Glutathione-dependent dechlorination of 1,6-dichloro-1,6-dideoxyfructose

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The metabolism of ^{14}C - and ^{36}Cl -labelled 1,6-dichloro-1,6-dideoxyfructose (DCF) was studied in the isolated perfused rat liver system. Dechlorination of DCF occurred in the liver and erythrocytes and was GSH-dependent. The GSH conjugate formed was identified by ^{13}C and ^1H n.m.r. as the 6-chlorofructos-1-yl-SG conjugate. It is proposed that the GS^- anion attacks the low steady-state concentration of the reactive keto form of DCF and that the conjugate formed cyclizes to the dominant β -anomer. 6-Chlorofructos-1-yl-SG conjugate of hepatic origin is excreted into bile, whereas that produced in erythrocytes does not enter the liver.

INTRODUCTION

Despite the well-documented role of GSH in the metabolism and dechlorination of chlorinated aliphatic and aromatic compounds (Booth *et al.*, 1961; Chasseaud, 1974, 1979; Kluwe *et al.*, 1981; Wolf *et al.*, 1984), little is known about the mammalian metabolism of chloro deoxy sugars, some of which are known to be neurotoxic and cause infertility in the male rat (Ford & Waites, 1978; Ford, 1982). Among others Sucralose (1,6-dichloro-1,6-dideoxy- β -D-fructofuranosyl-4-chloro-4-deoxy- α -D-galactopyranose) is of particular interest as a non-nutritive high-intensity sweetener (Br. Patent 1543167). The metabolism of its monosaccharide components 4-chloro-4-deoxygalactose and 1,6-dichloro-1,6-dideoxyfructose (DCF) have been studied in the rat. 4-Chloro-4-deoxygalactose is excreted mainly unchanged in the urine, whereas DCF undergoes extensive metabolism and dechlorination. About 80% of the radioactivity of an oral dose of [^{36}Cl]DCF (100 mg/kg) administered to rats was excreted in 14 days, approximately half as $^{36}\text{Cl}^-$. One of the major organochlorine metabolites in the urine was shown to have a mass-fragmentation pattern identical with that of 1,6-dichloro-1,6-dideoxymannitol (DCM) and/or its corresponding sorbitol isomer (J. W. Daniel, unpublished work). This reductive pathway for DCF metabolism was reproduced *in vitro* with the use of sorbitol dehydrogenase, where DCF could replace fructose as a substrate for the enzyme in the presence of NADH as cofactor. However, the reductive pathway does not result in the displacement of chlorine.

The present study describes the GSH-dependent dechlorination of DCF and identifies the liver and erythrocytes as two sites of dechlorination. The contribution of the liver was determined by using the isolated perfused rat liver system. The structure of the GSH conjugate of DCF was determined by ^{13}C - and ^1H -n.m.r. spectroscopy.

EXPERIMENTAL

Materials

1,6-Dichloro-1,6-dideoxyfructose (DCF), 1-chloro-1-deoxyfructose (ICF), 6-chloro-6-deoxyfructose (6CF), 1,6-dichloro-1,6-dideoxy[U- ^{14}C]fructose ([^{14}C]DCF) (specific radioactivity 2.03 $\mu\text{Ci}/\text{mg}$), 1,6-dichloro-1,6-dideoxy[U- ^{14}C]mannitol ([^{14}C]DCM) (specific radioactivity 0.51 $\mu\text{Ci}/\text{mg}$) and 1,6-di[^{36}Cl]chloro-1,6-dideoxyfructose ([^{36}Cl]DCF) (specific radioactivity 0.21 $\mu\text{Ci}/\text{mg}$) were supplied by Tate and Lyle Group Research and Development, Reading, Berks., U.K. Radioactive standard n-[1- ^{14}C]hexadecane (1.1×10^6 d.p.m./ml at 20 °C) was obtained from Amersham International, Amersham, Bucks., U.K. Lumagel and Lumasolve were obtained from May and Baker, Manchester, U.K. Thin-layer plates of silica gel (60 G) were obtained from BDH Chemicals, Poole, Dorset, U.K. GSH and bovine serum albumin were purchased from Boehringer Mannheim, Mannheim, West Germany. Heparin was supplied by Evans Medical, Greenford, Middx., U.K., and penicillin G from Sigma Chemical Co., Poole, Dorset, U.K. All other reagents were of the highest grade available and were obtained from either BDH Chemicals or from Sigma Chemical Co. Bovine blood from which erythrocytes were isolated was supplied by a local abattoir. Kodak Industrex CX X-ray film was obtained from Kodak, Hemel Hempstead, Herts., U.K. Phenisol developer and Hypam fixer were obtained from Ilford, Mobberley, Cheshire, U.K. Portex tubing was obtained from Portex, Hythe, Kent, U.K., and Centricon micro-concentrator units were from Amicon, Stonehouse, Glos., U.K.

