of substrate were used as controls.

Isolation and identification of metabolites. The important precautions taken to ensure minimal contamination of samples eluted from thin-layer chromatograms were those previously described.[1] The extracts, the preparation of which is described individually, were subjected to thin-layer chromatography (t.l.c.) on silicic acid (Merck, Kieselgel G) using plates of 20 × 5 cm. Radioactive bands were located with either a Panax or a Berthold radiochromatogram scanner.

Mass spectra were determined with an AEI-MS12 spectrometer, using the direct insertion technique, an ionizing voltage of 70 eV, and a source temperature not greater than 100°. The use of the lowest temperature consistent with the production of a reasonably intense spectrum was important in the case of the ethoxy derivatives (see Discussion) since spectral changes above 100° indicated thermal decomposition.

(a) *Bioassay results.* Table 1 shows the concentrations of cyclophosphamide and various synthetic metabolites required to cause a 75 per cent cell kill within the 2 hr incubation period. It can be seen from these results that the phosphoramide mustard (V) was by far the most toxic of the compounds tested. The stability of V, when incubated with microsomes and cofactors, was determined by bioassay and the results are shown in Fig. 2.

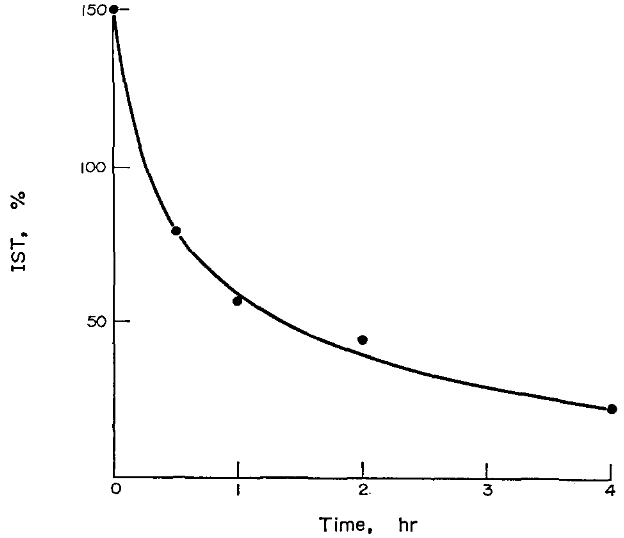


FIG. 2. Cytoxicity decay curve for phosphoramide mustard (V) (final concentration 2·64 μg/ml) incubated with microsomes and cofactors, determined by bioassay and expressed as percentage increase in survival time (per cent IST) vs duration of incubation.

TABLE 1. CONCENTRATION OF CYCLOPHOSPHAMIDE METABOLITES REQUIRED TO KILL 75% WALKER TUMOUR CELLS *in vitro*

Compound	Concn (μg/ml)
Cyclophosphamide (I)	400
Monochloroethylcyclophosphamide (VII)	>800
4-Ketocyclophosphamide (IV)	800
Carboxyphosphamide (III)	>200
Acrolein	3·5
Phosphoramide mustard (V)	0·5

(b) *Metabolism of cyclophosphamide.* $[^{32}P]$Cyclophosphamide (initially 3·1 mCi/m-mole, Radiochemical Centre, Amersham) appropriately diluted with unlabelled cyclophosphamide, was incubated at a concentration of 200–250 μg/ml with washed microsomes or with 9000 g_{av} supernatant as described above. Protein was precipitated by the addition of 4 vol. of redistilled ethanol and removed by centrifugation at 1200 g_{av}. After removal of most of the ethanol from the supernatant by concentration at 30°/10 mm the pH of the concentrate was adjusted to 4 by the addition of M HCl and the solution was extracted (3*x*) with alcohol-free chloroform. Aliquots of the combined extracts were dried with anhydrous magnesiusm sulphate and used for the following experiments.

