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THE METABOLISM OF CYCLOPHOSPHAMIDE BY ISOLATED RAT HEPATOCYTES

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Abstract—The metabolism of cyclophosphamide was studied *in vitro* using isolated rat hepatocytes and mass spectrometry. The major product of primary oxidative metabolism in hepatocytes from phenobarbital treated rats was 4-hydroxycyclophosphamide, isolated as the *O*-ethyl derivatives, but dechloroethylation was also a substantial pathway. 4-Hydroxycyclophosphamide was converted mainly into carboxy phosphamide and the formation of 4-ketocyclophosphamide was a minor pathway. Evidence is presented that under certain conditions a substantial amount of an *O*-glucuronide of 4-hydroxycyclophosphamide was formed. The pattern of metabolism in hepatocytes otherwise resembled qualitatively that observed previously *in vitro* using subcellular fractions and *in vivo*, but quantitative differences were found. The metabolism of cyclophosphamide by hepatocytes resembles more closely that *in vivo* than does the metabolism in subcellular fractions, and hepatocytes should be the preferred *in vitro* system for studying the metabolism of anti-tumour agents.

Cyclophosphamide (**1**) is a prodrug frequently employed in the chemotherapy of human tumours, particularly in combination therapy regimens. Cyclophosphamide requires metabolic activation to attain significant cytotoxic and alkylating activity [1, 2], which is probably effected by a microsomal cytochrome P-450 mono-oxygenase dependent process [3] occurring principally in the liver [1, 2, 5] but also in other tissues [4–7].

Current understanding of the major pathways of cyclophosphamide metabolism is depicted in Fig. 1. The primary product of metabolic activation is believed to be 4-hydroxycyclophosphamide (**2a**), which is in equilibrium with its cyclic tautomer aldophosphamide (**2b**). Both are intrinsically unstable but have been identified (after conversion into stable derivatives) in microsomal incubations with cyclophosphamide [8, 9] and in blood and urine of animals and humans given cyclophosphamide [9–12]. The ultimate alkylating metabolite responsible for anti-tumour activity is believed [9, 13] to be phosphoramide mustard (**6**) formed from aldophosphamide by non-enzymic β-elimination [14] of acrolein (**7**), which itself appears to contribute to the embryo- and bladder-toxicity of cyclophosphamide [15, 16]. Both phosphoramide mustard and acrolein are metabolites of cyclophosphamide *in vitro* [8, 13, 17] and *in vivo* [18–20]. 4-Hydroxycyclophosphamide/aldophosphamide probably (see [12]) serves as a transport form of the ultimate toxic metabolites and has been proposed [21] as the form conferring the oncostatic selectivity of cyclophosphamide.

Detoxification of cyclophosphamide is thought [22] to result principally from the conversion of 4-hydroxycyclophosphamide and aldophosphamide into the relatively non-cytotoxic metabolites 4-ketocyclophosphamide (**3**) and carboxyphosphamide (**4**) respectively, by soluble enzymes (notably aldehyde dehydrogenase, EC 1.2.1.5) present in liver and kidney [5, 22–24]. The products (**3**) and (**4**) are the major urinary metabolites of cyclophosphamide [10, 25, 26]. On incubation of cyclophosphamide with rat liver 9000 *g* supernatant [8] carboxyphosphamide and 4-ketocyclophosphamide were formed and aldophosphamide was converted into alcophosphamide (**5**). Whether the latter biotransformation, which may be catalysed [24] by cytosolic alcohol dehydrogenase (EC 1.1.1.1), or the conversion of cyclophosphamide into monochloroethylcyclophosphamide (**8**) observed in microsomal incubations [8] also contribute significantly towards detoxification has not been considered, although alcophosphamide and monochloroethylcyclophosphamide are relatively minor metabolites *in vivo* in mice [20].

Hydroxylated xenobiotics are often conjugated in the liver (e.g. with glucuronic acid) but the possibility that 4-hydroxycyclophosphamide may be conjugated, thus increasing its rate of excretion, has received little attention. Isolated hepatocytes provide a convenient *in vitro* system for studying the relative contributions of drug metabolising enzymes found in different compartments of the liver cell to the overall metabolism of a xenobiotic in the presence of physiological concentrations of cofactors [27] and are particularly useful for investigating the role of conjugation reactions [28]. Little use has been made of hepatocytes in metabolism studies of antitumour agents, although they have been used to investigate the metabolism-mediated toxicity of

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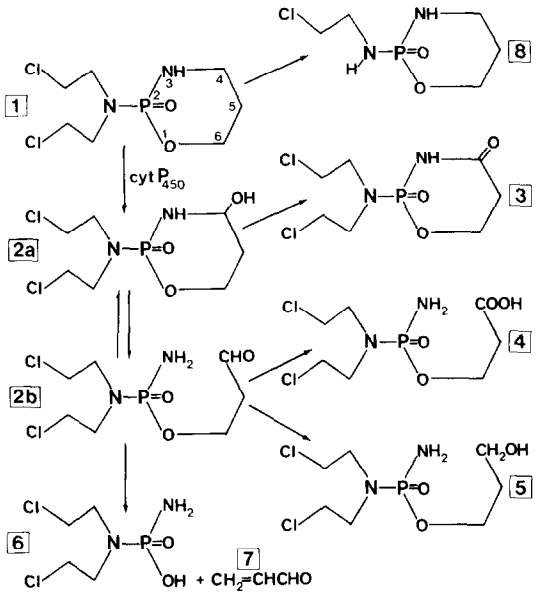
Abbreviations used: EGTA, ethyleneglycol-bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid; LDH, lactate dehydrogenase; EI, electron impact; CI, chemical ionisation.