Experimental animals

Adult male rats (350–450 g body wt.) of the Sprague–Dawley CD strain (bred within the Department of

Abbreviations used: DCF, 1,6-dichloro-1,6-dideoxyfructose; DCM, 1,6-dichloro-1,6-dideoxymannitol; ICF, 1-chloro-1-deoxyfructose; 6CF, 6-chloro-6-deoxyfructose.

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Biochemistry, University of Wales College of Cardiff, U.K.) were used throughout these studies. Animals were maintained on a standard laboratory diet (Diet 41B, Pilsbury, Birmingham, U.K.) and allowed water *ad libitum*.

Isolated perfused rat liver system

Rat livers were perfused in the recirculating perfusion system described by Curtis *et al.* (1970). The perfusate (120 ml) consisted of washed bovine erythrocytes resuspended in Krebs–Ringer bicarbonate buffer (Dawson, 1969) supplemented with 3% (w/v) bovine serum albumin to give a packed-cell volume of between 30% and 35%. Glucose (240 mg), heparin (500 i.u.) and penicillin G (3340 i.u.) were added to each 120 ml of perfusate (Hughes *et al.*, 1985). Heparinized whole rat blood was used as the perfusate on occasions. Blood was collected from anaesthetized rats via the dorsal aorta into a heparinized (2500 i.u.) container and filtered through several layers of surgical gauze before use. Penicillin G (3340 i.u.) was added to 120 ml of rat blood. Livers were isolated from donor rats under diethyl ether anaesthesia by using surgical procedures outlined by Miller (1973). The common bile duct was cannulated with Portex polyethylene tubing (internal diam. 0.28 mm; external diam. 0.61 mm) and the hepatic portal vein and superior vena cava with Portex polyethylene tubing (internal diam. 1.67 mm; external diam. 2.42 mm). The liver was attached to the perfusion apparatus via the hepatic portal-vein cannula. As soon as a satisfactory blood flow through the liver (40 ml/min) and bile flow (approx. 1 μ l/min per g of liver) had been established, a freshly prepared solution of [14 C]DCF (10 μ Ci; 60 mg) or [36 Cl]DCF (12 μ Ci; 60 mg) in Krebs–Ringer bicarbonate buffer (2 ml) was administered as a single pulse dose into the perfusate. The distribution and fate of radio-labelled DCF in the perfusion system was monitored over a 5 h period. The viability of the organ preparation over this time period has been thoroughly investigated in these laboratories (Curtis *et al.*, 1988).

Bile samples were taken every 15 min for 150 min and thereafter at 30 min intervals. Portions (2 ml) of perfusate were removed after 1, 5 and 15 min and thereafter at 30 min intervals up to 5 h. The packed-cell volume and the radioactivity content of each perfusate sample were determined. Plasma was separated from the erythrocytes by centrifuging (at 1500 *g* for 15 min at 20 °C) and plasma and cells were stored at –20 °C until analysed.

Radiochemical analysis of bile, perfusate and plasma

The radioactivity content of bile, perfusate and plasma samples was measured by counting the radioactivity of 5 μ l portions in 5 ml of Lumagel; efficiency of counting of 14 C was determined by using [14 C]hexadecane as internal standard. The channels-ratio method was used to determine d.p.m. values for samples containing the 36 Cl radioisotope.