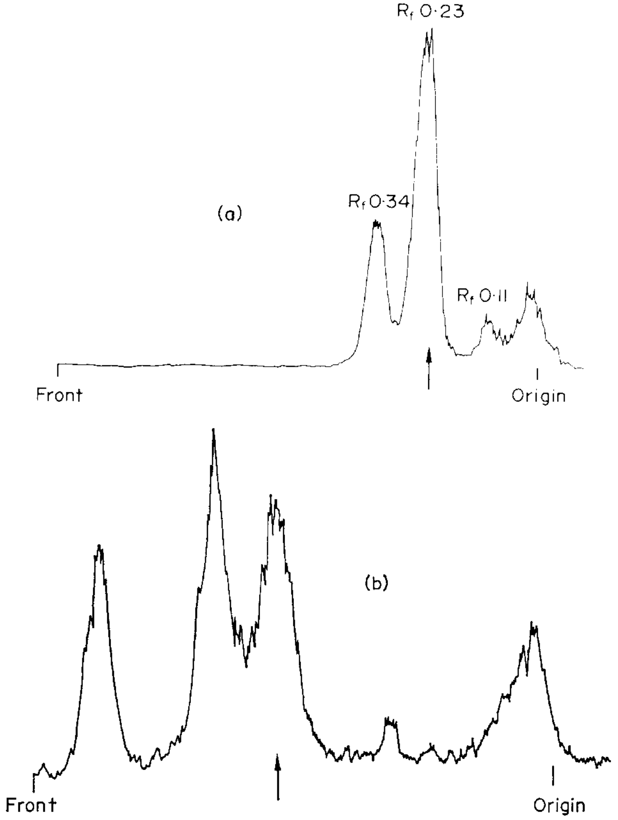


FIG. 3. Radiochromatogram scans of thin-layer chromatograms of cyclophosphamide metabolites produced with washed microsomes, after development in (a) chloroform–ethanol, (19 : 1), (b) ethanol–ether, (1 : 9, 2 developments). The mobility appropriate to authentic cyclophosphamide is indicated in each case by an arrow.

(i) *Metabolism by washed microsomes.* The products in the chloroform extract were subjected to t.l.c. in chloroform–ethanol (19 : 1). The developed plate was scanned for radioactivity (see Fig. 3a) and the silicic acid in the radioactive areas corresponding to the products was removed and eluted[15] with ethanol (*ca.* 0·1 ml). The product of R_f 0·11 gave a mass spectrum identical with that reported[16] for 2-(2-chloroethylamino)tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide (VII). The product of R_f 0·34 gave the mass spectrum shown in Fig. 4(a). Rechromatography of the product of R_f 0·23 in ether–ethanol (9 : 1, 2 developments) revealed two components, the faster of which gave the mass spectrum shown in Fig. 4(b), and the slower gave a mass spectrum identical with that of cyclophosphamide. The solvent system ether–ethanol (9 : 1, two developments) was also used to separate the components of the chloroform extract prior to application of the *in vitro* bioassay system (see following paper and Fig. 3b). The compounds the mass spectra of which are shown in Fig. 4 are hereinafter referred to as the "fast" (Fig. 4a) and the "slow" products (Fig. 4b), respectively. Each of these products could also be detected on thin-layer chromatograms either by the use of the Epstein test[17] (alkylating components afford blue spots) or by spraying

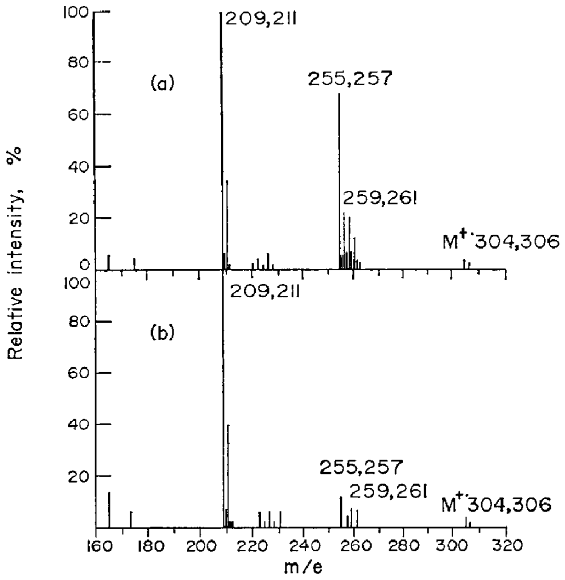


FIG. 4. Mass spectra of cyclophosphamide metabolites produced with washed microsomes: (a) fast product, (b) slow product.