Cyclophosphamide—1

4-Hydroxycyclophosphamide—2(a)

Aldophosphamide—2(b)

4-Ketocyclophosphamide—3

Carboxyphosphamide—4

Alcophosphamide—5

Phosphoramide mustard—6

Acrolein—7

Monochloroethylcyclo phosphamide—8

Fig. 1. Metabolic routes of cyclophosphamide.

cyclophosphamide [29, 30] and the uptake and polyglutamation of methotrexate [31, 32].

In 1976 a method of preparation of isolated hepatocytes not involving a perfusion stage was reported [33], which has subsequently been used to follow the metabolism of a number of xenobiotics [34–37]. Hepatocytes prepared from the rat by this method were capable of activating cyclophosphamide under defined conditions to determine whether the known pathways of its metabolism (Fig. 1) are operating in the intact cell and whether conjugation of 4-hydroxycyclophosphamide with glucuronic acid or sulphate also occur. The use of isolated hepatocytes in drug metabolism studies requires sensitive analytical procedures for the identification and assay of metabolites [27] and mass spectrometry is a key technique.

MATERIALS AND METHODS

Animals. Male Wistar albino rats (inbred colony) weighing 150–250 g were allowed access to standard rat chow and water *ad lib.* Where indicated sodium phenobarbital was introduced into their drinking water (0.5–1 g/l) at least 10 days preceding sacrifice to induce hepatic mixed function oxidase activity.

Isolated hepatocytes. Hepatocytes were prepared from rat liver slices essentially by the method of Fry *et al.* [33] with some modifications. After treatment with 0.5 mM EGTA, the liver slices were incubated at 37° for 30 min with Hank's Mg^{2+}-free balanced salt solution containing 5 mM $CaCl_2$ and 0.05% (w/v) collagenase, and then the liberated hepatocytes were collected by filtration through 125 μm pore Nybolt nylon mesh and by centrifugation at 50 g_{av} for 1 min. The washed pellet was finally resuspended in Buffer A or Buffer B, containing 5 mM sodium pyruvate and 1% (w/v) fatty acid-poor albumin where indicated. Buffer A consisted of 137 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 3.5 mM Na_2HPO_4, 0.81 mM $MgSO_4$ and 1.3 mM $CaCl_2$, pH 7.4. Buffer B consisted of 118 mM NaCl, 4.8 mM KCl, 0.96 mM KH_2PO_4, 1.2 mM $MgSO_4$, 23.8 mM $NaHCO_3$ and 2.5 mM $CaCl_2$, gassed with O_2:CO_2 (95:5) to bring the pH to 7.4. All solutions were made with double glass-distilled water and analytical reagents.

The yield and percentage of viable cells were determined using an improved Neubauer counting chamber, according to their ability to exclude trypan blue. The effect of incubation with cyclophosphamide on cell viability was assessed by measuring [38] leakage of intracellular LDH (L-lactate:NAD oxidoreductase, EC 1.1.1.27), which was found in 11 cell preparations of differing viability to correlate negatively with the percentage of trypan blue excluding cells ($r = -0.70$, $P < 0.05$). The drug metabolising capacity of the isolated hepatocytes was evaluated with two model substrates, 7-ethoxycoumarin [36] and harmine [39], using reported procedures, and standard curves were constructed using 7-hydroxycoumarin and harmol. Protein was determined [40] using bovine serum albumin as the standard.

Cell incubations. Immediately after isolation the hepatocytes were incubated at concentrations in the range 2–4 × 10^6 cells/ml as described below. Incubation of cells in Buffer A was performed at 37° in 25 ml or 50 ml Ehrlenmeyer flasks in a water bath shaking at 100 cycles/min, whereas incubations in Buffer B were performed at 37° in 50 ml round bottom flasks gassed with O_2:CO_2 (95:5) rotating at 30 rpm in a water bath as described by Moldéus, Högberg and Orrenius [38].

Hepatocytes prepared from phenobarbital-pretreated rats were used in incubations with cyclophosphamide (Expt. 1–3), which where initiated by the addition of the drug in a small volume of ethanol ($\leq$10 μl/ml of cell suspension). [4-^{14}C] Cyclophosphamide was diluted appropriately with

Table 1. Chromatographic characteristics of authentic cyclophosphamide derivatives in t.l.c.*

	R_f			
Solvent	1	3	4	5
cyclophosphamide	0.41	0.09	0.32	0.69
4-ketocyclophosphamide	0.48	0.30	-	0.73
monochloroethyl-cyclophosphamide	0.25	0	-	0.57
carboxyphosphamide	0.01	-	0.13	0.12
acrolein	-	-	-	0.63

* The chromatography and locating of spots are described in Methods.

carrier cyclophosphamide in ethanolic solution and added to 50 ml flasks containing 5 ml of cell suspension (2×10^6 cells/ml, Buffer A containing pyruvate and albumin) to give a final concentration of 50 μg/ml. Control incubations consisted of cyclophosphamide added to the medium alone. After 60 min these incubations were terminated by the addition of 5 vol. of distilled water followed by 4 vol. of redistilled ethanol to denature protein, which was removed by centrifugation at $1200\,g_{av}$. In one case (Expt. 2) the incubation was performed with cells suspended in Buffer B containing pyruvate and albumin (2×10^6 cells/ml) and ethanol was added without prior dilution of the suspension.

