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ORIGINAL INVESTIGATION

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Bone marrow and renal injury associated with haloalkene cysteine conjugates in calves

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Abstract Almost 40 years ago, it was reported that cattle-feed which had been extracted with hot trichloroethylene and then fed to calves produced renal injury and a fatal aplastic anaemia. The toxic factor was subsequently identified as *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC). These original findings have been confirmed, a single intravenous dose of DCVC at 4 mg/kg, or 0.4 mg/kg intravenously per day administered for 10 days to calves produced aplastic anaemia, and renal injury after a single dose of 4 mg/kg. The toxicity to calves of a number of other haloalkene cysteine conjugates has been examined to ascertain whether, like DCVC, they produce bone marrow and renal injury. Intravenous administration of the *N*-acetyl cysteine conjugate of DCVC produced renal but not bone marrow injury at a molar equivalent dose to DCVC, indicating that the calf can deacetylate the mercapturic acid and further that sufficient chemical had reached the kidney to be a substrate for the enzyme cysteine conjugate β-lyase. However, intravenous administration of the α-methyl analogue of DCVC, which cannot undergo metabolism via the enzyme cysteine conjugate β-lyase, was without toxicity at doses about five-fold higher than DCVC. These latter findings provide strong evidence that metabolism of DCVC via the enzyme β-lyase is necessary for bone marrow and renal injury to occur. The cysteine conjugates of perchloroethylene and hexachloro-1,3-butadiene(HCBD) when given intravenously to calves at molar equivalent doses to DCVC, or above, did not produce either bone marrow or renal injury. In contrast, intravenous administration of the cysteine conjugate of tetrafluoroethylene (TFEC) produced severe renal tubular injury in calves without affecting the bone marrow. In vitro studies with these haloalkene cysteine conjugates showed, like DCVC, that they were good substrates for calf renal cysteine conjugate β-lyase and toxic to renal cells as judged by their ability to reduce organic anion and cation transport by slices of calf renal cortex and inhibit the renal enzyme glutathione reductase. Calves were also dosed either orally or intravenously with HCBD to assess its toxicity. HCBD at higher molar equivalent doses than DCVC produced mid-zonal necrosis in the liver, renal tubular necrosis but no bone marrow injury in calves. The key findings emerging from these studies are (1) that none of the other cysteine conjugates, at molar equivalent doses to DCVC and above, produce bone marrow injury in calves, (2) TFEC produced only renal injury, suggesting that sufficient of the other conjugates had not reached the kidney for metabolism by β-lyase to produce cytotoxicity and (3) that HCBD itself is more toxic than its cysteine or mercapturic acid conjugate, suggesting that pharmacokinetics and disposition are important factors in determining the toxicity of these conjugates to calves. Further studies are needed to understand the basis for the selective toxicity of DCVC to the bone marrow of calves.

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Introduction

Fatal haemorrhagic disease has been reported in cattle fed trichloroethylene-extracted soybean oil meal (Stockman 1916). The toxic factor in this feedstuff was identified as *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) (McKinney et al. 1957, 1959). In the calf, intravenous or oral administration of DCVC in doses as low as 0.33 mg/kg given daily for 10 days, or a single dose of 4 mg/kg body weight induced fatal aplastic anaemia (McKinney et al. 1959; Schultze et al. 1959). In the single dose studies there was no apparent affect until about 2 weeks had elapsed, when a severe blood disorder developed that caused the death of the animal 1 or 2 days later. Following the smaller daily injections of 0.3–0.4 mg/kg for 10 days, the calves developed the haematological disorder within about 3 weeks, which proved fatal in some cases. At higher doses DCVC produced anuria and severe renal tubular necrosis prior to the onset of aplastic anaemia (Schultze et al. 1959). Subsequent work by Anderson and Schultze (1965) reported that DCVC was metabolised by an enzyme (now called cysteine conjugate β-lyase) present in bovine liver and kidney to generate pyruvate, ammonia and a reactive thiol moiety. The chemical reactivity of the thiol containing moiety was such that it readily reacted with protein and DNA in vitro (Bhattacharya and Schultze 1972, 1974) and produced irreversible changes in the properties of DNA, very similar to those found in samples of DNA isolated from the thymus, lymph nodes and bone marrow of calves with DCVC-induced aplastic anaemia (Bhattacharya and Schultze 1971a, b). In contrast, the rat, guinea pig, dog or cat did not develop aplastic anaemia following exposure to DCVC (Terracini and Parker 1965). The basis for the species difference in response is not currently understood.

Since this early work, a number of chlorinated and fluorinated chemicals have been shown to undergo conjugation with glutathione followed by further processing to their cysteine conjugates (see reviews by Anders et al. 1988; Lock 1988; Dekant et al. 1993). The cysteine conjugates of hexachlorobutadiene, *S*-(1,2,3,4,4-pentachloro-1,3-butadienyl)-L-cysteine (PCBC) (Nash et al. 1984); chlorotrifluoroethylene, *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine (Dohn et al. 1985) and tetrafluoroethylene, *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFEC) (Odum and Green 1984) are all nephrotoxic in rats, the nephrotoxicity being due to metabolism by the renal enzyme cysteine conjugate β-lyase. This paper reports studies conducted to determine whether, like DCVC, other haloalkene derived cysteine conjugates produce aplastic anaemia and renal injury in the calf by a similar mechanism. We have also studied the toxicity of hexachloro-1,3-butadiene in the calf. Preliminary findings from these studies have been reported (Seawright et al. 1994).

Materials and methods

Chemicals

S-(1,2-Dichlorovinyl)-L-cysteine (DCVC), *S*-(1,1,2-trichlorovinyl)-L-cysteine (TCVC), *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFEC), *S*-(1,2,3,4,4-pentachloro-1,3-butadienyl)-L-cysteine (PCBC), *N*-acetyl-*S*-(1,2,3,4,4-pentachloro-1,3-butadienyl)-L-cysteine (PCB-NAC) and *N*-acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine (DCV-NAC) were synthesised as described by Moore and Green (1988). *S*-(1,2-Dichlorovinyl)-DL-α-methylcysteine (DCVMC) was prepared as previously described by Elfarra et al. (1986). Hexachloro-1,3-butadiene (HCBD) was purchased from British Drug Houses, Poole, Dorset. Other reagents were of the highest purity available from commercial sources. Log P values for the conjugates were calculated using CLOGP, Daylight version 3.64 software.

Animal studies

Male and female Guernsey or Friesian calves of about 50 kg body weight (range 43–60 kg) were provided by the dairy farm in the Faculty of Veterinary Science at the University of Queensland. They were fed initially with whole cow's milk and later given ground grain sorgum and chaffed lucerne hay ad libitum. Before and during the study, blood samples were collected usually two or three times a week for the duration of the study. Blood urea nitrogen, creatinine, sorbitol dehydrogenase, ALP, AST and determination of platelet and white cell counts were performed by routine procedures in the diagnostic service laboratory in the School of Veterinary Science.

Twenty-four calves were dosed with the haloalkene conjugates or HCBD as described in Table 1. DCVC, DCV-NAC and DCVMC were dissolved with slight warming in sterile isotonic saline at 4–5 mg/ml and injected into the jugular vein. TCVC was dissolved in a minimal amount of dimethylsulphoxide, diluted with isotonic saline until the solution was faintly opalescent and then administered i.v. (calf 10). For calf 11, 1 g TCVC was dissolved in water made slightly acidic with HCl and then injected in three separate volumes over 1 h. TFEC and PCBC were dissolved in a minimal amount of dimethylsulphoxide, diluted with isotonic saline and administered intravenously. PCB-NAC was dissolved in dimethylsulphoxide and then diluted with isotonic saline until the solution was faintly opalescent and then administered i.v. to calves 17 and 18. For calf 19 PCB-NAC was dissolved in sodium bicarbonate, diluted to 10 ml and then administered i.v. while for calf 20 PCB-NAC was dissolved in sodium carbonate and administered orally in a gelatin capsule. HCBD was administered orally, undiluted, in a gelatin capsule for calves 21–23 and by i.v. administration for calf 24.