Radiochemical analysis of liver

The vascular bed of the liver was flushed with 0.154 M-NaCl, and the organ was weighed, finely minced and homogenized in 0.154 M-NaCl (20%, w/v) in a Potter–Elvehjem homogenizer. Portions (200 μ l) of the homogenate were solubilized with 1 ml of Lumasolve at 50 °C overnight. Propan-2-ol (0.2 ml) was added, and the solutions were bleached with 30% (v/v) H₂O₂ (0.6 ml).

Samples were left overnight in the dark, acidified with 1.5 M-HCl (1 ml) and mixed with 15 ml of Lumagel. The vials were stored in the dark to allow chemiluminescence to decay, and then assayed for radioactivity.

T.l.c. of bile, plasma and lysed erythrocytes

Bile (5 μ l) was applied to t.l.c. plates and chromatograms were developed with butan-1-ol/acetic acid/water (3:1:1, by vol.). Plasma samples were deproteinized by filtration in Centricon microconcentrator units (30000 *M_r* cut-off) under centrifugation at 3000 *g* for 30 min at 20 °C. Samples (10 μ l) of filtrates were subjected to t.l.c. The erythrocytes were lysed by the addition of an equal volume of water followed by vigorous mixing, centrifuging and freezing and thawing until microscopic examination showed complete lysis of cells. The ghosts were separated by centrifuging (1500 *g* for 10 min at 20 °C) and the supernatant was deproteinized by filtration as above. Samples (15 μ l) of the filtrate were subjected to t.l.c. Areas of radioactivity were detected by autoradiography with Kodak Industrex CX X-ray film. The amount of radioactivity associated with each image on the exposed plate was determined by dividing the chromatogram into sections. The sections (approx. 1 cm) were transferred to scintillation vials containing 5 ml of Lumagel and analysed for radioactivity.

Determination of free inorganic 36 Cl[–] in bile and plasma

36 Cl[–] in bile and plasma was determined by difference after precipitation with AgNO₃. Bile (40 μ l) and protein-free plasma (40 μ l) were acidified with 1 M-HNO₃ (10 μ l), and 1 M-AgNO₃ (20 μ l) was added with thorough mixing at room temperature. The AgCl precipitate was removed by centrifuging (3000 *g* for 10 min at 20 °C), and the radioactivity content of the supernatant was determined by counting the radioactivity of sample (20 μ l) in 5 ml of Lumagel. Appropriate controls were included in which the AgNO₃ solution was replaced by water (Daniel, 1963).

Incubation of [14 C]DCF, [36 Cl]DCF, [14 C]DCM, 6CF and 1CF with GSH

[14 C]DCF (2 mM), [36 Cl]DCF (2 mM), [14 C]DCM (2 mM), 6CF (2 mM) and 1CF (2 mM) were incubated separately with GSH (20 mM) in Krebs–Ringer bicarbonate buffer, pH 7.4, at 37 °C for 1 h. Samples (5 μ l) of the radiolabelled incubation mixtures were removed, and the distribution of radioactivity following t.l.c. was determined as described above. Samples (10 μ l) of the non-radiolabelled incubation mixtures were analysed by t.l.c. and the sugars were detected by spraying with an ethanolic solution of 10% (v/v) H₂SO₄ and heating at 130 °C for 5 min.

Synthesis and purification of the GSH conjugate of DCF

[14 C]DCF (4 μ Ci, 9 μ mol) was incubated with GSH (90 μ mol) in 1 ml of Krebs–Ringer bicarbonate buffer, pH 7.4, for 1 h at 37 °C, and the conjugate formed was separated by t.l.c. The appropriate section of the plate (*R*_{DCF} 0.24–0.26), located by autoradiography, was removed and the radioactivity was extracted from the silica gel with water. The extract was centrifuged (15000 *g* for 30 min at 20 °C). The supernatant was removed, filtered (0.2 μ m pore size) and freeze-dried. The 14 C-labelled conjugate (3.6 μ Ci, 8 μ mol of DCF equivalents) was

redissolved in Krebs–Ringer bicarbonate buffer, pH 7.4 (1 ml), and shown by t.l.c to be 96 % pure.