For the purpose of LDH leakage determinations, at the end of 60 min incubation with cyclophosphamide aliquots of the cell suspension were taken and centrifuged at $50\,g_{av}$ in the absence or presence of 0.2% (w/v) Triton X-100, and the activity of the enzyme in the supernatants assayed spectrophotometrically [38].

Extraction and analysis of cyclophosphamide metabolites. After protein precipitation most of the ethanol was evaporated from the supernatant at 30° under vacuum, the concentrate was adjusted to pH 4 by the addition of 2 M HCl then extracted with chloroform (3×1 vol.). In one case (Expt. 3) the concentrate was extracted again at pH 2 and in another (Expt. 2) it was extracted first at pH 7 with ethyl acetate (3×1 vol.) and then at pH 4. The combined extracts in each case were dried over anhydrous sodium sulphate, concentrated to dryness under vacuum and the residue was redissolved in a small volume of redistilled ethanol. Each aqueous phase remaining after extraction was lyophilised (Edwards High Vacuum, Modulyo Freeze Drier) and the residue was redissolved in a small volume of AR methanol. Precipitated protein was solubilised in 1 ml of tetraethylammonium hydroxide.

The distribution of radioactive label in each fraction was determined by counting aliquots in Fisofluor '1' scintillant (Fisons Scientific Apparatus, Loughborough, U.K.) using a Packard 3380 liquid scintillation counter. Samples of each fraction were also subjected to t.l.c. on 5×20 cm or 20×20 cm glass plates coated with Kieselgel G (Merck). The solvent systems used (as indicated in Results) were: (1) $CHCl_3$:CH_3CH_2OH (9:1), (2) $CHCl_3$:CH_3CH_2OH (19:1), (3) ethyl acetate, (4) $CHCl_3$:CH_3OH:CH_3COOH (94:3:3), and (5) $CHCl_3$:CH_3OH (3:1). Radioactive bands were located with a Berthold LB 2723 radiochromatogram scanner and the proportion of label in each band was determined from the area under the peaks. The substances in the bands were identified by cochromatography with authentic cyclophosphamide derivatives (see Table 1) and/or by mass spectrometry after elution from the silicic acid with ethanol or methanol. Components on t.l.c. plates were also located by exposure to iodine vapour, by spraying with acidic saturated 2,4-dinitrophenylhydrazine [8] and by the Epstein test [41]. Radioactive components corresponding in R_f value with 4-ethoxycyclophosphamide were treated after elution with acidic 2,4-dinitrophenylhydrazine as described previously [8].

The presence of conjugates in the incubates was investigated by incubating separate concentrates at 37° for 2 hr with pure β-glucuronidase (2500 U) and aryl sulphatase (160 U) in 0.2 M sodium acetate buffer (pH 4.5) prior to extraction, and comparing thin layer radiochromatograms of the resulting fractions with those from incubations performed in the absence of either enzyme. Contaminating β-glucuronidase activity in the sulphatase preparation was inhibited with 15 mM D-glucosaccharic acid-1,4-lactone.

Mass spectrometry. The direct insertion technique was used. EI mass spectra were determined with an AEI-MS12 spectrometer with an ionizing voltage of 70 eV and an ion source temperature of ~100°. CI mass spectra were determined with a VG 7070 spectrometer (ion source temp. ~120° unless otherwise stated) using isobutane as reagent gas. Methylated derivatives were obtained either by treatment with ethereal diazomethane for 20 min [8] or with silver oxide and methyl iodide [42].

Materials. [4-^{14}C]Cyclophosphamide (sp. act. 52.5 mCi/mmole) was obtained from New England Nuclear, Swindon, U.K., and was chromatographically homogenous (t.l.c. in solvent 1). Cyclophosphamide monohydrate was obtained from Koch-Light Laboratories Ltd., Colnbrook, U.K., and acrolein from Aldrich Chem. Co. Ltd., Gillingham, U.K. Collagenase (catalogue No. C2139), β-glucuronidase (Glucarase), aryl sulphatase (type H-1), EGTA, Triton X-100, sodium pyruvate, D-gluco-

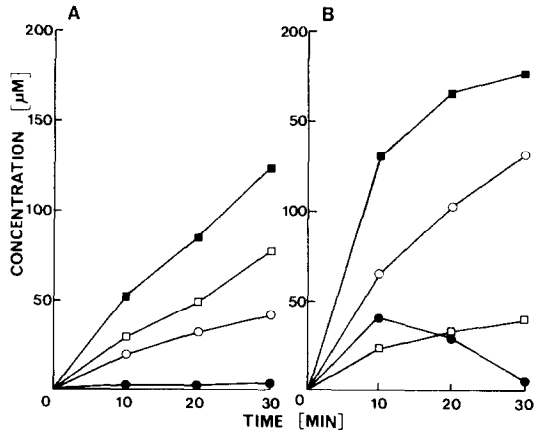


Fig. 2. Metabolism of harmine by isolated hepatocytes prepared from (A) normal and (B) phenobarbital pretreated rats. Hepatocytes (4×10^6 cells/ml, Buffer B) were incubated with 200 μM harmine and the formation of harmol (●), harmol glucuronide (○), harmol sulphate (□) and total metabolites (■) were determined as described in Methods.

saccharic acid-1,4-lactone, harmine HCl, harmol-HCl and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co., Poole, U.K. Fatty acid-poor albumin was prepared [43] from Sigma bovine serum albumin. 7-Ethoxycoumarin and 7-hydroxycoumarin were kindly provided by Dr. J. R. Fry and monochloroethylcyclophosphamide was a gift from Dr. W. J. Stec. 4-Ketocyclophosphamide and carboxyphosphamide were synthesised by chemical oxidation of cyclophosphamide with $KMnO_4$ [44].