The calves were examined daily for signs of toxicity, and humanely killed when showing marked signs of toxicity at the discretion of the veterinary pathologist. Necropsy was performed immediately and selected tissues, typically kidney, sternal bone marrow and liver, fixed in buffered neutral formalin. Sternal bone marrow was decalcified in formic acid by standard methods prior to processing. Paraffin sections (5 μm) were prepared and stained with haematoxylin and eosin for histopathological examination.

Biochemical studies

The ability of slices of calf renal cortex to accumulate the organic anion *p*-aminohippuric acid (PAH) or the organic cation tetraethylammonium (TEA) was examined as described by Lock and Ishmael (1979). Fresh calf kidneys were obtained from the local abattoir, stored in ice during transportation and then thin slices of the cortex cut by hand using a Stadie-Riggs tissue slicer. The slices

Table 1 Treatment of calves with cysteine conjugates. The compounds were administered via the jugular vein, except those marked with an *asterisk* which were given orally

Compound	Calf number	Dosing regimen
DCVC	1	0.4 mg(1.85 μmol)/kg per day for 10 days
	2	0.4 mg(1.85 μmol)/kg per day for 12 days
	3 & 4	4 mg(18.5 μmol)/kg
DCV-NAC	5	0.5 mg(1.94 μmol)/kg per day for 10 days
	6	0.75 mg(2.9 μmol)/kg per day for 12 days
	7	5 mg(19.4 μmol)/kg
DCVMC	8	2 mg(8.7 μmol)/kg per day for 8 days
	9	20 mg(87 μmol)/kg
TCVC	10	10 mg(40 μmol)/kg then 8 mg(36 μmol)/kg on day 25
	11	20 mg(80 μmol)/kg
TFEC	12	10 mg(45 μmol)/kg then 9 mg(41 μmol)/kg on day 25
	13	18 mg(90.4 μmol)/kg
PCBC	14	0.6 mg(1.69 μmol)/kg per day for 11 days then 4.2 mg(12 μmol)/kg on day 12
	15	10 mg(29 μmol)/kg then 8 mg(23 μmol)/kg on day 25
	16	8 mg(23 μmol)/kg per day for 5 days
PCB-NAC	17	0.5 mg(1.29 μmol)/kg per day for 12 days
	18	5 mg(12.9 μmol)/kg
	19	5 mg(12.9 μmol)/kg per day for 8 days
	20	7.5 mg(19.4 μmol)/kg per day for 10 days*
HCBD	21	50 mg(192 μmol)/kg*
	22	5 mg(19.2 μmol)/kg per day for 7 days*
	23	2.5 mg(9.6 μmol)/kg per day for 10 days then 5 mg(19.2 μmol)/kg per day for 8 days*
	24	5 mg(19.2 μmol)/kg per day for 8 days

(40–50 mg wet weight) were then incubated in 3 ml of modified Krebs-Ringer phosphate buffer glucose medium, pH 7.4. containing 0.1 μCi [^{3}H] PAH at 75 μM and 0.1 μCi [^{14}C] TEA at 2 μM plus the required concentrations of unlabelled PAH and TEA and cysteine conjugate. The incubations were performed at 25°C under an atmosphere of oxygen with shaking for 90 min. The slices were then blotted, weighed and dissolved in 1 ml Soluene (Packard Instrument Company, Downers Grove Ill.) and the radioactivity determined after the addition of 10 ml of scintillation fluid. Aliquots of medium (0.1 ml) were diluted to 10 ml with water and the radioactivity determined after the addition of 10 ml of scintillation fluid. The efficiency of counting was determined by external standards. Results are expressed as slice to medium concentration ratio (dpm per 100 mg wet weight of slice at the end of the incubation period divided by dpm per 100 μl incubation medium).

Fresh bovine renal cortex or bone marrow from the tibial bone was homogenised with 2 vol ice-cold 0.32 M sucrose using an Ultraturax homogeniser and then centrifuged at 105 000 *g* at 4°C for 60 min. The cytosol fraction was then removed and stored at −70°C prior to assay for enzyme activity. The protein content was determined by the method of Lowry et al. (1951). Cysteine conjugate *β*-lyase activity with DCVC, TCVC and TFEC as substrates was measured by monitoring the release of pyruvic acid as described by Stevens and Jakoby (1983). Glutathione reductase activity in renal and bone marrow cytosol and the effect of various cysteine conjugates on the activity was determined as described by Lock and Schnellmann (1990).

Statistics

Statistical analysis was by Students' *t*-test and *p* values of < 0.05 were considered significant.

Results and discussion

Studies with DCVC and related chemicals

Our initial studies aimed to produce aplastic anaemia and renal impairment in the calf analogous to that reported by Schultze and co-workers (1959). DCVC was administered at 0.4 mg/kg per day i.v. for 10 days (calf 1) or 12 days (calf 2) and blood samples taken on a regular basis to monitor for haematological changes and renal impairment. About 15 days after the start of dosing the platelet count started to decline, being markedly reduced from day 20 onwards (Fig. 1). Similarly, the white cell count also started to decline being reduced by about 50% by day 23 and 90% by day 26 (Fig. 1). Clinical signs of toxicity were evident by day 25 (calf 1) and day 23 (calf 2) with petechial haemorrhages visible on all mucous membranes and copious blood in the faeces. The animals were killed on day 26 (calf 1) and day 24 (calf 2). Blood urea and creatinine remained within the normal range until the final day (Fig. 1). The post-mortem revealed extensive sub-serosal haemorrhages in all parts of the alimentary tract, with some intramuscular and subpleural haemorrhages. Examination of the bone marrow showed marked acellularity (Fig. 2), while the kidneys were histopathologically normal.

Two claves were also given a single i.v. dose of 4 mg/kg DCVC (calves 3 and 4) and monitored on a regular basis

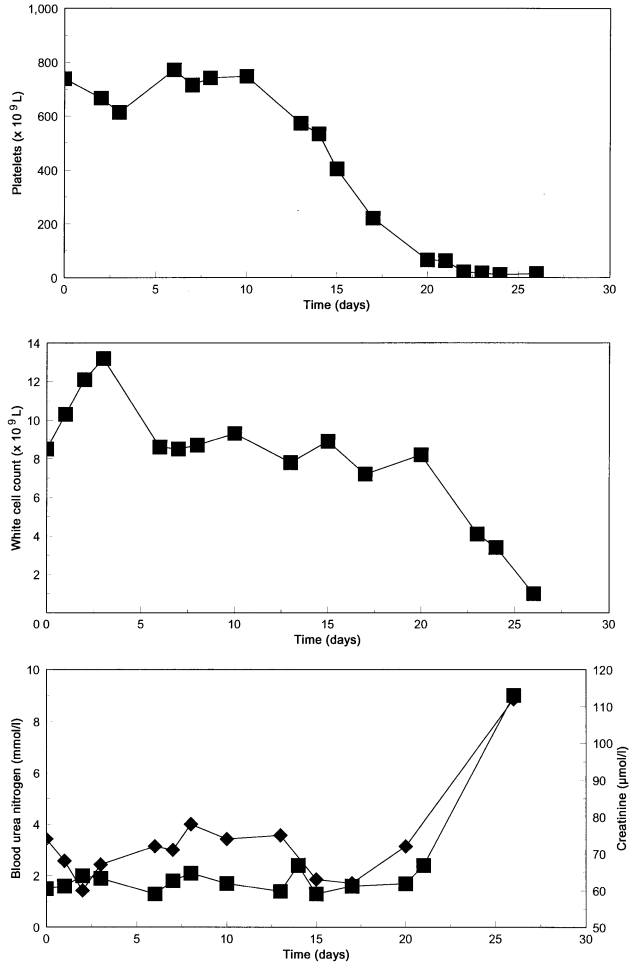

Fig. 1 The effect of intravenous injection of 0.4 mg DCVC/kg per day for 10 days on platelet and white cell counts and blood urea nitrogen and creatinine concentrations in the calf. Results are shown for calf 1; the time course of changes was essentially the same for calf 2, given the same dose. Blood urea nitrogen (■), creatinine (◆)

