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SHORT COMMUNICATIONS

Novel metabolic products of cyclophosphamide in human urine

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As part of an investigation into the metabolism and mode of action of cyclophosphamide {2-[*bis*(2-chloroethyl)amino]-tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide} [1–3], the urine of patients treated with the drug has been studied. Cyclophosphamide is ineffective as an anti-tumour agent before it is metabolized [4]. Metabolism is known to occur primarily in the liver, initially by hydroxylation at C4 [2]. The first metabolite can then break down spontaneously to yield the ultimate alkylating agent, phosphoramide mustard, or can be further oxidized to less toxic products, 4-ketocyclophosphamide and carboxyphosphamide. Thus the routine screening of human carcinoma cultures for senstivity to cyclophosphamide is complicated by the requirement for metabolism, and the use of urine from cyclophosphamide-treated patients, containing "activated cyclophosphamide", has been suggested [5]. However, the principal urinary metabolites, 4-keto-cyclophosphamide and carboxyphosphamide are relatively non-toxic to tumour cells, and no attempt has been made to isolate these compounds in this study. Nor-HN2, *bis*-(2-chloroethyl)amine, which could account for the cytotoxicity of urine, has been reported as a urinary metabolite [6]. The results reported here conclusively identify this compound and also two other products not previously isolated.

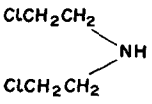
A small glass column (11 × 1·3 cm) was packed with Amberlite XAD2 resin. Distilled water (100 ml) was used to wash the resin in the column, after which u.v.-absorbing materials were eluted with methanol (600 ml). Finally, the column was equilibrated with water.

To test the retention of cyclophosphamide by the XAD2 resin, [^{32}P]cyclophosphamide (sp. act. initially 5·7 mCi/mmole) in aqueous solution (0·4 ml) was loaded onto the column and eluted with water (25 ml) and methanol (45 ml), and 5 ml fractions were collected. 0·3 per cent of the radioactive nucleide applied to the column was eluted in the aqueous fractions (1–6), compared with 92·8 per cent in the methanol fractions (7–14). Chromatography of the combined methanol eluate on silicic acid (Merck, Kieselgel G) with chloroform–ethanol (9:1) showed that the radioactivity was unchanged in mobility by this procedure using Amberlite XAD2 resin.

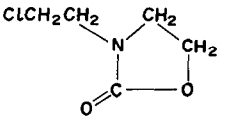
Patients being treated for ovarian carcinoma were used in this study. Cyclophosphamide (1 g) was administered intravenously, chlorpromazine being used as a tranquillizing agent. The patients' bladders were voided prior to administration of the cyclophosphamide and the subsequent 4-hr urines collected, frozen at −30° and stored at this temperature.

Urine was thawed and run through the XAD2 column, after which the column was washed with water. Subsequently, materials held on the resin were eluted with methanol. In one case, 270 ml of urine were used and 400 ml of aqueous eluate and 30 ml of methanolic eluate were collected. The methanol extract was then subjected to either thin-layer or column chromatography.

In the case of thin-layer chromatography (t.l.c.) the inherent 'stickiness' of the extract was reduced by diluting it to 50 ml with methanol and mixing thoroughly with silicic acid (5 g). The suspension was filtered and the filtrate was concentrated to a small volume (200 μl) under reduced pressure (10 torr). This treatment has the effect of removing materials of low mobility on t.l.c. The extract was then applied as a streak to a t.l.c. plate (20 × 20 cm) coated with silicic acid, and the plate was developed in chloroform–ethanol (9:1). The silicic acid was removed in 1 cm deep bands over most of the width of the plate. The remaining area of silicic acid was then sprayed with Epstein reagent [1% 4-(*p*-nitrobenzyl)-pyridine in acetone], heated at 110° for 20 min and developed with 3% ethanolic potassium hydroxide. Those bands of silicic acid corresponding to alkylating species (blue staining) were individually eluted with ethanol. Each eluate was then subjected to t.l.c. on plates 7·5 × 2·5 cm, with appropriate synthetic standards.