RESULTS

Characteristics of the hepatocytes

The concentration of cytochrome P-450 in hepatocytes prepared from phenobarbital-pretreated rats was 0.64 ± 0.10 nmole/10^6 cells (n = 6), which represents a 3-fold increase over levels in hepatocytes from normal rats (0.23 ± 0.05 nmole/10^6 cells, n = 4). The viability of hepatocytes used in the metabolism studies was always greater than 80% and their yield was in the range 5–13 × 10^6 cells/g tissue.

The drug metabolizing activity of hepatocytes prepared from normal and phenobarbital-treated rats is shown in Figs. 2 and 3. The formation of harmol from harmine (Fig. 2) and of 7-hydroxycoumarin from 7-ethoxycoumarin (Fig. 3) demonstrates that microsomal cytochrome P-450 mixed function oxidase activity was intact in the hepatocytes, whilst the appearance of conjugates of each product indicates that the hepatocytes were capable of conjugating hydroxylated compounds with glucuronic acid and sulphate. Initial rates of oxidative metabolism of harmine and 7-ethoxycoumarin were respectively 0.99 and 0.30 nmole/min/10^6 cells (0.42 and 0.13 nmole/min/mg protein), and the resulting products were conjugated preferentially with sulphate (Figs. 2A and 3A). The rate of metabolism of each substrate was markedly increased by phenobarbital pretreatment (4- and 3-fold stimulation for harmine and 7-ethoxycoumarin, respectively) and this resulted in an increased rate of glucuronidation so that it exceeded the rate of sulphation (Figs. 2B and 3B). These results agree with those obtained by other workers using isolated rat hepatocytes [36, 39, 45].

The hepatocytes used in the present study were also capable of conjugating added harmol with glucuronic acid and sulphate, and the conjugates formed were hydrolysed within 2 hr at 37° by treatment with β-glucuronidase and sulphatase respectively (Fig. 4). The deconjugating enzymes specifically hydrolysed only one type of conjugate (Fig. 4) using the procedure described in Methods.

Metabolism of cyclophosphamide by isolated rat hepatocytes

Effect of cyclophosphamide on hepatocyte viability. Since cyclophosphamide is a potentially toxic agent, it was important to ensure that hepatocyte viability remained unimpaired during incubation with cyclophosphamide. Incubation for 60 min with

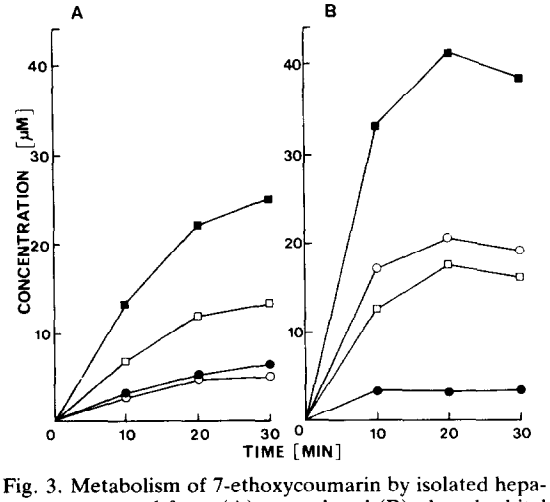


Fig. 3. Metabolism of 7-ethoxycoumarin by isolated hepatocytes prepared from (A) normal and (B) phenobarbital pretreated rats. Hepatocytes (4×10^6 cells/ml, Buffer B) were incubated with 70 μM 7-ethoxycoumarin and the formation of 7-hydroxycoumarin (●), hydroxycoumarin glucuronide (○), hydroxycoumarin sulphate (□) and total metabolites (■) were determined as described in Methods.

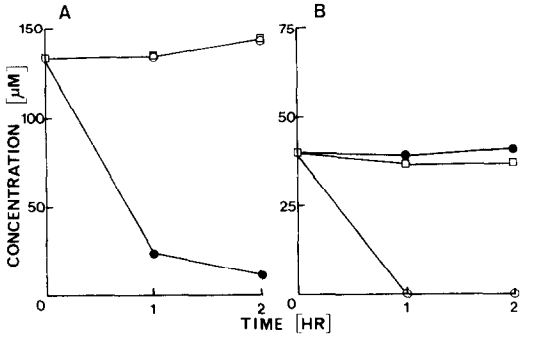


Fig. 4. The effect of incubation with sodium acetate buffer alone (□), β-glucuronidase (●) or aryl sulphatase (○) at pH 4.5 on harmol glucuronide (A) and harmol sulphate (B) formed by hepatocytes from a phenobarbital pretreated rat after incubation (2×10^6 cells/ml, Buffer B) for 30 min with 200 μM harmol. All procedures were as described in Methods.