for haematological changes and renal functional impairment. The haematological picture remained normal until about day 15 when the white cell and platelet count started to decline (Fig. 3). Calf 3 was killed on day 19 and calf 4 on day 24. Calf 3 had a marked elevation in both blood urea nitrogen and creatinine concentrations when monitored on day 4 after dosing which persisted for the remainder of the study (Fig. 3), while for calf 4 (data not shown) blood urea nitrogen was in the normal range for the entire period of the study. At necropsy the main findings were pale swollen kidneys in both animals, while the only indications of possible haemorrhagic disease were sub-serous haemorrhages in the coronary groove. Histopathological examination of the kidneys showed focal areas of interstitial fibrosis and tubular degeneration (Fig. 4), while the bone marrow showed a marked acellularity. These findings confirmed the original observations of Schultze et al. (1959) showing that DCVC produces aplastic anaemia, and at higher doses renal tubular necrosis.

Limited information is available on the metabolism and distribution of DCVC in the calf. Metabolism studies in the rat with [^{35}S] DCVC showed that [^{35}S] sulphate and [^{35}S] DCV-NAC were the major metabolites in urine (Derr and Schultze 1963; Finklestein et al. 1995). However, in the calf neither DCVC nor *N*-acetyl DCVC was detected in the urine following an i.v. dose of 1.5 mg/kg [^{35}S] DCVC (Derr et al. 1963), suggesting this species may be a poor *N*-acetylator. In rats, the balance between *N*-acetylation and activation via the enzyme cysteine conjugate β-lyase seems to be an important determinant of the extent of renal injury seen with certain cysteine conjugates (Commandeur et al. 1991). The α-methyl analogue of DCVC, which cannot undergo a pyridoxal phosphate-dependent elimination reaction to generate a reactive electrophile via the enzyme cysteine conjugate β-lyase, is not nephrotoxic to rats (Elfarra et al. 1986; Anders et al. 1987). We therefore decided to determine the toxicity of the *N*-acetyl DCVC (DCV-NAC) and the α-methyl analogue of DCVC (DCVMC) to calves, to assess the role of deacetylation and cysteine conjugate β-lyase activation in the toxicities.

In total, three calves were dosed with DCV-NAC; the first (calf 5) was given 0.5 mg/kg per day for 10 days, a molar equivalent dose to 0.4 mg/kg per day DCVC, and the platelet count and blood urea nitrogen monitored on a regular basis for 23 days from the start of dosing. No clinical signs of toxicity were seen and all the blood parameters were normal (Fig. 5). Calf 6 was given 0.75 mg/kg per day for 12 days and the blood monitored for 36 days from the start of dosing. No signs of toxicity were observed and blood urea remained in the normal range (Fig. 5). The platelet count was low from day 23 to 36 when the animal was killed (Fig. 5), but was still within the normal range. At post-mortem the bone marrow and kidney were normal. Calf 7 was given a single i.v. dose of 5 mg/kg DCV-NAC. The platelet count remained within the normal range for the entire period of the study; however, the calf developed renal impairment, blood urea nitrogen being elevated at the first time examined (day 5) and this progressively increased until the animal died from renal failure on day 22 (Fig. 5). At post-mortem there were clear signs of renal damage, which was confirmed by histopathological examination, while the bone marrow appeared normal.

Two calves were dosed with DCVMC, one at 2 mg/kg per day for eight consecutive days (calf 8) while the other was given a single dose of 20 mg/kg (calf 9). No clinical signs of toxicity were seen in either animal nor were there any changes in any of the blood parameters suggestive of renal injury or aplastic anaemia (data not shown). The animals were not subjected to post-mortem examination.

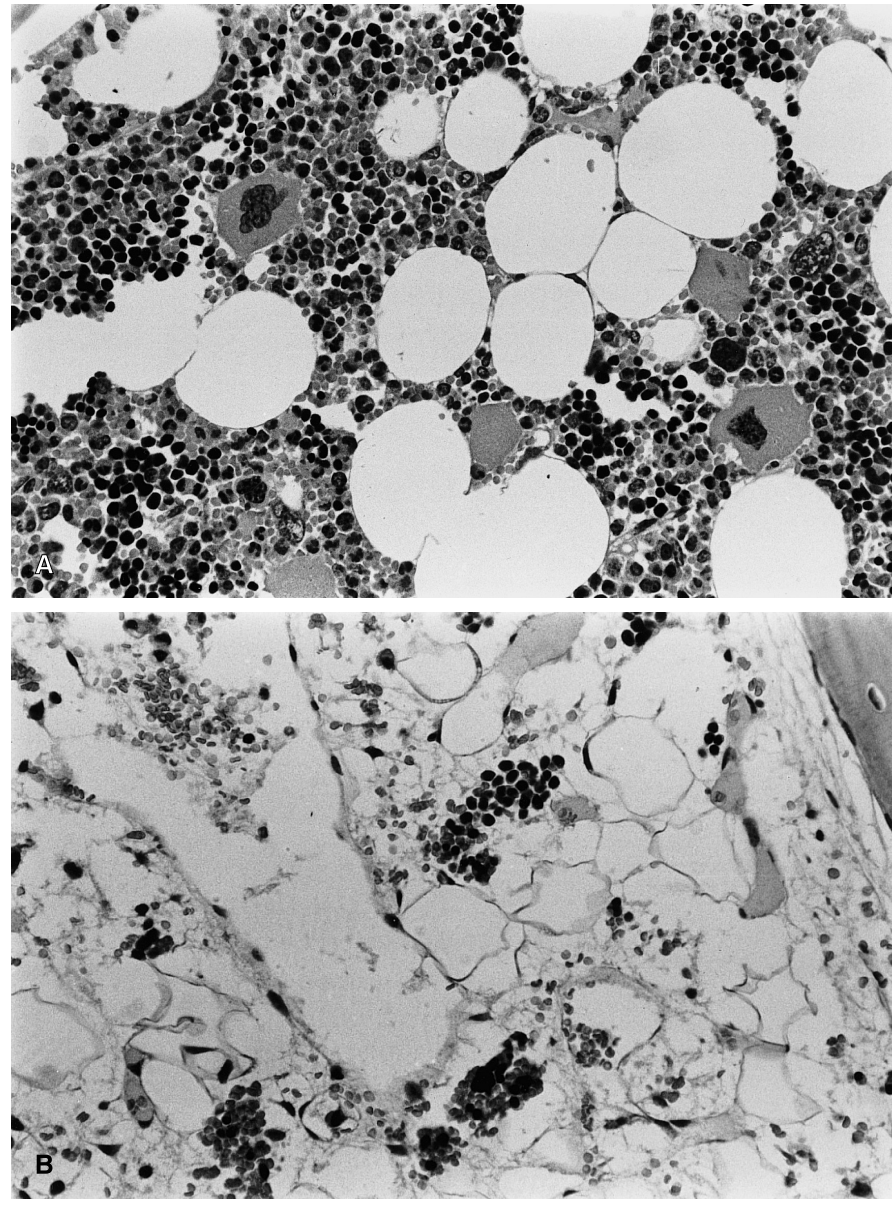

Fig. 2A, B Appearance of bone marrow sections from calves dosed with DCVC. The sections were stained with haematoxylin and eosin and were from an untreated calf (**A**) and calf 1 treated with DCVC at 0.4 mg/kg per day for 10 days, the sample being taken at death on day 26 (**B**). ×250