(I)



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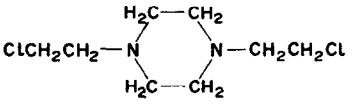

(III)

In both chromatographic methods, two main Epstein-positive bands were detected. The slower running material (R_f 0·44, chloroform–ethanol 9:1) was identical to cyclophosphamide on t.l.c. The faster running material (R_f 0·55, chloroform–ethanol 9:1) appeared to be similar to *bis*-(2-chloroethyl)amine (I, nor-HN2) on chromatography in three different solvent systems and identical in two other systems (Table 1). However, mass spectral data showed no evidence for a molecular ion at *m/e* 141/143 and only a small peak at *m/e* 92/94 (1 Cl, relative intensity 20%) attributable to the loss of CH_2Cl from nor-HN2. The other principal peaks in the spectrum were *m/e* 149 (1 Cl, 20% rel. int., with accompanying ^{37}Cl isotope peak at *m/e* 151, of 6% rel. int.), 100 (100%), 56 (70%), 44 (40%) and 42 (25%). A possible structure for this compound is 3-(2-chloroethyl)-oxazolidone (II). This compound was synthesized by the method of Arnold and Beckel [7] scaled down 20-fold, with a yield of 400 mg (19%) of a pure pale yellow oil (b.p. 116–117° at 0·35 torr). It was shown to behave in a similar way on t.l.c. to nor-HN2 and the product obtained from urine. Its mass spectrum contained only four signals of relative intensity greater than 5%,

Table 1. Thin-layer chromatographic properties of product of cyclophosphamide metabolism from human urine

Solvent system	R_f value		
	Cyclophosphamide	Nor-HN2	Urine product
Chloroform–ethanol (9·1)	0·40	0·50	0·50
Acetone	0·34	0·45	0·47
Diethyl ether–ethanol (9:1)	0·30	0·51	0·47
Benzene–acetone (7:3)	0·13	0·40	0·40
n-Butanol–water (86:14)	0·48	0·52	0·46

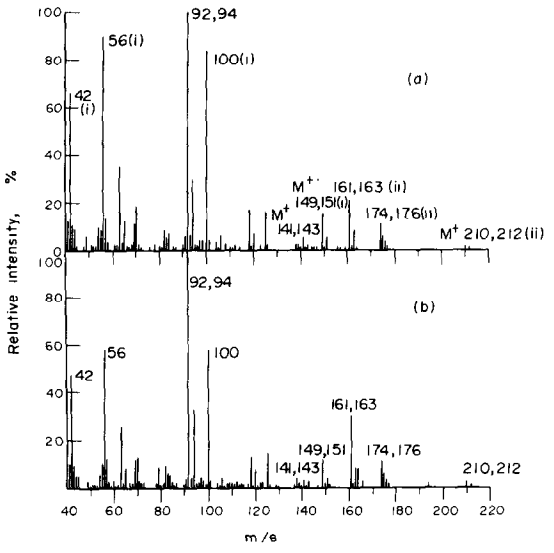

Fig. 1. Mass spectra of (a) a 1:1:1 mixture (by weight) of nor-HN2, 3-(2-chloroethyl)oxazolidone and 1,4-di-(2-chloroethyl)piperazine and (b) a mixture of products isolated from the urine of a patient treated with cyclophosphamide. Major identifiable peaks are marked with the appropriate *m/e* value, and with (i) where derived from the oxazolidone and (ii) where derived from the piperazine.

at *m/e* 149 (M^+, 16%, with accompanying ^{37}Cl isotope peak at *m/e* 151, 5% rel. int.), 100 (100%, $[M—CH_2Cl]^+$), 56 (41%) and 42 (16%). Thus the presence of these signals and the comparable relative intensities between synthetic and metabolically derived product confirm the identity of the metabolic product. As a control, nor-HN2 was added to normal urine (1 mg/ml) and reisolated by the techniques described; t.l.c. and mass spectrometry showed less than 10% conversion of nor-HN2 to oxazolidone during isolation.