with a saturated solution of 2,4-dinitrophenylhydrazine in 2 M HCl (aldehydes give orange spots).

There was also material which was immobile in the solvent system chloroform–ethanol (19:1). The silicic acid in the radioactive area at the origin (Fig. 3(a)) was eluted with methanol and conventionally methylated (cf. ref. 18) by treatment with ethereal diazomethane for 20 min. On t.l.c., the reaction mixture afforded a radioactive component of R_f 0·41 in chloroform–methanol (9:1), which gave a mass spectrum (Fig. 5) appropriate for the methyl ester of phosphoramide mustard (V) (see Discussion).

(ii) *Metabolism by* 9000 g_{av} *supernatant.* The products in the chloroform extract were subjected to t.l.c. in chloroform–methanol (19:1). There were, in addition to the fast

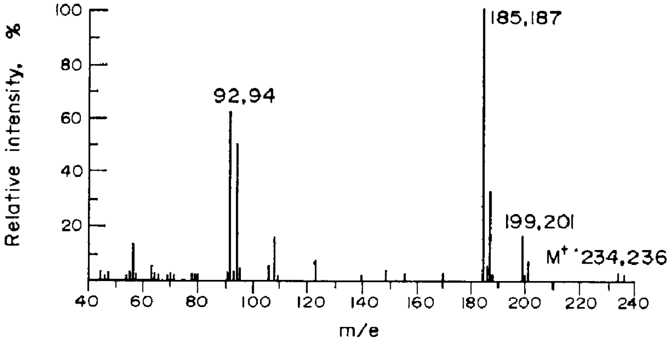


FIG. 5. Mass spectrum of a methylated cyclophosphamide metabolite believed to be the methyl ester of (V).

and slow products, further components of R_f 0·23, the mass spectrum of which was identical with that reported[18] for 4-ketocyclophosphamide, at R_f 0·04 with a mass spectrum similar to that reported[16] for 3-hydroxypropyl *N*,*N*-bis(2-chloroethyl)phosphorodiamidate (VIII), and at the origin (R_f 0·26, chloroform–methanol, 1 : 1), which after treatment with ethereal diazomethane for 10 min gave a product with the mass spectrum[18] of the methyl ester of carboxyphosphamide (III). Unchanged cyclophosphamide (R_f 0·27) was not detected on the radiochromatogram.

(iii) *Reaction with* 2,4-*dinitrophenylhydrazine of the fast and the slow products from cyclophosphamide.* Ethanol solutions of the fast and the slow products, separated as in (i) above, were concentrated to dryness and the residues were each treated with a saturated solution of 2,4-dinitrophenylhydrazine in 2 M HCl (0·4 ml). After 10 min the solutions were adjusted to pH 9–10 (M NaOH), extracted with chloroform, and the extracts were subjected to t.l.c. in benzene. An orange component was present in each case at the R_f value appropriate for acrolein 2,4-dinitrophenylhydrazone[19] and which, after elution, gave a mass spectrum containing all the signals for this compound, although the spectrum of highest quality (i.e., containing fewest signals attributable to contaminants) was that obtained from the fast isophosphamide product (see Fig. 7).

(iv) *Effect of water on the fast and the slow products from cyclophosphamide.* An ethanolic solution of the fast product, prepared as in (i) above, was concentrated to dryness and a solution of the residue in water (pH 4·3) was monitored by t.l.c. in chloroform–ethanol (19 : 1) followed by scanning for radioactivity. The formation from the fast component (R_f 0·38) of a single product (R_f 0·10) was complete within 12 hr. Similar hydrolysis of the slow component (R_f 0·23) also yielded a single product of R_f 0·10 but the reaction took *ca.* 46 hr.