These findings demonstrate that the calf is able to deacetylate DCV-NAC and produce renal injury, analogous to that seen with DCVC. However, none of the calves dosed with DCV-NAC showed clear evidence of aplastic anaemia similar to that seen with the parent compound, even when given at a higher molar equivalent dose. Calf 6 which received 2.9 μmol DCV-NAC/kg per day compared to calf 1 at 1.85 μmol DCVC/kg per day did show a decrease in circulating platelets which recovered. These findings suggest (1) that the formation of the toxic metabolite from 2.9 μmol/kg per day of the *N*-acetyl conjugate must be close to the threshold required to produce bone marrow injury and thereby reduce the number of circulating platelets, and (2) sufficient of the toxic metabolite was formed in the kidney to produce necrosis. No toxicity was produced by DCVMC in either calf, when given at a dose 4–5 times higher than a toxic dose of DCVC, thus providing evidence that *β*-lyase cleavage is required for both the haematological and renal toxicity.

Studies with other haloalkene or haloalkane cysteine conjugates

The cysteine conjugates of tetrachloroethylene (TCVC), tetrafluoroethylene (TFEC) and hexachlorobutadiene (PCBC) are all substrates for the renal enzyme cysteine conjugate *β*-lyase in the rat and thereby produce renal tubular necrosis (see reviews by Lock 1988; Dekant et al. 1993). The rat does not respond with aplastic anaemia following DCVC administration (Schultze et al.

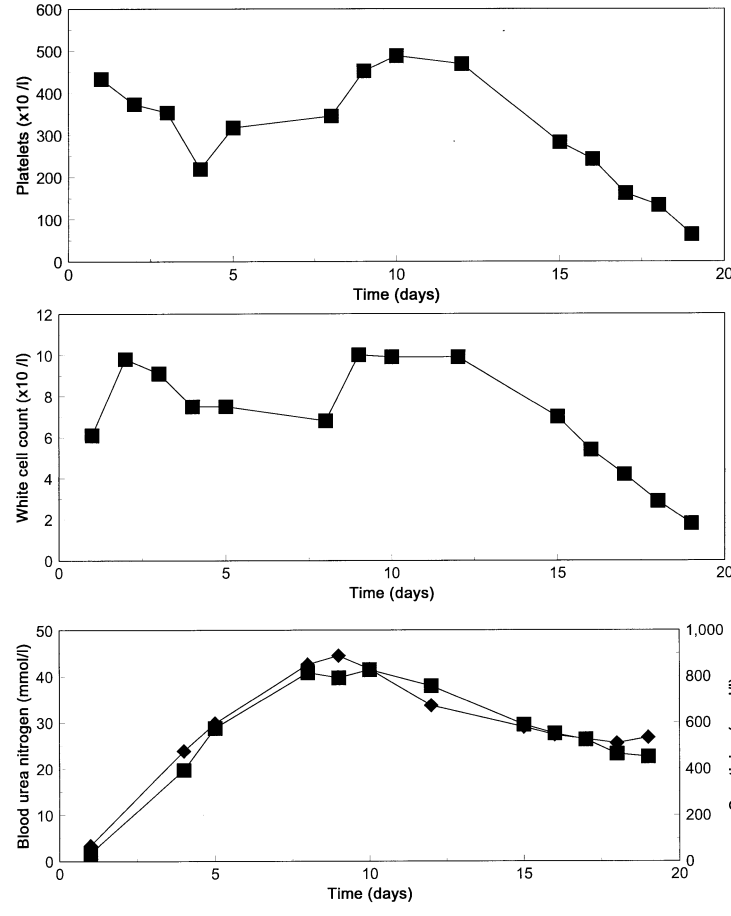

Fig. 3 The effect of intravenous injection of 4 mg DCVC/kg per day for 10 days on platelet and white cell counts and blood urea nitrogen and creatinine concentrations in the calf. Results are shown for calf 3; the time course of changes was essentially the same for calf 4, given the same dose except no alteration in renal function was observed. Blood urea nitrogen (■), creatinine (◆)

1959; Terracini and Parker 1965) nor has this condition been reported with these other conjugates in rats or studied in cattle. We therefore used the protocol developed with DCVC to ascertain whether these other chemicals, that require activation via the enzyme β-lyase, produce toxicity in calves.

TCVC was administered to two calves as a single dose. Calf 10 received 10 mg/kg i.v. (40 μmol/kg) and was observed for 25 days and then given a second dose of 8 mg/kg (36 μmol/kg) and observed for a further week. A second calf, no. 11, was given 18 mg/kg (72 μmol/kg) and observed for 20 days. Apart from an initial neutropenia seen with calf 10 during the first few days after dosing, no decline in platelet or neutrophil count or elevation in blood urea nitrogen was seen in either animal (data not shown). The calves appeared clinically normal and post-mortem examination and histopathology on the kidneys and bone marrow appeared normal.

Similar studies were conducted with TFEC. Calf 12 was given a single i.v. dose of 10 mg/kg (45 μmol/kg) and observed for 25 days and then given another dose of 9 mg/kg (41 μmol/kg) and observed for a further week. In general, the calf remained in good health throughout the study. The white cell count was elevated at early times after dosing but otherwise remained in the normal range (Fig. 6). The platelet count was also in the normal range for the first week and then was elevated for the remainder of the study (Fig. 6). Blood urea nitrogen was elevated about three-fold 4 days after dosing and then returned to normal, but was slightly elevated again following the second dose of TFEC (Fig. 6). At necropsy the kidneys were pale and congenital cysts were present in the left kidney. Histopathology on the kidneys showed marked atrophy of cortical nephrons (Fig. 4). The bone marrow appeared normal. A second calf (calf 13) was given 18.2 mg/kg i.v. TFEC and again blood samples taken on a regular basis. No signs of toxicity were seen over the first 24 h after dosing; however over the following 2 days the calf lost its appetite, was depressed and died 76 h after dosing. Blood urea nitrogen was markedly elevated while the platelet and white counts were in the normal range (Fig. 6). At post-mortem there was marked perirenal oedema, with the kidneys being pale and slightly swollen, while all other tissues appeared normal. Histopathological examination of the kidneys showed extensive tubular necrosis, disorganisation of the cortical parenchyma, interstitial oedema, haemorrhage and hyaline cast formation.