N.m.r. spectroscopy

The n.m.r. spectrum of DCF was examined as a solution in $^1\text{H}_2\text{O}$ or $^2\text{H}_2\text{O}$; GSH was examined in Krebs–Ringer bicarbonate buffer, pH 7.4, containing either $^2\text{H}_2\text{O}$ or $^1\text{H}_2\text{O}$ (Brown *et al.*, 1977). The two solutions were mixed and the pH was raised to approx. 8 with 5 M-NaOH. The reaction was monitored to completion by ^{13}C -n.m.r. spectroscopy at ambient temperature. The 100.6 MHz ^{13}C -n.m.r. spectra were obtained with a Bruker AM 400 instrument. In all 100–200 transients were acquired by using Waltz decoupling (Shaka *et al.*, 1983), 60° pulses and 2 s relaxation delays between acquisitions, 16384 data points being collected across a spectral width of 25 000 Hz. Gaussian multiplication or exponential line broadening was applied before Fourier transformation.

The 400 MHz ^1H -n.m.r. spectra were obtained (in Krebs–Ringer buffer, pH 7.4, containing $^1\text{H}_2\text{O}$ or $^2\text{H}_2\text{O}$) with a spin-echo sequence ($90^\circ-t-180^\circ-t$ -acquire) with

$t = 70$ ms, combined with presaturation of the solvent resonance. In all 128 transients were acquired with 8192 data points across a spectral width of 2133 Hz: a 4 s recycle time was used.

RESULTS

Dechlorination of DCF and biliary excretion of a GSH conjugate in the isolated perfused liver system

There was no difference in the relative distribution of radioactivity among bile, perfusate and liver following perfusion for 5 h with DCF labelled with ^{14}C or ^{36}Cl (Table 1). Most of the radioactivity in the bile was excreted within 60 min.

Experiments with [^{36}Cl]DCF showed that approx. 50 % of the radioactivity in plasma was present after 5 h as $^{36}\text{Cl}^-$ whereas it accounted for only 5 % and 9 % of the radioactivity in bile in the two experiments conducted.

Quantitative t.l.c. analysis of bile, perfusate and lysed erythrocytes showed the presence of a number of metabolites in addition to inorganic Cl^- . At least ten biliary metabolites were detected by autoradiography. The

Table 1. Distribution of radioactivity in the isolated perfused liver system 5 h after the addition of ^{14}C - or ^{36}Cl -labelled DCF (60 mg/120 ml of perfusate)

Results expressed as percentages of the added radioactivity. Radioactivity in perfusate associated with the plasma is denoted in brackets.

Compound	Radioactive dose (μCi)	Distribution of radioactivity (%)			Total recovery (%)
		In bile	In perfusate	In liver	
[^{14}C]DCF ($n = 4$)	10.53 ± 0.24	24.61 ± 0.88	58.18 ± 4.43 [93.06 $\pm$ 3.06]	10.27 ± 2.96	93.06 ± 2.77
[^{36}Cl]DCF ($n = 2$)	12.25 ± 0.35	20.35 ± 1.91	70.50 ± 5.94 [52.70 $\pm$ 1.41]	7.25 ± 2.05	98.10 ± 6.08

Fig. 1. Autoradiographs of chromatograms of bile, plasma and lysed erythrocyte samples from the isolated perfused rat liver after the addition of either [^{14}C]DCF or [^{36}Cl]DCF (60 mg/120 ml of perfusate)

I, [^{14}C]DCF; II, [^{36}Cl]DCF. (a) Bile; (b) plasma; (c) Lysed erythrocytes. Lanes 1, 2, 3 and 4 correspond to the 15 min, 30 min, 45 min and 60 min bile samples, lane 5 the 300 min plasma sample and lane 6 the 300 min lysed erythrocyte sample. [^{14}C]DCF, [^{14}C]DCM and [^{36}Cl]DCF were used as reference standards. Chromatograms were developed in butan-1-ol/acetic acid/water (3:1:1, by vol.). For all experimental details refer to the Experimental section.