(v) *Relationship between the fast and the slow products from cyclophosphamide.* A sample of the fast product was treated with 2 M HCl in ethanol (0·3 ml) at room temperature for 15 min. After dilution with ethanol, acid was removed by passage of the solution through a small column of Amberlite IR45 ion-exchange resin (HO^- form) which had previously been washed with ethanol. A concentrate of the eluate was subjected to t.l.c. in chloroform–ethanol (9 : 1). A radiochromatogram scan showed two peaks, with R_f-values appropriate to the fast and the slow products, in the ratio 1 : 1. The mass spectra of the products confirmed their identity.

(c) *Metabolism of isophosphamide by washed microsomes.* The conditions for metabolism and extraction of the metabolites with chloroform were as described for cyclophosphamide. The [^{32}P]isophosphamide used was initially of specific activity 13·4 mCi/m-mole.

(i) *Composition of the extract.* The procedure used was as described in [(b)(i)] except that the appropriate slow product was separated from isophosphamide by three developments in chloroform–ethanol (19 : 1). Three mobile components were observed in addition to unchanged isophosphamide. That of R_f 0·11 (after one development) gave a mass spectrum containing signals attributable to either of the isomeric monodechloroethylated products (VII) and (IX): it contained, *inter alia*, peaks at m/e 198/200 [$M^{+\cdot}$(^{35}Cl), $M^{+\cdot}$(^{37}Cl), 3 and 1 per cent], 149 [$(M\text{-}CH_2Cl)^+$, 100 per cent] and 120 [$(M\text{-}NHCH_2CH_2Cl)^+$, 10 per cent]. The mass spectra of the appropriate fast (R_f 0·33, after one development) and slow products (R_f 0·25, after one development, incompletely resolved from isophosphamide) are shown in Figs 6(a) and (b), respectively.

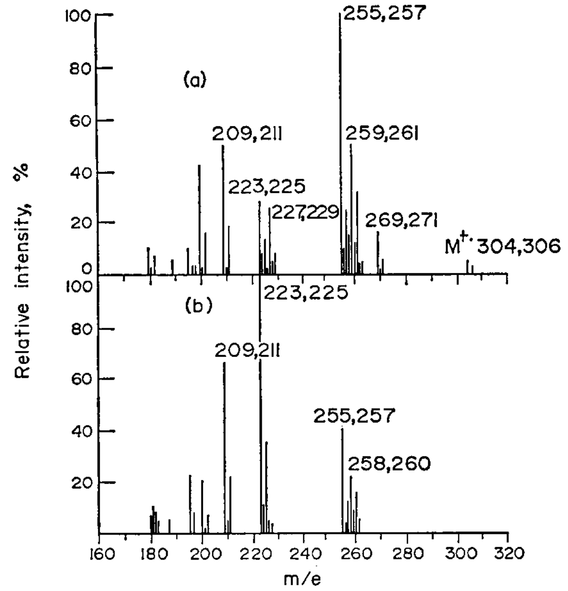


Fig. 6. Mass spectra of isophosphamide metabolites produced with washed microsomes: (a) fast product, (b) slow product.

(ii) *Reaction of the fast product from isophosphamide with* 2,4-*dinitrophenylhydrazine.* The conditions used were those described in [(b) (iii)]. The mass spectrum of the product, depicted in Fig. 7(a) may be compared with that of an authentic sample of acrolein 2,4-dinitrophenylhydrazone (Fig. 7b).