Three calves were dosed with PCBC. Calf 14 was given 0.6 mg/kg per day (1.69 μmol/kg per day) for 11

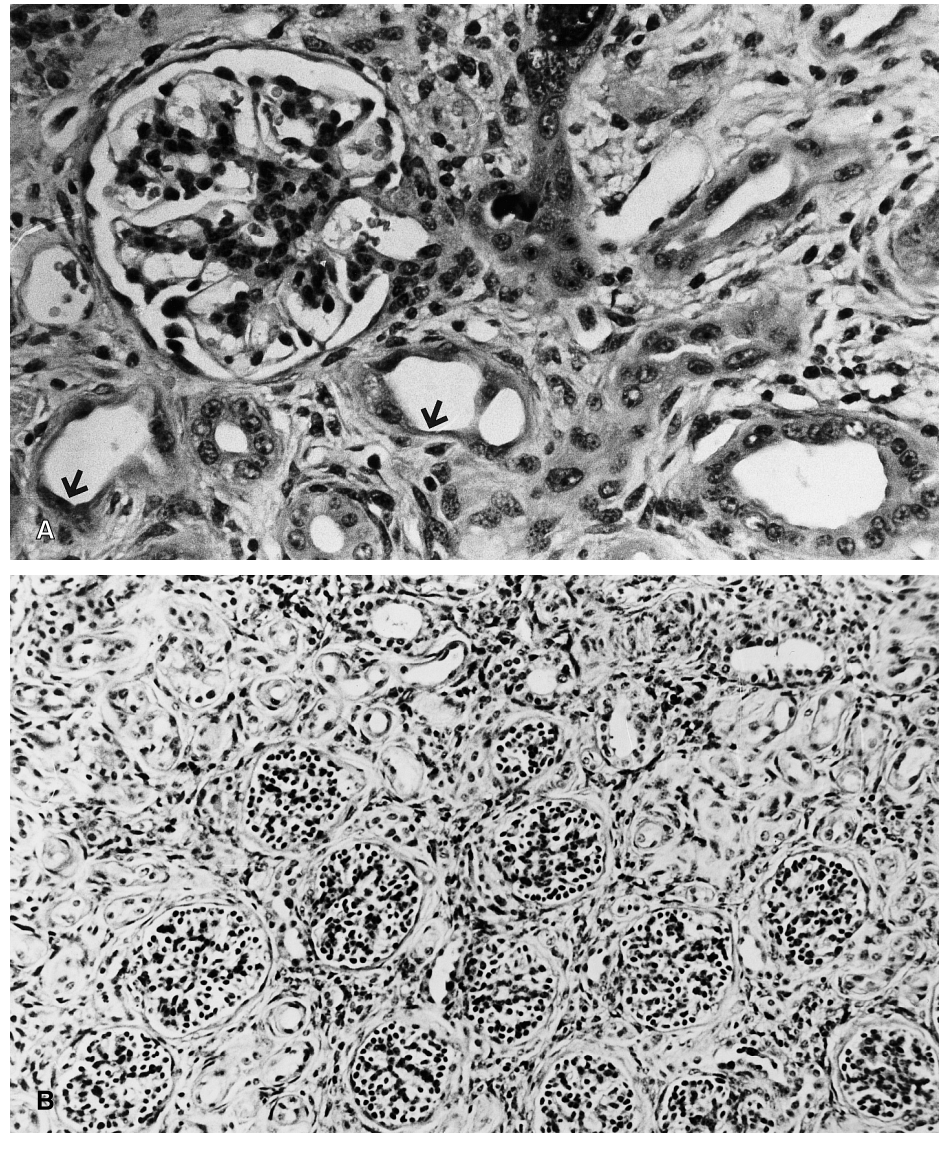

Fig. 4A, B Histological changes in the kidney seen following administration of either DCVC or TFEC to calves. Section of the kidney of calf 3 given a single intravenous dose of 4 mg DCVC/kg (**A**), showing marked tubular dystrophy, atrophy (↑) and interstitial fibrosis. (H & E ×250). Renal cortex of calf 12 dosed with TFEC at 10 mg/kg, i.v. initially and 25 days later at 9 mg/kg, i.v. (**B**). There is extensive atrophy of tubules and interstitial fibrosis as indicated by the large number of glomeruli per field (H & E ×100)

days and then given a single dose of 4.2 mg/kg (12 μmol/kg per day) on day 12 and observed for a further 2 weeks. No changes outside the normal range for blood platelets, white cell count or blood urea nitrogen were seen. No signs of toxicity were observed, the calf steadily gaining weight until the end of the study. At post-mortem no gross pathological changes were seen, although histopathological examination of the renal cortex showed a few focal areas of regenerating tubules surrounded by some interstitial fibrosis. No abnormalities were seen in the bone marrow. A second calf was given 10 mg/kg (29 μmol/kg) PCBC and observed for 25 days and then given another dose at 8 mg/kg (23 μmol/kg) and observed for a further week. With the exception of an initial neutropenia within the first week of dosing all blood platelet, white cell and blood urea nitrogen measurements were in the normal range. The calf appeared in good health throughout the study and the post-mortem examination and histopathology on the kidney and bone marrow showed no abnormalities. A third calf was dosed with 8 mg/kg per day (23 μmol/kg per day) for 5 days, and blood samples and clinical observation made on a regular basis. No adverse effects were seen. Post-mortem and histological examination of the kidney and bone marrow 32 days after the first dose revealed no compound related effects.

Thus it appears that PCBC, when given at a molar equivalent dose to DCVC over 10 days or when given at higher doses either as single 10 mg/kg injection or at 8 mg/kg per day for 5 days, does not produce toxicity analogous to that seen with DCVC. Surprisingly we did not see any renal injury, although there were some minor histological changes in the kidney of calf 14, indicating focal tubular necrosis may have occurred. PCBC is not soluble in aqueous solution and as an insoluble fine suspension in dimethylsulphoxide/isotonic saline may not reach the kidney. Studies were therefore conducted with the *N*-acetyl cysteine

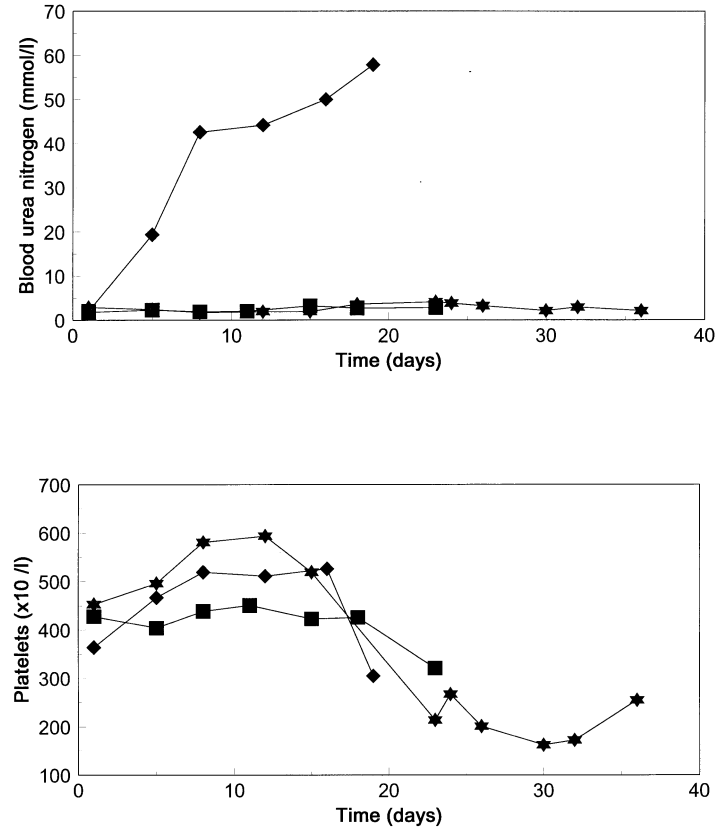

Fig. 5 The effect of intravenous administration of the *N*-acetylcysteine conjugate of DCVC on platelet and white cell counts and blood urea nitrogen and creatinine concentrations in the calf. Results are shown for calf 5 given 0.5 mg/kg per day for 10 days and monitored for 23 days (■), calf 6 given 0.75 mg/kg per day for 12 days and monitored for 36 days (★) and calf 7 given 5 mg/kg and monitored for 20 days (◆)

conjugate, PCB-NAC, which is soluble in aqueous solution and is known in the rat, to be readily de-acetylated in the kidney (Pratt and Lock 1988; Vamvakas et al. 1988) to produce renal toxicity (Nash et al. 1984; Ishmael and Lock 1986). Studies presented in this paper, have shown that in the calf DCV-NAC is nephrotoxic, indicating that de-acetylation to DCVC had occurred.