Fig. 2. Autoradiograph of chromatogram of plasma after the addition of the GSH conjugate of DCF to the isolated perfused rat liver system

6-Chloro[U- ^{14}C]fructos-1-yl-SG ($2.2\ \mu\text{Ci}$; $4.8\ \mu\text{mol}$ of DCF equivalents/100 ml of perfusate) was added at zero time. Lanes a and b correspond to the deproteinized plasma samples obtained after 1 min and 180 min respectively.

major ^{14}C - and ^{36}Cl -labelled biliary metabolites had the same R_{DCF} values (Fig. 1a), demonstrating that the products retained at least one chlorine atom. The major biliary metabolite (R_{DCF} 0.26) accounted for between 41 % and 64 % of the total radioactivity in bile and was shown to have the same chromatographic mobility as 6-chlorofructos-1-yl-SG.

T.l.c. of deproteinized plasma samples collected after 5 h perfusion showed three major metabolites labelled with either ^{14}C or ^{36}Cl (Fig. 1b). One metabolite, with R_{DCF} 0.96, co-chromatographed with authentic DCM and accounted for 26 % (^{14}C) and 33 % (^{36}Cl) of the total plasma radioactivity. The metabolite with R_{DCF} 0.26 was the GSH conjugate and accounted for 16 % (^{14}C) and 6 % (^{36}Cl) of the radioactivity. The third metabolite, with R_{DCF} 0.44, accounted for 17 % of the radioactivity after [^{14}C]DCF administration. After [^{36}Cl]DCF administration, however, this metabolite, if present, was masked by $^{36}\text{Cl}^-$ (R_{DCF} 0.33–0.53).

T.l.c. of lysed erythrocytes showed that the major radiolabelled component was the GSH conjugate (R_{DCF} 0.26) and the other two minor components corresponded in mobility to DCM and inorganic $^{36}\text{Cl}^-$ (Fig. 1c).

Site of formation of the GSH conjugate of DCF excreted in bile

The GSH conjugate appearing in the bile may be synthesized entirely in the liver and transported directly into the bile, or it may be formed in the erythrocytes from which it is released into plasma and subsequently excreted in bile. These possibilities were investigated by

Fig. 3. Incubation of [^{14}C]DCF, [^{36}Cl]DCF and [^{14}C]DCM with excess GSH

[^{14}C]DCF (2 mM), [^{36}Cl]DCF (2 mM) and [^{14}C]DCM (2 mM) were incubated separately with GSH (20 mM) in Krebs–Ringer bicarbonate buffer, pH 7.4, at 37°C . After 1 h, samples were removed and subjected to t.l.c. (butan-1-ol/acetic acid/water, 3:1:1, by vol.) and autoradiography. Lane a, [^{14}C]DCF and GSH; lane b, [^{36}Cl]DCF and GSH; lane c, [^{14}C]DCM and GSH.

preincubating DCF with whole rat blood, before attaching the liver to the system, thereby allowing the formation of the GSH conjugate within the cell, and subsequently by supplying the isolated perfused liver system with purified GSH conjugate. The extent of biliary elimination was determined in both cases.

Whole rat blood (120 ml) was incubated at 37°C in the perfusion apparatus with [^{14}C]DCF ($10\ \mu\text{Ci}$; 60 mg) for 20 min before attachment of the liver. In two experiments 84 % and 92 % of the radioactivity was taken up by the cells during the preincubation period and the distribution between cells and plasma was unchanged after perfusion. In these experiments 2.1 % and 2.7 % of the radioactivity was excreted in the bile, whereas when [^{14}C]DCF was added to the perfusate without preincubation between 19 % and 26 % of the radioactivity was eliminated in the bile.

The decrease in the excretion of the GSH conjugate of DCF in bile by preincubating DCF with blood may have been due to the slow release of the conjugate from erythrocytes and/or the lack of uptake of the conjugate by the liver. The latter was confirmed by adding the purified ^{14}C -labelled conjugate ($2.1\ \mu\text{Ci}$, $4.8\ \mu\text{mol}$ equivalents of DCF) to 100 ml of perfusate, when only 1.3 % of the radioactivity was secreted in bile over 3 h. Most of the radioactivity (90 %) was present in the perfusate plasma as the unchanged conjugate (Fig. 2). These results indicate that neither the liver nor the erythrocytes have a mechanism for uptake of the GSH conjugate of DCF. Thus the conjugate present in erythrocytes must be formed *in situ* from DCF and that excreted in the bile must be produced in the liver.