In one experiment, the isolated product of R_f 0·55 (chloroform–ethanol 9:1) gave a mass spectrum containing not only signals appropriate to I and II, but also signals at *m/e* 210/212, 174/176 and 161/163, as shown in Fig. 1b. These signals may have been due to a piperazine derivative [III, 1,4-di-(2-chloroethyl)piperazine] formed by dimerization [8] of I. Authentic III, synthesized and kindly supplied by Professor W. C. J. Ross, showed t.l.c. properties similar to those of nor-HN2. The mass spectrum of a 1:1:1 mixture (by weight) of nor-HN2 and its oxazolidone and piperazine derivatives is shown in Fig. 1a. These three compounds were found to be separable on t.l.c. using three developments with chloroform. The respective R_f values were 0·51 (nor-HN2), 0·65 (oxazolidone) and 0·31 (piperazine). Approximately 30 min exposure to iodine vapour was necessary to obtain equal intensity of staining of the three components. All three components also reacted with the Epstein spray reagent, although the oxazolidone derivative showed up only weakly after the 20-min heating period.

The toxicities of these compounds to Walker ascites tumour cells were tested *in vitro* by a method described previously [1]. Table 2 shows the concentrations required to kill 75 per cent of the cells, in comparison with one of the principal urinary metabolites, carboxyphosphamide and unchanged cyclophosphamide.

The relative importance of these three compounds identified in human urine to the metabolic pattern and toxicity of cyclophosphamide has yet to be assessed, and whether nor-HN2 arises by chemical or biological hydrolysis has yet to be established. Previous reports [6] have indicated

Table 2. Concentration of cyclophosphamide products required to kill 75 per cent Walker tumour cells *in vitro*

Compound	Concentration (μg/ml)
Cyclophosphamide	400
Carboxyphosphamide	>200
3-(2-Chloroethyl)oxazolidone (II)	≫32
Bis-(2-chloroethyl)amine (I)	2·2
1,4-Di-(2-chloroethyl)piperazine (III)	0·06

the presence of nor-HN2 in human urine at a level of about 10 per cent of the urinary products or cyclophosphamide. As this work relied mainly on thin-layer chromatography for identification, it is quite possible that these workers were studying mixtures. In fact, the ease of the reaction resulting in the oxazolidone product, which may be enzyme catalysed [9], and the high levels of bicarbonate anions in plasma strongly suggest that at least some of the nor-HN2 would be present as its oxazolidone and would be excreted as such.

Quantitation of the three components in biological fluids is being attempted using stable isotope dilution as recently described for cyclophosphamide [10]. It is possible that the relative concentrations of amine, oxazolidone and piperazine may be affected by the pH of the urine and by small variations in the isolation procedure, as has been shown for some more complex amines [11]. Indeed, it is not certain that the piperazine is formed *in vivo*, as dimerization of *N,N-bis*(2-chloroethyl)methylamine has been reported in concentrated aqueous solution [12].

It would appear that the alkylating activity found in patients' urine may be due in part to nor-HN2 and its piperazine. The latter compound has an LD_{50} of 0·6 mg/kg [8] and could, if formed, contribute significantly to the bladder toxicity frequently observed in humans following cyclophosphamide therapy. The oxazolidone formed may be regarded as a detoxification product of nor-HN2.

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Metabolism of [6-^{14}C]allopurinol—Lack of incorporation of allopurinol into nucleic acids

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Allopurinol [4-hydroxypyrazolo(3,4-d)pyrimidine] is an important therapeutic agent in the management of various types of hyperuricemia, including gout [1]. Plasma uric acid levels are lowered by virtue of the inhibition of xanthine oxidase by both allopurinol and its principal metabolite oxipurinol [4,6-dihydroxypyrazolo(3,4-d)pyrimidine], which persists in the plasma because of active reabsorption by the kidney tubule [2].

The ribonucleoside-5′-monophosphate derivatives of allopurinol (Alo-5′-P) and oxipurinol (1-Oxi-5′-P and 7-Oxi-5′-P) have been synthesized enzymatically *in vitro* [3, 4] and have been found at micromolar concentrations in tissues of rats given large intravenous doses of [6-^{14}C]allopurinol [5]. These ribonucleotide derivatives, particularly 1-Oxi-5′-P, inhibit orotidylate decarboxylase *in vitro* [4], and are thought to be responsible for the orotidinuria observed in animals and patients taking allopurinol [6, 7].