Two calves, 17 and 18, were given a single dose of 5 mg/kg (12.9 μmol/kg) i.v. or 0.5 mg/kg per day i.v. for 12 consecutive days in dimethylsulphoxide and were monitored for 32 days after the initial dose. Both calves appeared in good health for the duration of the study. Platelet and white counts and blood urea nitrogen were in the normal range for the entire period of the study. At post-mortem there were no signs of renal damage or haemorrhagic disease and the histopathology on the kidney and bone marrow revealed no abnormalities. Two further calves were dosed with PCB-NAC dissolved in aqueous solution as the sodium salt, one (calf 19) was given 5 mg/kg per day i.v. for 8 days and a second (calf 20) was given 7.5 mg/kg per day p.o. for 10 days. As with the previous animals, no clinical signs of toxicity or changes in blood urea, platelet or white cell counts were seen for the entire period of the study, 28 and 24 days, respectively. Post-mortem and histopathological examination of the target organs revealed no abnormalities.

Overall, these studies indicate that only DCVC produced aplastic anaemia. TFEC and DCVC were nephrotoxic whereas TCVC, PCBC and PCB-NAC were not.

In vitro studies with bovine tissue

As PCBC and TCVC did not produce renal injury at the doses studied, we decided to conduct studies in vitro to determine whether (1) calf renal cytosolic β-lyase could metabolise these substrates and (2) whether they were toxic in calf renal cortical slices as reported previously for rat renal slices (Green and Odum 1985). PCBC, TCVC, DCVC and TFEC all produced a dose-related decrease in both PAH and TEA transport, with the transport of PAH being slightly more sensitive (Table 2). DCVC and TCVC produced a statistically significant decrease of both transport systems at 500 μM, while TFEC and PCBC were more active producing a significant decrease at 100 μM. Thus, like DCVC, PCBC, TCVC and TFEC are able to reduce the transport of PAH and TEA into renal cortical slices, suggesting they have undergone metabolism to produce a reactive metabolite which can inhibit these transport systems.

Further support for this hypothesis was obtained by the demonstration that both TCVC and TFEC are metabolised by bovine renal cytosol to produce

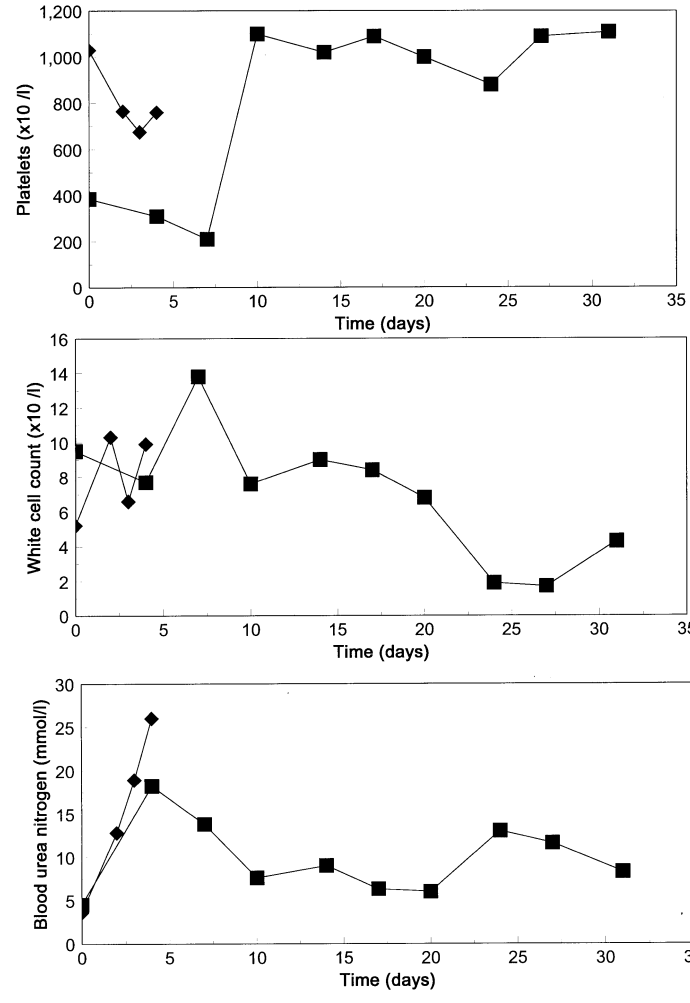

Fig. 6 The effect of intravenous administration of TFEC to calves on platelet and white cell counts and blood urea nitrogen concentrations. Results are shown for calf 12 given a single dose of 10 mg/kg and observed for 25 days and then given 9 mg/kg and observed until day 31 (■) and for calf 13 given a single dose of 18.2 mg/kg (◆)

Table 2 The effect of DCVC, TCVC, TFEC or PCBC on the accumulation of *p*-amino-hippuric acid (*PAH*) and tetraethylammonium (*TEA*) by bovine renal cortical slices. *DMF*, dimethylformamide

Cysteine conjugate	Concentration (μM)	PAH (slice to medium ratio)	TEA (slice to medium ratio)
None-buffer	0	8.18 ± 0.82	8.12 ± 0.88
DCVC	10	7.49 ± 0.73	8.10 ± 1.21
	50	6.64 ± 0.50	7.66 ± 1.21
	100	5.89 ± 0.63	7.52 ± 1.27
	500	3.22* ± 0.58	4.33* ± 0.34
TFEC	10	7.24 ± 0.57	8.02 ± 0.88
	50	6.80 ± 0.50	8.65 ± 1.29
	100	4.81* ± 0.31	7.55 ± 0.70
	500	1.71* ± 0.11	4.60* ± 0.29
None-DMF	0	8.26 ± 0.84	7.95 ± 0.72
TCVC	10	6.84 ± 0.30	6.62 ± 0.88
	50	6.25 ± 0.46	5.97 ± 0.80
	100	5.72 ± 0.72	6.07 ± 0.80
	500	1.74* ± 0.06	2.59* ± 0.36
PCBC	10	6.52 ± 0.59	5.96 ± 0.47
	50	4.47 ± 1.25	5.33 ± 0.29
	100	3.20* ± 1.26	4.52* ± 0.34
	500	1.47* ± 0.06	4.06* ± 0.48

Results are mean ± SE with three separate samples of renal cortex, $*p < 0.05$

pyruvate (Table 3). The kinetics of pyruvate formation from these substrates obeyed Michaelis-Menten kinetics, the K_m and V_{max} for TCVC and TFEC being slightly higher than those for DCVC (Table 3). The production of pyruvate from PCBC (or PCB-NAC) was below the limits of detection of the method used. The reactive metabolite formed following β-lyase cleavage of these substrates, by rat renal cytosol, inhibits the

enzyme glutathione reductase (Lock and Schnellmann 1990) and further, that this can be used as an indicator of metabolism via β-lyase. Studies were therefore conducted to examine whether incubation of DCVC or PCBC with bovine renal or bone marrow cytosol produced inhibition of glutathione reductase activity. Incubation of bovine renal cytosol for 30 min with either 1 mM DCVC or 0.1 mM PCBC produced about 75% inhibition of glutathione reductase activity (Table 4). In contrast, although bone marrow cytosol possessed glutathione reductase activity, it was not inhibited by the presence of either DCVC or PCBC (Table 4).