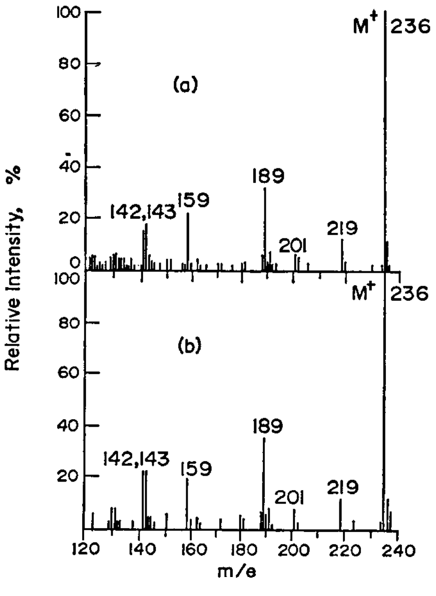


Fig. 7. Mass spectrum of 2,4-dinitrophenylhydrazones: (a) from the isophosphamide fast metabolite, (b) authentic acrolein 2,4-dinitrophenylhydrazone.

(iii) *Effect of water on the fast product of isophosphamide.* The conditions used were described in [(b) (iv)], and the results obtained were comparable. Thus, after 16 hr, t.l.c. revealed the starting material (R_f 0·32, 38 per cent) and a product (R_f 0·13, 62 per cent).

DISCUSSION

When cyclophosphamide or isophosphamide, each labelled with ^{32}P, was incubated with washed microsomes and the appropriate co-factors, followed by precipitation of protein with ethanol, a mixture of products could subsequently be extracted with chloroform. That these products were metabolites or derivatives thereof was shown by control incubations, where cyclophosphamide was added immediately prior to protein precipitation, and where cyclophosphamide was the sole substance subsequently extracted by chloroform. Thin-layer chromatography showed that the product mixture formed from cyclophosphamide contained in addition to non-mobile material, three mobile radioactive products two of which were markedly toxic when tested (on the microgram scale) by a bioassay system using Walker cells in culture (see following paper by B. J. Phillips). The third product, which had low toxicity, was identified by mass spectrometry as the *N*-(2-chloroethyl) derivative (VII). The metabolic loss of a 2-chloroethyl group from cyclophosphamide to give VII presumably occurs by oxene[20] insertion into the CH_2 group adjacent to the exocyclic nitrogen followed by loss of chloroacetaldehyde. This mechanism is formally analogous to that established for de-*O*-methylation.[21]

Since the metabolite VII is not active in the bioassay system and, moreover, is monofunctional, it is not likely to be an intermediate in the generation of a cytotoxic compound from cyclophosphamide. The metabolite VII appears in the urine of sheep following oral administration of cyclophosphamide.[16]

The two cytotoxic products derived from cyclophosphamide were shown by mass spectrometry to comprise a pair of chemically closely related substances which were readily separable from each other by t.l.c. For brevity and convenience in the subsequent discussion these products are called "fast" and "slow" by reference to their chromatographic mobilities and evidence is presented that they are derivatives of 4-hydroxycyclophosphamide (IIa).

A closely parallel series of results was obtained after the metabolism of isophosphamide. The product of low cytotoxicity was a de-2-chloroethylated product and could have been VII and/or IX. This product was not further investigated. As shown below the fast and slow products formed from isophosphamide are derivatives of the 4-hydroxy compound X.

Each member of the two pairs of fast and slow products gave a positive reaction for aldehyde with acidic 2,4-dinitrophenylhydrazine. Acrolein 2,4-dinitrophenylhydrazone was subsequently isolated (see Methods and Results) and identified by mass spectro-

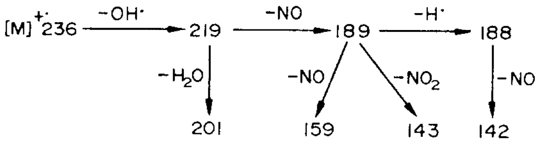


Fig. 8. Fragmentation pathways for acrolein 2,4-dinitrophenylhydrazone.