Ten years of clinical experience with allopurinol and extensive biologic and metabolic studies have shown allopurinol to be a safe and effective drug for prolonged administration. Nevertheless, an important and persistent question has been whether allopurinol or one of its metabolites may be incorporated into nucleic acids.

Previous studies with [6-^{14}C]allopurinol failed to demonstrate any radioactivity in the nucleic acids of either mouse liver [8] or cultured human fibroblast cells [9]. Moreover, examination of the acid-soluble nucleotides in rat tissues after a large intravenous dose of [6-^{14}C]allopurinol had revealed no measurable levels, i.e. $<10^{-9}$ M, of pyrazolopyrimidine analogs of AMP or GMP and no pyrazolopyrimidine nucleoside di- or triphosphates [5]. Nevertheless, it was considered important to rule out the possibility of incorporation unequivocally.

A large dose, 50 mg/kg, i.v. of [6-^{14}C]allopurinol of high specific activity (2·4 mCi/mmole) was given to female Sprague–Dawley rats and several tissues were examined, including a rapidly dividing one such as intestine. The animals were sacrificed at 4 hr, a time at which previous studies [5] had indicated the presence of a maximum concentration of allopurinol and oxipurinol ribonucleotides. Samples of liver, kidney, spleen and intestine were frozen with liquid nitrogen-cooled clamps and extracted by a phenol method described by Kimura *et al.* [10]. Nucleic acids obtained from the aqueous phase and from the "interphase" were differentially precipitated with cetyltrimethyl ammonium bromide (CTAB) to separate RNA and DNA [11] and to remove adsorbed low molecular weight contaminants [12] known to be present in phenol-extracted RNA. Several cycles of precipitation with CTAB gave a preparation of nucleic acid with a constant specific activity (Table 1). Liver RNA had the lowest specific activity, 44 dis/min/mg RNA, and intestinal RNA had the highest activity, 429 dis/min/mg RNA with the RNA of kidney and spleen being intermediate. These values correlate roughly with the mitotic activity of these tissues. The total amount of radioactivity in the intestinal RNA was 0·0025 per cent of the administered dose. The DNA isolated from the rat intestine contained less than 10 dis/min/mg, so that further analytical work was impossible.

The acid-soluble nucleotides in the rat intestine were separated by the method described by Nelson *et al.* [5] and the concentrations of 1-Alo-5′-P, 1-Oxi-5′-P and 7-Oxi-5′-P were 1·3, 0·9 and 1·0 nmoles/g wet wt. These levels are of the same order of magnitude as had previously been found in liver [5]. As in the earlier work, there was no radioactivity in the ATP or GTP fractions.

Since the intestinal RNA was available in the largest quantity and had the highest radioactivity, it was selected for identification of the radioactive species present. The intestinal RNA was hydrolyzed with KOH, 0·3 M, at 22° for 18 hr and the resulting mixture of 2′- and 3′-ribonucleoside monophosphates was separated by chromatography

Table 1. Incorporation of radioactivity from [6-^{14}C]allopurinol into nucleic acids of rat tissues*

	Specific activities	
	Expt. I	Expt. II
	(dis/min/mg)	
Liver RNA	44	
Kidney RNA	140	
Spleen RNA	226	
Intestinal RNA	513	429
Intestinal DNA	<10	<10
KOH hydrolysis of intestinal RNA	(dis/min/μmole)	
2′,3′-AMP	213	231
2′,3′-GMP	463	230
	(dis/min/mg)	
Calculated sp. act. of intestinal RNA	543	451

* In Expt. I, two female Sprague–Dawley rats were given [6-^{14}C]allopurinol, 1·7 μCi/μmole, at a dose of 50 mg/kg, i.v. In Expt. II, four rats were used and the specific activity of the allopurinol was 2·4 μCi/μmole. At 4 hr the animals were sacrificed and the nucleic acids were isolated and analyzed as described in the text.