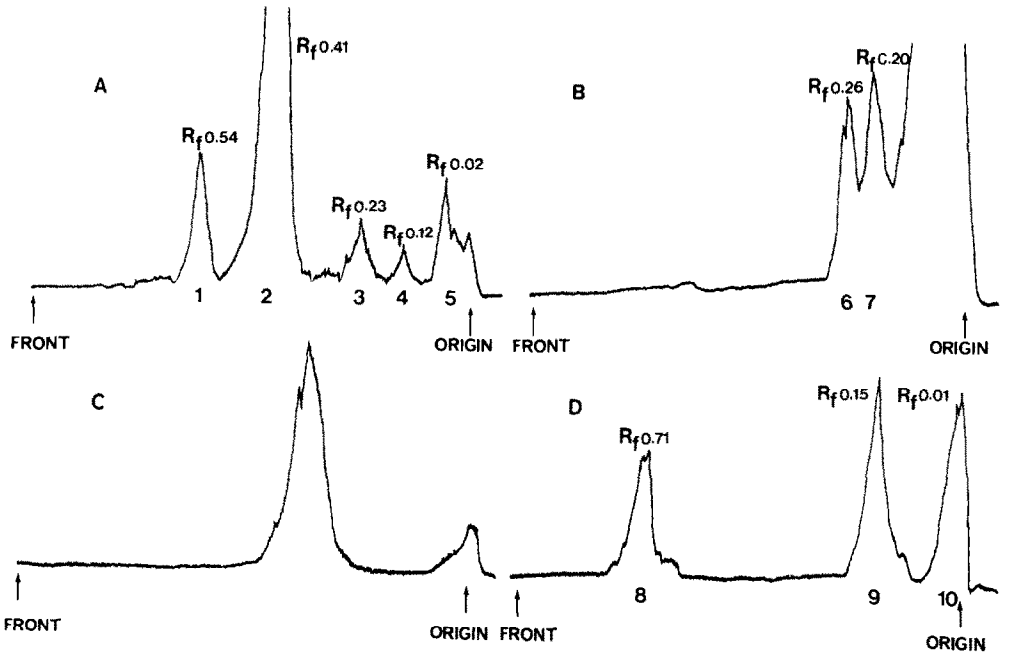


Fig. 5. Metabolism of [^{14}C]cyclophosphamide by isolated hepatocytes prepared from phenobarbital pretreated rats. Hepatocytes (2×10^6 cells/ml, Buffer A containing pyruvate and albumin) were incubated for 60 min with 50 μg/ml cyclophosphamide. The figure shows representative radiochromatograms from Expt. 1 of the pH 4 extract run in (A) solvent 1, (B) solvent 3, and (D) of the aqueous phase remaining after extraction run in solvent 5. The material in peak 5, after elution and methylation with diazomethane, gave the radiochromatogram (C) run in solvent 1. Full experimental details are given in Methods and Results.

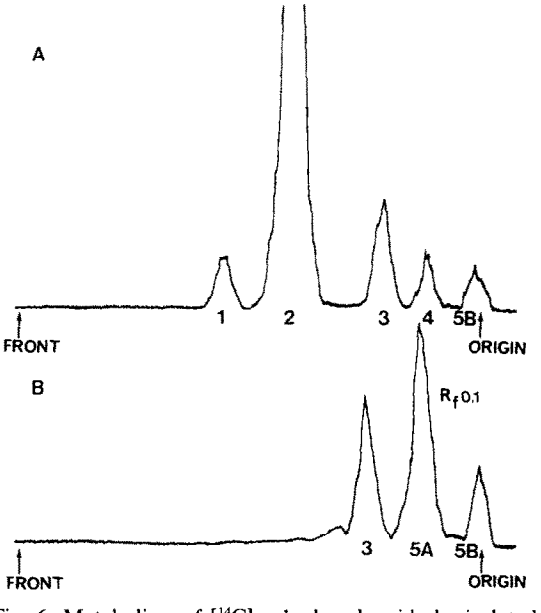


Fig. 6. Metabolism of [^{14}C]cyclophosphamide by isolated hepatocytes prepared from phenobarbital pretreated rats. Hepatocytes (2×10^6 cells/ml, Buffer A containing pyruvate and albumin) were incubated for 60 min with 50 μg/ml cyclophosphamide. The figure shows radiochromatograms from Expt. 2 of (A) the pH 7 extract run in solvent 1 and (B) the pH 4 extract run in solvent 4. In each case 20% of the entire extract was subjected to t.l.c. as described in Methods and the chromatograms were scanned at sensitivities of 30 and 10 counts/sec, respectively. Full experimental details are given in Methods.

cyclophosphamide up to a concentration of 200 μg/ml had no effect on LDH leakage from hepatocytes compared with control incubations (data not shown).

Identification of cyclophosphamide metabolites

The radiochromatograms obtained after incubation of hepatocytes with 50 μg/ml [^{14}C]-cyclophosphamide and extraction of metabolites are shown in Fig. 5 (Expt. 1) and Fig. 6 (Expt. 2). In addition to a large radioactive peak (peak 2) with mobility corresponding to that of authentic cyclophosphamide (Table 1), radiochromatograms of extracts made at pH 4 (Fig. 5A) exhibited several smaller radioactive peaks which were not present in extracts from control incubations.