Overall, these in vitro studies confirm previous findings that DCVC is a substrate for bovine renal cysteine conjugate β-lyase (Anderson and Schultze 1965; Bhattacharya and Schultze 1967) and also show that this metabolism does not occur to a measurable extent in bone marrow cytosol. We were unable to detect β-lyase activity in cytosol from the tibia; however, this source is very rich in adipose tissue and sampling of the red marrow from the sternum or vertebral bodies may reveal detectable activity. Another approach would be immunocytochemically to localise the enzyme with an antibody to β-lyase (Jones et al. 1989; MacFarlane et al. 1989) to ascertain whether it is selectively localised in a particular cell type. The early studies of Bhattacharya and Schulze (1971a,b) demonstrated that DNA, isolated from the bone marrow of calves given a single dose of DCVC and removed about 2 weeks later, showed abnormal physical properties. The abnormal properties of DNA could be reproduced in vitro by incubation of DNA with DCVC in the presence of β-lyase, suggesting that some metabolism of DCVC to a reactive metabolite had occurred in the bone marrow of treated calves (Bhattacharya and Schulze 1972). In this study we have shown that TCVC, TFEC and PCBC are substrates for bovine renal cytosolic β-lyase and that this produces a metabolite that is toxic to renal transport systems in cortical slices. Thus the basis for the renal toxicity with DCVC and TFEC would seem to be β-lyase mediated. It is, however, puzzling that both TCVC and PCBC did not produce renal damage in calves even when given at doses that are nephrotoxic in other species (Ishmael and Lock 1986). The basis for the lack of renal injury following either TCVC or PCBC is currently not clear. Presumably insufficient cysteine conjugate is concentrated in renal cells, at the doses given, to produce injury. TCVC and PCBC, with calculated log P values of 0.611 and 2.031, respectively, are more lipid soluble than TFEC or DCVC with calculated log P values of -0.111 and -0.242, respectively, suggesting that their pharmacokinetics may be different, as indeed could their rates of detoxification and/or elimination. The calf possesses a functional deacetylase enzyme which can convert DCV-NAC to DCVC as shown by the finding that the former produces renal injury, although not bone marrow damage, at the dose examined. However, administration of PCB-NAC which is water soluble and is presumably deacetylated did not result in renal injury. Further studies are needed to understand why bone marrow toxicity only resides with DCVC and further the basis for the lack of renal injury seen with certain conjugates.

Table 3 Metabolism of DCVC, TCVC and TFEC by bovine kidney cytosolic β-lyase. Values are mean $\pm$ SE for three separate renal samples. The values were calculated from Lineweaver-Burk plots, with at least five different substrate concentrations, using regression analysis to give the line of best fit

Substrate	K_m (mM)	V_{max} (nmol/min per mg protein)
DCVC	0.58 ± 0.09	9.71 ± 2.3
TCVC	1.63 ± 0.61	28.2 ± 11.3
TFEC	3.67 ± 0.25	17.1 ± 2.8

Studies with HCBD

Studies were conducted to ascertain whether (HCBD) itself was toxic to the kidney or bone marrow of calves. Administration of a single oral dose of 50 mg/kg HCBD produced marked toxicity such that the animal died 5 days after dosing, blood urea nitrogen was markedly elevated 3 days after dosing (Fig. 7), but no changes were seen in circulating white cells or platelets. At necropsy both the liver and kidneys were pale and swollen. Analysis of plasma aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activities showed elevations (Fig. 7). Histological examination of

Table 4 The effect of DCVC and PCBC on bovine renal cortical and bone marrow cytosolic glutathione reductase activity. Results are mean $\pm$ SE with three separate samples. The cytosol was incubated with the cysteine conjugate for 30 min at 37°C and then a sample (about 0.5 mg protein) was taken for the glutathione reductase assay

Tissue	Treatment	Glutathione reductase (nmol/min per mg)	Activity (% control)
Renal cortex	Buffer alone	58.1 ± 5.4	100
	DCVC 1 mM	$20.8^* \pm 2.7$	36
	PCBC 0.1 mM	$21.2^* \pm 6.7$	35
Bone marrow	Buffer alone	16.4 ± 2.5	100
	DCVC 1 mM	16.5 ± 2.6	100
	PCBC 0.1 mM	15.8 ± 2.3	97

*$p < 0.05$

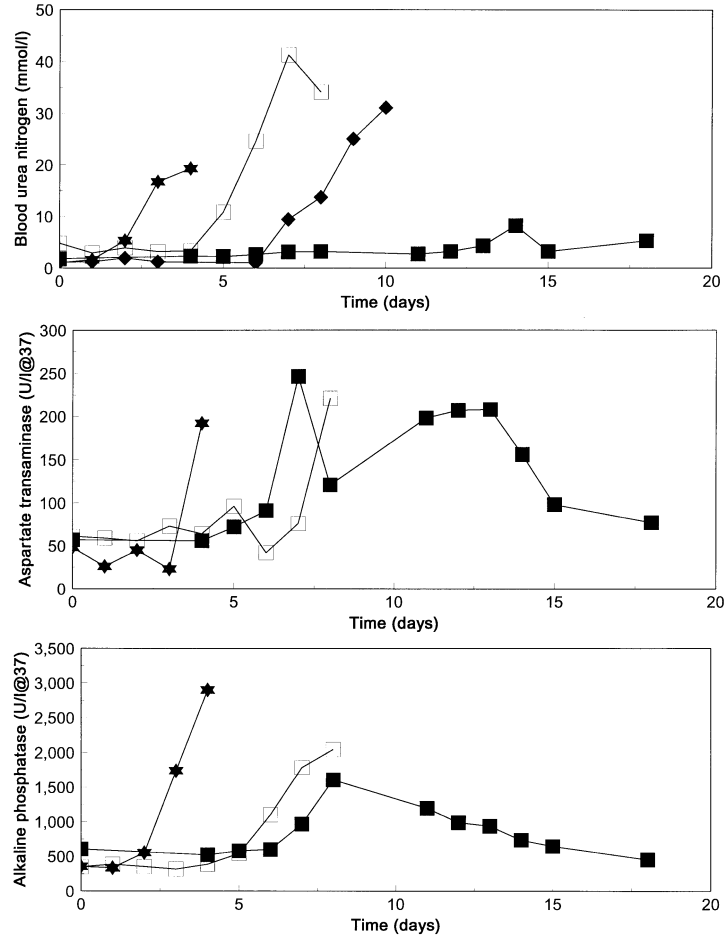

Fig. 7 The effect of oral or intravenous administration of HCBD on markers of renal and hepatic function in the calf. Blood urea nitrogen, AST and ALP were measured at the times shown. Results are shown for calf 21 given a single oral dose of 50 mg/kg (★), calf 22 given 5 mg/kg per day orally for 7 days and terminated on day 9 (□), calf 23 given 2.5 mg/kg per day orally for 10 days (not shown) followed by 5 mg/kg orally given on days 1, 4, 5, 6, 7, 8, 11 and 12 and observed until day 18 (■) and calf 24 given 5 mg/kg per day intravenously for 7 days and observed until day 10 (◆)