GSH-dependent dechlorination of DCF and other chloro deoxy sugars *in vitro*

Incubation of [^{14}C]DCF with excess GSH in buffer for 1 h (Fig. 3) resulted in the formation of one radioactive component (R_{DCF} 0.26), whereas [^{36}Cl]DCF under the same conditions gave rise to two radioactive components (R_{DCF} 0.26 and 0.32–0.52) in equimolar amounts. The

Table 2. Chemical shifts of fructofuranose, DCF, GSH and conjugate

Carbon atom	Chemical shift (p.p.m.)					
	Fructofuranose*		DCF		GSH	Conjugate
	β -Anomer	α -Anomer	β -Anomer	α -Anomer		
C-1	63.6	63.8	47.6	47.0		39.7
C-2	102.6	105.5	101.9	105.4		103.1
C-3	76.4	82.9	77.5	82.9		78.4
C-4	75.4	77.0	76.4	78.7		76.7
C-5	81.6	82.2	81.1	82.7		80.7
C-6	63.2	61.9	46.0	45.0		46.1
Cys α -C					56.2	54.3
Cys β -C					26.2	35.5

* From Bock & Pederson (1983).

component with R_{DCF} 0.32–0.52 corresponded in mobility in organic Cl^- , and AgNO_3 treatment of the incubation mixture showed that 43 % of the radioactivity was present as $^{36}\text{Cl}^-$. There was no reaction between GSH and either 6CF or ^{14}C -labelled DCM (Fig. 3). Incubation of 1CF with GSH resulted in the formation of a component with R_{DCF} 0.16, presumably a GSH conjugate of fructose.

These results show that DCF and 1CF can undergo a non-enzymic dechlorination in the presence of excess GSH. The lack of reactivity of both DCM and 6CF with GSH suggests that the reaction with DCF is dependent upon the carbonyl group at position 2.

N.m.r. spectroscopy

Initially, the structure determination of the GSH conjugate of DCF was achieved by ^{13}C -n.m.r. spectroscopy. Table 2 lists the observed ^{13}C chemical shifts of DCF, GSH and the conjugate together with the shifts of the two anomers of the furanose form of fructose. The spectrum of DCF (Fig. 4c), which clearly shows the presence of two anomeric forms in the approximate ratio 4:1, falls into three distinct regions. These regions are readily assigned: C-1 and C-6, carrying a chlorine substituent, resonate around 45–47 p.p.m.; C-3, C-4 and C-5, each bonded to a single oxygen atom, resonate in the range 75–85 p.p.m.; and C-2, bonded to two oxygen atoms, appears around 100 p.p.m. Detailed comparison of the shifts with those of fructofuranose reveals that the major anomer is the β -form, and also allows tentative assignment of C-3–C-5. It is clear that chlorine substitution has only a minor effect on the chemical shifts of remote carbon atoms.

The ^{13}C -n.m.r. spectrum of GSH (Fig. 4b) has been assigned previously (Huckerby *et al.*, 1985; Mason *et al.*, 1986), and only the cysteine α - and β -carbon shifts are listed in Table 2 because the remaining carbon resonances do not shift significantly on conjugation.

No reaction occurs on addition of DCF to an unbuffered GSH solution in $^2\text{H}_2\text{O}$ (pH 4). However, rapid reaction takes place when the pH of the mixture is raised to 8. Just before completion of the reaction, the spectrum shown in Fig. 4(a) was obtained. Some resonances of the unmodified cysteine residue are visible, but the bulk of the cysteine β -carbon resonance has moved downfield and the α -carbon resonance has moved upfield. This pattern is characteristic of *S*-alkylation in GSH (Mason *et al.*, 1986). The carbohydrate signals show minor shifts

Fig. 4. 100 MHz ^{13}C -n.m.r. spectra of (a) reaction mixture, (b) GSH and (c) DCF

in the 70–105 p.p.m. region, loss of one of the signals of chlorinated carbon atoms and the appearance of a weak and broadened new signal at 39.7 p.p.m.; only one anomeric form is detectable.