metry. Figure 7 shows the mass spectrum of the 2,4-dinitrophenylhydrazone product obtained from the fast metabolite of isophosphamide and that of authentic acrolein 2,4-dinitrophenylhydrazone, the fragmentation pathways of which (Fig. 8) are based on those established for crotonaldehyde 2,4-dinitrophenylhydrazone.[22] The production of a derivative of acrolein from the fast and slow products obtained from cyclophosphamide suggests that both may be closely related to 4-hydroxycyclophosphamide (IIa) or to its tautomer, aldophosphamide (IIb). Acrolein can be formed by a conventional β-elimination reaction of the enol form (IIc) of aldophosphamide. The formation of the acyclic tautomer of 6-hydroxycyclophosphamide could also precede the production of acrolein[8] and the mass spectral data (see below) for the fast and slow products obtained from cyclophosphamide is consistent with substitution at position 4 or 6. However, the biochemical evidence strongly favours 4-substitution since 4-hydroxycyclophosphamide (IIa) and aldophosphamide (IIb) are likely metabolic intermediates in the formation of 4-ketocyclophosphamide (IV)[6,7] and carboxyphosphamide (III) the structures of which have been established unequivocally. Compounds III and IV have now been identified as major products when cyclophosphamide is incubated with 9000 g_{av} rat liver supernatant (see Methods and Results).

The mass spectra of the fast and the slow products derived from cyclophosphamide (Fig. 4) and isophosphamide (Fig. 6) can be ascribed to compounds of MW 304 containing two (2-chloroethyl) amine substituents and an ethoxy group. Thus, the appropriate molecular ion, containing two chlorine substituents, was observed in three cases, and in all instances fragments attributable to the loss of C_2H_5O (at *m/e* 259) or C_2H_5OH (at *m/e* 258) and of CH_2Cl (at *m/e* 255) were prominent. The general fragmentation scheme is depicted in Fig. 9 and is consistent with the view that the fast and slow products are ethoxy derivatives (e.g., XI and XII, respectively) of cyclophosphamide and isophosphamide.

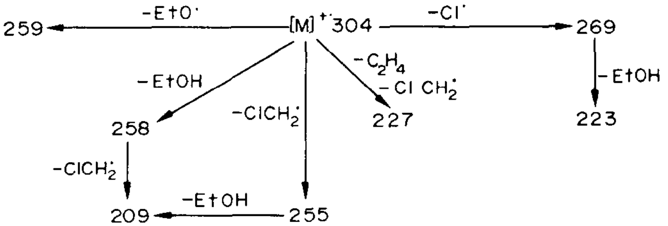
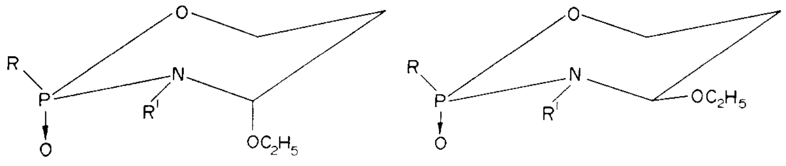


FIG. 9. Fragmentation pathways for the fast and the slow metabolites from cyclophosphamide and isophosphamide.

The possibility that one member of the pair of fast and slow ethoxy derivatives from cyclophosphamide could be a diethyl acetal (XIII) of the acyclic form, aldophosphamide (IIb) was also considered. However, the mass spectrum[23] of the acyclic derivative[24] (XIII) differed from those of the fast and slow products and afforded evidence that thermal cyclization of the acyclic diethyl acetal was not significant below 230°. The production of fast and slow products of the same MW from both cyclophosphamide and isophosphamide could reflect the stereochemistry of the oxazaphosphorine ring, which allows for both axial and equatorial substituents at the



(a) (e)

Fig. 10. Possible conformations for 4-ethoxycyclophosphamide (R = $N(CH_2CH_2Cl)_2$, R' = H) and isophosphamide (R = $NHCH_2CH_2Cl$, R' = CH_2CH_2Cl): (a) axial, (e) equatorial.