the liver showed midzonal necrosis (Fig. 8), while the kidney showed extensive areas of tubular necrosis with hyaline and granular cast formation (Fig. 9). A second calf (calf 22) was dosed orally at 5 mg/kg per day for 7 days by which time it had become sick and was killed 9 days after the first dose. Blood urea nitrogen started to rise after the fifth dose, being markedly elevated up to the time of death (Fig. 7), but there were no changes in the haematological parameters but based on previous observations with DCVC this would require more time to become apparent. Plasma markers of liver injury were increased (Fig. 7) and at necropsy the kidneys showed marked perirenal oedema and the liver was pale and swollen as seen with the single dose. Histopathological examination of the liver showed midzonal necrosis of a slightly milder form than seen after the single dose (Fig. 8), while in the kidney there was extensive swelling of the tubular epithelium with degenerative changes. There were few actual areas of tubular necrosis, but casts were plentiful in the tubules in the medulla. Again this multiple dosing regimen was too high, as the animals developed acute renal failure before any blood dyscrasia would become apparent. A third calf (calf 23) was dosed at 2.5 mg/kg per day orally for 10 days and blood samples regularly monitored for urea and platelet count for 20 days. The animal remained in good health and all the clinical chemical measurements were within the normal range (data not shown). The dose was then increased to 5 mg/kg per day with a total of eight doses given over 12 days. A marginal increase in blood urea nitrogen was seen on day 14 and AST and ALP were increased on day 7, gradually returning to normal on day 15 (Fig. 7). The animal was allowed to recover and terminated 18 days after the start of the second dosing regimen. At the necropsy the liver was normal, although histopathological examination revealed slight disruption of the midzonal architecture while the kidneys were pale and swollen and histology showed some mild renal tubular degeneration. Platelets remained within the normal range for the entire study and the bone marrow appeared normal.

Thus it appears that HCBD when given orally to calves produces marked necrosis to the renal proximal tubules which, at the higher doses, leads to acute renal failure, similar to that seen in common laboratory species (Harleman and Seinen 1979; Lock and Ishmael 1979). No evidence of any haematological disorder was seen in HCBD treated calves, indicating that this phenomenon appears to be selective to DCVC. Hepatocyte

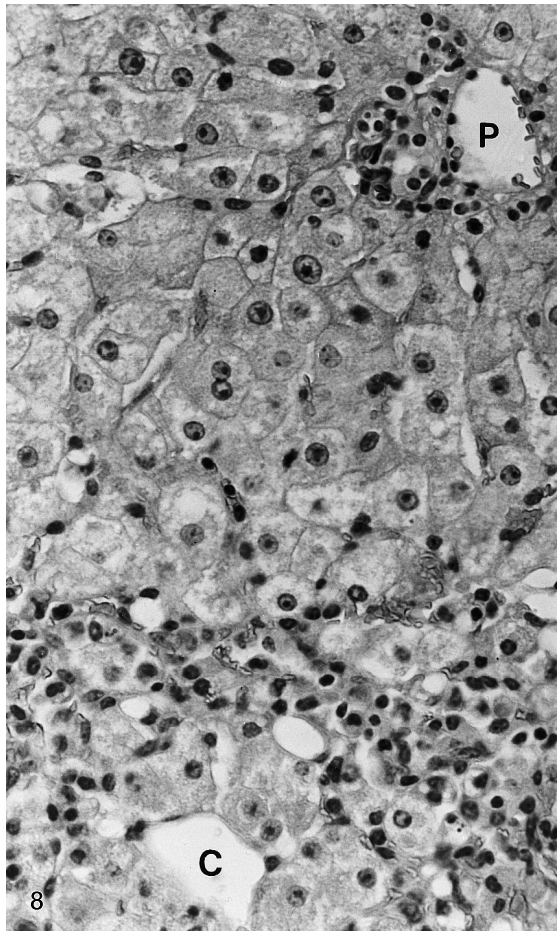

Fig. 8 Histological changes in the liver following HCBD administration to calves. Liver from calf 22 dosed with 5 mg/kg per day HCBD orally for 7 days, which died on day 9 showing an area of mid-zonal necrosis which is infiltrated with macrophages. *P* portal vein; *C* central vein. (H & E ×250)

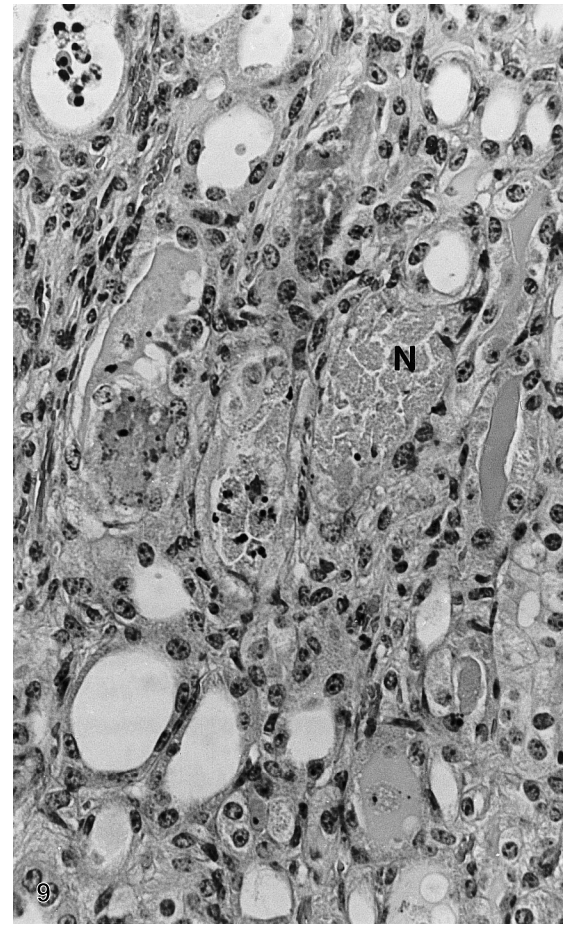

Fig. 9 Histological changes in the kidney following HCBD administration to calves. Kidney of calf 21 given a single oral dose of HCBD at 50 mg/kg showing necrotic tubular epithelium (*N*) and granular and hyaline casts. (H & E ×250)

injury was observed in the midzonal region of the liver in all calves treated with HCBD. This may be due to metabolism of the cysteine conjugate in the liver via β-lyase (Anderson and Schulze 1965). Sheep also develop mid-zonal liver necrosis and renal injury following HCBD administration (Ford and Evans 1985), whereas in rodents hepatocyte swelling is observed but this does not seem to progress to necrosis (Harleman and Seinen 1979; Lock et al. 1982, 1985). Thus HCBD is both nephrotoxic and hepatotoxic whereas the proximate metabolites PCBC and PCB-NAC are not. The most likely explanation lies in the pharmacokinetics and disposition of the conjugates. The cysteine conjugates were administered by the i.v. route, whereas HCBD was given orally. As the route of administration may influence the toxicity, we examined whether i.v. administration of HCBD on a daily basis would produce renal damage when given at an equivalent molar dose to PCBC (calf 16) or PCB-NAC (calf 20). HCBD was administered at 5 mg/kg per day i.v. for a total of 8 days (calf 24), at which time blood urea nitrogen started to rise (Fig. 7) followed by an elevation in plasma sorbitol dehydrogenase activity indicating liver damage. Thus, regardless of the route of administration, HCBD produces renal and hepatic injury. This suggests that when the HCBD conjugates are dosed i.v. sufficient chemical does not reach the kidneys to produce damage, whereas when formed in vivo in the liver and transported to the kidney, or formed directly in the kidney, sufficient chemical enters proximal tubular cells and undergoes activation to cause renal tubular necrosis. Early studies by McKinney et al. (1959) showed that administration of the glutathione conjugate of DCVC to the calf produced renal and bone marrow toxicity, supporting the view that these conjugates are transported to the kidney.

More studies are needed to understand the basis for the difference in sensitivity of the kidney to these different haloalkene cysteine conjugates and more importantly why only DCVC produces aplastic anaemia in the calf.

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