The product of R_f 0.54 (peak 1) gave a mass spectrum (CI, ion source ~200°) consistent with elimination of ethanol (pseudomolecular ion $[M+H]^+$ at m/z 259) from 4-ethoxycyclophosphamide and which showed a major high mass ion at m/z 223. In these respects its behaviour resembled that of the 'fast' ethoxy derivative of isophosphamide under electron impact [8]. Peak 2 had a shoulder indicating the presence of a metabolite with mobility slightly greater than that of cyclophosphamide in solvent 1. This material could be separated from cyclophosphamide by t.l.c. in solvent 3 (peak 7, Fig. 5B) and it did not migrate as fast as authentic 4-ketocyclophosphamide (Table 1).

When the materials in peaks 1 and 2 were eluted and treated with acidic 2,4-dinitrophenylhydrazine, chloroform extracts were found to contain an orange

component (t.l.c. in benzene) which cochromatographed with authentic acrolein 2,4-dinitrophenylhydrazone [8]. In another case (Expt. 2) a component was detected in the ethyl acetate extract (at pH 7) after methylation with Ag_2O/MeI which afforded a CI mass spectrum appropriate ($[M+H]^+$ at *m/z* 319/321) for *N*(3)-methylated 4-ethoxycyclophosphamide. Thus, it appears that the products migrating in t.l.c. ahead of cyclophosphamide (peaks 6 and 7, Fig. 5B) are the stable *O*-ethyl derivatives of 4-hydroxycyclophosphamide (**2a**) seen previously in microsomal incubations [8].

The product of R_f 0.23 (peak 3, fig. 5A) gave a EI mass spectrum with the characteristics ($M^{+\cdot}$ at *m/z* 198; $[M\text{-}CH_2Cl]$ at *m/z* 149; $[M\text{-}NHCH_2CH_2Cl]^+$ at *m/z* 120) of that reported [44] for monochloroethylcyclophosphamide (**8**), and its chromatographic mobility in solvent 1 also corresponded to that of authentic monochloroethylcyclophosphamide (Table 1). The material in peak 4 (R_f 0.12) had a mobility after elution and rechromatography in solvent 2 comparable to that [8] of alcophosphamide (**5**), and in Expt. 2 (Fig. 6A) this material after methylation with Ag_2O/MeI (which converted —NH and —OH functions into $-NCH_3$ and $-OCH_3$ respectively) afforded a CI mass spectrum with signals attributable to di- and tri-methyl derivatives of alcophosphamide (respectively $[M+H]^+$ at *m/z* 307/9 and $[M+H]^+$ at *m/z* 321/3).

The material remaining at the origin in solvent 1 (peak 5, Fig. 5A) appeared to contain mainly a radioactive component which was slightly mobile (R_f 0.02), together with an immobile component. These two components were extracted differentially at pH 7 and pH 4, and could be separated in solvent 4 (peaks 5A and 5B, Fig. 6), but neither was amenable to analysis by mass spectrometry. The product in peak 5 corresponding to peak 5A could also be separated in solvent 1 from that corresponding to peak 5B after methylation of the material in peak 5 with diazomethane (Fig. 5C).

The product of R_f 0.10 (peak 5A, Fig. 6) had the chromatographic characteristics in four different solvents (Table 1) of authentic carboxyphosphamide (**4**), but the origin material (peak 5B) was immobile in all the solvents. However, pre-treatment with β-glucuronidase, but not incubation alone at 37° and pH 4.5, resulted in the complete disappearance of peak 5B material from each extract together with the appearance of another radioactive peak with R_f values of 0.07, 0.07 and 0.44 in solvents 1, 4 and 5, respectively. The latter product could not be identified by CI mass spectrometry but its chromatographic properties indicate that it might be the same as the product believed to be 4-hydroxycyclophosphamide formed by hydrolysis of 4-ethoxycyclophosphamide [8], and since it did not appear in extracts of incubations performed in the absence of β-glucuronidase, it probably resulted from the action of the enzyme on the product in peak 5B. Thus, it may be concluded that the radioactively labelled material in this peak is the *O*-glucuronide of 4-hydroxycyclophosphamide.

When the material in the origin peak (Fig. 5A) was eluted and methylated with diazomethane, on one occasion an EI mass spectrum was obtained containing signals ($[M\text{-}CH_2Cl]^+$ at *m/z* 185/187; $[CH_2{=}NHCH_2CH_2Cl]^+$ at *m/z* 92/94) appropriate [8, 13] for the methyl ester of phosphoramide mustard (**6**), but this compound would not contain the labelled carbon atom.

The aqueous phase remaining after extraction at pH 4 (Fig. 5D) contained a radioactive component (peak 8) with R_f corresponding to that of authentic 4-ketocyclophosphamide (**3**). Further investigation (Expt. 3) showed that this component was extractable at pH 2 into chloroform and after elution it gave an EI mass spectrum identical with that reported [25] for 4-ketocyclophosphamide. The two most polar components (peaks 9 and 10, Fig. 5D) were not amenable to analysis by mass spectrometry. No evidence was obtained for the formation of a sulphate conjugate from cyclophosphamide by hepatocytes. Treatment with aryl sulphatase had no effect on these polar components. The possibility that one of the polar products might represent acrolein (**7**) was also discounted because of their low R_f in solvent 5. Their identity was therefore not established.