The fact that the product retains an anomeric carbon atom with conventional chemical shift and three carbon atoms with a single attached oxygen atom proves almost conclusively that the GSH is attached at C-1 or C-6. The spectrum alone does not prove unambiguously that substitution occurs at C-1 rather than at C-6, but the larger changes for C-2 and C-3 than for C-4 and C-5 are powerful indicators. However, mechanistic argument (see

the Discussion section) and the lack of reaction of 6CF leaves no real ambiguity: the product is the result of reaction of C-1. Given this structure, the shifts of C-3–C-5 show, by comparison with those of DCF and fructose itself, that the β -anomer is the predominant product.

The C-1 signal of the product is broad and weak because its attached protons have exchanged partly with deuterium in the solvent $^2\text{H}_2\text{O}$ (Abell & Staunton, 1983; Sanders & Hunter, 1987). When the reaction was repeated in $^1\text{H}_2\text{O}$, C-1 appeared as a sharp signal of normal intensity. Confirmation of the structure of the GSH conjugate of DCF and its deuterium-exchange properties was obtained from spin-echo ^1H -n.m.r. spectroscopy (Sanders & Hunter, 1987). After conjugation the cysteine β -carbon protons of GSH become markedly inequivalent, moving from 2.92 p.p.m. to 2.95 and 3.15 p.p.m. When the reaction was carried out in $^1\text{H}_2\text{O}$, the C-1 protons of DCF appeared as a singlet at 2.43 p.p.m., but this new signal was missing when $^2\text{H}_2\text{O}$ was used as the medium. The remainder of the ^1H -n.m.r. spectrum of DCF or of the DCF portion of the conjugate was too complex to interpret. The observation of exchange at C-1 lends further weight to the proposed mechanisms of reaction (see the Discussion section).

DISCUSSION

The present investigation has identified the liver and erythrocytes as sites that make a significant contribution to the GSH-dependent dechlorination of DCF, and the mechanism of the reaction with GSH has been described.

Conjugation of foreign compounds with GSH in the liver is well documented, but the contribution of the erythrocytes in conjugation has only recently been addressed. Erythrocytes have been reported as the site of formation of GSH conjugates of other chlorinated compounds such as 1-chloro-2,4-dinitrobenzene (Eckert & Eyer, 1986). However, the significance of such extrahepatic metabolism and the subsequent fate of the conjugates *in vivo* have been largely ignored. Clearly, the relative rates of GSH conjugation by the liver and

other sites may have important consequences for the distribution and rates of elimination.

The GSH conjugate of DCF that appears in bile must be formed in the liver because there is no mechanism for the hepatic uptake of 6-chlorofructos-1-yl-SG produced by extrahepatic tissues. Similarly, erythrocytes have no uptake mechanism for the conjugate, which must also be formed *in situ* from DCF.

It is not known if the reaction is entirely non-enzymic or if glutathione *S*-transferases are involved. Preliminary studies (A. Crawford & J. K. M. Sanders, unpublished work) with ^1H -n.m.r. spectroscopy have shown no enhancement in the rate of reaction between DCF and GSH (pH 6.0, 20 °C) in the presence of any one of six purified dimers of the glutathione *S*-transferases.

There are two possible mechanisms for the dechlorination of DCF by the GS^- anion: direct substitution of the cyclic isomer or attack on the open keto form (Scheme 1). Direct substitution of the cyclic isomer would probably be favoured at C-6 on steric grounds; however, this mechanism is unlikely because 6CF does not react with GSH. Furthermore, the absence of reaction of DCM with GSH argues strongly for the role of the carbonyl group.