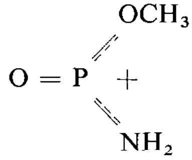
4-position. In Fig. 10 the appropriate structures are based on the probable conformation of cyclophosphamide in solution according to studies with analogues.[25]

When the fast product from cyclophosphamide was treated with ethanolic hydrogen chloride, partial conversion into the corresponding slow product was demonstrated by t.l.c. and mass spectrometry, thus validating the assumption that each compound carried an ethoxy substituent and affording a further demonstration of the close structural relationship between the two compounds.

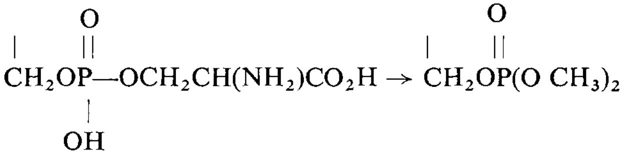
The conditions under which the ethoxy derivatives are formed have been partially defined. The critical factor is the use of ethanol as the protein precipitant following the incubation of cyclophosphamide or isophosphamide with microsomes. Precipitation of the proteins with zinc sulphate–barium hydroxide (1 : 1) afforded no radioactive peaks at the R_f-values appropriate to the fast and slow products on t.l.c. but did, however, reveal a component at R_f 0·30 (chloroform–ethanol, 9 : 1) which gave a positive reaction with acidic, 2,4-dinitrophenylhydrazine. The two ethoxy derivatives from cyclophosphamide showed slightly different extraction properties. Thus, the fast product was partially extracted by chloroform with the aqueous phase at pH 7·4 whereas the slow product was extracted only after pH adjustment to 4. The ethylation of the 4-hydroxy derivatives of the drugs may be subject to mild acid catalysis (possibly by traces of metabolically derived carboxyphosphamide) and the formation of ethoxy derivatives under mild conditions implies that reconversion into hydroxy derivatives might readily occur. Some evidence for this reconversion was afforded by the hydrolytic behaviour of the fast and slow products from cyclophosphamide, each of which was converted in aqueous solution (pH 4·3) into a product of R_f 0·10 which gave a positive reaction with acidic 2,4-dinitrophenylhydrazine. The times for essentially complete hydrolysis of the fast and slow products from cyclophosphamide were $\sim$12 and $\sim$46 hr, respectively. The fast and slow products are glycosides which are structural analogues of, for example, ethyl α- and β-D-hexopyranosides, for which a significant difference in the rate of acid hydrolysis has been recorded.[26] These products of hydrolysis (presumably IIa) could not be eluted from chromatograms without decomposition and attempts to obtain mass spectra from the residues of lyophilized aqueous solutions were unsuccessful.

The fast product from isophosphamide showed behaviour on hydrolysis similar to that of the fast and slow products from cyclophosphamide in that a single product (presumably X) was formed which gave a positive reaction with acidic 2,4-dintrophenylhydrazine.

The results thus far presented support the scheme (Fig. 1) proposed in the introduction, inasmuch as evidence is afforded which relates the previously described production of acrolein from cyclophosphamide and isophosphamide, mediated by liver microsomes, to a derivative of 4-hydroxycyclophosphamide or its isophosphamide analogue. Evidence was also obtained that phosphoramide mustard (V) was among the immobile products (on t.l.c.) isolated following the metabolism of cyclophosphamide with washed microsomes {see Methods and Results, [(b) (i)]}. Thus, methylation of this material with diazomethane afforded a product with mass spectral characteristics {$M^{+\cdot}$ at *m/e* 234/236, $(M - Cl)^+$ at *m/e* 199/201, $(M - CH_2Cl)^+$ at *m/e* 185/187, and $CH_2 = {}^+NHCH_2CH_2Cl$ at *m/e* 92/94} appropriate to the methyl ester of V (see Fig. 5). The relative intensity of the peak at *m/e* 94 is enhanced over that of the ^{37}Cl-containing analogue at *m/e* 92 because of a contribution from the ion