In summary, evidence was obtained for the formation by hepatocytes of the following metabolites of cyclophosphamide: the 'fast' and 'slow' ethyl derivatives of 4-hydroxycyclophosphamide (peaks 6 and 7), monochloroethylcyclophosphamide (peak

Table 2. Distribution of radioactivity in cyclophosphamide metabolites*

	Percentage of total label in metabolites†
'fast' ethoxycyclophosphamide	24.6 ± 2.5
'slow' ethoxycyclophosphamide	33.2 ± 3.3
monochloroethylcyclophosphamide	11.4 ± 3.5
alcophosphamide	5.4 ± 1.6
carboxyphosphamide + 4-hydroxy cyclophosphamide O-glucuronide	14.3 ± 1.6
4-ketocyclophosphamide	2.2 ± 0.3
unidentified polar metabolites	8.9 ± 0.3

* Identified as described in Results.

† Values are from Expt. 1 and given as means ± S.E. for 4 separate hepatocyte preparations.

3), alcophosphamide (peak 4), carboxyphosphamide (peak 5A), 4-ketocyclophosphamide (peak 8), phosphoramide mustard (non-radioactive material coincident with peak 5) and the glucuronide of 4-hydroxycyclophosphamide (peak 5B).

Distribution of radioactivity in cyclophosphamide metabolites

Of the radiolabelled cyclophosphamide incubated with the hepatocytes <1% was associated with the fraction precipitated with ethanol and 55% was recovered unchanged in chloroform extracts. The remainder was mainly extractable at pH 4 (40%) and metabolites non-extractable at this pH (including 4-ketocyclophosphamide) accounted for only 5% of the label.

The relative amounts based on distribution of radioactivity of the metabolites formed during incubation of hepatocytes for 60 min with 50 μg/ml cyclophosphamide (Expt. 1) are shown in Table 2. In this experiment it was not possible to distinguish between the two products identified as carboxyphosphamide and the glucuronide, and Table 2 shows their combined contribution to the total radioactive label in metabolites. However, in the two cases (Figs. 5C and 6) when their relative proportions could be determined (Table 3), the amount of the glucuronide appeared to be dependent on the concentration of Ca^{2+} in the incubation medium. At a concentration of 1.3 mM (Expt. 1) the amount of glucuronide was relatively small, but it was considerably elevated at 2.5 mM (Expt. 2). The relative amounts of alcophosphamide and monochloroethylcyclophosphamide were also 2-fold higher in the latter experiment and all the increases were at the expense of the ethoxy derivatives of cyclophosphamide (data not shown).

The major metabolite of cyclophosphamide in hepatocyte incubations (Table 2) was 4-hydroxycyclophosphamide (**2a**) isolated as the 'fast' and 'slow' ethyl derivatives. The major product of its further metabolism was carboxyphosphamide (**4**), formed presumably by oxidation of aldophosphamide (**2b**). Of the three other identified products, the formation of which could contribute to removal of the activated form of cyclophosphamide, only alcophosphamide (**5**) was present in substantial amount, although at the higher Ca^{2+} concentration the contribution made by the glucuronide was quite considerable (Table 3). dechloroethylation of cyclophosphamide, which would compete against the 4-hydroxylation reaction to yield monochloroethylcyclophosphamide (**8**), was also a substantial pathway in hepatocytes (Table 2). The two unidentified polar metabolites were present in approximately equal amounts.

DISCUSSION

Metabolism studies involving incubation of xenobiotics with subcellular fractions may misrepresent both qualitatively and quantitatively the *in vivo* situation [27]. Isolated hepatocytes provide a more suitable alternative, having the advantages of an *in vitro* preparation whilst retaining many of the characteristics of the liver cell *in vivo*. In the absence of added cofactors they catalyse numerous metabolic reactions at rates comparable to those in the intact liver and the coupling between Phase I and Phase II reactions remains intact [27–29, 33–39, 45–49]. The scheme shown in Fig. 1 has emerged from studies of the metabolism of cyclophosphamide *in vitro* using broken cell preparations from liver and *in vivo* by measuring blood and urine metabolites. The metabolism of cyclophosphamide has now been studied using freshly isolated rat hepatocytes in which the cytochrome P-450 mono-oxygenase system was active as were those which mediated conjugation with glucuronic acid and sulphate (Figs. 2 and 3). Several quantitative differences were found compared with metabolism in subcellular fractions and *in vivo*. Moreover, in addition to the previously identified metabolites (Fig. 1), the hepatocytes generated a glucuronide but not a sulphate of 4-hydroxycyclophosphamide, the first detectable product of cyclophosphamide metabolism. At a Ca^{2+} concentration in the physiological range (2.5 mM) and which is optimal for harmol glucuronidation in isolated rat hepatocytes [46], the glucuronidation pathway was substantial.

As in microsomal incubations [8] the major metabolite of cyclophosphamide was 4-hydroxycyclophosphamide, isolated as its *O*-ethyl derivatives. That large amounts of this metabolite were produced accords with it being [9–12] a major blood metabolite and is consistent with the view (see [12]) that it serves as a transport form of the ultimate toxic metabolites. Evidence was also obtained for the presence of phosphoramide mustard in one hepatocyte extract but the extent of its formation could not be assessed. Although no radioactive material could be attributed to acrolein, this might have been due to its instability or to conjugation with gluathione [18] since glutathione *S*-transferases are active in isolated hepatocytes [47]. Such a conjugate may be one of the

Table 3. Relative proportions of carboxyphosphamide and cyclophosphamide *O*-glucuronide*

Expt.	$[Ca^{2+}]$	Ratio of glucuronide : carboxyphosphamide:	Percentage of total label in metabolites: carboxyphosphamide	glucuronide
1	1.3	0.20	11.9	2.4
2	2.5	0.78	14.0	10.9

* Distinguished as described in Results.

unidentified polar components, representing at the most 5% of the total metabolites.