It is well established that halide atoms adjacent to carbonyl groups are exceptionally reactive. It is for this reason, for example, that active-site-directed inhibitors of proteinases are usually α -chloro ketones (Walsh, 1979). It would be expected, therefore, that in the keto form C-1 should be much more reactive than C-6 towards substitution and the formation of a GSH conjugate with 1CF but not with 6CF supports this mechanism. We propose, therefore, that GS^- attacks the low steady-state concentration of the reactive keto form of DCF to yield the corresponding conjugate, which then cyclizes to the dominant β -anomer (Scheme 1). The size of the GS group attached to C-1 accounts for the dominant formation of this single anomer. The keto form of DCF is not present in concentrations that can be detected directly, but the consumption of both anomers provides evidence for its existence. In the absence of GSH and under

Scheme 1. Mechanistic scheme for the reaction of DCF and GSH and for deuterium exchange via the enolate anion

alkaline conditions, C-1 is attacked by OH⁻ ions or water to give Cl⁻ and 6CF.

The exchange of deuterium into C-1 of the product but not into the starting material is readily explained. The sulphur atom will stabilize an adjacent negative charge more effectively than the chlorine atom, so the enolate anion of the keto form is present at a higher concentration in the product than in the starting material. Repeated enolization and protonation leads eventually to complete deuterium exchange of the starred proton (Scheme 1). This result further supports the presence of keto isomers in the solution.

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REFERENCES

- Abell, C. & Staunton, J. (1981) *J. Chem. Soc. Chem. Commun.* 856–858
- Bock, K. & Pederson, C. (1983) *Adv. Carbohydr. Chem. Biochem.* **41**, 27–66
- Booth, J., Boyland, E. & Sims, P. (1961) *Biochem. J.* **79**, 516–524
- Brown, F. F., Campbell, I. D., Kuchel, P. W. & Rabenstein, D. L. (1977) *FEBS Lett.* **82**, 12–16
- Chasseaud, L. F. (1974) *Drug Metab. Rev.* **2**, 185–220
- Chasseaud, L. F. (1979) *Adv. Cancer Res.* **29**, 175–274
- Curtis, C. G., Powell, G. M. & Stone, S. L. (1970) *J. Physiol. (London)* **213**, 14P–15P
- Curtis, C. G., Hughes, H. M. & Powell, G. M. (1988) in *Metabolism of Xenobiotics* (Gorrod, J. W., Oelschlanger, H. & Caldwell, J., eds.), pp. 295–302, Taylor and Francis, London
- Daniel, J. W. (1963) *Biochem. Pharmacol.* **12**, 795–802
- Dawson, R. M. C. (1969) in *Data for Biochemical Research* (Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M., eds.), p. 507, Oxford University Press, Oxford
- Eckert, K. G. & Eyer, P. (1986) *Biochem. Pharmacol.* **35**, 325–329
- Ford, W. C. L. (1982) in *Progress Towards a Male Contraceptive* (Jeffcoate, S. L. & Sandler, M., eds.), pp. 159–184, John Wiley and Sons, London
- Ford, W. C. L. & Waites, G. M. H. (1978) *Int. J. Androl. Suppl.* **2**, 541–564
- Huckerby, T. N., Tudor, A. J. & Dawber, J. G. (1985) *J. Chem. Soc. Perkin Trans. 2*, 759–763
- Hughes, H. M., Powell, G. M. & Curtis, C. G. (1985) *Biochem. Soc. Trans.* **13**, 760–761
- Kluwe, W. M., McNish, R., Smithson, S. & Hook, J. B. (1981) *Biochem. Pharmacol.* **30**, 2265–2271
- Mason, R. P., Sanders, J. K. M., Crawford, A. & Hunter, B. K. (1986) *Biochemistry* **25**, 4504–4507
- Miller, L. L. (1973) in *Isolated Liver Perfusion and its Applications* (Miller, L. L., ed.), pp. 1–52, Raven Press, New York
- Sanders, J. K. M. & Hunter, B. K. (1987) *Modern NMR Spectroscopy*, pp. 249–252, Oxford University Press, Oxford
- Shaka, A. J., Keeler, J. H., Frenkiel, T. & Freeman, R. (1983) *J. Magn. Reson.* **52**, 335–338
- Walsh, C. T. (1979) *Enzymatic Reaction Mechanisms*, p. 87, W. B. Freeman, San Francisco
- Wolf, C. R., Berry, P. N., Nash, J. A., Green, T. & Lock, E. A. (1984) *J. Pharmacol. Exp. Ther.* **228**, 202–208

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