The duration of treatment with diazomethane is important. Thus, brief treatment (10 min) of synthetic carboxyphosphamide (III) with diazomethane gave the methyl ester as the sole product, but a 10 hr treatment afforded the methyl ester of V as the sole product. Baer and Maurukan[27] have reported an analogous displacement reaction:



$$\overset{|}{C}H_2O\underset{\underset{OH}{|}}{\overset{\overset{O}{\|}}{P}}{-}OCH_2CH(NH_2)CO_2H \rightarrow \overset{|}{C}H_2O\overset{\overset{O}{\|}}{P}(O\,CH_3)_2$$

There is indirect evidence that compound (V) has at least some of the properties of the cytotoxic principle derived from cyclophosphamide.*

Carboxyphosphamide (III) and 4-ketocyclophosphamide (IV) are the principal urinary metabolites of cyclophosphamide,[7,16,18] but are non-toxic to Walker tumour cells in the *in vitro* bioassay system (Table 1). In contrast, phosphoramide mustard (V) is markedly toxic and the dose needed to kill 75 per cent of Walker tumour cells is low enough for it to be the toxic metabolite in "activated" cyclophosphamide.[10] The half-life for the decay of cytotoxicity of compound (V) (Fig. 2) is also similar to the half-life of the cytotoxicity of a microsmal incubate containing "activated" cyclophosphamide. In earlier experiments in this laboratory,[28] in which the *in vitro* system {Methods and Results, [(b) (i)]} was used for the activation of cyclophosphamide, an unidentified metabolite was isolated which appeared to decompose with the same half-life as that of the decay of cytotoxicity. The extraction procedures used to isolate this metabolite differed from those of the present experiments, and its relationship to phosphoramide mustard (V) or to the cytotoxic products now isolated remains to be established. Acrolein, which has been detected following microsomal incubation of cyclophosphamide[8] and isophosphamide[14] has been suggested as the

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* After this paper was submitted, phosphoramide mustard (V) was reported as an *in vitro* metabolite of cyclophosphamide and the salient features of the unifying scheme (Fig. 1) were presented [M. Colvin, C. A. Padgett and C. Fenselau, *Cancer Res.* **33**, 915 (1973)].

antitumour metabolite, but it is not as toxic in the bioassay system as phosphoramide mustard.

The highly selective anti-tumour action of cyclophosphamide[29,30] could be explained if normal cells, but not tumour cells, could efficiently convert the primary metabolite by further enzymatic oxidation (or dehydrogenation) into the non-toxic 4-keto (IV) and carboxy derivatives (III) (see Fig. 1). The formation of III and IV in *in vitro* systems containing 9000 g_{av} liver supernatant but not in those containing washed microsomes, provides indirect evidence that they arise by the action of enzymes in the soluble supernatant fraction on the primary metabolite. Tumour cells would be selectively killed if they did not efficiently perform the detoxification process. A proportion of the primary metabolite entering the tumour cell would break down spontaneously into the highly toxic phosphoramide mustard and acrolein.

The selective effect of cyclophosphamide could thus be due to intracellular release of phosphoramide mustard specifically in tumour cells while the whole animal toxicity could be due to breakdown of the primary metabolite in extracellular fluid.

The difficulty of obtaining direct evidence for the presence of 4-hydroxycyclophosphamide or of aldophosphamide, both from microsomal incubates and from hydrolysates of ethoxy derivatives may reflect the lability of these substances to atmospheric oxidation. Very recently a synthesis of 4-hydroxycyclophosphamide has been reported.[31] This compound was described as labile and apparently possesses cytotoxic properties appropriate to the active principle derived from cyclophosphamide. The observation (see following paper by B. J. Phillips) that both the fast and slow ethoxy products from cyclophosphamide were highly cytotoxic to cells in culture is of potential importance since these compounds, which are stable to oxidation, may represent a convenient latent form in which to administer the active metabolite of cyclophosphamide. Attention is therefore being directed towards the synthesis of ethoxy and related derivatives of cyclophosphamide and isophosphamide.

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