The major metabolism of 4-hydroxycyclophosphamide/aldophosphamide in hepatocytes was conversion into carboxyphosphamide, and the formation of 4-ketocyclophosphamide was a minor pathway. Both carboxyphosphamide and 4-ketocyclophosphamide are present in large amounts in blood [10, 20] and urine [10, 20, 25, 26] after administration of cyclophosphamide. However, in mice in which the renal arteries and veins were ligated only carboxyphosphamide was present in blood in substantial amount [12]. Since the intact hepatocyte produced considerably more carboxyphosphamide than 4-ketocyclophosphamide, the formation of the latter may occur principally in extrahepatic tissue, probably the kidney. The activity in kidney of aldehyde dehydrogenase, the enzyme thought to be responsible for the formation of 4-ketocyclophosphamide from 4-hydroxycyclophosphamide [22–24], is comparable to that of liver [22].

At the higher concentration of Ca^{2+} glucuronidation of 4-hydroxycyclophosphamide was marked in hepatocytes. Although no data are available on the cytotoxicity of this glucuronide its biosynthesis could promote the excretion of 4-hydroxycyclophosphamide and prevent the formation of aldophosphamide, the precursor of the ultimate cytotoxic agent phosphoramide mustard [9, 13]. However, if a tumour were high in β-glucuronidase activity, hydrolysis of the *O*-glucuronide of 4-hydroxycyclophosphamide would lead to the selective release of the cytotoxic precursor at that site has been observed for the *O*-glucuronide of *p*-hydroxyaniline mustard [50]. Thus, it is possible that the glucuronide of 4-hydroxycyclophosphamide could also serve as a transport form and contribute towards the oncostatic selectivity of cyclophosphamide.

Alcophosphamide production from aldophosphamide was also a substantial route of metabolism in hepatocytes, and as with the glucuronide its formation was greater in the incubation at 2.5 mM Ca^{2+} than at 1.3 mM Ca^{2+}. The rates of harmol glucuronidation and ethanol oxidation in isolated rat hepatocytes increase [46] with increasing Ca^{2+} concentration, reach a maximum at 2.5 mM Ca^{2+}, and then decline rapidly. That Ca^{2+} concentration has a similar effect on alcophosphamide formation is consistent with the proposed role [24] for alcohol dehydrogenase in this biotransformation, and since reactions catalysed by this enzyme are generally reversible, alcophosphamide may not play an important part in the deactivation of 4-hydroxycyclophosphamide/aldophosphamide.

Detoxification of cyclophosphamide could also be effected by dechloroethylation to monochloroethylcyclophosphamide, a non cytotoxic metabolite [8]. This metabolite was formed from cyclophosphamide *in vitro* by microsomes [8] but was present in negligible amounts compared with the 'fast' and 'slow' ethoxy derivatives of cyclophosphamide. By contrast, dechloroethylation was a more important route in isolated hepatocytes and the data suggest that it is a substantial detoxification pathway.

The present study has shown that, for cyclophosphamide metabolism, information obtained previously using subcellular fractions of liver is incomplete and misleading, and that the results obtained with isolated hepatocytes probably reflect better the interactions between metabolic reactions occurring in different parts of the liver cell. The nature and relative amounts of the metabolites of cyclophosphamide formed by hepatocytes are consistent with much of the data obtained from *in vivo* studies. The metabolism of other xenobiotics by hepatocytes resembles more closely their metabolism *in vivo* than does that seen with subcellular fractions [48, 49]. Therefore, isolated hepatocytes would appear to be the preferred experimental system for studying the metabolism of antitumour agents and other drugs *in vitro*. Hepatocytes isolated from samples of human liver would be particularly relevant to investigations of anti-tumour agents and this aspect is under investigation.

The use of isolated hepatocytes in drug metabolism studies requires sensitive techniques for identifying metabolites which are generally found in much lower concentrations in isolated hepatocyte incubations than in incubations with subcellular fractions [27]. EI and CI mass spectrometry provided direct evidence for the identity of most of the metabolites of cyclophosphamide formed by hepatocytes, but some could be identified only by indirect means (carboxyphosphamide and the *O*-glucuronide conjugate) or not at all. These metabolites, notably the most polar ones, were not amenable to conventional CI or EI mass spectrometry even after attempts to derivatise them. Field desorption or desorption CI [51] mass spectrometry may afford a more suitable method for detection of polar and thermolabile metabolites, since they allow direct analysis of these compounds without the need for derivatisation.

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Since submission of this paper it has come to our attention that evidence was presented at the 65th Annual Meeting of the Federation of American Societies for Experimental Biology, April 12–17 [J. P. Lehman, C. Fenselau, M. Colvin and G. S. Yost, *Fedn Proc.* **40** (3), Part 1, Abstract 2865 (1981)], for the formation of the glucuronide of alcophosphamide during incubation *in vitro* of cyclophosphamide with a solubilised preparation of hepatic microsomes, in which UDP-glucuronyl transferase activity was maintained. The product released after β-glucuronidase treatment of the material in peak 5B always migrated slower in t.l.c. than the material identified as alcophosphamide (peak 4). It seems likely, therefore, that the glucuronide reported in the present study is a separate species.

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